Amino Acids (2012) 42:1045–1053
DOI 10.1007/s00726-011-1019-7

ORIGINAL ARTICLE

Tissue transglutaminase regulates chondrogenesis in mesenchymal stem cells on collagen type XI matrices

Shobana Shanmugasundaram · Sheila Logan-Mauney · Kaitlin Burgos · Maria Nurminskaya

Received: 25 March 2011 / Accepted: 7 June 2011 / Published online: 10 August 2011

Abstract Tissue transglutaminase (tTG) is a multifunctional enzyme with a plethora of potential applications in regenerative medicine and tissue bioengineering. In this study, we examined the role of tTG as a regulator of chondrogenesis in human mesenchymal stem cells (MSC) using nanofibrous scaffolds coated with collagen type XI. Transient treatment of collagen type XI films and 3D scaffolds with tTG results in enhanced attachment of MSC and supports rounded cell morphology compared to the untreated matrices or those incubated in the continuous presence of tTG. Accordingly, enhanced cell aggregation and augmented chondrogenic differentiation have been observed on the collagen type XI-coated poly- (L-lactide) nanofibrous scaffolds treated with tTG prior to cell seeding. These changes implicate that MSC chondrogenesis is enhanced by the tTG-mediated modifications of the collagen matrix. For example, exogenous tTG increases resistance to collagenolysis in collagen type XI matrices by catalyzing intermolecular cross-linking, detected by a shift in the denaturation temperature. In addition, tTG autocrosslinks to collagen type XI as detected by western blot and immunofluorescent analysis. This study identifies tTG as a novel regulator of MSC chondrogenesis further contributing to the expanding use of these cells in cartilage bioengineering.

Keywords Transglutaminase · Collagen type XI · Adult mesenchymal stem cells · Tissue engineering · Cartilage

Abbreviations

BSA	Bovine serum albumin
Ca^{2+}	Calcium
CO_2	Carbon dioxide
cont-tTG	Collagen type XI films continuously treated with tTG
pre-TG	Collagen type XI films pre-treated with tTG
DSC	Differential scanning calorimetry
DMEM	Dulbecco's modified eagle medium
EDTA	Ethylenediaminetetraacetic acid
MSC	Human mesenchymal stem cells
HCl	Hydrochloric acid
PFA	Paraformaldehyde
PBS	Phosphate buffered saline
PLLA-coll XI	PLLA scaffold coated with collagen type XI
coll XI-pre tTG	PLLA-coll XI pre-treated with tTG
coll XI-cont tTG	PLLA-coll XI in the continuous presence of tTG
PLLA	Poly (L-lactide)
GAG	Sulphated glycosaminoglycan
3D	Three- dimensional
tTG	Tissue transglutaminase
2D	Two-dimensional

S. Shanmugasundaram, S. Logan-Mauney contributed equally.

S. Shanmugasundaram · M. Nurminskaya (✉)
Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, Baltimore, MD 21201, USA
e-mail: mnurminskaya@som.umaryland.edu

S. Logan-Mauney
Department of Anatomy and Cell Biology, Tufts University School of Medicine, Boston, MA 02111, USA

K. Burgos
Department of Biology, Towson University, Towson, MD 21252, USA

Introduction

Articular cartilage has a limited regenerative capacity, which is attributed to its avascular structure and low cell density, posing a challenging goal for researchers and clinicians to regenerate the tissue. Tissue engineering strategies utilizing human mesenchymal stem cells (MSC) are expected to solve this problem and have rapidly developed over the last decade, however, a reliable reproduction of the biological composition and biomechanical properties of hyaline articular cartilage has yet to be achieved (Pelttari et al. 2009). Lineage commitment and cell morphology of MSC are in large regulated by scaffold topography and fibril diameter in fibrous scaffolds (McBeath et al. 2004; Gao et al. 2010; Shanmugasundaram et al. 2011).

Collagen is one of the most popular biomaterials for scaffolds in tissue engineering and for implants in tissue repair.

Despite several advantageous features of the collagen-based materials, such as good biocompatibility, biodegradability, and only weak antigenicity, biomedical applications of collagen are limited in part by their susceptibility to enzymatic and thermal degradation in vivo. A common approach to stabilize collagen scaffolds is cross-linking, for example by the calcium dependent enzymes transglutaminase which catalyze formation of the ε-(γ-glutaminyl)-lysine isopeptide bonds (Lorand and Graham 2003). Transglutaminase treatment has been shown to enhance thermal stability and mechanical strength of the collagen type I/hydroxyapatite and collagen type I/elastin composites (Ciardelli et al. 2010; Garcia et al. 2009). In addition, transglutaminase-treated collagen type I-based scaffolds acquire selectivity toward the tissue-specific cell lineages and support enhanced cell adhesion, proliferation, viability, and differentiation (Chau et al. 2005; Ciardelli et al. 2010; Garcia et al. 2009). Based on these data, we propose that transglutaminase-mediated cross-linking of cartilage-specific scaffolds may enhance MSC differentiation into chondrogenic lineage.

Three major types of collagen in cartilage are collagens II, IX and XI which form heterologous II/IX/XI fibrils. Collagen type XI localizes to the surface of these fibrils where it controls the uniform fibril diameter (Blaschke et al. 2000; Eyre et al. 2006) and is accessible for cell contact. Collagen type XI is also the first cartilage collagen deposited by mesenchymal stem cells undergoing chondrogenic differentiation (Xu et al. 2008), suggesting its involvement in the regulation of cartilage formation. Inherited mutations in collagen type XI polypeptides have been linked to cartilage abnormalities, including stickler dysplasia and otospondylomegaepiphyseal dysplasia in human patients (Rimoin 1998). Collagen type XI has been identified as a potential substrate for tissue transglutaminase (tTG) by [^{3}H]-putrescine incorporation (Kleman et al. 1995), but the role of this cross-linking in chondrogenesis has not been addressed. The purpose of this study is to determine whether the modifications introduced by tTG in collagen type XI regulate chondrogenic differentiation in human bone marrow-derived MSC. These studies will provide the necessary background for the use of tTG in cartilage bioengineering.

Materials and methods

Scaffold fabrication

Lyophilized Collagen type XI (Chondrex, Inc.,WA) was solubilized at 0.5 mg/ml in 0.2% glacial acetic acid (Sigma-Aldrich, MO) overnight at 4°C. Two-dimensional (2D) films were made by drying 0.1 mg/cm^2 of collagen type XI in tissue culture plates for 48 h. Three-dimensional (3D) scaffolds of poly (L-lactide) (PLLA) (Sigma-Aldrich, MO) were fabricated with an average fiber diameter of 0.29 ± 0.08 μm by electrospinning as described previously (Shanmugasundaram et al. 2011). Collagen type XI was immobilized on the 3D scaffolds by drying 0.1 mg/cm^2 of collagen type XI overnight at room temperature. Tissue transglutaminase (tTG) from guinea pig liver (Sigma-Aldrich, MO) was used at a concentration of 0.1U/ml. Scaffolds were either treated with tTG continuously during in vitro culture (cont-tTG) or pre-treated with tTG (pre-tTG) prior to overnight cell seeding at 37°C Dulbecco's modified eagle medium (DMEM) containing 1.8mM Ca^{2+}, pH 7.4, 5% CO_2.

Differential scanning calorimetry

Collagen type XI films washed with phosphate buffered saline (PBS) were subjected to thermal analysis in a Differential Scanning Calorimeter (TA Instruments, CO) under continuous flow of dry nitrogen gas at a heating rate of 5°C/min from 0 to 200°C. The temperature at the endothermic peak of the thermogram was measured as the temperature of denaturation.

Collagenase assay

Collagen type XI films were washed with PBS and treated with 200 μl of 0.01% collagenase from *Clostridium histolyticum* (≥125 CD U/mg; Sigma-Aldrich, MO) in PBS for 3 h at 37°C, pH 7.5. The solution was removed after treatment, and films were incubated with 200 μl of 2 mM EDTA for 30 min at 37°C. The collagenase and EDTA solutions were mixed and quantified for protein using the BCA protein assay kit (Thermo Scientific, IL) with BSA as

standard. Three independent experiments were performed with three repeats per condition. Paired Student's *t* test was used for statistical analysis with p-value <0.05.

Electrophoresis and western blot analysis

Proteins were separated by SDS-PAGE on a gradient 4–20% Tris-Hcl gel under denaturing conditions. For western blots, the PVDF membranes were blocked in 5% non-fat milk in TBS-Tween (Teknova, CA) and incubated with primary goat anti-transglutaminase antibody (Millipore, MA) at 1:1,000 dilution overnight followed by an hour in secondary rabbit anti-goat HRP conjugated antibody (Sigma-Aldrich, MO) at 1:5,000 dilution and visualized with super signal west pico chemiluminescent substrate (Thermo Scientific, IL).

MSC expansion, culture and differentiation

Human mesenchymal stem cells (MSC) (Lonza Walkersville Inc., MD) were expanded in DMEM (Invitrogen, CA) supplemented with 10% fetal bovine serum (Thermo Scientific, IL) and 1% antibiotic–antimycotic (Invitrogen, CA). For MSC chondrogenesis, cells at the second passage were seeded at a density of $10^5/cm^2$, and maintained in DMEM–high glucose supplemented with 10^{-7} mM dexamethasone (Sigma-Aldrich, MO), 0.1 mM ascorbic acid (Wako Chemicals, VA), 1% ITS premix (BD Biosciences, NJ), 1 mM sodium pyruvate (Sigma-Aldrich, MO), 0.35 mM L-proline (Sigma-Aldrich, MO), 4 mM L-Glutamine (Invitrogen, CA) and 1% Pencillin–Streptomycin (Invitrogen, CA) supplemented with 10 ng/mL TGF-β3 (ProSpec, NJ) at 37°C, 5% CO_2. Medium was changed twice a week for 8 days. Sulfated glycosaminoglycan (GAG) synthesized by MSC cultured on 3D scaffolds for 8 days was detected by fixing them in 4% PFA and staining with 1% Alcian blue (8GX) dissolved in 0.1 N HCl. For quantitative analysis, Alcian blue dye bound to the scaffolds was extracted with 4 M guanidine hydrochloride and absorbance was measured in a spectrophotometer at 590 nm. Cell number was estimated by staining with crystal violet (Sigma-Aldrich, MO).

Cell attachment assay and cell spreading analysis

Attached MSC on collagen type XI films at 1, 3 and 5 h were detected with the calcein AM cell viability kit according to the manufacturer's instructions (Trevigen, MD). For quantitative assessment, cells were counted with a hemacytometer under a fluorescence microscope (Leica, IL). To analyze MSC morphology, three different areas in each image was chosen for counting the spread and round cells. They were compared to MSC on untreated films at the respective time-points. At least 60 cells per film were counted. Three replicates were used for quantifying total cell aggregates in collagen type XI, pre-tTG and cont-tTG scaffolds.

Immunohistochemical analysis

Collagen type XI films treated with tTG and tTG/EZ-Link pentylamine (Promega) were washed 3–5 times with PBS to remove unbound tTG, blocked for 1 h at room temperature in 2% solution of bovine serum albumin (BSA) in PBST and incubated with monoclonal anti-biotin mouse (Sigma-Aldrich, MO) and rhodamine-conjugated goat anti-mouse IgG (Sigma-Aldrich,MO) for imaging the EZ-Link bound sites. tTG was detected with polyclonal goat anti-transglutaminase (Millipore, MA) and FITC-conjugated rabbit anti-goat IgG (Sigma-Aldrich, MO). The antibodies were diluted in 1% BSA–PBS. The 4% PFA-fixed PLLA-collagen type XI scaffolds with differentiating MSC were stained with 1% Alcian blue (8GX) in 0.1 N HCl to visualize matrix synthesis.

Results

tTG regulates MSC attachment to collagen type XI scaffolds

Previous studies have shown increased cell attachment and spreading of smooth muscle cells and fibroblasts on tTG-treated collagen type I susbstrates (Telci and Griffin 2006; Spurlin et al. 2009), indicating that tTG-induced modifications in fibrillar collagens may affect the general mechanisms of cell adhesion. Therefore, we compared adhesion of human MSC on untreated collagen type XI films to films that were either pre-treated with tTG prior to cell seeding or incubated with cells in the continuous presence of tTG. To mimic the physiological conditions of cartilage implants, these experiments were performed in DMEM with 0.1 U/ml purified tTG.

First, we observed a higher number of MSC attaching to collagen type XI films either pre-treated overnight with 0.1 U/ml tTG (pre-tTG) or cultured in the continuous presence of 0.1 U/ml tTG (cont-tTG) when compared to untreated controls (Fig. 1a), indicating that tTG-induced modification of collagen type XI promotes cell adhesion. Further, analysis of cell morphology with calcein AM vital staining revealed a dramatic six-fold increase in the proportion of round cells versus spreading cells on the pre-tTG collagen type XI films, and a three-fold increase on scaffolds to which tTG was added at the time of cell seeding (Fig. 1b). Thus, after 5 h, 80% of MSC on films pre-treated with tTG and 42% cells cultured in the continuous presence of tTG retain round morphology when compared to 15% of

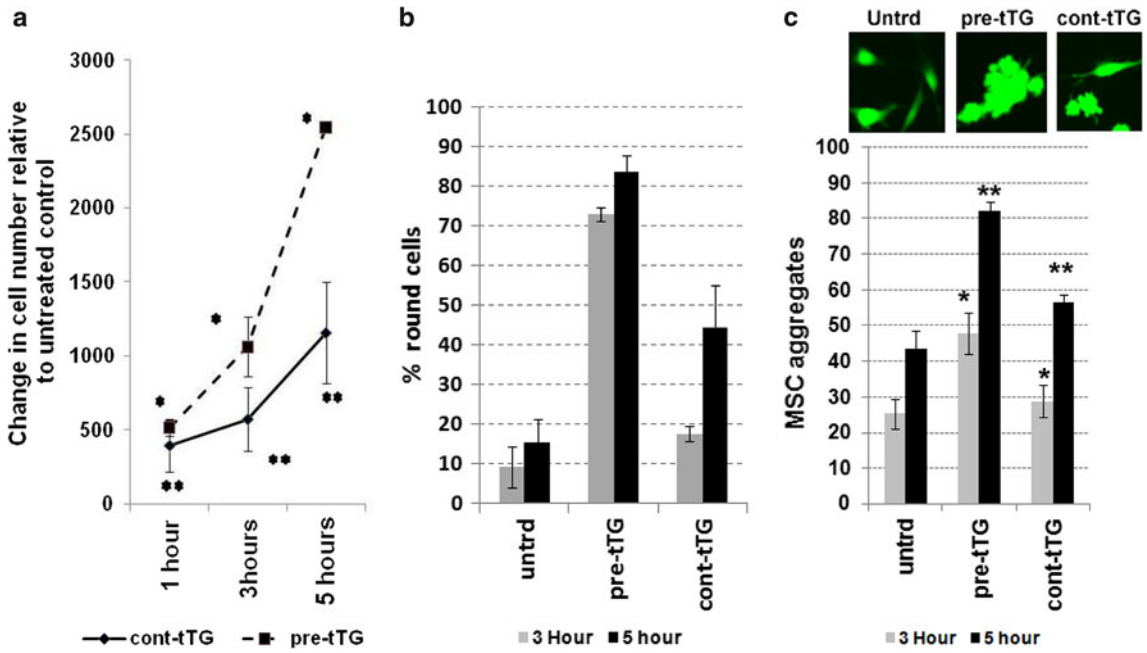

Fig. 1 MSC seeded on collagen type XI films either pre-treated (pre-tTG) or continuously treated (cont-tTG) with 0.1 U/ml of tTG were counted and analyzed for morphology at 3 and 5 h post-seeding. When compared to untreated collagen type XI films, they exhibited **a** increased cell adhesion **b** higher number of round than spread cells, and **c** more cell aggregates as visualized with calcein AM. $*p < 0.05$; $**p < 0.01$; $n = 3$. Magnification: ×200, *Scale bar* 10 µm

round cells on untreated collagen type XI scaffolds. We also noticed that with time, the number of cell aggregates was two-fold higher on the tTG-treated versus untreated collagen type XI scaffolds (Fig. 1c, top panel). These results suggest that tTG-mediated crosslinking of collagen type XI films enhances the adhesive properties of this matrix similar to tTG-treated collagen type I (Telci and Griffin, 2006; Spurlin et al. 2009; Ciardelli et al. 2010). However, contrary to collagen type I, the tTG-treated collagen type XI stimulates MSC to maintain rounded morphology characteristic of cartilaginous tissue. These changes should promote chondrogenesis in MSC seeded on the tTG-modified collagen type XI scaffolds, as suggested by our previous data on the enhanced differentiation in rounded versus spreading chondrocytes (Nurminsky et al. 2007; Shanmugasundaram et al. 2011). This hypothesis was tested in cells induced to undergo chondrogenic differentiation on the tTG-modified versus untreated 3D collagen type XI scaffolds.

Enhanced chondrogenic differentiation of MSC on tTG-modified collagen type XI scaffolds

Deposition of the glycosaminoglycan-rich cartilaginous matrix was analyzed as a hallmark of chondrogenesis. Cartilaginous matrix was detected with alcian blue dye and analyzed histologically and quantitatively by measuring the optical density of the scaffold-bound dye extracted with HCl. First, we observed that collagen type XI supports chondrocyte differentiation when used as a 2D film (Fig. 2a). Next, we analyzed the effects of exogenous tTG on MSC differentiation in 3D nanofibrous PLLA scaffolds using the following conditions: PLLA scaffold coated with collagen type XI (PLLA-coll XI), the same scaffold pre-treated with tTG prior to cell seeding (coll XI-pre tTG) and MSC seeded PLLA-coll XI scaffold cultured in the presence of tTG (coll XI-cont tTG). A three-fold increase in matrix synthesis was detected in cells differentiating on the PLLA-coll XI scaffolds pretreated with tTG before cell seeding when compared to PLLA-collagen type XI scaffolds (Fig. 2b). Propidium iodide staining was used to visualize the cells incorporated in the nanofibrous scaffolds following the alcian blue staining. MSC in the pre-tTG PLLA-collagen type XI scaffolds formed large aggregates which co-localized with the sites of positive alcian blue staining in contrast to uniform dispersion of cells anchored to the collagen type XI-PLLA nanofibers cultured without tTG or in its continuous presence (data not shown). This suggests that the observed increase in cartilaginous matrix production on the pre-tTG scaffolds was due to increased cell number and the higher number of aggregated cells nodules. Accordingly, continuous presence of tTG did not enhance chondrogenic differentiation and matrix deposition in MSC (Fig. 2b, coll XI-cont-tTG), in agreement with our previous study which demonstrated the inhibitory effects of elevated tTG levels on chondrogenesis in avian

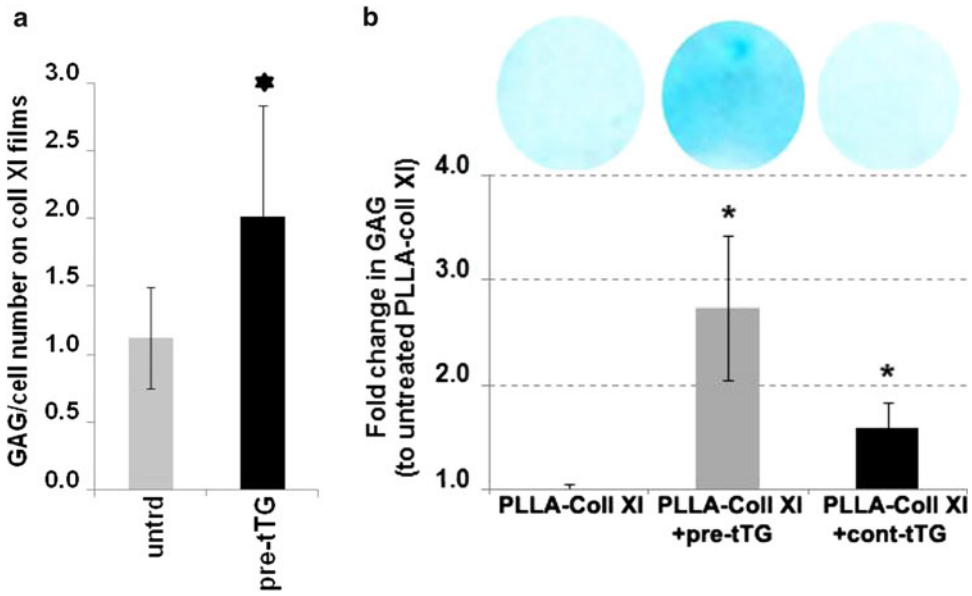

Fig. 2 Chondrogenic differentiation of MSC was enhanced on **a** 2D collagen type XI films, and **b** 3D PLLA scaffolds coated with collagen type XI that were pre-treated with tTG (pre-tTG) or cultured in the continuous presence of tTG (cont-tTG). GAG synthesis was detected with Alcian Blue staining (**b**, *upper panel*) and quantitatively analyzed after extraction of Alcian Blue dye, with Crystal Violet staining used as a control for cell number (**b**, *lower panel*). *$p < 0.05$, $n = 3$. Magnification: ×100, *Scale bar* 10 μm

limb bud mesenchymal cells and in the developing limbs in vivo (Nurminsky et al. 2010). These results indicate that tTG-mediated modification of the collagen type XI protein alter its adhesive properties and thus promote chondrogenic differentiation in MSC.

tTG cross-links collagen type XI films

Collagen cross-linking by various agents leads to increased resistance to collagenase digestion (Harris and Farrell 1972). Therefore, we analyzed whether tTG treatment confers increased resistance of collagen type XI to collagenolysis. We found that protein release from the tTG treated collagen type XI films upon treatment with bacterial collagenase was significantly decreased when compared to the untreated control films (Fig. 3a), indicating that tTG mediates inter- or intra-molecular cross-linking of pure collagen type XI.

To further confirm tTG-mediated cross-linking of collagen type XI, we employed differential scanning calorimetry (DSC) which determines the temperature at which collagen denatures from a triple helix to a random coil structure reflecting the degree of crosslinking (Christopher and Bailey 1999). The DSC thermograms of the control untreated collagen type XI film exhibited an endothermic peak at 102°C (Fig. 3b), while in the tTG-treated films the major endothermic peak shifted up to 105°C, demonstrating increased stability of the collagen fibrils, most likely resulting from the tTG-mediated cross-linking. The slight increase in the denaturation temperature from 102 to 105°C corresponds to crosslinking outside the collagen triple helical structure, since crosslinking in the triple helix would result in a substantial increase in the denaturation temperature. In addition, a new endothermic peak at 89°C appears in the tTG-treated collagen (Fig. 3b). The unfolding temperature of purified tTG has been estimated at 50–54°C for both catalytically inactive and Ca^{2+}-activated forms (Cervellati et al. 2009), indicating that auto-cross-linking activity of tTG (Birckbichler et al. 1977; Barsigian et al. 1991) does not affect thermal stability of this protein. These data implicate that the endothermic peak at 89°C does not represent pure tTG. Several possible explanations for the appearance of this peak may be offered. For example, the intramolecular cross-links of collagen type XI might weaken the structure of some collagen helix regions. However, taking into consideration the fact that glutamine and lysine residues in the triple helical region of the reconstituted collagen fibrils are inaccessible for transglutaminase (Jelenska et al. 1980) and that transglutaminase-mediated crosslinking is mostly directed toward the telopeptide sequences of collagen V/XI (Kleman et al. 1995), weakening of the collagen helix resulting from transglutaminase-mediated crosslinking appears unlikely. An alternative explanation for the 89°C peak accounts for cross-linked complexes comprised of tTG and collagen molecules. To test this further, we employed western blot and immunohistochemical analysis of tTG-treated collagen type XI scaffolds.

tTG auto-cross-links to collagen type XI

Collagen type XI films treated overnight with tTG and biotinylated pentylamine, were washed vigorously with PBS to remove the unbound compounds and then incubated with antibodies recognizing biotin and tTG to visualize their cross-linking into collagen matrix. Both antigens incorporated at specific sites on the collagen type XI films where their localization overlapped (Fig. 4a). Pentylamine

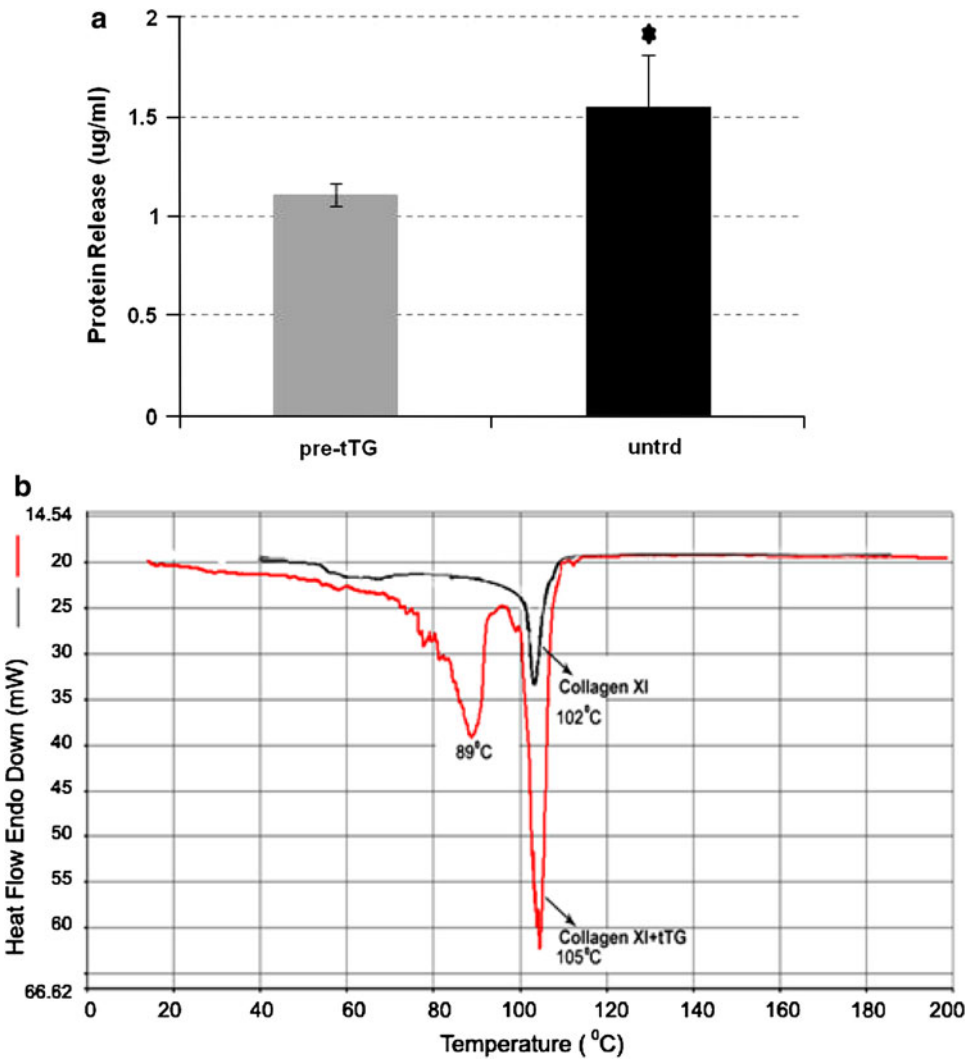

Fig. 3 tTG-mediated collagen type XI crosslinking increases resistance to collagenolysis (**a**) and thermal denaturation (**b**). (**a**) Protein release to the liquid phase from collagen type XI films treated with collagenase measured with the BCAS protein assay (**b**), endothermic heat flow profile; temperature shown on the horizontal axis in °C. pre-tTG, collagen type XI films pre-treated with tTG prior to cell seeding. $*p < 0.05$, $n = 3$

was cross-linked into the matrix through its terminal amino group and thus identified the acceptor regions in the collagen substrate susceptible for transamidating the reaction. Auto-crosslinking of tTG into the same region of collagen molecule supports the notion on spatial restriction of the tTG-accessible areas in polymerized collagen. Another plausible explanation is that tTG tethered to the film is more likely to modify the same or closely adjacent area owing to the physical proximity constraints.

To further confirm tTG crosslinking to collagen, tTG-treated collagen films were analyzed by gel electrophoresis and western blot. Following tTG treatment, collagen type XI films were vigorously washed with PBS to remove unbound tTG and boiled in the reducing denaturing buffer. Western blots detected tTG monomer at 78 kDa (Fig. 4b). Additionally, in the tTG-treated collagen type XI sample we detected a new tTG-positive protein band with a molecular weight of approximately 270 kDa (Fig. 4b, lanes 2 and 3) which was absent in the control tTG sample incubated without collagen (lane 4) or in the control untreated collagen (lane 1). We propose that this high molecular weight band represents a cross-linked complex of tTG with the 100–150 kDa alpha chains of collagen XI, although a portion of it may also represent the auto-catalyzed polymerization of tTG described previously in the reaction mixture containing DTT and 5 mM Ca^{2+} (Birckbichler et al. 1977). However, this high molecular weight band is barely detected in tTG incubated without collagen XI (Fig. 4b, lane 4), supporting the perception that collagen XI molecules are included into the 250 kDa complex.

Discussion

Human MSC (in particular, the bone marrow-derived MSC) are an excellent cell source for tissue engineering because they are readily available, exhibit no immunogenicity if used in autologous implant applications, and

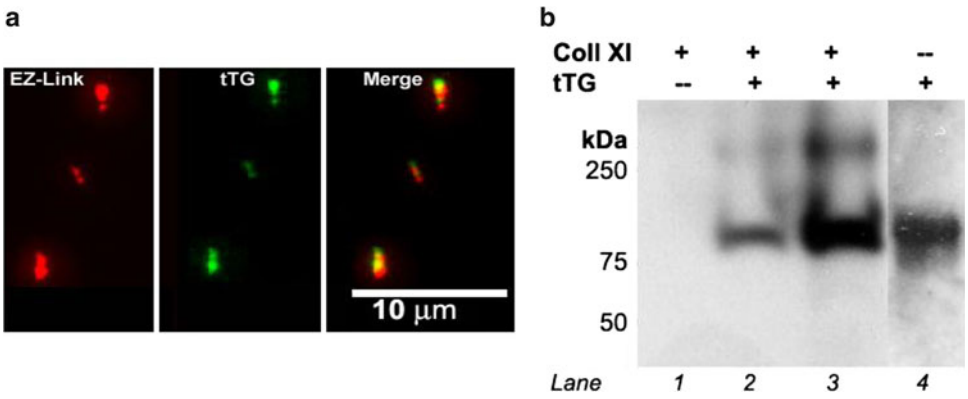

Fig. 4 Cross-linking of tTG to collagen type XI. (**a**) Incorporation of tTG protein (*green*) and of the biotinylated pentylamine substrate for tTG (*red*) was visualized by double immunofluorescence. Right panel–merged image (*yellow*). Magnification: ×200, *Scale bar* 10 μm (b) western blot with tTG antibody. *Lane 1* coll XI, *lane 2*coll XI + 0.01U tTG, *lane3* coll XI + 0.1 U tTG, *lane 4* 0.01U tTG

possess the ability to differentiate into chondrocytes. Initial attachment of MSC to the scaffolds is the key early event that directs cell differentiation and cartilage formation. Previous studies have demonstrated that treatment of collagen matrices with tissue transglutaminase (tTG) increases adhesion of diverse cell types including fibroblasts, endothelial cells, vascular smooth muscle cells and osteoblasts (Jones et al. 1997; Verderio et al. 2001; Chau et al. 2005; Garcia et al. 2009; Spurlin et al. 2009; Ciardelli et al. 2010). Previous publications supported a model in which elevated levels of overexpressed cell-surface tTG mediate increased cell adhesion through interaction of tTG with integrins and diverse matrix proteins (Akimov et al. 2000; Aeschlimann and Thomazy 2000). However, even though a positive effect of tTG overexpression on integrin-mediated MSC survival has been reported (Song et al. 2007), regulation by exogenous tTG of cell behavior of the genetically unaltered MSC on tissue-specific matrices remains underinvestigated. At the same time, purified tTG is readily available and has already been introduced into the bioengineering arsenal (Jürgensen et al. 1997). The notable findings of our study are that tTG-induced modifications of the cartilage-specific collagen type XI scaffolds, introduced either by pre-treatment of the scaffolds with tTG prior to cell seeding or by tTG continuously present in the growth medium, promote MSC attachment and supports round chondrocytic morphology on collagen type XI. Incorporating tTG into the matrix bypasses the need for genetic manipulations with MSCs, such as forced expression of the elevated tTG levels (Verderio et al. 2001; Song et al. 2007), and offers substantial technological and procedural advantages in MSC-based bioengineering.

To our knowledge, this study is the first direct demonstration of tTG incorporation into collagen type XI films. Several collagens have been identified as tTG substrates, mostly based on the in vitro incorporation of [^{3}H]-putrescine. The potential [^{3}H] putrescine-binding glutamine sites have been identified in aminopropeptide of type III collagen, the non-triple-helical telopeptides of *a*1(V) and *a*1(XI) chains, and the N-terminal noncollagenous domain of *a*1(XVI) chain (Bowness et al. 1989; Kleman et al. 1995; Akagi et al. 2002). However, no partnering lysine residues for natural cross-link formation have been defined in collagen molecules. Therefore, in addition to the established ability of tTG to act as both amine donor and anime acceptor substrate in an auto-crosslinking reaction (Birckbichler et al. 1977), it is feasible to suggest that tTG can serve as a lysine-donor in cross-linking reactions with collagen type XI. The very close proximity of the sites incorporating two independent lysine donors—pentylamine and tTG—in the collagen type XI scaffold is consistent with clustering of the glutamine residues in the N- and C-telopeptides in alpha1(XI) chain (Kleman et al. 1995). Auto-crosslinking of tTG to collagen type XI is a novel finding of the present investigation, although not entirely unexpected in light of the earlier reports demonstrating that tTG cross-links itself to other substrates including beta2-macroglobulin (Fésüs et al. 1981) and fibronectin (Barsigian et al. 1991). Accounting for possible auto-cross-linking of tTG to various collagen-based bioengineered scaffolds treated with this enzyme to increase their mechanical stability (Orban et al. 2004; Chau et al. 2005; Garcia et al. 2009; Spurlin et al. 2009; Ciardelli et al. 2010) is important to foresee the potential effects of the retained tTG on cell behavior and lineage commitment.

We demonstrate that tTG-treated collagen type XI used as a coating for the 3D PLLA nanofibrous scaffolds enhances cell attachment and supports chondrocytic cell morphology. A plausible model accounts for the tTG-integrin interactions (Isobe et al. 1999; Akimov et al. 2000), which may regulate both the cell adhesion and cell morphology through integrin association with cytoskeleton. This results in increased deposition of the cartilaginous matrix on the tTG-treated collagen type XI scaffolds, further confirming the interdependence of cytoskeleton, cell shape, and chondrocyte maturation (Nurminsky et al. 2007), and the role of the lineage oriented cell shape in MSC commitment and differentiation (Goldring et al.

2006; McBeath et al. 2004; Gao et al. 2010). However, continuous presence of elevated levels of tTG prevents augmentation of matrix synthesis in differentiating human MSC, similar to its inhibitory effect on the in vitro and in vivo cartilage formation by avian mesenchymal limb bud cells (Nurminsky et al. 2010). Our previous study implicated the PKA-dependent synthesis of xylosyltransferase as a major intracellular target of this phenomenon (Nurminsky et al. 2010). It is reasonable to assume that cell-surface receptors for tTG change in the process of MSC differentiation into chondrocytes, and this change defines the difference in biological effects of exogenous tTG during lineage commitment in stem cell versus maturation in committed chondrogenic cells. The established repertoire of tTG receptors, including the α_{1B} adrenergic receptors (Nakaoka et al. 1994), integrins (Akimov et al. 2000), the atypical G-protein-coupled receptor GPR56 (Xu et al. 2006), the VEGF receptor 2 (Dardik and Inbal 2006), and the LRP5/6 receptors (Faverman et al. 2008), probably determines the wide range of tissue-specific biological activities of this multifunctional protein.

In addition to direct regulation of MSC adhesion and differentiation, the tTG protein cross-linked collagen type XI may change the structure of collagen molecule in the vicinity of bound enzyme and therefore, alter cell binding to collagen. The tTG-induced cross-linking in collagen type XI may affect the cell attachment mediated by integrins, cell surface discoidin domain receptors and CD44 glycoprotein, all of which bind collagens and regulate various intracellular signaling pathways (Shrivastava et al. 1997; Woods et al. 2007). Therefore, tTG may have the potential to regulate chondrogenic differentiation in MSC via diverse signaling conduits. Stability of the cross-linked tTG-collagen type XI complexes has not been addressed in the present study, although it may be a matter of interest in evaluating the stability of the cell-seeded modified scaffolds both in vitro and in vivo. An elaborate examination of longer time-points, gene expression profiles and relevant signaling pathways will be necessary to further elucidate the long-term effects of the tTG-mediated modification of the collagen type XI scaffolds for enhanced chondrogenesis of MSC in vitro. Nevertheless, this study demonstrates the significance of tTG in MSC-based cartilage bioengineering, and contributes to further characterization of the tTG-extracellular matrix interactions.

Acknowledgments We would like to thank Dr. Kimberly Griswold, of Picatinny Arsenal, NJ for DSC measurements. Also, Dr. Michael Jaffe and Dr. George Collins of New Jersey Institute of Technology, NJ for helpful discussions on DSC data. This work was supported by NIH grants R56DK071920 and R03AR057126 and a grant from Maryland Stem Cell Research Fund to M. Nurminskaya.

Conflict of interest The authors declare that they have no conflict of interest.

References

Aeschlimann D, Thomazy V (2000) Protein crosslinking in assembly and remodelling of extracellular matrices: the role of transglutaminases. Connect Tissue Res 41(1):1–27. doi:10.3109/03008200009005638

Akagi A, Tajima S, Ishibashi A, Matsubara Y, Takehana M, Kobayashi S, Yamaguchi N (2002) Type XVI collagen is expressed in factor XIIIa+ monocyte-derived dermal dendrocytes and constitutes a potential substrate for factor XIIIa. J Invest Dermatol 118(2):267–274. doi:10.1046/j.0022-202x.2001.01666.x

Akimov SS, Krylov D, Fleischman LF, Belkin AM (2000) Tissue transglutaminase is an integrin-binding adhesion coreceptor for fibronectin. J Cell Biol. 21;148(4):825-38. doi:0021-9525/2000/02/825/14

Barsigian C, Stern AM, Martinez J (1991) Tissue (type II) transglutaminase covalently incorporates itself, fibrinogen, or fibronectin into high molecular weight complexes on the extracellular surface of isolated hepatocytes. Use of 2-[(2-oxopropyl)thio] imidazolium derivatives as cellular transglutaminase inactivators. J Biol Chem 266(33):22501–22509. Retrieved from http://www.jbc.org/

Birckbichler PJ, Orr GR, Carter HA, Patterson MK Jr (1977) Catalytic formation of epsilon-(gamma-glutamyl)lysine in guinea pig liver transglutaminase. Biochem Biophys Res Commun. 78(1):1–7. Retrieved from http://www.sciencedirect.com/science/journal/0006291X

Blaschke UK, Eikenberry EF, Hulmes DJ, Galla HJ, Bruckner P (2000) Collagen XI nucleates self-assembly and limits lateral growth of cartilage fibrils. J Biol Chem 275(14):10370–10378. doi:10.1074/jbc.275.14.10370

Bowness JM, Tarr AH, Wiebe RI (1989) Transglutaminase-catalysed cross-linking: a potential mechanism for the interaction of fibrinogen, low density lipoprotein and arterial type III procollagen. Thromb Res 54:357–367. doi:10.1016/0049-3848(89)90094-7

Cervellati C, Franzoni L, Squerzanti M, Bergamini CM, Spinozzi F, Mariani P, Lanzara V, Spisni A (2009) Unfolding studies of tissue transglutaminase. Amino Acids 36(4):633–641. doi:10.1007/s00726-008-0161-3

Chau DY, Collighan RJ, Verderio EA, Addy VL, Griffin M (2005) The cellular response to transglutaminase-cross-linked collagen. Biomaterials 26(33):6518–6529. doi:10.1016/j.biomaterials.2005.04.017

Christopher AM, Bailey AJ (1999) Thermal denaturation of collagen revisited. Proc Indian Acad Sci (Chem Sci) 111:71–80. doi:10.1007/BF02869897

Ciardelli G, Gentile P, Chiono V, Mattioli-Belmonte M, Vozzi G, Barbani N, Giusti P (2010) Enzymatically crosslinked porous composite matrices for bone tissue regeneration. J Biomed Mater Res A 92(1):137–151. doi:10.1002/jbm.a.32344

Dardik R, Inbal A (2006) Complex formation between tissue transglutaminase II (tTG) and vascular endothelial growth factor receptor 2 (VEGFR-2): proposed mechanism for modulation of endothelial cell response to VEGF. Exp Cell Res 312(16):2973–2982. doi:10.1016/j.yexcr.2006.05.019

Eyre DR, Weis MA, Wu JJ (2006) Articular cartilage collagen: an irreplaceable framework? Eur Cell Mater 12:57–63. Retrieved from http://www.ecmjournal.org/

Faverman L, Mikhaylova L, Malmquist J, Nurminskaya M (2008) Extracellular transglutaminase 2 activates beta-catenin signaling in calcifying vascular smooth muscle cells. FEBS Lett 582(10):1552–1557. doi:10.1016/j.febslet.2008.03.053

Fésüs L, Falus A, Erdei A, Laki K (1981) Human beta 2-microglobulin is a substrate of tissue transglutaminase: polymerization in solution and on the cell surface. J Cell Biol 89(3):706–710. doi:0021-9525/81/06/0706/05

Gao L, McBeath R, Chen CS (2010) Stem cell shape regulates a chondrogenic versus myogenic fate through Rac1 and N-cadherin. Stem Cells 28(3):564–572. doi:10.1002/stem.308

Garcia Y, Hemantkumar N, Collighan R, Griffin M, Rodriguez-Cabello JC, Pandit A (2009) In vitro characterization of a collagen scaffold enzymatically cross-linked with a tailored elastin-like polymer. Tissue Eng Part A. (4):887-99. doi:10.1089/ten.tea.2008.0104

Goldring MB, Tsuchimochi K, Ijiri K (2006) The control of chondrogenesis. J Cell Biochem 97(1):33–44. doi:10.1002/jcb.20652

Harris ED Jr, Farrell ME (1972) Resistance to collagenase: a characteristic of collagen fibrils cross-linked by formaldehyde. Biochim Biophys Acta. 1972 Aug 31;278(1):133–141. doi:10.1016/0005-2795(72)90114-6

Isobe T, Takahashi H, Ueki S, Takagi J, Saito Y (1999) Activity-independent cell adhesion to tissue-type transglutaminase is mediated by alpha4beta1 integrin. Eur J Cell Biol. 78(12):876-883. Retrieved from http://www.sciencedirect.com/science/journal/01719335

Jeleńska MM, Fesüs L, Kopeć M (1980) The comparative ability of plasma and tissue transglutaminases to use collagen as a substrate. Biochim Biophys Acta 616(2):167–178. doi:10.1016/0005-2744(80)90135-7

Jones RA, Nicholas B, Mian S, Davies PJ, Griffin M (1997) Reduced expression of tissue transglutaminase in a human endothelial cell line leads to changes in cell spreading, cell adhesion and reduced polymerisation of fibronectin. J Cell Sci. 110(Pt 19):2461–2472. Retrieved from http://jcs.biologists.org/

Jürgensen K, Aeschlimann D, Cavin V, Genge M, Hunziker EB (1997) A new biological glue for cartilage–cartilage interfaces: tissue transglutaminase. J Bone Joint Surg Am 79(2):185–193. Retrieved from http://www.jbjs.org/

Kleman JP, Aeschlimann D, Paulsson M, Van der Rest M (1995) Transglutaminase-catalyzed cross-linking of fibrils of collagen V/XI in A204 rhabdomyosarcoma cells. Biochemistry 34(42):13768–13775. doi:10.1021/bi00042a007

Lorand L, Graham RM (2003) Transglutaminases: crosslinking enzymes with pleiotropic functions. Nat Rev Mol Cell Biol 4(2):140–156. doi:10.1038/nrm1014

McBeath R, Pirone DM, Nelson CM, Bhadriraju K, Chen CS (2004) Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. Dev Cell 6(4):483–495. doi:10.1016/S1534-5807(04)00075-9

Nakaoka H, Perez DM, Baek KJ, Das T, Husain A, Misono K, Im MJ, Graham RM (1994) Gh: a GTP-binding protein with transglutaminase activity and receptor signaling function. Science. 264(5165):1593–1596. doi:10.1126/science.7911253

Nurminsky D, Magee C, Faverman L, Nurminskaya M (2007) Regulation of chondrocyte differentiation by actin-severing protein adseverin. Dev Biol 302(2):427–437. doi:10.1016/j.ydbio.2006.09.052

Nurminsky D, Shanmugasundaram S, Deasey S, Michaud C, Allen S, Hendig D, Dastjerdi A, Francis-West P, Nurminskaya M (2010) Transglutaminase 2 regulates early chondrogenesis and glycosaminoglycan synthesis. Mech Dev doi:10.1016/j.mod.2010.11.007

Orban JM, Wilson LB, Kofroth JA, El-Kurdi MS, Maul TM, Vorp DA (2004) Crosslinking of collagen gels by transglutaminase. J Biomed Mater Res A 68(4):756–762. doi:10.1002/jbm.a.20110

Pelttari K, Wixmerten A, Martin I (2009) Do we really need cartilage tissue engineering? Swiss Med Wkly 139(41–42):602–609

Rimoin D, Francomano C, Giedion A (1998) International nomenclature and classification of the osteochondrodysplasias: international working group on constitutional diseases of bone. Am J Med Genet 79(5):376–382. doi:10.1002/(SICI)1096-8628(19981012)79:5<376:AID-AJMG9>3.0.CO;2-H

Shanmugasundaram S, Chaudhry H, Arinzeh TL (2011) Microscale versus nanoscale scaffold architecture for mesenchymal stem cell chondrogenesis. Tissue Eng Part A 17(5–6):831–840. doi:10.1089/ten.tea.2010.0409

Shrivastava A, Radziejewski C, Campbell E, Kovac L, McGlynn M, Ryan TE, Davis S, Goldfarb MP, Glass DJ, Lemke G, Yancopoulos GD (1997) An orphan receptor tyrosine kinase family whose members serve as non-integrin collagen receptors. Mol Cell 1:25–34. doi:10.1016/S1097-2765(00)80004-0

Song H, Chang W, Lim S, Seo HS, Shim CY, Park S, Yoo KJ, Kim BS, Min BH, Lee H, Jang Y, Chung N, Hwang KC (2007) Tissue transglutaminase is essential for integrin-mediated survival of bone marrow-derived mesenchymal stem cells. Stem Cells 25(6):1431–1438. doi:10.1634/stemcells.2006-0467

Spurlin TA, Bhadriraju K, Chung KH, Tona A, Plant AL (2009) The treatment of collagen fibrils by tissue transglutaminase to promote vascular smooth muscle cell contractile signaling. Biomaterials 30(29):5486–5496. doi:10.1016/j.biomaterials.2009.07.014

Telci D, Griffin M (2006) Tissue transglutaminase (TG2)-a wound response enzyme. Front Biosci. 11:867-82. doi: http://dx.doi.org/10.2741/1843

Verderio E, Coombes A, Jones RA, Li X, Heath D, Downes S, Griffin M (2001) Role of the cross-linking enzyme tissue transglutaminase in the biological recognition of synthetic biodegradable polymers. J Biomed Mater Res 54(2):294–304. doi:10.1002/1097-4636(200102)54:2<294:AID-JBM17>3.0.CO;2-Q

Woods A, Wang G, Beier F (2007) Regulation of chondrocyte differentiation by the actin cytoskeleton and adhesive interactions. J Cell Physiol 213(1):1–8. doi:10.1002/jcp.21110

Xu L, Begum S, Hearn JD, Hynes RO (2006) GPR56, an atypical G protein-coupled receptor, binds tissue transglutaminase, TG2, and inhibits melanoma tumor growth and metastasis. Proc Natl Acad Sci USA 103(24):9023–9028. doi:10.1073/pnas.0602681103

Xu J, Wang W, Ludeman M, Cheng K, Hayami T, Lotz JC, Kapila S (2008) Chondrogenic differentiation of human mesenchymal stem cells in 3D alginate gels. Tissue Eng Part A 14(5):667–680. doi:10.1089/tea.2007.0272