

induced by dSyno overexpression was not affected by Dmp53 in the double overexpressing flies (Figure 6Dii and iii, arrow). These results confirm that dSyno regulates Dmp53 protein level *in vivo* and such regulation might be accomplished through ubiquitination of dDmp53.

To determine whether this phenotypic suppression of Dmp53 by dSyno is specific to Dmp53, we investigated the interaction between dSyno and the upstream activators of p53 such as dATM, CHK2, using the same strategy. None of these activators showed interaction with dSyno (data not shown), suggesting that dSyno regulates Dmp53 protein level directly *in vivo*.

Discussion

We provide concrete evidence for the first time of the functional relationship between Synoviolin and p53. As a target for Synoviolin, p53 is evidently a non-ERAD substrate. In this regard, Doa10p, the RING finger E3 ubiquitin ligase, is known not only to be involved exclusively in removing ER proteins in the ERAD, but also to eliminate cytoplasmic targets, especially the soluble transcriptional factor Mat α 2, which translocates into the nucleus similar to p53 (Swanson *et al*, 2001; Laney and Hochstrasser, 2003). Thus, our finding can be viewed within the same framework of yeast though in higher eukaryotes. In the meantime, it was proposed that the ERAD in yeast is composed of two distinct surveillance mechanisms, that is, the folded state of luminal domains and the cytosolic domains are monitored by ERAD-luminal (ERAD-L) and ERAD-cytosolic (ERAD-C) pathways, respectively (Vashist and Ng, 2004; Nishikawa *et al*, 2005). Hrd1p is recognized as an ERAD-L ligase; however, this classification is not applicable to Synoviolin as a human homolog of yeast Hrd1, because Synoviolin can target both ERAD-L substrate and cytoplasmic p53 (ERAD-C substrate). Therefore, we propose the novel regulatory system of Synoviolin as a different classification of the ERAD-L/C pathway.

Maintenance of homeostasis is an important cellular function, and cells are equipped with various processes to maintain their conditions. Transcriptional alteration mediated by p53 results in a variety of cell fate changes, including growth arrest and apoptosis (Vousden and Lu, 2002; Meek, 2004). Normally, the cell maintains low levels of p53 through rapid protein degradation via the UPS by the function of ubiquitin ligases. In contrast, under genotoxic stress conditions, stabilization of p53 is promoted and the diffusely distributed p53 translocates to the nucleus owing to growth inhibition and apoptosis by its transcriptional activity. Thus, adjusting the level and nuclear localization of p53 are two essential processes for cells in order to maintain the physiological state. Although p53 mutations have been documented in more than half of all human tumors (Hollstein *et al*, 1999), it is also known that tumor cells retain WT p53. In this regard, functional inactivation of WT p53 by abnormal cytoplasmic sequestration is frequently observed in many tumor types (Moll *et al*, 1992, 1996; Schlamp *et al*, 1997). The RING finger protein Parc is considered to act as a cytoplasmic anchoring molecule of p53, but this clone does not have a p53 ubiquitination activity (Nikolaev *et al*, 2003). On the other hand, our present findings demonstrated that Synoviolin not only anchors p53 in the cytoplasm, but also ubiquitinates it, and thus differs from Parc (Figure 4). Moreover, Synoviolin

diverges from other ligases for p53; each of the three ubiquitin ligases for p53 (MDM2, Pirh2 and Cop1) forms an auto-regulatory negative feedback loop, resulting in lower p53 activity upon its expression, but these three ligases are target for the p53 transcriptional pathway (Dornan *et al*, 2004; Leng *et al*, 2003), whereas the expression of Synoviolin is not regulated by p53 (Figure 5F). Indeed, the *synoviolin* promoter region does not have a p53 target sequence, whereas it contains the ER stress responsive element (Tsuchimochi *et al*, 2005) and responds to the stress (Figure 5F). The reason for the multiple post-translational steps for p53 is the enormous importance of this molecule in maintaining cellular homeostasis. p53 is negatively regulated by various ubiquitin ligases, such as MDM2, MdmX, HAUSP, ARF, COP1, Pirh2 and ARF-BP1 (Brooks and Gu, 2006), and it is assumed that each molecule has its specific roles in p53 control. Among them, Synoviolin is also a unique regulator of p53 because of its independency from other ligases and transcriptional regulation by p53, ER localization and canonical function in ERAD.

In the present study, we demonstrated that Synoviolin participates in genotoxic stress-mediated p53 signaling, and its participation in the ER stress-induced apoptosis is also well known (Bordallo *et al*, 1998; Kaneko *et al*, 2002; Kikkert *et al*, 2004; Yagishita *et al*, 2005). Therefore, Synoviolin seems to regulate two distinct apoptotic pathways and the ubiquitination of p53 by Synoviolin may be another target for crosstalk between them. Another linkage between ER stress and p53 pathway is also implicated by our finding that UPR markers are increased in cells with *synoviolin* knockdown (data not shown). Two reports described a crosstalk of p53- and ER stress-induced apoptosis pathways, that is, ER stress antagonizes p53-mediated apoptosis through the cytoplasmic localization of p53 due to phosphorylation by glycogen synthase kinase-3 β (GSK-3 β) (Qu *et al*, 2004), and p53 destabilization utilized the cooperative action of MDM2 and GSK-3 β in ER-stressed cells (Pluquet *et al*, 2005). In this regard, it is important to note that UPR activation upon Synoviolin knockdown in RKO cells may be related to ER stress with impaired ERAD system. Since Synoviolin null cells show upregulation of p53, it is possible that the effect of p53 stabilization by Synoviolin knockdown exceeds the p53 destabilization effect of UPR induced by Synoviolin knockdown. This hypothesis may be supported by the finding that *synoviolin* siRNA treatment seemed to restore the expression of p53 at least in part, which was suppressed by ER stress (Figure 5F). The regulatory action of Synoviolin on p53 under ER stress is obviously more complex, because ER stress also induces Synoviolin expression. Further studies are necessary to determine the physiological regulatory role of Synoviolin in p53 expression under ER stress conditions.

The function of p53 in patients with RA is still controversial (Firestein *et al*, 1997; Reme *et al*, 1998; Inazuka *et al*, 2000; Muller-Ladner and Nishioka, 2000; Sun and Cheung, 2002). Mice lacking p53 do not develop spontaneous arthropathy but have severe collagen-induced arthritis (CIA) (Yamanishi *et al*, 2002; Simelyte *et al*, 2005). As we reported previously, overexpression of Synoviolin resulted in spontaneous arthropathy and its deficiency resulted in resistance to CIA in mice (Amano *et al*, 2003). Therefore, we assumed that the severity of arthritis could be determined by the Synoviolin-p53 control pathway and that the onset of spon-

taneous arthropathy may be caused by p53-independent pathway in these models. The influence of these relationships on arthritis is currently being examined in our laboratories, using *synoviolin* and p53 double null mutant mice. We hope that our research could uncover new pathogenic mechanisms of RA. Furthermore, since p53 is an important tumor suppressor gene, we believe that Synoviolin could be a useful therapeutic target for not only RA but also cancer based on its cytological and biochemical features, i.e., cytoplasmic localization and enzymatic activity (Hopkins and Groom, 2002).

In conclusion, we demonstrated that Synoviolin acts as an ERAD E3 ubiquitin ligase that controls cellular p53 and thus opens, a new concept for proliferative disorders such as RA and cancer.

Materials and methods

Plasmids

pcDNA3/Synoviolin WT or C307S-FLAG, pcDNA3/HA- and FLAG-p53, pcDNA3/MDM2 and pcDNA/HA-Ub plasmids have been described previously (Amano et al, 2003; Matsushita et al, 2005). Deletion of 53BD, MBP- and GST-fusions in Synoviolin deletion mutants was performed by PCR-based method in this study. To clone a cDNA encoding *Drosophila* homolog of human Synoviolin (dSyno), 2282 bp of CG1937 was cut out from EST GH11117 with *EcoRI/XhoI*, and subcloned into pUAST vector (Brand and Perrimon, 1993). The sequences of all plasmids generated by PCR were confirmed by ABI auto-sequencer.

Cells and transfections

RKO and HEK293 cells were cultured in Minimum Essential Medium (Sigma) and U2OS and Saos-2 in Dulbecco's modified Eagle's medium (Sigma). The sense sequences of siRNA oligonucleotides to *synoviolin* are (1) GGUGUUCUUUGGGCAACUG, (2) GCUGUGACAGAUCCAUCA, (3) GGUUCUGCUGUACAUGGCC. Changes in p53 protein in RKO cells were determined by all these siRNAs. The sense sequence of siRNA oligonucleotides to *GFP* is GGCUACGUCCAGGAGCGC.

GST pull-down assay

GST-fusion proteins were expressed in *Escherichia coli* strain BL21 (Invitrogen) and purified by using glutathione-Sepharose beads (Amersham Biosciences). *In vitro*-translated ³⁵S-labeled p53 was pre-cleaned with 10 μg GST protein for 1 h at 4°C, followed by incubation with 10 μg of each GST-fusion protein in binding buffer (20 mM N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid (HEPES), pH 7.9, 150 mM NaCl and 0.2% TritonX-100) for 1 h at 4°C. After washing, bound proteins were separated by SDS-PAGE and detected by BAS.

Immunoprecipitation assay

For co-immunoprecipitation assay between exogenous Synoviolin and exogenous p53, HEK293 cells were co-transfected with HA-p53 and pcDNA3-Synoviolin WT-FLAG, pcDNA3-SynoviolinΔ53BD-FLAG or pcDNA3-Synoviolin C307S-FLAG plasmids. Cell extracts were prepared with high-salt buffer (20 mM HEPES pH 7.2, 420 mM NaCl, 10% glycerol, 0.5% NP-40, 0.5 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride (PMSF)) and diluted at three-fold with 0.5 mM DTT and a protease inhibitor solution, followed by incubation with mouse IgG or anti-FLAG antibody. Precipitated proteins were detected by anti-HA or anti-FLAG antibodies.

To detect the interaction between endogenous Synoviolin and p53, HEK293 cells were lysed in 100 mM Tris-HCl, 80 mM NaCl, 1 mM EDTA, 5 mM EGTA, 5% (w/v) digitonin, 0.1% Brij 35, protease inhibitor cocktail and 20 μM of MG132.

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Immunoprecipitation was carried out in the presence or absence of SDS by using anti-p53 antibodies, anti-Synoviolin antibodies or control IgG. The immunoprecipitated samples were analyzed by western blot by using anti-p53 or anti-Synoviolin antibodies.

In vitro and in vivo ubiquitination assays

The *in vitro* ubiquitination assay was conducted as described previously (Amano et al, 2003). For the peptide inhibition assay, reaction solutions lacking no MBP-SynoviolinΔTM-6xHis and ATP were incubated with 53BD or control peptides (50, 100, and 200 μM) for 30 min at 4°C. Reactions were started by addition of MBP-SynoviolinΔTM-6xHis and ATP and incubating at 37°C.

For the *in vivo* ubiquitination assay, HEK293 cells were transfected with pcDNA3/HA-Ubiquitin, pcDNA3/FLAG-p53, and pcDNA3/Synoviolin WT, C307S or pcDNA3/MDM2. At 24 h post-transfection, cells were treated with MG132 (10 μM) for 1 h, then the cells were lysed in SDS containing buffer (50 mM Tris, pH 7.5, 0.5 mM EDTA, 1% SDS, and 1 mM DTT) and boiled for 5 min to denature the proteins. The denatured samples were diluted with immunoprecipitation buffer (50 mM Tris, pH 7.5, 2 mM EDTA, 150 mM NaCl, and 0.1% NP-40 and protease inhibitor cocktail) and the p53 protein was immunopurified by using anti-p53 antibody. Ubiquitinated p53 was detected by western blotting by using anti-HA antibody.

Immunostaining of fly wing discs

Fly wing discs were dissected in PBS, fixed in a buffer containing 50 mM Tris-HCl, pH 6.8, 1 mM EGTA, 1% Triton X-100, 2 mM MgSO₄, 150 mM NaCl, and 2.2% formaldehyde for 15 min, and blocked using a blocking buffer (50 mM Tris-HCl, pH 6.8, 150 mM NaCl, 0.5% NP-40 and 5 mg/ml BSA). The fixed wing discs were incubated overnight at 4°C in a 1:200 dilution of anti-Dmp53 (d-200) antibody. After washing in a wash buffer (50 mM Tris-HCl, pH 6.8, 150 mM NaCl, 0.5% NP-40 and 1 mg/ml BSA), they were incubated for 3 h at 4°C in donkey anti-rabbit FITC at 1:200 dilution, washed with the wash buffer and then mounted in a mounting solution (50 mM Tris-HCl, pH 6.8, 30% glycerol, 150 mM NaCl, and 5 mg/ml phenylethylenediamine). The fluorescence intensity of each disc was quantified with Quantity One software (Bio-Rad Laboratories). Acridine orange staining was performed as reported previously (Brodsky et al, 2000).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We are grateful to MR Montminy, G Verdine, R Nagata, H Shimizu, I Hishinuma, H Yokohama, H Kato, S Kitamura, K Yoshimatsu, Yuichiro ITAKURA OFFICE and ES Takagi, for advice and encouragement, and to H Takahashi, M Sato, S Otani, A Sugamiya, N Takagi, S Shinkawa, Y Nakagawa, Y Sato, M Yamanashi and members of Toshi's Laboratory for the excellent technical assistance. This study was supported in part by LocomoGene Inc., Eisai Co., Ltd, National Institute of Biomedical Innovation, the Japanese Ministry of Education, Culture, Sports, Science and Technology, the Japanese Ministry of Health, Labour and Welfare, the Kato Memorial Trust for Nanbyo Research, the Japan Medical Association, Nagao Memorial Fund, Kanae Foundation for Life & Socio-medical Science, Japan Research Foundation for Clinical Pharmacology, Kanagawa Nanbyo Foundation, Kanagawa Academy of Science and Technology Research Grants, Japan College of Rheumatology, the Nakajima Foundation, Japan Society for Promotion of Science, New Energy and Industrial Technology Development Organization, Mochida Pharmaceutical Co. Ltd, Kanto Bureau of Economy, Trade and Industry, and the Uehara Memorial Foundation. HF is supported by Japan Society for the Promotion of Science.

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Resistance to endoplasmic reticulum stress is an acquired cellular characteristic of rheumatoid synovial cells

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Received January 10, 2006; Accepted February 24, 2006

Abstract. Synoviolin is an endoplasmic reticulum (ER)-resident E3 ubiquitin ligase which plays a critical role in ER-associated degradation (ERAD). We found that Synoviolin is a novel causative factor for rheumatoid arthritis (RA), which is especially up-regulated in proliferating synovial cells in the disease. We attempted to examine the role of Synoviolin in ER stress-induced apoptosis and proliferation of synovial cells. RA synovial cells (RSCs) were refractory to ER stress-induced apoptosis compared with HEK293 or HeLa cells. RSCs were also more resistant to the apoptosis than synovial cells from osteoarthritis patients, significantly. Down-regulation of Synoviolin by siRNA increased the susceptibility to ER stress-induced apoptosis in RSCs. Knock-down of Synoviolin by siRNA did not only induce apoptosis of RSCs but also inhibited their proliferation *in vitro*. These data suggest that RSCs are extraordinarily refractory to ER stress-induced apoptosis, and we termed this special property 'hyper-ERAD'. Since Synoviolin is overexpressed in RSCs, and is known to play a critical role in the ERAD system as E3 ubiquitin ligase, hyper-ERAD is likely to present in these cells. Subsequently, the hyper-ERAD may cause synovial hyperplasia through its anti-apoptotic effect in RA. Further analyses are necessary to address this point, however, resistance to ER stress-induced apoptosis, or hyper-ERAD is a noteworthy new cellular characteristic of RSCs.

Introduction

Rheumatoid arthritis (RA) is characterized by abnormal immunity and synovial fibroblasts overgrowth followed by bone and cartilage destruction. While the immunological aspects of RA are well understood, the molecular basis for promoting overgrowth of rheumatoid synovial cells (RSCs) remains elusive (1,2). We recently succeeded in identifying Synoviolin as a possible pathogenic factor for RA (3). Synoviolin is an endoplasmic reticulum (ER)-resident ubiquitin ligase with N-terminal six transmembrane domains and RING finger motif (4,5). Synoviolin is involved in ER-associated degradation (ERAD), which is essential for maintenance of ER function, and activation of ERAD is important to eliminate proteins with an incorrect three-dimensional structure (unfolded protein) through the ubiquitin and proteasome system (3-6). The importance of Synoviolin in the ERAD system is clearly confirmed by this gene being embryonically lethal in the phenotypes of homozygous knock-out mice (3,7).

Both 'loss of function' and 'gain of function' using transgenic technology, Synoviolin is proved to be a pathogenic factor for arthropathy at least in mice (3). Detailed examination of synovial tissue from *synoviolin* heterozygous knock-out mice (*syno*^{+/-}) with collagen-induced arthritis (CIA) revealed that there is an aberrantly increased apoptosis of synovial cells, which inhibited the progression of synovial hyperplasia in the mice, in spite of comparable productions of anti-type II collagen antibodies, tumor necrosis factor α (TNF α), and interleukin 1 (IL-1) to those of wild-type mice. Conversely, there was remarkable synovial cell overgrowth in Synoviolin overexpressing mice. These results indicate that Synoviolin accelerates synovial cell overgrowth, and consequently leads to arthropathy in mice. Synoviolin overexpressing mice can be regarded as a novel RA model that firstly demonstrates the possible importance of hyperactivation of the ERAD system (hyper-ERAD) in overgrowth of RSCs, because RSCs express high levels of Synoviolin protein (3). To address this hypothesis, we examined whether RSCs are equipped with the hyper-ERAD system by Synoviolin, and

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Key words: Synoviolin, Hrd1, endoplasmic reticulum-associated degradation, apoptosis, proliferation

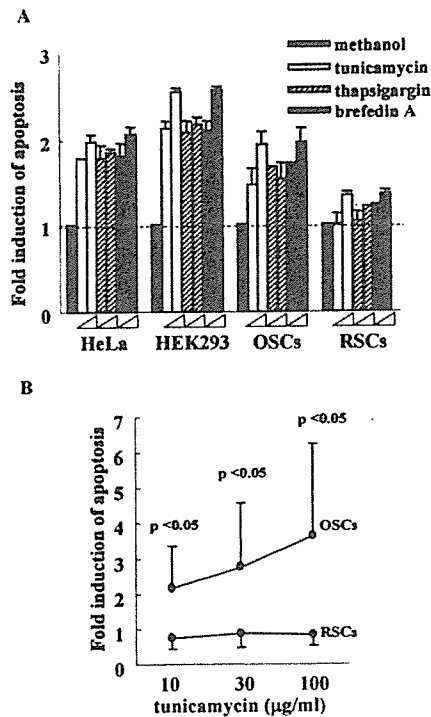


Figure 1. Rheumatoid synovial cells are refractory to ER stress-induced apoptosis. (A) HeLa, HEK293, and RSCs or OA synovial cells (OSCs) were treated with tunicamycin (10 or 100 $\mu\text{g/ml}$), thapsigargin (1 or 10 μM), breferdin A (10 or 100 $\mu\text{g/ml}$) or methanol (negative control) for 48 h. Apoptosis was evaluated using an ssDNA apoptosis ELISA kit. (B) RSCs ($n=5$) and OSCs ($n=5$) were treated with tunicamycin for 48 h. Apoptosis was evaluated by ssDNA Apoptosis ELISA kit.

how hyper-ERAD affects the proliferation and apoptosis of RSCs in this study.

Materials and methods

Reagents. ER stress inducers, including tunicamycin, thapsigargin and breferdin A, were obtained from Sigma (St. Louis, MO) to induce ER stress in various cells (8).

Synovial cell isolation and culture. Synovial cells were isolated from synovial tissue obtained from 10 patients with rheumatoid arthritis (RA) who met the American College of Rheumatology criteria for RA at the time of orthopedic surgery (9). In some experiments, synovial cells isolated from patients with osteoarthritis (OA) were also used. These cells were cultured in DMEM containing 1% penicillin, 1% streptomycin and 10% fetal bovine serum. The adherent synovial cells used in this study at third to fifth passages were <1% reactive with monoclonal antibodies, including CD3, CD68, CD20, and von Willebrand factor.

RNA interference assay. Small interfering RNA (siRNA) with 21 nucleotides was chemically synthesized (Hokkaido System Science Co., Japan). The siRNA for synoviolin was created by annealing 5'-CGU UCC UGG UAC GCC GUC ATT-3' (sense sequence) and 5'-UGA CGG CGU ACC AGG AAC GTT-3' (anti-sense). The sense sequence corresponds to the sequence

of synoviolin cDNA (238-256), and the TT of 3' end is overhanging nucleotides. Transfection was carried out by using Oligofectamine (Invitrogen, CA) at the concentration of 20 nM annealed RNA duplex, according to the instructions provided by the supplier. The siRNA for green fluorescent protein (GFP) was used as negative control.

Western blot analysis. Cell cultures were harvested and lysed in buffer containing 25 mM Tris-HCl (pH 6.8), 50 mM NaCl, 1% NP-40, 0.25% SDS and protease inhibitors, and aliquots of clear cell lysates were separated on SDS-polyacrylamide gels, transferred onto nitrocellulose membrane, and immunoblotted with anti-Synoviolin monoclonal antibodies (Abs) (3). Bound antibodies were detected by peroxidase-conjugated goat anti-mouse Abs and ECL detection system (Amersham Pharmacia Biotech).

Apoptosis assay. Apoptosis of RSCs was determined using an ssDNA apoptosis ELISA kit (Chemicon International, Inc., CA). ELISA was carried out according to the manufacturer's protocol. The extent of apoptosis was evaluated as a fold induction compared with control cells. In some experiments, apoptosis was detected by staining cells with propidium iodide and analyzing them for DNA content on a FACScan to detect hypodiploid DNA.

Proliferation assay. The proliferation of RSCs was evaluated using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the instructions provided by the manufacturer.

Statistical analysis. Data are expressed as mean \pm SD. Differences between control and treated cells were examined for statistical significance using the Student's t-test. A P-value <0.05 denoted the presence of a statistically significant difference.

Ethical considerations. All human experimental protocols described in this study were approved by the Ethics Review Committees of St. Marianna University School of Medicine. Before obtaining human tissue, a signed consent form was obtained from each subject participating in the study.

Results

RSCs are refractory to ER stress-induced apoptosis. First, we simply questioned whether RSCs are refractory to apoptosis induced by excess accumulation of unfolded proteins in ER. RSCs are expected to have high ability to eliminate these unfolded proteins from ER through the 'hyper-ERAD' system by their high expression levels of Synoviolin. To address this issue, pharmacological analysis was performed by using ER stress inducers to evaluate the ERAD function of RSCs. We compared the sensitivities of RSCs to ER stress inducers with that of OA synovial cells (OSCs), human embryonic kidney (HEK293) cell, or HeLa cells, which are known to express a lower amount of Synoviolin (3; data not shown). These cells were treated with ER stress-inducing reagents (tunicamycin, thapsigargin and breferdin A) to load unfolded proteins in ER, and the susceptibility of these cells to ER stress-induced apoptosis was determined by ELISA. As expected, RSCs

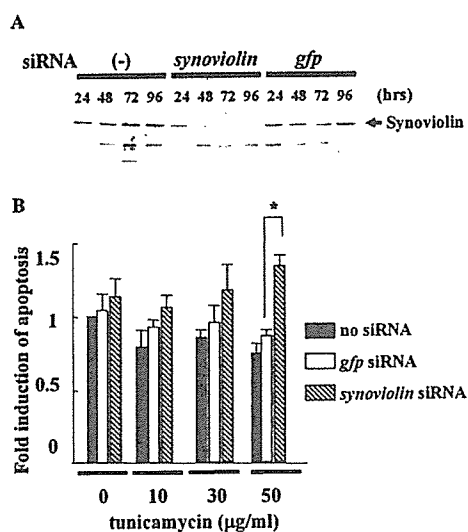


Figure 2. Synoviolin siRNA increase the sensitivity to ER stress-induced apoptosis. (A) Down-regulation of Synoviolin in RSCs by siRNA. (B) RSCs were treated with siRNA for 72 h, and the cells were further treated with an indicated dose of tunicamycin for 48 h. Apoptosis was evaluated by using ELISA. * $P < 0.05$.

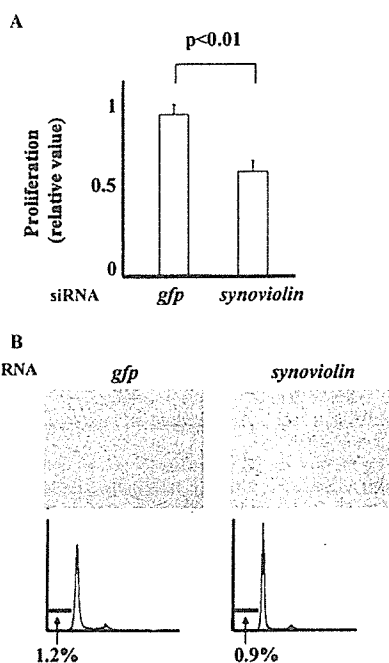


Figure 3. Down-regulation of Synoviolin inhibits RSC proliferation. (A) RSCs were treated with siRNA for 96 h, and the proliferation of the cells was evaluated using Cell Counting kit-8. (B) Morphological change (upper) and apoptosis assay (lower) of RSCs treated with siRNA for Synoviolin for 96 h. RSCs were treated with siRNA for 96 h, and the apoptosis of the cells was detected by FACScan after propidium iodide staining.

whether RSCs are more resistant to ER stress, and found that RSCs were more refractory to ER stress-induced apoptosis than OSCs, significantly (Fig. 1B; 10 $\mu\text{g/ml}$: RSCs 0.73 ± 0.30 , OSCs 2.18 ± 1.19 ; 30 $\mu\text{g/ml}$: RSCs 0.84 ± 0.35 , OSCs 2.76 ± 1.84 ; 100 $\mu\text{g/ml}$: RSCs 0.81 ± 0.28 , OSCs 3.65 ± 2.53). It should be noted that apoptosis of RSCs was not induced at a considerably high concentration of tunicamycin (8). These data clearly indicate that RSCs are distinctly equipped with a high ability to deal with a large amount of unfolded proteins in ER.

Down-regulation of Synoviolin promotes ER stress-induced apoptosis of RSCs. Next, we addressed whether 'hyper-ERAD' is achieved by high expression of Synoviolin in RSCs. We administered an indicated dose of tunicamycin to RSCs after the down-regulation of Synoviolin expression by 72-h treatment with siRNA for Synoviolin. Treatment of RSCs with siRNA for Synoviolin efficiently suppressed the protein expression of Synoviolin (Fig. 2A). The siRNA for Synoviolin alone did not enhance the apoptosis of RSCs (Fig. 2B). Treatment with tunicamycin did not induce significant apoptosis again in RSCs treated with control siRNA. However, *synoviolin* knock-down significantly enhanced the apoptosis of RSCs induced by tunicamycin at a concentration of 50 $\mu\text{g/ml}$ compared with those treated with control siRNA, even though the sensitivity of these cells to apoptosis is still very low compared with that of other cells (Fig. 2B). This data indicated that RSCs restored the sensitivity to ER stress-induced apoptosis by Synoviolin knock-down. Thus, it can be said that Synoviolin is important, at least in part, for the hyper-ERAD of RSCs, and for RSC survival in ER stress milieu.

Down-regulation of Synoviolin inhibits proliferation of RSCs. One of the most striking data of our previous report about Synoviolin is that Synoviolin overexpressing mice develop arthropathy with synovial cell overgrowth. These results strongly suggest that Synoviolin is also required for synovial cell overgrowth. To verify the importance of Synoviolin in RSC proliferation, we carried out more detailed examinations of the proliferation of Synoviolin down-regulated RSCs. As we expected, the proliferation of RSCs was significantly inhibited by 96 h after treatment with siRNA for Synoviolin compared with RSCs treated with siRNA for GFP (Fig. 3A). Since the appearance of RSCs treated with siRNA for Synoviolin by phase contrast microscopic examination shows morphological change (Fig. 3B), we examined whether there are apoptotic cells in them by flow cytometry. There was no increment of hypodiploid DNA in Synoviolin down-regulated RSCs by 96-h treatment with siRNA for Synoviolin (Fig. 3B). These results are consistent with the data shown in Fig. 2B, and indicate that down-regulation of Synoviolin inhibits the proliferation of RSCs without inducing apoptosis of them.

Discussion

were found to be remarkably refractory to ER stress-induced apoptosis compared with OSCs, HEK293 cells or HeLa cells (Fig. 1A). Since RSCs were apparently more resistant to ER stress-induced apoptosis than OSCs, we further evaluated

Based on our serial experiments, we propose 'hyper-ERAD' as a novel pathogenic mechanism that promotes RSC survival and proliferation, and that this machinery can be mediated by up-regulation of Synoviolin in RA. RSCs, equipped with

'hyper-ERAD', are proved to be a unique cell with high ability to accommodate severe ER stress (Fig. 1). Down-regulation of Synoviolin resulted in aberrant apoptosis of synovial cells in CIA-induced *syno*^{+/+} mice (3). Likewise, down-regulation of the gene in RSCs resulted in increased apoptosis induced by ER stress inducers (Fig. 2). These accumulating data strongly support our novel disease concept that synovial cell overgrowth is triggered, at least in part, by hyper-ERAD, which consequently leads to synovial hyperplasia in RA. The importance of Synoviolin in 'hyper-ERAD' is also confirmed in this study.

Another remarkable aspect of Synoviolin is its ability to regulate the proliferation of synovial cells both *in vivo* and *in vitro*. Synoviolin overexpressing mice show spontaneous overgrowth of synovial cells (3), and siRNA for Synoviolin abrogates the proliferation of RSCs *in vitro* (Fig. 3). In addition, our recent analyses of the *synoviolin* promoter also demonstrated that transcriptional regulation of the gene is critical for cell growth and apoptosis *in vitro* (10), which strongly suggests that the amount of Synoviolin is critical to the regulation of synovial cell overgrowth.

Further examination is required to understand how Synoviolin regulates the proliferation of RSCs on a molecular basis. First, it is necessary to determine the alternation of unfolded protein response (UPR) by Synoviolin. It is believed that dual response UPR and the ERAD are triggered by the ER dysfunction or so-called 'ER stress', and both responses cooperate to maintain ER homeostasis (6,11). The ER-resident kinase, PERK-like ER kinase (PERK), is known to be activated as one UPR response. PERK phosphorylates the eukaryotic initiation factor (eIF2 α) that attenuates global translation to block the influx of newly synthesized proteins into ER (12). Therefore, protein synthesis for cell proliferation becomes insufficient if the ERAD system does not work sufficiently enough to avoid ER stress and eventually triggers UPR. GADD153 is another candidate molecule that explains the importance of Synoviolin in RSC proliferation because it is thought to be activated in cells with ER stress, and causes cell cycle arrest (13). Actually, cell cycle study by staining cells with propidium iodide demonstrates that the cell treated with siRNA for Synoviolin seems to be arrested at G1 or G0 phase (Fig. 3B). Further detailed studies are necessary to clarify the whole mechanism of UPR activation in RSCs with Synoviolin down-regulation.

Is 'hyper-ERAD' really necessary for overgrowth of RSCs? It is easy to assume that 'hyper-ERAD' is a very favourable function for RSCs because the articular milieu in inflamed joints of RA is extremely hostile to ER. It is not difficult to find the cause of ER stress in inflamed joints of RA. For example, hypoxia frequently occurs in inflamed joints of RA, which is known to inhibit ER function (12). In addition, pro-inflammatory cytokines promote the synthesis of various proteins, including hyaluronate, cytokines and proteases in RSCs, which inevitably accelerates the accumulation of unfolded protein in ER. Indeed, nuclear translocation of ATF6, an ER-resident transcriptional factor that translocates to the nucleus under ER stress conditions (14), was certainly confirmed in RA synovia (data not shown). A report from another laboratory also implicates the existence of ER stress by demonstrating the high expression of BiP, a well-known

transcriptional target for ATF6 (15). Thus, it is rational that 'hyper-ERAD' evoked by Synoviolin overexpression in RSCs helps RSCs to prevent these extra- and/or intra-cellular environmental stresses and subsequent apoptosis.

Here, we propose a concept that hyper-ERAD is a novel pathogenic factor for RA, which is especially important in RSC overgrowth. Historically, the autonomous proliferation of RSCs is thought to be due to high expression of proto-oncogenes, such as *c-myc*, *fos*, *ras*, or virus transforming gene *tax* or activation of specific transcriptional factors, such as NF- κ B (16,17). Synoviolin is also a member of the genes responsible for synovial cell overgrowth. Moreover, Synoviolin is one of the most convincing molecules for the RA therapy targeting RSCs because it is an enzyme and enzymes are regarded as the most appropriate target for drug development. The detailed molecular mechanisms are still unclear and various approaches are required to prove our hypothesis; however, it should be noted that resistance to ER stress-induced apoptosis is a novel cellular characteristic of RSCs.

Acknowledgements

We thank Drs T. Ohta and K. Tanaka for the critical comments. We are grateful to S. Toriyama, N. Furuya, H. Takahashi, N. Takagi, M. Hinata, S. Shinkawa, A. Sugamiya, and Y. Nakagawa for the excellent technical assistance. This study was supported by LocomoGene Inc. and grants from Ministry of Education, Culture, Sports, Science and Technology, Japan Society for the Promotion of Science, Regional Science Promotion Program in Kanagawa, The Kanagawa High-Technology Foundation, Kanagawa Academy of Science and Technology Research Grants, Mochida Pharmaceutical Co., Ltd., Pharmaceuticals and Medical Devices Agency, and New Energy and Industrial Technology development organization.

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Comparative Analysis of Gene Expression Profiles in Intact and Damaged Regions of Human Osteoarthritic Cartilage

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Objective. To analyze the differences in gene expression profiles of chondrocytes in intact and damaged regions of cartilage from the same knee joint of patients with osteoarthritis (OA) of the knee.

Methods. We compared messenger RNA expression profiles in regions of intact and damaged cartilage (classified according to the Mankin scale) obtained from patients with knee OA. Five pairs of intact and damaged regions of OA cartilage were evaluated by oligonucleotide array analysis using a double in vitro transcription amplification technique. The microarray data were confirmed by real-time quantitative polymerase chain reaction (PCR) amplification and were compared with previously published data.

Results. About 1,500 transcripts, which corresponded to 8% of the expressed transcripts, showed ≥ 2 -fold differences in expression between the cartilage tissue pairs. Approximately 10% of these transcripts (n = 151) were commonly expressed in the 5 patient

samples. Accordingly, 114 genes (35 genes expressed in intact > damaged; 79 genes expressed in intact < damaged) were selected. The expression of some genes related to the wound-healing process, including cell proliferation and interstitial collagen synthesis, was higher in damaged regions than in intact regions, similar to the findings for genes that inhibit matrix degradation. Comparisons of the real-time quantitative PCR data with the previously reported data support the validity of our microarray data.

Conclusion. Differences between intact and damaged regions of OA cartilage exhibited a similar pattern among the 5 patients examined, indicating the presence of common mechanisms that contribute to cartilage destruction. Elucidation of this mechanism is important for the development of effective treatments for OA.

Osteoarthritis (OA) is the most prevalent form of arthritis in the elderly and is characterized primarily by the degeneration and loss of articular cartilage. OA is considered a heterogeneous group of disorders with a variety of pathogenic factors, all of which result in similar patterns of cartilage degeneration (1). In OA of the knee, the medial compartment of the articular cartilage is the most susceptible to degeneration, whereas the lateral compartment remains relatively unaffected (2). This phenomenon appears in a single joint despite the same OA susceptibility of the cartilage matrix and the same genetic background.

There is now abundant evidence that chondrocytes play a critical role in cartilage degeneration. For example, OA chondrocytes secrete a variety of matrix breakdown products and cytokines, and cleavage of type II collagen is observed primarily around chondrocytes (3). Therefore, changes in the gene expression patterns of chondrocytes in response to various exogenous stimuli could affect the integrity of articular cartilage. Based on

Supported in part by Santen Pharmaceutical Company Ltd., the Japanese Ministry of Education, Science, Culture, and Sports, the Japanese Ministry of Health and Welfare, the Japan Science and Technology Corporation, the Kanagawa Academy of Science and Technology, and the Japan College of Rheumatology.

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Submitted for publication January 5, 2005; accepted in revised form November 10, 2005.

this concept, many groups of investigators have reported that gene expression in certain molecules differ from region to region within a single OA joint (4–7).

Because comprehensive gene expression profiles in intact and damaged regions of human OA cartilage have never been compared, the following questions remain unresolved. What percentage of the expressed transcripts shows obvious differences in expression levels in intact and damaged regions? What percentage of the differentially expressed transcripts shows the same expression pattern in different patient samples? What are the molecular functions involved in such groups of genes?

To provide answers to these questions, we used Affymetrix high-density oligonucleotide array analysis to compare the gene expression profile of chondrocytes in intact regions of joint cartilage with the profile of chondrocytes in damaged regions of joint cartilage from the same knee. We also evaluated the validity of our oligonucleotide array data according to the results of real-time quantitative polymerase chain reaction (PCR) amplification as well as gene expression data reported by other groups of investigators (4–7).

MATERIALS AND METHODS

Cartilage samples. Specimens of human articular cartilage were obtained from a total of 15 OA patients; 5 of the initial 9 patients' samples were used for microarray analysis, based on the results of the histologic assessment, and 4 of the 6 patients' samples used to confirm the microarray results were used for real-time PCR analysis after histologic assessment. Patients were undergoing total knee replacement surgery at St. Marianna University Hospital. OA was diagnosed according to the findings of the clinical history and physical examination, as well as radiographic findings. Within 4 hours after surgery, 8 pieces of cartilage tissue (4 apparently intact cartilage pieces and 4 damaged cartilage pieces) were dissected from each specimen. Each piece of cartilage was further divided into 2 fragments. One of the fragments was rapidly frozen in liquid nitrogen and stored at -80°C until RNA isolation was performed. The other fragment was frozen in TissueTek OCT compound (Sakura, Tokyo, Japan) and was used for histologic examination.

This study was performed after obtaining approval from the Ethics Committee for Human Studies, St. Marianna University Hospital. All patients provided informed consent.

Histopathologic assessment. Cartilage sections were stained with Safranin O–fast green. Sections were then evaluated histopathologically and scored according to the Mankin scale (8), which assesses the following 4 features: cartilage structure (0–6 scale, where 0 = normal, 1 = surface irregularity, 2 = pannus and surface irregularity, 3 = clefts to transitional zone, 4 = clefts to radial zone, 5 = clefts to calcified zone, and 6 = complete disorganization), cellularity (0–3 scale, where 0 = normal, 1 = hypercellularity, 2 = cloning, and 3 =

hypocellularity), Safranin O staining (0–4 scale, where 0 = normal, 1 = slight reduction, 2 = modest reduction, 3 = severe reduction, and 4 = no dye noted), and tidemark integrity (0–1 scale, where 0 = intact and 1 = blood vessels cross the tidemark). Cartilage sections in the present study were scored for the first 3 features (structure, cellularity, and Safranin O staining). Analysis of tidemark integrity was not evaluated because of the absence of a calcified zone in the cartilage samples we examined.

Based on the Mankin scores, we defined intact and damaged regions of the OA cartilage samples for comparative analysis. Intact cartilage regions were defined as follows: normal cartilage structure (score of 0), no cell clusters (score of 0 or 1), and a total score <4 . Damaged cartilage regions were defined as follows: clefts within the cartilage structure (score of 3 or 4), presence of several cell clusters (score of 2), and a total score >6 . Of the 9 pairs of cartilage samples obtained, 5 met these definitions. These paired samples were obtained from 5 OA patients (4 women and 1 man; age range 64–81 years). For real-time quantitative PCR, 4 of 6 additional pairs of cartilage samples met the above definitions. These additional samples were obtained from another group of 4 OA patients (4 women; age range 64–74 years).

RNA extraction. Frozen cartilage tissue (~ 50 mg) was rapidly minced with ophthalmic scissors in 1 ml of Isogen (Nippon Gene, Tokyo, Japan) and then immediately homogenized using a Polytron homogenizer (Hitachi Koki, Tokyo, Japan). Total RNA was isolated from the homogenized solution according to the manufacturer's instructions, except that the aqueous phase after initial separation was mixed with 0.25 ml of isopropanol, 0.25 ml of a high-salt precipitation solution (1.2M NaCl and 0.8M sodium citrate), and 3 μl of Ethachinmate (Nippon Gene) instead of with 0.5 ml of isopropanol. This extraction process was performed once again before RNA cleanup and DNase digestion using the RNeasy Mini kit (Qiagen, Crawley, UK).

Normalization of the amount of RNA. Because the amount of total RNA obtained from the piece of cartilage tissue was too small to measure accurately using a spectrophotometer, a Human β -Actin Competitive PCR Set (Takara Bio, Kyoto, Japan) was used according to the manufacturer's instructions to normalize the RNA amount that would be available for microarray analysis. Total RNA isolated from cultured chondrocytes was used as a control. To confirm the integrity of the total RNA, 60 ng (normalized value) of denatured total RNA was loaded in each lane of a 1% nondenaturing (formaldehyde-free) agarose gel, subjected to electrophoresis, and stained with ethidium bromide (data not shown). Smaller amounts of total RNA can be detected in nondenaturing agarose gels than in formaldehyde-denaturing agarose gels.

High-density oligonucleotide array analysis. Because the amount of RNA was too small to be applied to the standard protocol of the GeneChip system (Affymetrix, Santa Clara, CA), we used a double in vitro transcription technique, which can amplify the signals considerably. Biotin-labeled complementary RNA (cRNA) fragments were prepared as the target hybridized to the oligonucleotide array according to the Affymetrix protocol (GeneChip Eukaryotic Small Sample Target Labeling Technical Note). Briefly, double-stranded complementary DNA (cDNA) was synthesized from 50 ng (nor-

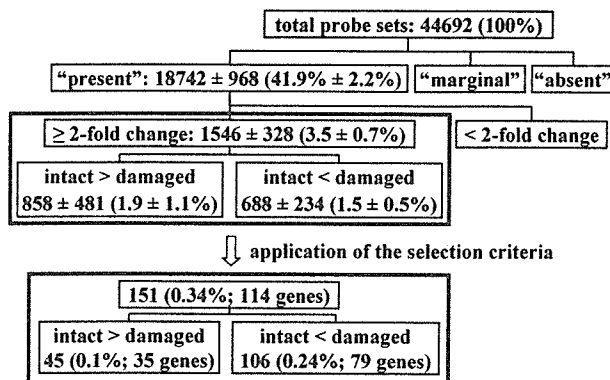


Figure 1. Flow chart showing the procedure for identifying genes with different expression levels in intact and damaged regions of human osteoarthritic cartilage. The terms “present,” “marginal,” and “absent” represent expression levels of the transcripts described in Materials and Methods. Values are the mean \pm SD number and percentage of transcripts. See Materials and Methods for details of the selection criteria.

malized value) of total RNA as the starting material. The cDNA was amplified by initial *in vitro* transcription, resulting in unlabeled cRNA. Double-stranded cDNA was also synthesized from the amplified cRNA. A second *in vitro* transcription was performed to produce biotin-labeled cRNA from the double-stranded cDNA. The biotin-labeled cRNA was fragmented before hybridization. Finally, 15 μ g of the fragmented cRNA was hybridized to human U133A and U133B arrays, stained with streptavidin–phycoerythrin, and scanned with a GeneArray scanner.

Analysis of the data. We normalized all intensity data by scaling the average signal intensity of 100 maintenance genes on each array to a value of 2,000 and then determined whether the transcript was detected (“present”), undetected (“absent”), or at the limit of detection (“marginal”) using Microarray Suite 5.0 software (Affymetrix). Comparison analysis was performed using Data Mining Tool 3.0 software (Affymetrix).

To identify the genes that showed the same expression pattern in different samples, the selection criteria (Figure 1) were defined as follows. First, selected transcripts had to show a ≥ 2 -fold difference in the level of expression among all 5 samples or a ≥ 3 -fold difference among 4 of the 5 samples. Second, selected transcripts had to be detected (“present”) and have normalized signal intensities > 100 in either the intact or the damaged region. The “fold change” value represents the ratio of the average signal intensity in intact regions to the average signal intensity in damaged regions of all 5 samples. The standard deviation of the mean was calculated from the standard deviation of the fold change value and was determined by applying to the following formula each mean and SD of the signal intensities in the intact and damaged regions of all 5 samples:

$$CV = \sqrt{CV1^2 + CV2^2}$$

where CV represents the coefficient of variation (the SD divided by the mean), CV1 represents the CV of the signal

intensities in intact regions, and CV2 represents the CV of the signal intensities in damaged regions.

The significance of the difference in levels of expression between the intact and damaged groups of cartilage was determined by paired *t*-test. Hierarchical clustering analysis was performed using the Cluster and TreeView software from Stanford University (available at <http://rana.lbl.gov/EisenSoftware.htm>).

Real-time quantitative PCR. Total RNA was prepared in the same way as for the oligonucleotide array analysis. These RNA samples were converted into cDNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and random primer. Quantitative PCR reactions were performed using the ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Primer and probe sets were purchased as TaqMan Gene Expression Assays for the set of genes to be studied: Hs00248808 (*CHRD2*), Hs00234422 (*MMP2*), Hs00155794 (*APOD*), Hs00182807 (*FGF13*), Hs00243202 (*SI00A4*), Hs00932737 (*TGFBI*), Hs00610420 (*PTGES*), and Hs00200180 (*TNFAIP6*). The quantities of target genes were calculated using the standard curve method and were normalized with 18S RNA (ABI item no. 4319413E) as an internal control. The fold change and SEM values were calculated as described above.

RESULTS

Findings of the histopathologic assessment of cartilage samples. First, we evaluated histopathologically the dissected cartilage fragments and scored them using the Mankin scale. We then determined whether each cartilage fragment satisfied the criteria for intact or damaged tissue as defined in Materials and Methods. Briefly, cartilage samples with a smooth surface and hyaline cartilage architecture and without fibrocartilage were defined as an intact region of OA cartilage. Cartilage samples with fibrillation, clefts, and several cell clusters were defined as a damaged region of OA cartilage. The histologic score for the damaged region (mean \pm SD 7.8 ± 0.8) was significantly higher than that for the intact region (2.6 ± 0.5).

Findings of the genome-wide screening. We used the Affymetrix GeneChip system to compare gene expression profiles in intact and damaged regions of the same OA knee joint. There are 44,692 probe sets on the Affymetrix human U133 chip set, covering $> 39,000$ transcript variants that represent 33,000 human genes. The expression analysis detected $\sim 40\%$ (mean \pm SD $18,742 \pm 968$) of all probe sets corresponding to the transcripts in each cartilage sample (Figure 1), but little difference was noted between the percentage of transcripts expressed in intact and damaged regions of cartilage (intact $42.1 \pm 2.5\%$ [$18,813 \pm 1,106$] versus damaged $41.8 \pm 2.1\%$ [$18,671 \pm 933$]). About 3.5% ($1,546 \pm 328$) of all probe sets indicated a ≥ 2 -fold difference in expression per sample (intact $>$ damaged

Table 1. Representative genes with higher expression in intact regions than in damaged regions of human osteoarthritic cartilage*

Category, gene name	Gene symbol	Public ID	Fold change	SEM	P†
Transcription factors					
<i>v-ets</i> erythroblastosis virus E26 oncogene homolog 1	<i>ETS1</i>	NM_005238	3.64	1.35	0.007
Signal transduction					
Guanylate cyclase 1, soluble, $\alpha 3$ subunit	<i>GUCY1A3</i>	NM_000856	8.17	2.60	0.028
Membrane proteins/receptors					
Glypican 5	<i>GPC5</i>	NM_004466	4.82	1.46	0.010
Transporters/carrier proteins					
Apolipoprotein D	<i>APOD</i>	NM_001647	6.24	1.15	0.004
Cytokines/growth factors					
Fibroblast growth factor 13	<i>FGF13</i>	NM_004114	4.47	0.73	0.001
Proteases					
Matrix metalloproteinase 2	<i>MMP2</i>	NM_004530	7.30	2.90	0.017
Secreted inhibitors/antagonists					
Chordin-like 2	<i>CHRDL2</i>	NM_015424	8.95	5.35	0.060
Immunity/defense					
Complement component 4 binding protein α subunit	<i>C4BPA</i>	NM_000715	6.44	3.53	0.121
Extracellular matrix proteins					
Vitron	<i>VIT</i>	NM_053276	9.34	3.09	0.031
Miscellaneous					
Expressed sequence tag	<i>EST</i>	BF002625	14.01	6.14	0.056

* See Materials and Methods for the definition of “fold change” and for the method of calculating the SEM. Complete data for all of the selected genes are available upon request from the corresponding author. Public ID = accession number in public databases (RefSeq or GenBank).

† All *P* values were significant (<0.05), as determined by paired *t*-test, except for the *P* values for genes *CHRDL2*, *C4BPA*, and *EST*.

Table 2. Representative genes with higher expression in damaged regions than in intact regions of human osteoarthritic cartilage*

Category, gene name	Gene symbol	Public ID	Fold change	SEM	P†
Transcription factor					
Sex determining region Y-type high mobility group box 11	<i>SOX11</i>	NM_003108	30.19	7.66	0.009
Cell cycle					
Cyclin D1	<i>CCND1</i>	NM_053056	6.96	2.25	0.006
Metabolism					
Phosphoserine aminotransferase 1	<i>PSAT1</i>	NM_058179	9.82	4.77	0.009
Collagen synthesis					
Prolyl 4-hydroxylase α polypeptide III	<i>P4HA3</i>	NM_182904	9.66	3.44	0.021
Transporters/carrier proteins					
Uncoupling protein 2	<i>UCP2</i>	NM_003355	10.56	5.88	0.017
Glycosylation					
UDP- <i>N</i> -acetyl- α -D-galactosamine:polypeptide <i>N</i> -acetylgalactosaminyltransferase-like 1	<i>GALNTL1</i>	XM_031104	12.16	2.97	0.010
Cell motility/invasion					
S100 calcium binding protein A4	<i>S100A4</i>	NM_002961	5.33	1.73	0.007
Signal transduction					
Down syndrome critical region gene 1	<i>DSCR1</i>	NM_004414	4.69	0.82	0.008
Membrane proteins/receptors					
Triggering receptor expressed on myeloid cells 1	<i>TREM1</i>	NM_018643	15.38	4.43	0.020
Secreted inhibitors/antagonists					
Tumor necrosis factor α -induced protein 6	<i>TNFAIP6</i>	NM_007115	25.34	5.78	0.001
Growth factors/cytokines					
Cytokine receptor-like factor 1	<i>CRLF1</i>	NM_004750	22.90	7.98	0.031
Immunity/defense					
Prostaglandin E synthase	<i>PTGES</i>	NM_004878	12.44	3.08	0.013
Proteases					
A disintegrin and metalloproteinase domain 12	<i>ADAM12</i>	NM_003474	4.43	1.59	0.024
Structural proteins					
Erythrocyte membrane protein band 4.1-like 3	<i>EPB41L3</i>	NM_012307	5.36	2.88	0.070
Adhesion proteins					
Transforming growth factor β -induced 68 kd	<i>TGFBI</i>	NM_000358	12.05	3.74	0.001
Extracellular matrix proteins					
C-type lectin domain family 3, member B	<i>CLEC3B</i>	NM_003278	11.16	5.45	0.030
Miscellaneous					
Hypothetical protein FLJ37034	<i>FLJ37034</i>	H16258	7.65	2.46	0.019

* See Materials and Methods for the definition of “fold change” and for the method of calculating the SEM. Complete data for all of the selected genes are available upon request from the corresponding author. Public ID = accession number in public databases (RefSeq or GenBank).

† All *P* values were significant (<0.05), as determined by paired *t*-test, except for the *P* value for gene *EPB41L3*.

1.9 ± 1.1% [858 ± 481]; intact < damaged 1.5 ± 0.5% [688 ± 234]). This corresponds to ~8% of the expressed transcripts (1,546/18,742 = 0.082). Furthermore, ~10% of differentially expressed transcripts were commonly detected in all 5 OA samples (151/1,546 = 0.098).

Thus, we identified 114 genes, which consisted of 35 genes expressed in intact > damaged cartilage and 79 genes expressed in intact < damaged cartilage. These genes were then subdivided into functional categories: the 35 genes with higher expression in intact regions than in damaged regions were subdivided into 10 categories, and the 79 genes with higher expression in damaged regions than in intact regions were subdivided into 17 categories. Since ~18% of all selected genes (21 of 114) have unknown function, these were assigned to the miscellaneous group, representing the largest category in the list. (Complete data for all of the selected genes are available upon request from the corresponding author.)

Differentially expressed genes in intact versus damaged regions. Tables 1 and 2 show a representative gene from each functional subcategory: 10 genes that were highly expressed in intact regions and 17 genes that were highly expressed in damaged regions, respectively.

The following genes were highly expressed in the intact region of OA cartilage (Table 1). The v-Ets-1 erythroblastosis virus E26 oncogene homolog (*ETS1*; 3.64-fold) is a member of the Ets transcription factor family and can activate the expression of many matrix metalloproteinases (MMPs) (9,10). Soluble guanylate cyclase 1 α 3 subunit (*GUCY1A3*; 8.17-fold) functions as the main receptor for nitric oxide. Glypican 5 (*GPC5*; 4.82-fold) is a glycosyl phosphatidylinositol (GPI)-anchored heparan sulfate proteoglycan and functions as a "coreceptor" for several "heparin-binding" growth factors (11). Apolipoprotein D (*APOD*; 6.24-fold) is a member of the lipocalin superfamily of transporter proteins that bind progesterone and arachidonic acid (12). Fibroblast growth factor 13 (*FGF13*; 4.47-fold), which is also called fibroblast growth factor homologous factor 2, is an intracellular protein that unlike other FGFs, cannot activate any FGF receptors (13). MMP-2 (*MMP2*; 7.3-fold) is an enzyme known to degrade denatured collagen. Chordin-like 2 (*CHRD2*; 8.95-fold) is a novel chordin-like bone morphogenetic protein inhibitor expressed preferentially in chondrocytes of developing cartilage and OA cartilage (14). C4 binding protein α subunit (*C4BPA*; 6.44-fold) is a regulatory protein of the classical pathway of complement. Vitron (*VIT*; 9.34-fold) is a member of the von Willebrand factor A superfamily, and it might serve as a bridge between collagen fibrils

and the hyaluronan network (15). The function of gene BF002625 (14.01-fold) remains unknown. Hierarchical clustering of this group of miscellaneous genes did not show characteristic changes (Figure 2A).

The following genes were highly expressed in the damaged region of OA cartilage (Table 2). Sex determining region Y-type high mobility group box 11 (*SOX11*; 30.19-fold) plays an important role in tissue remodeling, including craniofacial and skeletal tissue remodeling (16). Cyclin D1 (*CCND1*; 6.96-fold) is an essential component of chondrocyte proliferation in the growth plate (17). Phosphoserine aminotransferase 1 (*PSAT*; 9.82-fold) is an enzyme involved in the biosynthesis of L-serine, and the messenger RNA (mRNA) level for *PSAT* appears to be up-regulated to support cell proliferation (18). Prolyl 4-hydroxylase α polypeptide III (*P4HA3*; 9.66-fold) is a component of prolyl 4-hydroxylase, a key enzyme in collagen synthesis (19). Uncoupling protein 2 (*UCP2*; 10.56-fold) is a mitochondrial anion carrier protein that also protects against oxidative stress-induced cell death (20). Polypeptide N-acetylgalactosaminyltransferase-like 1 (*GALNTL1*; 12.16-fold) is an enzyme that catalyzes the initial reaction in the biosynthesis of O-linked oligosaccharide. S100 calcium binding protein A4 (*S100A4*; 5.33-fold) is involved in cell proliferation and tumor metastasis (21). Down syndrome critical region gene 1 (*DSCR1*; 4.69-fold) acts as a negative regulator of calcineurin signaling, which is known to induce chondrogenesis (22,23). Triggering receptor expressed on myeloid cells 1 (*TREML1*; 15.38-fold) plays a role in acute inflammation. Tumor necrosis factor α -induced protein 6 (*TNFAIP6*; 25.34-fold) forms complexes with inter- α -inhibitor (a serine protease inhibitor) and inhibits MMP activation via antiplasmin activity (24,25). Cytokine receptor-like factor 1 (*CRLF1*; 22.9-fold) is expressed in chondrocytes and osteoblasts and plays a role in cartilage and bone formation (26). Prostaglandin E (PGE) synthase (*PTGES*; 12.44-fold), which is also called microsomal PGE synthase 1 (27), is responsible for the production of PGE₂, which has a wide variety of biologic activities. A disintegrin and metalloproteinase domain 12 (*ADAM12*; 4.43-fold) is one of the candidate genes in OA susceptibility and progression (28). Erythrocyte membrane protein band 4.1-like 3 (*EPB41L3*; 5.36-fold) is a structural protein that links transmembrane proteins to the actin cytoskeleton. Transforming growth factor β -induced 68 kd (*TGFB1*; 12.05-fold) may play an important role in cell-collagen interactions in cartilage (29). C-type lectin domain family 3, member B (*COLEC3B*; 11.16-fold) is a plasminogen-binding protein that plays a role in tissue

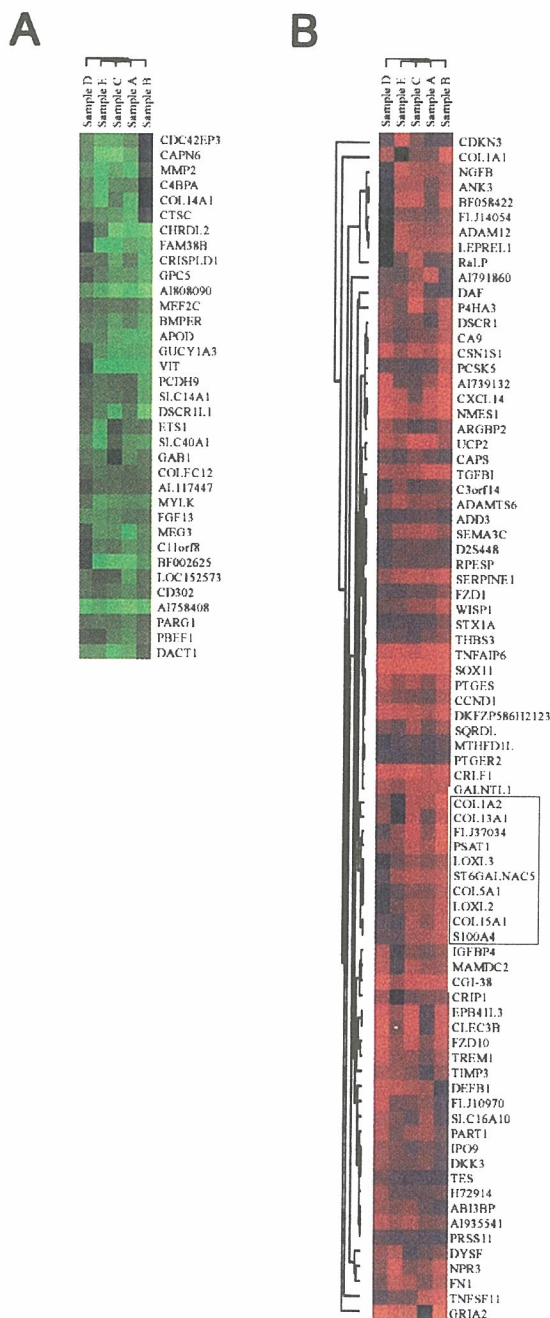


Figure 2. Hierarchical clustering of **A**, 35 genes that were highly expressed in intact regions of human osteoarthritic (OA) cartilage (green) and **B**, 79 genes that were highly expressed in damaged regions of human OA cartilage (red). Black areas indicate no difference in the level of expression between intact and damaged regions of human OA cartilage.

growth and remodeling (30). The function of gene FLJ37034 (7.65-fold) remains unknown.

There is a characteristic change in the group of

genes with higher expression in damaged cartilage. Three interstitial collagen genes (collagen, type I, $\alpha 1$ [*COL1A1*]; collagen, type I, $\alpha 2$ [*COL1A2*]; and collagen, type V, $\alpha 1$ [*COL5A1*]) together with 4 collagen biosynthetic enzymes (*P4HA3* [19]; lysyl oxidase-like 2 [*LOXL2*] [31]; leprecan-like 1 [*LEPREL1*] [32], and *LOXL3* [33]) were expressed at significantly higher levels in chondrocytes from the damaged region of OA cartilage than those from the intact region (Complete data for all of the selected genes are available upon request from the corresponding author.) Furthermore, hierarchical clustering analysis indicated that *COL1A2*, *COL5A1*, *LOXL2*, and *LOXL3* as well as other minor collagen genes belonged to the same cluster in the tree diagram (Figure 2B). This cluster also contained 2 proliferation-associated genes (*S100A4* and *PSAT1*).

Results of real-time quantitative PCR of selected 8 genes. To validate the oligonucleotide array data, real-time quantitative PCR was performed on 8 selected genes using additional 4 pairs of cartilage samples (Figure 3). For these 8 genes, the region of high expression as determined by real-time quantitative PCR was consistent with the region of high expression as determined by oligonucleotide array analysis. We found that the levels of expression of mRNA for *FGF13*, *TGFBI*, *PTGES*, and *TNFAIP6* genes in intact and damaged regions of OA cartilage were significantly different as determined by both methods. In the present study, however, the fold change in gene expression estimated using real-time quantitative PCR tended to

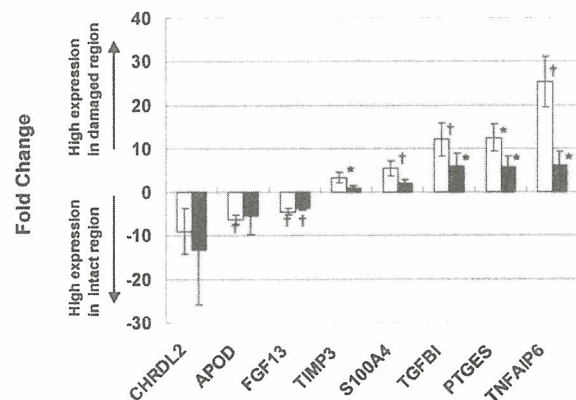


Figure 3. Histogram showing levels of expression of 8 selected genes, as measured by oligonucleotide array (open bars; $n = 5$) and real-time quantitative polymerase chain reaction (solid bars; $n = 4$). Values are the mean \pm SEM (see Materials and Methods for further details). * = $P < 0.05$; † = $P < 0.01$ for intact region versus damaged region, by paired t -test.

Table 3. Expression patterns of genes reported to be differentially expressed in intact versus damaged regions of cartilage as well as in normal versus OA cartilage*

Comparison, expression pattern	Ref.	Gene name (alias)	Gene symbol	Fold change†	SEM	P		
Intact versus damaged regions of cartilage from the same joint	Intact < damaged	4	Nerve growth factor, β polypeptide (NGF)‡	<i>NGFB</i>	5.08	1.54	0.011	
		4	Neurotrophic tyrosine kinase receptor type I (p140 TrkA)	<i>NTRK1</i>	ND			
	Intact > damaged	5	Insulin-like growth factor binding protein 3	<i>IGFBP3</i>	5.94	2.71	0.056	
		5	Insulin-like growth factor binding protein 4‡	<i>IGFBP4</i>	3.93	1.20	0.006	
		5	Insulin-like growth factor binding protein 5	<i>IGFBP5</i>	1.17	0.33	0.611	
		6	Heparan sulfate proteoglycan 2 (perlecan)	<i>HSPG2</i>	2.22	0.80	0.074	
		7	Matrix metalloproteinase 13	<i>MMP13</i>	-9.51	9.34	0.32	
		7	Hyaluronan and proteoglycan link protein 1 (link protein)	<i>HAPLN1</i>	-1.94	0.45	0.043	
		7	B cell CLL/lymphoma 2	<i>BCL2</i>	1.05	0.34	0.828	
		7	Sex determining region Y-type high mobility group box 9	<i>SOX9</i>	1.08	0.18	0.685	
	Normal versus OA cartilage	Normal < OA	34	Tumor necrosis factor α -induced protein 6 (TSG-6)‡	<i>TNFAIP6</i>	25.34	5.78	0.001
			35	Serine protease 11 (HtrA1)‡	<i>PRSS11</i>	3.08	0.77	0.012
36			Prostaglandin E synthase‡	<i>PTGES</i>	12.44	3.08	0.013	
37			S100 calcium binding protein A4‡	<i>S100A4</i>	5.33	1.73	0.007	
37			Fibronectin 1‡	<i>FN1</i>	6.16	1.49	0.002	
Normal > OA		37	Transforming growth factor β -induced 68 kd (BIGH3)‡	<i>TGFBI</i>	12.05	3.74	0.001	
		37	Collagen, type I, α 2‡	<i>COL1A2</i>	5.67	3.19	0.027	
		37	Matrix metalloproteinase 2‡	<i>MMP2</i>	-7.30	2.90	0.017	
		14	Chordin-like 2 (CHL2)‡	<i>CHRDL2</i>	-8.95	5.35	0.06	
		38	Serine proteinase inhibitor, clade E, member 1 (PAI-1)‡	<i>SERPINE1</i>	14.38	8.09	0.016	

* OA = osteoarthritic; ND = not detected.

† Positive numbers represent high expression in damaged regions; negative numbers represent high expression in intact regions.

‡ Included in the list of 114 genes selected by our criteria. Complete data for all of the selected genes are available upon request from the corresponding author.

be smaller than that estimated using oligonucleotide array analysis.

Expression patterns of genes reported to be differentially expressed in intact versus damaged regions of OA cartilage and in normal versus OA cartilage. We assessed the microarray data for genes that in previous studies were shown to be differentially expressed in intact versus damaged regions of OA cartilage from the same joint or in normal versus OA cartilage from different patients (4–7,14,34–38). The name and symbol of the reported genes are shown in Table 3. Table 3 also shows the sample characterization (comparison being made) and the gene expression pattern reported in previous studies, as well as the results of oligonucleotide array analysis for each gene.

Consistent with studies that compared intact and damaged regions of OA cartilage from the same joint, we confirmed high expression of *NGFB* (4), *IGFBP3*, *IGFBP4* (5), and *HSPG2* (6) in the damaged region, as

well as high expression of *MMP13* and *HAPLN1* (7) in the intact region of OA cartilage. However, we did not observe *NTRK1* gene expression itself or differential expression of *IGFBP5*, *BCL2*, and *SOX9*.

We also evaluated whether the results of comparisons between intact and damaged regions of cartilage from the same joint resembled the results of comparisons between normal and OA cartilage obtained from different individuals. At least 7 genes reported to be highly expressed in OA cartilage compared with normal cartilage (*TNFAIP6* [34], *PRSS11* [35], *PTGES* [36], *S100A4*, *FN1*, *TGFBI*, and *COL1A2* [37]) were highly expressed in the damaged region compared with the intact region of OA cartilage. However, there were some exceptions. According to our data, the 2 genes reported to be highly expressed in OA cartilage (*MMP2* [37] and *CHRDL2* [14]) were highly expressed in the intact region, and *SERPINE1* (38), which was reported to be expressed at low levels in OA

cartilage, was highly expressed in the damaged region of OA cartilage.

DISCUSSION

In this study, we compared the gene expression profiles in intact versus damaged regions of OA cartilage. Transcripts with a ≥ 2 -fold difference in mRNA expression between these 2 regions accounted for an average of 8% of all expressed transcripts per OA cartilage tissue sample, $\sim 10\%$ of which were commonly detected in the 5 patient samples. The former observation indicates that the gene expression profile of chondrocytes in the intact region is quite different from that of chondrocytes in the damaged region, even though both regions are in contact with the same synovial fluid and have the same genetic background. The latter finding suggests that the gene expression profiles of chondrocytes in OA cartilage change in a region-specific manner. This also suggests the presence of common molecular mechanisms of OA development, assuming that the changes in gene expression patterns of chondrocytes lead to cartilage degeneration.

Using our selection criteria, we identified 114 genes that were differentially expressed in the intact region versus damaged region of OA cartilage. Thirty-five of these genes were up-regulated in the intact region, and 79 genes were up-regulated in the damaged region. The validity of our data was confirmed using real-time quantitative PCR and comparing our findings with those of previous studies. The possible roles of these genes with significantly altered mRNA expression are discussed here in terms of the reaction patterns of chondrocytes in order to determine the underlying mechanisms that may participate in the pathogenesis or progression of OA. Sandell and Aigner (39) described 5 categories of cellular reaction patterns related to OA development: phenotypic modulation of articular chondrocytes, formation of osteophytes, chondrocyte proliferation and apoptosis, matrix synthetic activity of chondrocytes, and matrix degradation activity of chondrocytes. The latter 3 are discussed below.

With regard to chondrocyte proliferation and apoptosis, the *CCND1*, *PSAT*, and *S100A4* genes are known to be involved in cell proliferation, as mentioned above (17,18,21). These genes were more highly expressed in damaged regions than in intact regions of OA cartilage. This finding is consistent with the pathologic feature showing some clusters of chondrocytes in the surface layer of damaged cartilage, and it supports the hypothesis that changes in gene expression patterns of

chondrocytes lead to the pathologic condition of OA in cartilage. Yet, among the 114 genes we identified, no genes were clearly related to apoptosis. (Complete data for all of the selected genes are available upon request from the corresponding author.) However, we cannot suggest that there are no differences in the activation of the apoptosis signaling pathway between intact and damaged regions of OA cartilage, because the proteolytic cascade, rather than transcriptional regulation, is important in apoptotic signaling (40).

With regard to the matrix synthetic activity of chondrocytes, our microarray data showed that although the signal intensities of type II collagen and aggrecan were very high in both intact and damaged regions of OA cartilage, there were no differences in gene expression between these 2 regions (data not shown). However, we found that 3 interstitial collagen genes (*COL1A1*, *COL1A2*, and *COL5A1*) and 4 genes for enzymes involved in the collagen biosynthetic pathway (*P4HA3*, *LOXL2*, *LEPREL1*, and *LOXL3*) were highly expressed in the damaged region of OA cartilage. Interestingly, hierarchical clustering analysis showed that 4 of these 7 genes (*COL1A2*, *COL5A1*, *LOXL2*, and *LOXL3*) belonged to the same cluster as proliferation-associated genes (*S100A4* and *PSATI*). This suggests that wound healing, including the process of cell proliferation and interstitial collagen synthesis, occurs in damaged OA cartilage, where the expression of genes related to wound healing might be regulated in the same manner.

With regard to the matrix degradation activity of chondrocytes, the most obvious pathologic feature in the damaged region is advanced cartilage destruction. Therefore, we predicted that expression levels of protease genes would be higher in damaged regions than in intact regions. Consistent with this expectation, we identified 4 proteases (*DKFZP586H2123*, *ADAMTS6*, *ADAM12*, and *PRSS11*) with high expression in the damaged region of OA cartilage among the selected 114 genes. However, MMP-2, which can degrade the extracellular matrix, is only one protease showing high expression in the intact region of OA cartilage. In particular, the *PRSS11* (HtrA1) gene was recently reported to enhance cartilage degeneration via digestion of major cartilage components (41), and the single-nucleotide polymorphisms of the *ADAM12* gene are associated with the progression of knee OA (28). However, there is a discrepancy between the function of the detected genes and the histopathologic features. The expression levels of 3 genes known to inhibit degradation of the extracellular matrix (*TNFAIP6*, *SERPINE1*, and *TIMP3*) were

significantly high in the damaged region compared with the intact region of OA cartilage. These molecules are probably up-regulated to prevent the progression of cartilage destruction in the damaged areas of OA cartilage.

We subsequently examined whether the results of comparisons between intact and damaged regions of OA cartilage from the same joint resembled the results of comparisons between normal and OA cartilage obtained from different persons. We found that comparisons between normal and OA cartilage and between intact and damaged regions of OA cartilage yielded similar results with regard to the expression pattern of 7 of the 10 genes examined (Table 3). This suggests that during the transition from normal cartilage to OA lesional cartilage, the gene expression profile changes before there is any apparent damage to the cartilage. Because the expression levels of some genes probably change during the transitional period from normal to normal-appearing cartilage, our study design might have allowed us to miss some important genes that show no differences in expression levels between intact and damaged regions. To overcome this problem, further studies comparing these samples with normal cartilage samples from normal joints are needed.

What causes the OA-specific pattern of gene expression? Several possible mechanisms have been investigated, such as mechanical stress, cytokine stimulation, cell-matrix interaction, hypoxia, and reoxygenation. One of the strongest candidates is mechanical stress, because the damaged cartilage region is usually subjected to mechanical loading, whereas the intact region is not. In particular, chondrocytes residing in damaged regions are susceptible to mechanical stress because the tensile properties of the damaged cartilage are lost as a result of destruction of the collagen network (42). Proinflammatory cytokines, especially interleukin-1 (IL-1) and TNF α , are also closely related to the development of OA (3). We thought that these cytokines might be accessible to the chondrocytes in damaged cartilage. However, some IL-1-induced genes were detected in both regions (in the intact region, *ETS1*, *GUCY1A3*, *CABPA*, *PBEF*, and *APOD*; in the damaged region, *TNFAIP6*, *PTGES*, *FNI*, *NGFB*, and *TNFSF11*). Furthermore, although MMP-2 mRNA is not significantly up-regulated by treatment with IL-1 (43), our microarray data showed that MMP-2 mRNA was expressed 7.3-fold higher in intact regions than in damaged regions of OA cartilage. Therefore, the effects of the cytokine alone could not account for these complex conditions of OA in vivo. Up-regulation of MMP-2 mRNA might be the result of other factors,

including hypoxia/reoxygenation, cell-matrix interactions, and intermittent hydrostatic pressures (44–46).

In conclusion, our study demonstrated a clear difference in the gene expression profile in damaged regions of human OA cartilage compared with that in intact regions. The pattern of differences between these 2 regions was similar among 5 pairs of OA cartilage samples. This finding implies that there is a common mechanism responsible for the destruction and maintenance of the articular cartilage in OA. Elucidation of this mechanism is important for the development of effective treatments for OA.

ACKNOWLEDGMENTS

The authors thank Dr. A. Shibakawa for providing the human cartilage specimens, and N. Furuya, S. Asada, N. Takagi, S. Shinkawa, Y. Nakagawa, T. Mogi, H. Ogasawara, and M. Yamanashi for excellent technical assistance.

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Role of synoviolin in rheumatoid arthritis: possible clinical relevance

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The symptoms of rheumatoid arthritis (RA) are derived from the process of chronic inflammation and the overgrowth of synovial cells. However, the mechanisms of RA flares are not fully understood. To clarify these mechanisms, the authors carried out immunoscreening using anti-rheumatoid synovial cell antibodies and identified and cloned synoviolin – an endoplasmic reticulum-associated degradation (ERAD) E3 ubiquitin ligase. This molecule is overexpressed in the rheumatoid synovium and approximately 30% of littermates of *synoviolin*-overexpressing mice developed spontaneous arthropathy. Moreover, *synoviolin*^{+/-} mice were resistant to collagen-induced arthritis through enhanced apoptosis of synovial cells. Based on the gain- and loss-of-function, the authors consider synoviolin to play a critical role in the crisis of arthritis, and propose that RA is a hyper-ERAD disease. These findings provide a new pathogenetic model of RA, and suggest that synoviolin could be targeted as a therapeutic strategy for RA.

Rheumatoid arthritis (RA) is a disease associated not only with painful joints, but also generalized symptoms related to the whole body such as febricula, malaise and anorexia. RA affects approximately 1% of the population worldwide [1,2]. Although RA is a serious condition, a specific cure that leads to the improvement of quality of life is not yet available, mainly because the exact etiology of RA is still poorly understood.

The pathological features of RA include the chronic inflammation of systemic joints associated with overgrowth of synovial cells, which eventually causes cartilage and bone destruction in the affected joint [3,4]. It is thought that inflammation results from the activation of the cytokine system regulated by inflammatory cells [5]. During the course of inflammation, activated macrophages produce tumor necrosis factor (TNF)- α , interleukin (IL)-1 and IL-6. These cytokines, in turn, stimulate overgrowth of synovial cells to form a mass of synovial tissue called pannus, which invades the bone and cartilage through osteoclast activation and protease production [6-9]. As RA is considered to be an autoimmune disease, medical treatment targeting inflammation has been applied. However, nearly 25% of RA patients do not respond to anticytokine or anti-inflammatory therapies [10-12]. This article will focus on synovial cells and discuss the mechanisms of hyperplasia of rheumatoid synovial cells (RSCs).

Molecular cloning of synoviolin

The authors' laboratory first cloned RSCs and found that these cells bear autonomous proliferation properties with aberrant cytokine production

in a culture system [13,14]. Moreover, it was found that human T-cell leukemia virus type I (HTLV-I), one of the epidemic human retroviruses associated with arthropathy (HAAP) [15], and *tax*, the viral transforming gene that causes HAAP and its product, pp40Tax, could transform synovial cells into RSCs in patients and overexpressing mice [16-18]. However, expression of pp40Tax is not observed in human RSCs. Thus, to determine the functionally equivalent endogenous gene products in RSCs, comprehensive immunoscreening was carried out using anti-RSC antibodies, and synoviolin was cloned successfully [19], a human homolog of the yeast ubiquitin ligase (E3) Hrd1p/Del3p [20]. Synoviolin is an endoplasmic reticulum (ER)-resident membrane protein with a RING-H2 motif and is highly expressed in the rheumatoid synovium [19]. Since it is expected that this endogenous molecule might elucidate the cause of RA, the distribution and functional properties of synoviolin was investigated further.

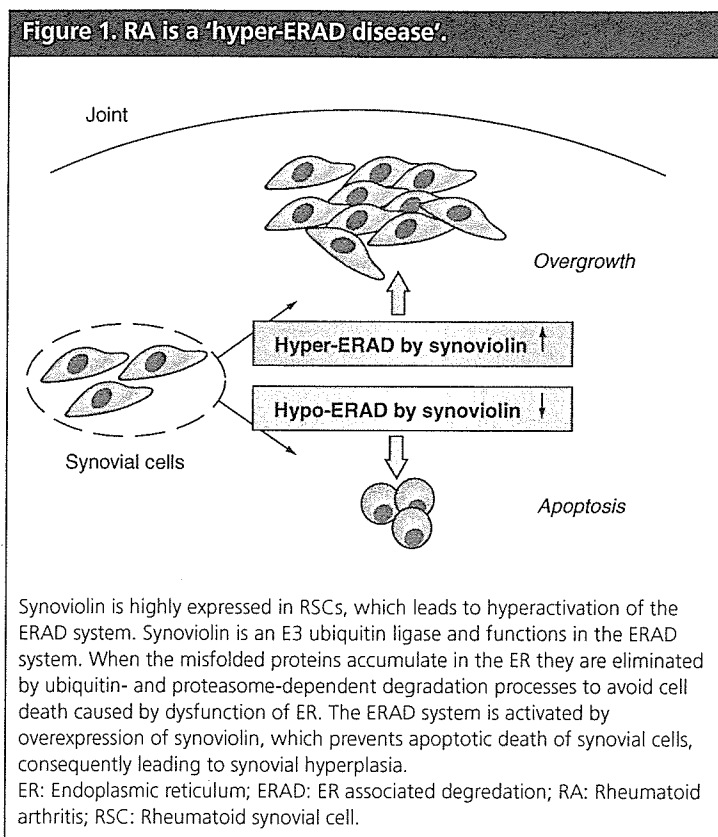
Arthropathy in *synoviolin*-overexpressing mice

To investigate physiologically the role of this molecule, the tissue distribution of synoviolin was first examined by northern blot analysis. The results in mice showed that *synoviolin* was ubiquitously expressed in various tissues. This was an unexpected result, since synoviolin was cloned in RSCs and it was predicted that *synoviolin* was specifically expressed in the rheumatoid synovium.

To gain insight into the function of synoviolin *in vivo*, the *synoviolin*-overexpressing mouse was generated. Essentially, it is desirable to analyze the

Keywords: apoptosis, endoplasmic reticulum-associated degradation, endoplasmic reticulum stress, hyper-endoplasmic reticulum-associated degradation disease, rheumatoid arthritis, synovial cell, synoviolin

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synoviolin function by using a promoter expressed specifically in synovial cells. However, such a promoter has not yet been identified. Thus, human *synoviolin*-overexpressing mice were established using a β -actin promoter, which drives systemic protein expression, including synovial cells. Strikingly, approximately 30% of *synoviolin*-overexpressing mice developed spontaneous arthropathy with marked joint swelling even in the C57BL/6 strain. Interestingly, no other abnormality was apparent in these mice throughout their life, apart from the spontaneous arthritis. In spite of the ubiquitous expression of *synoviolin*, this phenotype of *synoviolin*-overexpressing mice exhibited pathological features similar to those of patients with RA, indicating that these mice are a suitable animal model of RA.

Collagen-induced arthritis model of *synoviolin*-deficient mice

The results of the gain-of-function study suggested that synoviolin acts as an inducer of synovial cell hyperplasia. An attempt to verify this hypothesis was subsequently made in *synoviolin*-deficient mice, that is, loss-of-function. Mice with type II collagen-induced arthritis (CIA) are used

commonly as a model of experimental arthritis. This model can also be considered suitable for analyzing the function of synoviolin in RA. Thus, *synoviolin*-deficient (*syno*^{-/-}) mice were generated by gene-targeted disruption.

Unfortunately, all fetuses lacking synoviolin died *in utero* at around E13.5 [21], although Hrd1p/Del3p, a yeast ortholog of synoviolin, was described as nonessential for survival [22]. *Syno*^{-/-} were anemic owing to enhancement of apoptosis in the fetal liver [21]. In the next series of studies, the relationship between synoviolin and flares of RA was clarified. The CIA model was applied to *synoviolin*-heterozygous mice (*syno*^{+/-}). The results showed that *syno*^{+/-}, treated for induction of CIA, were resistant to the development of arthritis. Furthermore, CIA-*syno*^{+/-} mice exhibited intact immunoreactions and demonstrated inflammatory cell infiltration but lacked advanced synovial cell hyperplasia [19]. These results indicate that the synovial cell hyperplasia process is independent of preceding immunoreactions, and is an indispensable process in the pathogenesis of arthropathy. This conclusion was confirmed *in vitro*; *synoviolin* small interfering RNA (siRNA) suppressed the growth of synovial cells even when these cells were stimulated by cytokines [19]. When combined, these results of loss-of-function indicate that synoviolin is essential for the crisis of arthritis.

New disease concept: hyper-ERAD disease

How does synoviolin, an E3 ubiquitin ligase resident in the endoplasmic reticulum (ER), participate in synovial cell hyperplasia? In the ER of eukaryotic cells, newly synthesized proteins are transported for correct folding. Under normal conditions, the transport and folding processes in the ER match the requirement of the secretory pathway. Alternatively, since various environmental insults can overwhelm the efficacy of intracellular protein folding, cells have a self-protective mechanism for survival following an increased demand for protein folding. ER stress can trigger a cellular response termed the unfolded protein response (UPR) [23], during which the synthesis of new proteins is inhibited globally and genes encoding the ER chaperone proteins are also upregulated to refold the misfolded proteins correctly [24]. However, when the UPR fails to deal with this problem, misfolded proteins are eliminated by ubiquitin- and proteasome-dependent degradation processes, known as the ER-associated degradation (ERAD) system, and thus spare