Factor XIIIA mobilizes transglutaminase 2 to induce chondrocyte hypertrophic differentiation

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Summary

Two transglutaminases (TGs), factor XIIIA (FXIIIA) and TG2, undergo physiologic upregulation in growth plate hypertrophic chondrocytes, and pathological upregulation in osteoarthritic cartilage. Externalization of guanine-nucleotide-bound TG2 drives chondrocyte maturation to hypertrophy, a state linked to matrix remodeling and calcification. Here, we tested the hypothesis that FXIIIA also promotes hypertrophic differentiation. Using human articular chondrocytes, we determined that extracellular FXIIIA induced chondrocyte hypertrophy associated with rapid movement of TG2 to the cell surface. Site-directed mutagenesis revealed that FXIIIA Pro37 bordering the thrombin endoproteolytic Arg38-Gly39 site, but not intrinsic TG catalytic activity, were necessary for FXIIIA to induce chondrocyte hypertrophy. TGs have been demonstrated to interact with certain integrins and, during osteoarthritis (OA), α1β1 integrin is upregulated and associated with hypertrophic chondrocytes. FXIIIA engaged α1β1 integrin in chondrocytes. Antibody crosslinking of α1β1 integrin mobilized TG2. Conversely, an α1β1-integrin-specific blocking antibody inhibited the capacity of FXIIIA to induce TG2 mobilization to the cell surface, phosphorylation of p38 MAP kinase, and chondrocyte hypertrophy. Our results identify a unique functional network between two cartilage TG isoenzymes that accelerates chondrocyte maturation without requirement for TG-catalyzed transamidation by either TG.

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introduction

Transglutaminases (TGs) catalyze a Ca^{2+}-dependent transamidation reaction that generates covalent crosslinks of available substrate glutamine residues with primary amino groups (EC 2.3.2.13), thereby modifying proteins and protein-protein interactions (Lorand and Graham, 2003). Expression and cellular release of the most widely expressed TG isoenzyme (TG2), and of the TG isoenzyme FXIIIA – the homodimeric tissue form of the heterotetrameric plasma coagulation protein factor XIII – have been identified in bone and cartilage (Aeschlimann et al., 1996; Nurminskaya and Linsenmayer, 1996; Nurminskaya et al., 1998). Moreover, FXIIIA and TG2 have both been implicated in modification of the extracellular matrix that modulates the capacity of osteoblasts to mature and form bone mineral (Nurminskaya and Kaartinen, 2006). Changes in FXIIIA and TG2 expression and release also have been identified in the physiological maturation of growth plate chondrocytes, a matrix metalloproteinase 13 (MMP13)-dependent process that occurs in a temporal and spatially organized manner and progresses through resting, proliferative and pre-hypertrophic differentiation, to terminal hypertrophic differentiation and cell death (Aeschlimann et al., 1993; Borge et al., 1996).

The growth plate chondrocyte hypertrophy gene-expression program promotes remodeling of the extracellular matrix, partly through a shift in cartilage-specific collagen expression from type II to type X and by enhanced expression of MMP13 (Drissi et al., 2005). Functional consequences of chondrocyte hypertrophy in the growth plate include calcification mediated partly by matrix-vesicle shedding and angiogenesis mediated in part by VEGF expression (Kirsch et al., 1997). Although articular cartilage chondrocytes are in a physiological, maturity-arrested differentiation state, pathologic hypertrophic differentiation is observed to develop among osteoarthritic (OA) chondrocytes in situ and has the potential to promote OA progression through dysregulated matrix repair (Tchetina et al., 2006) and pathological calcification (Kirsch et al., 2000). Significantly, both FXIIIA and TG2 are molecular markers of chondrocyte hypertrophic differentiation in the growth plate (Aeschlimann et al., 1993; Nurminskaya and Linsenmayer, 1996). Furthermore, TG2 and FXIIIA expression, as well as total TG catalytic activity, are upregulated in human knee OA cartilage chondrocytes (Johnson et al., 2001).

Despite lacking a signal peptide, TG2 and FXIIIA both are released by chondrocytes and osteoblasts (Nurminskaya and Kaartinen, 2006). Transamidation of proteins in the extracellular matrix by secreted TGs has the potential to alter cell differentiation and function, specifically exemplified by TG2-catalyzed transamidation of extracellular matrix collagen to promote calcification (Chau et al., 2005). TG2 is essential to accelerate chondrocyte maturation to hypertrophy in response to signals provided by retinoic acid and CXCL1 (Johnson et al., 2003; Merz et al., 2003). Moreover, exogenous nanomolar TG2 is sufficient to directly induce hypertrophic differentiation in chondrocytes in articular cartilage organ culture (Johnson and Terkeltaub, 2005). In addition, paracrine and juxtacrine effects of TG2 released from chondrocytes modulate osteoblast differentiation through extracellular TG2-induced PKA signaling (Nurminskaya et al., 2003; Nurminskaya and Kaartinen, 2006).

Cardinal aspects of the multifunctionality of TG2 include the potential for TG2 to function as an unconventional GTPase and as

a cell adhesion protein (Lorand and Graham, 2003). TG2 interconverts in a reciprocally regulated manner between a TG catalytically latent guanine nucleotide-bound state and TG catalytically active Ca^{2+}-bound state (Fesus and Piacentini, 2002). Cell surface TG2 serves as an integrin co-receptor for fibronectin (Hang et al., 2005) and extracellular TG2 promotes integrin clustering that induces RhoA activation (Janiak et al., 2006). In this context, exogenous TG2 must be in a guanine nucleotide-bound state and employ β1-integrin-mediated signaling and rapid activation of p38 MAPK pathway signaling to induce chondrocyte hypertrophic differentiation in vitro (Johnson and Terkeltaub, 2005; Tanaka et al., 2007). TG2 does not require transamidation activity, GTPase activity or fibronectin binding to promote chondrocyte maturation to hypertrophy (Johnson and Terkeltaub, 2005).

Increased FXIIIA expression and extrusion coincide with and directly stimulate matrix calcification in chondrocytes in situ and in vitro (Johnson et al., 2001; Nurminskaya et al., 1998; Nurminskaya et al., 2002). Here, we examined molecular structure and function of FXIIIA in chondrocyte differentiation. FXIIIA, similar to TG2, exerts both transamidation-dependent and transamidation-independent activities, binds integrins and functions to modulate both cell adhesion and differentiation. Seminal work by Dardik and colleagues has revealed that FXIIIA binds αVβ1 and αVβ3 integrins and has the potential to covalently and noncovalently complex them with other receptors, exemplified by vascular endothelial growth factor receptor 2 (VGFR2, hereafter referred to as VEGFR-2) (Dardik et al., 2002; Dardik et al., 2005; Dardik et al., 2007). Moreover, provision to monocytes and endothelial cells of catalytically active FXIIIA (FXIIIa) modulates cell signaling and impacts on functions such as angiogenesis, migration and apoptosis (Dardik et al., 2005; Dardik et al., 2007). In OA articular cartilage, α1β1 integrin expression is augmented, particularly in hypertrophic chondrocytes (Zemmyo et al., 2003), and this study implicates α1β1 integrin function as essential to the capacity of FXIIIA to promote chondrocyte hypertrophic differentiation. Our results further identify a unique functional network between FXIIIA and TG2 that accelerates chondrocyte maturation without the requirement for either TG isoenzyme to catalyze transamidation.

Results

Molecular FXIIIA structure and function in the induction of type X collagen

To test the hypothesis that extracellular FXIIIA can promote chondrocyte hypertrophy, we first treated cultured human articular chondrocytes with soluble recombinant FXIIIA (sFXIIIA) in comparison with recombinant TG2 (sTG2). Treatment with either of the TG isoenzymes at 100 ng/ml was sufficient to increase expression of vascular endothelial growth factor (*VEGF*) and *MMP13* (genes typically expressed by hypertrophic chondrocytes), and to markedly augment the ratio of type X collagen (the stereotypic marker of chondrocyte hypertrophy) to type II collagen mRNA within 8 hours (Fig. 1A). In bovine knee articular cartilage in organ culture, sTG2 and sFXIIIA both induced type X collagen (Fig. 1B). Under these conditions, sTG2 but not sFXIIIA stimulated enlargement of chondrocyte-containing lacunae (36.54±5.46 μm vs 19.25±5.44 μm in diameter, P=0.043, n=5 donors, with one section containing ten lacunae counted from each donor) in articular cartilage explants (Fig. 1B), which suggested differential effects on chondrocyte hypertrophy.

To test whether TG2 stimulated chondrocyte hypertrophy in a manner mediated by FXIIIA or vice versa, we studied cultured knee

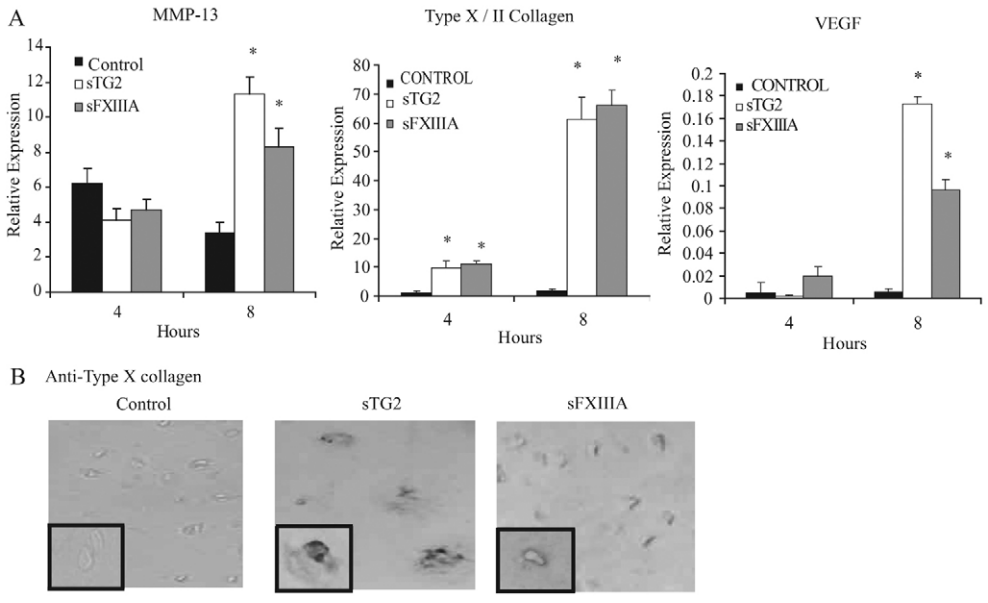

Fig. 1. Both exogenous FXIIIA and TG2 induce chondrocyte hypertrophic differentiation. We studied induction by TG2 and FXIIIA of MMP13, VEGF and type X collagen in cultured chondrocytes. Normal human knee articular chondrocytes plated in 12-well dishes (at 10^5 cells per well) were incubated for 4 or 8 hours with 100 ng/ml of recombinant WT TG2 or FXIIIA in the ascorbate-containing medium A described in Materials and Methods. (A) Quantitative PCR. Using quantitative RT-PCR, we determined chondrocyte *GAPDH* mRNA expression levels relative to those of *MMP13* and *VEGF*, and the mRNA expression ratio of type X collagen to type II collagen, studying data collected from three separate human donors (n=9, *P<0.05). (B) Bovine articular cartilage explants. Assessment of the induction by TG2 and FXIIIA of type X collagen in cartilage organ culture. Normal bovine articular cartilage explants in organ culture were incubated with 100 ng/ml recombinant WT TG2 or FXIIIA for 5 days in medium A, and frozen 10-μm sections were stained for expression of type X collagen (representative of five donors).

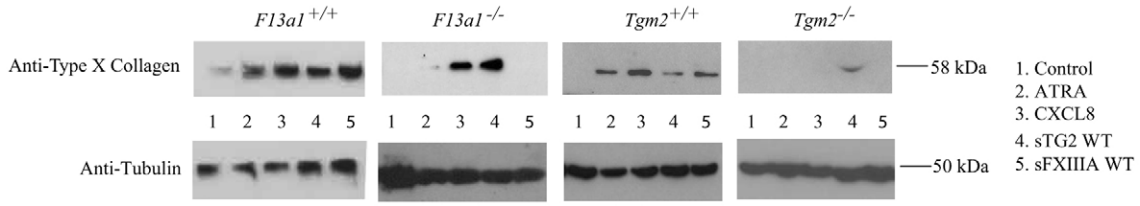

Fig. 2. FXIIIA-stimulated expression of type X collagen is dependent upon TG2. Assessment of TG2- and FXIIIA-knockout mouse cells. Primary knee chondrocytes were removed from *F13a1*$^{+/+}$, *F13a1* $^{-/-}$, *Tgm2*$^{+/+}$ and *Tgm2*$^{-/-}$ mice. After two weeks in culture, aliquots of 5×10^3 cells in Medium A were stimulated for 5 days with 10 nM ATRA, 10 ng/ml CXCL8 or 100 ng/ml of sTG2 or sFXIIIA, and then type X collagen was examined by SDS-PAGE and western blotting, as described in the Materials and Methods. Representative of three experiments.

articular chondrocytes from *Tgm2*- and *F13a1*-knockout mice and congenic wild-type (WT) controls. We observed that sFXIIIA failed to induce expression of type X collagen in *Tgm2*$^{-/-}$ chondrocytes, whereas sTG2 did not require FXIIIA to induce type X collagen (Fig. 2). In parallel studies, we observed that sFXIIIA also required endogenous FXIIIA to induce type X collagen, and that all-trans retinoic acid (ATRA) required both endogenous TG2 and FXIIIA to induce type X collagen (Fig. 2). By contrast, chondrocytes did not require FXIIIA expression to develop type X collagen expression in response to CXCL8, under conditions where TG2 was necessary (Fig. 2).

To elucidate FXIIIA structure and function in chondrocyte hypertrophy induction, we designed a site-directed mutagenesis strategy that factored in the constitutive latency of FXIIIA as a TG. Specifically, binding of Ca^{2+} to FXIIIA is required for TG catalytic activity triggered by endoproteolysis of FXIIIA at the Arg38-Gly39 bond by thrombin (or alternatively by excess Ca^{2+} alone) removes a 4-kDa peptide to expose the conserved TG family catalytic triad that includes Cys314 (Aeschlimann et al., 1996). FXIIIa TG activity is subsequently inactivated by thrombin-catalyzed proteolysis at Lys514 (Lai et al., 1999) or, alternatively, by a decrease in ambient Ca^{2+} We observed catalytic activity possessed by recombinant WT FXIIIA protein, most probably due to the presence of Ca^{2+} during amplification (supplementary material Fig. S1). Our strategy for functional analysis of FXIIIA was to leave intact the previously defined amino acids at which thrombin cleaves, but to potentially inhibit the action of thrombin on FXIIIA catalytic activity by mutating one amino acid immediately adjacent to the amino acid residues at which thrombin cleaves the molecule. Thus, residue Pro37 was chosen based upon it significance together with Arg38 (Aeschlimann et al., 1996) in the thrombin-binding site, as determined by crystal structure analysis (Sadasivan and Yee, 2000), and Met513 chosen because of its immediate proximity to the endoproteolytic thrombin K514 'inactivation site' (Lai et al., 1999). As schematized in Fig. 3A, we prepared and isolated recombinant forms of FXIIIA site mutants; specifically, one that contained the previously characterized mutation of the catalytic site (C314A) (Hettasch and Greenberg, 1994), a mutation immediately adjacent to the Arg38-Gly39 endoproteolytic thrombin 'activation site' (P37A), and a mutation immediately adjacent to the endoproteolytic thrombin Lys514 'inactivation site' (M513G).

Importantly, direct comparison of equal amounts of these human FXIIIA-mutant proteins for TG catalytic activity (supplementary

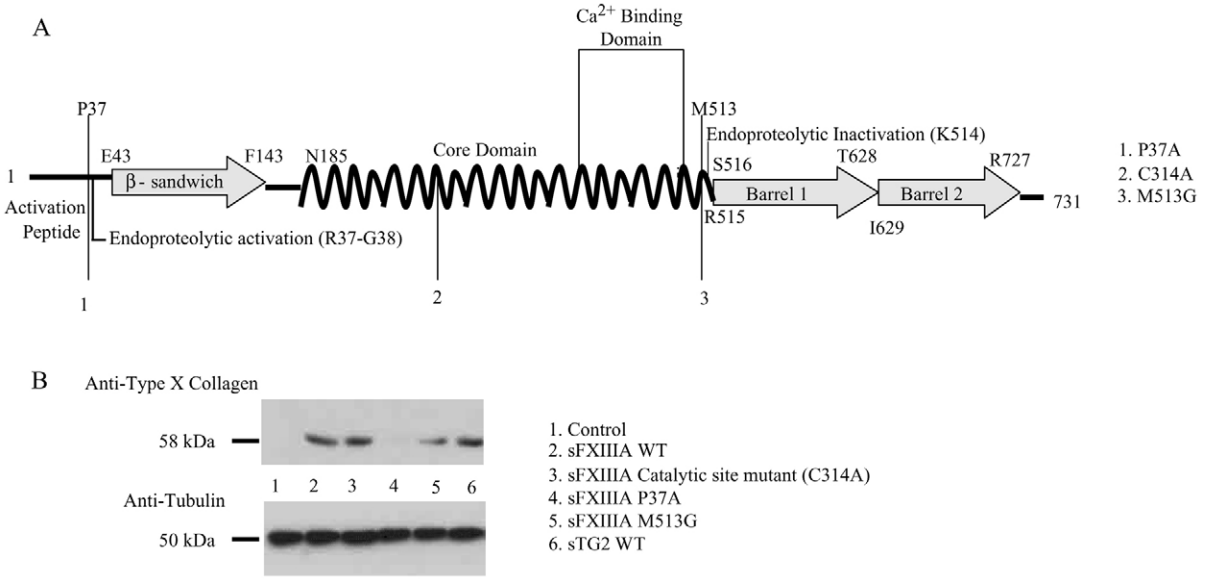

Fig. 3. (A) FXIIIA. Site-directed FXIIIA mutations generated for structural analysis examination. Depicted are features of the primary structure of WT FXIIIA and the site-directed FXIIIA mutations generated and studied, listed her as 1-3. (B) Type X collagen. Aliquots of human articular chondrocytes (10^5 cells) were incubated with 100 ng/ml of each recombinant protein in medium A for 72 hours, and type X collagen assessed in cell lysates by SDS-PAGE and western blotting. Representative of three donors in three separate experiments (n=9).

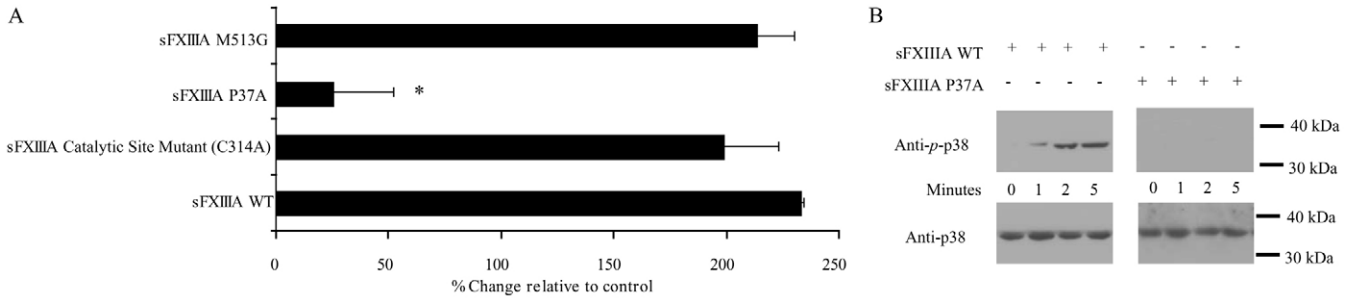

Fig. 4. Rapid mobilization of TG2 by FXIIIA is essential for stimulation of p38 phosphorylation. (A) Plasma-membrane-bound TG2. Aliquots of 5×10^3 human articular chondrocytes were starved in serum-free high-glucose DMEM for 2 hours and then stimulated with WT or mutant sFXIIIA. TG2-specific antibodies were used to detect membrane-bound TG2, quantified through successive incubations with biotin anti-rabbit and streptavidin-AP antibodies as described in Materials and Methods (n=9). (B) p38 MAP kinase phosphorylation. Aliquots of 3×10^5 human articular chondrocytes were starved in serum-free high-glucose DMEM for 2 hours and then stimulated with WT or mutant sFXIIIA for the time indicated. Cell lysates were analyzed by western blotting for phosphorylated p38 (p-p38) and total p38. Representative of three donors in three separate experiments (n=9).

material Fig. S1), revealed that individual mutants possessed the relative changes in TG activity in the presence and absence of thrombin indicative of the intended interference with thrombin action**.** Incubation of chondrocytes with recombinant forms of FXIIIA mutants (100 ng/ml) revealed dependence on the P37 residue bordering the thrombin 'activation site' for extracellular sFXIIIA to induce type X collagen expression (Fig. 3B).

FXIIIA induces p38 MAPK signaling dependent on rapid externalization of TG2

Release of TG2 by articular chondrocytes is required for TG2-dependent hypertrophic differentiation, and recombinant TG2 induces phosphorylation of p38 MAP kinase within minutes (Johnson and Terkeltaub, 2005). Signaling through the p38 MAP kinase pathway has a central role in transducing maturation to hypertrophy under certain conditions in cultured chondrocytes (Merz et al., 2003; Wang and Beier, 2005; Zhang et al., 2006). Because FXIIIA appeared to require TG2 to induce hypertrophic differentiation, we hypothesized that FXIIIA mediates the localization of TG2 to the cell surface to promote the rapid initiation of chondrocyte hypertrophic differentiation.

To detect TG2 on the cell surface, chondrocytes were serum starved and then stimulated with sFXIIIA. The non-permeabilized and fixed cells were incubated with a biotinylated monoclonal antibody to TG2 (Balklava et al., 2002). First, a 200-fold change in the amount of TG2 localized on the cell surface was detected within 5 minutes of treatment of primary human articular chondrocytes with WT sFXIIIA (WT sFXIIIA) (Fig. 4A). These results were not owing to transcriptional regulation of TG2, because there was no significant change in levels of mRNA expression for TG2 (supplementary material Fig. S2). Second, the P37A mutant of FXIIIA (which had a depressed capacity to induce type X collagen; Fig. 3B) retained the capacity to induce an increase in the cell surface TG2 expression but at significantly lower levels than that of not only WT sFXIIIA but also the M513G and the catalytic site FXIIIA mutants (Fig. 4A). Next, we determined that

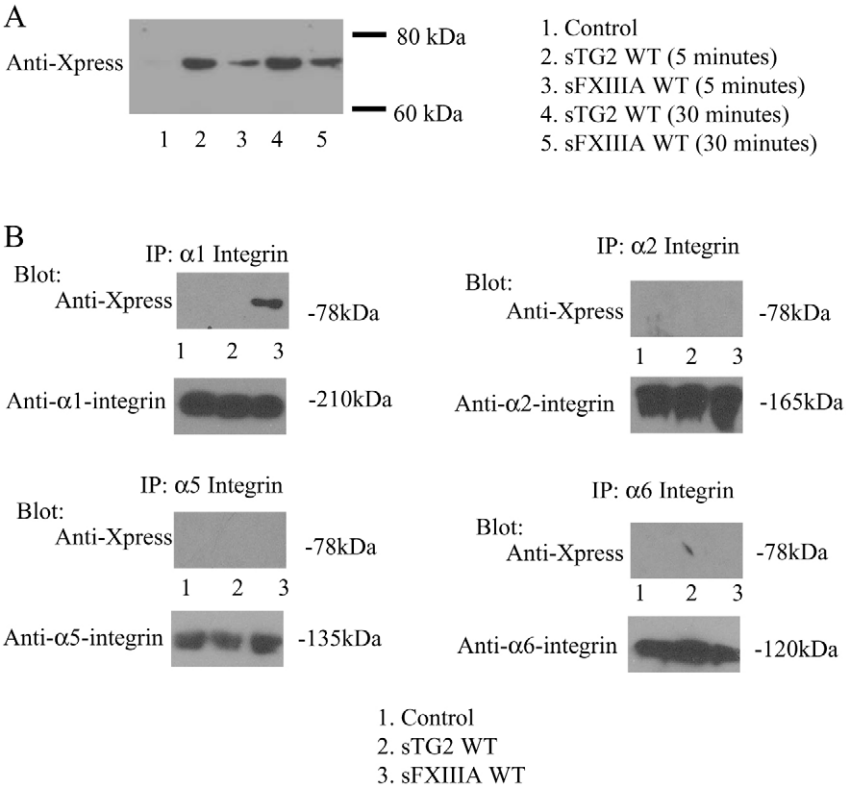

Fig. 5. Recombinant FXIIIA engages the α1-integrin subunit in human chondrocytes. (A) Western blot. Aliquots of 10^5 human articular chondrocytes were starved for 2 hours in serum-free DMEM and then incubated for the indicated times with 100 ng/ml sTG2 or sFXIIIA. The cells were washed prior to lysis and then examined for the presence of the Xpress epitope on the recombinant proteins by SDS-PAGE and western blotting. (B) Integrin immunoprecipitation. Aliquots of 10^6 human articular chondrocytes were incubated for 3 days in medium A containing 100 ng/ml of sTG2 or sFXIIIA where indicated. Cell lysates (200 μg protein) were immunoprecipitated using 1 μg/ml of α1- (clone TS2/7), α2-, α5- or α6-integrin-subunit-specific antibody, and precipitated proteins were analyzed for the Xpress tag or each respective α-integrin subunit by western blotting. Representative of three separate experiments using three different donors.

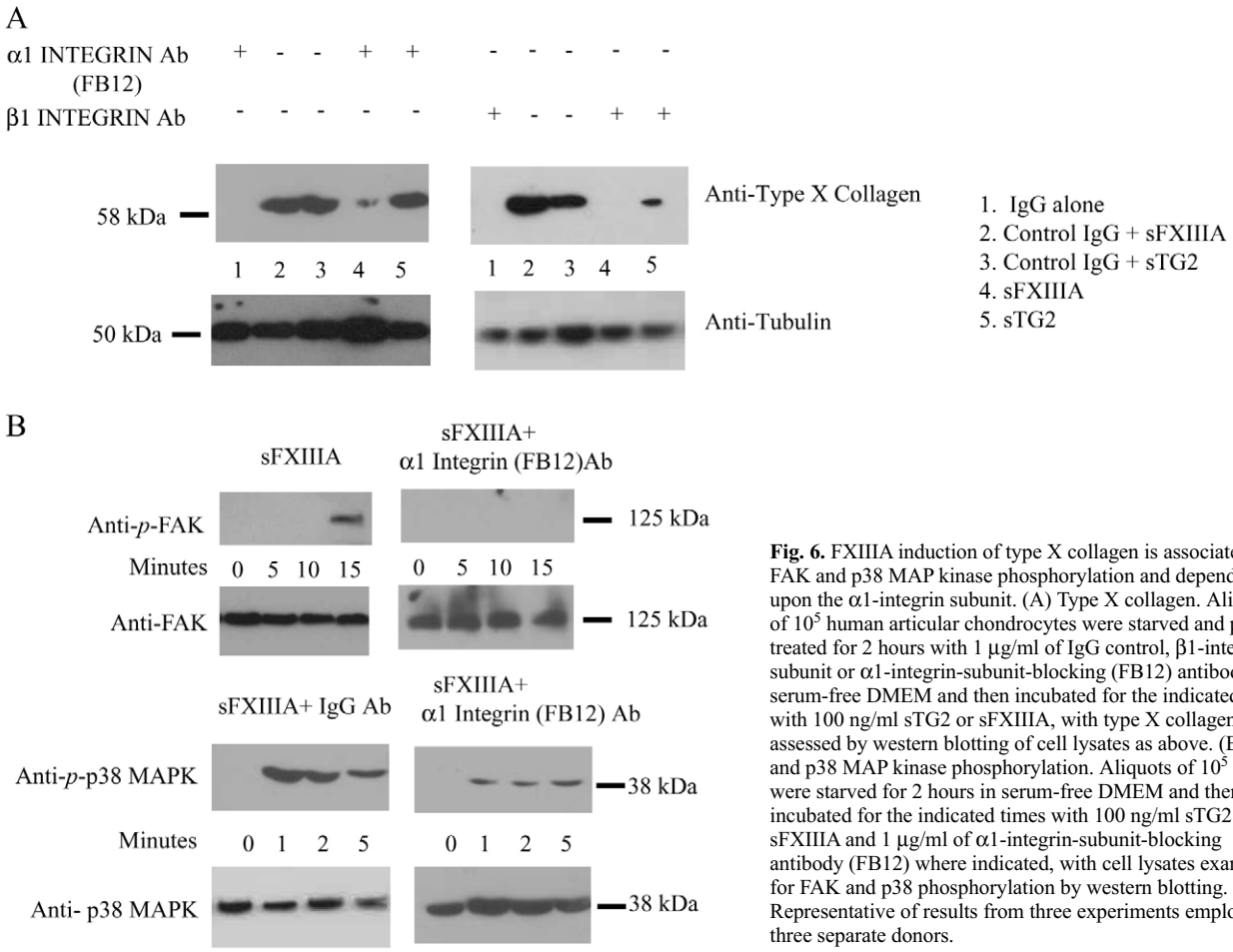

Fig. 6. FXIIIA induction of type X collagen is associated with FAK and p38 MAP kinase phosphorylation and dependent upon the α1-integrin subunit. (A) Type X collagen. Aliquots of 10^5 human articular chondrocytes were starved and pre-treated for 2 hours with 1 μg/ml of IgG control, β1-integrin subunit or α1-integrin-subunit-blocking (FB12) antibodies in serum-free DMEM and then incubated for the indicated times with 100 ng/ml sTG2 or sFXIIIA, with type X collagen assessed by western blotting of cell lysates as above. (B) FAK and p38 MAP kinase phosphorylation. Aliquots of 10^5 cells were starved for 2 hours in serum-free DMEM and then incubated for the indicated times with 100 ng/ml sTG2 or sFXIIIA and 1 μg/ml of α1-integrin-subunit-blocking antibody (FB12) where indicated, with cell lysates examined for FAK and p38 phosphorylation by western blotting. Representative of results from three experiments employing three separate donors.

sFXIIIA stimulated phosphorylation of p38 MAP kinase within minutes, an activity not shared by the P37A mutant (Fig. 4B). Finally, neither the catalytic site mutant nor the M513G mutation affected the capacity of FXIIIA to induce phosphorylation of p38 (Fig. 4B and supplementary material Fig. S3).

The α1-integrin subunit in induction of p38 MAPK signaling by FXIIIA in chondrocytes

After application of TG2 and FXIIIA to the chondrocytes, we observed binding and internalization (within minutes) of the TG isoenzymes by chondrocytes (Fig. 5A). In fibroblasts, TG2 can bind to the extracellular domain of β1- and β3-integrin subunits (Akimov et al., 2000). Activated FXIIIA can bind to αVβ3 integrin in HUVECs (Dardik et al., 2002). Chondrocytes themselves express multiple integrins, including α1β1, α2β1, α5β1, α6β1, α10β1 and αVβ3 (Loeser, 2000). Previous studies with blocking antibodies indicated a role of β1 integrins in TG2-induced chondrocyte hypertrophy, whereas inhibition of β3-integrin subunit had no effect (Johnson and Terkeltaub, 2005; Tanaka et al., 2007). Therefore, we treated chondrocytes with recombinant TG2 and FXIIIA and assessed for interaction between TGs and four α-integrin subunits. The exogenous sTG2 did not detectably bind to α1-, α2-, α5- or α6-integrin, but exogenous sFXIIIA engaged the α1-integrin subunit (Fig. 5B).

To assess whether the binding of α1-integrin subunit is crucial in FXIIIA-induced hypertrophy, chondrocytes were pre-treated with the α1-integrin-subunit-blocking antibody FB12, which inhibited the capacity of sFXIIIA but not sTG2 to induce type X collagen (Fig. 6A). By contrast, the blocking antibody to the β1-integrin subunit, suppressed type X collagen induction in response to both TG isoenzymes under these conditions (Fig. 6A). Additionally, sFXIIIA-induced p38 MAP kinase activation, together with FAK phosphorylation assessed as a readout for integrin signaling, were inhibited by pretreatment of chondrocytes with α1-integrin-subunit-blocking antibody FB12 (Fig. 6B).

Crosslinking of the α1-integrin subunit antibody (TS2/7) with a mouse IgG mimicked the enhanced movement of TG2 to the plasma membrane seen with WT sFXIIIA (Fig. 7). Furthermore, pre-treating chondrocytes with the α1-integrin-subunit-blocking antibody FB12 inhibited the ability of FXIIIA to increase TG2 mobilization to the plasma membrane (Fig. 7). Hence, the function of α1β1 integrin was required for FXIIIA to both mobilize TG2 and induce chondrocyte hypertrophy.

Discussion

Chondrocytes have the capacity to release FXIIIA (Nurminskaya et al., 1998; Nurminskaya et al., 2002; Rosenthal et al., 2001). In this study, we identified novel functional implications of upregulation of FXIIIA expression and release in both the growth plate chondrocyte hypertrophic differentiation program and in OA cartilage chondrocytes (Linsenmayer et al., 1998; Nurminskaya and Linsenmayer, 1996). As summarized in the model depicted in Fig. 8, we demonstrated that stimulation of chondrocytes with recombinant FXIIIA stimulated the release of TG2. We previously observed TG2 effects on

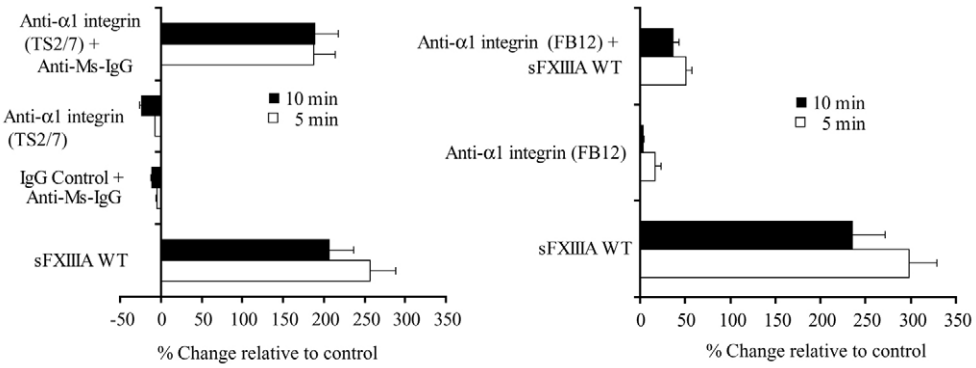

Fig. 7. Rapid mobilization of TG2 to the cell surface by either sFXIIIA or antibody crosslinking of α1-integrin subunit. To determine whether FXIIIA-induced movement of TG2 to the cell surface is α1-integrin-subunit dependent, aliquots of 5×10^3 human articular chondrocytes were starved in serum-free high-glucose DMEM for 2 hours and then stimulated with sFXIIIA, the α1-integrin-subunit antibody TS2/7 (with and without crosslinking by anti-mouse IgG) versus an IgG control antibody. Additionally, after starvation the chondrocytes were pre-treated with the blocking α1-integrin-subunit antibody, FB12 and then stimulated for 5 or 10 minutes with WT sFXIIIA. After fixation of the cells, TG2-specific antibodies were used to detect membrane-bound TG2, quantified through successive incubations with biotin anti-rabbit and streptavidin-AP antibodies as described in Materials and Methods.

chondrocyte maturation to hypertrophy to be dependent on TG2 externalization (Johnson and Terkeltaub, 2005). In the absence of TG2 or lack of the promotion of TG2 externalization, FXIIIA was unable to induce hypertrophy. Both mineralizing hypertrophic growth plate chondrocytes and mineralizing osteoblasts robustly release FXIIIA (Nurminskaya et al., 1998; Nurminskaya and Kaartinen, 2006). Our results suggest that one of the functions of FXIIIA release by bone-forming cells is to fine-tune TG2 release and thereby regulate TG2-dependent effects on chondrocyte and osteoblast differentiation (Aeschlimann et al., 1996; Al-Jallad et al., 2006; Heath et al., 2001) and transamidation-catalyzed extracellular matrix remodeling in the skeleton (Aeschlimann et al., 1993; Nurminskaya and Kaartinen, 2006).

In this study, recombinant sTG2 was able to induce chondrocyte hypertrophy in the absence of FXIIIA expression, but not vice versa. These findings indicated TG2 to be the driving force of the functional network of two TG isoenzymes that stimulated chondrocyte maturation. Once outside the cell, FXIIIA rapidly mobilized TG2, evidenced by ~200-fold enrichment of TG2 on the surface of chondrocytes within minutes of sFXIIIA addition. In vitro binding assays have not revealed a direct association between TG2 and FXIIIA (our unpublished observations). As such, there was no evidence that TG2 mobilization to the cell surface by FXIIIA was caused by direct mutual interaction.

Changes in subcellular localization of TG2 modulate wound healing, partly through interactions of plasma-membrane-bound TG2 with fibronectin and certain integrins that regulate cell adhesion and migration (Akimov and Belkin, 2001). Additionally, plasma-membrane-associated TG2 and FXIIIA, in conjunction with increased FXIIIA expression (Johnson et al., 2001), may contribute to the upregulated activity of the p38 MAP kinase signaling pathway in OA cartilage chondrocytes in situ (Chun, 2004).

Significantly, both sFXIIIA and sTG2 were observed to directly induce type X collagen in non-proliferating single chondrocytes within lacunae in articular explants in this study. Thus, robust release of TG2 and FXIIIA in OA cartilage potentially bypasses the conventional growth-plate-chondrocyte-maturation sequence. The capacity of TG2, but not FXIIIA, to promote enlargement of the chondrocytes in the lacunae could be attributed to the fact that TG2 and FXIIIA preferentially mediate the transamidation of distinct protein sequences (Sugimura et al., 2006).

In cultured chondrocytes, activation of the p38 MAP kinase signaling pathway in response to signals including sTG2, and certain calgranulins and chemokines, has a central role in promoting maturation to hypertrophy (Johnson and Terkeltaub, 2005; Merz et al., 2003; Wang and Beier, 2005; Zhen et al., 2001). However, it has been noted that p38 MAP kinase signaling does increase the

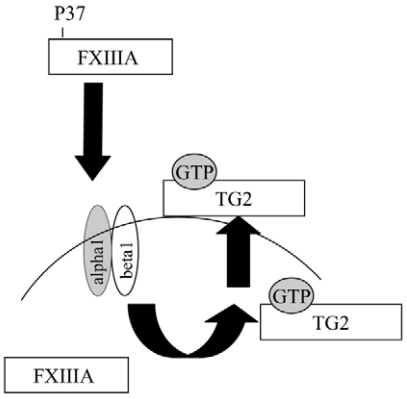

Fig. 8. Model for how extracellular FXIIIA mobilizes TG2 in promoting chondrocyte hypertrophic differentiation that is dependent on both α1β1 integrin and endogenous FXIIIA. Extracellular GTP-bound TG2 was previously demonstated to induce β1-integrin-dependent signaling to stimulate chondrocyte maturation to hypertrophy, and associated with p38 MAP kinase activation. In this model, which summarizes results of this study, TG2 is rapidly mobilized from the cytosol to the extracellular surface of the plasma membrane by FXIIIA in a manner dependent on α1β1-integrin-dependent signaling, and reproduced by anti-α1-antibody-induced α1β1-integrin crosslinking. To promote chondrocyte hypertrophy, FXIIIA requires endogenous TG2 to be mobilized, and FXIIIA structurally requires the Pro37 residue that mediates thrombin induction of TG catalytic activity in latent FXIIIA. By contrast, TG catalytic activity of exogenous FXIIIA is not required for induction of hypertrophy, nor is the Met513 residue that mediates thrombin inactivation of FXIIIa TG catalytic activity. Exogenous FXIIIA is shown to be a ligand of the α1-integrin subunit, but α1β1-integrin signaling in response to FXIIIA is possibly mediated by other α1β1-integrin ligand(s) or trough their complex-formation with FXIIIA and α1β1 integrin. Last, we observed that exogenous FXIIIA (as well as retinoic acid) fail to induce chondrocyte hypertrophy in the absence of endogenous FXIIIA. Hence, endogenous FXIIIA modulates chondrocyte differentiation, including responsiveness to exogenous FXIIIA.

transcriptional activity of the chondrogenic master transcription factor Sox9, an inhibitor of chondrocyte maturation to hypertrophy in vivo and in vitro. Moreover, constitutively activated p38 MAP kinase signaling in MKK6 transgenic mice is associated with reduced chondrocyte proliferation and inhibition of hypertrophic chondrocyte differentiation in growth plates in situ (Zhang et al., 2006).

Further studies will be needed to elucidate which downstream signaling pathways, beyond FAK and p38, transduce chondrocyte maturation in response to FXIIIA and TG2. The functional role of the FXIIIA-induced chondrocyte p38-pathway-activation within growth plate and articular cartilages might depend crucially on the timing and spatial organization of upregulated FXIIIA release by chondrocytes, as well as the concurrent expression of TG2. In addition, internalization of secreted TG2 followed by nuclear localization of TG2 and its receptor could mediate signaling by TG2 in chondrocytes, as demonstrated with VEGFR-2 in endothelial cells (Dardik and Inbal, 2006).

Upregulation of α1β1 integrin develops in the superficial and upper mid-zone in murine OA cartilage, a condition in which α1β1 integrin appears to mediate cartilage-matrix remodeling (Zemmyo et al., 2003). Moreover, α1β1-integrin expression is augmented particularly in hypertrophic chondrocytes in OA articular cartilage (Zemmyo et al., 2003). Here, we uncovered a physical interaction between exogenous FXIIIA and the α1 subunit of α1β1 integrin. Antibody-induced crosslinking of α1β1 integrin induced TG2 mobilization. Conversely, a blocking antibody specific for the α1-integrin subunit suppressed the capacity of FXIIIA to induce both TG2 mobilization and type X collagen expression, but it did not block TG2-induced type X collagen expression. TG2 has been shown to cluster cell surface integrins and lead to integrin-dependent signaling and activation (Janiak et al., 2006). Furthermore, α5β1 integrin has been implicated as being essential for exogenous TG2 to promote chondrocyte hypertrophy (Tanaka et al., 2007). However, the absence of detectable engagement of α5 integrin by exogenous TG2 in this study is potentially instructive, as it is possible that the described α5β1-integrin effects (Tanaka et al., 2007) also are indirect. Specifically, it remains possible that FXIIIA clusters α1β1 integrin and thereby primes chondrocytes for TG2-dependent induction of hypertrophy, but it also is possible that the role of α1β1 integrin in FXIIIA-induced TG2 mobilization and chondrocyte hypertrophy is indirect. For example, it might be that both binding by α1β1 integrin of its previously recognized matrix ligands (collagen and/or matrilin-1), and binding of α5β1 integrin to ligands other than TG2, are crucial to drive chondrocyte hypertrophy. Alternatively, FXIIIA might bind an integrin-associated protein that binds or otherwise complexes with or clusters α1β1 integrin. This last scenario merits further study in chondrocytes, because ternary-complex formation of FXIIIA with αVβ3 integrin and VEGFR-2 on the endothelial cell surface mediates angiogenesis through VEGF-independent VEGFR-2 activation (Dardik et al., 2005).

In both the *F13a1*$^{-/-}$ and *Tgm2*$^{-/-}$ mouse chondrocytes, sFXIIIA was not able to induce an increase in the expression of type X collagen. This finding suggests that intracellular FXIIIA exerts significant effects on signaling and differentiation in chondrocytes. FXIIIA dimerization of the type 1 angiotensin II receptor through crosslinking the cytosolic tails of the receptor is a notable example of such FXIIIA intracellular activity (AbdAlla et al., 2004). Other secondary findings in this study were that retinoic acid required both endogenous TG2 and FXIIIA to induce type X collagen. By contrast, chondrocytes did not require FXIIIA expression to express type X collagen in response to CXCL8 under conditions where TG2 was necessary. We have not begun to probe for α1β1-integrin-subunit expression or the many other potential changes in FXIIIA-knockout chondrocytes that could affect their differentiation in response to either FXIIIA or retinoic acid.

Limitations of this study included the use of monolayer-culture conditions for these experiments, necessary not only because of the low yields of primary mouse articular chondrocytes but also to allow subsequent comparative studies with human chondrocytes. Monolayer-culture conditions might have imposed significant cell-cell and cell-matrix interactions that would not take place in growth plate and articular cartilages in vivo. Another limitation within much of this study was the treatment of cultured chondrocytes with nanomolar amounts of exogenous recombinant FXIIIA and TG2 to assess mechanisms for TG effects on differentiation. Endogenous chondrocyte TG2 and FXIIIA typically reach only high picomolar extracellular concentrations in chondrocytes (K.A.J., unpublished). However, the movement of secreted endogenous TG isoenzymes to the cell surface is likely to be more efficient than for exogenous TGs. It should be noted that this study employed both catalytically active and inactive sFXIIIA at a concentration 500 times lower than the 50 μg/ml amounts of sFXIIIa previously added to vascular cells and fibroblasts (Dardik et al., 2002; Dardik et al., 2005; Dardik et al., 2007). We assessed changes in mutant FXIIIA TG catalytic activity in response to thrombin, but we did not investigate effects of the P37A and M513G mutations on endoproteolytic cleavage of FXIIIA by either thrombin alone or by chondrocytes without exogenous thrombin. This study did not attempt to define specific effects of endogenous intracellular TG2 or FXIIIA on chondrocyte differentiation. In this light, guanine-nucleotide bound TG2 does functionally engage several α-integrin-subunit cytosolic tails (Kang et al., 2004). We also did not specifically test potential roles in mediating FXIIIA and TG2 effects on chondrocyte differentiation, of binding or transamidation of TGFβ (Verderio et al., 1999), soluble integrin ligands or other extracellular matrix proteins. Last, we did not assess for signaling effects by intracellular TG2 (Dardik and Inbal, 2006).

In summary, we have established that two TG isoenzymes act together to promote and accelerate chondrocyte maturation, but do so in a transamidation-independent manner. Our results add to growing evidence that direct interactions between certain TG isoenzymes and integrins alter cell signaling and differentiation. Our results point to the biological significance of the robust expression of two TG isoenzymes in association with chondrocyte hypertrophy in OA cartilage in vivo that might lead to a rapid signal amplification and progression of OA. Our results identify relative timing of FXIIIA and TG2 upregulation in cartilages as a novel mechanism for modulation of chondrocyte differentiation under both physiological and pathological conditions.

Materials and Methods

Reagents

All chemicals and other reagents were obtained from Sigma (St Louis, MO), unless otherwise indicated.

Generation of FXIIIA cDNA mutants and soluble recombinant forms of FXIIIA

Human FXIIIA cDNA in pcDNA4/HisMax was the template for the generation of the FXIIIA mutants and the recombinant forms. Recombinant TG2 and FXIIIA were prepared through overexpression in mammalian cells (HEK 293 cells) and harvested under sterile conditions. After purification through binding to nickel columns, dialysis and concentration, the preparations were determined to be >90% pure by SDS-PAGE Coomassie-Blue staining, tested using the Limulus Amebocyte Lysate QCL 1000

assay (Cambrex, Baltimore, MD) and determined to have <0.1EU/ml (or below the detection limits) of endotoxin (Johnson and Terkeltaub, 2005). Purified, soluble, recombinant TG2 protein was used following treatment to generate Mg^{2+}-GTP complexes, as described (Johnson and Terkeltaub, 2005).

Tgm2$^{-/-}$ and *F13a1*$^{-/-}$ mice

Tgm2$^{+/+}$ and *Tgm2*$^{-/-}$ mice have originally been generated and described by Nanda et al. (Nanda et al., 2001). *F13a1*$^{-/-}$ mice and WT congenic littermate controls were bred from *F13a1*$^{+/-}$ mice previously generated, characterized, and generously provided by Gerhard Dickneite and colleagues (Aventis Behring GMBH, Germany) (Lauer et al., 2002). All animal experimentation was assessed and approved by the IACUC (Institutional Animal Care and Use Committee) of the San Diego Veterans Affairs Medical Center.

Cell and explant culture isolation and conditions

Primary articular chondrocytes were isolated by dissection of the tibial plateaus and femoral condyles of *Tgm2*$^{+/+}$, *Tgm2*$^{-/-}$, *F13a1*$^{+/+}$ and *F13a1*$^{-/-}$ mice at two months of age, as described previously (Johnson et al., 2003). Human articular chondrocytes from normal donor knees were isolated as described (Merz et al., 2003). First-passage human articular chondrocytes and mouse chondrocytes were cultured in high-glucose DMEM supplemented with 10% FCS, 1% glutamine, 100 U/ml penicillin, 50 μg/ml streptomycin (Mediatech, Herndon, VA) and maintained at 37°C. Studies on differentiation and function were performed in medium A (high-glucose DMEM supplemented with 1% FCS, 1% glutamine, 100 U/ml Penicillin, 50 μg/ml Streptomycin, and 50 μg/ml of ascorbic acid) with 100 ng/ml of sFXIIIA and sTG2 added where indicated.

For cartilage organ culture studies, 2×2 mm slices of articular cartilage were removed from the patellar groove and femoral condyles of normal bovine knees (Animal Technologies, Tyler, TX). Explants were cultured, treated, sectioned and stained as previously described (Johnson and Terkeltaub, 2005).

For immunocytochemical analysis of human articular chondrocytes, aliquots of 10^5 cells were plated on 18 mm circular glass coverslips in medium A. The cells were then fixed for 20 minutes at room temperature with 4% paraformaldehyde and washed with PBS. All primary antibodies were used at a 1:100 dilution. For light-microscopy, bound antibodies were detected by the ABC method. All light-microscopy images were visualized on a Nikon microscope using the 4× and 10× objective lenses and with 10× binoculars, and Nikon digital camera images were captured using ACT-2U software. The camera images were captured as TIFF files, cropped and arranged using Adobe Photoshop and Illustrator software. All imaging was performed at room temperature.

SDS PAGE, western blotting and RT-PCR

For SDS-PAGE and western blotting analyses, conditioned medium and/or cell lysates were collected and treated as described (Johnson and Terkeltaub, 2005). Primary antibodies against type X collagen (Calbiochem, San Diego, CA), TG2 and FXIIIA (Neomarkers, Freemont, CA), phosphorylated FAK ($Try^{567,577}$), FAK, phosphorylated p38, p38 (Cell Signaling, Beverly, MA), Xpress (Invitrogen, San Diego, CA) and tubulin were used at 1:1000 dilution in western blotting studies and detected as described (Johnson et al., 2003). The monoclonal antibody against α1-integrin subunit (TS2/7) (Genetex, San Antonio, TX) was used for immunoprecipitation in addition to immunofluorescent staining. The antibody against FB12 α1-integrin subunit (Chemicon/Millipore, Billerica, MA), a validated blocking antibody, was used to pre-treat chondrocytes for 1 hour prior to stimulation, as indicated.

Total RNA was isolated as described (Johnson et al., 2003). For quantitative RT-PCR, 1 μl of a 1:5 dilution of the cDNA from reverse-transcription reactions was amplified using the LightCycler FastStart DNA MasterPlus SYBR Green I kit (Roche Diagnostics, Indianapolis, IN) with addition of 0.5 μM of each primer in the LightCycler 2.0 (Roche Diagnostics, Indianapolis, IN). Following amplification, a monocolor relative quantification of the target gene and reference (GAPDH) analysis determined the normalized target gene: *GAPDH* mRNA copy ratios by the manufacturer's LightCycler Software (Version 4.0). The primers employed where designed using LightCycler Probe software, version 2.0 (Roche, Diagnostics, Indianapolis, IN).

Assays of transamidation activity and TG externalization

TG transamidation activity was determined as previously described (Johnson et al., 2003). TG2 on the plasma membrane was quantified in cells fixed with 4% PFA for 15 minutes, followed by detection by direct ELISA using biotin-labeled TG2-specific antibody CUB7402 or FXIIIA-specific antibody (Neomarkers, Freemont, CA).

Statistical analyses

Statistical analyses were performed using the Student's *t*-test (paired two-sample testing for means). Error bars, where indicated, represented standard deviation.

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