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Title: Endothelin-1 induces chondrocyte senescence and cartilage damage via endothelin receptor type B in a post-traumatic osteoarthritis mouse model

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Running title: ET-1/ET_BR axis in PTOA

Abstract

Objectives this study is to investigate the role of endothelin-1 (ET-1) and its receptors, the potent vasoconstrictor, in chondrocyte senescence and osteoarthritis development.

Method Temporal changes of ET-1 and its receptors were characterized in a well-received DMM murine model at time zero, 1 and 4 months after surgery. A transgenic mouse model overexpressing ET-1 (TET-1) was deployed to investigate the effect of upregulation of ET-1 to chondrocyte senescence and OA development. Rescue effects of selective endothelin receptor antagonists for cartilage degradation was then examined in DMM murine model. ET-1 H2O2 induce chondrocyte senescence models

Results Plasma ET-1 level increased from 3.18 ± 0.21pg/ml at time zero to 6.47 ± 0.34pg/ml in 4-month post-DMM mice. Upregulation of endothelin type B receptor (ET_BR) expression ~~[-30.50, 95%CI (-43.77 to -17.23)]~~ ~~and oxidative stress~~ ~~[-7.09, 95%CI (-13.53 to -0.64)]~~ p16INK4a+ senescent chondrocytes accumulation at articular cartilage started at 1-month after DMM prior to cartilage loss at 4-month post-surgery. Overexpressed endothelial ET-1 promoted ~~oxidative stress [22.38, 95%CI (9.955 to 34.80)] and~~ chondrocyte senescence ~~[0.2820, 95%CI (0.166 to 0.398)] in chondrocytes,~~ and ultimately led to cartilage degradation in TET-1 mice. Selective blockade of ET_BR, but not ET_AR, lowered the expression of p16INK4a in ET-1 or H2O2-induced chondrocytes senescence both *in vitro*, and mitigated chondrocyte senescence and the severity of murine PTOA *in vivo* ~~associated with reduction of ROS and removal of senescent chondrocytes. *In vitro* experiment further confirms ET_BR antagonist mitigated ET-1-induced chondrocyte senescence through lowering ROS production possibly via restoration of mitochondrial dynamics.~~
Increased ROS production and altered mitochondrial dynamics.

Conclusion

ET-1 could induce chondrocytes senescence and cartilage damages via ET_BR in PTOA.

Key words: Osteoarthritis, Endothelin-1, endothelin type B receptor, reactive oxygen species, senescence

Introduction

Osteoarthritis (OA) is one of the most common causes of disability in adults[1]. The hallmark of OA is cartilage degradation. In human OA cartilage lesions, senescent cells are detected near the cluster of chondrocytes[2]. Adult articular chondrocytes have limited proliferation capacity. In response to altered mechanical loading[3 4] or oxidative stress[5], articular chondrocytes undergo premature senescence with shortening of telomeres, which provokes the onset of OA[6]. Overexpressed senescence marker $p16^{INK4a}$ was sufficient to induce production of two major cartilaginous matrix remodelling enzymes: matrix metalloproteinase (MMP)-1 and -13[7]. Targeted ablation of $p16^{INK4a}$-positive senescent chondrocytes (SnCs) could mitigate OA in genetically modified mice[6]. Collectively, senescent chondrocytes are the emerging therapeutic targets for OA[20].

Vascular aetiology of OA has been proposed for decades[8]. Increased newly-formed blood vessels were found in OA synovium and osteochondral junction in both human and rodents[9]. Our previous studies have demonstrated the association of endothelial dysfunction in synovium and subchondral bone with articular cartilage damage in murine posttraumatic OA[10 11]. Yet the underlying molecular mechanism remains poorly understood.

Endothelin-1 (ET-1), the most potent vasoconstrictor predominantly expressed in endothelium, plays a pivotal role in vascular tone maintenance and a plethora of age-related pathologies including OA[12 13]. ET-1 transduces its biological functions through transduction via two G protein-coupled receptors, endothelin type A receptor (ET_AR) and type B receptor (ET_BR). ET-1 has been recently shown to promote formation of ROS and induce cellular senescence while blocking ET_BR lowered ET-1-induced ROS and senescent cells accumulation in endothelium[14]

[15]. Meanwhile, serum and synovial fluid ET-1 was found to associate with the severity of radiographic knee OA in human[16]. Moreover, ET-1 induces NFκB activation in a variety of cells such as macrophages and chondrocytes[17]; ET_AR or ET_BR antagonism attenuates NFκB activation and mitigates senescence-associated secretory phenotypes (SASPs) including MMP-13 *in vitro*[18 19].

Collectively, we hypothesize that ET-1 leads to chondrocyte senescence and cartilage damage through its receptors. We will test this hypothesis with the following aims 1) to characterize the spatiotemporal changes of local and systemic ET-1 with OA development in a DMM-induced PTOA mouse model; 2) to evaluate the effect of ET-1 overexpression or endothelin receptor blockade on chondrocyte senescence and cartilage degradation in murine PTOA; 3) to investigate the underlying molecular mechanism of ET-1-induced chondrocyte senescence.

Method

Institutional animal ethics committee approved all the experiments listed below (ASESC Case # 15-16/29-BME-R-GRF).

DMM-induced PTOA mouse model

Animals were raised in constant temperature at 25°C, with 12/12 light-dark cycle and supplied with food and water ad libitum. To generate a post-traumatic OA model, destabilization of medial meniscus (DMM) surgery was performed on 6-month old balb/c male mice according to an established protocol[20]. Briefly, mice were put in general anaesthesia by intraperitoneal injection of an anaesthetic cocktail [ketamine (100mg/ml): Xylazine (20mg/ml): saline = 1:0.5:8.5]. The medial meniscus was destabilized under general anaesthesia. Animals were allowed to recover on a heating pad until they are fully recovered from the anaesthetics. Drug treatment started one day after the surgery. Sample size estimation was performed based on the difference in OARSI histopathological score between the control and endothelin receptor antagonist treatment group as previously reported[21]. Assuming a pooled standard deviation of 8 units, the study would require a sample size of 7 for each group to achieve a power of 80% and a level of significance of 5% (two sided), for detecting a 12.25-unit difference in the means between surgery and treatment group using two-sample independent *t* test. To allow for 10% ineligibility, 8 mice were used in each group. Animals were randomly divided into 7 groups: a baseline group without DMM surgery (n=4); one-month (n=8) and four-month (n=7) post-DMM groups with vehicle injection; one-month (n=8) and four-month (n=8) post-DMM with 1 mg/kg BQ123 (a selective ET_AR blocker) (Sigma, USA) treatment; one-month (n=8) and four-month (n= 7) post-DMM with 1 mg/kg BQ788 (a selective ET_BR blocker) (Sigma, USA) treatment. Vehicles and drugs were administered intraperitoneally on a daily basis. Two mice died due to unexpected general anaesthesia accident during imaging examination, therefore were excluded in the analysis.

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Transgenic ET-1 overexpression mouse model

Transgenic mice overexpressing ET-1 in endothelial cells (TET-1) were given as a gift from Prof. Sookja Kim CHUNG form the University of Hong Kong. The transgenic mouse model was generated by microinjection of ET-1 construct, which contains mouse ET-1 cDNA with SV40 polyA driven by Tie-1 promoter. Genotyping was carried out to confirm the homogeneity of the animals used in the study (Fig. S1). Five TET-1 and four wildtype from the same litter at the age of 9-month were used in this experiment.

Evaluation of OA severity using multiple imaging modalities

Vascular volume and function measurement by power doppler (PD) and photoacoustic (PA) imaging

The vascular volume from PA (PAVV) and PD (PDVV) imaging as well as the tissue oxygenation level (sO_2) were measured using Vevo2100 high-frequency Micro-Imaging System (VisualSonics, Canada) in order to evaluate angiogenesis, vessel function as well as oxygenated haemoglobin in synovium respectively[11]. PD images were acquired using 50 MHz ultrasound transducer with a bandwidth of 30-70 MHz (MS700), while PA images were acquired using 30 MHz ultrasound transducer with a bandwidth of 18-38 MHz (LZ400). Briefly, a tendon–tibia–femur (TTF) triangle in murine knee joint was defined as the region of interest (ROI) in 2D ultrasonic and PA images. The vascular volume (%) was measured by linear translocation of the transducer perpendicular to the sagittal plane of the 2D image, and then calculated by multiplying the number of colour pixels by 0.032mm (slice thickness) and divided by total number of voxels in volume of interest.

Microstructure analysis of tibia subchondral bone using Microcomputed Tomography (μCT)

Knees were scanned using Viva CT40 (Scanco, Switzerland) and analysed by a built-in software. Isotropic voxel size for the scan was 10.5 μm. X-ray voltage and filter used were 70 kV and 1.0 respectively. Bone volume over total volume (BV/TV), trabecular number (Tb. N.) and trabecular thickness (Tb. Th.) were generated from the ROI in the subchondral bone of tibia.

Measurement of plasma ET-1 level

Endothelin-1 ELISA kit (Abcam, UK) was used to quantify endothelin-1 in plasma of mice according to the manufacturer's protocol. Blood was withdrawn from the animal on the day of sacrifice. Plasma was obtained by centrifuging blood at 1,600 x g for 15 minutes at 0˚C. The concentration of ET-1 was determined by the assay. Results were expressed as mean ± SEM of plasma samples.

Histological/immunohistochemical evaluation of OA knee joints

Samples were fixed and embedded following standard protocols. Samples were cut into 5 µm sections using microtome. Sections were stained with haematoxylin and eosin (H&E) and Safranin O/Fast green according to standard staining protocols for evaluation of histomorphology of the knee sections. Severity of OA was graded by a modified OARSI score (cartilage) [22 23] (Fig. S2) by two independent observers (one carried out blinded assessment). The agreement between the data obtained by observers was assessed by Cohen's kappa coefficient using online calculator QuickCals from Graphpad. The average scores from two independent observers were obtained for analysis. ImageJ was used to measure the thickness of hyaline cartilage. All H&E and Safranin O images were taken using Nikon Eclipse 80i Microscope (Nikon, Japan).

Immunohistochemical staining was performed to detect specific proteins in our samples. Antigen retrieval and quenching of endogenous peroxidase activity were performed. After blocking, primary antibodies were incubated with the samples at 4°C overnight. Primary antibodies used: Endothelin 1 (1:2000; Abcam, ab117757), Endothelin type A Receptor (1:2000; Abcam, ab117521), Endothelin type B Receptor (1:2000; Abcam, ab117529), CDKN2A/p16^{INK4a} (1:1000; Abcam, ab211542), NFκB p65 (1:500; Abcam, ab16502), 4-Hydroxynonenal (4HNE) antibody (1:50; Abcam, ab46545) and MMP13 antibody (1:200; Abcam, ab39012). For DAB staining, Vectastain ABC kit and DAB substrate kit for peroxidase (Vector Labs, USA) were used to stain targeted antigens followed by counterstaining using Harris Haematoxylin. For fluorescence staining, visualization of positive signals was performed using Donkey anti-Rabbit IgG H&L (DyLight® 488), Goat anti-Mouse IgG (H+L) (Alexa Fluor® 488) and Goat anti-Mouse IgG (H+L) Superclonal™ (Alexa Fluor® 647) secondary antibodies. The negative immune controls underwent the same procedure without primary antibody. Quantification using percentage of positive cells or fluorescence intensity was performed when deemed appropriate. Images were taken using Leica TCS SPE Confocal Microscope at a magnification of 200X.

Chondrocyte senescence model using ATDC5

ATDC5 cell line was received as a gift from Prof. Rong from Sun Yat-Sen University and cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12 1:1; Gibco Life Technologies, USA) supplemented with 5% (v/v) Fetal Bovine Serum and 1% (v/v) Penicillin/Streptomycin. The medium was changed every 2-3 days. All cells were incubated at 37°C in a humidified 5% CO_2 atmosphere.

ATDC5 were seeded on coverslips at a density of 10^4 cells/cm^2. After 24 hours in serum-free medium, drug treatment started when the cells reached 60-70% confluence. Working solution was prepared right before use. The final working concentrations of drugs used in this study were as follows: 100 μM hydrogen peroxide (H_2O_2) 100 nM Endothelin-1 (ET-1; Sigma), 1 μM BQ123 (Sigma, USA), 1 μM BQ788 (Sigma, USA), unless specified. Blocker treatment was performed 30 minutes before H_2O_2 or ET-1 stimulation for 24 hours. Various concentrations of vitamin C were added to the cells 1 hour before addition of H_2O_2 or ET-1. Technical replicates (n=3~5) were obtained for each assay in subsequent analyses.

Immunostaining was performed on the cells using the antibodies described above. Cells were fixed and permeabilized if deemed necessary. NFκB p65 and $p16^{INK4a}$ was stained to evaluate the H_2O_2- or ET-1-induced inflammation and SnCs accumulation respectively with blockers' pre-treatment.

Evaluation of mitochondrial oxidative stress and dynamics

For measurement of mitochondrial superoxide, 5 μM MitoSOX™ Red mitochondrial superoxide indicator (Molecular Probes, Eugene, USA) was applied to cells, incubated for 10 minutes at 37°C. Signal was visualized by excitation/emission at 510/580 nm. For evaluation of mitochondrial morphology, mitochondria were labelled with 100 nM MitoTracker® Red CMXRos for 30 minutes at 37°C. Excitation and emission wavelengths are 579 nm and 599 nm respectively. Fixation and mounting with ProLong® Gold antifade reagent with DAPI were performed in these two types of staining. Images were captured using Leica TCS SPE Confocal Microscope (Leica, Mainz, Germany) at a magnification of 630X. ImageJ was used to quantitatively measure the signal intensity and length of mitochondria.

Statistical analysis

All data were presented as mean ± S.E.M, with the mean difference with 95%CI given in the result. The comparisons of signal intensities and histomorphometric data among different groups were performed using one-way ANOVA or Kruskal-Wallis test when deemed appropriate. Respective post-hoc tests were carried when overall significance was detected between groups. Correlation between plasma ET-1 and modified OARSI score (cartilage degradation) was assessed by using Spearman's correlation. Two-tailed Student's *t*-test (unpaired) was performed for comparison between WT and TET-1. The level of significance was set at $p < 0.05$. Analyses and graphs were generated using Prism 8 (GraphPad).

Results

Plasma ET-1 positively correlated with articular cartilage damage in murine PTOA

Destabilization of medial meniscus (DMM) surgery was performed to generate a post-traumatic murine OA model. From H&E and safranin O/fast green staining, 4-month post-DMM surgery caused disruption of articular surface and proteoglycan loss (Fig.1a). Thickness of hyaline cartilage decreased substantially from 183.3 ± 11.05 µm in baseline to 77.73 ± 15.02 µm in 4-month post-DMM group [Mean difference (MD) = 105.6, 95%CI (21.76 to 189.4)] (Fig. 1b). More severe OA developed after 4-month of surgery as demonstrated by assessment of the modified OARSI score (cartilage) by two independent observers [Kappa=0.735; 95%CI (0.472 to 0.999)] (Fig. 1c).

To study the expression of ET-1 with OA development, local and systemic ET-1 was evaluated using immunostaining and ELISA respectively. With increasing severity of OA, ET-1 expression increased in subchondral bone and synovium but decreased in articular cartilage (Fig. 1d). Plasma ET-1 was found to increase significantly from 3.18 ± 0.21 pg/ml at baseline to 6.47 ± 0.34 pg/ml at 4-month post-DMM [-3.289, 95%CI (-4.771 to -1.807)] (Fig. 1e). A moderate positive relationship [$r^2 = 0.64$, 95%CI (0.349 to 0.9510)] was observed between the modified OARSI score (cartilage) and plasma ET-1 level, indicating ET-1 positively related to OA severity (Fig. 1f).

For receptors expression, we observed alteration of endothelin receptors expression prior to cartilage damage. Before cartilage damage at 1-month post-DMM, ET_AR expression decreased [22.91, 95%CI (8.723 to 37.1)] while ET_BR expression increased [-30.50, 95%CI (-43.77 to -17.23)] when compared to baseline. After cartilage damage at 4-month post-DMM, expression

of ET_AR remained at a low level while ET_BR dropped significantly [59.12, 95%CI (45.85 to 72.39)] (Fig. 1g).

Oxidative stress and senescent chondrocytes (SnCs) accumulation with OA development

Oxidative damage and chondrocytes senescence, as indicated by 4HNE and $p16^{INK4a}$ respectively, increased with the progression of PTOA. Similar to ET_BR, most of the 4HNE- or $p16^{INK4a}$-positive chondrocytes were located on the superficial layer of articular cartilage (Fig. 1h). Increase of 4HNE [-7.09, 95%CI (-13.53 to -0.64)] and $p16^{INK4a}$ [-17.29, 95%CI (-23.01 to -11.58)] expression in cartilage were observed from 1-month post-DMM onwards together with the upregulation of ET_BR. Similarly, we observed an increased in NFκB p65 translocation from cytoplasm to nucleus [-6.471, 95%CI (-12.69 to -0.2473)] (Fig. 1i) and MMP13 production [-13.34, 95%CI (-21.49 to -5.183)] (Fig. 1j) starting from 1-month after DMM surgery.

Overexpressed endothelial ET-1 led to SnCs accumulation and articular cartilage damage

A transgenic mouse model overexpressing ET-1 in endothelial cells (TET-1) was used to study the effect of ET-1 in OA pathogenesis. It was reported that TET-1 developed OA phenotypes at the age of 9-month. Damage of hyaline cartilage (HC), thickening of calcified cartilage (CC) and loss of proteoglycan were observed in 9-month TET-1 mice when compared to wildtype (WT) littermates using H&E and safranin O/fast green staining (Fig. 2a). However, no significant changes were observed in tibial subchondral bone plate (Fig. 2b). Fewer ET-1 and ET_AR but more ET_BR expression were found at articular cartilage of TET-1 mice (Fig. 2c), with a trend similar to OA cartilage in DMM model. Similar to DMM-induced OA cartilage, TET-1 cartilage showed a significant increase in 4HNE [22.38, 95%CI (9.955 to 34.80)] and

279 $p16^{INK4a}$ expression [0.2820, 95%CI (0.1656 to 0.3984)] (Fig. 2d-e), showing high level of
280 oxidative stress and senescent cells at cartilage when ET-1 is overexpressed. TET-1 mice also
281 displayed significantly more NFκB translocation [9.907, 95%CI (1.174 to 18.64)] and MMP13
282 production [72.36, 95%CI (53.35 to 91.36)] at articular cartilage than WT animals (Fig. 2f-g).
283
284 **H_2O_2 or ET-1-induced ROS accumulation and chondrocyte senescence via ET_BR *in***
285 ***vitro***
286 ATDC5 treated with 100 μM H_2O_2 for 24 hours showed an increase of mitochondrial
287 superoxide [-12.76, 95%CI (-15.72 to -9.794)] compared to control group. Thirty minutes pre-
288 treatment of BQ788 but not BQ123, inhibited the increase [10.09, 95%CI (6.641 to 13.54)]
289 (Fig. 3a). Immunostaining of NFκB p65 and $p16^{INK4a}$ showed that both endothelin receptor
290 blockers could reduce the nuclear translocation of NFκB p65, while only BQ788 could reduce
291 H_2O_2-induced chondrocyte senescence [12.92, 95%CI (4.066 to 21.77)] (Fig. 3b-c).
292
293 Similar to H_2O_2, 100 nM ET-1 stimulated production of mitochondrial oxidative stress in
294 ATDC5 after 24 hours of incubation, Again, pretreatment of BQ788 but not BQ123 prevented
295 oxidative stress accumulation in mitochondria [18.89, 95%CI (10.44 to 27.33)] (Fig. 3d). ET-
296 1 also promoted NFκB p65 translocation and $p16^{INK4a}$ accumulation (Fig. 3e-f). Similar to the
297 observations *in vivo*, both selective blockers decreased NFκB p65 translocation from
298 cytoplasm to nucleus, indicating endothelin receptors blockade suppressed ET-1-induced
299 NFκB activation (Fig. 3e). Senescent cells accumulation was also lowered by selective ET_BR
300 blockade [13.16, 95%CI (2.625 to 23.7)] (Fig. 3f), suggesting ET-1 causes increase of ROS
301 and senescent cells possibly through ET_BR. ET-1 also caused changes in mitochondria
302 morphology from tubular to blob form, this indicates the stress caused by ET-1 to the cells can
303 be reflected by shape change of mitochondria. ET-1 also reduced the length of mitochondria

304 from ~4 μm to ~0.6 μm. Blocking ET_BR selectively restored ET-1-induced change in
305 mitochondrial dynamics and increased the length of mitochondria (Fig. 3g).
306
307 **Selective blockade of ET_BR, but not ET_AR could lower ROS, mitigate chondrocyte**
308 **senescence and articular cartilage *in vivo***
309 To investigate the effect of drugs *in vivo*, BQ123 or BQ788 was intraperitoneally injected to
310 DMM mice. Our result showed that ET_BR but not ET_AR antagonism rescued cartilage
311 degradation and proteoglycan loss after 4-month of DMM surgery (Fig. 4a). Thickness of
312 hyaline cartilage in BQ788-treated group was restored to a level (186.4±26.24 μm) that is
313 insignificant to baseline (183.3±11.05 μm) (Fig. 4b). Only BQ788 but not BQ123 lowered
314 4HNE [24.17, 95%CI (18.94 to 29.39)] and $p16^{INK4a}$ expression [69.19, 95%CI (57.81 to 80.57)]
315 (Fig. 4c-d), indicating an important link between ET_BR, oxidative stress accumulation and
316 chondrocyte senescence. However, both selective receptor blockers reduced NFκB p65
317 translocation and MMP13 production (Fig, 4e-f). These findings indicated selective blockade
318 of endothelin type B receptor rescued OA cartilage phenotypes possibly through reduction of
319 oxidative stress and senescence accumulation in chondrocytes. While NFκB may play a role
320 in ET-1-induced MMP13 increase via both endothelin receptors.
321
322 **Endothelin receptor antagonism improves DMM-induced subchondral bone changes and**
323 **synovial vessels function**
324 Micro-CT analysis of DMM tibia subchondral bone showed osteophytes formation and
325 thickening of tibial plateau with reduction in trabecular number and increase in trabecular
326 thickness compared to baseline. Both BQ123 and BQ788 treatment reversed DMM-induced
327 changes of tibia subchondral bone plate (Fig. 5a-d), suggesting blocking either one of the
328 endothelin receptors could rescue osteoarthritic changes of subchondral trabecular bone. Using
329 power doppler and photoacoustic imaging techniques reported by our group previously[11], we

demonstrated better synovial vascular function, reduced synovial angiogenesis and higher tissue oxygenation level in OA knee after endothelin receptor blockers treatment (Fig. 5e). Besides, histological analysis showed a reduction of blood vessels per area in synovium after 1-month of blockers treatment (Fig. 5f), which further confirms endothelin receptors play a role in endothelial dysfunction.

ET-1 induced chondrocytes senescence through ROS accumulation

To elucidate the causal relationship between ROS accumulation and ET-1-induced chondrocyte senescence, different concentrations of vitamin C (Vit C), a typical ROS scavenger, were added to the cells one hour before ET-1 stimulation. We found that 100 μM or higher concentration of vitamin C significantly lowered ET-1-induced mitochondrial superoxide by more than half [100 μM Vit C: 13.41, 95%CI (8.27 to 18.55); 200 μM: 17.18, 95%CI (12.04 to 22.32)] (Fig. 6a). The same concentration, i.e. 100 μM of vitamin C, also reduced senescence maker $p16^{INK4a}$ by half [100 μM Vit C: 8.519, 95%CI (3.454 to 13.58); 200 μM: 14.43, 95%CI (9.035 to 19.83)] (Fig. 6b). This implicated ET-1-induced chondrocyte senescence could be reduced by removing oxidative stress in cells. We inferred that ET-1-induced chondrocyte senescence occurs via ROS accumulation.

Discussion

Endothelium-derived ET-1 induces not only cellular senescence in blood vessels but also in bone and joint. Firstly, our data showed that both H_2O_2 and ET-1 led to accumulation of oxidative stress and chondrocyte senescence *in vitro.* Secondly, we demonstrated ET-1 induced chondrocyte senescence through accumulation of ROS via ET_BR but not ET_AR. Thirdly, ET_BR is responsible for replicative senescence and SASPs production that lead to cartilage damage in OA development, while ET_AR is responsible for SASPs production only (Fig. 6C). Fourthly, selective blockade of ET_BR successfully alleviated OA-like cartilage changes in a PTOA murine model, indicating cartilage damage on OA development is more likely to be driven by replicative senescence via ET_BR but not SASPs production via ET_AR. For the first time, a direct effect of endothelial dysfunction and the role of endothelin type B receptor in OA pathogenesis have been reported.

Despite an increase of ET-1 expression in subchondral bone, synovium and plasma with increasing OA severity, expression of ET-1 decreased in articular cartilage. Intriguingly, we observed an upregulation of ET_BR in cartilage when OA develops. ET_BR, has been known as a clearance receptor to remove excess ET-1[24]. Angiogenesis in synovium and subchondral bone was reported in OA knee joints in some of our previous studies[10 11]. This is a possible explanation why ET-1 is highly expressed in these highly vascularized structure instead of avascular articular cartilage in OA joints. Due to the abundance of blood vessels in these structures, the increase of ET_BR may not be sufficient to clear away the excess ET-1 secreted by the endothelial cells. This explains the tissue-specific property of ET-1 expression within a joint.

Given overexpression of ET-1 leads to OA phenotypes, blocking endothelin receptors to prevent transduction of ET-1 via ET_AR or ET_BR would be a possible way to alleviate OA symptoms. A study showed improvement in radiographic indices of OA after treating ACLT-induced rat with ET_AR blocker BQ123[21]. Their study mainly focused on bone changes and nociceptive tolerance, yet cartilage changes were less studied. In our study, we studied the effect of both selective ET_AR or ET_BR blockade to rescue of OA. However, our results suggested that ET_BR blocker BQ788 but not BQ123 brought genuine protective effect to OA cartilage from injury. The discrepancy in result may due to the different drug administration regimes, weekly injection was used in Kaufman's study while we employed daily injection. The half-life of BQ123 is less than 15 minutes[25], which raised our concern on the effectiveness of weekly administration of this drug to OA joint. In our study, we observed that ET_AR antagonist lowered NFκB and MMP-13, yet failed to remove SnCs and preserve cartilage integrity in OA joint. It indicates that it is not sufficient to rescue OA by only targeting SASPs. Together with unsatisfactory results from a variety of clinical trials on IL-1β, IL-6[26 27], our work implies that replicative senescence of chondrocytes might contribute more to OA pathology compared to SASPs.

However, the role of replicative senescence of chondrocytes in OA pathology remains controversial. A recent study by Jeon and colleagues showed local clearance of $p16^{INK4a}$-positive cells at articular joint attenuated cartilage damage in a PTOA model[6]. However, the other study showed conflicting results that conditioned knockout of $p16^{INK4a}$ in aggrecan-positive cells, i.e. chondrocytes, failed to alleviate cartilage damage and joint destruction after DMM[28]. Our findings well aligned with Jeon's findings. Moreover, our pharmaceutical treatment not only removed senescent cells in articular cartilage but also in subchondral bone

(Fig. S3). It indicates that it is critical to remove senescent cells from whole joint tissues, rather than simply eliminating them from articular cartilage for rescue of OA.

A few limitations of the study should be noted. Firstly, OA is a whole joint disease, not limited to articular cartilage. We suggested removal of senescent cells from the whole joint can alleviate PTOA. In this study, we focused on the direct effect of endothelial dysfunction on cartilage, with only some changes in synovium reported. Our results suggest endothelin receptor blockers can rescue endothelial dysfunction in both cartilage and synovium, driving removal of senescent cells. Further investigation is needed to confirm our postulation. Secondly, we reported the effect of two selective endothelin receptor blockers to cartilage damage but the effect of dual receptors blocker was not included. Both selective endothelin receptor blockers led to reduction of MMP13, which is a major SASP causing OA progression. Bosentan, a dual endothelin receptor blocker, lowered the increase of p16 and SA-β-Gal expression in endothelial cells[15]. In future, we will investigate the effect of dual receptors blockade on rescue of OA.

To our best knowledge, we for the first time, decipher the molecular basis of endothelial dysfunction, i.e. ET-1/ET_BR signalling, in the pathogenesis and management of posttraumatic OA. It provides us a new insight into the vascular aetiology of OA and opens a door for mechanism-based discovery for disease-modifying OA drugs targeting endothelial dysfunction. In the past decade, the focus of our research was placed on the bone-cartilage crosstalk; the findings generated from the present study highlight the importance of synovial angiogenesis in chondrocyte senescence and cartilage damage. Apart from the structural damage, our work warrants further investigation into the role of ET-1/ET_BR in synovitis and OA pain.

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Author Contributions

MTA, ZYL and CYW designed the study. MTA and ZYL performed experiments and analysed data. MTA performed all the staining and *in vitro* study. ZYL performed the surgery, contributed to ultrasound imaging data collection, measured plasma ET-1 concentration and evaluated severity of OA. YPZ contributed to ultrasound imaging data collection, 3D data analysis and interpretation. LMR contributed to study design and data interpretation from clinical perspective. MTA prepared the draft of the manuscript, which was revised by CYW. All authors have read and approved the final version of the manuscript.

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Competing interest statement

We declare that there are no conflicts of interest.

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