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Tissue Transglutaminase Regulates Chondrogenesis in Mesenchymal Stem Cells on Collagen Type XI Matrices

Shobana Shanmugasundaram, PhD*,

Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, Baltimore, MD 21201

Sheila Logan-Mauney, PhD*,

Department of Anatomy and Cell Biology, Tufts University School of Medicine, Boston, MA 02111

Kaitlin Burgos, and

Department of Biology, Towson University, Towson, MD 21252

Maria Nurminskaya, PhD

Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, Baltimore, MD 21201, mnurminskaya@som.umaryland.edu, Phone: 410-706-7469, Fax: 410-706- 8297

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. 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 auto-crosslinks 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

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

Correspondence to: Maria Nurminskaya.

*these authors contributed equally

Conflict of interest

The authors declare that they have no conflict of interest

regenerate the tissue. Tissue engineering strategies utilizing human mesenchymal stem cells (MSC) are expected to solve this problem and are rapidly developing over the last decades, 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 L 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 scaffold is cross-linking, for example by the calcium dependent enzymes transglutaminases which catalyze formation of the ϵ -(γ -glutaminy)-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 towards 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 the 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 fibril 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 Otopospondylomegalapiphyseal 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 modifications introduced by tTG in collagen type XI regulate chondrogenic differentiation in human bone marrow-derived mesenchymal stem cells (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 0.5mg/ml in 0.2% glacial acetic acid (Sigma-Aldrich, MO) overnight at 4° C. Two dimensional (2D) films were made by drying 0.1mg/cm² of collagen type XI in tissue culture plates for 48 hours. Three dimensional (3D) scaffolds of poly (L-lactide) (PLLA) (Sigma-Aldrich, MO) were fabricated with an average fiber diameter of 0.29±0.08µm by electrospinning as described previously (Shanmugasundaram et al. 2011). Collagen type XI was immobilized on the 3D scaffolds by drying 0.1mg/cm² 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 cell seeding overnight at 37° C in 1.8 mM Ca²⁺ Dulbecco's modified eagle medium (DMEM), pH7.4, 5% CO₂.

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 denaturation temperature is the temperature at the maximum of the peak.

Collagenase Assay

Collagen type XI films were washed with PBS and treated with 200 µl of 0.01% collagenase from *Clostridium histolyticum*, (≥ 125 CDU/mg; Sigma-Aldrich, MO) in PBS for 3 hours at 37° C, pH 7.5. The solution was removed after treatment, and films were incubated with 200 µl of 2mM EDTA for 30 minutes at 37° C. The collagenase and EDTA solutions were mixed and quantified for protein using the BCA protein assay kit (Thermo Scientific, IL) using BSA as standard. Three independent experiments were performed with 3 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 in a gradient 4–20% SDS-PAGE 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 1:1000 dilution overnight followed by an hour in secondary rabbit anti-goat HRP conjugated antibody (Sigma-Aldrich, MO) at 1:5000 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 Dulbecco's modified eagle medium (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 $10^5/\text{cm}^2$, and maintained in DMEM-high glucose supplemented with 10^{-7} mM dexamethasone (Sigma-Aldrich, MO), 0.1mM ascorbic acid (Wako Chemicals, VA), 1% ITS premix (BD Biosciences, NJ), 1mM sodium pyruvate (Sigma-Aldrich, MO), 0.35mM L-proline (Sigma-Aldrich, MO), 4mM L-Glutamine (Invitrogen, CA) and 1% Pencillin –Streptomycin (Invitrogen, CA) supplemented with the 10 ng/mL TGF- β 3 (ProSpec, NJ) at 37° C, 5% CO₂. 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 staining with 1% Alcian blue (8GX) in 0.1N HCl in samples fixed with 4% PFA. For quantitative analysis, Alcian blue dye bound to the scaffolds was extracted with 4M guanidine hydrochloride and absorbance was measured in a spectrophotometer at 590nm. Cell number was estimated with crystal violet staining (Sigma-Aldrich, MO).

Cell Attachment Assay and Cell Spreading Analysis

Attached MSC on collagen type XI films at 1, 3 and 5 hours 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 each timepoint. 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 hour 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 0.1N HCl to visualize matrix synthesis and subsequently stained with propidium iodide to visualize cells.

Results

1. 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 substrates (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, which approximates the average 0.08U/ml serum levels of transglutaminase activity (D'Argenio et al. 1990).

First, we observed a higher number of MSC attaching to collagen type XI films either pre-treated overnight with 0.1U/ml tTG (pre-tTG) or cultured in the continuous presence of 0.1U/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 calceinAM vital staining revealed a dramatic 6-fold increase in the proportion of round cells versus spreading cell on the pre-tTG collagen type XI films, and a 3-fold increase on scaffolds to which tTG was added at the time of cell seeding (Fig. 1b). Thus, after 5 hours, 80% of MSC on films pre-treated with tTG and 42% cells cultured in the continuous presence of tTG retain round morphology compared to 15% of round cells on untreated collagen type XI scaffolds. We also noticed that with time, number of cell aggregates was 2-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 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 tTG-modified versus untreated 3D collagen type XI scaffolds.

2. 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 3-fold increase in matrix synthesis was detected in cells differentiating on the PLLA-coll XI scaffolds pretreated with tTG before cell seeding 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 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 demonstrating the inhibitory effects of elevated tTG levels on chondrogenesis in avian limb bud mesenchymal cells and in the developing limbs *in ovo* (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.

3. 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 shift 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°C 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 Ca²⁺-activated forms (Cervellati et al. 2009), indicating that auto-cross-linking activity of tTG (Birkbincher 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 cross-linking 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.

4. tTG auto-cross-links to collagen type XI

Collagen type XI films treated overnight with tTG and biotinylated pentylamine, and washed vigorously with PBS to remove the unbound compounds, were 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 is cross-linked into the matrix through its terminal amino group and thus identifies the acceptor regions in the collagen substrate susceptible for transamidating reaction. Auto-cross-linking of tTG into the same region of collagen molecule supports a 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 78kDa (Fig. 4b). In addition, in the tTG-treated collagen type XI sample we detect a new tTG-positive protein band with molecular weight of approximately 270kDa (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 5mM Ca²⁺ (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 mesenchymal stem cells (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 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 2004). 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 under-investigated. At the same time, purified tTG is readily available and has been already introduced into the bioengineering arsenal (Jürgensen et al. 1997; Mehta et al. 2006). 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 the cell seeding or by tTG continuously present

in the growth medium, promote MSC attachment and supports chondrocytic round 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 [³H]-putrescine. The potential [³H]putrescine-binding glutamine sites have been identified in aminopropeptide of type III collagen, the non-triple-helical telopeptides of $\alpha 1(V)$ and $\alpha 1(XI)$ chains, and the N-terminal noncollagenous domain of $\alpha 1(XVI)$ 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 amine acceptor substrate in an auto-crosslinking reaction (Brickbichler et al. 1977), it is feasible to suggest that tTG can serve as a lysine-donor in cross-linking reaction 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 $\alpha 1(XI)$ chain (Kleman et al. 1995). Auto-cross-linking 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 (Fesus 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 cell adhesion and cell morphology through integrins association with cytoskeleton. These result 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 2010). However, continuous presence of the elevated tTG prevents augmentation of matrix synthesis in differentiating human MSC, similar to its inhibitory effect on the *in vitro* and *in ovo* cartilage formation by avian mesenchymal limb bud cells (Nurminsky et al. 2010). Our previous study has 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 α_{1B} adrenergic receptors (Nakaoka et al. 1994), integrins (Akimov et al. 2000), the atypical G-protein-coupled receptor GPR56 (Xu et al. 2006), VEGF receptor 2 (Dardik and Inbal 2006), and 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, tTG cross-linked to collagen type XI may change the structure of the collagen in vicinity of the bound enzyme

and therefore, alter cell binding to collagen molecules. The tTG-induced cross-linking in collagen type XI may affect 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 regulate chondrogenic differentiation in MSC via siverse 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 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 tTG-mediated modification of 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.

Abbreviation list

BSA	Bovine serum albumin
Ca²⁺	Calcium
CO₂	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

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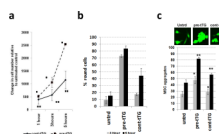


Fig. 1. MSC seeded on collagen type XI films either pre-treated (pre-tTG) or continuously treated (cont-tTG) with 0.1U/ml of tTG were counted and analyzed for morphology at 3 and 5 hours post-seeding. When compared to untreated collagen type XI films, they exhibited (a) increased cell adhesion, (b) higher 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: 200X, Scale bar: 10 μm

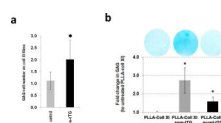
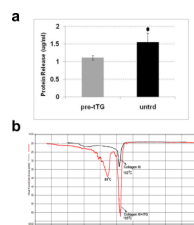


Fig. 2. Chondrogenic differentiation of MSC was enhanced on (a) 2D collagen type XI films, and (b) 3D PLLA scaffolds coated with collagen type XI that were pre-treated with tTG (pre-tTG) or cultured in the continuous presence of tTG (cont-tTG). GAG synthesis was detected with Alcian Blue staining (b, 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: 100X, Scale bar: 10 μ m

**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 BCS 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. * $p < 0.05$, $n = 3$

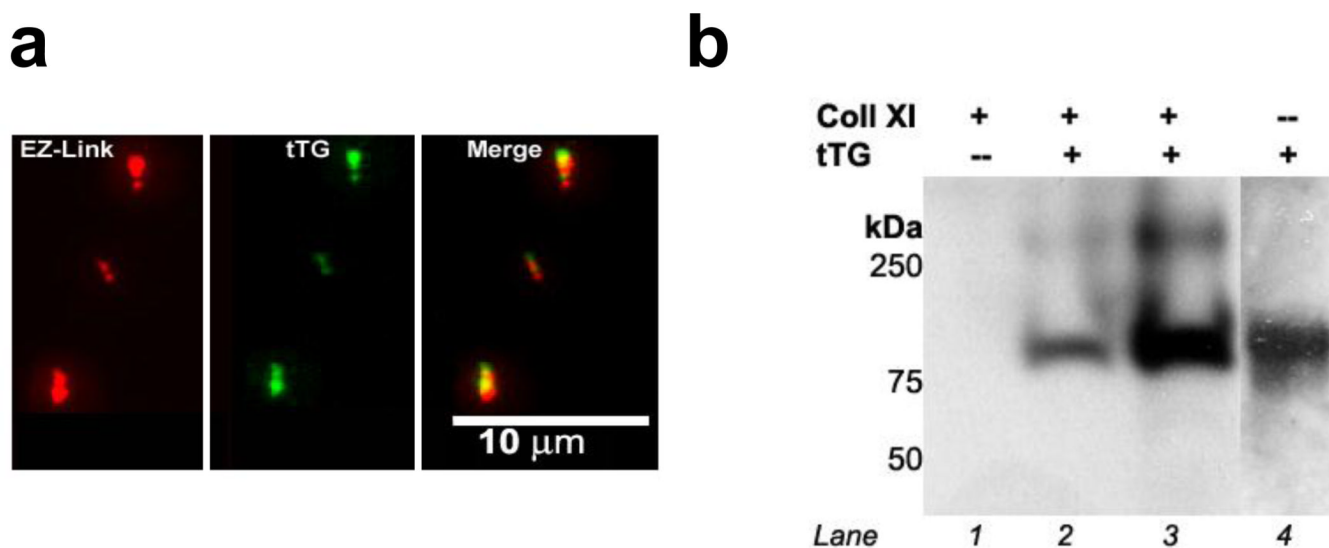


Fig. 4. Cross-linking of tTG to collagen type XI. (a) Incorporation of tTG protein (green) and of the biotinylated pentylamine substrate for tTG (red) was visualized by double immunofluorescence. Right panel - merged image (yellow). Magnification: 200X, Scale bar: 10 μ m (b) western blot with tTG antibody.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