# ENDOTHELIAL SENESCENCE MODULATES CHONDROCYTE FATE IN JOINT AGEING AND OSTEOARTHRITIS

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# Endothelial Senescence modulates Chondrocyte Fate in Joint Ageing and Osteoarthritis

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## Certificate of Originality

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# Abstract

Osteoarthritis (OA) is a prevalent joint disease leading to disability. Despite decades of effort in OA research, current treatment for the disease is limited to pain relief. Its complex pathogenesis and aetiology have hindered the therapeutic discovery. Understanding the cellular and molecular perspectives of the disease hence become an urge in the field.

Cartilage lesion is the typical hallmark of OA. As the primary constituent of hyaline cartilage, chondrocytes have been of particular interest. Chondrocyte hypertrophy and senescence are two important cellular states that have both been implicated in OA onset and progression. While they co-exist frequently in OA joints, little is known about the relationship between the two cellular processes. This study provides a pioneering idea of the role of chondrocyte fates in joint homeostasis by establishing a novel model to distinct the two cellular states. It has been demonstrated that hypertrophic chondrocytes were more prone to senescence, and could be driven into senescence state under oxidative stress. Hypertrophy also seemed to be a critical stage for oxidative-stress induced senescence. Interference with chondrocyte hypertrophy has been shown to protect cells from becoming senescence. As an age-related degenerative disease, cellular senescence has been targeted for OA therapy. The findings of the transition from hypertrophy to senescence in chondrocytes therefore provided new insight into the anti-senescence strategy in OA.

OA is now conceived as a whole-joint disease with the interplay between systemic and local risk factors. Amongst all metabolic conditions, hypertension has been the most prominent comorbidity to knee OA epidemiologically. This study fills the gap of the lack of experimental proof of the causal association between them. We demonstrated that the secretome of senescent endothelial cells could trigger chondrocyte senescence, potentially via hypertrophy. Noticeably,

such senescence phenotypes could be alleviated by improving vascular health. Antihypertensive drug, captopril, has exhibited its senolytic effect on vascular ageing. It seemed to mitigate endothelial senescence and its senescence-associated secretory phenotypes. Our findings provided the first experimental evidence of vascular dysfunction-induced cartilage ageing through biochemical crosstalk. The finding has also strengthened the notion of hypertension being a risk factor of OA as observed in clinics.

In short, this project has opened up new avenues for OA management strategy. Microscopically, our data has pointed in the direction of targeting chondrocyte hypertrophy for senescence elimination in age-related OA. Macroscopically, we have evidenced the role of vascular health in cartilage homeostasis. We demonstrated the senolytic effect of antihypertensive drug in endothelium and its subsequent beneficial effect on chondrocytes. This work hints at the potential of targeting local joint dysfunction systemically and would thus lay a foundation for upcoming scientific advances in OA management.

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## 1.1 Osteoarthritis

### 1.1.1 Prevalence and Burden

Osteoarthritis, also known as OA, is a common musculoskeletal disease that affects over 500 million people worldwide (1). While population ageing continues to trend, the number of OA cases has continued to prevail and has increased by nearly 50% in the past fifty years without any signs to cease. Being responsible for 2% of global years lived with disability (YLD) in 2019, OA is recognised as the leading cause of disability in older adults (1, 2).

OA usually occurs in load-bearing joints, including the hip and knee joints, and it is reported that approximately 80% of patients experienced movement restriction while a quarter of them could not perform major daily living activities (3). Limited activities and chronic pain, in turn, obstruct the social inclusion of OA patients. Additionally, increased mortality was reported in the OA population even though OA is considered to be a chronic, non-lethal disease (4).

To date, OA remains incurable. The obstinacy of the disease, therefore, brings not only individual but also socioeconomic burden. Direct costs of OA are mostly attributed to hospitalisation and surgical operations. The joint replacement expense was up to a total of 15 billion in the United States in the past decade, and was projected to increase at an accelerating rate (5). On the other hand, indirect costs of OA, including workforce absenteeism, premature death and early retirement also comprised a large proportion of the total burden. For example, the average aggregate annual cost of absenteeism of OA workers exceeded 10 billion in the US (6). With the growing prevalence of the disease, the burden of OA is substantial and could be a challenge to the healthcare system.  Noticeably, knee OA has been reported to account for

83% of total OA burden, highlighting the compelling need for promising knee OA treatment in near future (7).

### 1.1.2 Pathophysiology of knee Osteoarthritis

OA is traditionally conceived as a non-inflammatory disease, which distinguishes it from another common chronic inflammatory joint disorder, rheumatoid arthritis (8). It is widely known that OA is a simple disease where articular cartilage that cushions the ends of bones wears and tear over time. With the advancement of imaging modalities, knee OA is currently recognised as a whole-joint disease with not only cartilage erosion, but also synovial lining inflammation and subchondral abnormalities (9).

*Structural (Radiographic) changes in knee OA*

Knee OA is primarily diagnosed by radiography. Most common techniques used for OA detection include X-ray imaging and computer tomography (CT) for rigid tissues, and magnetic resonance imaging (MRI) for both rigid and soft tissues (10). The Kellgren-Lawrence (K-L) scale, a semi-quantitative grading system, is often used to evaluate the severity of OA based on radiographic changes (11, 12). This scoring system takes osteophyte formation, joint space narrowing, bone sclerosis and deformity into account and categorises the disease into grades 0 to 4, with grade 4 being the most severe stage (12). Another routine method for OA assessment is the Osteoarthritis Research Society International (OARSI) atlas criteria (11, 13). It defines the presence of OA when there is either moderate joint space narrowing (grade $\geq 2$), moderate osteophyte formation (grade $\geq 2$), or mild joint space narrowing (grade 1) along with mild osteophyte (grade 1) (13). The two systems define OA in similar manner but are not always interchangeable (14, 15).

## Articular cartilage

The hyaline cartilage lines the surfaces where the femur and tibia articulate with each other. It is mainly composed of water, type II collagen and proteoglycans (16). Articular cartilage erosion is a definite hallmark of OA. Currently, MRI is often used to capture the changes of cartilage contour during OA progression by assessing spatial distribution of water content (16, 17). Regions with abnormal water content are usually associated with chondral lesions. Hence, the heterogeneity of water distribution indicates the severity and location of damage sites. With the help of contrast agents such as gadolinium, MRI can reveal biochemical changes of glycosaminoglycans at sites of lesions (16). Yet it remains technically challenging to capture detailed microscopic changes within the 4mm-thick cartilage layer (17).

## Subchondral Bone

Subchondral bone is the layer of bone lying underneath the articular cartilage. It consists of the subchondral bone plate which directly connects to the calcified cartilage, and trabecular bones which arise from the bone plate and provide shock-absorbing function (18). Subchondral bone is a highly dynamic tissue that responds to mechanical stress by modulating bone modelling and remodelling. Changes in bone dynamics are therefore typically used to assess the severity of OA.

Joint space narrowing and osteophytosis can be readily detected under X-ray (19). The former can be diagnosed by measuring the distance between two bone ends on X-ray film. It is usually caused by the wear-out of articular cartilage. The increased friction between bones may then cause the formation of osteophytes, the outgrowth of bony structures. Appearance of the two features is particularly useful for grading the stage of

OA development via the K-L grading system and OARSI atlas (12, 13). Other radiographic changes of bone structure in knee OA include subchondral bone cyst formation and sclerosis, which can be seen under X-ray and bone marrow oedema-like lesion under MRI (19, 20). All of the features reflect disruption in bone microarchitecture and structure.

Synovium

The synovial membrane is a connective tissue that can be found on the inner surface of the joint capsule. It produces synovial fluid to lubricate and nourish the articular cartilage (21). Low-grade inflammation of synovial lining is frequently reported in OA knees. Synovitis can be detected by noticing the thickening of tissue under MRI. Affected synovium also often shows synovial fluid drainage defect and causes joint effusion. The excess accumulation of fluid can be diagnosed with contrast-enhanced MRI (22).

*Functional (symptomatic) changes in knee OA*

While radiographic changes are always observed in OA knees, not every patient experience symptomatic changes and knee pain. Many studies have tried to correlate radiographic with symptomatic OA but the results widely varied (23, 24). Knee pain can poorly predict structural damage of joint and is not necessarily associated with the severity of disease. The reason behind this remains unelucidated but the discrepancy between the two could sometimes cause confusion about research findings.

### 1.1.3. Aetiology of knee Osteoarthritis

OA has multifactorial aetiologies involving both local and systemic risk factors. Aside from the conventional concept of the impact of mechanical loading, metabolic factors and genetic influence are gaining more attention in the field. The complex aetiology of knee OA complicates the discovery of an effective treatment for the disease. It is therefore pivotal to understand the causes of OA from different perspectives.

*Local risk factors*

Knee OA was once conceived as a local problem of the joint. As a load-bearing tissue, the impact of mechanical force on the knee became a particular interest while studying the disease. Moderate mechanical stimuli are found to be crucial for musculoskeletal system homeostasis including cartilage and bone (25). However, it could be deteriorative when excessive mechanical stress is present.

Physical Activity and Mechanical Stress

Regeneration capacity of articular cartilage is highly limited. Repetitive and intensive exercise that involves weight-bearing, squatting and lifting potentially poses higher biomechanical stress to the knee joint and contributes to cartilage worn out, a representative hallmark of OA. Population with strenuous occupation has been found to have a higher risk of suffering from OA (26, 27). Similar findings have been demonstrated by rodent studies. Moderate and regular exercises showed an anabolic effect on young mice's cartilage. In contrast, forced and excessive exercise seemed to promote catabolism of cartilage, especially in aged mice (28).

### Injury and Trauma

Although knee OA is well known as an age-related degenerative disease, it is not uncommon in the younger population. Most OA cases in young patients are post-injury and post-surgery (29, 30). Specifically, early-age anterior cruciate ligament (ACL) tear has been identified as a risk factor for OA development at a later age. ACL runs diagonally to connect the femur and tibia and helps stabilise the knee joint. A high incidence of ACL injuries is seen in adolescents engaging in sports activities. The injury alters the biomechanics of the joint and often poses excessive stress on the medial compartment of the knee, leading to the onset of OA. The reported rate of post-traumatic OA (PTOA) following ACL tear was over 80% (31). The situation did not seem to reverse after reconstruction surgery (29). One of the theories suggests unresolved chronic inflammation after trauma initiates early onset of OA but further investigation is required (32).

## *Systemic risk factors*

Understanding in OA aetiology has evolved from taking it as a simple local disorder to realising the interplay between local and systemic risk factors in disease onset and progression. Ageing and obesity have been of particular interest in OA research. Despite being identified as primary risk factors, both of them are insufficient to account for the growth of the OA population (33). Research focus is then shifted to other metabolic factors that might predispose to OA.

### Genetics

Studies suggested genetics as an influential determinant of OA, with an influence between 39% to 65% of radiographic hand and knee OA in women (34, 35). The disproportionated racial difference in the OA population also suggests the role of

genetic factors in disease development. Genes that have been implicated in OA include cartilage matrix proteins *COL2A* and *COL1A1*, signalling molecules *TGFβ* and *IGF-1*, and immunological interleukin (IL) genes and human leukocyte antigen genes (34, 36, 37). Some of the genes identified from genome-wide association studies have been extensively researched in OA pathogenesis but some may need further experimental studies.

Ageing

Ageing remains the primary risk factor of OA. The rate of knee OA keeps increasing with age and peaks at age of around 75 regardless of gender (38). However, the mechanism behind ageing and the high incidence of OA is poorly understood. Some theories suggest cytokines such as IL-6 involved in low-grade inflammation during the ageing process contribute to OA development; some hypothesise the reduced capacity of joint tissues to adapt to biomechanical changes leads to disease onset; and some try to look into the molecular perspective by taking cellular ageing and senescence into account for the tissue degeneration (2, 39-41).

Metabolic syndrome

Metabolic syndrome (MetS) is a cluster of at least three out of the following five conditions: central obesity, high blood pressure, high blood glucose, high cholesterol and low high-density lipoprotein levels (42). Amongst all, obesity is the most discussed metabolic condition in OA pathogenesis.

The chance of over-weight subjects having OA was 2.5 fold higher than a normal-weight person; while the risk for obese subjects with body mass index (BMI) $\geq$ 30 was

6 fold higher (43). In addition to the extra mechanical burden to joints, an increase in adipokines level in obesity seems to promote cartilage catabolism. A higher concentration of leptin was detected in OA synovial fluid and was associated with cartilage damage severity. Current evidence suggests that leptin induces the production of pro-inflammatory cytokines and matrix-degrading enzymes matrix metallopeptidase (MMP) 9 to 13 (44, 45).

Hyperglycaemia and hypertension are the other frequently encountered conditions in the OA cohort (46). Oxidative stress, advanced glycation end products and inflammatory caused by hyperglycaemia were demonstrated to associate with local cartilage toxicity and destruction (41, 47, 48). Yet the epidemiological association between high blood glucose and knee OA remains controversial with contrasting data reported (49). While the conclusion of hyperglycaemia as an independent risk factor of OA cannot be drawn by case-control studies, a comprehensive meta-analysis revealed hypertension as the only component of metabolic syndrome positively associated with knee OA (49, 50). However, neither are the weights of individual components contributing to MetS-associated OA known, nor are all these components as well-studied as obesity and also hyperglycaemia to date.

## 1.2 Comorbidity of Hypertension and OA

The first positive association between hypertension and knee OA was reported in 1995 (51). Many studies have since then been done attempting to decipher the correlation between the two. The latest Framingham OA study reported that after adjustment for weight or BMI, all metabolic syndrome components except hypertension have no significant association with the occurrence of OA (50). In other words, hypertension is highly likely a key factor in the pathogenesis of MetS-associated OA. This brings up a debate on the stake of vascular aetiology in OA pathogenesis which has long been proposed.

### 1.2.1 Epidemiology evidence

Epidemiologically, most studies agreed that there is a connection between hypertension and knee OA, in particular radiographic knee OA. Hypertension is a regularly associated comorbidity to knee OA, and may lead to an increased risk of joint deterioration (52, 53). Most published meta-analyses and systematic reviews had pointed out that even though both radiographic knee OA and symptomatic knee OA are associated with hypertension, radiographic knee OA clearly showed a more significant correlation in comparison. A meta-analysis showed that hypertension was associated with higher radiographic knee OA than symptomatic knee OA risks, another systematic review performed in 2021 also concurred that a more convincing relationship between hypertension and radiographic knee OA than symptomatic knee OA (53, 54).

Nonetheless, the diversity in experimental parameters and OA definition have caused discrepancies in results reported. For example, only increased systolic blood pressure and pulse pressure showed a significant relationship with the progression of radiographic knee OA in a study with data from the Osteoarthritis Initiative (55). On contrary, both systolic and diastolic

blood pressure has been taken into account in the Framingham Osteoarthritis Study, but they were proved to be related to symptomatic knee OA instead (56).

While hypertension seems to be more likely to relate to structural deterioration in OA knees than subjective discomfort experienced by OA patients, the interference of other confounding factors cannot be overlooked. Only a minority of studies have included gender and race as cofounding factors, while the relationships between these factors and knee OA are noticeable. Examples include knee OA being more prevalent in females and Asian populations (54, 56). As most studies performed were descriptive researches, the interrelation between knee OA and hypertension cannot be established.

A study in 2019 had reported an inverse relationship between genetically determined blood pressure and the risk of knee OA, hip OA and surgical joint replacements (57). The study acquired data from the UK Biobank and employed a big data analytic tool, Mendelian randomization, which allows investigation of confounding and inverse causation (57, 58). The study presented hypertension as a repercussion rather than a cause of knee OA. While the experimental setting might be powerful, only symptomatic knee OA had been considered in the research. Based on the fact that radiographic changes in knee OA progression have poor correlations with symptomatic severity of the disease, the claims of the study remains controversial.

All in all, there is a significant research gap regarding the causal relationship between hypertension and knee OA. Given the technical difficulty to standardise research settings in epidemiological studies, benchwork experiments may be a more direct way to provide experimental evidence of the correlation between the two pathologies.

### 1.2.1 Vasculature in joint health and disease

While knee OA has now been recognised as a whole-joint disease, the contributions of the vascular system in knee OA pathogenesis may have been under-researched. Studies on vasculature in joint homeostasis were mostly done in healthy joints without inflammatory or degenerative symptoms. Reduction in blood flow was reported to lead to both reduced angiogenesis and osteogenesis, while the administration of bisphosphonate, a class of drugs mainly used to treat osteoporosis, led to increased blood flow and enhances the growth of blood vessels (59). However, the study on metaphyseal bones and diaphyseal bones may not represent the situation in subchondral bones.

To study vascular performance in OA knees, another research performed microangiography on the subchondral bone tissue of post-traumatic OA mouse samples. They demonstrated angiogenesis in OA knees by reporting the elevated blood flow volume and the number of blood vessels (60). Visualisation and analysis of the vascular system and angiogenesis in animals and humans remain technically challenging. Advanced techniques such as optical clearing could be helpful to investigate how changes in the vasculature of subchondral bones affect the progression of OA.

*Cartilage*

As the hyaline cartilage is avascular and aneural in nature, the continuation of cartilage depends greatly on the subchondral bones and synovium. Synovial fluid allows nutrients and oxygen to diffuse across the synovial capillaries to the chondrocytes lying in the superficial zone of hyaline cartilage (61, 62); while the subchondral bone replenishes nutrients for the calcified cartilage (63). Despite being a barrier between the superficial layer of cartilage and subchondral bone, the calcified cartilage is permeable to tiny molecules such as glucose. The biochemical

interaction between subchondral bone and cartilage was proven by the presence of bone-derived proteins in articular cartilage (64). These interactions may occur via microcracks between the bone end and cartilage, where subchondral bone blood vessel invasion was documented in OA (65).

*Subchondral bone*

Trabecular bone is highly vascularised with a dense network of capillaries and sinusoids. While microvessels are in the form of sinusoids in haematopoietic bone marrow, they appear to be ordinary capillaries in adipose bone marrow (66). Cortical bones are also filled with extensions of marrow space that contain small vascular channels. These channels are in nature thin-walled capillaries encapsulated by concentric bone shells and are responsible for transporting nutrients to cartilage and regulating remodelling at the osteochondral intersection (67). Angiogenesis has been observed in both subchondral bone and synovium during OA progression (68-70). Angiogenesis often couples with osteogenesis in the process of bone modelling and remodelling upon mechanical stimuli (71). Therefore, vascular modification is anticipated given the abnormal mechanical loading between bone ends caused by cartilage erosion in OA.

Other than subchondral angiogenesis, vascular invasion could also be discovered between calcified and non-calcified cartilage in OA knees (71). The extent of invasion could also be linked to the severity of cartilage erosion and subjective pain (68, 72). Other than vascular invasion, sympathetic and sensory nerve endings could also infiltrate the cartilage via vascular channels, causing symptomatic changes (73). This may explain the clinical correlation between vascular invasion and knee pain (68, 74). The delay of joint deterioration and reduction of joint pain after angiogenesis inhibition also reflected the role of vasculature in knee joint homeostasis and health (75).

*Synovium*

Synovium is a structure with a dense network of capillaries. Vascularisation varies across anatomical locations in the synovial tissue (76). They are found dense in the superficial layer of the synovial membrane, areolar and adipose tissues (77). The proximity between the synovial capillaries and hyaline cartilage facilitates the transport of nutrients and biochemical signalling molecules (78). Endothelial modification has been documented in joint arthritis. Microvessel formation accompanied by endothelial cell proliferation could be seen in OA synovium (68, 69). Meanwhile, secretory profile of synoviocytes, cells that are responsible for synovial fluid production, altered in OA. Cells extracted from the inflammatory areas have shown great angiogenic potential with increased secretion of vascular endothelial growth factors (VEGF) (79). Hence, the extent of angiogenesis in the synovium may be highly related to the progression of inflammation in OA knees.

### 1.2.3 Antihypertensive drug in OA treatment

Repurposing of existing antihypertensive drugs could be a potential OA treatment given the intimate relationship between the two diseases. This strategy provides immediate clinical value and therefore has been gaining attention in OA research. Potassium-sparing diuretics and adrenergic antagonists are two types of drugs which are used most often among all as they possess inflammation and pain reduction effects.

*Potassium-sparing diuretics*

Potassium-sparing diuretics help to excrete water and sodium while allowing potassium to be reabsorbed. They lower blood pressure by regulating ion concentration in body fluid. Although their antihypertensive effect is relatively mild, they could be used to treat hypertension which is non-responsive to other drugs (80). Potassium-sparing diuretics could be further divided into

aldosterone antagonists and epithelial sodium channel blockers. Spironolactone is an example of an aldosterone antagonist which could be helpful in OA patients who has normal blood pressure. Low dosage of spironolactone had been proved to improve knee joint effusion and reduce pain even more efficiently than ibuprofen, a common NSAID used in treating OA, while not affecting the blood pressure in normotensive patients (81). Eplerenone is another example of an aldosterone antagonist which could be helpful in protecting metabolic-related OA joint damage, as it had successfully reduced cartilage deterioration, osteophytes emergence and synovial inflammation in mouse models with obesity-induced hypertensive heart failure (82). Amiloride is an example of epithelial sodium channel blockers. Inflammation and a drop in pH value could signal abnormal activation of acid-sensing ion channels, enhancing pain and progression in OA models (83). As an acid-sensing ion channel inhibitor, amiloride suppresses cartilage erosion caused by acids and enhances type II collagen expression (84).

*Adrenergic antagonists*

Adrenergic antagonists inhibit hypertension by counteracting adrenaline and could be divided into alpha and beta adrenergic antagonists. Clonidine is an example of an alpha-adrenergic antagonist which acts on the central nervous system. It was found to be more effective in reducing joint pain in OA rat models when administered systemically compared to intra-articular injection (85). However, some may argue that local anaesthesia could suffice in humans (86). Beta-blockers are beta adrenergic antagonists. However, its effect on knee OA is still under debate as a study had reported reduced pain in OA subjects after the use of beta-blockers while another reported the contrary (87, 88).

## 1.3 Chondrocyte development and fate in cartilage homeostasis

Chondrocytes constitute the cartilage which acts as a template in the early stage of skeletal development and persists as resilient tissue afterwards. In the knee joint, chondrocytes are abundant in both articular cartilage and epiphyseal growth plate. The organization and homeostasis of cartilage depend solely on the growth and differentiation of chondrocytes. Chondrocytes arise from mesenchymal stem cells (MSCs) and undergo chondrogenic differentiation and maturation. They eventually reach their terminal differentiation state and become hypertrophic cells. The fate of hypertrophic chondrocytes, however, remains controversial. Conventionally, it has been suggested that hypertrophic cells will either undergo apoptosis or transdifferentiate into bone cells. Yet, recent evidence proposed the ability of hypertrophic chondrocytes to persist in senescence state, providing new insight into cartilage research (89).

### 1.3.1 Condensation and Chondrogenic differentiation

MSCs undergo chondrogenesis and give rise to chondrocytes. Condensation of MSCs begins with the increase of hyaluronidase that digests hyaluronan in the cartilage matrix and thereby allows cell-cell interaction (89). The expression of adhesion proteins such as neural cadherin (N-cadherin) and neural cell adhesion molecule (N-CAM) in MSCs further facilitates cellular contact. Simultaneously, the extracellular matrix (ECM) component, fibronectin, is upregulated to mediate MSC translocation (90). In condensation and chondrogenesis, the expression of transcription factor *Sox9* is critical. It activates the transcription of Col2a1 gene and initiates the synthesis of type II collagen, the basis of articular cartilage (90).

### 1.3.2 Proliferation

Bone morphogenic protein (BMP) signalling is one of the pathways in controlling chondrocyte proliferation. BMP activates SMAD transcriptional factors and thereby promoting cell proliferation (91). The induction can be antagonised by fibroblast growth factors (FGFs) which stimulate expression of cell cycle inhibitor p21 (92).

Chondrocytes show a dynamic development profile with regard to their anatomical location. Cells located in the superficial surface of cartilage remain undifferentiated while those located in the deeper zone tend to commit to a terminally differentiated state (93). This columnar organisation of cells in various stages is stringently regulated by parathyroid hormone-related protein (PTHrP) and Indian hedgehog (IHH) signalling. PTHrP is expressed by proliferating chondrocytes to keep cells from getting into prehypertrophic state while IHH is produced by prehypertrophic cells to stimulate PTHrP production in periarticular cells (94, 95). The negative feedback loop prevents the onset of chondrocyte hypertrophy and maintains cells in the proliferating stage.

### 1.3.3 Terminal hypertrophic differentiation

Terminal differentiation of chondrocytes is a critical process for endochondral ossification in long bones. Hypertrophic cells locate at the growth plate but are not commonly found in the permeant hyaline cartilage layer. The hypertrophic differentiation is governed by the IHH/PTHrP signalling. Cells committed to hypertrophic fate show distinct markers, including expression of collagen type X (*COL10*), *MMP13*, *RUNX2* and *RUNX3* (95). While *RUNX2* can induce *IHH*, it is negatively regulated by *SOX9* (96). The interaction between proliferation, prehypertrophy and hypertrophy genes assures controlled regulation of chondrocyte proliferation and differentiation.

### 1.3.4 Transdifferentiation

Endochondral ossification is a classic example of transdifferentiation of chondrocytes. It usually happens at the growth plate during the long bone development. Hypertrophic chondrocytes are transdifferentiated into osteoblasts. The process starts with the apoptosis of hypertrophic chondrocytes and the enclosure of chondrocytes by the bone collar. The process is followed by vascular invasion which brings in mesenchymal stromal cells. The cells then differentiate into COL1-producing osteoblasts. Other bone matrix proteins are also produced to facilitate the mineralisation of tissue (97).

### 1.3.5 Dedifferentiation and Redifferentiation

While the direct transdifferentiation model describes the direct changes of hypertrophic chondrocytes into osteoblast, a dedifferentiation-to-redifferentiation process has also been described in fracture healing. The theory suggests dedifferentiation of mature chondrocytes into immature ones and redifferentiates into osteoblasts. A study has demonstrated the expression of stem-like markers such as OCT4, NANOG and SOX2 in COL10-expressing cells isolated from fracture callus (98). Those COL10-expressing cells were found to be able to differentiate into osteogenic cells with OSX expression in a subsequent experiment (99). This hints at the possibility of hypertrophic chondrocytes dedifferentiation and redifferentiation.

### 1.3.6 Senescence

Chondrocytes in hyaline cartilage normally remain in quiescence state. Cellular quiescence is a reversible cell growth arrest. This mechanism maintains the turnover rate of chondrocytes at low level and thereby protecting cartilage integrity (100). In contrast, senescence is a process associated with ageing. It describes an irreversible cell cycle arrest. Cells in senescence state exit the cell growth cycle permanently and no longer be able to divide.

Replicative senescence, also known as Hayflick's limit, is a concept first introduced in 1961. It defines the limit of the replicative lifespan of cells (101). It is primarily caused by the shortening of telomere during cell division. This intrinsic senescence phenotype is usually observed in cells undergoing excessive subculture in laboratories. It also happened in aged organs where cells have reached their natural proliferation capacity. Senescence can also be induced by exogenous stimuli. Extrinsic senescence is a type of cellular senescence caused by stress, including DNA damage, oxidative stress, and chronic inflammation (102). Although stress-induced premature senescence is not directly linked to telomere shortening, stressors such as oxidative stress can often cause telomeric damage (103).

Regardless of the type of senescence, expression of catabolic factors and pro-inflammatory cytokines is recorded. The catabolic secretory profile is described as senescence-associated secretory phenotype (SASP). Other markers of senescent cells include upregulation of $p16^{INK4a}$, which is also known as cyclin-dependent kinase inhibitor 2A (CDKN2A). It binds to CDK4/6 to inhibit Rb phosphorylation and stops cells from proceeding to S phase from G1 (104). The p16 pathway is, therefore, being extensively studied in cancer research as well. Senescence-associated beta-galactosidase (SA-β-Gal) expression is another commonly used senescence marker. It distinguishes senescent cells by their ability to hydrolyse β-galactosides into monosaccharides (105). Noticeably, matrix-degrading enzyme, MMP, upregulation is a marker shared by both hypertrophic and senescent chondrocytes, highlighting their possible roles in cartilage degeneration (106, 107).

### 1.3.7 Apoptosis and Necrosis

Chondrocyte death can be either apoptotic or necrotic. Apoptosis is programmed cell death. It is a normal physiological process in tissue homeostasis. It is a regulated event starting with

cytoplasm and nucleus condensation. Fragmented cellular content is packed in apoptotic bodies. On the other hand, necrosis refers to immediate cell death in response to injury and stress. Rupture of the cell membrane and leakage of content is reported in necrotic cells (108). Although apoptosis is a natural cellular process, it has been implicated in degenerative diseases, including OA. Loss of cellularity and lacunar emptying were observed more frequently in OA than in normal cartilage (109). A possible explanation is the accumulation of nitric oxide in OA cartilage which can trigger apoptotic response (110).

## 1.4 Cellular senescence links hypertension to OA

A study on progeroid mice shed light on the triggering effect of vascular dysfunction in systemic ageing and joint phenotype. The group generated a mouse model that recapitulates progerin accumulation in the natural ageing process (111). The malformed protein cannot integrate into the nuclear lamina properly and the structural disruption of nuclear membrane entails genomic instability by interfering with the DNA double strand break repair, which represents genome instability, one of the hallmarks of ageing. It was shown that an endothelial-specific mutation led to atherosclerosis and fibrosis in blood vessels. Noticeably, the endothelial dysfunction accelerated systemic ageing in progeroid mice and led to skeletal degeneration such as osteoporosis-like change (112). This work has described endothelial ageing as a trigger of whole-organismal ageing and joint disruption which further strengthened the causal relationship between vascular and skeletal pathology. Still, the responsible pathogenic factor and underlying mechanism remain to be elucidated. Macroscopically, ageing is a common primary risk factor for both OA and cardiovascular diseases. The role of ageing in both diseases therefore should not be overlooked.

### 1.4.1 Endothelial senescence in hypertension

A retrospective study has shown that increment of age-associated endothelial senescence correlates with vascular impairment in elder adults(113). In both *in vivo* senescent-accelerated mice and *in vitro* cell models, senescent cells were associated with endothelial vasoactive factor dysregulation which leads to hypercontractility; actin polymerization, calcification, fibrosis and extracellular matrix deposition that result in vascular stiffening and remodelling; and reactive oxygen species (ROS) and proinflammatory cytokines production that contributes to endothelial inflammation and oxidative stress(114).

Conversely, vascular dysfunction can induce the accumulation of senescent cells. Of note, young hypertensive patients exhibit similar vascular dysfunctional features as elder normotensive patients(115). This suggests that hypertension gives rise to the onset of premature vascular ageing. Almost all hypertensive animal models and human hypertensive patients showed cellular senescence in various organs. Dahl Salt-sensitive rats, deoxycorticosterone acetate (DOCA)-salt hypertension rat and spontaneously hypertensive rats all showed senescence in the aorta and myocardium; while human patients exhibited both endothelial and renal senescence. Studies also showed that the administration of anti-hypertensive drugs could reduce the expression $p16^{Ink4a}$ in DOCA rats (114, 116). Although the chronological sequence of endothelial senescence and endothelial dysfunction remains elusive and it is unsure if vascular ageing is the trigger or the consequence of vascular dysfunction, it can be concluded that endothelial senescence is a pathogenic factor for tissue degeneration.

### 1.4.2 Chondrocyte senescence and hypertrophy in OA

*Chondrocyte senescence*

Cellular senescence is a typical hallmark of ageing and is associated with many age-related diseases (39, 117). It refers to the irreversible arrest of cell cycle (101). Despite the incapability of replicative division, senescent cells remain alive but exhibit altered biochemistry. They exhibit SASP that consists of pro-inflammatory cytokines, chemokines and proteinases which contribute to age-associated diseases including OA (39, 118). Chondrocytes from OA patients showed elevated expression of $p16^{INK4a}$, a cell cycle inhibitor that is strongly associated with cellular senescence, compared with age-matched normal adults (39, 119). Meanwhile, matrix-degrading enzymes such as matrix metalloproteinase (MMP) 1 and 13 were overexpressed in OA joints (119, 120). Severity of OA was also found to correlate with senescence-associated

beta-galactosidase (SAβGal) expression which catalyses the hydrolysis of β-galactosides into monosaccharides only in senescent cells (105, 120). *p16$^{INK4a}$*, along with SA-β-Gal, therefore become common biomarkers for senescent cells. The latest research found that p16$^{Ink4a}$–positive senescent cells accumulated in articular cartilage and synovium upon injury or ageing in which the elimination of using genetically modified mice could ameliorate OA (121). A study showing the induction of OA-like phenotype in mice by senescent cell transplantation further supports that OA onset can be initiated by cellular senescence (122). Some publications reported that clearance of senescent cells using genetically modified mice successfully delayed the onset of several age-related diseases including OA (121, 123, 124). In addition, intra-articular injection of UBX0101, a BCL-2 family protein, suppressed the expression of SASP mediators and thus attenuated joint erosion (125). In contrast, another research group demonstrated that the loss of senescent chondrocytes did not necessarily protect joints from OA (126).

*Chondrocyte hypertrophy*

Hypertrophic differentiation of chondrocytes is another hallmark of OA. Increment of hypertrophic chondrocytes was found in disrupted cartilage. They produce mineralized extracellular matrix protein at the fibrillated site of cartilage. Calcium deposition eventually calcifies chondrocytes at the end stage of hypertrophy. Apoptosis of hypertrophic chondrocytes leaving empty lacunae is thus often found in OA (127). The final consequence of this series of events is the thickening of calcified cartilage and worn out of non-calcified hyaline cartilage. Clinically, cartilage calcification was correlated with the severity of OA in patients (128). When hypertrophic chondrocytes recruit osteogenic factors, osteophytes may form, causing pain sensation (127). Overexpression of COL10 by hypertrophic was also found to associate with OA phenotypes. Indeed, COL10 has been a major marker for hypertrophy detection. It is

a unique type of collagen that is not usually found in hyaline cartilage. High expression of COL10 was also reported in OA cartilage (129).

*Correlation between senescence and hypertrophy*

Both chondrocyte senescence and hypertrophy play a pivotal role in OA initiation and progression. They represent the homeostasis of chondrocytes. Their imbalance results in disrupted joint physiology and degeneration. Of note, they share some markers including MMP13 and VEGF, which have both been implicated in OA pathogenesis. However, the relationship between senescence and hypertrophy remains one of the major mysteries in the field. It is unsure if senescence or hypertrophy comes first in OA pathogenesis.

## 1.5 Shared molecular pathway between hypertension and OA

The pathogenic factors in hypertension including the renin-angiotensin system (RAS) and endothelin (ET) system have already been implicated in the pathogenesis and management of OA (130-132). RAS and ET system regulate vascular tone in the body. They are interrelated pathways rather than independent ones. Angiotensin (ANG) II in RAS is an important transcriptional inducer for Endothelin-1 (ET-1), a vasoconstricting peptide in the ET system.

### 1.5.1 Renin-angiotensin system

RAS is the centre of immediate blood pressure regulation (133). The kidney produces renin that is being released when reduction of pressure in afferent arteriole is detected. Major RAS components include renin, angiotensin I (ANG I) and II (ANG II), angiotensin-converting enzyme (ACE) and angiotensin receptors (ATR). Renin catalyses the hydrolysis of ANG I from angiotensinogen. ACE in endothelial cells then hydrolysed ANG I to form ANG II, leading to vasoconstriction (134). ANG II enhances the secretion of aldosterone and sodium reabsorption (135). This in turn causes water retention in the kidney followed by an increment in blood pressure. Although RAS components were first discovered in the vascular system, they also show tissue-specific function locally (136). Specific expression and function of RAS components are cell-type specific. In the skeletal system, local RAS is particularly important for chondrocytes.

*RAS in chondrocyte hypertrophy*

RAS components are involved in different stages of chondrocyte differentiation. However, the respective roles of AT1R and AT2R remain controversial. In a cellular model using ATDC5, AT1R is dominantly expressed during chondrocyte differentiation while AT2R is expressed in hypertrophic chondrocytes. The two receptors counteract with each other in which AT1R

represses hypertrophic-related genes while AT2R upregulates them (137). In contrast, AT1R is found to be the responsible receptor for chondrocyte hypertrophy in murine models. AT1R is dominantly expressed in hypertrophic chondrocytes while proliferating and pre-hypertrophic chondrocytes have shown weak signals (138). In the case of OA with extensive chondrocyte hypertrophic differentiation, the level of *AT1R* mRNA was elevated while that of *AT2R* was repressed (139). Despite the ambiguity of the function of two ANG II receptors, current research has evidenced the role of local RAS in chondrocyte hypertrophy. The theory is further supported by the exclusive expression of RAS components in hypertrophic chondrocytes but not in hyaline cartilage i*n vivo* (140).

Upregulation of hypertrophy-related genes such as *Runx2* and *MMP13* was reported upon ANG II infusion and AT2R activation (137). On the other hand, administration of hypertrophy stimulants interleukin-1β and TNFβ could initiate expression of ATRs (141). However, contradictory to current understanding, the induced hypertrophic differentiation by ANG II protected cells from apoptosis, possibly via the overexpression of anti-apoptotic genes *Bcl-2* and *Bcl-xl*. This result contradicted general findings that ANG II promotes cell death via activation of AT2R (138).

*RAS in chondrocyte senescence*

Most hypertensive animal models and human hypertensive patients showed cellular senescence in various organs possibly through the activation of AT1R (142). Elevated expression of senescence markers was found in both subchondral bone and articular cartilage in DOCA hypertensive rat and SHR; and the phenotype could be attenuated by captopril treatment (143). Since local RAS also takes part in maintaining chondrocyte homeostasis, the potential role of RAS in chondrocyte senescence should not be neglected.

*Targeting RAS for OA treatment*

Since the discovery of the contribution of local RAS in rheumatoid arthritis and possibly OA, RAS components have been targeted for joint disease treatment (144, 145). An FDA-approved renin inhibitor, Aliskiren, has shown the chondroprotective effect by *IL-1*, *TNF-*$\alpha$ and *Runx2* downregulation. It inhibits chondrocyte hypertrophy and reduces local RAS expression, and thereby preserving cartilage integrity in an OA model (146). Another approved antihypertensive drug, captopril, an ACE inhibitor, demonstrated a similar effect. It suppresses renin, ACE and Ang II in OA rats by altering the expression of AT1R and AT2R (139). The hypertrophic zone was greatly reduced in either treatment method, suggesting that the chondroprotective effect of hypertensive drugs was brought by suppression of the local RAS (139, 146).

### 1.5.2 Endothelin family

The endothelin system and RAS function together to regulate vascular tone. Ang II itself is an important transcriptional inducer for ET-1, a vasoconstricting peptide in the ET system. Infusion of Ang II in Wistar Kyoto (WKY) rat enhances both endothelin converting enzyme activity and renal ET-1 level (147). In contrast, ET-1 inhibits renin secretion from juxtaglomerular cells directly and indirectly by impacting adrenocortical zona glomerulosa cells (148).

ET-1 is the predominant form in the cardiovascular system amongst the three isoforms in the ET family. It is identified as an aggravating factor in endothelial dysfunction. Production of ET-1 mainly comes from endothelial cells. Binding of ET-1 on two receptors on endothelial cells, $ET_AR$ and $ET_BR$, triggers an opposing effect. The former results in vasoconstriction and the latter causes vasodilation. The countereffect of receptors ensures precise control of vascular

tone (149). In addition to the role in vascular tone regulation, all three isoforms of ETs have been studied on the regulation of DNA and proteoglycan synthesis of rat articular chondrocytes (150). Both ET-1 and ET-2 stimulate DNA and proteoglycan synthesis of rat articular chondrocytes, whereas ET-3 has no significant effect. This biological function of ET-1/ET-2 in articular chondrocytes is mainly through $ET_AR$ than $ET_BR$ (150).

*ET in chondrocyte hypertrophy and senescence*

ET-1 has been implicated in vascular hypertrophy and senescence. Administration of $ET_AR$ blockers, but not $ET_BR$, showed a beneficial effect on restoring cardiomyocyte autophagic function and rescuing vascular ageing (151). ET-1 also enhanced endothelial and fibroblast senescence (152, 153). Likewise, ET-1 promotes cardiomyocyte hypertrophy possibly via MAPK signalling (154-156) which could be recused by $ET_AR$ antagonist (155). Although the role of the endothelin system in chondrocytes is less defined, ET-1 has shown an association with chondrocyte hypertrophy and senescence. Transgenic endothelial endothelin-1 overexpressed (TET-1) mice exhibited OA-like phenotypes compared to their littermates. Hypertrophic changes in cartilage were also documented (157, 158). Simultaneously, a recent study has demonstrated that ET-1 could induce cellular senescence in murine cell line ATDC5 which could be rescued by $ET_BR$ blockade(159). Considering that the endothelin system is intercalated with RAS, the effect of endothelins on chondrocyte fate is anticipated.

# Chapter 2 Hypertrophic Chondrocytes are more prone to Senescence

## 2.1 Introduction

Both chondrocyte hypertrophy and senescence have been implicated in OA onset and progression. Although the chronological sequence and relationship between chondrocyte senescence and hypertrophy are still under debate, understanding the regulation between the two would definitely provide novel insight into possible therapeutic approaches for joint degenerative diseases including OA (127).

### Aim and Approaches

The goal of this experiment is to unravel the relationship between chondrocyte hypertrophy and senescence. Co-existence and changes of hypertrophy and senescence phenotypes were demonstrated along with OA progression *in vivo.* To isolate cells at different stages for downstream analysis, a cell sorting technique was utilised to distinguish COL10-expressing cells. A novel starvation cell culture was then developed and employed to examine the chronological changes of cell fates *in vitro.* The hypothesis of the correlation between chondrocyte hypertrophy and senescence was then tested by PTHrP intervention.

*Post-traumatic, metabolic and ageing OA models*

Three OA models were employed to examine the general expression of hypertrophy and senescence phenotypes in disease progression and identify any differences among various types of OA. The post-traumatic OA model was generated by destabilization of medial meniscus (DMM) surgery. This surgical model mimics clinical post-traumatic OA by disrupting the structural integrity of joint. This leads to disturbed mechanical loading on

cartilage and eventually initiates OA onset and progression. The metabolic OA used was an obese model, in which mice in the obese group were fed with high-cholesterol and high-fat diet and control lean group received normal diet. This model aimed to recapitulate the obese-associated OA that is frequently observed clinically. The ageing model allows the longitudinal study of the normal ageing process where no special treatment was given. Mice were raised to different ages under ordinary caring and diet. Generation of these models was done by members of our group. Validation of OA phenotypes has been proved with either preliminary or published data (160).

*Parathyroid hormone-related protein (PTHrP)*

PTHrP belongs to the parathyroid hormone (PTH) family. It is expressed in many tissues including the cartilage. It is primarily secreted by periarticular chondrocytes and acts on pre-hypertrophic chondrocytes via its receptor PTH1R. While the ablation of PTHrP could promote hypertrophy, chondrocyte-specific overexpression of PTHrP was found to delay the terminal differentiation of cells (161, 162). Together with IHH produced by pre-hypertrophic cells, the IHH-PTHrP regulatory axis maintains the proliferation capacity of cells and prevents them from getting into hypertrophic state (163). While the antihypertrophic effect of PTHrP is well-characterised, anti-inflammatory and anti-senescence properties of the protein have been reported in human osteoblast culture. The treatment with PTHrP peptides decreased expression of proinflammatory cytokine IL-1β, senescence markers $p16^{INK4a}$, p21, p53 and SA-β-gal (164). However, it is unclear if the protein functions in a similar manner in chondrocytes.

*Starvation model*

Serum starvation is a frequently performed procedure in molecular biology studies. There is no consensus on the exact definition of serum deprivation. It can be done by reducing the

concentration of serum, total removal of serum, or in a serum- and protein-free manner. The exact protocol highly varies depending on the cell type and purpose of the experiment. The concentration of serum used can be as low as below 1% to up to 5%; and the timeframe could range from minutes to weeks. Starvation is usually employed to study cellular responses under nutrition stress, autophagy and apoptosis process, and to synchronise cell cycles for circadian rhythm study (165). The idea of culturing cells in low-serum condition (1% FBS) in this project originated from hoping to generate a population of cells with a more homologous response to $H_2O_2$.

*Oxidative stress-induced senescence*

Hydrogen peroxide ($H_2O_2$) is a frequently used reagent for premature senescence induction. $H_2O_2$ produces ROS that can cause DNA damage in cells. This triggers the DNA repair mechanism and activates p53/p21/Rb pathway, thereby causing cell cycle arrest. Prolonged sublethal treatment of $H_2O_2$ has been reported to lead to cellular senescence with upregulation of senescence markers (166). It is therefore being used as a senescence inducer in this experiment. It should also be noted that the effect of oxidative stress on telomere is controversial. Although it has been generally reported that there was no significant shortening of telomere length in $H_2O_2$-induced senescent cells, some researches have proposed the possible telomeric damage caused by ROS (103, 166).

## 2.2 Materials and Method

*Immunohistochemistry*

Post-traumatic OA, ageing and obese mice model were analysed. Knee samples were previously harvested, processed and embedded in paraffin by our lab. They were sectioned with a microtome with a thickness of 5 μm. Sample slides were dewaxed and rehydrated in decreasing ethanol concentration from 100% to 70% prior to staining. Heat mediated antigen retrieval was performed in sodium citrate buffer of pH 6.0 following the manufacturer's instruction. Endogenous peroxidase activity was quenched with 3% (v/v) hydrogen peroxide. Sections were then blocked with 10% (v/v) horse serum for 1 hour. Overnight incubation was carried out with antibodies against p16ink4a (ab189034, Abcam, 1:500 dilution). On the next day, target antigens were stained with Vectastain ABC kit and DAB substrate kit (Vector Labs, USA). Harris Haematoxylin was used for counterstain. Images were taken using Nikon Eclipse 80i Microscope (Nikon, Japan). Histomorphometry was employed to distinguish hypertrophic cells.

*Cell culture*

The C28/I2 cell line was received as a gift from Prof. Danny CHAN from the School of Biomedical Sciences at The University of Hong Kong. Cells were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% (v/v) Foetal Bovine Serum (FBS) and 1% (v/v) Penicillin/Streptomycin antibiotic solution (Gibco Life Technologies, USA). Cells were incubated in a humidified chamber with 5% $CO_2$ at 37ºC. The medium was changed every 3-4 days.

*Fluorescence-Activated Cell Sorting (FACS)*

Cells were grown until confluent before sorting. They were harvested and resuspended a sorting buffer of 1% FBS in 1X phosphate buffered saline (PBS) at a density of 1 x $10^6$ cells/ml. Incubation with primary antibody against collagen X (ab49945, Abcam, 1:200 dilution) was done on ice for 1 hour. Cells were then washed with PBS twice and incubated in secondary antibody using Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (A-11001, Invitrogen, 1:200 dilution) on ice for 30 minutes. After washing with PBS, cells were filtered through a 0.4 μm cell strainer to obtain a single-cell suspension. Sorting was performed using BD FACS Aria III Cell Sorter (BD Bioscience). Cells were gated and sorted into collagen X positive and negative based on their fluorescence signal intensity.

*Immunocytochemistry*

After the removal of the culture medium, cells were washed with PBS twice. Cells were then fixed by 4% (w/v) paraformaldehyde (PFA). Permeabilization was performed using 0.25% Triton X-100 if deemed necessary. Cells were then incubated overnight with antibodies against collagen X (ab49945, Abcam, 1:1000 dilution), p16ink4a (ab189034, ab201980, Abcam, 1:500 dilution). Secondary antibodies used were Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (A-11001, Invitrogen, 1:200 dilution) and Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 (A-11012, Invitrogen, 1:200 dilution). Cells were mounted with ProLong® Gold antifade reagent with 4', 6-diamidino-2-phenylindole (DAPI). Images were taken using Leica TCS SPE Confocal Microscope. Quantitatively analysis was done by Fiji Image J.

*Senescence-associated β-galactosidase (SA-β-gal) activity assay*

Cells were stained for senescence using β-Galactosidase Staining Kit (#9860, Cell Signalling Technology) according to the manufacturer's instruction. Cells were examined after overnight incubation.

*Quantitative PCR*

Total RNA was isolated using E.Z.N.A.® Total RNA Kit I (R6834-02, Omega Bio-tek) and reversely transcribed using RevertAid First Strand cDNA Synthesis Kit (K1622, Thermo Scientific). Real-time PCR was then performed on Bio-Rad CFX96 Touch System with QuantiNova SYBR Green PCR Kit (Qiagen). 1 μg of cDNA was used in each qPCR reaction with 32 cycles. All samples were normalized to GAPDH mRNA expression and analysed in triplicate. Primers for specific genes are as follow.

| Genes | Forward Primer (5'- 3') | Reverse Primer (3'- 5') |
|---|---|---|
| *GAPDH* | CTGACTTCAACAGCGACACC | CCACCCTGTTGCTGTAGCC |
| *COL10A1* | GCTTCAGGGAGTGCCATCATC | CTCACATTGGAGCCACTAGGAATC |
| *MMP13* | GACCCTGGAGCACTCATGTTTC | TCGGAGACTGGTAATGGCATCA |
| *IHH* | CCTCAGTTGATGCTGCTAAATTC | AACAGTCTCTGGATGTGTCTTG |
| *p16 INK4a* | GCACCAGAGGCAGTAACCAT | AACTACGAAAGCGGGGTGG |
| *p21* | GGTGGACCTGGAGACTCTCAG | CCTCTTGGAGAAGATCAGCCG |
| *SOX9* | GCAGGCGGAGGCAGAG | GGAGGAGGAGTGTGGCG |

**Table 1. List of human genes and their repecitve primer sequences.**

*Starvation model*

Chondrocytes were passaged and cultured in normal growth medium for a day prior to starvation. Starvation medium was prepared by supplementing DMEM/F12 with 1% (v/v) FBS and 1% (v/v) Penicillin/Streptomycin antibiotic solution (Gibco Life Technologies, USA). Cells were cultured in starvation medium for a total of 48 hours to induce hypertrophy. To establish senescent phenotypes, 150 μM of hydrogen peroxide ($H_2O_2$) was added to starvation medium after 42 hours and incubated for another 6 hours until the 48-hour timeline was fulfilled. Two treatment strategies were employed to investigate the preventive and rescue

effect of interventions. For the preventive treatment group, 100 ng/ml recombinant human PTHrP (SRP4651, Sigma-Aldrich) was added before the start of starvation for 24 hours in normal culture condition and was then replaced by drug-free starvation medium; while for the treatment group, intervention was done at the start of starvation and drugs were replaced every 24 hours to maintain the concentration.

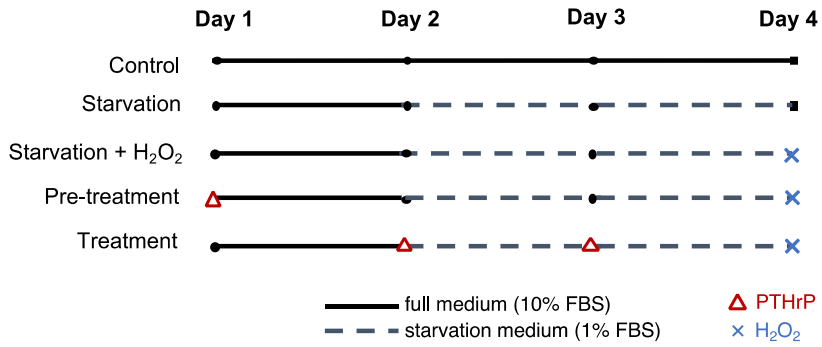



**Fig 1. Design of starvation model.** Schematic diagram of the setup of starvation experiment. Groups other than the control group were starved for a total of 2 days. $H_2O_2$ was added 6 hours before the starvation ended. 100 ng/ml of PTHrP was added at different time points according to the experiment grouping.

*Oxidative stress detection*

CellROX™ Deep Red Reagent (Invitrogen) was added to cells at a final concentration of 5μM and incubated at 37ºC for 30 minutes. Cells were then washed with PBS twice and fixed in 4% (w/v) PFA for 15 minutes. ProLong® Gold antifade reagent with DAPI was used to counterstain the cells. ). Images were taken using Leica TCS SPE Confocal Microscope and analysed by Fiji Image J.

## 2.3 Results

**Hypertrophic and senescent chondrocytes accumulated in various OA models**

Hypertrophic cells are characterised by enlarged cell size. Distinguishment between hypertrophic and non-hypertrophic cells was made manually by separating the superficial layer which contained mainly flattened cells, with the deeper layer which consisted of cells with large lacunae. Immunohistochemistry showed the differential expression of $p16^{INK4a}$ in chondrocytes in various anatomical locations. An increase in the total number of $p16^{INK4a}$ positive cells with the progression of OA was observed in all models. The composition of $p16^{INK4a}$ positive cells also changed from mainly flat cells to round cell dominant (Fig 2).

The percentage of senescent cells increased after 1 month of DMM surgery and was 3-fold higher after 4 months post-surgery than the baseline. The finding was consistent with published data that mice started showing more severe OA phenotypes 4 months after DMM (160). While flat cells remained dominant in $p16^{INK4a}$ positive cell population in baseline and 1-month post-DMM mice, more than half of them (53.47 ± 3.78 %) were round cells in 4-month post-DMM group. A similar shift in the dominating cell type was also seen in the ageing model. 4-month old mice showed the least amount of $p16^{INK4a}$ positive cells (20.82 ± 3.30 %) with flat cells comprising most of them. In 9-month and 16-month old mice, the percentages of senescent cells were 54.66 ± 4.32 and 56.4 ± 7.21 respectively. Markedly, the ratio of flat cells to round cells among the $p16^{INK4a}$ positive population is inverted, with hypertrophic cells being the majority. In the metabolic model, the overall number of $p16^{INK4a}$ positive cells did not show significant change statistically but the elevation of the percentage of $p16^{INK4a}$ positive round cells was observable.

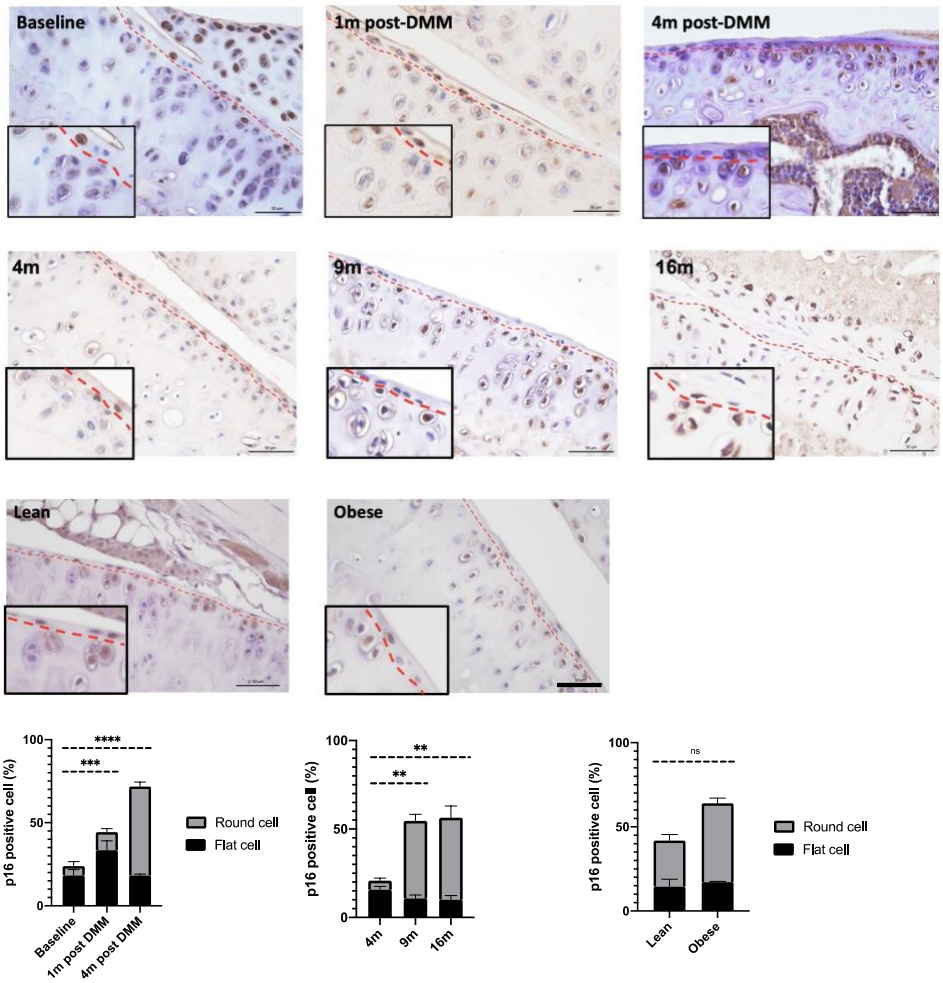



**Fig 2. Majority of accumulating senescent cells during OA progression were hypertrophic-like.** Representative images of p16$^{INK4a}$ immunohistochemistry staining on post-traumatic model from baseline (n=3), 1-month post-DMM (n=3) to 4-month post-DMM (n=3); ageing model from 4-month-old (n=3), 9-month-old (n=3) to 16-month-old (n=4); and metabolic OA mice model from lean (n=3) and obese (n=3) mice. Scale bar. 100 μm. Red dashed line distinguishing the superficial layer of articular cartilage. Inset digital magnification showing a close-up view of chondrocytes. (b) Quantification data of the percentage of p16$^{INK4a}$ positive cells in each group. All data are shown as means ± SEM, and each data point represents an individual mouse. One-way ANOVA with Tukey's multiple-comparisons test was employed for statistical analysis. * p<0.05; ** p<0.005; *** p<0.001; **** p<0.0001

There were three possible explanations for the inversion of dominating cell type in the senescent population. Firstly, the percentages were calculated from the proportion of p16$^{INK4a}$ positive cells among all cells. Therefore, the number did not take the changes within the flat cell and round cell population into account. The reduction in percentage of p16$^{INK4a}$ positive

flat cells could be because of the decline in flat cell population; similarly, the elevation of percentage of $p16^{INK4a}$ positive round cells may reflect the expansion of the round cell population. On the other hand, the shift of composition of the senescent population may indicate existing round cells were more likely to be $p16^{INK4a}$ positive. The last hypothesis would be senescent flat cells became round during OA development. However, the current characterisation of senescent cells is mostly based on the expression of nuclear proteins. The lack of invasive markers hinders the isolation senescent cell for downstream experimentation and analysis. This project would therefore attempt to study the interplay between hypertrophy and senescence from a single direction. Although this histomorphometry assay could not verify the theory behind the changes, the results have sufficiently demonstrated the alteration of chondrocyte fates during OA progression.

**Collagen X-positive chondrocytes exhibited more senescent phenotypes**

Results from histological assay illustrated that majority of the senescent chondrocytes found accumulated with OA progression were hypertrophic-like cells. To further validate these findings, hypertrophic cells were purposely isolated and screened for senescence phenotypes.

Expression of collagen X (COL10) was used to characterize hypertrophic and non-hypertrophic cells. Markedly, COL10+ cells tended to higher values of forward scatter (FSC) and side scatter (SSC), implying they were larger in size and had higher granularity (Fig 3a). Cell size increment is a common phenotype shared by both hypertrophy and senescence; while high granularity may reflect the increase of lysosome in senescent cells. Several cellular assays were performed to further assess the expression of senescence marker in COL10+ and COL10- cells.

At transcriptional level, qPCR was carried out to examine the expression of senescent-associated genes including *p16$^{INK4a}$* and *p21*. Hypertrophy markers, *COL10*, *MMP13* and *IHH*, were also included to evaluate the specificity of cell sorting. The result showed that COL10+ cells had higher expression of some of the hypertrophy markers but they did not seem to be more senescent-like. The mRNA levels of *p16$^{INK4a}$* and *p21* were comparable in COL10+ and COL10- groups (Fig 3b). Yet one of the hallmarks of cellular senescence, telomere shortening, was more profound in COL10+ cells (0.80 ± 0.05) (Fig 3c). At translational level, COL10+ cells showed significantly higher expression of both COL10 (13.91 ± 0.87) and p16$^{INK4a}$ (10.96 ± 0.86) (Fig 3d, e). Functional assay assessing the SA-β-gal activity was also performed. More cells with high enzyme activity were observed in the COL10+ group, indicating that hypertrophic cells exhibited senescence phenotypes (Fig 3f).

To validate if hypertrophy predisposes to senescence, PTHrP was added to interfere with cellular hypertrophy. The intervention with PTHrP significantly lowered the expression of COL10 protein in COL10+ cells. The signal intensity decreased to 8.15 ± 0.97. This implied the reversion of established hypertrophy in culture, which was unanticipated but not unreported before (167). Reduced expression of p16$^{INK4a}$ (6.71 ± 0.82) was found to accompany the relieved hypertrophy phenotype (Fig 3d, e). The result hinted at a plausible association between the two cell fates.

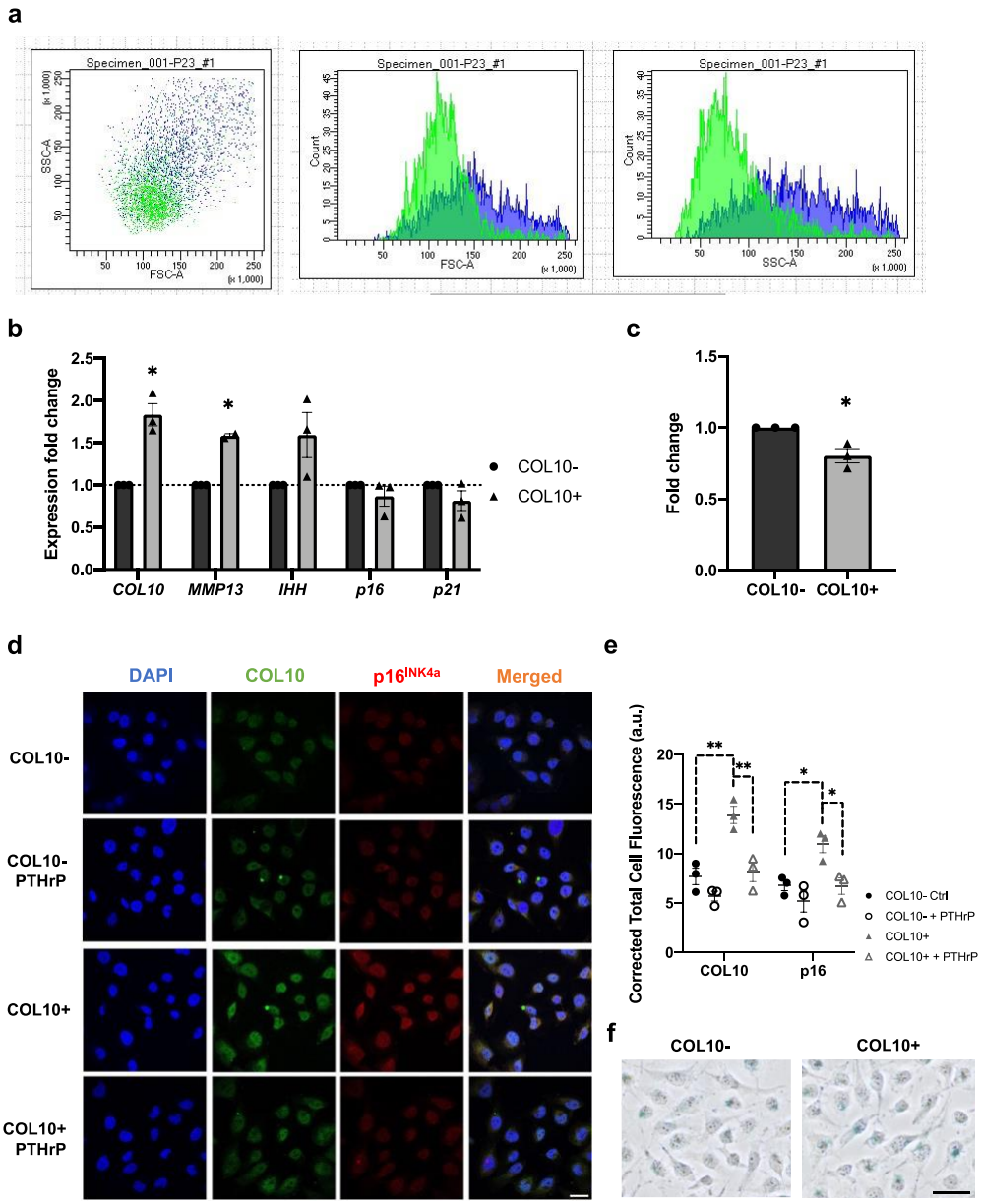



**Fig 3. COL10+ chondrocytes were more hypertrophic and senescent.** (a) FACS data showing the FSC and SSC of COL10+ (blue) and COL10- cells (green). COL10+ cells had slightly higher values of FSC and SSC, hinting at the changes in cellular morphology. (b) Relative mRNA expression of *COL10*, *MMP13*, *IHH*, *p16* $^{INK4a}$ and *p21* in sorted cells (n=3). All data were normalised to *GAPDH* expression. (c) Relative telomere length of COL10+ cells with COL10- group as control (n=3). (d) Representative images of immunofluorescence staining of COL10 (green) and p16$^{INK4a}$ (red) for each group with DAPI (blue). Scale bar. 25 μm. (e) Quantification of fluorescence signal of immunocytochemistry (n=3). COL10+ cells had a higher baseline of COL10 expression, which could be reversed by PTHrP treatment. (f) Representative images of SA-β-gal assay (n=2). Cells with SA-β-gal activity were stained in blue. Scale bar. 25 μm. All data are shown as means ± SEM. Each data point represents an individual experiment. T-test was employed for statistical analysis. * $p<0.05$; ** $p<0.005$; *** $p<0.001$; **** $p<0.0001$

**Starvation model demonstrated temporal changes of hypertrophy and senescence**

The co-existence of hypertrophy and senescence markers was observed in the previous experiment. To study the association between the two distinct stages, a cellular model that could dissect the temporal sequence along the changes of cell fate is critical.

While the original purpose of culturing cells in low-serum condition was to generate a population of cells with a more homologous response to $H_2O_2$, it was then realised starvation alone was capable of inducing hypertrophic changes phenotypes in chondrocytes. The protocol was then optimised for hypertrophy induction.

Hypertrophic changes were observed after 48 hours of serum depletion. The expression of *COL10* and *IHH* genes increased by 1.78 ± 0.24 and 1.60 ± 0.03 fold while that of *MMP13* was 5.14 ± 0.03 fold higher than the control (Fig 4a). Upregulation of COL10 was also revealed in immunostaining but not as convincing as that of $p16^{INK4a}$, whose signal increased to 13.72 ± 1.12 (Fig 4c). Markedly, senescence markers including upregulation of $p16^{INK4a}$ (1.49 ± 0.15) and *p21* (2.31 ± 0.46) genes, telomere shortening (0.59 ± 0.08) and significant SA-β-gal activity were only documented after adding $H_2O_2$ in starvation medium (Fig 4a, b, e). Signal intensity of COL10 and $p16^{INK4a}$ also rose to 24.83 ± 4.04 and 24.56 ± 2.76 respectively (Fig 4c). The data implied the starvation approach could induce only hypertrophy while the starvation in combination with $H_2O_2$ treatment could further drive cells into senescence state. The establishment of this model provided information on the possible relationship between hypertrophy and senescence by showing the capability of hypertrophic cells turning into senescent. The step-wise changes of cell fate in this model also provided a timeframe for intervention.

In the pre-treatment group, 100 ng/ml of PTHrP was added to culture medium before starvation. The aim was to mitigate existing hypertrophy phenotypes so as to understand if hypertrophy predisposes to senescence. The expression of hypertrophy and senescence genes *COL10*, *p16$^{INK4a}$* and *p21* reduced to levels statistically comparable to the control group (Fig 4a). Fluorescence signal intensity of both COL10 (22.56 ± 1.99) and p16$^{INK4a}$ (10.06 ± 0.74), as well as SA-β-gal activity in pre-treated cells also decreased (Fig 4c, e). A similar trend was observed in PTHrP treatment group. The design of this group was to examine if cellular senescence is hypertrophy-dependent. Notably, intervention during starvation seemed to be a more potent way to improve chondrocyte hypertrophy. Expression of hypertrophy markers *COL10* (0.74 ± 0.12) and *IHH* (0.92 ± 0.03) restored to similar levels as the control group (Fig 4a). Protein expression of COL10 and p16$^{INK4a}$ also dropped to 10.03 ± 1.74 and 10.11 ± 1.70 respectively (Fig 4c). Although the treatment group showed more profound results in suppressing hypertrophic changes in chondrocytes, its efficacy in preventing cellular senescence was no different from the pre-treatment group.

Starvation with $H_2O_2$ was also found to upregulate the accumulation of ROS in chondrocytes (Fig 4d). Signal of CellROX staining showed a slight increase upon starvation but became more pronounced after $H_2O_2$ induction. Fluorescence signal diminished in both pre-treatment and treatment of PTHrP, reflecting a reduction of oxidative stress in cells. The staining result of ROS concurred with the expression of p16$^{INK4a}$ in immunostaining.

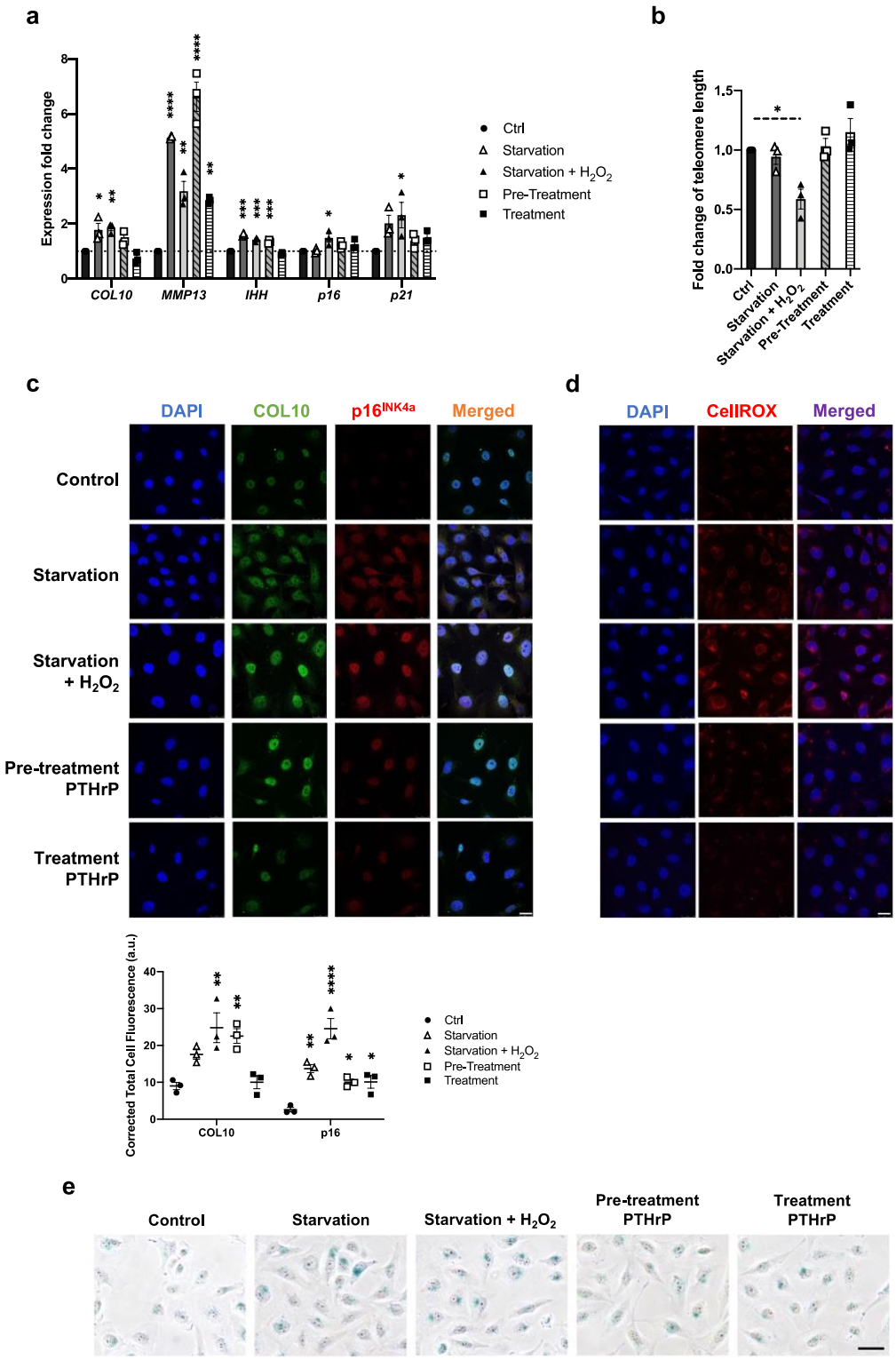



**Fig 4. PTHrP could mitigate both starvation-induced hypertrophy and senescence phenotypes.** (a) Relative mRNA level of *COL10*, *MMP13*, *IHH*, *p16* $^{INK4a}$ and *p21* in each group (n=3). Statistical comparison was performed between the experimental groups and the control group. (b) Relative telomere length of starved cells with and without $H_2O_2$ compared to control (n=3). Telomere only shortened after $H_2O_2$ treatment, indicating concrete cellular

senescence. (c) Representative images of immunofluorescence staining and quantification of COL10 (green) and $p16^{INK4a}$ (red) for each group with DAPI (blue) (n=3). The fluorescence signal intensity of COL10 and $p16^{INK4a}$ always showed a consistent trend, implying the intimate relationship in-between. Scale bar. 25 μm (d) Representative images of CellROX staining (red) of each group (n=2). Oxidative stress built up in starvation with $H_2O_2$ group and was alleviated by both pre-treatment and treatment of PTHrP. Scale bar. 25 μm (e) Representative images of SA-β-gal assay (n=2). Scale bar. 50 μm. All data are shown as means ± SEM, and each data point represents an individual experiment. One-way ANOVA with Tukey's multiple-comparisons test was employed for statistical analysis to compare between each experimental group and control group. * $p<0.05$; ** $p<0.005$; *** $p<0.001$; **** $p<0.0001$

Attention should also be drawn to the discrepancy of *MMP13* expression among groups. MMP13 is a mutual marker for both hypertrophy and senescence. Yet its expression did not follow the trend of either of them. The translational expression of *MMP13* was not examined in this experiment. MMP13 functions as degrading enzyme in the cartilage matrix. Its expression might only be reflected at protein but not mRNA level, which was one of the limitations of this experiment.

## 2.4 Discussion

Chondrocyte hypertrophy and senescence were frequently documented in OA knees. The two distinct stages of cells contribute to cartilage degradation and disease progression. Their individual impact on cartilage homeostasis have been extensively studied; however, the relationship between the two has never been concluded. Part of the reason is the simultaneous appearance of the two phenotypes *in vivo* and in *in vitro*. The mixed population of cells made research on individual processes unfeasible. While the histology results have shown the senescence marker in hypertrophic-like cells in rodents, a similar phenomenon was also seen in human knees (Fig 5). $p16^{INK4a}$ expression was more prominent in cells with large lacunae than cells in the superficial layer of cartilage.

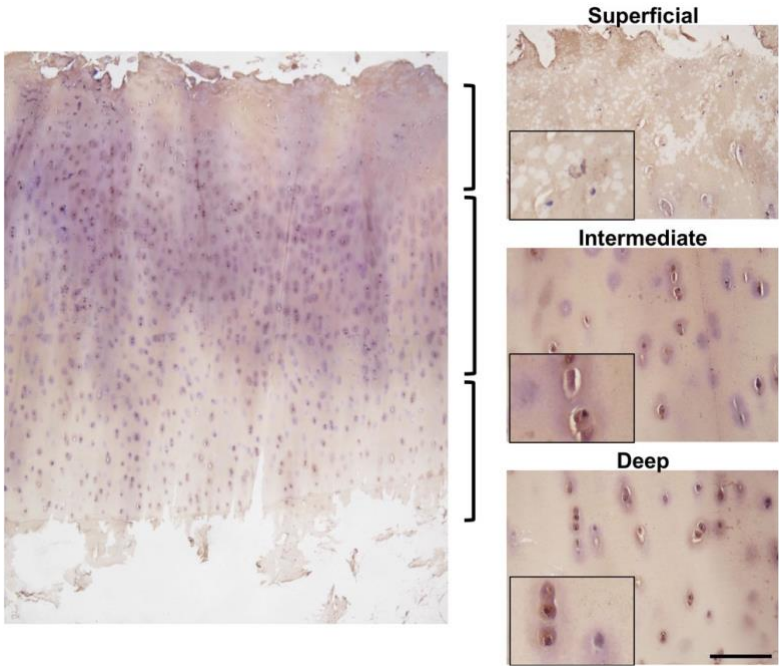



**Fig 5. Deep zone chondrocytes showed higher $p16^{INK4a}$ expression in OA human knee.** Representative images of $p16^{INK4a}$ immunohistochemistry staining in different zones of articular cartilage of OA knee samples. Positive cells are mainly located in the deeper zones of cartilage. Scale bar. 50 μm

Senescence marker expression observed in COL10+ cells indicated the overlap of hypertrophy and cellular senescence events. This implied the possibility of transition between two cellular

processes. Cells in the intermediate stage between hypertrophy and senescence may therefore express both markers. It also indicates the tendency of hypertrophic cells to become senescence and vice versa. The simultaneous expression of both markers could only hint that conversion between two but was unable to give more information on the chronological sequence of events.

The gap of knowledge was filled by our starvation model, which provided information on the possible temporal changes of chondrocyte fate upon stresses. It successfully separated the two cellular states and demonstrated the feasibility of pushing hypertrophic cells into senescence. It, therefore, verified the correlation between chondrocyte hypertrophy and senescence. Although the PTHrP treatment did not eliminate all senescence phenotypes, the significant reduction of senescence markers upon inhibition of hypertrophy has shown hypertrophy as a critical step for cells committing to senescence. This finding may provide new insight into cartilage erosion treatment in OA management.

However, this experiment could only demonstrate the single-direction progression from hypertrophy to senescence. Non-invasive isolation of senescent cells for downstream experiment and analysis remains technically challenging. It is therefore difficult to employ the same approach and examine the hypertrophy marker expression in senescent chondrocytes. Extensive researches have been carried out for senescence biomarkers discovery. Possible markers include cell size and stiffness and senescence marker protein 30 (SMP30) (168). Yet no consensus has been made due to the cell-type and condition-specific nature of some of the markers.

# Chapter 3 Captopril attenuated endothelial senescence and the subsequent chondrocyte senescence

## 3.1 Introduction

Comorbidity of hypertension and OA was reported both epidemiologically and histopathologically in animal models. However, there is a lack of evidence that could demonstrate a causal relationship between the two. Cardiovascular pathologies including hypertension are often accompanied by endothelial senescence. Hence, the effect of endothelial dysfunction was presented as endothelial senescence to study its effect on chondrocyte homeostasis. Unravelling their association and possible mechanism gives new insight into the aetiology of OA. The discovery of the role of vasculature on joint homeostasis pave a way for a novel therapeutic approach that targets not only local joint disruption but systemic vascular dysfunction.

**Aim and Approaches**

This experiment aims to provide direct scientific evidence of the association between endothelial senescence and chondrocyte phenotypes to demonstrate the interplay between endothelium and cartilage in physiological conditions.

*Indirect co-culture*

Chemical crosstalk is the primary mean of communication between the endothelium and cartilage. To examine the impact of vascular senescence on chondrocyte fate and homeostasis, co-culture system with conditioned medium was employed. The indirect co-culture method enables the study of chemical exchange between two types of cells without having physical

contact as a confounding factor. It also allows investigation in a single direction of the effect of endothelial dysfunction on chondrocytes but not vice versa.

*Captopril*

Captopril is a widely used anti-hypertensive drug targeting an angiotensin converting enzyme (ACE) and thereby suppressing the production of ANG II, a potent vasoconstrictor. The anti-inflammatory and anti-senescence properties of captopril have also been demonstrated in different disease models by attenuating oxidative stress (169, 170). Treatment with captopril also mitigated OA phenotypes in hypertensive rats (143). The potential endo- and chondroprotective effect of the drug make it a worth-investigating candidate for hypertension-associated OA research.

## 3.2 Materials and Method

*Cell culture*

The Human Umbilical Vein Endothelial Cell (HUVEC) was a gift from Dr. Youhua TAN from Department of Biomedical Engineering in The Hong Kong Polytechnic University. Cells were cultured in Endothelial Cell Medium (ECM) supplemented with 5% (v/v) FBS, 1% (v/v) endothelial cell growth supplement and 1% (v/v) Penicillin/Streptomycin antibiotic solution (ScienCell, CA). Cells were incubated in a humidified chamber with 5% $CO_2$ at 37ºC. The medium was changed every 3-4 days. For senescence induction, an appropriate amount of $H_2O_2$ was added into the culture medium to make up a final concentration of 150 μM. Cells were incubated in the dark for 24 hours to minimise the decomposition of chemicals. Cells in captopril treatment groups were incubated in $H_2O_2$-free medium with either 100 μM or 1mM of captopril (#62571-86-2, Aladdin) for one more day.

*Conditioned medium preparation and co-culture*

HUVECs were subcultured at a density of 4.5 x $10^5$ cells per T-75 flask and maintained in 6 ml of the medium at all times. Conditioned medium (CM) was collected from the same population of cells at different time points. After passaging, cells were allowed to recover for 24 hours. Fresh medium was then replenished; and was collected as "normal CM" after a day. Cells were then incubated in 150 μM of $H_2O_2$ for 24 hours and the medium was retrieved as "$H_2O_2$ CM". To harvest "captopril CM", cells were treated with either 100 μM or 1 mM of captopril (#62571-86-2, Aladdin) for one day after $H_2O_2$ induction. CM harvested from different phrases therefore reflected the temporal changes of the secretome of HUVECs.

All CM was centrifuged at 200g for 5 minutes to remove cells. They were aliquoted into small vials and kept at -20ºC for up to 2 weeks. CM was mixed with fresh DMEM/F12 medium in

1:2 ratio for C28/I2 culturing. Comparison of cellular phenotypes was always done between chondrocytes culturing in the same batch of CM (harvested from the same flask of HUVECs).

Immunocytochemistry staining

Fixation and permeabilization were performed as previously mentioned. Cells were then incubated overnight with antibodies against collagen X (ab49945, Abcam, 1:1000 dilution), p16ink4a (ab189034, ab201980, Abcam, 1:500 dilution). Secondary antibodies used were Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (A-11001, Invitrogen, 1:200 dilution), Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 (A-11005, Invitrogen, 1:200 dilution), Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (A-11008, Invitrogen, 1:200 dilution) and Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 (A-11012, Invitrogen, 1:200 dilution). Cells were mounted with ProLong® Gold antifade reagent with 4', 6-diamidino-2-phenylindole (DAPI). Images were taken using Leica TCS SPE Confocal Microscope.

## 3.3 Results

### Captopril alleviated $H_2O_2$-induced HUVEC senescence

Oxidative stress is a gold standard for senescence induction and $H_2O_2$ is a commonly used reagent for this purpose. Treatment with 150μM of $H_2O_2$ efficiently induced upregulation of *p16$^{INK4a}$* and *p21* at transcriptional level, which showed 2.25 ± 0.15 and 1.45 ± 0.11 fold increase (Fig 6a). The changes were accompanied by overexpression of p16$^{INK4a}$ protein in immunofluorescence staining (15.24 ± 1.49) (Fig 6b). The number of SA-β-gal positive cells (55.14 ± 1.14 %) was also showed a significant increase after the addition of $H_2O_2$ (Fig 6c).

Different concentration of captopril was added to HUVECs after $H_2O_2$ induction to evaluate its senolytic effect. No difference in efficacy was found between 100 μM and 1 mM of captopril. Both groups showed similar downregulation of senescence-associated gene expression. Levels of expression of *p16$^{INK4a}$* and *p21* were 1.11 ± 0.07 and 0.91 ± 0.11 respectively in 100 μM captopril group; while that in 1 mM captopril group were 0.80 ± 0.26 and 0.67 ± 0.07 respectively (Fig 6a). Consistent findings of reduction of senescence markers were observed in immunocytochemistry and SA-β-gal activity assay (Fig 6c). Fluorescence signal rose upon $H_2O_2$ induction and diminished to 5.61 ± 1.47 following low dose captopril treatment, and to 3.98 ± 1.21 after high dose treatment (Fig 6b). SA-β-gal activity also decreased in treatment groups, after peaking in $H_2O_2$ group. The percentage of positive cells declined to 23.45 ± 2.72 and 23.29 ± 3.43 in 100 μM and 1 mM groups respectively (Fig 6c). All senescence phenotypes dropped to the level comparable to the control group, implying the addition of captopril effectively restored cellular phenotypes regardless of drug concentration.

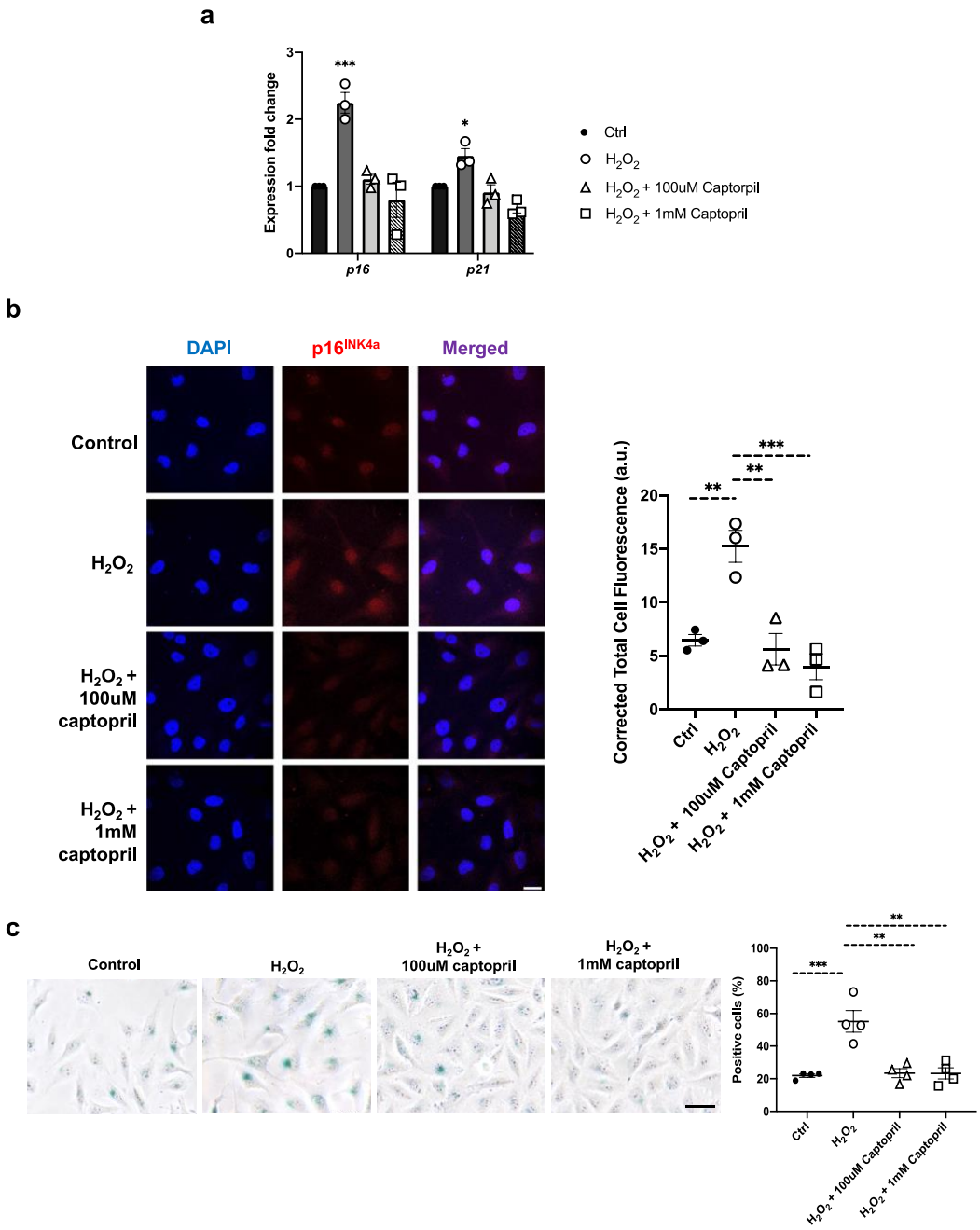



**Fig 6. Senolytic property of captopril was dose-independent.** (a) Relative mRNA level of *p16*$^{INK4a}$ and *p21* in HUVECs under various conditions (n=3). A sharp increase in the expression of both genes was reported after 24-hour $H_2O_2$ treatment. The two captopril treatments with different doses lowered senescence gene expression in a similar manner. (b) Representative images of immunocytochemistry and quantification of p16$^{INK4a}$ (red) for each group with DAPI (blue) (n=3). Signal peaked in $H_2O_2$-treated group but diminished after captopril treatment. Scale bar. 25 μm (c) Representative images and quantification of SA-β-gal assay (n=4). The highest number of SA-β-gal positive cells (blue) were observed in $H_2O_2$ group amongst all groups. Scale bar. 50 μm. All data are shown as means ± SEM, and each data point represents an individual experiment. One-way ANOVA with Tukey's multiple-comparisons test was employed for statistical analysis to compare between each experimental group and control group. * p<0.05; ** p<0.005; *** p<0.001; **** p<0.

**Conditioned medium from senescent HUVEC induced chondrocyte senescence**

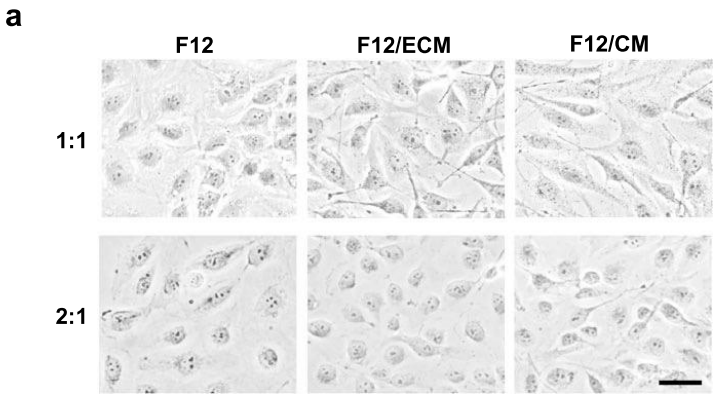
HUVECs were cultured in a medium supplemented with endothelial growth factors, which could potentially affect the growth and behaviour of C28/I2 cells. Optimisation of co-culture method was therefore performed by testing the ratio of F12, medium for chondrocytes, to ECM, medium for HUVECs. Morphological changes were observed when C28/I2 were culture in F12/ECM in 1:1 ratio (Fig 7a). Cells were elongated and gained a fibroblast-like morphology. The phenotype became more significant when F12/CM medium was used. This may imply the de-differentiation of chondrocytes in culture. To minimise the morphological changes of cells. the ECM and CM were further diluted with 2 volumes of F12. It had proven to be a more gentle protocol. Chondrocytes remained their chondrocyte-like appearance. Hence, the 2:1 ratio was adopted in the following experiments.

CM harvested from various experimental conditions was mixed with fresh F12 medium and used to culture chondrocytes for 24 hours. When cells were cultured in normal CM, their expression of both COL10 (8.39 ± 1.01) and $p16^{INK4a}$ (8.18 ± 1.08) did not differ from the F12/ECM group (Fig 7b). A similar observation was made in the SA-β-gal activity, where the percentage of positive cells in F12/ECM and F12/CM were 11.34 ±1.62 and 16.48 ± 1.48 respectively (Fig 7c). As no significant difference of hypertrophy and senescence markers was found between the F12/ECM and F12/CM groups, qPCR was done using F12/CM as the control group for simplicity (Fig 7d).

CM recovered from $H_2O_2$-induced senescent HUVECs, hereafter regarded as $H_2O_2$ CM, was used to examine if the secretome of senescent endothelium affects chondrocyte behaviour. After one-day culture, chondrocytes expressed a higher level of COL10 (15.67 ± 1.22) and $p16^{INK4a}$ (21.22 ± 3.01) proteins (Fig 7b). This is accompanied by the elevation of SA-β-gal

positive cells (40.76 ± 2.89 %) and upregulation of hypertrophy and senescence genes (Fig 7c). The levels of *COL10*, *MMP13*, $p16^{INK4a}$ and *p21* increased by 1.84 ± 0.30, 1.85 ± 0.33, 2.46 ± 0.37 and 2.36 ± 0.44 fold respectively (Fig 7d). No significant difference in *SOX9* expression was found between groups, indicating the chondrogenesis was not altered by CM treatment.

Remarkedly, captopril CM demonstrated senolytic effect upon co-culture. CM from captopril-treated HUVECS was labelled as "captopril CM". Cells that received 100 μM captopril CM treatment had diminished signal of COL10 (7.35 ± 1.78). The expression of $p16^{INK4a}$ (3.44 ± 0.69) was even lower than the normal CM group (Fig 7b). SA-β-gal assay also demonstrated population decline of senescent cells (Fig 7c). The trend of reduction in hypertrophy and senescence markers concurred with the observation in the 1 mM captopril CM treatment group. Fluorescence signal intensities of COL10 and $p16^{INK4a}$ were 5.66 ± 2.01 and 4.32 ± 0.84 respectively; while the percentage of SA-β-gal positive cells was 11.36 ± 2.06. From transcriptional perspective, both captopril CM groups showed down-regulation of $p16^{INK4a}$ and *p21* (Fig 7b, c, d). Expression levels of the two genes in both groups dropped to comparable levels to that of the control group. The result suggested that the captopril treatment not only restored HUVECs phenotypes, but also its secretome.



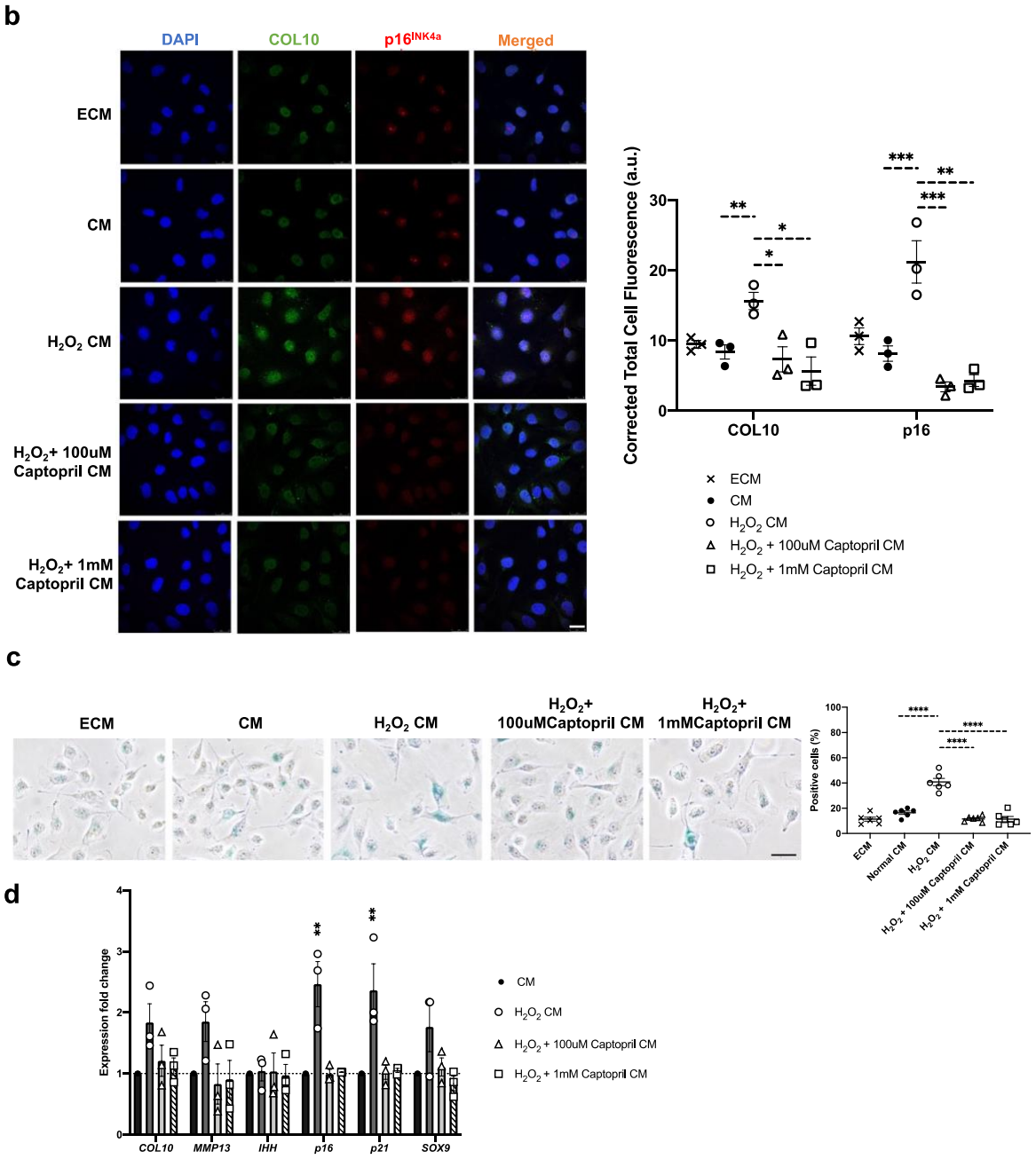



**Fig 7. $H_2O_2$ CM induced chondrocyte hypertrophy and senescence which could be attenuated by captopril CM.** (a) Phase contrast images of chondrocytes under different culture conditions. Fibroblast-like morphology was observed when cells were cultured in F12/ECM and F12/CM in 1:1 ratio. Diluting the ECM and CM with F12 in 1:2 ratio helped mitigate the changes in cell morphology. Scale bar. 50 μm (b) Representative images of immunocytochemistry and quantification of COL10 (green) and $p16^{INK4a}$ (red) for each group with DAPI (blue) (n=3). (c) Representative images and quantification of SA-β-gal assay (n=5). Scale bar. 50 μm. (d) Relative mRNA level of *COL10*, *MMP13*, *IHH*, *p16* $^{INK4a}$, *p21* and *SOX9* in each group (n=3). Statistical comparison was performed between the experimental groups and the CM group. All data are shown as means ± SEM, and each data point represents an

individual experiment. One-way ANOVA with Tukey's multiple-comparisons test was employed for statistical analysis. * $p<0.05$; ** $p<0.005$; *** $p<0.001$; **** $p<0$.

**Captopril had no direct anti-hypertrophic or senolytic effect on chondrocytes**

Harvested CM was frozen directly without further processing. This helped preserve the secretory profile of HUVECs but could potentially carry leftover drugs from the previous experiment and interfered with the results of co-culture. Given the rapid decomposition rate and light sensitivity of $H_2O_2$, it was assumed that the $H_2O_2$ in $H_2O_2$ CM was fully depleted when used for co-culturing. However, the degradation of captopril was uncertain. To evaluate the potential senolytic effect of leftover drugs on chondrocytes, captopril was added to cells after $H_2O_2$ induction.

From the SA-β-gal assay, it could be seen that the number of positive cells elevated after the addition of $H_2O_2$, and remained in captopril treated groups (Fig 8a). Immunocytochemistry result aligned with the observation that captopril could not mitigate the expression of senescence marker. $H_2O_2$ slightly upregulated the production of COL10 (13.66 ± 0.61) and $p16^{INK4a}$ (10.87 ± 1.22) (Fig 8b, c). The expression of genes upon $H_2O_2$ treatment showed huge variations. The only significant change detected was the level of *p21*, which increased by 2.27 ± 0.33 fold (Fig 8d). The signal intensities of COL10 and $p16^{INK4a}$ were at similar levels of that after $H_2O_2$ treatment regardless of the concentration of captopril used. No changes in mRNA levels of hypertrophy and senescence genes were reported after drug treatment. The expression levels of them showed no statistically significant difference compared to either the control or the $H_2O_2$ group (Fig 8d).

Although the senescence induction by $H_2O_2$ was not distinguished, this experiment demonstrated a major finding that captopril did not play a role in chondrocyte fate decisioning.

Neither 100 μM nor 1 mM of captopril showed anti-hypertrophic or senolytic effect on chondrocytes. Considering captopril had been metabolised by HUVECs for a day before CM was harvested, the concentration of the remaining drug in CM should be lower than the two tested doses. Therefore, it could be assured that the senolytic effect of captopril CM reported in the previous experiment was not brought by the leftover drugs, but by the changes in the secretory profile of HUVECs.

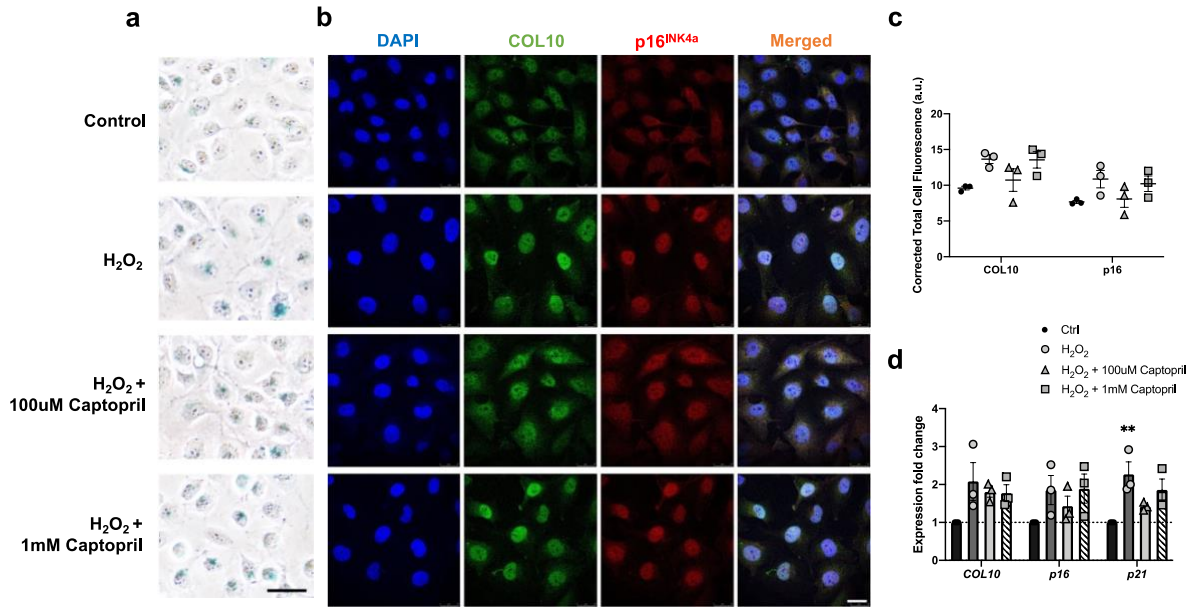



**Fig 8. Captopril did not directly attenuate hypertrophy and senescence phenotypes of chondrocytes.** (a) Representative images of SA-β-gal assay (n=2). Scale bar. 50 μm. (b) Representative images of immunocytochemistry and quantification of COL10 (green) and $p16^{INK4a}$ (red) for each group with DAPI (blue) (n=3). (c) Quantitative data of immune fluorescence signal intensity of each group. No significant difference was detected across groups. (d) Relative mRNA level of *COL10*, *p16* $^{INK4a}$ and *p21* in each group (n=3). Statistical comparison was performed between experimental groups and the control. All data are shown as means ± SEM, and each data point represents an individual experiment. One-way ANOVA with Tukey's multiple-comparisons test was employed for statistical analysis. * $p<0.05$; ** $p<0.005$; *** $p<0.001$; **** $p<0$.

## 3.4 Discussion

The senolytic effect of captopril on HUVECs aligned with the previous study done on human coronary artery endothelial cells which showed the attenuation of oxidative stress-induced endothelial dysfunction. The anti-senescence effect property, together with the antihypertensive effect, make captopril a potent medication for hypertension. It does not only lower the blood pressure but may also alleviate endothelial dysfunction-associated cellular senescence. Given the beneficial outcome of senescent cell elimination, captopril may potentially help restore tissue functions.

The suppression of senescence phenotypes by captopril altered the secretory profile of HUVECs. While secretion from senescent HUVECs could induce chondrocyte hypertrophy and senescence, such phenomenon could not be observed after captopril treatment on HUVECs. Although the content of secretion was not being investigated, it could be deduced that senescent HUVECs exhibited SASP and produced proinflammatory cytokines that could trigger changes in chondrocytes. Captopril eliminated senescent HUVECs and thereby resolved the SASP. Normal chondrocyte phenotypes were then restored. This evidenced the biochemical crosstalk between endothelium and chondrocytes in disease. Our findings may also provide a possible explanation for the comorbidity of hypertension and OA in the context of cellular senescence.

It has been demonstrated in Chapter 2 that chondrocyte hypertrophy predisposes to senescence and anti-hypertrophic intervention could mitigate senescence phenotypes. In the previous experiment, the co-existence of hypertrophy and senescence markers was frequently encountered in CM-induced chondrocyte changes. Hence, it is worth investigating if the hypertrophy also partakes in the endothelium-induced chondrocyte senescence. The same approach using 100 ng/ml PTHrP was employed. It was added into chondrocytes during the

24-hour incubation in $H_2O_2$ CM. Hence, it resembled the "treatment" group setting in Chapter 2 but using $H_2O_2$ CM instead of starvation.

The number of SA-β-gal positive cells declined after the PTHrP treatment in comparison to the $H_2O_2$ CM group (Fig 9a). Yet the efficacy was not as promising as captopril CM. At transcriptional level, the expression of *COL10* slightly dropped and became comparable to the CM group. Meanwhile, the $p16^{INK4a}$ expression (1.07 ± 0.22) was significantly downregulated compared to the $H_2O_2$ CM group (Fig 9b). The reduction was not observed in *p21* due to the large variation across experiments. Although the treatment could not eliminate all senescence markers completely, the results have indicated partial senolytic effect of PTHrP in $H_2O_2$ CM-induced chondrocyte senescence. This may also imply hypertrophy is one of, but not the only, player in endothelium-induced chondrocyte senescence.

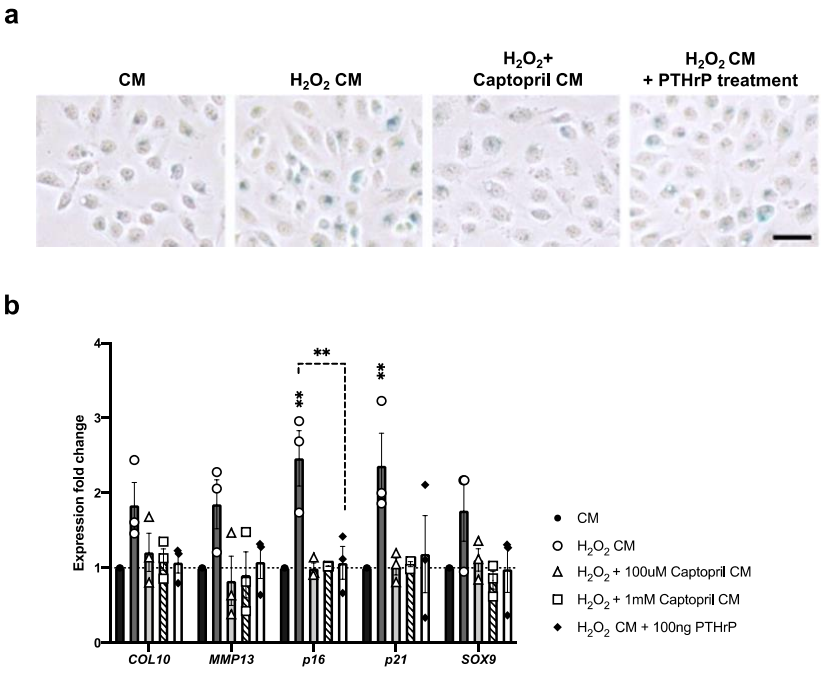



**Fig 9. PTHrP partially alleviated $H_2O_2$ CM-induced chondrocyte senescence.** (a) Representative images of SA-β-gal assay (n=2). Scale bar. 50 μm. (b) Relative mRNA level of *COL10, MMP13, IHH, p16* $^{INK4a}$*, p21* and *SOX9* in each group (n=3). Statistical comparison was performed between experimental groups and the CM group, and between experimental

groups and $H_2O_2$ CM groups. All data are shown as means ± SEM, and each data point represents an individual experiment. One-way ANOVA with Tukey's multiple-comparisons test was employed for statistical analysis. * $p<0.05$; ** $p<0.005$; *** $p<0.001$; **** $p<0$.

Although the HUVEC-C28/I2 model has pinpointed the impact of endothelial SASP on chondrocytes, model that can reflect endothelial-chondral crosstalk *in vivo* requires a more sophisticated study design. In a normal knee joint, microvessels in subchondral bone and synovium would be of closest proximity to articular cartilage and are responsible for the majority of biochemical signalling from vascular system. Heterogeneity has been among different types of endothelial cells from different tissues (171). The secretory profile of HUVECs, which are large vessel cells, may not sorely represent the cellular behaviour of microvessels in joint cavity. Nonetheless, both HUVECs and synovial microvessel endothelial cells express major hypertension-related receptors and proteins such as RAS components (172). The data obtained from our HUVEC model therefore still provide insight into endothelium-cartilage interaction in the context of hypertension.

# Chapter 4 Pathogenic Factors involved in Endothelial Senescence-induced Chondrocyte Hypertrophy and Senescence

## 4.1 Introduction

While endothelial senescence has been proven to induce chondrocyte hypertrophy and senescence via biochemical means, it is important to reveal the pathogenic factors in endothelial secretome that disrupt chondrocyte homeostasis for therapeutic discovery. Overactivation of RAS and ET pathway is often documented in hypertension. They are also implicated in joint and skeletal health and diseases. The two blood pressure regulatory systems were therefore the focuses of this experiment.

**Aim and Approaches**

The aim of this experiment was to determine the pathogenic factors from senescent HUVEC CM that contributed to chondrocyte changes. Specifically, we would like to examine if the two main vascular tone regulatory centres, RAS and ET, partook in the endothelial-chondral interaction.

*Spontaneous hypertensive rat (SHR) model*

RAS overactivation was often reported in hypertensive patients. As a proof of concept, the level of one of the RAS components, Ang II, in rat models were examined. SHR is bred from normotensive Wistar Kyoto (WKY) rat and is a commonly used animal model with primary vascular pathology. The animal will eventually develop hypertension after 3- to 4-week-old. Along with high systolic pressure, SHR shows other characteristics of cardiovascular disorder such as cardiac hypertrophy and heart failure, which makes them a representative candidate for vasculature dysfunction (173).

*ANG II-induced HUVEC senescence model*

Although oxidative stress is a common practice to promote premature senescence *in vitro*, it may not recapitulate the cellular responses of vasoconstrictor-induced endothelial senescence observed *in vivo*. Therefore, ANG II was used to replace $H_2O_2$ in this experiment to resemble clinical situations.

## 4.2 Materials and Method

*Animals*

SHR and WKY rats were raised in standard condition until 13-month-old. They were sacrificed and blood sample was taken via cardiac puncture by lab members. Plasma samples were then collected after centrifugation and kept in -80$^{O}$C.

*Immunocytochemistry staining*

Staining procedures followed previously described. Primary antibody against angiotensinogen (ab97381, Abcam, 1:500 dilution). Secondary antibodies used were Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (A-11008, Invitrogen, 1:200 dilution) Cells were mounted with ProLong® Gold antifade reagent with DAPI. Images were taken using Leica TCS SPE Confocal Microscope and analysed with Fiji Image J.

*Enzyme-linked Immunosorbent Assay (ELISA)*

ANG II level was detected with Angiotensin II ELISA Kit (ADI-900-204, Enzo). Assay was performed according to the manufacturer's instruction. The protein level was calculated using the interpolation method. ET-1 level was detected using Endothelin-1 (ET-1) Human ELISA Kit (EIAET1, Invitrogen). The amount of ANG II and ET-1 was then normalised to the total protein concentration in the corresponding CM detected by Pierce™ BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's instruction.

*In vitro ANG II treatment*

Human Angiotensin II peptide (ab120183, Abcam) solution was prepared by diluting with cell culture medium. It was added to cells for 24 hours before any further analysis was performed.

*ANG II conditioned Medium preparation and co-culture*

CM was prepared as described in the previous chapter. "Control CM" was collected 24 hours after the recovery of HUVECs from passaging. Cells were then incubated in either 1 μM or 10 μM of ANG II peptide (ab120183, Abcam) for 24 hours and the medium was retrieved as "ANG II CM". All CM was aliquoted into small vials and kept at -20$^{o}$C for up to 2 weeks. CM was mixed with fresh DMEM/F12 medium in 1:2 ratio for C28/I2 culturing. Comparison of cellular phenotypes was always done between chondrocytes culturing in the same batch of CM (harvested from the same flask of HUVECs).

## 4.3 Results

**ANG II induced HUVEC senescence but was not secreted by oxidative stress-induced senescent cells**

Although RAS overactivation was reported in hypertension, no significant difference in the plasma Ang II level was found between normotensive WKY rats and hypertensive SHR. The result was ambiguous considering the large variation of Ang II level detected in SHR (Fig 10a).

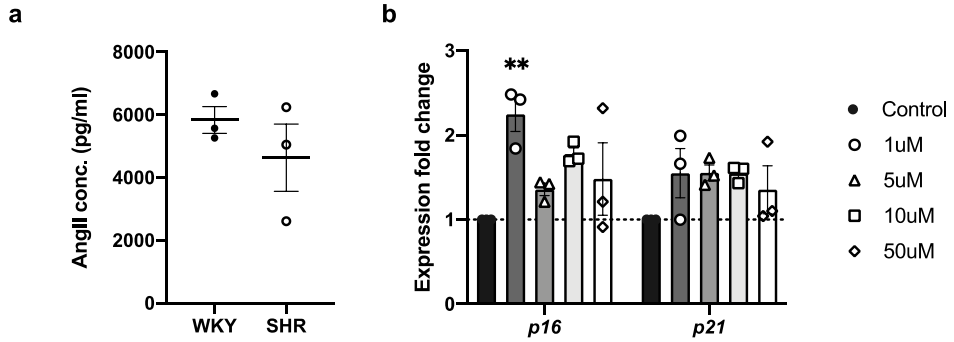
Since plasma ANG II elevation was frequently reported and agreed in hypertension condition, subsequent experiments examining the effect of ANG II were performed. Various concentration of ANG II peptide was added to HUVECs. It should be pinpointed that the expression of senescence genes did not follow a dose-dependent pattern (Fig 10b). The expression of senescence gene $p16^{INK4a}$ (2.25 ± 0.20) showed the most significant fold change after treatment with 1 μM of ANG II. Although upregulation of $p16^{INK4a}$ (1.78 ± 0.07) was also observed in the 10 μM group, the change did not pass the statistical test. The result was generally consistent with the published data that ANG II infusion could induce endothelial senescence.

While ANG II was reported to be a senescence inducer, little is known about the intrinsic ANG II production from senescent endothelial cells. The major source of circulating ANG II is endothelium (174). Therefore, an excessive amount of ANG II found in hypertensive patients should be produced from endothelial cells. To test this hypothesis, endogenous level of AGT, precursor of ANG II, was examined in HUVEC senescence model. Expression of AGT (17.9 ± 3.9) rose after $H_2O_2$ induction and diminished upon captopril treatment (Fig 10c). While the expression of AGT may not directly reflect the final concentration of ANG II, the result demonstrated the activation of RAS in endothelial cells in response to oxidative stress. On the

other hand, the reduction of fluorescence signal in captopril-treated group may hint at the decline in ANG II level. Captopril is an ACE inhibitor that covert the ANG I from precursor AGT into ANG II, which could, in turn, stimulate AGT mRNA and protein production, forming a positive feedback loop (175). Therefore, the signal decline after captopril treatment may reflect the break of this loop.

To investigate if the endogenous overexpression AGT correlates with the secretory ANG II level, ELISA was performed on CM. The levels of ANG II in CM showed high variation across individual experiments (Fig 10d). Some samples in $H_2O_2$ CM consisted elevated amount of ANG II while some showed a decline in ANG II. A similar phenomenon was observed in the 100 μM and 1 mM captopril CM group. Therefore, no conclusion could be drawn from this set of data.

Despite the discrepant result in ANG II detection, level of ET-1 in CM showed a consistent decreasing trend upon treatments (Fig 10e). The level of ET-1 decreased by 50% in $H_2O_2$ CM and further declined to -72.42 ± 4.69 % in 100 μM captopril CM and -80.18 ± 12.03 % in 1 mM captopril CM. The results contradicted the previous findings that oxidative stress-induced ET-1 production from HUVEC (176). The further drop of ET-1 concentration after captopril treatment may be accounted for the decline in ANG II, which acts as a transcriptional inducer of ET-1.



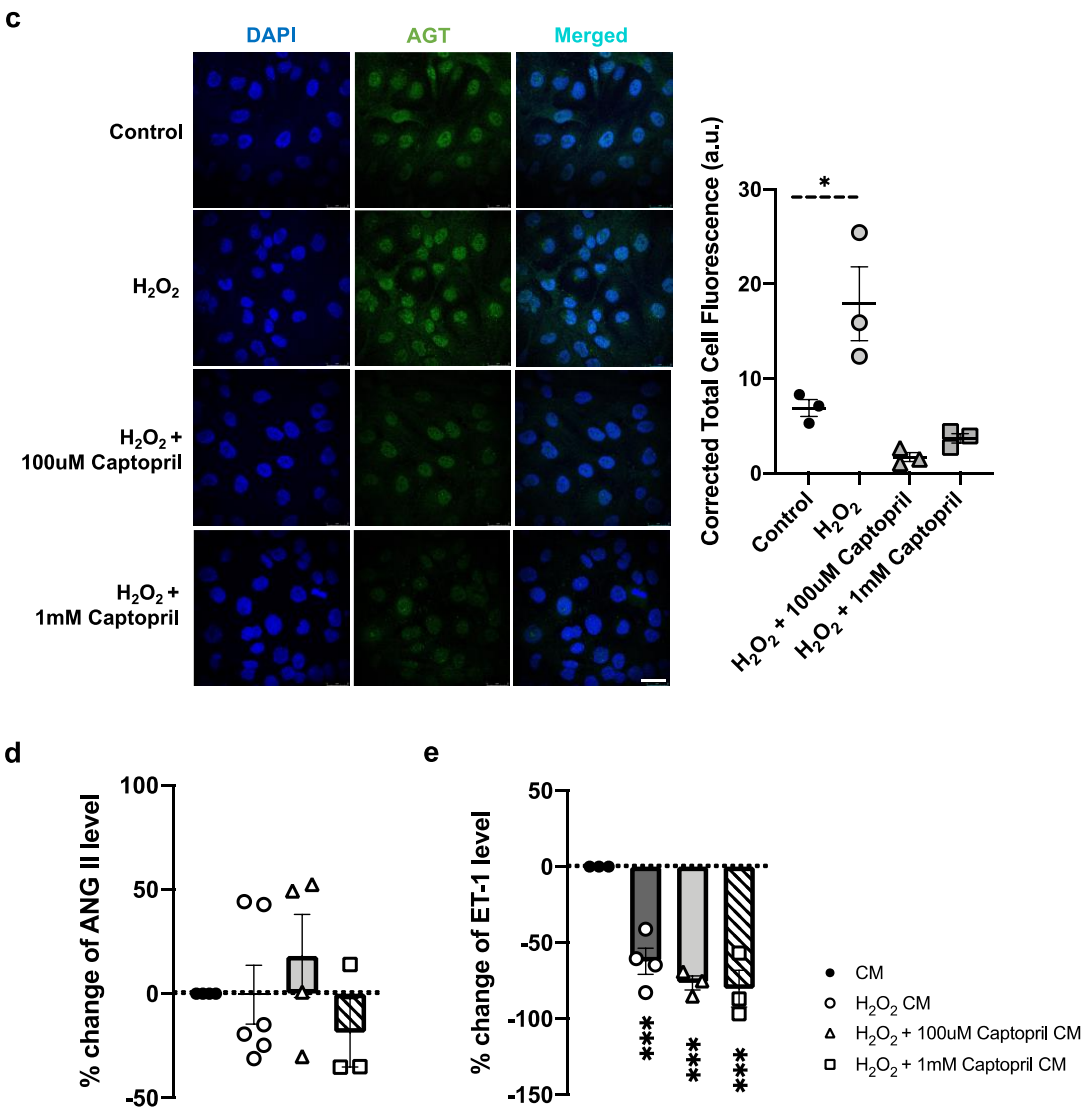



**Fig 10. Senescent HUVECs expressed more AGT but not secretory ANG II.** (a) Plasma Ang II concentration in WKY rat and SHR (n=3). A huge variation of Ang II level was observed in SHR. (b) Relative mRNA level of *p16* $^{INK4a}$ and *p21* in HUVECs under different concentrations of ANG II treatment (n=3). The changes in gene expression did not show dose-dependent trend. (c) Representative images of immunocytochemistry and quantification of AGT (green) for each group with DAPI (blue) (n=3). Fluorescence signal intensity increased significantly upon oxidative stress challenge. Scale bar. 25 μm (d) Percentage changes of ANG II level in different CM compared to normal CM. Levels of ANG II were normalised to total protein concentration. Discrepant results were obtained within each group. No statistical significant conclusion could be drawn. (e) Percentage changes of ET-1 level in different CM compared to normal CM. Levels of ET-1 were normalised to total protein concentration. ET-1 expression dropped in $H_2O_2$ CM group and showed a continuous decline upon increasing concentration of captopril treatment. All data are shown as means ± SEM, and each data point represents one sample. One-way ANOVA with Tukey's multiple-comparisons test was employed for statistical analysis. * $p<0.05$; ** $p<0.005$; *** $p<0.001$; **** $p<0$.

**ANG II CM from HUVECs induced chondrocyte senescence but direct addition of ANG II could not**

In Chapter 3, we demonstrated that oxidative stress-induced endothelial senescence could promote hypertrophic and senescent changes in chondrocytes. This evidenced the casual relationship between endothelial dysfunction and chondrocyte fate alteration. To further recapitulate clinical vascular dysregulation, ANG II was used to replace $H_2O_2$ in HUVEC senescence model.

The chosen doses of ANG II were 1 μM and 10 μM as they had been proven to sufficiently drive HUVEC senescence in the previous experiment. Dissimilar to the findings in $H_2O_2$ model, ANG II CM did not cause chondrocyte hypertrophy (Fig 11a). Expression of *COL10* in 1 μM and 10 μM ANG II CM was 1.10 ± 0.27 and 1.39 ± 0.11 respectively. Markedly, *MMP13* expression decreased in ANG II CM groups. It showed a significant decline (0.48 ± 0.10) in high dose ANG II CM. Although the level of *p21* did not fluctuate across groups, that of $p16^{INK4a}$ increased by 2.28 ± 0.16 fold after treating with 1 μM ANG II CM. Upregulation of senescence genes, however, was not reported in 10 μM ANG II CM. *SOX9* expression remained constant in all groups, indicating no changes in chondrogenesis upon CM treatment.

Experiment on the direct impact of ANG II on chondrocytes was also performed to exclude the effect of leftover ANG II in CM. Expression of hypertrophy and senescence genes including *COL10*, $p16^{INK4a}$ and *p21* was examined (Fig 11b). No consistent changes were observed due to the discrepancy in results. Although there seemed to be an increasing trend of $p16^{INK4a}$ expression with elevating ANG II concentration treatment, the fold changes of mRNA level were subtle and not significant. Together with the SA-β-gal activity assay, it could be

concluded that all tested doses of ANG II were not able to trigger chondrocyte senescence (Fig 11c).

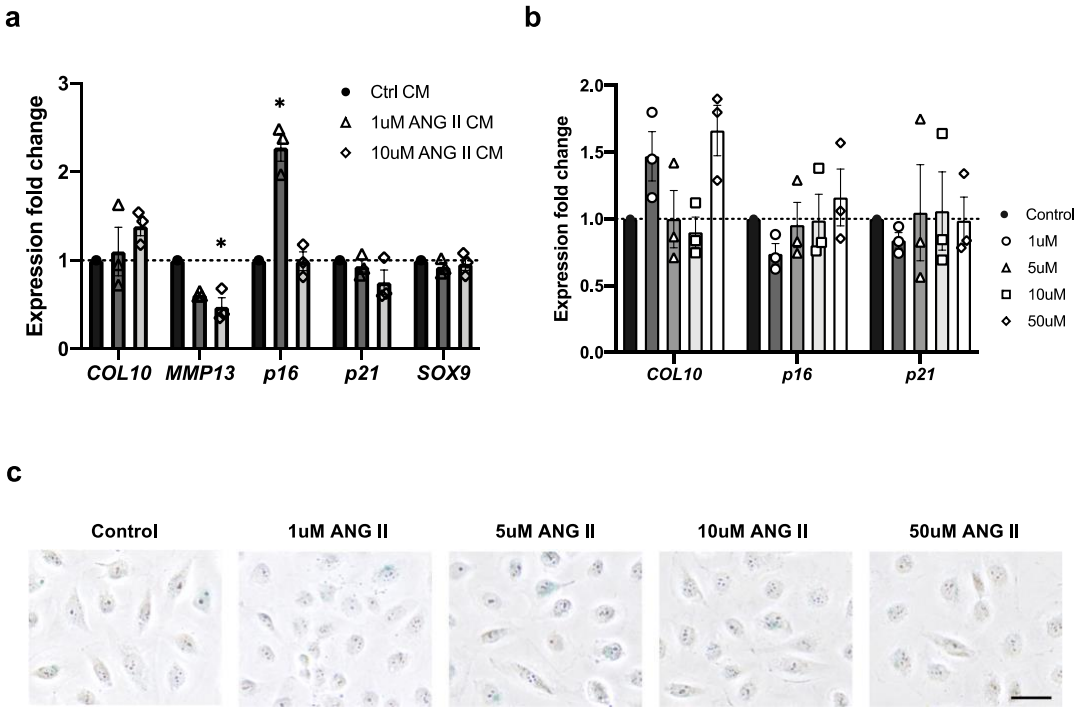



**Fig 11. ANG II CM induced chondrocyte senescence.** (a) Relative mRNA level of *COL10, MMP13, p16 INK4a, p21* and *SOX9* in each group (n=3). (b) Relative gene expression of *COL10, p16 INK4a* and *p21* in chondrocytes under different concentrations of ANG II treatment (n=3). (c) Representative images of SA-β-gal assay of chondrocytes after ANG II treatment (n=2). Scale bar. 50 μm. No positive signal was detected in all groups.

## 4.4 Discussion

The ambiguous plasma levels of Ang II hypertensive rats are unanticipated as RAS components are often found upregulated in hypertensive models (177). Still, our data has demonstrated ANG II as a senescence inducer which has also been reported in animal models. While ANG II has been known for triggering endothelial senescence via ROS production, oxidative stress did not appear to stimulate ANG II secretion in endothelial cells according to our results (178). The level of ANG II in $H_2O_2$ did not show increments. This may imply ANG II is a cause rather than a consequence of endothelial senescence. The same deduction may also be applicable on ET-1, which has also been proved to activate ROS generation.

The negative result of direct addition ANG II on chondrocyte behaviour gave another proof of ANG II being the cause of endothelial dysfunction but not chondrocyte senescence. While ANG II may promote cellular senescence in endothelial in hypertension, it may not be the pathogenic factor causing cartilage degradation in the endothelial-chondral crosstalk given that chondrocyte only showed senescence phenotypes upon ANG II CM but not ANG II treatment. This notion is also supported by the negative effect of captopril on chondrocytes mentioned in Chapter 3. Captopril suppresses the production of ANG II by inhibiting its converting enzyme. Since ANG II may only be responsible for endothelial senescence, senolytic effect of captopril was only demonstrated in HUVECs but not C28/I2, whose senescence phenotypes might be contributed by other factors. Yet it is interesting to note that ANG II did not stimulate hypertrophic changes in the C28/I2 cell line as it did on chondrogenic cell line ATDC5 (137).

To determine the pathogenic secretory factors involved in the CM that caused chondrocyte hypertrophy and senescence, a secretome analysis has been carried out using mass spectrometry. The experiment was performed with the help of Dr. ZHU Lin from the Hong

Kong Baptist University (HKBU). The CM samples were prepared with the addition of protease inhibitors and sent to HKBU for analysis. Data analysis and graphic illustration were also done by Dr Zhu.

Certain proteins were overexpressed in $H_2O_2$ groups (H1-4) compared to the control CM groups (C1-4), and downregulated again in captopril CM groups (D1-4) (Fig 12). They are more likely to be the factors responsible for the phenotypic changes observed earlier and therefore should be paid more attention to. Some of them did not seem to be involved in skeletal health such as constituent of microtubules, TUBA1C, Ras-associated protein, RAPH1, and proteoglycan GPC1. Amongst those that have been implicated in joint homeostasis and disease, some of them such as CILP2, COL3A1 and PLOD1 are either matrix proteins or matrix crosslinker that might not seem to fit in the context of endothelium-cartilage crosstalk.

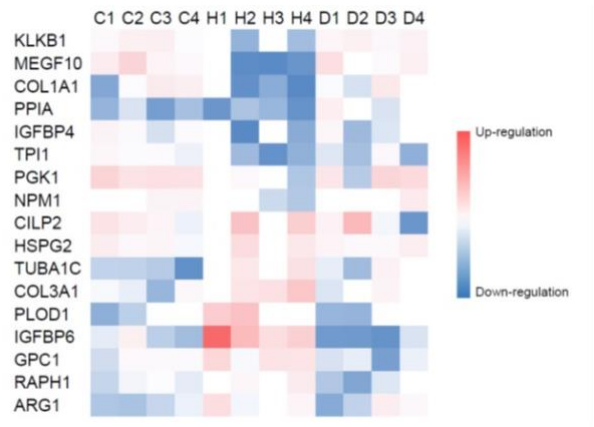



**Fig 12. Heatmap showing changes of protein expression in each group of CM.** Proteins such as IGFBP6 and HSPG2 that show upregulation upon oxidative stress might be potential pathogenic factors contributing to endothelial senescence-induced chondrocyte changes.

Proteins that may be worth noticing are IGFBP6, which showed the strongest expression upon oxidative stress. Insulin-like growth factor binding protein 6 (IGFBP6) is a specific inhibitor of IGF-II. Given the high circulating level of IGF-II in humans, interest in the protein and its inhibitor IGFBP6 has sparked an interest. Noticeably, the level of IGFBP6 was higher in both

serum and synovial tissue of RA patients than in OA patients and healthy individuals. The protein also demonstrated chemotactic properties that promote the migration of T lymphocytes (179, 180). Overexpression of the *IGFBP6* gene was also observed in leptin-induced rat OA model, implying the role of the protein in obesity-associated OA (181). However, little research has been done on the cellular and molecular impact of IGFBP6 on cartilage integrity and joint disease pathogenesis.

Attention should also be drawn to heparan sulphate proteoglycan 2 (HSPG2), which is also known as Perlecan. The role of perlecan in both vascular and skeletal system has been investigated. Deficiency of perlecan led to endothelial dysfunction in aortic endothelial cells; however, knockdown of it could also protect mice from hypoxic pulmonary hypertension by limiting excessive growth of vascular cells (182, 183). In the knee joint, perlecan seems to correlate with chondrocyte hypertrophy. It modulates VEGF signalling during endochondral ossification for proper vascularisation (184). Perlecan may also act as an effector for the regulation of chondrocyte proliferation and differentiation by hypertrophic chondrocyte-specific gene product (185).

The importance of perlecan in chondrocyte hypertrophy has led to further investigation of the protein in joint diseases. Perlecan-knockout mice have shown a significant reduction of OA phenotype after DMM surgery. The severity of cartilage erosion, osteophyte formation and synovitis have been mitigated with perlecan ablation. The chondroprotective effect of perlecan is possibly attributed to the alteration of fibroblast growth factor (FGF) signalling. Given its roles in vascular health, chondrocyte hypertrophy and the beneficial effect of the protein knockout, perlecan could be a strong candidate for the pathogenic factor involved in the endothelium-cartilage crosstalk.

# Chapter 5 Conclusion and Perspectives

## 5.1 Conclusion

This project started with the observation of changes in chondrocyte fate during OA progression. Both hypertrophic and senescent cells accumulated in affected knees, regardless of the types of OA. The findings initiated the research of the association between chondrocyte hypertrophy and senescence in joint health.

Aside from demonstrating the senescence-like phenotypes in hypertrophic cells, we established a novel cellular model to study the chronological sequence of hypertrophy and senescence. Our model dissected the co-existence of the two events into step-wise changes, allowing manipulation of the individual process. This model would be a critical protocol for the study of chondrocyte fates in the field of joint research. It has been shown that hypertrophic cells are more prone to senescence. Transition from hypertrophy to senescence with the upregulation of $p16^{INK4a}$ and *p21* expression was also observed under oxidative stress. Considering the accumulation of ROS in OA joints, the conversion from hypertrophy to senescence may also happen *in vivo*. In addition, the inhibition of hypertrophy using PTHrP has been proved to mitigate senescence phenotypes in cells. This implies that chondrocyte hypertrophy may be an essential step for cells to become senescent. The understanding of the interaction between the two frequently-encountered cellular phenotypes in OA progression helps devise treatment strategy for the disease.

The complex aetiology of OA is another major challenge in the field. This project provided the first experimental evidence of the impact of endothelial dysfunction on chondrocyte homeostasis. We demonstrated how hypertension-associated endothelial senescence induced chondrocyte hypertrophy and senescence by biochemical means. The secretome of oxidative

stress-induced endothelial senescence significantly stimulated the expression of hypertrophy senescence makers in chondrocytes. More importantly, such phenotypes could be alleviated by restoring vascular health. We examined the senolytic effect of antihypertensive drug, captopril. The medication successfully eliminated senescent endothelial cells in culture and seemed to improve the secretome of the remaining cells given that the conditioned medium from treated cells did not drive chondrocyte hypertrophy and senescence. The experiment provided concrete proof of the causal relationship between vascular dysfunction and cartilage degeneration.

To propose potential treatment for the endothelial senescence-associated chondrocyte damage, we have attempted to uncover the pathogenic factors involved in the process. We identified ANG II as the inducer of vascular ageing and rejected its direct role in chondrocyte homeostasis. It is proposed that the elevation of plasma ANG II in hypertensive individuals contributes to endothelial senescence, which in turn trigger catabolic changes in chondrocytes. Based on the secretome analysis, matrix protein, perlecan, could be a possible candidate responsible for the endothelial-induced changes. It has been implicated in both cardiovascular diseases and OA. Considering that perlecan plays a role in chondrocyte hypertrophy, which has been demonstrated to partake in endothelial senescence-induced chondrocyte senescence, it is highly likely to be the pathogenic factor responsible.

All in all, this project has provided novel insight into OA pathogenesis by unravelling the association between two important cellular processes, hypertrophy and senescence. It also explained the comorbidity of hypertension and OA by evidencing the causal relationship between endothelial and chondrocyte senescence. Technical wise, we established a cell culture model that allows the study of chondrocyte hypertrophy and senescence individually. This

model would hopefully benefit the study of chondrocyte fate, and thereby OA pathogenesis in the field.

## 5.2 Perspectives

This study provided evidence for the potential correlation between chondrocyte hypertrophy and senescence. However, more experimental proves are required to validate these novel findings. Although the inhibition of chondrocyte hypertrophy and senescence have both shown beneficial effects on joint integrity and protected animals from OA, none of those studies had looked at both cell fates (121, 186). It is unsure if the clearance of hypertrophic cells affects chondrocyte senescence and vice versa. *In vivo* studies that take both cellular processes into account would be critical to validate the association between the two cell fates.

At molecular level, the underlying mechanism linking hypertrophy and senescence remains unknown. Although the two processes shared several markers, they seem to be regulated by different sets of genes (127). The plausible link between them may be oxidative stress. It is well-established that ROS can induce both chondrocyte hypertrophy and senescence, however, via distinct pathways. Oxidative stress is currently known to induce hypertrophy by VEGF and RUNX2 expression, and triggers cellular senescence through NF-κB signalling (187, 188). As the investigation on the two cellular processes is a pioneering idea, not much research has been done to purposely look at their correlation. Nonetheless, based on previous findings that ROS accumulation in OA joints could initiate chondrocyte hypertrophy and our findings that oxidative stress can drive hypertrophic cells into senescence state, it is reasonable to put more focus on the role of ROS in the conversion of two cell fates.

Meanwhile, the scope of this project only covered the transition from hypertrophy to senescence but not the other way round. The isolation of senescence cells remains technically challenging without the discovery of non-invasive biomarkers. A new model would also have to be generated for the study of senescence-to-hypertrophy conversion.

The endothelial-chondral crosstalk is another important finding of this project. As mentioned in the previous chapter, we have excluded ANG II as a pathogenic factor and proposed perlecan as a candidate. Once the target molecule has been identified, subsequent experiments can be done to uncover the mechanism underneath. The understanding of the interplay between vascular and skeletal pathologies would open up a new direction for hypertension-associated OA and possibly other metabolic OA.

On the other hand, it would also be interesting to look at the chondrocyte-endothelial cell crosstalk to understand the endothelial feedback in response to chondrocyte hypertrophy and senescence. Hypertrophic chondrocytes are known to express VEGF and promote vascular growth (189). While vascular invasion is essential for endochondral ossification, it is also a hallmark of OA (190). The physical contact between endothelial cells and chondrocytes would therefore be worth investigating. Also, no studies have been done on the cellular impact of chondrocyte hypertrophy and senescence on endothelial cells. Some epidemiological studies have found that patients with OA confer a higher risk of hypertension (57, 191). Therefore, the impact of chondrocyte phenotypes on vascular health should not be overlooked. Studying the crosstalk between vascular and skeletal systems in both directions would give a whole picture of the impact of systemic factors on local tissue. This would benefit not only OA research, but other multi-aetiology diseases.

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