EXTENDED REPORT

Deletion of the receptor tyrosine kinase Tyro3 inhibits synovial hyperplasia and bone damage in arthritis

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ABSTRACT

Objective To test whether the tyrosine kinase Tyro3 affects arthritis. Tyro3, the ligand of growth arrest-specific protein 6 (GAS6) is a receptor tyrosine kinase involved in cell survival. Tyro3 and GAS6 are expressed in the arthritic synovium, and in vitro studies have shown their role in osteoclast differentiation.

Methods Bone was assessed by micro CT and histomorphometry in Tyro3-deficient (Tyro3^{-/-}) and wild-type mice. Arthritis was induced in both genotypes, and GAS6 level was measured by ELISA. Synovitis, synovial hyperplasia, bone erosion, osteoclast activation and osteoclast gene expression were assessed by histomorphometry and reverse transcriptase-PCR, respectively. In vitro osteoclast differentiation assays were performed in Tyro3^{-/-} and wild-type mice. Furthermore, effects of Tyro3 and GAS6 on human synovial fibroblast proliferation and osteoclastogenesis were assessed in human cells.

Results Tyro3^{-/-} mice had significantly higher bone mass than wild-type littermates. Induction of arthritis increased GAS6 serum levels. Arthritic Tyro3^{-/-} mice showed less synovial hyperplasia, osteoclast numbers and bone damage compared with controls. In vivo expression of osteoclast-associated receptor and receptor activator of nuclear factor- κ B and in vitro osteoclastogenesis were impaired in Tyro3^{-/-} mice. GAS6 also induced synovial fibroblast proliferation and osteoclast differentiation in human cells in Tyro3-dependent manner.

Conclusions These findings indicate that Tyro3 is a critical signal for synovial hyperplasia, osteoclast differentiation and bone erosion during arthritis. GAS6 and Tyro3 therefore constitute therapeutic targets to inhibit synovial hyperplasia and associated bone erosion.

INTRODUCTION

Receptor tyrosine kinases (RTKs) are plasma membrane receptors, which essentially mediate the effect of growth factor on cells. Examples for RTKs include the insulin receptor as well as the receptors for vascular endothelial growth factor and platelet-derived growth factor. A less known RTK is Tyro3, which is expressed on the plasma membrane of a variety of different cells and acts as receptor for growth arrest-specific protein 6 (GAS6).¹ Tyro3 is not the only receptor for GAS6, which also activates the receptor tyrosin kinases Axl and Mer, transmitting anti-apoptotic and survival signals to cells.² Activation of Tyro3 by GAS6 binding

controls a variety of cellular processes such as toll-like receptor (TLR)-mediated and type I interferon-mediated regulation of cytokine production,^{3–5} haematopoiesis,⁶ thrombus formation⁷ and cell proliferation.⁸ Both Tyro3 and GAS6 are expressed in the synovial membrane of patients with rheumatoid arthritis (RA) and appear to play a role in the survival of endothelial cells in the synovial tissue of RA patients.⁹ Furthermore, GAS6 expression appears to be stimulated by inflammatory reaction, since increased serum levels of GAS6 have been described in sepsis and other types of systemic inflammatory responses.¹⁰ Interestingly, there is also a role of Tyro3 in bone metabolism: binding of GAS6 to Tyro3 leads to enhanced differentiation of bone-forming osteoclasts.^{11–13}

RA is characterised by chronic inflammation and hyperplasia of the synovial membrane, which leads to progressive destruction of bone and the adjacent articular cartilage. Inflammatory cytokines are considered as the essential triggers for bone loss in RA.^{14 15} Bone destruction in RA is closely linked to the presence of an activated synovial inflammatory tissue as well as synovial hyperplasia, which provides the essential signals for the development of bone-resorbing osteoclasts.^{16 17} Synovial-derived molecules, which trigger the generation of bone-resorbing osteoclasts, include inflammatory cytokines such as tumour necrosis factor- α (TNF- α), interleukin (IL)-6/IL-6R complexes and IL-1 as well as essential growth and differentiation factors for osteoclasts such as macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor- κ B ligand (RANKL).¹⁸

GAS6–Tyro3 interactions may be an interesting link between synovial inflammation and bone loss for several reasons: (1) GAS6 as well as its receptor Tyro3 are expressed in the synovial membrane of patients with RA⁹; (2) engagement of Tyro3 by GAS6 has been identified as direct inducer for osteoclastogenesis^{11–13}; and (3) GAS6–Tyro3 interactions play a role in the survival of cells involved in synovial inflammation such as fibroblasts, monocytes/macrophages, endothelial cells and chondrocytes.^{4 9 19 20} Apart from expression of Tyro3 and GAS6 in human RA and in vitro studies showing a role of GAS6–Tyro3 interactions in osteoclast differentiation, nothing is known about a potential in vivo role of the GAS6–Tyro3 system in arthritis. Based on the function of Tyro3, we hypothesised that Tyro3 activation may play a key role in

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inflammatory bone loss. To test this hypothesis, we assessed the bone phenotype of mice deficient for Tyro3 (Tyro3^{-/-}) and also induced arthritis in these mice and respective controls to analyse inflammatory bone changes.

MATERIAL AND METHODS

Animals

Mice deficient for Tyro3 (Tyro3^{-/-}) (B6.129-Tyro3^{tm1GRL/J}) has been described previously⁵ and were purchased from Jackson Laboratory (Bar Harbour, Maine, USA). Mice were backcrossed into the C57Bl6 background for six generations. Littermate wild-type mice expressing Tyro3 (Tyro3^{+/+}) were used as controls for all the experiments. Approval for all animal procedures was obtained by the ethical committee of the University of Erlangen-Nuremberg.

Arthritis model

Eight-week-old Tyro3-deficient animals (n=10) and littermate wild-type controls (n=10) were used for induction of arthritis. Two independent experiments with each five wild-type and five mutant mice were conducted. Arthritis was induced by intraperitoneal injection of 250 µl serum obtained from K/BxN mice as described previously.²¹ Disease progression was assessed by measuring joint swelling, grip strength and body weight every other day. After 12 days, animals were sacrificed by cervical dislocation.

Paw histology

Hind paws were fixed overnight in 4% paraformaldehyde and decalcified using EDTA. The material was then embedded in paraffin, cut and 1–2 µm sections were stained with H&E, toluidine blue or tartrate-resistant acid phosphatase (TRAP) for histological evaluation of articular inflammation, proteoglycan loss, bone erosions and osteoclast assessment. Analysis was performed using a microscope (Nikon, Berlin, Germany) equipped with a digital camera and digital analysis system (OsteoMeasure; OsteoMetrics, Decatur, Georgia, USA).

Histomorphometry

Ten-week-old Tyro3^{-/-} mice (N=10) and 10 wild-type littermate controls were sacrificed, and tibial bones were prepared and fixed overnight with 4% paraformaldehyde. Right tibial bones were embedded in methylmetacrylate (Technovit; Heraeus Kulzer, Wehrheim, Germany), cut and stained by Goldner trichrome and Van Kossa for histomorphometry. Bone histomorphometry was performed using a microscope (Nikon, Japan) equipped with a video camera and digital analysis system (OsteoMeasure; OsteoMetrics, Decatur, California, USA). The fraction of bone volume of the total sample volume (bone volume per total volume (BV/TV)), trabecular number (Tb. N), trabecular thickness (Tb. Th), trabecular separation (Tb.Sp), number of osteoclasts per bone perimeter (N.Oc/B.Pm), number of osteoblasts per bone perimeter (N.Ob/B.Pm) were measured according to international standards.²² In vivo bone formation was assessed by dynamic histomorphometry after injecting 0.6 mg calcein (Sigma-Aldrich, St Louis, Missouri, USA) subcutaneously 9 and 2 days before sacrificing the mice. The mineral apposition rate (MAR; µm/day) was determined by fluorescent microscopy. Left tibias were decalcified with EDTA and stained using TRAP staining for osteoclast assessment.

Micro-computed tomography

Tibias were analysed by CT using a high-resolution CT scanner (GE explore Locus SP Specimen Scanner; GE Healthcare,

London, Canada). Bones were placed in a sealed acrylic specimen holder containing phosphate-buffered saline and a hydroxyapatite phantom, which was used to calibrate Hounsfield units to mineral density (mg/ml). After scanning, images were reconstructed to an isotropic voxel size of 13.2 µm³ using a standard Feldkamp Conebeam algorithm and a threshold of 585 mg/ml. The following parameters were analysed: bone volume per total volume (BV/TV), Tb.Th, Tb.N and Tb.Sp.

Serum analyses

Serum was analysed using commercially available ELISA kits for Rat Laps (IDS Immunodiagnostic Systems), TRAP 5b (IDS Immunodiagnostic Systems) and GAS6 (AB Adipobiotech).

Osteoclast differentiation assay

Bone marrow cells were isolated and cultured for 3 days in 50 ng/ml of M-CSF. Cells (1×10⁶/well) were plated on a 24-wells cell culture plate (Costar-Corning, New York, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 50 ng/ml of M-CSF and 30 ng/ml of RANKL (both from R&D Systems) for another 3–4 days. Osteoclasts were detected by staining for TRAP staining using a commercial kit (Sigma-Aldrich) following manufacturer's instructions. Osteoclasts were identified as multinucleated purple-stained cells.

Real-time PCR

RNA was isolated from arthritic knee joints or osteoclast cultures by using Trizol (Invitrogen, Grand Island, New York, USA) following manufacturer's instructions. RNA concentration was measured before genomic DNA was digested with DNase I (Fermentas, St Leon-Rot, Germany), and cDNA was synthesised in the presence of RNase inhibitor. For SYBR green-based detection, a dissociation curve was carried out by one cycle following the last amplification cycle to control for the specificity of PCR amplification: 95°C for 15 s, 60°C for 30 s and 95°C for 15 s. Relative quantification was performed by calculating the difference in cross-threshold values (ΔC_t) of the gene of interest and a housekeeping gene according to the equation 2^{-ΔC_t}. For primers details, see online supplementary table 1.

Human osteoclast culture

Peripheral blood mononuclear cells were isolated from 10 ml EDTA blood of healthy donors using a Ficoll gradient (Lyphoflot, BioRad) and incubated in 48-well plates (7.5×10⁵ cells per well) in α-minimum essential medium (MEM) (Gibco) with 1% PenStrep (Gibco). After 3 h, the cells were washed for monocyte enrichment and cultivated for 14 days in α-MEM supplemented with 10% heat-inactivated fetal bovine serum (Biocrom AG), 1% penicillin/streptomycin (Gibco), 10 ng/ml M-CSF and submaximal concentrations of 1 ng/ml RANKL (both Peprotech). Every 3 days, medium was replaced with fresh medium. Various concentrations of recombinant GAS6 (R&D), Tyro3:Fc (also termed DTK:Fc), Axl:Fc (both Sigma Aldrich) or OPG:Fc (R&D Systems) were added as indicated. For coculture experiments with synovial fibroblasts, osteoclasts were isolated and cultured according to the same protocol as described above with the exception that no RANKL was added. Osteoclast differentiation was evaluated by staining cells for TRAP using a Leukocyte Acid Phosphatase Kit (Sigma-Aldrich) according to the manufacturer's instructions.

Human synovial fibroblast culture

Synovial tissue was retrieved from three patients with RA receiving synovectomy of the knee joint. Synovial tissue was minced and digested in dispase II (1.5 mg/ml; Roche Diagnostics Corporation, Indianapolis, Indiana, USA) dissolved in DMEM (without any supplements) for 2 h with vortexing steps each 15 min. The tissue suspension was then filtered through a 70 µm cell strainer. The cell suspension was collected, centrifuged, resuspended and cultured in DMEM (Invitrogen, Carlsbad, California, USA) supplemented with 10% heat-inactivated fetal bovine serum (Biocrom AG), 1% penicillin/streptomycin (Gibco). Cells from passages 3 to 5 were used for in vitro assays. Cells were used for coculture experiments as described above and as monocultures for assessing cell proliferation. The proliferation rate was determined at passage 4 using the CyQUANT Cell Proliferation Assay Kit (Invitrogen), according to the manufacturer's instructions. Fluorescence measurements were made with excitation at 485 nm and emission detection at 530 nm.

Statistical analysis

Data are presented as the mean±SEM. For group comparison, we used the non-parametric Mann–Whitney U test. A p value of less than 0.05 was considered significant.

RESULTS

Increased bone mass in mice deficient in tyro3

First, we analysed the bone phenotype of tyro3-deficient mice. Using 10-week-old tyro3^{-/-} mice, we performed µCT examination of the tibial bones. Analysis revealed a significant increase (p=0.031) in bone mass in tyro3^{-/-} mice in comparison to tyro3^{+/+} littermates. Bone microstructure analysis showed that increased bone mass in tyro3^{-/-} is based on enhanced Tb.Th, whereas Tb.N and Tb.Sp were not different among tyro3^{-/-} mice and littermate controls (figure 1A,B).

We also performed histomorphometric analysis of trabecular bone to further define bone microstructure of tyro3^{-/-} mice. Similar to the data obtained from µCT measurements, bone mass (BV/TV) as well as Tb.Th were significantly increased in tyro3^{-/-} mice compared with wild-type littermates. Evaluation of Tb.N and separation did not show major differences between tyro3^{-/-} mice and the controls (figure 1B). With respect to bone cells, tyro3^{-/-} mice and littermate controls were not different in osteoblast parameters such as osteoblast numbers and osteoblast-covered bone surface. Also, dynamic histomorphometry showed no significant difference in the MARs between the two groups (tyro3^{-/-}, 1.07±0.16 µm/day; tyro3^{+/+}, 1.15±0.09 µm/day). In contrast, osteoclasts were significantly reduced in their numbers (No.c/B.Pm) in tyro3^{-/-} mice compared with littermate controls (figure 1C,D).

Upregulation of the tyro3 ligand GAS6 in arthritis

We next attempted to define a potential role of tyro3 in pathological bone loss, since the ligand of tyro3 and GAS6 is expressed in the joints of patients with human RA.⁹ We induced arthritis by injection of serum from K/BxN mice into tyro3^{-/-} mice and wild-type littermate controls and measured the serum level of GAS6. Arthritis indeed led to a significant increase in GAS6 levels in tyro3^{-/-} mice (mean±SD before arthritis: 1131±2.00 ng/ml and after arthritis: 13.51±1.65 ng/ml) and wild-type controls (before arthritis: 10.05±1.20 ng/ml and after arthritis: 13.58±2.81 ng/ml) (figure 2A). Moreover, GAS6 levels were comparable in tyro3^{-/-} mice and wild-type controls.

Attenuated arthritis and arthritis-related bone loss in tyro3-deficient mice

Based on the phenotype of higher bone mass in conjunction with impaired bone resorption in tyro3^{-/-} mice as well as enhanced GAS6 levels in arthritis, we evaluated whether GAS6–tyro3 interaction affects bone loss during arthritis. After induction of arthritis in tyro3^{-/-} mice and wild-type littermates, we analysed clinical disease activity in tyro3^{-/-} mice and tyro3^{+/+} controls. Paw swelling was milder in tyro3^{-/-} mice than in the controls (figure 2B). Histological analysis of the hind paws showed mild but significant reduction of synovial inflammation in tyro3^{-/-} mice compared with controls (figure 3A and B). Most strikingly, however, synovial hyperplasia was almost absent in tyro3^{-/-} mice (figure 3A and C), and significant (p=0.045) reduction of bone erosions was found compared with controls (figure 3D and E). Osteoclast numbers were significantly lower (p=0.049) in tyro3^{-/-} mice compared with controls (figure 3F).

To further analyse bone protection in tyro3^{-/-} mice during arthritis, we measured systemic bone resorption in the serum of tyro3^{-/-} mice as well as local expression of osteoclast-specific markers in the joints. In accordance with histological findings, serum level of collagen type I cleavage products, a marker for bone resorption, was significantly (p=0.015) lower in tyro3^{-/-} mice (41.5±6.8 ng/ml) compared with wild-type controls (89.55±54.27 ng/ml). Similar results were obtained for the serum levels of matrix metalloproteinase 3 (figure 3G). Furthermore, we analysed the expression of osteoclast genes in the arthritic joints of tyro3^{-/-} mice and respective controls by real-time PCR analysis. We could show significantly lower expression of two essential receptors for osteoclast activation, OSCAR and, in tyro3^{-/-} arthritic mice compared with littermate controls (figure 3H).

Impaired osteoclast differentiation in tyro3^{-/-} mice

We next tested whether tyro3^{-/-} monocytes show an intrinsic defect to differentiate into osteoclasts. Monocytes were isolated from the bone marrow of tyro3^{-/-}-deficient mice and littermate wild-type controls and differentiated into osteoclast in vitro. Osteoclast differentiation was significantly impaired in monocytes of tyro3^{-/-}-deficient mice compared with those from wild-type controls. The number of multinucleated TRAP-positive cells, resembling osteoclasts, which could be generated from bone marrow monocytes of tyro3^{-/-}-deficient mice was significantly lower than in wild-type littermate controls (figure 4A,B). When analysing the expression of the osteoclast receptors, RANK and OSCAR, which were downregulated in tyro3^{-/-} mice in vivo, we found similar differences in cultured osteoclast in vitro. Expression of RANK and OSCAR was significantly lower in tyro3^{-/-} cultures than in wild-type controls (figure 4C). Moreover, induction of RANK and OSCAR expression by TNF-α was seen only in wild-type but not in tyro3-deficient cells (figure 4D).

Direct and indirect induction of human osteoclastogenesis by GAS6 in Tyro3-dependent manner

We next wanted to translate these findings to human disease and tested whether GAS6–Tyro3 interactions have an impact on human osteoclast differentiation. When human osteoclast precursor cells were exposed to GAS6, a dose-dependent stimulation of osteoclast differentiation was observed (figure 5A,B). This effect was particularly pronounced when cells were cultured under suboptimal concentrations of RANKL. GAS6 also induced osteoclastogenesis in monocytes from wild-type but not

Figure 1 High bone mass due to low osteoclast numbers in tyro3^{-/-} mice (A) Microphotographs showing trabecular structure of the tibial bone of tyro3^{-/-} mice and littermate controls assessed by micro-CT. (B) Results from micro-CT in tyro3^{-/-} mice (N=10) and littermate controls (N=10) showing bone volume per total volume (BV/TV), trabecular thickness (Tb.Th) and number (Tb.N) and separation (Tb.S). (C) Microphotographs show trabecular structure of the tibial bone of tyro3^{-/-} mice and littermate controls. Data are the mean±SEM. Asterisks indicate significant differences (p<0.05). (D) Results from histomorphometry of tyro3^{-/-} mice (N=5) and littermate controls (N=5); the following parameters were analysed: BV/TV, trabecular number (Tb.N), trabecular thickness (Tb.Th), number of osteoclast per bone perimeter (N.Oc/B.Pm), number of osteoblast per bone perimeter (N.Ob/B.Pm) and mineral apposition rate (MAR).

Figure 2 Role of the GAS6-Tyro3 pathway in arthritis (A) Circulating levels of GAS6 in *tyro3*^{-/-} mice (N=10) and littermate controls (N=10) with and without induction of arthritis. Data are the mean±SEM. Asterisks indicate significant differences (p<0.05). (B) Clinical evaluation of joint swelling of control mice (n=10) and *tyro3*^{-/-} mice (n=10) induced for arthritis by serum transfer.

in Tyro3-deficient mice (data not shown); however, it was not able to promote osteoclastogenesis in the absence of RANKL either in murine or in human cells. The pro-osteoclastogenic effect of GAS6 in human osteoclasts could be dose dependently abolished by addition of its soluble receptor Tyro3:Fc but not Axl:Fc (figure 5C). Aside from promoting osteoclastogenesis GAS6 also stimulated the proliferation of synovial fibroblasts. Human synovial fibroblasts did show better proliferation when exposed to GAS6 (figure 5D). This effect could be overcome by adding Tyro3:Fc or Axl:Fc, suggesting both receptors being involved in the autocrine stimulation of fibroblasts. Interestingly, soluble Axl (Axl:Fc) yielded similar effects to Tyro3:Fc on fibroblasts but did not affect GAS6-induced osteoclastogenesis (figure 5E). Finally, we assessed whether induction of osteoclastogenesis by synovial fibroblasts depends on GAS6. We performed coculture experiments with synovial fibroblasts, acting as a source for RANKL¹⁶ and monocytes. We could show that addition of Tyro3:Fc dose dependently inhibited osteoclastogenesis by synovial fibroblasts, suggesting that Gas6-Tyro3 interactions are essential for fibroblast-mediated stimulation of osteoclast differentiation (figure 5F).

DISCUSSION

The knowledge on the role of Gas6 and Tyro3 in bone biology was so far confined to in vitro observations. Three independent studies in the late 1990s and early 2000s have shown that the Gas6/Tyro3 system induces the differentiation of osteoclasts.^{11–13} Two of these studies have also shown that activation of the ERK mitogen-activated protein kinase pathway is an essential step for induction of osteoclast differentiation upon binding of Gas6 to Tyro3.^{12 13} However, these findings have not prompted investigators to assess the role of Tyro3 in bone homeostasis in vivo and to search for potential relevance of the Gas6/Tyro3 system in pathological bone loss. In this study, we have shown that deletion of *tyro3*, which disrupts Gas6-Tyro3 interaction, results in (1) significant increase of bone mass and (2) protection from bone loss during arthritis.

Interestingly, the Tyro3 ligand, GAS6, has been originally described as a gene upregulated in fibroblasts.¹⁹ GAS6 facilitates the proliferation and survival of fibroblasts, which may partly explain the mechanism of synovial hyperplasia in RA, which is characterised by the accumulation of activated synovial fibroblasts associated with cartilage and bone destruction in RA.^{14 18 23} Indeed, we found that the anti-inflammatory effect of Tyro3 deletion is based on the inhibition of synovial hyperplasia. In addition, our experiments showed that GAS6 induces the proliferation of human synovial fibroblasts by interaction with its receptors Tyro3 and Axl. In this context, it is interesting that O'Donnell and colleagues have shown that GAS6 is upregulated in the synovium of patients with RA.⁹ Expression of GAS6 by human synovial fibroblasts could thus represent a link between synovial hyperplasia and bone and cartilage destruction in RA. Our data support this concept by showing that inflammatory bone loss is regulated by Gas6 binding to Tyro3 expressed on the surface of monocyte/osteoclast lineage cells. In addition, our human data clearly showed that GAS6 induce osteoclast differentiation in human monocytes in a Tyro3-dependent manner and also that fibroblasts isolated from the joints of patients with RA support osteoclast differentiation in GAS6-Tyro3-dependent manner.

Earlier studies by Loeser and colleagues²⁰ have also suggested a role of Gas6-Tyro3 interaction in chondrocytes. Gas6 can stimulate chondrocyte proliferation and survival, which would be beneficial in arthritis. However, in our studies, we could not observe increased cartilage damage in Tyro3-deficient mice, but rather a protection from cartilage damage in conjunction with arthritis. Abrogation of GAS6-Tyro3 interaction may not essentially hamper chondrocyte survival, but allow protection from articular cartilage damage due to less synovial hyperplasia as well as due to the preservation of bone. Aside from bone-cartilage interactions with the synovium, our study opens new possibilities for future research on synovial hyperplasia in arthritis: (1) Stanczyk and colleagues²⁴ have shown that synovial fibroblast activation is at least in part controlled by microRNAs (eg, microRNA-203). It remains to be determined whether GAS6 affect the expression of these microRNAs, and thereby, control proliferation and activation of these cells. Interactions of GAS6 with certain microRNAs occurs in cancer.²⁵ (2) Mesenchymal stem cells (MSCs) have been described in the synovium²⁶ and may represent an important source for proliferating synovial fibroblasts. The effect of GAS6 on MSC is unclear, but it has been shown that GAS6 inhibits the differentiation of these cells into mature chondrocytes.^{27 28} GAS6 could maintain the MSC pool as a source for synovial hyperplasia. (3) Finally, previous studies suggest that GAS6 influences the

Figure 3 Less synovial hyperplasia and bone erosion in *tyro3*^{-/-} mice (A) Microphotographs showing H&E-stained hind paw section of wild-type littermates and *tyro3*^{-/-} mice. Left: talonavicular joint (original magnification $\times 100$), right: close-up showing the synovial lining (black bars; (original magnification $\times 400$); (B) quantification of synovitis and (C) synovial hyperplasia in the two groups (each $n=10$); (D) microphotographs showing TRAP-stained hind paw section of wild-type littermates and *tyro3*^{-/-} mice. Left: talonavicular joint (original magnification $\times 100$; black arrows: osteoclasts), right: close-up showing osteoclasts and erosion (original magnification $\times 400$); (E) quantification of bone erosion and (F) osteoclast numbers in the two groups (each $n=10$); (G) Serum levels of collagen type I cleavage products, a marker for bone resorption in *tyro3*^{-/-} mice ($N=10$) and littermate controls ($N=10$) induced for arthritis by serum transfer. (H) Expression of osteoclast receptors OSCAR and RANK in the knee joints of arthritic *tyro3*^{-/-} mice ($N=10$) and littermate controls ($N=10$) measured by reverse transcriptase-PCR. Data are the mean $\pm$ SEM. Asterisks indicate significant differences ($p<0.05$).

Figure 4 Impaired bone resorption, osteoclast differentiation and gene expression in *tyro3*^{-/-} mice (A,B) Microphotographs and graphs showing the results of osteoclast differentiation assays from *tyro3*^{-/-} mice (N=10) and littermate controls (N=10). Osteoclasts appear as purple-stained cells TRAP staining. (C) Expression of osteoclast receptors OSCAR and RANK in osteoclast cultures from *tyro3*^{-/-} mice (N=5) and littermate controls (N=5). (D) Expression of osteoclast receptors OSCAR and RANK in osteoclast cultures from *tyro3*^{-/-} mice (N=5) and littermate controls (N=5) with and without exposure to TNF-α. Data are the mean±SEM. Asterisks indicate significant differences (p<0.05).

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Figure 5 GAS6-induced human osteoclast differentiation and human synovial fibroblast proliferation in Tyro3-dependent manner. (A) Human osteoclast precursors were cultured with 10 ng/ml M-CSF and 1 ng/ml RANKL as well as various amounts of GAS6 for 14 days. (B) Human osteoclast precursors were cultured with 10 ng/ml M-CSF and 50 ng/ml RANKL as well as various amounts of GAS6 for 14 days. (C) Dose-dependent inhibition of GAS6-induced osteoclast differentiation by Tyro3:Fc in the presence of 1 ng/ml RANKL. (D) Proliferation assay of human synovial fibroblasts exposed to GAS6. (E) Inhibition of GAS6 (25 ng/ml)-induced proliferation of human synovial fibroblasts by Tyro3:Fc (0.5 µg/ml; yellow line) and Axl:Fc (0.5 µg/ml; blue line) and a combination of both agents (each 0.5 µg/ml; purple line). Controls were stimulated with 25 ng/ml GAS6 and Fc-fusion protein not related to the GAS6–Tyro3 pathway (osteoprotegerin (OPG):Fc; 0.5 µg/ml; red line) or were left without GAS6 stimulation (black dotted line). (F) Coculture of human synovial fibroblasts and osteoclasts in the presence of various amounts of Tyro3:Fc. OPG:Fc (0.5 µg/ml) was used as positive control for inhibition of osteoclastogenesis. All data are from three independent experiments. Data are the mean±SEM. Asterisks indicate significant differences (*p<0.05, **p<0.01).

Figure 6 Scheme on the role of GAS6 in synovial hyperplasia and osteoclastogenesis. Synovial fibroblasts proliferate by secreting GAS6 and autocrine stimulation of its receptors Tyro3 and Axl. In addition, GAS6 produced by synovial fibroblasts leads to Tyro3-mediated osteoclast differentiation.

chemoattractive behaviour of fibroblasts, allowing prolonged interactions with haematopoietic cells.²⁹ It can be speculated that GAS6 is at least partly responsible for the distinct chemokine and cytokine expression patterns found in synovial fibroblasts.³⁰

Strategies that inhibit GAS6 or its receptor Tyro3 could thus emerge as tools to reduce synovial hyperplasia and limit structural damage, particularly in RA. In contrast, diseases with a strong on IL-17/IL-23 signature, such as psoriasis and potentially also spondyloarthritis (SpA), may profit from GAS6 agonists rather than antagonists. van den Brand and colleagues³ have shown that GAS6 controls IL-12 and IL-23 production and that fostering GAS6 activity suppresses the expression of these two cytokines and mitigates disease activity in collagen-induced arthritis. Considering that GAS6 acts as a proliferation signal, however, direct neutralisation of IL-17 or IL-23 may be a more feasible strategy to treat disease like psoriasis and SpA, than an indirect approach through GAS6.

In summary, our data suggest that GAS6–Tyro3 ligand–receptor interactions affect bone mass in vivo. GAS6 directly induces osteoclast differentiation, and disruption of GAS6–Tyro3 interaction leads to impaired osteoclast differentiation and higher bone mass. This mechanism appears being particularly relevant in diseases with high local production of GAS6, such as RA, where synovial fibroblast produce this survival-associated and proliferation-associated protein, which links synovial hyperplasia with osteoclastogenesis (figure 6).

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