Amino Acids (2013) 44:209–214
DOI 10.1007/s00726-011-1131-8

ORIGINAL ARTICLE

Transglutaminase 2 and Factor XIII catalyze distinct substrates in differentiating osteoblastic cell line: utility of highly reactive substrate peptides

Kazuya Watanabe · Kanako Tsunoda · Miho Itoh · Mina Fukui · Hitoshi Mori · Kiyotaka Hitomi

Received: 30 May 2011 / Accepted: 20 October 2011 / Published online: 2 November 2011

Abstract Differentiated osteoblastic cell line, MC3T3-E1 expresses transglutaminase 2 (TG2) and Factor XIII (FXIII). In previous studies, we identified isozyme-specific and highly reactive glutamine-donor substrate peptides (pepF11KA and pepT26) for each isozyme. Using these peptides, we compared the reaction products with lysine-donor substrates for each isozyme in differentiating MC3T3-E1 cells. By this analysis, distinct substrates for the activated TG2 and FXIII were detected in cultured cellular extract. Possible substrates that incorporated biotin-labeled peptides were further purified using streptavidin-affinity chromatography. Several isozyme-specific substrates were identified by mass spectrometry analysis of the purified fractions. These analyses also indicate the benefit of the substrate peptides for obtaining distinct substrates in a reaction mixture where two isozymes co-exist.

Keywords Transglutaminase · Osteoblast · TG2 · Factor XIII

Abbreviations

Asc-P	L-ascorbic acid 2-phosphate
CBB	Coomassie Brilliant Blue
DTT	Dithiothreitol
TG2	Transglutaminase 2
FXIII	Factor XIII

K. Watanabe · K. Tsunoda · M. Itoh · M. Fukui · K. Hitomi (✉)
Department of Applied Molecular Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya 4648601, Japan
e-mail: hitomi@agr.nagoya-u.ac.jp

H. Mori
Department of Bioengineering Sciences, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 4648601, Japan

Introduction

Transglutaminase (TGase; E.C. 2.3.2.13) is a family of enzymes that catalyze cross-linking reaction between two proteins through glutamine and lysine residues in a calcium ion-dependent manner (Griffin et al. 2002; Lorand and Graham 2003; Beninati and Piacentini 2004). In addition, by the catalytic reaction, glutamine residue is replaced to glutamic acid residue by deamidation or modified by the attachment of a primary amine. In humans, eight isozymes have been identified: TG1–TG7 and Factor XIII (FXIII). Among these, both TG2 and FXIII have been extensively investigated in several cells and tissues. FXIII, circulating in plasma, is essential for cross-linking fibrin for blood coagulation, but this isozyme is also actively expressed as an intracellular protein in macrophages and osteoblasts (Nakano et al. 2007). TG2 is ubiquitously expressed as multifunctional proteins and hence, it has been implicated in several physiological roles (Chen and Mehta 1999; Fesus and Piacentini 2002).

Over the years, osteoblasts and chondrocytes have been shown to express both TG2 and FXIII (Nurminskaya and Kaartinen 2006; Johnson et al. 2008). Investigations have been performed to elucidate the significance of TGases in bone formation (Kaartinen et al. 2002; Forsprecher et al. 2009). MC3T3-E1, a mouse calvaria-derived cell line, is a useful in vitro model system for studying the process of osteoblastic differentiation, because this cell line displays a temporal sequence of development similar to in vivo bone formation (Quarles et al. 1992). FXIII and TG2 have been expressed in differentiating MC3T3-E1 cells, where FXIII

is induced depending on differentiation and TG2 is constitutively expressed (Al-Jallad et al. 2006). In this cell model, the enzymatic activities of both isozymes contribute to the stabilization of extracellular matrix at the secreted form and also to mineralization. Although a couple of extracellular matrix proteins are known to act as substrate proteins (Kaartinen et al. 2002), the intracellular substrates for TGases cross-linking activity are under investigation.

Generally, to investigate substrate proteins for TGases, chemically modified primary amines are used for the identification of the glutamine-donor substrate proteins. For example, the amounts of substrates were determined using biotin-labeled cadaverine and based on their affinity for avidin-conjugated molecules (Esposito and Caputo 2005). However, in this case, the glutamine-donor substrate can only be obtained without the restriction of isozyme specificities. Over years of research, we have obtained isozyme-specific glutamine-donor substrate sequences from a phage-displayed random peptide library (Sugimura et al. 2006): Peptide sequences for F11KA (DQMML PWPAVAL) and T26 (HQSYVDPWMLDH) have been identified for FXIII and TG2, respectively. These synthetic peptides are useful tools for isozyme-specific research involving in microtiter plate assays, immobilization of functional proteins, and the detection of in situ activity of tissue sections (Perez-Alea et al. 2009; Hitomi et al. 2009a, Itoh et al. 2011). In this study, taking advantage of the high reactivities and specificities, we attempted to identify possible distinct substrates for FXIII and TG2 in differentiating MC3T3-E1 cells.

Experimental procedures

Materials

Biochemical reagents used in the experiments were from Sigma (Sigma-Aldrich, MO, USA), Promega (Madison, WI, USA), and Wako Chemicals (Osaka, Japan). The MC3T3-E1 cells were obtained from RIKEN BRC Cell Bank (Tsukuba, Ibaragi, Japan). Monoclonal antibodies against factor XIII (AC-1A1) and TG2 (Cub7402) were purchased from Thermo Fisher Scientific (Fremont, CA, USA) and Quartett (Berlin, Germany), respectively. Recombinant human TG2 was obtained from Zedira (Darmstadt, Germany). Fibrogaminn, as a zymogen form of FXIII, was purchased from CSL Behring (Tokyo, Japan). Streptavidin-coated Sepharose gels were from GE Healthcare (Tokyo, Japan). Biotinylated peptides (pepF11KA; DQMMLPWPAVAL, pepF11KAQN; DNM MLPWPAVAL, pepT26; HQSYVDPWMLDH, pepT26 QN; HNSYVDPWMLDH) were synthesized by Bio-synthesis (Lewisville, TX, USA).

Characterization of the expressed TGases in differentiating osteoblastic cell line

The MC3T3-E1 cells were cultured in DMEM containing 10% fetal bovine serum and antibiotics. Induction of differentiation of the subcultured cells, we initiated by the addition of L-ascorbic acid 2-phosphate (Asc-P) (2 mM) and β-glycerophosphate (10 mM).

The cultured MC3T3-E1 cells were lysed with lysis buffer (10 mM Tris–HCl, pH 8.0, 0.5 mM β-mercaptoethanol, 0.5 mM EDTA, 0.1% Triton-X100, and protease inhibitors; pepstatin and E-64) and sonication. NaCl was added to be isotonic in solution (150 mM) in order to stabilize proteins and then centrifuged. The soluble fraction was used for immunoblotting by a standard method. Protein concentration of each extract was determined by Bradford method (Bio-Rad).

Biotin-incorporation by endogenous activities of TGs in the cellular extract and analysis of the possible substrate proteins

Subcultured MC3T3-E1 cells were treated for differentiation by the addition of Asc-P and β-glycerophosphate. On days 12 after induction, cells were harvested and lysed with lysis buffer as described above.

To carry out the TGase cross-linking reactions by endogenous enzymatic activities, the cellular extract was incubated with 5 mM CaCl2 and 1 mM dithiothreitol (DTT), in the presence of 10 μM biotinylated-peptide (pepF11KA or pepT26) or 1 mM biotin-cadaverine. In the case of cross-linking reaction using pepF11KA, thrombin was simultaneously added to enhance the FXIII enzyme activity. After incubation of the mixture at 37°C for 40 min, the extract was subjected to analyses or purification.

The small aliquot of the reacted cellular extract was mixed with SDS-sample buffer and subjected to 5–20% gradient SDS-PAGE. In the case of two-dimensional electrophoresis analysis, the part of the reaction mixture was mixed with ninefold volume of rehydration buffer (6 M Urea, 2 M thiourea, 2% CHAPS, 50 mM DTT, and 0.2% Bio-lyte). Two-dimensional electrophoresis was performed according to the standard method (Bio-Rad IEF system, Bio-Rad).

Affinity purification of the possible substrates

The cellular extract after the enzymatic reaction was applied to the streptavidin-Sepharose gel. The gel was extensively washed by washing buffer (10 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM β-mercaptoethanol) and then mixed with SDS-containing sample buffer. The gel-containing solution was separated from the column and

heated for 2 min. After centrifugation, the supernatant separated from the gel was harvested for further analysis. For mass spectrometric analysis, the gels were stained by Coomassie Brilliant Blue (CBB).

Mass spectrometry analysis

The purified cross-linked products were subjected to gradient SDS-PAGE (5–20%) following CBB staining. The proteins in the excised gel were digested by trypsin in the presence of 0.01% Max surfactant (Promega) at 50°C for 1 h. Then, the trypsinized peptides were fractionated by a Dina nanoHPLC system with a reverse-phase chromatography using C18 column (KYA Technologies, Tokyo, Japan). Each fraction was directly spotted on MALDI plate with a-cyano-4-hydroxycinnamic acid.

MALDI-TOF mass spectrometry was carried out on a 4700 Proteomics Analyzer. MS and MS/MS data for each peptide were analyzed by the MASCOT searching software in the NCBI non-redundant public protein database (http://www.matrixscience.com/search_form_select.html).

Results

MC3T3-E1 is a model system for osteoblastic cells, because the addition of ascorbic acid and β-glycerophosphate to the cultured cells induces differentiation and mineralization. Using this system, the expression patterns of TG2 and FXIII in differentiating MC3T3-E1 were investigated by immunoblotting. After induction using Asc-P instead of ascorbic acid and β-glycerophosphate, an increase in the enzymatic activity of the alkaline phosphatase, a well-known differentiation marker protein, was confirmed in the cellular extract (data not shown).

The cellular extract of differentiating MC3T3-E1 mainly contained intracellular fraction and extracellular matrix. Assayed samples showed TG2 constitutively expressed at the molecular size of 76 kDa (Fig. 1). In contrast, the expression of FXIII was inducible depending on the differentiation: its size was 80 kDa, which corresponds to that as a zymogen form.

Because the differentiating cells cultured on day 12 showed significant levels of both FXIII and TG2, we further analyzed the possible reaction products by the catalytic action of the endogenous enzyme (Fig. 2). Initially, biotin-labeled cadaverine, which reacts with glutamine-donor substrates, was incubated with the cellular extract of differentiated MC3T3-E1. The biotin-incorporated proteins were then detected by avidin-conjugated peroxidase. When compared to the product in the presence of EDTA, as a negative control, 5–9 specific bands were detected as possible glutamine-donor substrates in a calcium-dependent manner.

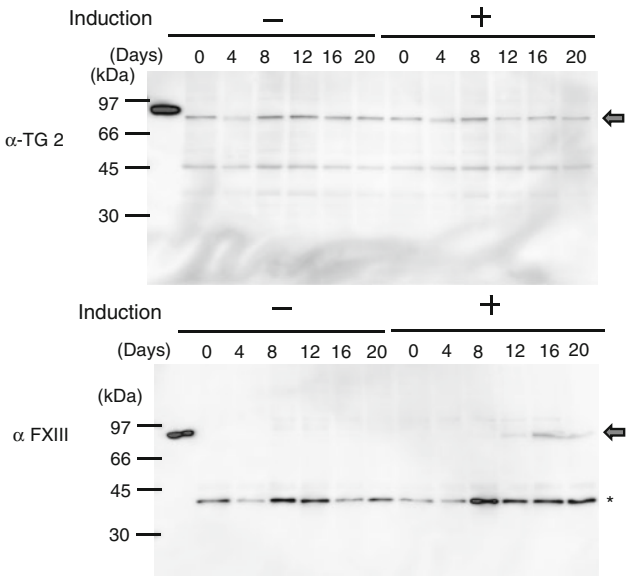

Fig. 1 Analyses of the expression levels for TG2 and FXIII in the differentiating osteoblastic cell line, MC3T3-E1. The cells were split and cultured for the indicated periods in either an Asc-P-free medium (Induction −) or a medium containing Asc-P and glycerophosphate (Induction +). The cellular extract at the indicated days was subjected to immunoblotting using anti-TG2 (upper) and anti-FXIII monoclonal antibodies (*lower*). Proteins for recombinant TG2 and FXIII zymogen were also subjected to each gel (*left*). *Asterisk* indicates non-specific binding

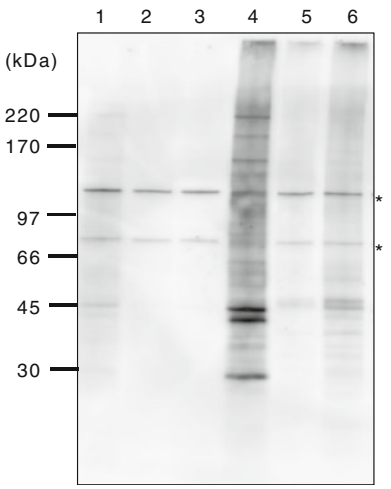

Fig. 2 Incorporation of biotinylated-cadaverine and biotinylated-peptides into the possible substrates in differentiating MC3T3-E1 cellular extract. The cellular extract from differentiating MC3T3-E1 was incubated in the presence of biotin-cadaverine, biotinylated-peptides. Samples were subjected to 5–20% SDS-PAGE following immunoblotting. Then the biotin-incorporated proteins were detected by peroxidase-conjugated streptavidin and chemiluminescent reagent. Reactions were carried out using following substrates. *lane 1* biotin-cadaverine in the presence of EDTA, *lane 2* biotin-pepT26QN mutant, *lane 3* biotin-pepF11KAQN mutant, *lane 4* biotin-cadaverine, *lane 5* biotin-pepT26, *lane 6* biotin-pepF11KA. *Asterisks* indicate the endogenous biotin-conjugated proteins

In the case of biotin-labeled peptides, pepF11KA and pepT26, a couple of protein bands, as glutamine-acceptor substrates, were detected. However, no specific signals were detected for the reaction products with mutant peptides, pepF11KAQN and pepT26QN. In each case, approximately 100 and 70 kDa proteins along with other minor bands reacted non-specifically, because they are possible biotin-conjugating proteins in the cell. Next, in order to further comprehend the difference between the reaction products for pepF11KA and pepT26 in the cellular extracts, we performed a two-dimensional electrophoresis (Fig. 3). In the case of pepF11KA, several proteins reacted as glutamine-donor substrates. In contrast, in the case of pepT26, there was less reaction but significant amounts of cross-linked substrates compared with the reaction products for pepT26QN. These results suggest that pepF11KA and pepT26 reacted distinctly with their respective substrates for FXIII and TG2.

We subsequently attempted to purify these possible substrate proteins from the reaction products using affinity purification. The extract fraction from the differentiated cells were incubated with biotin-peptides in the presence of calcium ion, and then applied to a streptavidin-column for chromatography. The captured proteins that cross-linked to the biotin-labeled substrate peptides were eluted by the addition of an SDS-containing buffer and then subjected to SDS-PAGE. To observe the specific substrate eluates clearly, the same experiments were paralleled using the mutant substrate peptides. The CBB staining demonstrated several protein bands in the eluate samples for pepF11KA and pepT26 (Fig. 4).

Next, we analyzed the purified proteins by mass spectrometry. The obtained bands from the wild type- and the mutant peptide reaction products were excised. The trypsinized peptides were then subjected to the analysis that provides the data for each peptide. After comparison of the peptide pattern, the specific substrate proteins corresponding to the pepF11KA and pepT26 reaction were selected as candidates.

As shown in Fig. 5, for pepF11KA reaction products, several possible proteins were identified, as follows: glucose-regulated protein, ubiquitin carboxyl-terminal hydrolase 47, Ras GTPase-activating-like protein, IQGAP1, glutamate dehydrogenase 1, ATP synthase subunit α and tubulin (α and β). In the case of pepT26 reaction products, distinct proteins were obtained as follows: serpin H1, hsp60, lysozyme C-1, endoplasmin, collagen α-1 chain (type III), and elongation factor 1-α1. A couple of substrate proteins were commonly observed for both enzymes: vimentin, actin, hsp71, hsp90, and β-actin like protein 2. These results suggested that two peptides can be used for the identification of substrates specific to each isozyme even contained in the same cellular extracts.

Discussion

Identification of possible substrates is an initial step in clarifying physiological significance of enzymes. For TGase investigations, several approaches have been attempted to screen and identify substrates such as

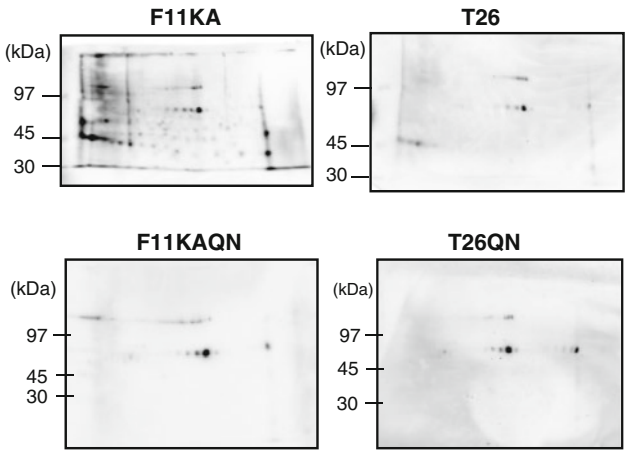

Fig. 3 Two-dimensional electrophoresis of the reaction products with biotinylated peptides. To further compare the distinct patterns of possible substrates by TG2 and FXIII in osteoblast, the samples analyzed as described in the legend to Fig. 2 (pepF11KA, pepF11KAQN, pepT26, and pepT26QN) were subjected to two-dimensional electrophoresis (pH 5–8; from *left* to *right*). The transferred proteins were reacted with peroxidase-conjugated streptavidin for detection of biotin-containing proteins

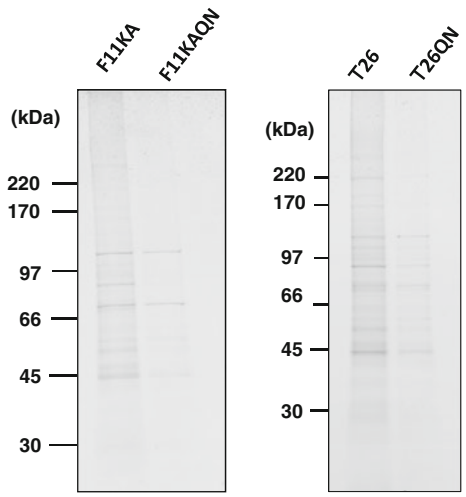

Fig. 4 Affinity purification of possible substrate proteins incorporating biotinylated substrates. Possible glutamine-acceptor substrates incorporating the biotinylated-peptide were affinity purified using streptavidin-Sepharose chromatography from the reaction mixture of the cellular extract and the biotin-peptide (*left* pepF11KA, *right* pepT26). The reactions using mutant peptides were also paralleled. The eluted samples for the reaction mixtures were analyzed by 5–20% SDS-PAGE followed by CBB staining

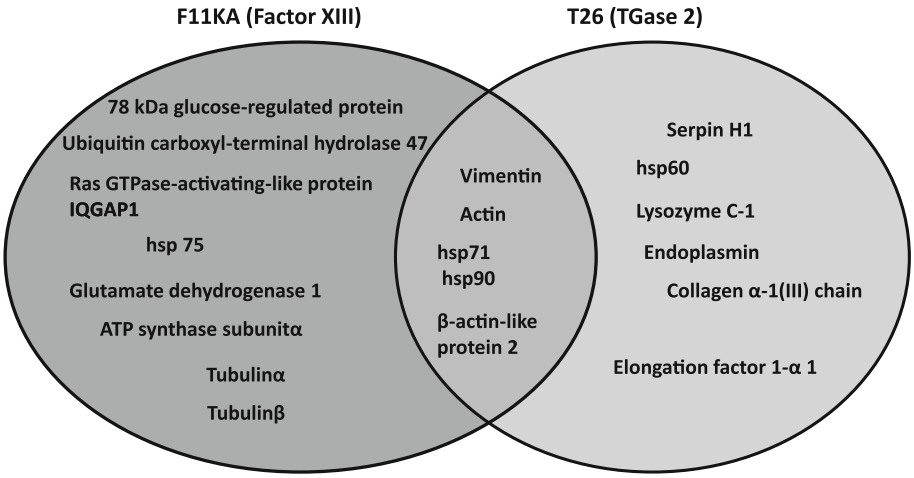

Fig. 5 Candidate substrate proteins in differentiated osteoblast cell extracts. Proteins identified by mass spectrometry were listed for each affinity-purified samples incorporated biotin-pepF11KA (FXIII) or -pepT26 (TG2). Proteins in the *dark* and *light gray circles* indicate possible substrates for FXIII and TG2, respectively. Candidates for both the TGases were described in the *center area*

proteomic analysis for cross-linked products or affinity-purified proteins that incorporated a labeled primary amine (Ruoppolo et al. 2003). With this approach, it is generally difficult to identify substrates specific for each isozyme in TGase family because multiple isozymes are most active in cells and tissues. Additionally, the molecules that have been attached to the lysine-donor substrates are impossible to identify using primary amine as the probe. Therefore, it has been necessary to use the biotin-labeled glutamine-containing peptides, in an isozyme-dependent manner.

Recently, we obtained highly reactive and isozyme-specific glutamine-donor substrate peptides from a random peptide library (Sugimura et al. 2006; Hitomi et al. 2009a). Two peptides, pepF11KA and pepT26, specific for FXIII and TG2 respectively, appeared to be useful for in vitro isozyme-specific assay and for in situ detection of activity in tissue sections (Hitomi et al. 2009b; Itoh et al. 2011). This result led us to attempt for utilizing the peptides to identify cellular target substrates in an isozyme-specific manner.

In this study, we identified several substrates in MC3T3-E1 cells, because this osteoblastic cell line expresses both FXIII and TG2 during differentiation. The substrates and physiological significance of the enzymes in MC3T3-E1 has been investigated, reporting that TGases are involved in the mineralization and matrix formation through cross-linking activity and phosphatase activity (Al-Jallad et al. 2006; Nakano et al. 2010). We initially confirmed the expression patterns of these enzymes through differentiation, and then attempted to determine the possible substrates for each isozyme. Using this cell system, we first demonstrated the detection of different glutamine-acceptor substrates using the isozyme-specific substrate peptides in the cellular extract where the endogenous TGases are active (Figs. 2, 3). In Fig. 2, when compared to those of biotin-labeled primary amine, less but specific proteins were identified in the cases of detection using the biotin-labeled pepF11KA and pepT26. Most of the labeled proteins could be evaluated as possible substrates, because less protein was detected with two-dimensional gel electrophoresis using the QN-mutant peptides.

The possible substrates were purified from the total cell extract where endogenous TGases catalyzed the cross-linking reaction by taking advantage of the biotin-streptavidin affinity. As described in the results, several proteins that were identified by proteomic analysis were candidates for glutamine-acceptor substrates. Among the proteins analyzed, tubulin, actin, and collagen have been already reported as substrates for these TGases (Esposito and Caputo 2005). Common proteins that were identified for TG2 and FXIII reactions were possible candidates for the cross-linking through these enzymatic activities that cooperatively depend on osteoblastic differentiation.

Candidate substrates include both intracellular and extracellular proteins. Both TGases are possibly involved in cross-linking of substrate proteins to sustain the extracellular matrix formation and mineralization, whereas the significance of the cross-linking reactions for intracellular proteins is unknown. Elucidation of the cross-linking products and/or polyamine-conjugated molecules would provide some clarity.

In conclusion, we established a system in which two isozymes co-existed for the identification of isozyme-specific substrate proteins in cells. Using this system, several substrate proteins were identified from the differentiating osteoblastic cell lines. Further studies are ongoing to investigate the cross-linking pattern of these proteins and their contribution to the cell functions.

Acknowledgments We greatly appreciate Dr. Masatoshi Maki and Dr. Hideki Shibata in our laboratory for providing valuable suggestions. This work was supported by a Grant-in-Aid for Scientific Research on Innovative Areas (No. 20200072) (to K. H.) from the Ministry of Education, Sports, Science and Technology (MEXT, Japan).

References

Al-Jallad HF, Nakano Y, Chen JL, McMillan E, Lefebvre C, Kaartinen MT (2006) Transglutaminase activity regulates osteoblast differentiation and matrix mineralization in MC3T3-E1 osteoblast cultures. Matrix Biol 25:135–148

Beninati S, Piacentini M (2004) The transglutaminase family: an overview. Amino Acids 26:367–372

Chen JSK, Mehta K (1999) Tissue transglutaminase: an enzyme with a split personality. Int J Biochem Cell Biol 31:817–836

Esposito C, Caputo I (2005) Mammalian transglutaminases: identification of substrates as a key to physiological function and physiological relevance. FEBS J 272:615–631

Fesus L, Piacentini M (2002) Transglutaminase 2: an enigmatic enzyme with diverstic functions. Trends Biochem Sci 27:534–539

Forsprecher J, Wang Z, Nelea V, Kaartinen MT (2009) Enhanced osteoblast adhesion on transglutaminase 2-crosslinked fibronectin. Amino Acids 36:747–753

Griffin M, Casadio R, Bergamini CM (2002) Transglutaminases: nature's biological glues. Biochem J 368:377–396

Hitomi K, Kitamura M, Sugimura Y (2009a) Preferred substrate sequences for transglutaminase 2: screening using a phage-display peptide library. Amino Acids 36:619–624

Hitomi K, Kitamura M, Perez-Alea M, Ceylan I, Thomas V, El-Alaoui S (2009b) A specific colorimetric assay for measuring transglutaminase 1 and factor XIII activities. Anal Biochem 394:281–283

Itoh M, Kawamoto T, Tatsukawa H, Kojima S, Yamanishi K, Hitomi K (2011) in situ detection of active transglutaminases for keratinocyte (TGase 1) and tissue type (TGase 2) using fluorescence-labeled highly reactive substrate peptides. J Histochem Cytochem 59:180–187

Johnson KA, Rose DM, Terkeltaub RA (2008) Factor XIIIA mobilizes transglutaminases 2 to induce chondrocyte hypertrophic differentiation. J Cell Sci 121:2256–2264

Kaartinen MT, El-Maadawy S, Rasanen NH, Mckee MD (2002) Tissue transglutaminase and its substrate in bone. J Bone Miner Res 17:2161–2173

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

Nakano Y, Al-Jallad HF, Mousa A, Kaartinen MT (2007) Expression and localization of plasma transglutaminase factor XIIIA in bone. J Histochem Cytochem 55:675–685

Nakano Y, Forsprecher J, Kaartinen MT (2010) Regulation of ATPase activity of transglutaminase 2 by MT1-MMP: implications for mineralization of MC3T3-E1 osteoblast cultures. J Cell Physiol 223:260–269

Nurminskaya M, Kaartinen MT (2006) Transglutaminase in mineralized tissues. Front Biosci 11:1591–1606

Perez-Alea M, Kitamura M, Martin G, Thomas V, Hitomi K, El Alaoui S (2009) Development of an isoenzyme-specific colorimetric assay for tissue transglutaminase 2 cross-linking activity. Anal Biochem 389:150–156

Quarles LD, Yohay DA, Lever LW, Caton R, Wenstrup RJ (1992) Distinct proliferative and differentiated stages of murine MC-3T3-E1 culture; an in vitro model of osteoblast development. J Bone Miner Res 7:683–692

Ruoppolo M, Orru S, D'Amato A, Francese S, Rovero P, Marino G, Esposito C (2003) Analysis of transglutaminase protein substrates by functional proteomics. Protein Sci 12:1290–1297

Sugimura Y, Hosono M, Wada F, Yoshimura T, Maki M, Hitomi K (2006) Screening for the preferred substrate sequence of transglutaminase using a phage-displayed peptide library: identification of peptide substrates for TGase 2 and Factor XIIIa. J Biol Chem 281:17699–17706