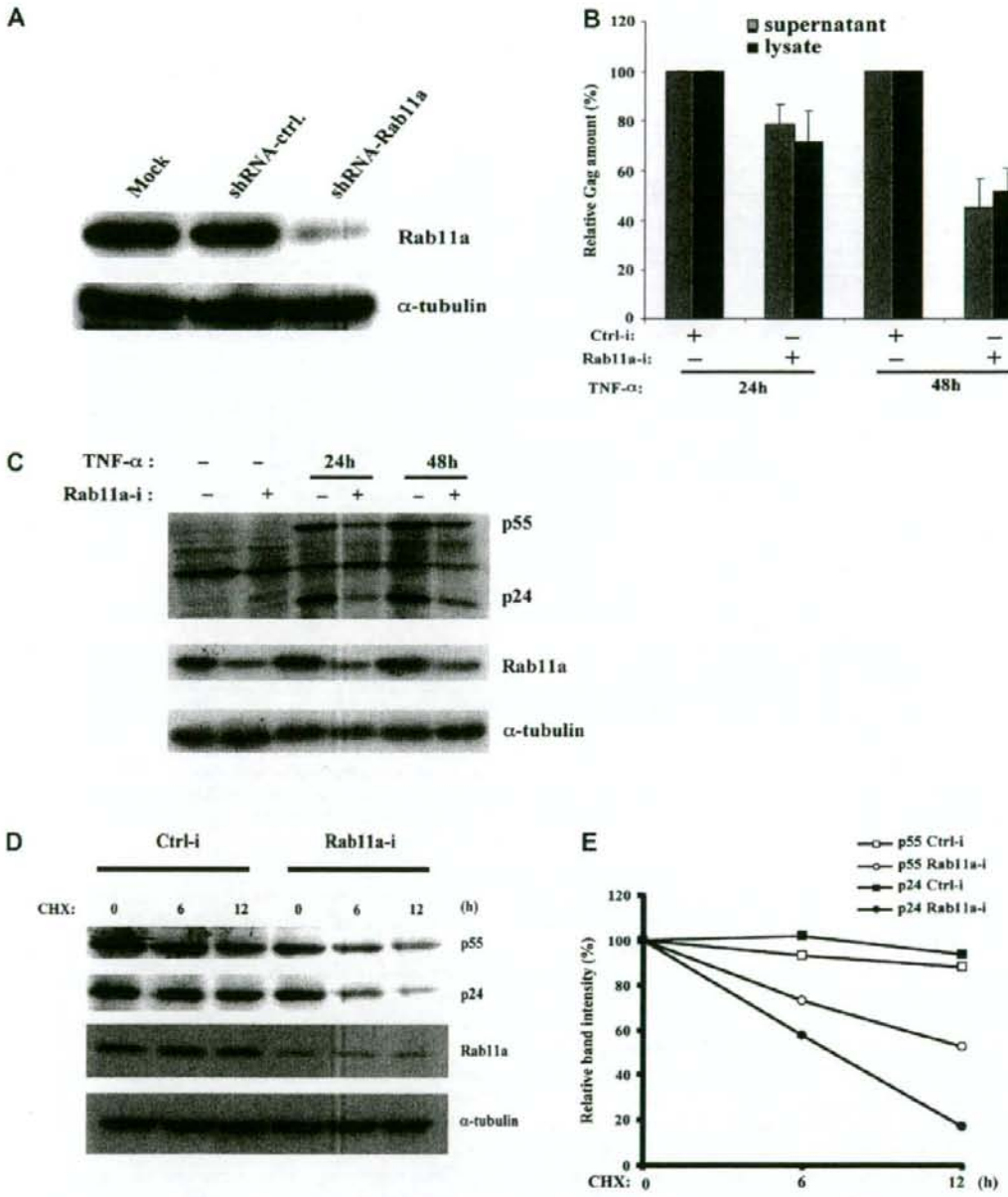

Fig. 5. Depletion of Rab11a destabilized HIV-1 Gag in U1 cells. (A) Whole-cell lysates (30 μg) prepared from U1 cells expressing control (Ctrl-i) or *rab11a*-specific (Rab11a-i) shRNA were subjected to 12% SDS–PAGE and immunoblotting with anti-Rab11a and anti-α-tubulin antibodies. (B) U1 cells expressing Ctrl-i or Rab11a-i were stimulated with TNF-α (1 ng/ml) for 24 or 48 h. Gag in supernatants and cell lysates was quantified, and the relative amounts of Gag are shown in percentage of that for cells expressing Ctrl-i (arbitrarily set at 100%). (C) Whole-cell lysates (30 μg) were prepared 24 or 48 h after TNF-α stimulation, and subjected to immunoblotting with the HIV-1-infected patient's serum, anti-Rab11a or anti-α-tubulin antibodies. (D) U1 cells expressing Ctrl-i or Rab11a-i were stimulated with TNF-α (1 ng/ml) for 24 h. Cells were then treated with cycloheximide (50 μM) for the indicated periods of time and whole-cell lysates (30 μg) were subjected to immunoblotting with the HIV-1-infected patient's serum, anti-Rab11a or anti-α-tubulin antibodies. (E) The relative band intensities of Gag (p55 and p24) normalized by that of α-tubulin are shown in percentage of that of cells before the addition of cycloheximide (arbitrarily set at 100%).

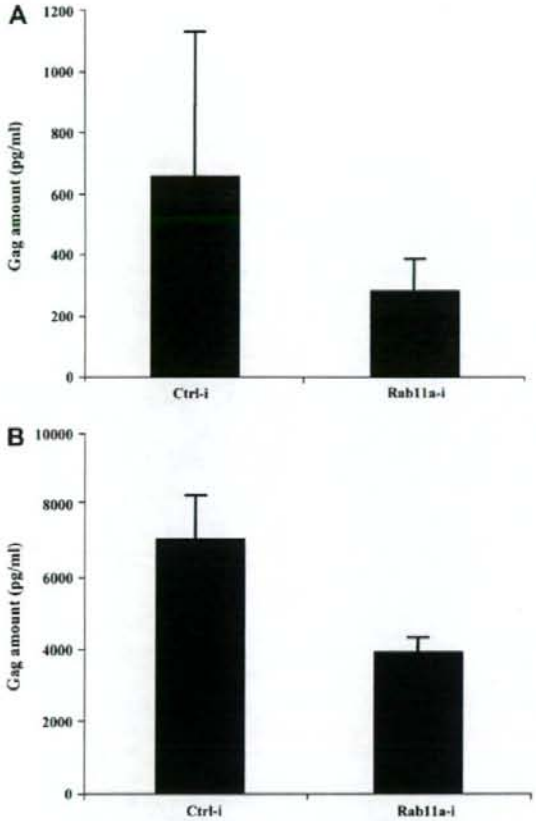

Fig. 6. Depletion of Rab11a reduced HIV-1 production in NOG mice. NOG mice (four mice in each group) were injected intraperitoneally with approximately 2.5×10^6 U1 cells expressing either Ctrl-i or Rab11a-i. The amounts of Gag in serum (A) and ascites (B) were quantified 2 weeks after cell inoculation.

be therapeutically more beneficial, because GGTIs do not work specifically on particular GTPases.

In summary, the present study revealed a critical role for protein geranylgeranylation in HIV-1 virion release from chronically HIV-1-infected promonocytic cells, and suggest that geranylgeranyltransferase-1 or its substrates, small GTPases involved in Gag trafficking, could be attractive molecular targets for controlling HIV-1 replication.

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The prolyl isomerase Pin1 stabilizes the human T-cell leukemia virus type 1 (HTLV-1) Tax oncoprotein and promotes malignant transformation

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ABSTRACT

The HTLV Tax protein is crucial for viral replication and malignant transformation. We investigated the possible role of peptidyl prolyl isomerase Pin1 in the positive regulation of the human T-cell leukemia virus type 1 Tax. Pin1 is highly expressed in adult T-cell leukemia (ATL) cells expressing Tax protein and forced expression of Pin1 in turn increases the Tax protein expression. Pin1 prolonged the protein half-life of Tax by suppressing the ubiquitination and subsequent lysosomal degradation of Tax. Pin1 interacts with phosphorylated Tax on its Ser160-Pro motif at the mitotic phase. Finally, we found that Pin1 plays a supporting role in Tax-mediated cell transformation. Our current study demonstrates an important role for Pin1 in the post-translational regulation of Tax and suggests that the targeting of Pin1 may offer a new insight into the pathogenesis of HTLV-1 related diseases.

Introduction

The peptidyl-prolyl *cis/trans* isomerase (PPIase) Pin1 binds only to phospho-Ser/Thr-Pro motifs on its substrate proteins, thereby catalyzing the *cis/trans* isomerization of the peptide bond and acting as a post-phosphorylation catalyst in the regulation of protein function [1]. Pin1 is highly expressed in various cancers and its deregulation of Pin1 may play a pivotal role in these diseases [1,2]. For instance, it has been reported that Pin1 positively regulates both colorectal and mammary tumorigenesis by increasing β-catenin and cyclin D1 expression [3]. Also, our recent studies have demonstrated that Pin1 acts as a putative anti-apoptotic molecule by the negative regulating Daxx in malignant tumor cells [4].

Human T-cell leukemia virus type 1 (HTLV-1) is the etiologic agent of the aggressive and fatal malignancy of CD4+ lymphocytes known as adult T-cell leukemia (ATL) [5]. During the carrier state in HTLV-1-mediated malignancies such as ATL, the oncoprotein Tax has been shown to play an essential role in the cell proliferation and oncogenesis [6]. Tax is a 40-kDa viral regulatory protein and plays critical roles in the activation of various cellular genes and for viral gene expression, replication and transformation [7]. Tax is a phosphoprotein with a predominately nuclear subcellular localization that accomplishes multiple functions via protein–protein interactions [8].

In our current study, we have investigated the regulation of Tax by Pin1 through phosphorylation-dependent prolyl isomerization. Our results demonstrate that Pin1 physically interacts with Tax, especially during the mitotic phase there by inhibiting both the ubiquitination and the lysosomal degradation of Tax. We also demonstrate that the phosphorylation of Tax on Ser160-Pro motif by mitotic kinase(s) is crucial for both its interaction with Pin1 and its prolonged stabilization. Of importance, a soft agar colony transformation assay using CTLL-2 cells demonstrated that Pin1 increases the transformation activity of Tax whereas the targeted inhibition of Pin1 significantly suppressed the Tax-mediated cell transformation. Hence, our current data provide the evidence that Pin1 plays a critical role in the post-translational regulation of HTLV-1 Tax.

Materials and methods

Cells. HTLV-1-transformed cells and T-lymphocytes were maintained in RPMI supplemented with 10% fetal calf serum, 2 μM L-glutamine and penicillin/streptomycin. 293T cells were maintained in DMEM supplemented with 10% fetal calf serum, 2 μM L-glutamine and penicillin/streptomycin. Mouse T-lymphocyte CTLL-2 cells were kindly provided by Dr. Masahiro Fujii (Niigata University, Japan) and maintained in RPMI supplemented with 10% fetal calf serum, 1 nM IL-2, 50 nM 2-ME, 2 μM L-glutamine and penicillin/streptomycin.

Transfection and luciferase assay. Transient transfections were carried out using Effectene Transfection Reagent (Qiagen). Luciferase assays have been described previously [9].

RT-PCR. Total RNA was isolated using the RNeasy Mini kit (Qiagen). RT-PCR was performed using the Takara one-step RT-PCR kit, following the manufacturers' instructions.

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Protein degradation assay. Protein degradation assays were performed as described previously [10]. Briefly, cycloheximide (100 μg/ml) was added to the medium 24 h after cell transfection, and the cells were harvested at different time points.

In vitro and in vivo protein binding assays. GST pull-down and co-immunoprecipitation assay were carried out as described previously [4].

Cell cycle analyses. Cell cycle was analyzed for DNA content using FACSCalibur (Becton Dickinson Bioscience). Cells were stained with propidium idodide (50 μg/ml) after RNase treatment as described previously [11].

Colony formation assay. Colony formation assays were performed with CTLL-2 cells infected with retrovirus carrying pMRX-ires-blst-Tax and/or pMRX-ires-puro-Pin1, and pMRX-ires-blst-Tax and/or pSuper-retro-puro-Pin1 siRNA in soft agar media using a CytoSelect 96-well cell transformation assay kit (Cell Biolabs) according to the manufacturer's instructions.

Results

Pin1 increases HTLV-1 Tax-mediated transactivation

To examine whether Pin1 influences Tax-mediated transcriptional activation, 293T cells were transiently transfected with luciferase reporter constructs, HTLV-1 LTR- or NF-κB-Luc in the absence or presence of Tax and/or Pin1. As expected, the expression of Tax increased the activation of both the HTLV-1 LTR and NF-κB (Fig. 1A and B, lane 2). In contrast, the expression of Pin1 had no significant effect upon the viral LTR and NF-κB (lane 3). Interestingly, the co-expression of Tax and Pin1 produced a significant increase in the reporter activity for both HTLV-1 LTR- and NF-κB-Luc, indicating that Pin1 increases Tax-mediated transcriptional activation (lane 4). These data were further confirmed in HTLV-1-transformed Hut 102 cells expressing endogenous Tax (Fig. 1C). Hut 102 cells infected with a retroviral vector carrying Pin1 siRNA decreased the HTLV-1 LTR luciferase activity compared with cells infected with control siRNA. These data thus demonstrate that Pin1 enhances Tax-mediated transactivation in HTLV-1-transformed cells.

Pin1 overexpression facilitates the protein stability of HTLV-1 Tax

Next, we sought to examine the expression of Pin1 in HTLV-1-transformed cells and T-lymphocytes (Fig. S1A). Pin1 was found to be highly expressed in Tax-expressing HTLV-1-transformed cells M8166, Hut 102, MT-1, MT-2 and MT-4 compared with Tax-non-expressing HTLV-1-transformed cells ATL-43Tb(−) and T-lymphocytes Jurkat, Molt-4 and CEM cells. To further confirm this contention, we performed Western blot analysis and RT-PCR using 293T/Pin1 siRNA cells transfected with Pin1 and Tax. Pin1 significantly increased the protein expression of Tax in a dose-dependent manner (Fig. S1B) but the Tax mRNA levels showed no significant change (Fig. S1C). These results suggest that Pin1 regulates the expression of the Tax protein via a post-translational mechanism.

To analyze whether Pin1 can stabilize the Tax protein, we performed a protein degradation assay by treating cells with cycloheximide (CHX). First, 293T cells stably expressing either control or Pin1 siRNA cells were transiently co-transfected with Tax and HA-LacZ as an internal control (Fig. 2A). Cycloheximide was then added 24 h post-transfection. Cells were collected at 0, 2, 4, 6 or 8 h and Western blot analyses were performed for Tax, Pin1 and HA-LacZ. This experiment revealed that the protein stability of Tax was significantly reduced in 293T/Pin1 siRNA cells compared with control cells, indicating that Pin1 indeed enhances the protein stability of Tax. Consistent with this result, the stability of Tax was found to be restored by Pin1 overexpression in 293T/Pin1 siRNA cells (Fig. 2B).

Two different pathways, proteasome- and lysosome-dependent, are principally responsible for intracellular protein degradation [12]. To address whether Tax degradation is mediated by either pathway, we performed parallel experiments using either the proteasome inhibitor MG-132 or the lysosome inhibitor NH_4Cl (Fig. S2A). Treatment with NH_4Cl significantly inhibited the rapid degradation of Tax in 293T/Pin1 siRNA cells, whereas the treat-

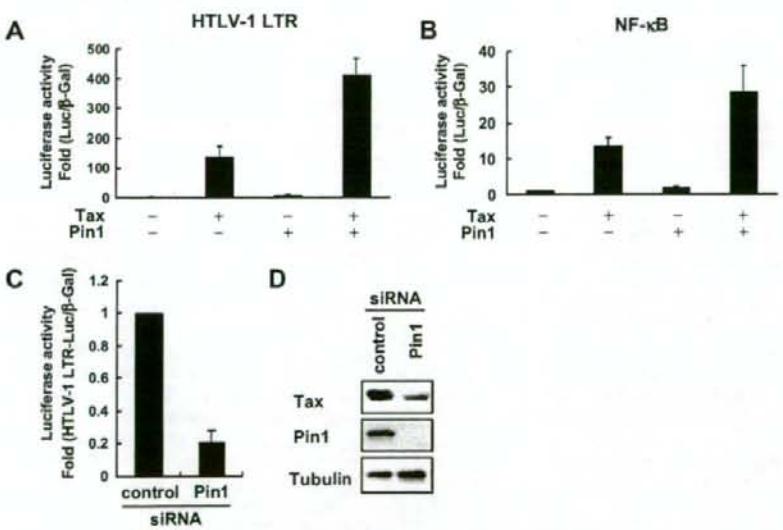

Fig. 1. Pin1 enhances HTLV-1 Tax-mediated transcriptional activation. (A,B) 293T cells were transiently transfected with plasmids expressing Tax, Pin1 and the reporter constructs HTLV-1 LTR- (A) or NF-κB-Luc (B). (C) Hut 102 cells were infected with either control or Pin1 siRNA molecules using the pSuper-Retro-puro-vector and then transiently transfected with HTLV-1 LTR-Luc. Forty-eight hours post-transfection, cells were collected and luciferase activities were measured. Luciferase values were adjusted for transfection efficiency using RSV β-galactosidase. (D) Western blot analysis of Hut 102 cells was performed for Tax, Pin1 and tubulin.

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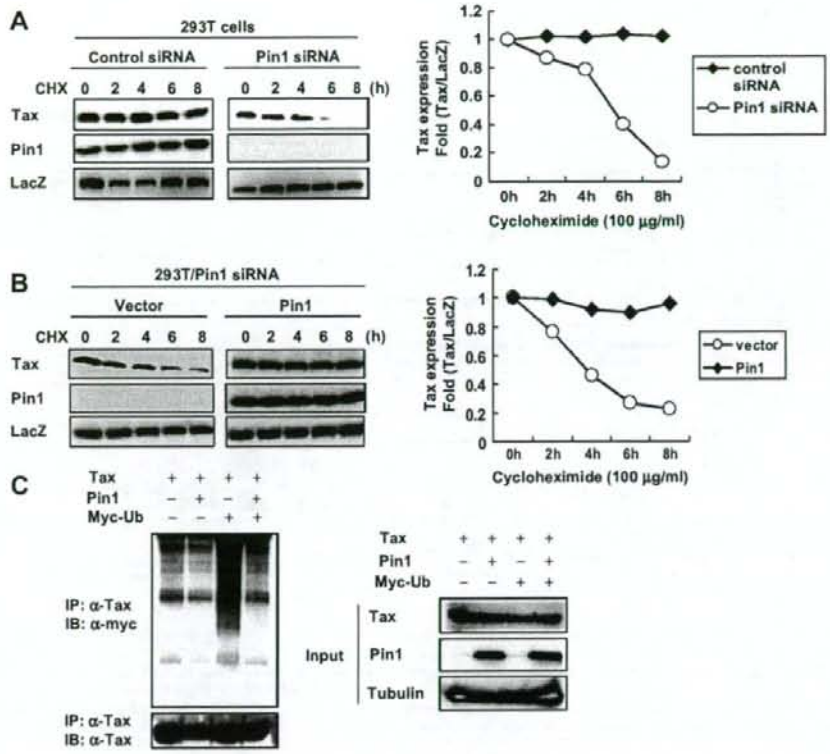

Fig. 2. Pin1 overexpression inhibits both the degradation and the ubiquitination of Tax. (A,B) 293T cells stably expressing control siRNA or Pin1 siRNA molecules were transfected with Tax and HA-LacZ (A). 293T/Pin1 siRNA cells were transfected with empty vector or Pin, Tax and HA-LacZ (B). Cells were treated with cycloheximide (CHX) after 24 h, and harvested at the indicated time points. This was followed by Western blotting analysis with anti-Tax, Pin1 and HA antibodies. The data from the quantitative analyses are shown in the graphs. (C) 239T cells were transfected with either empty vector or Tax in the absence or presence of Myc-Ub. Whole cell lysates were immunoprecipitated with anti-Tax or anti-Myc and immunoblotted with these same antibodies.

ment with MG-132 did not prolong the protein half-life of Tax compared with DMSO control. Additional lysosome inhibitors (leupeptin and chloroquine) further confirmed that the protein degradation of Tax is lysosome-dependent (Fig. S2B).

Pin1 overexpression inhibits the ubiquitination of Tax

To explore the effects of Pin1 upon the ubiquitination of Tax immunoprecipitation analyses of 293T/Pin1 siRNA cells transfected with Tax and/or Pin1 in the absence or presence of ubiquitin revealed that Pin1 overexpression blocks the ubiquitination of Tax (Fig. 2C). In an additional experiment to examine the importance of Pin1 in the suppression of Tax ubiquitination, HTLV-1-transformed Hut 102 cells were treated with the Pin1 inhibitor Juglone or infected with retroviral Pin1 siRNA and processed for immunoblot analysis with anti-Tax antibody which allow the detection of both wild type and ubiquitinated Tax (Fig. S3). The results indicated an increase in the level of polyubiquitinated Tax resulting from the blockage of Pin1. Our data thus confirm that Pin1 suppresses the destabilization and also the ubiquitination of Tax.

Pin1 interacts with Tax during the mitotic phase

The interaction between Tax and Pin1 was determined by GST pull-down assay. The results demonstrate that GST-Pin1, but not GST interacts with Tax in cell lysates derived from DMSO control cells (Fig. 3A). Of interest, this interaction was significantly increased when cells had been treated with nocodazole (M-phase blocker), but not with hydroxyurea (G1–S-phase blocker), suggesting that cell cycle arrest at mitotic phase facilitates this interaction.

The results of a co-immunoprecipitation assay provide further evidence for the interaction between Pin1 and Tax (Fig. 3B). Hut 102 cells were incubated with or without nocodazole to induce mitotic arrest and immunoprecipitated with either control IgG or anti-Pin1 antibody. The results demonstrate that a significant interaction between Pin1 and Tax occurs at the mitotic phase. The mitotic arrest of cell cycle with nocodazole was determined by the level of cyclin B1 (Fig. 3B) and flow cytometry analysis (Fig. 3C), respectively. In addition, the interaction between Pin1 and Tax was significantly decreased by the treatment with alkaline phosphatase (AP), suggesting that Pin1 binds to only phosphorylated Tax (Fig. S4A). A parallel analysis of Tax and Pin1 binding was performed to test whether either the binding or catalytic activity of Pin1 is necessary for the interaction between Pin1 and Tax. We used 293T/Pin1 siRNA cells transfected with Tax and either empty vector, Pin1 wild type or the Pin1 mutants W34A (WWdomain mutant) and K63A (PPIase domain mutant) (Fig. S4B). The results demonstrate that the WW domain of Pin1 is required for the binding to Tax.

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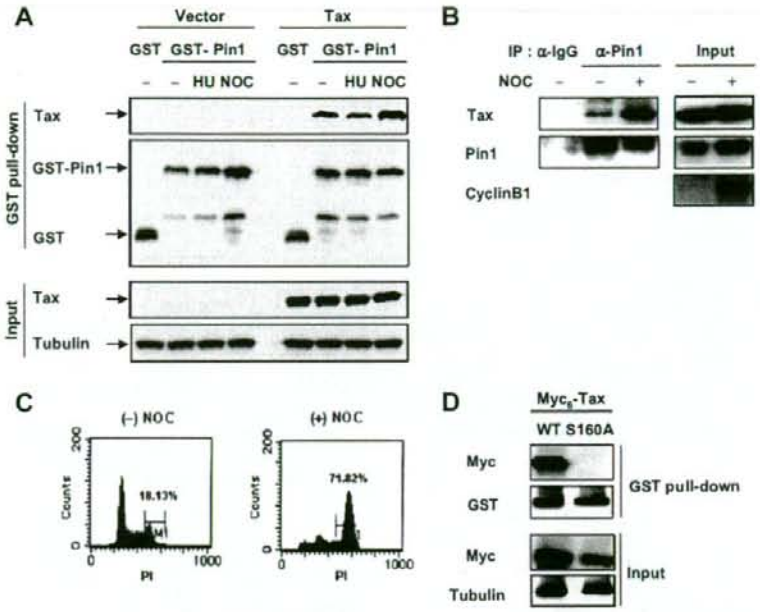

Fig. 3. Pin1 physically interacts with phosphorylated Tax in the mitotic phase *in vitro* and *in vivo*. (A) 293T cells were transfected with either empty vector or Tax and treated with hydroxyurea (HU) or nocodazole (NOC). Cell lysates were prepared, subjected to a pull-downed with purified GST or GST-Pin1 and immunoblotted with anti-Tax and GST antibodies. (B) Hut 102 cells were treated with or without NOC. Whole cell lysates were immunoprecipitated with IgG control or anti-Pin1 antibody and then immunoblotted with anti-Tax and anti-Pin1 antibodies. (C) Hut 102 cells treated with or without NOC were fixed in 70% ethanol, stained with propidium iodide (PI) and their cell cycle distribution was analyzed by flow cytometry. (D) 293T cells were transfected with Myc_6-Tax wild type or the Tax S160A mutant for 48 h. Cell lysates were prepared, pulled-down with purified GST or GST-Pin1 and immunoblotted with anti-Myc and GST antibodies. A Western blot for Tax and tubulin is also shown.

The HTLV-1 Tax Ser160-Pro motif is important for the interaction with Pin1 and subsequent protein stabilization by Pin1

To identify the region of Tax that binds to Pin1, we generated several Tax deletion mutants (Fig. S5A). Results of the GST pull-down assay shown in Fig. S5B indicate that Pin1 binds to Tax in the region between amino acids 157 and 190. Pin1 binds to only phosphorylated Ser/Thr-Pro motifs within its target proteins [1,13]. Since there is only single site of Ser/Thr-Pro motif (Ser160-Pro161) between amino acids 157 and 190 in Tax, we introduced a site-specific mutation into Ser160 site by substitution of Ala for Ser (S160A). GST pull-down analysis revealed that Pin1 does not bind to Tax S160A, indicating that Pin1 binds to phosphorylated Ser160-Pro motif of Tax (Fig. 3D). In addition, cycloheximide analysis demonstrated that Pin1 fails to stabilize the S160A mutant of Tax (Fig. S6A). Mutant S160A decreased the transcription of both HTLV-1 LTR and NF-κB by 50% compared with wild type Tax, suggesting that the phosphorylation of Tax on Ser-160-Pro motif indeed plays a role in regulating the transactivation of HTLV-1 LTR (Figs. S6B and C).

Taken together, these data suggest that the direct interaction between Pin1 and Tax via the Ser 160-Pro motif suppresses the degradation of Tax and thereby enhances its transcriptional activity.

Pin1 promotes Tax-mediated transformation in CTLL-2 cells

Cellular transformation assay was performed using the mouse T-lymphocytes CTLL-2 cells, which are interleukin (IL)-2 dependent cells to explore whether Pin1 can regulate Tax-mediated transformation. Results showed that CTLL-2 cells infected with retroviral Tax alone showed increased their transformation whereas Pin1 alone had no detectable effect (Fig. 4A). Significantly, co-infection with the retroviral Tax and Pin1 vectors resulted in an increase in transformation, suggesting that Pin1 positively affects the Tax-mediated transformation of CTLL-2 cells. We found by Western blot analysis that Tax expression was stabilized in the presence of Pin1.

A parallel assay was performed to further confirm the importance of Pin1 for Tax-mediated transformation using CTLL-2 cells infected with retroviral vectors expressing either control or Pin1 siRNA and Tax. The results showed that the suppression of Pin1 significantly decreased the transformation efficiency of Tax (Fig. 4B). Taken together, these results indicate that Pin1 plays a positive role in the induction of Tax-mediated transformation via its stable interaction with HTLV-1 Tax protein.

Discussion

The peptidyl prolyl isomerase Pin1 regulates a variety of cellular processes, including cell growth, cell cycle progression and cellular stress responses [14,15] and is linked to the progression of several diseases, including cancer, Alzheimer's disease and asthma [1,13,16,17]. In this regard, it is of interest that in recent studies Pin1 has also been shown to play an important role in virus-related diseases [18,19]. Of interest, Pelopenese et al. reported that Pin1 interacts with the HTLV-1 Tax which is a key factor in leukemogenesis and modulates its activation of NF-κB [20]. In agreement with this study, we also show that Pin1 binds to phosphorylated Tax and positively regulates the stabilization of Tax, thus promoting Tax-mediated transformation. In addition, we identified a novel phosphorylation site on Ser160 of Tax and showed that it is targeted

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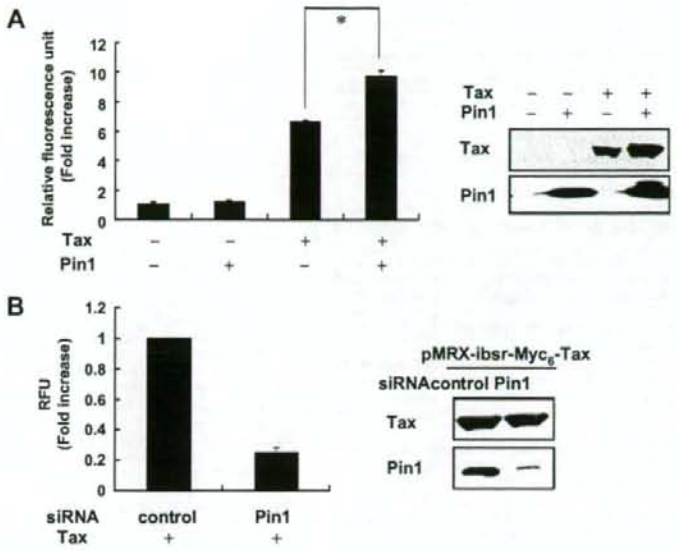

Fig. 4. Pin1 promotes the transformation of CTLL-2 cells by Tax. CTLL-2 mouse T-lymphocytes were infected with retroviruses expressing Tax and/or Pin1 (A) and control or Pin1 siRNA, and Tax (B) and incubated in soft agar media for 6–8 days. The formation of colonies was detected with the plate reader using a 485/520 nm filter set. A Western blot analysis for Pin1 and Tax is also shown. Asterisk means the value of Student's *t*-test ($^{*}p < 0.001$).

by Pin1-mediated prolyl isomerization by a mapping of Tax binding region to Pin1.

It was recently reported that, by phosphoryl mapping of Tax that both positive and negative phosphorylation signals for this protein result in the maintenance of an "active" subfraction, suggesting the importance of phosphorylation in the regulation of Tax activity [21]. However, it has not been well characterized how the activity of Tax is further regulated by phosphorylation in the cells. Our data demonstrate that Pin1 fails to stabilize a Tax mutant with alanine substitution at Ser160 (S160A), suggesting that the interaction between Pin1 and Tax is critical for the protein stability of Tax. Similarly, it has been demonstrated previously that Pin1 directly binds to phosphorylated cyclin D1 that has been phosphorylated by glycogen synthase kinase-3β (GSK-3β) and thereby prevents its nuclear export and ubiquitin-mediated degradation of cyclin D1 [22]. Pin1 also targets the phosphorylated Thr254-Pro motif of NF-κB p65 and inhibits the binding of p65 and IκB, which subsequently prevents its degradation by a ubiquitin-mediated pathway [10].

Intracellular protein stabilization is mainly regulated by the ubiquitin-proteasome pathway [23]. Several studies have been shown that Tax is ubiquitinated and physically interacts with the proteasome [24–26]. Of interest, Chiari et al. reported that Tax is not degraded by ubiquitination and that Tax conjugation to ubiquitin in fact mediates a non-proteolytic function of this protein [24]. Our current data suggest that the degradation of Tax is accomplished by a ubiquitin-dependent lysosomal pathway. The degradation of Tax was blocked by the treatment with the lysosome inhibitors NH_4Cl, leupeptin and chloroquine, but not by the proteasome inhibitors MG-132, MG-115 and lactacystin. Importantly, we observed that Pin1 overexpression inhibits the ubiquitination of Tax, further indicating that Pin1 prevents Tax degradation via the ubiquitin–lysosome pathway. Marques et al. previously reported the ubiquitin-dependent lysosomal degradation of the 3-hydroxynonenal (HNE)-modified protein in lens epithelial cells [27]. The authors in this study speculated that the ubiquitin-conjugating system may serve as a common mechanism for recognizing various types of modified or damaged proteins. These ubiquitinated proteins can then be degraded by either the proteasome or through lysosomes although the mechanisms underlying the different fates of these ubiquitinated proteins remain unclear.

Because the HTLV-1 Tax protein is essential for the cellular transformation as well as viral replication [7], it was important from a clinical perspective to identify the mechanisms underlying the activities of this protein. To elucidate the physiological role of Pin1 in the regulation of Tax, we performed a colony transformation assay using CTLL-2 primary mouse T-lymphocytes. Our results demonstrate that Pin1 promotes Tax-mediated cellular transformation and also stabilizes the Tax protein.

In conclusion, we herein demonstrated a novel regulatory mechanism for HTLV-1 Tax that is mediated via Pin1-catalyzed-phosphorylation-dependent prolyl isomerization. Phosphorylation of Tax at Ser160-Pro motif promotes its interaction with Pin1 during the mitotic phase of cell cycle, which stabilizes the Tax protein by inhibiting its ubiquitination and thereby promotes malignant transformation. Further studies will be required to identify the specific kinase(s) responsible for phosphorylation of the Ser160-Pro Pin1 binding motif of Tax. Our present analyses suggest that the targeting of Pin1 may offer a new insight into the pathogenesis and treatment of HTLV-1 related diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.02.024.

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Research article

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Low-intensity pulsed ultrasound activates the phosphatidylinositol 3 kinase/Akt pathway and stimulates the growth of chondrocytes in three-dimensional cultures: a basic science study

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Abstract

Introduction The effect of low-intensity pulsed ultrasound (LIPUS) on cell growth was examined in three-dimensional-cultured chondrocytes with a collagen sponge. To elucidate the mechanisms underlying the mechanical activation of chondrocytes, intracellular signaling pathways through the Ras/mitogen-activated protein kinase (MAPK) and the integrin/phosphatidylinositol 3 kinase (PI3K)/Akt pathways as well as proteins involved in proliferation of chondrocytes were examined in LIPUS-treated chondrocytes.

Methods Articular cartilage tissue was obtained from the metatarso-phalangeal joints of freshly sacrificed pigs. Isolated chondrocytes mixed with collagen gel and culture medium composites were added to type-I collagen honeycomb sponges. Experimental cells were cultured with daily 20-minute exposures to LIPUS. The chondrocytes proliferated and a collagenous matrix was formed on the surface of the sponge. Cell counting, histological examinations, immunohistochemical analyses and western blotting analysis were performed.

Results The rate of chondrocyte proliferation was slightly but significantly higher in the LIPUS group in comparison with the control group during the 2-week culture period. Western blot analysis showed intense staining of type-IX collagen, cyclin B_1 and cyclin D_1, phosphorylated focal adhesion kinase, and phosphorylated Akt in the LIPUS group in comparison with the control group. No differences were detected, however, in the MAPK, phosphorylated MAPK and type-II collagen levels.

Conclusion LIPUS promoted the proliferation of cultured chondrocytes and the production of type-IX collagen in a three-dimensional culture using a collagen sponge. In addition, the anabolic LIPUS signal transduction to the nucleus via the integrin/phosphatidylinositol 3-OH kinase/Akt pathway rather than the integrin/MAPK pathway was generally associated with cell proliferation.

Introduction

The degenerative abrasion of cartilage tissue due to aging and a malalignment of the lower extremities causes osteoarthritis. Moreover, articular cartilage is a tissue that is difficult to

3D = three-dimensional; DMEM = Dulbecco's modified Eagle's medium; FAK = focal adhesion kinase; FBS = fetal bovine serum; LIPUS = low-intensity pulsed ultrasound; MAPK= mitogen-activated protein kinase; PBS = phosphate-buffered saline; PCNA = proliferating cell nuclear antigen; PI3K= phosphatidylinositol 3-OH kinase.

regenerate once damaged. Many attempts have therefore been made to achieve regeneration of damaged cartilage tissue. Conservative treatments include physiotherapy, such as quadriceps muscle training, or the intra-articular injection of hyaluronic acid. The regeneration of normal cartilage tissue, however, has not yet been achieved [1]. The elements that promote the regeneration of cartilage include growth factors [2], soluble mediators [3], corrections of any malalignment and mechanical stimulation [4-6].

Surgical treatments include a high tibial osteotomy, the microfracture method, transplantation of osteocartilaginous plugs [7], and transplantation of cultured cartilage [8]. During the transplantation of cultured cartilage, a key part of the procedure is the *in vitro* preparation of high-quality cartilage tissue prior to transplantation [9]. Mechanical stimulation is one of the essential factors that promotes the differentiation and proliferation of intact chondrocytes as well as *in vitro* cultures for transplantation. Various methods of mechanical stimulation of chondrocytes have been reported, such as loading with hydrostatic pressure [10], the application of tensile stress against the culture scaffold [11], oscillation using a vibrator [12] and low-intensity pulsed ultrasound (LIPUS) [13-15].

The matrix surrounding the chondrocytes also plays an important role in the proliferation and survival of chondrocytes. Through this extracellular matrix, chondrocytes receive various kinds of extracellular information such as mechanical signals and hormonal mediators. Mechanical stimulation has been reported to activate chondrocytes and to promote their synthesis of the extracellular matrix. Few reports have focused on the signal transmission, however, which results in chondrocyte activation. To characterize these mechano-transduction pathways in chondrocytes, we have previously established a new three-dimensional (3D) culture system, which forms a tissue architecture similar to the structure of articular cartilage tissue *in vivo* [12]. The effects of vibration on chondrocytes were previously examined in this system, and the involvement of a mechano-transduction pathway via the integrin/mitogen-activated protein kinase (MAPK) pathway and of another signaling pathway via β-catenin was evaluated. Although many previous studies reported that osteoblasts are activated by LIPUS, which has been widely used in clinical settings to accelerate the process of fracture healing, its practical use for cartilage repair in a clinical setting is so far limited [16-18].

The present study demonstrates that the combination of the 3D chondrocyte culturing technique with LIPUS not only promotes the production of type-IX collagen, but also significantly increases the number of chondrocytes. In addition, the results indicate the potential involvement of the integrin/phosphatidylinositol 3-OH kinase (PI3K)/Akt pathway downstream of LIPUS exposure, rather than the integrin/MAPK/MAPK kinase pathway, which is generally involved in the induction of cellular proliferation.

Materials and methods

Cell cultures

Articular cartilage tissue was obtained from the metatarsophalangeal joints of freshly slaughtered 6-month-old pigs in a slaughterhouse. Articular cartilage slices were cut into smaller pieces, and the cartilage specimens were washed well in PBS (pH 7.4) and digested with 0.25% trypsin–ethylenediamine tetraacetic acid (Gibco, Grand Island, NY, USA) for 20 minutes. The resultant chondrocyte preparations were washed again with PBS to remove the trypsin, and were then incubated for about 8 hours in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 0.1% type-II collagenase (Worthington Biochemical Co., Lakewood, OH, USA), 10% heat-inactivated FBS (Equitech-Bio, Inc., Kerrville, TX, USA) and antibiotics. The chondrocytes were subsequently isolated and washed with culture medium, collected by centrifugation (2,000 rpm, 37°C, 5 min), and then mixed with 0.2% atelocollagen gel (type-I collagen derived from bovine tendons; Koken Co., Tokyo, Japan) containing culture medium (DMEM; Gibco).

Twenty-four-well plates containing type-I honeycomb collagen sponges (discs with a diameter of 15 mm and thickness of 2 mm; Koken Co.) at the bottom of each well were used as 3D carriers of the chondrocyte culture [19]. Chondrocytes in the atelocollagen gel and also chondrocytes in the culture medium composites were added to each sponge and were incubated at 37°C for 1 hour. The final cell density was adjusted to 2×10^6 cells/well/ml [12]. After the collagen sponge and cell–collagen gel composites became stiff, they were then incubated with 2 ml complete DMEM in 5% CO_2/95% air at 37°C, and the cultured medium was replaced with fresh DMEM containing L-ascorbic acid (50 μg/ml) twice weekly.

Low-intensity pulsed ultrasound stimulation

The sonic accelerated fracture healing system (Exogen Inc., Piscataway, NJ, USA), a LIPUS apparatus, was used to deliver an ultrasound signal. The sonic accelerated fracture healing system is one of the instruments in current clinical use in cases of delayed repair of a fracture. The temporal average intensity was 30 mW/cm² and the frequency was 1.5 MHz with a 200-μs tone burst repeated at 1.0 KHz. LIPUS was applied to the chondrocytes after 24 hours in culture through the bottom of the culture dish (24-well plate) via a coupling gel and silicon rubber that had been placed between the LIPUS transducer and the dish. LIPUS was administered for 20 minutes every day in a span of this experiment. Control samples were prepared in the same manner without LIPUS. Thereafter, the cultured tissues and their supernatant medium were harvested at days 3, 7, 10 and 14.

Cell counting

The cartilage tissues were harvested 1, 3, 7, 10 and 14 days after culture (2 hours after the last LIPUS) and were cut into smaller pieces. Each sample was then incubated for about 8

hours in DMEM (Gibco) supplemented with 0.1% type-II collagenase (Worthington Biochemical Co.), 10%-heat-inactivated FBS (Equitech-Bio, Inc.) and antibiotics. The chondrocytes were then isolated, washed with culture medium, and collected by centrifugation (2,000 rpm, 37°C, 5 min). After the supernatant medium was removed, a solution containing 0.1 M. citric acid and 0.1% crystal violet was added to the cells and then the cells were counted using a hemocytometer (Burker-Turk, Tokyo, Japan).

Histological examinations

Histological evaluations of the specimens were conducted at weeks 1 and 2 post culture. The specimens were fixed overnight in 4% paraformaldehyde in PBS, paraffin-embedded, sectioned to a 5 μm thickness, and were stained with Alcian blue and Safranin O. For each sample, at least two different section levels and two histological sections for each level were analyzed. The sections were analyzed and photographed using an Olympus photomicroscope BX-50 (Olympus Co., Tokyo, Japan).

Immunohistochemistry

Immunohistochemical analyses were conducted with antibodies raised against anti-type-II collagen antibody (1:100; Fuji Pharm. Lab., Toyama, Japan) and against anti-type-IX collagen (1:100; Chemicon International, Billerica, MA, USA) using week 1 and week 2 postcultures to evaluate the expression of the chondrocyte phenotype and also to assess the type-II and type-IX collagen production levels. The specimens from the 1-week and 2-week postcultures that were harvested 2 hours after the last LIPUS were fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.4), and 16-μm cryostat sections were made.

For further confirmation of chondrocyte growth, Ki67 staining was performed because this factor has been shown to be a very reliable proliferation marker [20]. The monoclonal mouse anti-human antibody Ki67 (MIB1; DAKO, Glostrup, Denmark), which also shows cross-reactivity with porcine tissues, was used to determine the extent of proliferation. The sections cultured at day 7 were incubated with this Ki67 primary antibody, followed by a secondary biotinylated anti-rabbit antibody and horseradish peroxidase–avidin complex (DAKO). The color reaction was developed by 3,3'-diaminobenzidine substrate, followed by counterstaining with hemalaun (Merck, Frankfult, Germany). Chondrocytes showing a definite nuclear staining pattern were scored as positive. All slides were reviewed by two investigators without any prior knowledge of the experiment. Five different randomly chosen areas were reviewed in five different specimens, and the number of Ki67-positive cells per 100 chondrocytes was counted in each slice. The percentages of positive cells (MIB1 index) were then calculated.

Quantitative evaluations were conducted using specimens stained with an anti-β-catenin antibody (Acris, Herford, Germany). The nuclear translocation of β-catenin was visible by brown staining. After counting 100 cells, the ratio of the cells whose nuclei were stained brown was compared between the ultrasound group and the control group. All slides were reviewed by two investigators without any prior knowledge of the experiment. In five different randomly chosen areas in five different specimens, the number of β-catenin antibody-positive stained cells per 100 chondrocytes was counted in each slice. The percentages of positive cells were then calculated.

Western blotting analysis

For the western blotting analysis of the specimens cultured for 1 week, cartilage tissues specimens were harvested 2 hours after the last LIPUS and were cut into smaller pieces. Each sample was then incubated for about 8 hours in DMEM (Gibco) supplemented with 0.1% type-II collagenase (Worthington Biochemical Co.), 10%-heat-inactivated FBS (Equitech-Bio, Inc.) and antibiotics. The chondrocytes were then isolated, washed with culture medium, and collected by centrifugation (2,000 rpm, 37°C, 5 min). After the supernatant medium was removed, the cells were rinsed with 200 μl PBS, filtered by centrifugation, and added to a 200 μl aliquot of 2× sample buffer (62.5 mmol/l Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mmol/l dithiothreitol, 0.01% bromophenol blue). The cell lysates were then boiled for 10 minutes at 75°C.

Equal amount of the proteins were separated on a 10% SDS–polyacrylamide gel at 200 V, 25 mA for 80 minutes and were transblotted to nitrocellulose membranes (Millipore, Billerica, MA, USA) using a wet transfer system (BIO-RAD, Hercules, CA, USA) at 200 V, 150 mA for 60 minutes. The membranes were blocked with blocking buffer (5% skimmed milk in TBS and 0.05% Tween 20 and Blocking One–P; Nacalai Tesque Inc., Kyoto, Japan) and were incubated with the following antibodies: anti-Akt (Rockland, Gilbertsville, PA, USA), anti-phospho-Akt (Cell Signaling Technology, Beverly, MA, USA), anti-MAPK and anti-phospho-MAPK (Cell Signaling Technology), anti-cyclin D_1 (Biosource, Camarillo, CA, USA), anti-cyclin B_1 and anti-focal adhesion kinase (anti-FAK; Upstate Cell Signaling Solutions, NY, USA), anti-phospho-FAK (Rockland), anti-collagen-II (Chemicon International), and anti-collagen-IX (Cell Signaling Technology).

After incubation with the corresponding horseradish peroxidase-conjugated secondary antibodies (dilution: 1/5,000), membranes were finally incubated with a chemiluminescent reagent (NEL103; Perkin Elmer Life Science, Fremont, CA, USA) and the signals produced were recorded on X-ray film (BIOMAX XAR Film, Rochester, Minesota, USA) for a densitometric analysis. The effects of PI3K inhibitor (LY294002; Cell Signaling Technology) and MEK1 inhibitor (PD98059; Cell Signaling Technology) for cell growth were studied. Chondrocytes were pretreated with MEK1 inhibitor (250 μM/ml) and PI3K inhibitor (250 μM/ml) for 12 hours and 24 hours, followed by stimulation with LIPUS for 20 minutes. Each sample was harvested 2 hours after LIPUS stimulation and the

influence of these inhibitors was judged by western blotting analysis of proliferating cell nuclear antigen (PCNA; DAKO).

Statistical analysis

Data are expressed as the mean ± standard deviation. Quantitative evaluations of Ki67-positive cells and β-catenin-positive cells were assessed by Mann–Whitney's U test. The change in the number of chondrocytes was assessed using repeated-measures analysis of variance. $P < 0.05$ was considered significant.

Results

Histological specimens

After 1 week of culture, cartilaginous tissue consisting of at least five cell layers was formed on the collagen sponges in both the control group and the LIPUS group (Figure 1a to 1d). Simultaneously, both the penetration of chondrocytes and the formation of an extracellular cartilage matrix were observed inside the collagen sponge. In addition, an extracellular matrix rich in proteoglycans and intensively stained with Alcian blue and Safranin O was observed surrounding the chondrocytes.

During week 2 of culture in the 3D system, the cartilaginous tissue in each specimen appeared thicker in comparison with week 1, and the volume of the extracellular matrix had also increased and formed a stable cartilaginous tissue. The thickness of the tissue in week 2 was found to be greater in the LIPUS group than in the control group, and the staining of the matrix, especially near the surface, was also more intense in the LIPUS group (Figure 1e to 1h). The ratio between the number of cells in the cartilage layer that had formed on the collagen sponge and in the sponge was approximately 2:1.

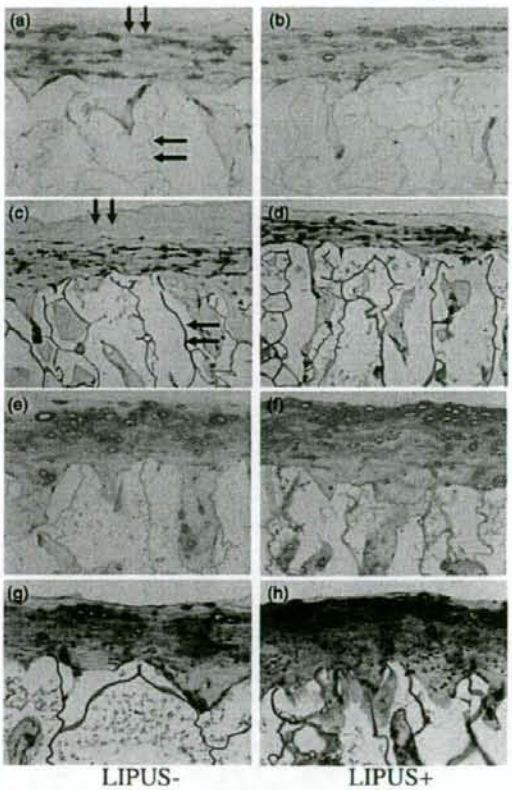
Growth curves of the chondrocytes

The initial results demonstrated that LIPUS facilitates the formation of a 3D structure of cartilage tissue, suggesting that increased cell proliferation had occurred. The effect of LIPUS on cell proliferation was therefore examined in the culture system. The number of live chondrocytes on day 0 was $(13.6 \pm 0.8) \times 10^5$ and $(12.9 \pm 0.6) \times 10^5$ cells in the control and LIPUS groups, respectively. A time-dependent increase in the total number of chondrocytes was noted, and on day 14 the cell counts were $(30.4 \pm 0.8) \times 10^5$ and $(33.0 \pm 1.7) \times 10^5$ in the control group and the LIPUS group, respectively. There was therefore a small but significantly greater increase in the cell number observed in the LIPUS group in comparison with the control group ($P < 0.01$; Figure 2).

Type-II collagen and type-IX collagen

Collagen is essential for the formation of cartilage tissue and also for the proliferation of chondrocytes. Furthermore, the current results demonstrated the formation of a thicker cartilaginous structure following LIPUS – suggesting that the increased secretion of extracellular matrix components such as the collagens had occurred.

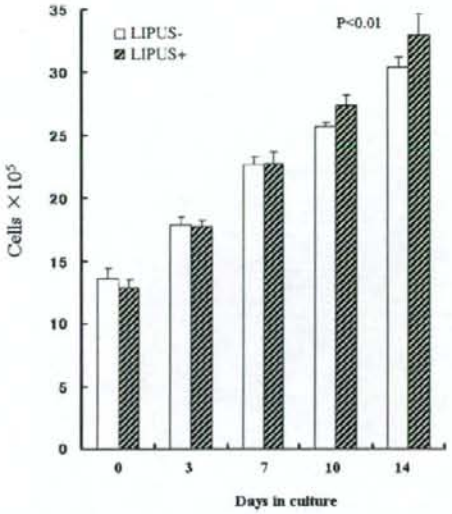
Figure 1



High-magnification sections of chondrocyte–collagen sponges 1 week after culture. Cartilage layers with a laminar structure on the collagen sponges (green arrows) and a grey structure, which represents the walls of collagen sponges (black arrows), are visible at high magnification (100×). **(a)** to **(d)** Specimens at week 1 of culture. (a) and (b) Alcian blue staining, and (c) and (d) Safranin O staining: many chondrocytes with blue-stained and red-stained peripheral matrices could be observed, respectively. The chondrocytes exhibited a layer structure, and their infiltration into the sponge can also be observed. **(e)** to **(h)** Specimens at week 2 of culture. (e) and (f) Alcian blue staining, and (g) and (h) Safranin O staining: many chondrocytes with blue-stained and red-stained peripheral matrices can be observed, respectively. The layer of chondrocytes that formed on the surface of the sponge was found to be thicker in comparison with the week 1 cultures, and the volume of the extracellular matrix had also increased. The cartilage tissue that formed on the surface of the sponge consisted of more than 10 layers of chondrocytes. The staining of the extracellular matrix in the LIPUS group was also found to be stronger than in the control group.

Following type-II collagen antibody staining, both the chondrocyte layers, which formed cartilaginous tissue on the collagen sponge, and the matrix formed inside the sponge were strongly positive in both the LIPUS group and the control group. There were also no apparent differences in the

Figure 2



Growth curves of the cells in the chondrocyte–collagen sponges (n = 7). A time-dependent increase in the number of chondrocytes can be seen in both the low-intensity pulsed ultrasound (LIPUS) group (US+) and in the control group (US-). The rate of increase in the chondrocytes number was significantly greater, however, in the LIPUS group in comparison with the control group ($P < 0.01$). The change in the number of chondrocytes was assessed using repeated-measures analysis of variance.

intensities of this staining between these two groups (Figure 3a to 3d).

Type-IX collagen antibody staining of the culture specimens showed the intensity of this staining in the chondrocyte layers on the sponge to be far stronger in the LIPUS group than in the control group after 2 weeks of culture, thus indicating an accumulation of type-IX collagen (Figure 3e to 3h).

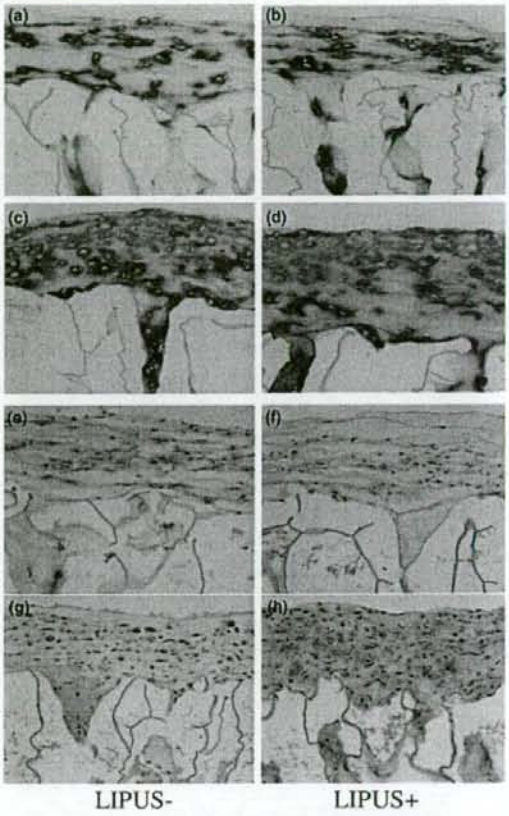
Ki67 and β-catenin

Immunohistochemical staining with Ki67 revealed distinctive labeling in the chondrocyte nuclei (Figure 4a,b). The cells with brown-stained nuclei were considered Ki67-positive. The large number of Ki67-positive cells indicated that LIPUS stimulated cell proliferation. In the cells in which β-catenin had translocated to the nucleus, brown nuclear staining with an anti-β-catenin antibody was evident (Figure 4c,d).

Quantitative evaluation of both Ki67-positive cells and β-catenin-positive cells

The Ki67 index of the chondrocytes exposed to LIPUS was found to be 48 ± 3.7%, in comparison with 41 ± 3.0% in the control group (Figure 5a), which was significantly different. The average percentage of β-catenin-positive cells with brown-stained nuclei (that is, positive cells) was determined to

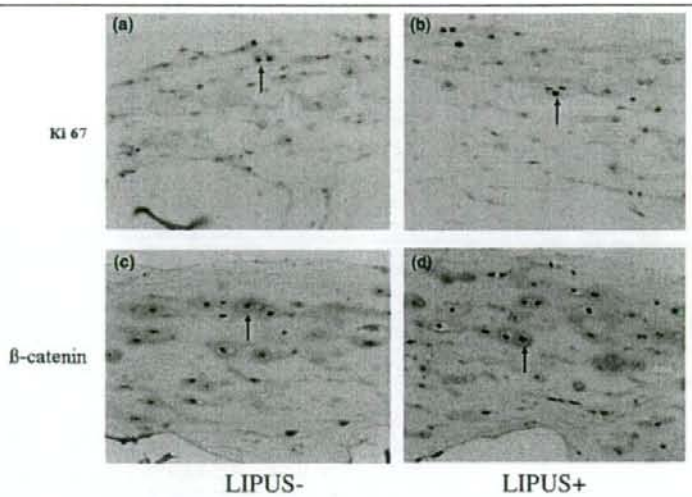
Figure 3



High-magnification sections of chondrocyte–collagen sponges 1 and 2 weeks post culture. Sections of chondrocyte–collagen sponges 1 and 2 weeks post culture at high magnification (anti-collagen antibody type-II and type-IX stain, 100×). **(a)**, **(b)** Anti-type-II collagen antibody staining of specimens after week 1 of culture. Brown staining of the matrix with anti-collagen type-II antibodies can be observed around the chondrocytes, indicating production of this collagen. **(c)**, **(d)** Anti-type-II collagen antibody-stained specimens after week 2 of culture. Strong brown staining of the matrix can be observed around the cells at a similar level in both groups. **(e)**, **(f)** Anti-type-IX collagen antibody-stained specimens after week 1 of culture. Positive brown staining of the matrix with anti-type-IX collagen antibodies can be observed around the cells, thus indicating the production of this collagen around the chondrocytes. **(g)**, **(h)** Anti-type-IX collagen antibody stained specimens after week 2 of culture. Positive brown staining of the matrix with anti-type-IX collagen antibodies can be observed around the cells, thus indicating production of this collagen around the chondrocytes.

be 42 ± 4.9% in the LIPUS group and 32 ± 2.7% in the control group. This indicated a significant difference between the two groups (Figure 5b).

Figure 4



Ki67 and β-catenin antibody staining. (a), (b) Anti-Ki67 antibody staining of week 2 cultures (200× magnification). The nuclei are positively stained with an anti-Ki67 antibody in both the control group (US-) and the low-intensity pulsed ultrasound (LIPUS) group (US+) (black arrows). **(c), (d)** Anti-β-catenin antibody staining of week 2 cultures (200× magnification). The nuclei are positively stained with an anti-β-catenin antibody in both the control group (US-) and the LIPUS group (US+) (black arrows).

Western blotting analysis

Collagen type-II

A western blot analysis showed immunoreactive bands for collagen type-II were observed at about 200 kDa, and were found to be present at a similar intensity in the LIPUS group and the control group (Figure 6a).

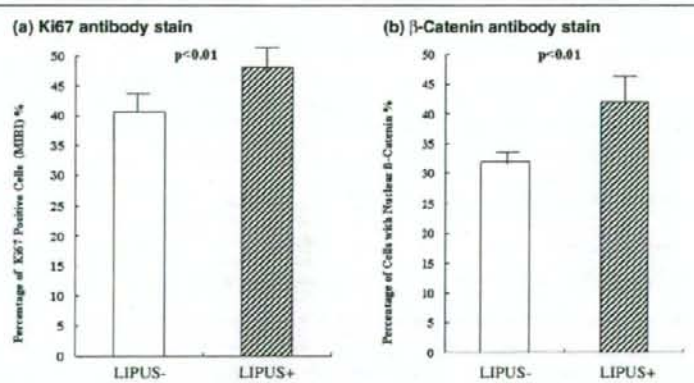
Collagen type-IX

An immunoreactive western band for collagen type-IX of about 110 kDa was detected at a higher level in the LIPUS group in comparison with the control group (Figure 6b).

FAK, phosphorylated FAK, Paxillin and phosphorylated Paxillin

Immunoreactive bands corresponding to FAK and phosphor-

Figure 5



Quantitative evaluation of Ki67-positive cells and β-catenin-positive cells. After counting 100 cells in each specimen in the low-intensity pulsed ultrasound (LIPUS) group (US+) and the control group (US-), the numbers of cells with positively stained nuclei were compared for both **(a)** Ki67 and **(b)** β-catenin. There were significantly more brown stained cells in the LIPUS group in both cases ($P < 0.01$).

ylated FAK were detected by western blotting at about 125 kDa (Figure 6c). Positive bands for Paxillin and its phosphorylated form were observed at about 68 kDa (Figure 6d). Although the levels of total FAK and Paxillin were similar with and without LIPUS exposure, the staining of their phosphorylated counterparts was stronger in the LIPUS group than in the control group (Figure 6c,d). These data thus indicate that LIPUS exposure results in the activation of both FAK and Paxillin.

MAPK and phosphorylated MAPK

Whereas MAPK and phosphorylated MAPK (p-42, p-44) were both detected in both the LIPUS group and the control group, there were no evident differences in the intensity (Figure 6e).

Akt and phosphorylated Akt

Akt, a cell survival signal, was found to be similarly expressed in both the LIPUS group and the control group but was observed to be phosphorylated to a greater extent in the LIPUS group (Figure 6f). These results indicate that LIPUS increased cell proliferation in this culture system by preferentially activating the PI3K/Akt pathway rather than the MEK/MAPK pathway.

Cyclin B_1 and cyclin D_1

Consistent with the increased chondrocyte growth, the expression of the cell proliferation markers cyclin D_1 and cyclin B_1 was enhanced in both cases by LIPUS. The expression of both of these cyclins was also detected at higher levels in the LIPUS group in comparison with the control group (Figure 6g). These results confirm that the increase in cell numbers in response to LIPUS coincide with the enhanced expression of these two cyclins.

Changes of proliferating cell nuclear antigen using MEK1 inhibitor and PI3K inhibitor

The influence of the MEK1 inhibitor (PD98059) and of the PI3K inhibitor (LY294002) was judged in western blotting analysis of PCNA. The expression of PCNA at 12 hours was decreased by PD98059 in the LIPUS-negative group and was detected at higher level in the LIPUS-positive group in comparison with the LIPUS-negative group, but the expression of PCNA at 24 hour was completely decreased by this inhibitor in both the LIPUS-negative and LIPUS-positive groups. Cell growth according to LIPUS is hypothesized to depend not only on a MAPK cascade but also on the effect of other signal transductions. The expression of PCNA at 12 and 24 hours, however, was completely decreased by PI3K inhibitor (LY294002).

Discussion

LIPUS promotes proliferation of chondrocytes

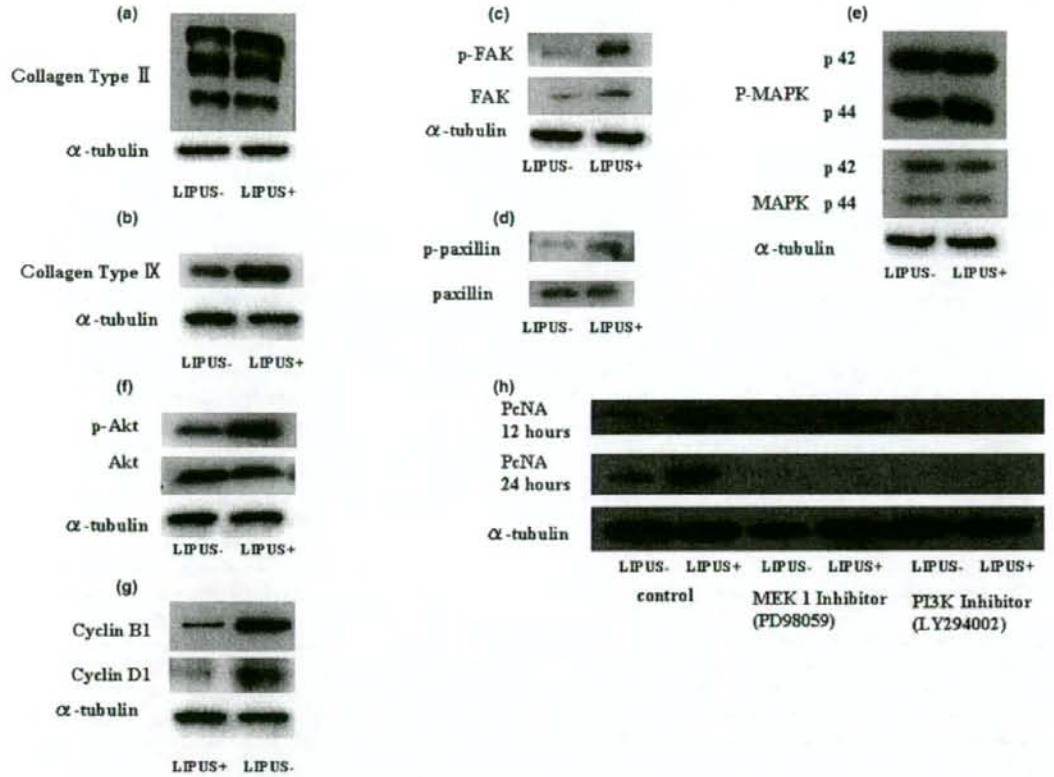
Previous studies indicated that LIPUS increases the production of the extracellular matrix around chondrocytes, but not the actual proliferation of the chondrocytes themselves. Zhang and colleagues have reported that although pulsed low-intensity ultrasound increases the number of hypertrophic chondrocytes around the callus of healing fractures, it does not alter the hyaline cartilage [15]. Nishikori and coworkers have also reported that chondrocytes can be grown in a 3D collagen gel without loss of their chondrogenic phenotype but that LIPUS did not enhance cell proliferation in either a monolayer culture or a 3D culture [13]. In this same study, ultrasound exposure was found to be advantageous in inducing chondrocyte production of collagen gel composites with mature aggrecan.

Parvizi and colleagues irradiated the rat monolayer culture cells at 1 MHz to investigate the [^{3}H]thymidine incorporation levels, the DNA contents, the mRNA levels of α(I) and α(II) procollagens and the mRNA contents of proteoglycans inducing aggrecan. The group reported that the irradiation increased the aggrecan mRNA and proteoglycan levels without any significant effects upon the proliferation of chondrocytes [14].

A number of studies have reported a slight increase in the number of chondrocytes following the use of the same therapeutic low-intensity pulsed ultrasound, which may be called PLIUS. Zhang and colleagues previously irradiated cultured chondrocytes at 2 mW/cm^2 and 30 mW/cm^2, and measured the cell count and volume of the extracellular matrix over time. At 2 mW/cm^2, they reported that the extracellular matrix as well as the cell number increases significantly but only transiently on day 3 of culture, in comparison with the control group [15]. In the current study with a 3D culture system, the number of chondrocytes doubled by the end of the 2-week incubation in both groups. This rate of increase was slightly but significantly higher in the LIPUS group.

To further confirm these findings, Ki67 staining of sections from these cultures was performed because it has been shown to be a very reliable proliferation marker. The Ki67 index in the LIPUS group, also significantly higher in comparison with the control group, again indicated that LIPUS promotes the proliferation of chondrocytes slightly but significantly. In terms of cartilage regeneration, even a slight increase in the number of chondrocytes is very important. In a previous study performed *in vivo* by Cook and colleagues, cartilage defects in New Zealand rabbits were artificially induced by drilling holes. These defects treated by LIPUS regenerated articular cartilage earlier than the control group, with a hint of increased numbers of chondrocytes [21]. In many previous *in vitro* studies, the cartilage of small animals such as mice and rats has been used. Chondrocytes in cartilage of these animals have a tendency to proliferate more easily, and therefore the regeneration of cartilage is easier than in higher animals. The current study utilized porcine cartilage on the assumption that this is a more appropriate animal model system for the development of future treatments in human cartilage.

Figure 6



Western blotting analysis. **(a)** Type-II collagen. **(b)** Type-IX collagen. **(c)** Focal adhesion kinase (FAK) and phosphorylated FAK (p-FAK). **(d)** Paxillin and phosphorylated Paxillin (p-Paxillin). **(e)** Mitogen-activated protein kinase (MAPK) and phosphorylated MAPK (p-MAPK). There are no evident differences in the expression levels of total MAPK and p-MAPK between the two groups. **(f)** Akt and phosphorylated Akt (p-Akt). There were no differences found in the intensity the total Akt expression between the two groups, but p-Akt was found at higher levels in the LIPUS group (US+) in comparison with the control group (US-). **(g)** Cyclin B_1 and cyclin D_1. **(h)** Changes of proliferating cell nuclear antigen (PCNA) using MEK1 inhibitor (PD98059) and phosphatidylinositol 3-OH kinase (PI3K) inhibitor (LY294002). Chondrocytes were pretreated with MEK1 inhibitor (PD98059, 250 μM/ml) and PI3K inhibitor (LY294002, 250 μM/ml) for 12 hours and 24 hours followed by stimulation with LIPUS for 20 minutes. Each sample was harvested 2 hours after LIPUS stimulation and the influence of these inhibitors was judged in western blotting analysis of the expression of PCNA.

LIPUS promotes production of collagen type-IX

The immunoblotting analysis in the present study indicated that LIPUS increases the production of collagen type-IX, but not collagen type-II. These results suggest that LIPUS transduces the signals through the intracellular signaling pathway that transactivates the collagen type-IX gene. Although the major constituent of the cartilage matrix is type-II collagen, this matrix also contains collagen types of smaller molecular weights, including type VI, type-IX, type X, type XI, and type XII. These collagens all play regulatory roles in maintaining cartilage. Type-IX collagen is present in zones 1 and 2, and it is said to be involved in promoting chondrocyte proliferation and in the expansion of the cartilage layer [22].

In addition, Eyre and colleagues have earlier reported that type-IX collagen accounts for at least 10% of the collagenous protein in fetal cartilage, but only about 1% to 2% of adult hyaline cartilage – and that the ratio of type-IX collagen to type-II collagen decreases as the cartilage matures [23].

Jarmo and coworkers reported that type-IX collagen has unique cell adhesion properties in comparison with other collagen types, and that it provides a novel mechanism for cell adhesion to the cartilaginous matrix [24]. They demonstrated that the type-IX collagen is a superior cell adhesion protein for chondrocytes. In addition to these reports, Wu and colleagues and Blaschke and colleagues suggested that type-IX collagen may be an important stabilizing factor for cartilage type-II col-

lagen fibrils, since it determines the resistance of the fibrils to swelling in the framework of cartilage [25,26]. Hu and colleagues have also reported that type-IX collagen-deficient mice are prone to developing osteoarthritis [27].

The present results suggest that the chondrocyte proliferation in response to LIPUS is associated with the increase in collagen type-IX expression. Eyre and colleagues reported that the ratio of collagen type-IX to collagen type-II in immature cartilage tissue is greater than that in mature cartilage tissue [23]. The results of the current study support their findings. It is likely that the production of collagen type-IX increases in the current system because of an increase in the number of immature chondrocytes in the cultures. In immature chondrocytes, it was reported that the construction of a peripheral matrix with collagen type-IX also promotes the attachment between the cells and the matrix [26].

Activation of the PI3K/Akt pathway but not the MEK/MAPK pathway by LIPUS

It is very probable that LIPUS transmits signals into the cell via an integrin that acts as a mechanoreceptor on the cell membrane. When ultrasound is transmitted to integrin molecules, this promotes the attachment of various focal adhesion adaptor proteins. Both FAK and Paxillin are in turn phosphorylated as a result of LIPUS exposure initiating this signal transduction.

The integrin/Ras/MAPK/nucleus pathway is considered a general pathway involved in cell proliferation. In the current study, however, MAPK was shown to be similarly activated and phosphorylated regardless of the LIPUS exposure. The results confirmed that MAPK is constitutively activated in both LIPUS-stimulated cells and control cells, probably due to the culture conditions in which the medium is supplemented with 10% FBS. This observation suggests that the significant increase in cell numbers observed in relation to the elevation of type-IX collagen expression is attributable to a signal transduction pathway other than the Ras/MAPK pathway.

The PI3K/Akt pathway, on the other hand, is known to be involved in various functions such as cell survival, proliferation, motility, control of cell size and metabolism [28,29]. In the present experiments, this pathway was found to be newly activated by LIPUS. A previous report also showed that phosphorylated Akt inhibits glycogen synthase kinase-3, which otherwise phosphorylates β-catenin [30]. A high intracellular concentration of β-catenin therefore accumulates when glycogen synthase kinase-3 is inhibited by phosphorylated Akt. In turn, β-catenin translocates to the nucleus and promotes the transcription of its target genes.

The Wnt signaling pathway may also be involved in the increase in the intracellular β-catenin levels [31]. In the current study, LIPUS was found to significantly increase the number of β-catenin-positive cells during enhanced cell proliferation. Both the PI3K/Akt pathway and the Akt/β-catenin pathway are therefore strongly implicated in this process (Figure 7). Moreover, the expression of the cyclin B_1 and cyclin D_1 was found to be elevated in the LIPUS group, providing further evidence that LIPUS promotes the active division of chondrocytes [32,33]. In this regard, Li and colleagues have demonstrated that transforming growth factor beat stimulates cyclin D_1 expression in chondrocytes in part through the activation of β-catenin signaling [34].

Wnt/β-catenin signaling has been reported to play a crucial role in cell proliferation and in the morphogenesis of chondrocytes [35]. Since there is some functional interaction between the PI3K/Akt pathway and Wnt/β-catenin signaling, LIPUS may activate β-catenin signaling via the PI3K/Akt pathway. As indicated in Figure 4c,d, the nuclear localization of β-catenin, as a marker of the β-catenin signaling, was more prominent in LIPUS-stimulated cells than in the control cells, thus indicating this to be the case.

Conclusion

LIPUS promotes type-IX collagen accumulation and enhances the proliferation of cultured chondrocytes. In addition to the general growth factor signaling via the Ras/MAPK pathway, mechanical signal transduction to the nucleus through the integrin/PI3K/Akt pathway is activated by LIPUS, thus resulting in an increased matrix production and proliferation of chondrocytes. Akt seems to control the metabolism of β-catenin via glycogen synthase kinase-3, which phosphorylates β-catenin, and also raises the intracellular β-catenin concentration, which in turn promotes its translocation to the nucleus.

In future studies it will be necessary to elucidate the signals or transcription factors that operate downstream of Akt in this system. Certain membrane receptors or ion channels other than integrins, which may reside upstream of the transcription factors that promote the production of collagen type-IX, should also be investigated.

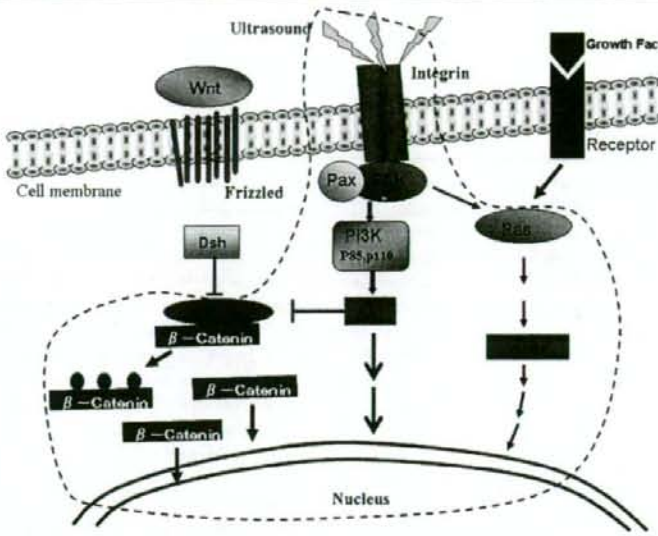
Competing interests

The authors declare that they have no competing interests.

Authors' contributions

RT performed planning of this study, the *in vitro* experiment, and generalization. AR performed the immunohistochemistry. NK performed western blotting analysis. YM-T was a senior advisor. AF performed cell counting and histological examinations. YT performed western blotting analysis. TS performed ultrasound stimulation. SM was a senior advisor. YY performed histological examinations. KK performed planning and cell culture. IA was a senior advisor. TS was a senior advisor. All authors participated in the conception and design of the study. All authors read and approved the final manuscript.

Figure 7



Signal transduction pathways activated by low-intensity pulsed ultrasound. Area enclosed with a black broken line is the signaling pathway specified in the present experiment. One of the receptors of low-intensity pulsed ultrasound (LIPUS) is through integrin, and the integrin/mitogen-activated protein kinase (MAPK) pathway is activated to the same extent in both the LIPUS group and the control group. The integrin/phosphatidylinositol 3 kinase (PI3K)/Akt pathway, however, was further activated by LIPUS. The expression of β-catenin, which is downstream of the Akt signaling pathways, is also increased by LIPUS. FAK, focal adhesion kinase; GSK-3, glycogen synthase kinase-3; Pax, Paxillin.

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