TRPA1 as a Novel Factor and Drug Target in Osteoarthritis

ELINA NUMMENMAA

Tampere University Dissertations 210
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TRPA1 as a Novel Factor and Drug Target in Osteoarthritis

ACADEMIC DISSERTATION
To be presented, with the permission of the Faculty of Medicine and Health Technology of Tampere University, for public discussion in the auditorium F115 of the Arvo building, Arvo Ylpön katu 34, Tampere, on 7 February 2020, at 12 o’clock.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADAMTS</td>
<td>A disintegrin and metalloproteinase with thrombospondin motifs (aggrecanase)</td>
</tr>
<tr>
<td>AITC</td>
<td>allyl isothiocyanate (TRPA1 activator)</td>
</tr>
<tr>
<td>ARD</td>
<td>ankyrin repeat domain</td>
</tr>
<tr>
<td>[Ca^{2+}]_i</td>
<td>intracellular free calcium</td>
</tr>
<tr>
<td>CFA</td>
<td>complete Freund’s adjuvant</td>
</tr>
<tr>
<td>CGRP</td>
<td>calcitonin gene-related peptide</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>DAMP</td>
<td>damage-associated molecular pattern</td>
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<tr>
<td>DMARD</td>
<td>disease-modifying antirheumatic drug</td>
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<tr>
<td>DMOAD</td>
<td>disease-modifying OA drug</td>
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<td>DRG</td>
<td>dorsal root ganglia</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FC</td>
<td>fold change</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
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<tr>
<td>FGFR</td>
<td>fibroblast growth factor receptor</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>gp130</td>
<td>glycoprotein 130</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>GR</td>
<td>glucocorticoid receptor</td>
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<tr>
<td>HA</td>
<td>hyaluronan</td>
</tr>
<tr>
<td>HC-030031</td>
<td>2-(1,3-Dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-isopropylphenyl)acetamide (selective TRPA1 blocker)</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IL-1R</td>
<td>interleukin-1 receptor</td>
</tr>
<tr>
<td>IL-1Ra</td>
<td>interleukin-1 receptor antagonist</td>
</tr>
<tr>
<td>IL-6R</td>
<td>interleukin-6 receptor</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IRAK</td>
<td>Interleukin-1 receptor-activated protein kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>JAK</td>
<td>janus kinase</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>KO</td>
<td>knock-out</td>
</tr>
<tr>
<td>LIF</td>
<td>leukemia inhibitory factor</td>
</tr>
<tr>
<td>LN</td>
<td>natural logarithm</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MIA</td>
<td>monosodium iodoacetate</td>
</tr>
<tr>
<td>MKP-1</td>
<td>mitogen-activated protein kinase phosphatase 1</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MSU</td>
<td>monosodium urate</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor κB (transcription factor)</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NSAID</td>
<td>non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>OA</td>
<td>osteoarthritis</td>
</tr>
<tr>
<td>PDTC</td>
<td>ammonium pyrrolidinedithiocarbamate (NF-κB inhibitor)</td>
</tr>
<tr>
<td>PGE₂</td>
<td>prostaglandin E₂</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
</tr>
<tr>
<td>RNA-Seq</td>
<td>RNA sequencing</td>
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<tr>
<td>RNS</td>
<td>reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RPKM</td>
<td>reads per kilobase per million</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean</td>
</tr>
<tr>
<td>SP</td>
<td>substance P</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription (transcription factor)</td>
</tr>
<tr>
<td>TCS 5861528</td>
<td>2-(1,3-Dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-[4-(1-methylpropyl)phenyl]acetamide (selective TRPA1 blocker)</td>
</tr>
<tr>
<td>TG</td>
<td>trigeminal ganglia</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of metalloproteinases</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TRP</td>
<td>transient receptor potential</td>
</tr>
<tr>
<td>TRPA1</td>
<td>transient receptor potential ankyrin 1</td>
</tr>
<tr>
<td>TRPV1</td>
<td>transient receptor potential vanilloid 1</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
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Osteoarthritis (OA) is the most common joint disease and one of the leading causes of disability worldwide. The hallmark of the pathogenesis of OA is cartilage degradation, which is driven by an imbalance between the production of catabolic, anabolic and inflammatory factors within the joint. Chondrocytes are considered to be essential in this reaction, and during OA they produce increased amounts of inflammatory and catabolic agents, such as matrix degrading enzymes, reactive oxygen species, cytokines and growth factors. There is currently no disease-modifying drug treatment available to prevent or retard the progression of OA. In order to develop improved treatment modalities, identification of novel drug targets, and a better understanding of the molecular pathways involved in the pathogenesis of OA are imperative.

Transient receptor potential ankyrin 1 (TRPA1) is a membrane bound ligand-gated cation channel, which has primarily been studied in sensory neurons for its roles in pain and neurogenic inflammation. TRPA1 was initially found to function as a chemosensor for potentially harmful exogenous compounds. However, more recent studies indicate that it can possibly also be expressed in tissue cells and be activated by endogenous factors formed during inflammation and hypoxia. Due to these new findings, we hypothesized that TRPA1 may also be expressed and functional in nonneuronal cells, particularly in chondrocytes, within the OA joint.

The aim of the present study was to examine the expression and effects of TRPA1 in chondrocytes and cartilage, in order to produce novel data on the factors involved in the pathogenesis of OA, and ultimately to assess the potential of TRPA1 as a novel drug target in OA. In addition, the effects of anti-inflammatory drugs on TRPA1 expression were investigated. We utilized cartilage and chondrocytes from OA patients undergoing knee replacement surgery, immortalized human T/C28a2 chondrocytes, as well as cartilage and chondrocytes from TRPA1 knock-out and corresponding wild-type mice.
TRPA1 was discovered to be functionally expressed in human OA chondrocytes and an immortalized chondrocyte cell line. The expression of TRPA1 was increased following stimulation with OA-related inflammatory factors, most notably the cytokine interleukin (IL)-1β, which is traditionally considered one of the significant inflammatory mediators driving the pathogenesis of OA. TRPA1 was shown to be involved in the upregulation of inflammatory and catabolic factors IL-6, IL-11, leukemia inhibitory factor (LIF), fibroblast growth factor (FGF)-2, matrix metalloproteinase (MMP)-1, MMP-3, MMP-13, and prostaglandin E2 in chondrocytes/cartilage. The effects of FGF-2 in cartilage and chondrocytes were also examined further, and FGF-2 was shown to exert catabolic and anti-anabolic effects, which were reversed by specific FGF receptor antagonists. Additionally, the clinically used anti-inflammatory drugs dexamethasone and aurothiomalate, as well as inhibition of the transcription factor NF-κB, downregulated the expression of TRPA1 in chondrocytes.

This is the first study to show the expression of a functional TRPA1 channel in chondrocytes. The enhanced expression of TRPA1 by OA-related inflammatory mediators, and the TRPA1-mediated increase in the production of pro-inflammatory and catabolic factors in chondrocytes and cartilage, point to a detrimental role for TRPA1 in the pathogenesis of OA. Further, the clinically used anti-inflammatory drugs dexamethasone and aurothiomalate decreased the expression of TRPA1 in chondrocytes, suggesting that TRPA1 may already be a previously unidentified target of anti-inflammatory drug treatment. Taken together, this study has produced novel data on the inflammatory factors involved in the pathogenesis of OA and presents TRPA1 as a potential novel target for future drug development. Additionally, this thesis has produced valuable information on the nonneuronal expression of TRPA1, expanding our knowledge of this multifunctional ion channel.
lääkeaineiden vaikutuksia TRPA1:n ilmentymiseen. Tutkimuksessa
hyödynnettiin nivelrikkopotilaista tekonivelleikkauksen yhteydessä poistettavaa
rustokudosta ja siitä eristettyjä rustosoluja, ihmisen rustosolulinjasoluja sekä
TRPA1 poistogeenisiltä hiiriltä ja vastaavilta villityypin hiiriltä eristettyä
rustokudosta ja rustosoluja.

Tutkimuksen tulokset osoittivat funktionaalisen TRPA1-ionikanavan
ilmentyvän ihmisen nivelrikkorustosolussa sekä rustosolulinjan soluissa.
TRPA1:n ilmentyminen lisääntyi, kun soluja stimuloitiin nivelrikon patogeneesin
kannalta keskeisillä tulehdustekijöillä, kuten interleukiini (IL)-1:llä. TRPA1:n
havaittiin välittävän usean tulehduskellisen ja kataboliaan liittyvän tekijän tuottoa
rustosolussa/rustokudoksessa. TRPA1:n estäminen vähensi sytiinien IL-6, IL-
11 ja LIF (engl. leukemia inhibitory factor), fibroblastikasvutekijä-2:n (FGF-2:n),
prostaglandiini E₂:n (PGE₂:n), sekä rustokudosta hajottavien
matriksimetalloproteinaasi (MMP)-1, MMP-3 sekä MMP-13 entsyyymien tuottoa.
Tutkittaessa tarkemmin FGF-2:n vaikutuksia rustossa ja rustosolussa, havaittiin
sillä olevan rustokudokselle haitallisia katabolisia ja anti-anabolisia vaikutuksia.
Lisäksi tulehdusta vaimentavat lääkkeet deksametasoni ja aurotiomalaatti estivät
TRPA1-kanavan ilmentymistä ihmisen rustosolussa.

Tämä on ensimmäinen tutkimus, jossa on osoitettu funktionaalisen TRPA1-
ionikanavan ilmentyvän rustosolussa. Tutkimuksen tulokset osoittivat TRPA1:n
ilmentymisen lisääntyvän nivelrikkonivelelle tyypillisten tulehdustekijöiden
vaikutuksesta, sekä TRPA1:n välittävän useiden tulehduskellisten ja katabolisten
tekijöiden tuottoa rustosolussa/rustossa. Tulokset viittaavat siihen, että TRPA1
voi välittää nivelrustovaurioita edistäviä vaikutuksia nivelrikkonivelessä. Tämä
tutkimus on tuottanut uutta tietoa nivelrikon tautimekanismeista sekä uudesta
mahdollisesta lääkevaikutuskohteesta. Lisäksi tämän väitöskirjan
tutkimustulokset tuovat uutta arvokasta tietoa TRPA1:n ilmentymisestä
hermoston ulkopuolella.
1 INTRODUCTION

Osteoarthritis (OA) is the leading cause of musculoskeletal disability and pain worldwide (Hunter et al. 2019; Mobasher and Batt 2016). OA is traditionally a late-onset disease. The incidence of knee OA is reported to peak at around 60 years of age, affecting around 10% of men and 18% of women over the age of 60 (Arokoski et al. 2007; Losina et al. 2013). The global prevalence of OA is estimated to keep rising due to the world’s aging population, presenting a significant and mounting socioeconomic burden on communities (Hunter et al. 2019; Hunter et al. 2014).

OA is a slowly progressing disease, which ultimately leads to the degradation of cartilage from articular surfaces. According to current knowledge, a key feature of OA pathogenesis is the development of an inflammatory and hypoxic state within the joint (Berenbaum 2013; Glyn-Jones et al. 2015). Increased production of inflammatory mediators drives cartilage degradation by shifting the balance of catabolic and anabolic responses towards catabolism. Chondrocytes, the only cells expressed in cartilage, are considered to be essential in this reaction. During OA progression, the normally quiescent chondrocytes are activated to produce increased amounts of inflammatory and catabolic agents, such as cytokines, growth factors, matrix degrading enzymes and reactive oxygen species (Goldring 2012a; Lepetsos and Papavassiliou 2016; van der Kraan and van den Berg 2012).

There are currently no disease modifying drugs available to prevent or retard the progression of OA. Treatment is based on alleviation of pain, improving joint function and preventing disability by pharmacological and non-pharmacological means. In most severe cases, joint replacement surgery is utilized as the last-line treatment. Therefore, there is an urgent need for the development of disease-modifying OA drugs (DMOADs). Important aspects for the successful development of novel therapies are an increased understanding of the mediators
and molecular pathways involved in the pathogenesis of OA as well as the identification of novel drug targets.

Transient receptor potential ankyrin 1 (TRPA1) is a membrane-bound ligand-gated ion channel, which was discovered in 1999 (Jaquemar et al. 1999). Since then, the expression and function of TRPA1 has primarily been studied in sensory neurons, where it functions as a chemosensor for potentially harmful exogenous compounds, and mediates pain, nociception and neurogenic inflammation (Nilius et al. 2012; Zygmunt and Högestätt 2014). In addition to its wide expression in neurons, the expression of TRPA1 has also been shown in some nonneuronal cells, such as keratinocytes and synoviocytes (Jain et al. 2011; Yin et al. 2018). Intriguingly, endogenous factors produced in inflammatory and hypoxic conditions, such as those found in OA joints, can also activate TRPA1, leading to increased production of inflammatory factors by the activated cells.

Due to its multiple functions in mediating pain and inflammation, TRPA1 has attracted considerable interest as a potential drug target for painful and inflammatory conditions (Chen, J. and Hackos 2015; Koivisto et al. 2018; Moran 2018). Very recently, the potential role of TRPA1 as a mediator and drug target in inflammatory arthritides has also began to raise interest (Galindo et al. 2018). Accordingly, TRPA1 has been shown to mediate pain, inflammation and cartilage degradation in experimental models of OA (Horvath et al. 2016; McGaraughty et al. 2010; Moilanen, Hämäläinen, Nummenmaa et al. 2015b), and further studies are needed to elucidate the exact mechanisms behind these effects.

The aim of the present study was to investigate the expression and effects of TRPA1 in human osteoarthritic chondrocytes and cartilage, with the ultimate goal of expanding our knowledge on the factors involved in the pathogenesis of OA, as well as exploring the potential of TRPA1 as a novel drug target in the treatment of OA.
2 REVIEW OF THE LITERATURE

2.1 Osteoarthritis

Osteoarthritis (OA) is the most common joint disease and one of the leading causes of disability worldwide (Glyn-Jones et al. 2015; Mobasher and Batt 2016). According to the Finnish Health 2000 survey, 5% of men and 7% of women over the age of 30 suffer from clinically diagnosed knee OA. The prevalence of knee OA increases with age, and at 65-74 years, around 11% of men and 18% of women suffer from knee OA (Arokoski et al. 2007). These Finnish statistics are similar to global estimates, which state that the incidence of knee OA peaks at around 60 years of age, with a prevalence of around 10% for men and 18% for women over the age of 60 (Losina et al. 2013; Woolf and Pfleger 2003).

The etiology of OA remains partly unknown, but many risk factors have been identified. Risk factors are commonly divided into systemic and local factors. Systemic risk factors increase the risk of OA to all joints, whereas local factors may increase the risk to single joints. The two major systemic risk factors of OA are aging and obesity. Obesity contributes to the pathogenesis of OA by increasing load on weight-bearing joints. However, obesity has additionally been shown to be associated with OA of non-weight bearing joints of the hand and fingers (Haara et al. 2003; Kloppenburg and Kwok 2011; Yusuf et al. 2010), suggesting that there is also a metabolic link. These systemic effects of obesity on OA pathogenesis are currently under intense investigation, and obesity-associated low-grade inflammation as well as metabolic syndrome are considered to have a key role (Berenbaum et al. 2013; Berenbaum et al. 2017). Other systemic risk factors include the female sex, genetic predisposition, race, nutritional factors, and bone density (Blagojevic et al. 2010; Felson et al. 2000;
Firestein et al. 2016). Systemic risk factors may render the articular cartilage more susceptible to degradation through local risk factors, which include obesity through increased loading, extreme strenuous physical activity, trauma to the joint, and congenital malformations of the joint (Blagojevic et al. 2010; Felson et al. 2000; Firestein et al. 2016).

Osteoarthritis can affect any joint, but the most commonly affected joints are the knee, hip, hand interphalangeal and carpometacarpal I joints and the spine (Firestein et al. 2016; Hunter et al. 2019). The leading symptom of OA is pain. In early OA, the pain typically worsens during movement and weight bearing, and is alleviated by rest. As the disease progresses, pain may become constant. The nature of OA-related pain is mostly considered to be nociceptive, caused by tissue injury. However, cumulative data suggests that there may also be a neuropathic component to OA pain, and the use of drugs targeting neuropathic pain could be beneficial in such cases (Fingleton et al. 2015; Hochman et al. 2011). Distinct disease exacerbation phases may occur, which are characterized by excessive pain, inflammation, and effusion of the affected joint, and may require arthrocentesis along with intra-articular injection of glucocorticoids (Marty et al. 2009; Parry et al. 2018). Other symptoms of OA include limited range of motion, crepitus, swelling, and stiffness after inactivity. In advanced OA, the changes in cartilage and bone can ultimately lead to joint deformity (Firestein et al. 2016; Knee and hip osteoarthritis: Current Care Guidelines 2018).

Treatment of OA is based on alleviation of pain through pharmacological and non-pharmacological means and maintaining the ability to perform activities of daily living. Exercise and physical therapy are the first-line treatments for OA. These are accompanied by weight loss of obese patients. Physical therapy can include cold therapy of the joint, transcutaneous electrical nerve stimulation (TENS) therapy, therapeutic ultrasound or acupuncture. If exercise and physical therapy are not effective enough in pain alleviation, analgesic drugs are used.

Paracetamol (acetaminophen) and nonsteroidal anti-inflammatory drugs (NSAIDs) are used as first-line medication, and if they are not effective enough, tramadol and possibly other opioids can be considered. Paracetamol is a centrally acting analgesic and antipyretic drug. The mechanisms of action of paracetamol
are not known in detail but may partially be explained by inhibition of cyclooxygenase (COX) enzyme and prostaglandin production in the central nervous system, even though it does not inhibit peripheral prostanoid synthesis. Paracetamol has also been proposed to modify serotonin, opioid, endocannabinoid and TRP pathways in the central nervous system in a manner contributing to its analgesic effect. NSAIDs such as ibuprofen exert analgesic, antipyretic and anti-inflammatory effects through the inhibition of COX enzymes and the resulting inhibition of prostaglandin synthesis (Rang et al. 2019).

Topical analgesics (NSAIDs, capsaicin) and intra-articular injections of glucocorticoids or hyaluronate are also used. Glucocorticoids are potent anti-inflammatory drugs, which regulate the expression of numerous inflammatory genes. Glucocorticoids generally exert their effects by entering the cell, binding to intracellular glucocorticoid receptors (GRs), and migrating into the nucleus to interact with DNA to modify gene transcription. In this way, glucocorticoids activate the expression of innumerable genes, including many anti-inflammatory genes. Additionally, glucocorticoids downregulate the expression of an array of pro-inflammatory genes by inhibiting the activity of inflammatory transcription factors, particularly nuclear factor (NF)-κB and activator protein (AP)-1, through various direct and indirect mechanisms (Hartmann et al. 2016; Rang et al. 2019). Intra-articular injections of hyaluronic acid are used in the treatment of OA; however, evidence of its efficacy is somewhat controversial. According to meta-analyses, hyaluronate seems to alleviate OA pain and improve joint function better than placebo, but more studies are needed to elaborate its clinical effectiveness as OA treatment. The mechanisms of action of hyaluronate remain unclear and have been speculated to involve changes in the viscosity of synovial fluid and reduction of inflammation (Altman et al. 2018; Santilli et al. 2016).

If none of the other forms of treatment have been effective, or the state of the joint has worsened considerably, the last-line treatment is joint replacement surgery (Hochberg et al. 2012; Knee and hip osteoarthritis: Current Care Guidelines 2018; McAlindon et al. 2014).

There is currently no treatment that can prevent or retard disease progression and cartilage degradation. Therefore, there is an urgent need for novel disease
modifying OA drugs (DMOADs) to help prevent, retard or even reverse cartilage degradation. In addition, improved analgesic treatment modalities are needed to effectively treat the debilitating pain associated with OA.

2.1.1 Pathogenesis of Osteoarthritis

The etiology and pathogenesis of OA remain unknown in many aspects. Previously OA was considered a “wear and tear” disease, in which cartilage becomes eroded due to mechanical stress and increased pressure on the joints. Now it is known that OA is a more complex inflammatory condition, which includes the release of inflammatory mediators from cartilage, bone, and synovium as well as characteristics of metabolic syndrome, innate and adaptive immunity, chondrosenescence, and inflammaging i.e. changes in the function of the immune system during aging (Berenbaum 2013; Glyn-Jones et al. 2015; Haseeb and Haqqi 2013; Mobasher et al. 2019).

OA is a disease that affects the entire joint structure. Changes associated with OA include fibrillation and degradation of articular cartilage, thickening of subchondral bone, formation of osteophytes, synovial inflammation, i.e. synovitis and degeneration of ligaments and menisci of the joint (Figure 1) (Loeser et al. 2012; Robinson et al. 2016). The most severe changes in OA are seen in cartilage, which is affected by degradative mediators produced in all tissues of the joint. Despite this, OA-associated pain, which is the most severe symptom of OA, is mediated through other joint tissues, as cartilage is aneural.
Articular cartilage is a thin ($\leq 7$ mm), specialized, connective tissue, which provides a smooth, lubricated surface for the bone ends. The tensile and viscoelastic properties of articular cartilage enable it to resist shear, tensile and compressive forces. Articular cartilage is avascular and aneural and receives its nutrition via diffusion from the synovial fluid and vasculature in the subchondral bone. Normal mature articular cartilage contains 65-80% water, and most of its dry weight consists of collagens and proteoglycans. The extracellular matrix (ECM) also contains small amounts of other molecules including lipids, phospholipids, noncollagenous proteins and glycoproteins. The most abundant
macromolecule in cartilage is collagen, of which 90-95% is type II. Type II collagen fibrils form a highly cross-linked network, which interacts with other ECM components in cartilage (Firestein et al. 2016; Heinegård and Saxne 2011; Fox et al. 2009). Proteoglycans represent the second-largest group of macromolecules in the articular cartilage. The major proteoglycan in articular cartilage is the large aggregating proteoglycan, or aggrecan, which is composed of a core aggrecan (ACAN) protein and multiple chondroitin and keratan sulphate glycosaminoglycan side chains. These bottle brush-like molecules are bound to a hyaluronan (HA) backbone forming large HA proteoglycan aggregates. Negatively charged proteoglycan aggregates attract water into the cartilage tissue, which is important for the normal biomechanical function of cartilage. Collagen and aggrecan also form a stiff network structure in the ECM, which is resistant to deformation (Heinegård and Saxne 2011; Kiani et al. 2002; Fox et al. 2009). Chondrocytes, which are the only cells present in cartilage, compose only 1-2% of the total volume of cartilage (Fox et al. 2009).

Normal articular cartilage is composed of four distinct regions: the superficial (tangential) zone, the middle (transitional) zone, the deep (radial) zone, and the calcified cartilage zone (Figure 2). The superficial zone contains a relatively high amount of flattened chondrocytes and thin collagen fibrils, which are tightly packed and aligned parallel to the cartilage surface. The middle zone, which composes 40 to 60% of the cartilage volume, contains rounded chondrocytes and bundles of thick collagen fibrils that are situated obliquely and radially around the chondrocytes. The deep zone contains the highest amount of proteoglycan and the lowest amount of collagen. In this zone, the collagen fibers are typically arranged perpendicularly to the articular surface, and the chondrocytes are arranged in columns or clusters. The deep zone is distinguished from the calcified cartilage by a histologically defined tidemark. The calcified zone functions as a mechanical buffer between uncalcified cartilage and subchondral bone. The chondrocytes in this zone are scarce and typically hypertrophic (Firestein et al. 2016; Fox et al. 2009).
Figure 2. Structure of normal cartilage and structural and functional changes caused by OA in cartilage. Factors commonly produced by osteoarthritic chondrocytes. Normal cartilage is divided into superficial, middle, deep and calcified zones, which have characteristic macromolecule networks and chondrocyte phenotypes. During osteoarthritis, the cartilage macromolecules start to break down leading to reduced compressive stiffness and fibrillation of the cartilage surface. Osteoarthritic chondrocytes undergo transient clonal expansion and shift towards a hypertrophic phenotype, characterized by increased production of a variety of inflammatory and degradative factors. Ultimately, the changes in chondrocyte function and cartilage structure lead to severe ulceration and erosion of cartilage. ADAMTS - a disintegrin and metalloproteinase with thrombospondin motifs, COX - cyclooxygenase, CSF-G - colony stimulating factor, FGF - fibroblast growth factor, H₂O₂ - hydrogen peroxide, IFNα - interferon α, IL - interleukin, LIF - leukemia inhibitory factor, LOX - lipoxygenase, MMP - matrix metalloproteinase, NO - nitric oxide, PAF - platelet activation factor, OA – osteoarthritis, OH - hydroxyl radical, PDGF - platelet derived growth factor, PLA - phospholipase A, PTH - parathyroid hormone, RNS - reactive nitrogen species, ROS - reactive oxygen species, TGFβ - transforming growth factor β, TNF - tumor necrosis factor, VEGF
- vascular endothelial growth factor (Modified from Heinegård and Saxne 2011, Li et al. 2013, Attur et al. 2002).

The initial changes in OA cartilage are seen as a decrease in proteoglycan content, length and level of aggregation, followed by a weakening in the collagen network. This leads to reduced compressive stiffness of the tissue and is initially seen as softening and fibrillation of the cartilage surface. Changes in the cartilage surface are accompanied by an increase in water content leading to swelling and alterations in the biomechanical properties of cartilage. During the initial stages of OA, chondrocytes attempt to repair the cartilage damage by producing increased amounts of cartilage matrix components. This however often leads to the production of matrix that is inferior in structure and function to normal cartilage. At the same time, proteolytic cartilage matrix degradation is increased, and, as OA progresses, the degradation eventually exceeds the enhanced matrix synthesis. This leads to reduced thickness, as well as increasingly severe fibrillation, ulceration, and cleft formation in the cartilage tissue, and may ultimately result in the cartilage becoming fully eroded leading to the exposure and eburnation of the subchondral bone (Firestein et al. 2016; Goldring and Goldring 2010; Heinegård and Saxne 2011; Lorenzo et al. 2004; Tchetina 2011). Figure 2 represents common changes seen in OA cartilage.

The proteolytic degradation of cartilage matrix macromolecules is mediated by matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTSs). The most important MMPs in the pathogenesis of OA include MMP-1 and -13, which cleave fibrillary collagens, as well as MMP-3, which mainly cleaves proteoglycans and activates latent collagenases (Cawston and Wilson 2006; Troeberg and Nagase 2012). MMP-enzymes are produced by chondrocytes and synovial cells, and their production is increased in OA by inflammatory factors such as IL-1 and TNF-α (Kobayashi et al. 2005; Shlopov et al. 1997; Tetlow et al. 2001). Initially, MMP-enzymes were thought to be the main aggrecan-degrading enzymes in OA, until it was found that aggrecan fragments are mostly cleaved at a site distinct from the MMP cleavage site. It was found that aggrecanases, later named ADAMTS-4 and ADAMTS-5, cleave aggrecan at a site distinct from that of MMP-enzymes, and they are now considered to be important factors in proteoglycan degradation in
OA (Malfait et al. 2002; Troeberg and Nagase 2012). Tissue inhibitors of metalloproteinases (TIMPs) act as endogenous inhibitors of MMPs and several ADAMTSs, and therefore function as key regulators of the matrix degrading metalloproteinases (Brew and Nagase 2010). Recently, the breakdown products of ECM have gained interest as potential damage associated molecular patterns (DAMPs). Once released from the ECM, these DAMPs can activate inflammatory receptors such as members of the toll-like receptor (TLR) family in surrounding cells and thereby accelerate the inflammation and cartilage degradation in OA (Henrotin et al. 2014; Lees et al. 2015; Sokolove and Lepus 2013).

2.1.1.2 Chondrocyte

The chondrocyte is the only cell type present in adult articular cartilage, and it constitutes around 1 to 2 % of the total volume of articular cartilage (Firestein et al. 2016; Fox et al. 2009). Mature chondrocytes embedded in the ECM of cartilage are considered to be terminally differentiated, with a low rate of synthetic activity (Firestein et al. 2016; Goldring 2012b). Since cartilage is avascular, chondrocytes rely on the exchange of nutrients and metabolites through diffusion from the articular surface (Archer and Francis-West 2003; Bhosale and Richardson 2008). The oxygen consumption of chondrocytes is low, and their energy metabolism relies heavily on glucose. Accordingly, chondrocytes generally contain low amounts of mitochondria (Archer and Francis-West 2003; Firestein et al. 2016). Chondrocytes also maintain active membrane transport systems for the exchange of cations, including Na\(^+\), K\(^+\), Ca\(^{2+}\), and H\(^+\) (Firestein et al. 2016). Chondrocytes are responsible for the production of the major components of extracellular matrix, including collagen type II and aggrecan, along with several other molecules of the ECM (Akkiraju and Nohe 2015; Goldring 2000). Under normal conditions, chondrocytes maintain a low turnover rate of cartilage matrix components, retaining a stable equilibrium between their synthesis and degradation (Goldring 2000).
In OA, the activity of chondrocytes increases and becomes aberrant, and their phenotype shifts towards hypertrophy (Akkiraju and Nohe 2015; van der Kraan and van den Berg 2012). Chondrocytes respond to abnormal environmental insults, mechanical stress, alterations in ECM proteins, cartilage degradation products and inflammatory mediators derived from cartilage, synovial fluid or subchondral bone (Goldring and Otero 2011; Heinegård and Saxne 2011). In early OA, structural changes of the surrounding matrix result in a transient increase of chondrocyte proliferation, hypertrophy, and the production of cartilage matrix components, along with an increase in the production of matrix degrading enzymes and inflammatory cytokines (Firestein et al. 2016; Goldring 2000; van der Kraan and van den Berg 2012). After the initial proliferative response of early OA, a decrease in the number of chondrocytes has been reported (Haseeb and Haqqi 2013). Increased apoptosis of chondrocytes in OA has been attributed to local inflammation and especially IL-1β (Heraud et al. 2000; Lopez-Armada et al. 2006). During OA, the gene expression profiles of chondrocytes become altered causing the overall balance of catabolic and anabolic responses to shift to the catabolic side (Aigner et al. 2007; Akkiraju and Nohe 2015; Heinegård and Saxne 2011). Figure 2 demonstrates factors commonly produced by OA-affected chondrocytes.

2.1.1.3 Inflammation in OA

Inflammation is a host defense mechanism, which is triggered by numerous harmful stimuli, such as invading pathogens and factors released during tissue damage. Inflammation is aimed at the removal of harmful agents, the repair of damaged tissue, and restoration of host homeostasis. It is commonly divided into acute and chronic inflammation. In acute inflammation, there is increased local blood flow due to blood vessel dilation, increased capillary permeability resulting in leakage of fluids and plasma proteins into the interstitial spaces, and migration of neutrophils and monocytes into the affected tissues. This reaction results in the cardinal signs of acute inflammation: redness (rubor), swelling (tumor), heat (calor) and pain (dolor) (Kumar et al. 2014). These classical signs of
inflammation are not generally present in osteoarthritic joints, with the exception of specific exacerbation periods that can result in joint effusions, synovial swelling, and increased pain (Marty et al. 2009; Parry et al. 2018). Instead, the inflammatory reaction in osteoarthritic joints represents a local low-grade chronic inflammation, with prolonged production of inflammatory and catabolic mediators causing tissue damage in the affected joint (Robinson et al. 2016; Sokolove and Lepus 2013).

It is not yet known what initiates the inflammatory reaction in the OA joint, but several tissues and cells of the joint are known to contribute to the pathogenesis of the reaction. Synovitis, i.e., inflammation of the synovial membrane, is considered to be a significant part of OA pathogenesis. It is positively correlated with the severity and progression of OA (Ayral et al. 2005; MacFarlane et al. 2019; Roemer et al. 2011) and is considered to be an important factor in OA-related pain (Neogi et al. 2016; Wang, X. et al. 2018). Neurons in the synovial membrane contribute to inflammation and pain by releasing neuropeptides such as bradykinin, substance P (SP) and calcitonin gene-related peptide (CGRP) (Henrotin et al. 2014). Synovial cells and infiltrating macrophages produce inflammatory factors in the synovium, from which they can diffuse into the surrounding synovial fluid. Via the synovial fluid, they can further activate cells in the surrounding tissues and contribute to chondrocyte activation and cartilage degradation. Inflammatory and catabolic factors are also produced by chondrocytes in cartilage, osteoblasts in subchondral bone, adipocytes in the infrapatellar fat pad and leukocytes in synovial fluid. The released inflammatory mediators can act in a paracrine, juxtacrine or autocrine fashion, and also the degradation products of cartilage ECM can further stimulate the inflammatory and catabolic reactions in the OA joint (Henrotin et al. 2014; Rahmati et al. 2016; Robinson et al. 2016).
During osteoarthritis, increased amounts of inflammatory cytokines and catabolic enzymes are produced by chondrocytes and other cells of the joint and found in the synovial fluid of OA patients (Beekhuizen et al. 2013; Tsuchida et al. 2014). Cartilage degradation, which is the hallmark of pathogenesis in OA, is mediated by a complex interplay of inflammatory and catabolic factors released in all parts of the joint. The next paragraphs give a brief overview of OA-related inflammatory factors that were used in this thesis either as stimulants or as measured factors reflecting inflammation in OA.

The cytokines IL-1β and IL-6 are regarded as major players in OA pathogenesis and cartilage degradation. IL-1β production is elevated in OA joints, where it stimulates the release of catabolic enzymes MMP-1, -3 and -13 and suppresses the expression of type II collagen and aggrecan. It also stimulates the production of other inflammatory factors including IL-6, leukemia inhibitory factor (LIF), and PGE₂ (Kapoor et al. 2011; Rahmati et al. 2016).

In concert with IL-1β, IL-6 upregulates the expression of MMP-1 and -13 and reduces the expression of type II collagen. In addition to IL-6, LIF and IL-11 are also members of the IL-6 cytokine family and are implicated in the pathogenesis of OA. LIF, which is produced in response to IL-1β and elevated in the synovial fluid of OA patients, participates in cartilage matrix destruction by stimulating proteoglycan degradation, MMP-enzyme synthesis and nitric oxide (NO) production (Jiang, Y. et al. 2014; Kapoor et al. 2011; Lotz et al. 1992). IL-11, unlike most cytokines found in OA joints, seems to have both pro- and anti-inflammatory properties. It has been found to stimulate inflammatory cells, but also to induce TIMP production and reduce PGE₂ release (Alaaeddine et al. 1999; Maier et al. 1993; Wong et al. 2003).

Prostaglandin PGE₂ is a significant mediator of OA-related pain and inflammation. It has been reported to promote chondrocyte apoptosis and stimulate the production of IL-6 in synergism with IL-1β (Kapoor et al. 2011; Lee, AS. et al. 2013).
The cytokine IL-17 in part feeds forward the inflammatory and catabolic reactions in the OA joint by stimulating the production of IL-1β and IL-6, and by upregulating MMP enzymes and downregulating proteoglycan levels (Honorati et al. 2002; Kapoor et al. 2011).

Several growth factors are also involved in the pathogenesis of OA. One of these is fibroblast growth factor-2 (FGF-2), which is found at elevated levels in the synovial fluid of OA patients, and exerts catabolic and anti-anabolic effects in cartilage, especially as a result of cartilage damage (Ellman et al. 2013; Vincent et al. 2002).

In addition, the innate immune system, and particularly the toll-like receptors (TLRs), are involved in driving the pathogenesis of OA. TLRs are expressed in chondrocytes, and when activated by factors such as bacterial lipopolysaccharide (LPS) or DAMPs, they trigger major inflammatory pathways (Bobacz et al. 2007; Sillat et al. 2013; Sokolove and Lepus 2013). The adipocytokine resistin, which is produced by macrophages and found in OA joints, has also been found to signal through TLR4 and induce catabolic responses (Koskinen et al. 2014; Tarkowski et al. 2010).

The next section describes some of these inflammatory mediators considered to be essential in the cartilage degradation and pathogenesis of OA in more detail.

2.1.2.1 Interleukin-1β (IL-1β)

Interleukin-1β is a proinflammatory cytokine, which is considered to be one of the key players in the pathogenesis of OA. IL-1β is produced in the joints by chondrocytes, osteoblasts, mononuclear cells and cells of the synovial tissue (Farahat et al. 1993; Goto et al. 1988; Keeting et al. 1991; Massicotte et al. 2002; Melchiorri et al. 1998; Moos et al. 1999; Smith et al. 1997). Elevated levels of IL-1β have been detected in the synovial fluid of OA patients. However, IL-1β is not consistently elevated or even detectable in all OA patients (Beekhuizen et al. 2013; Scanzello and Goldring 2012; Tsuchida et al. 2014; Westacott et al. 1990).
IL-1β is originally formed as a cytosolic precursor protein (pro-IL-1β), which is intracellularly cleaved by the enzyme Caspase 1 (also known as IL-1β converting enzyme, ICE) to the active form of IL-1β, after which the cytokine is secreted out of the cell (Wojdasiewicz et al. 2014). The activation of cells by IL-1β is mediated through a ubiquitously expressed membrane receptor, IL-1 receptor type I (IL-1R1). The expression of IL-1R1 is increased in human OA chondrocytes compared to chondrocytes in the normal state (Martel-Pelletier et al. 1992). IL-1β can also bind a type II receptor (IL-1R2). However, this receptor is unable to transduce a signal, and is accordingly termed a decoy receptor. Several cell types including chondrocytes and synovial fibroblasts produce an IL-1 receptor antagonist (IL-1Ra), which functions as a natural antagonist of IL-1β. IL-1Ra is capable of binding IL-1R1 with similar specificity and affinity as IL-1β, but does not activate signal transduction (Kapoor et al. 2011; Weber et al. 2010).

Binding of IL-1β to IL-1R1 initiates a complex intracellular cascade. Required for signal transduction of the IL-1/IL-1R1 complex is the co-receptor IL-1 receptor accessory protein (IL-1RacP). Binding of IL-1 to IL-1R1 causes the recruitment of IL-1RacP and the subsequent assembly of two intracellular signaling proteins, myeloid differentiation primary response gene 88 (MYD88) and interleukin-1 receptor-activated protein kinase (IRAK), thereby forming an active IL-1R signaling complex. IRAK activates tumor necrosis factor-associated factor 6 (TRAF6). Oligomerization of TRAF6 results in the activation of the NF-κB, c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) pathways eventually leading to modification of the transcription of target genes (Weber et al. 2010; Wojdasiewicz et al. 2014).

IL-1β plays an important role in cartilage degradation in OA by exerting both catabolic and anti-anabolic effects. IL-1β stimulates the production of cartilage matrix degrading enzymes including MMP-1, MMP-3 and MMP-13, and aggrecanases ADAMTS-4 and ADAMTS-5. Additionally, IL-1β stimulates the production of proinflammatory factors such as IL-6 and IL-8, along with OA-related inflammatory enzymes COX-2 and inducible nitric oxide synthase (iNOS) leading to the production of PGE₂ and nitric oxide (Kapoor et al. 2011;
IL-1β exerts its anti-anabolic responses in chondrocytes and cartilage by suppressing the expression of type II collagen and aggregan, which are major cartilage ECM components (Haseeb and Haqqi 2013; Kapoor et al. 2011). IL-1β can also induce the production of reactive oxygen species (ROS) and downregulate the expression of antioxidant enzymes that scavenge ROS (Afonso et al. 2007; Mathy-Hartert et al. 2008), thereby contributing to the oxidative stress and cartilage degradation in OA. Additionally, IL-1β has been shown to induce the apoptosis of chondrocytes in OA (Heraud et al. 2000; Lopez-Armada et al. 2006).

Since IL-1β is considered one of the key inflammatory mediators in OA, it is not surprising that there have been high hopes for anti-IL-1-therapy in the treatment of OA. Inhibiting IL-1 with IL-1Ra has produced promising results in animal models, where disease activity and progression of structural changes associated with OA have been reduced (Jotanovic et al. 2012). However, despite these encouraging results, the use of anti-IL-1-therapy in human studies has mostly been disappointing, showing only minor symptom-relieving effects (Chevalier et al. 2013; Jotanovic et al. 2012; Kapoor et al. 2011). Fortunately, there have recently been some progressive advances in this field. Promising results were obtained from a Phase 1 clinical trial with the novel human dual variable domain immunoglobulin, ABT-981, which simultaneously binds and inhibits IL-1α and IL-1β. In the trial, subcutaneous injections of ABT-981 significantly reduced absolute neutrophil counts and serum IL-1α and IL-1β concentrations in knee OA patients, without serious adverse events (Wang, SX. et al. 2017). Additionally, the plant-based drug diacerein, which is available in some European countries for the treatment of OA, has been shown to have IL-1-inhibitory activity. Diacerein has been reported to have a modest effect on OA-related pain, and more research is needed to examine its possible effects on disease progression (Panova and Jones 2015; Pelletier et al. 2000).
Interleukin-6 (IL-6)

Interleukin-6 is a proinflammatory cytokine involved in rheumatic diseases. It has long been investigated as a major factor in rheumatoid arthritis (RA) and has recently also raised interest as a key inflammatory factor in OA. IL-6 is produced by chondrocytes, synoviocytes, osteoblasts, macrophages and adipocytes in OA joints (Wojdasiewicz et al. 2014). The production of IL-6 is predominantly stimulated by cytokines IL-1β and TNF-α but can also be upregulated by other inflammatory factors including transforming growth factor β (TGF-β) and PGE2 (Haseeb and Haqqi 2013; Kapoor et al. 2011). IL-6 is found in the serum and synovial fluid of OA patients, with the concentration of IL-6 being significantly higher in synovial fluid compared to serum (Doss et al. 2007; Laavola et al. 2018). IL-6 levels in the synovial fluid of OA patients are reported to correlate positively with radiographic disease severity as well as levels of MMP-enzymes and bradykinin (Bellucci et al. 2013; Laavola et al. 2018).

IL-6 mediates its effects through receptor IL-6R, which occurs in transmembrane and soluble (sIL-6R) forms. IL-6 signaling via membrane-bound IL-6R is termed classical, while signaling via sIL-6R is called trans-signaling. Trans-signaling is important as the membrane-bound receptor is only found in a small number of cell types such as hepatocytes and some leukocytes (Calabrese and Rose-John 2014). In classical signaling IL-6 binds to the transmembrane receptor, after which the IL-6–IL-6R complex associates with a second, ubiquitously expressed transmembrane glycoprotein gp130, causing its dimerization and initiation of intracellular signaling. In trans-signalling, the sIL-6R is formed either by cleaving the membrane-bound IL-6R or by alternative splicing of IL-6R mRNA. IL-6 binds the sIL-6R and the IL-6–sIL-6R complex binds to membrane-bound gp130, causing its dimerization (Calabrese and Rose-John 2014). In both signaling routes, the dimerization of gp130 leads to the activation of Janus kinases (JAKs), which are constitutively bound to the gp130 proteins. Activation of JAKs results in the phosphorylation of tyrosine residues in the intracellular portion of gp130 proteins, leading to the recruitment, phosphorylation and nuclear translocation of STAT1 and STAT3, and the subsequent transcription of IL-6 target genes. IL-6 dependent JAK activation also
leads to the activation of MAPK/ERK (extracellular signal-regulated kinase) and PI3K (phosphoinositide 3-kinase)/Akt (protein kinase B) pathways (Calabrese and Rose-John 2014; Kapoor et al. 2011).

IL-6 has been shown to induce catabolic effects by upregulating the expression of MMP-1 and MMP-13 together with IL-1β and oncostatin M (OSM) in human and bovine cartilage explants (Haseeb and Haqqi 2013; Kapoor et al. 2011). It has also been shown that mechanical injury can potentiate the effects of IL-6, together with TNF-α, on proteoglycan degradation in articular cartilage (Sui et al. 2009). IL-6 is considered the key cytokine involved in subchondral bone remodeling in OA. This is largely attributed to the promotion of osteoclast formation and subsequent bone resorption, which is stimulated by IL-6 in synergism with IL-1β and TNF-α (Chenoufi et al. 2001; Kwan Tat et al. 2004). Osteophyte-derived osteoblasts stimulated with IL-6 also produce elevated amounts of MMP-13, which may contribute to cartilage degradation near the activated osteoblasts (Sakao et al. 2009). Increased levels of circulating IL-6 along with high body mass index (BMI) have been reported to predict the development of radiographic knee OA in a prospective population study conducted in Britain (Livshits et al. 2009). In the same study, IL-6 levels were found to correlate positively with the grading of radiographic knee OA (Livshits et al. 2009). IL-6 also induces anti-anabolism by inhibiting the expression of type II collagen in cartilage (Haseeb and Haqqi 2013; Kapoor et al. 2011).

Despite being a significant factor in the pathogenesis of OA, the use of IL-6 or IL-6R inhibitors in the treatment of OA has only recently begun to be investigated in clinical trials. The humanized anti-IL-6R antibody tozilicumab is currently being investigated in a phase 3 clinical trial for efficacy on pain and function in patients with refractory hand OA (NCT02477059). No results have been published yet, but there are high hopes as tozilicumab is already effectively used in the treatment of RA along with other rheumatic diseases (Rubbert-Roth et al. 2018). As discussed earlier, the effects of IL-6 are mediated by the JAK-STAT signaling pathway. Therefore, certain JAK inhibitors are also used to inhibit the effects of IL-6 in rheumatoid diseases. JAK inhibitors, which are orally administered targeted synthetic DMARDs (tsDMARDs), are the newest drugs in
the treatment of arthritis. Currently in use are the first-generation JAK inhibitors tofacitinib and baricitinib, which target a wide range of cytokines through multiple JAK subtypes. Research is ongoing to develop new, more selective JAK inhibitors. Two second-generation JAK inhibitors upadacitinib and filgotinib, with high selectivity for JAK1, are currently in clinical trials for RA and psoriatic arthritis (O'Shea and Gadina 2019).

2.1.2.3 Fibroblast growth factor-2 (FGF-2)

FGF-2 belongs to a large family of fibroblast growth factors, which have various functions in the growth, differentiation, migration and survival of a wide variety of cell types. FGF-2 (also called basic FGF or bFGF) has been shown to regulate cartilage matrix homeostasis and has therefore attracted interest as a potential mediator in OA (Ellman et al. 2008). FGF-2 is produced in the cartilage, where it is sequestered by a heparan sulfate proteoglycan perlecan in the ECM (Vincent et al. 2002; Vincent et al. 2007). Upon cartilage damage, FGF-2 is released from the ECM, and subsequently activates ERK, p38 and JNK signaling pathways (Figure 3) (Im et al. 2007; Vincent et al. 2002; Yan et al. 2012). FGF-2 is found at elevated levels in the synovial fluid and serum of OA patients compared to healthy controls (Honsawek et al. 2012; Im et al. 2007).
Figure 3. Schematic representation of FGF-2 signaling pathways in articular chondrocytes. FGF-2 binds to FGFR1 and FGFR3 with high affinity. Binding of FGF-2 to FGFR1 results in the phosphorylation of the receptor, and the subsequent activation of the signaling molecules Ras and PKCδ, which converge on the Raf-MEK1/2-ERK1/2 MAPK pathway. PKCδ also activates MAP kinases p38 and JNK. Activation of MAP kinases causes the upregulation of catabolic gene expression. Signaling through FGFR3 is associated with anabolism, but more research is needed to reveal the connection between FGF-2 and FGFR3-associated anabolic responses. ADAMTS - a disintegrin and metalloproteinase with thrombospondin motif, ECM - extracellular matrix, ERK - extracellular signal-regulated kinase, FGF - fibroblast growth factor, FGFR - fibroblast growth factor receptor, JNK - c-Jun N-terminal kinase, MEK - MAPK/ERK kinase, MMP - matrix metalloproteinase, PKC - protein kinase C, Raf - rapidly accelerated fibrosarcoma kinase, Ras - rat sarcoma GTPase (Modified from Ellman et al. 2013)
The role of FGF-2 in cartilage matrix homeostasis has been investigated in various species and cell models, but the results have been somewhat contradictory, and the exact role of FGF-2 remains controversial. FGF-2 has been shown to have a potent mitogenic effect on growth plate cartilage and adult articular cartilage (Ellman et al. 2008). Various studies investigating the role of FGF-2 in human articular cartilage and chondrocytes have shown FGF-2 to induce catabolism and anti-anabolism. FGF-2 has been shown to trigger proteoglycan depletion in cartilage explants and inhibit proteoglycan accumulation in articular chondrocytes both in vitro in alginate beads and in vivo in cultured human articular cartilage explants (Ellman et al. 2013). FGF-2 also inhibits bone morphogenetic protein-7 (BMP-7) and insulin-like growth factor-1 (IGF-1)-mediated proteoglycan production in human articular cartilage (Loeser et al. 2005). In addition, FGF-2 induces the production of cartilage matrix degrading enzymes MMP-13 and ADAMTS-5 and downregulates the expression of aggrecan in human articular chondrocytes (Im et al. 2007; Wang, X. et al. 2004; Yan et al. 2011).

Surprisingly, FGF-2 seems to have an opposing role in murine cartilage compared to human articular cartilage. FGF-2 has exerted anabolic and chondroprotective effects in murine models of OA. In these, intra-articular injections of FGF-2 reversed the progressive cartilage degradation caused by the DMM (destabilization of the medial meniscus) model of OA (Li et al. 2012), and FGF-2 deficiency was shown to increase the development of OA, a finding that was counteracted by subcutaneous injection of FGF-2 (Chia et al. 2009).

The discrepancies in the reported effects of FGF-2 have been suggested to result from differing receptor expression profiles between species, tissues and/or cell types. FGFs signal through four FGF receptor (FGFR) subtypes: FGFR1-4 (Ornitz and Itoh 2015). Human articular chondrocytes express all four subtypes, with the expression of FGFR1 and FGFR3 being significantly higher that FGFR2 and FGFR4 (Yan et al. 2011). FGF-2 has been shown to signal primarily via FGFR1 and FGFR3 in human chondrocytes, and its catabolic properties are shown to be mediated selectively by FGFR1 (Yan et al. 2011). Conversely, activation of FGFR3 is associated with anabolic effects (Figure 3) (Davidson et
al. 2005; Ellman et al. 2013). It has also been shown that in OA cartilage the expression of FGFR1 is increased while the expression of FGFR3 is decreased, which may contribute to the FGF-2-dependent catabolism seen in OA cartilage (Weng et al. 2012; Yan et al. 2011). Unlike in human OA cartilage, FGFR2 and 4 have been shown to be the most prominent receptors in murine knee articular cartilage, although their precise roles are not yet clearly understood (Li et al. 2012). This predominant expression of FGFR2 and 4, coupled with the low expression of catabolic FGFR1 and the finding that FGFR3 is upregulated in response to FGF-2 may explain why FGF-2 has been reported to have anabolic effects on murine cartilage (Li et al. 2012).

Due to its catabolic nature, FGFR1 has gained interest as a potential drug target in OA. FGFR1 antagonists and genetic deletion of *fgfr1* have been investigated in murine chondrocytes and experimental models of OA. Inhibiting FGFR1 genetically and pharmacologically was found to attenuate the degradation of articular cartilage in multiple models of experimental arthritis, and this was largely attributed to the diminished production of MMP-13 and increased expression of the anabolic receptor FGFR3 (Weng et al. 2012; Xu et al. 2016). Despite promising results in experimental murine models, no clinical trials for FGFR1 antagonists with indications for OA had been conducted at the time of this thesis.

Another member of the FGF growth factor family, FGF-18, is currently under intense investigation. The recombinant human FGF-18 drug sprifermin is used in clinical trials for osteoarthritis and cartilage injury of the knee. Sprifermin is administered as intra-articular injections, and has undergone a randomized, double-blind, placebo-controlled proof of concept study, which showed statistically significant, dose-dependent reductions in loss of total and lateral femorotibial cartilage thickness in patients with OA, with no safety concerns (Lohmander et al. 2014; Roemer et al. 2016). Research with sprifermin *in vitro* and *ex vivo* has shown that sprifermin exposure leads to expansion of hyaline-cartilage producing chondrocytes and modulation of ECM turnover (Gigout et al. 2017; Reker et al. 2017). The drug is currently in a Phase 2 clinical trial to
investigate the safety and effectiveness of different doses in patients with knee OA.

2.2 Transient receptor potential ion channels

Transient receptor potential (TRP) ion channels represent a large family of ion channels comprising more than 56 subtypes. In mammals, TRPs contain 28 membrane-associated proteins, which mainly function as non-selective cation permeable channels (Nassini et al. 2014). Mammalian TRP channels are divided into seven subfamilies based on differences in amino acid sequence homology between the different gene products: TRPC (Canonical), TRPV (Vanilloid), TRPM (Melastatin), TRPP (Polycystin), TRPML (Mucolipin), TRPN (no mechanoreceptor potential, NOMP-C) and TRPA (Ankyrin) with TRPA1 being the only known mammalian TRPA channel (Pan et al. 2011).

TRP subunits generally consist of six putative membrane-spanning segments (S1-S6), a pore-forming loop between S5 and S6, and intracellularly located NH2- and COOH-termini. Channel subunits are assembled into homo- or heterotetramers, which result in the formation of cation-selective ion channels (Nilius et al. 2007). Most characterized TRP channels are permeable, though poorly selective, to Ca2+. TRPs are gated by diverse stimuli, which include binding of extracellular and intracellular messengers, osmotic stress, and changes in temperature. There are also some TRP channels that seem to be constitutively open (Clapham 2003).

2.3 TRPA1

The trpa1 gene was originally cloned from lung fibroblasts in 1999 by Jaquemar et al., and it was reported to be located on human chromosome 8 in band 8q13. The N-terminal domain was reported to consist of 18 repeats that are related to the cytoskeletal protein ankyrin, and the C-terminal domain was said to contain six putative transmembrane segments resembling many ion channels (Jaquemar
et al. 1999). Due to its multiple ankyrin repeats in the amino-(N-)-terminal part, TRPA1 was initially named ANKTM1: Ankyrin-like with transmembrane domains 1 (Story et al. 2003). The channel was later renamed TRPA1.

2.3.1 TRPA1 structure and biophysical properties

TRPA1 structure

The trpa1 gene is composed of 27 exons and spans 55,701 base pairs (bp) of the human chromosome 8q13. TRPA1 homologues have been found in a number of vertebrates and invertebrates. Mammals seem to contain only one trpa1 gene, whereas other classes of the Animalia Kingdom contain multiple TRPA1 homologues (Nilius et al. 2012). The ability of TRPA1 to sense harmful electrophilic compounds has been conserved for around 500 million years, but it seems that the thermosensitive properties have diverged later (Kang, K. et al. 2010; Panzano et al. 2010).

The trpa1 gene encodes a protein, which is roughly 1,100 amino acids and 120-130 kDa in size, varying slightly between species. The TRPA1 protein consist of six putative transmembrane segments (S1-S6), intracellular N- and C-termini and a pore loop between S5 and S6. The N-terminus contains 14-18 ankyrin repeat domains (ARDs) composed of 33 amino acids and a large number of cysteines (Nilius et al. 2012; Nilius et al. 2011; Story et al. 2003) (Figure 4). The TRPA1 pore diameter is estimated to be 1.10 nm under basal conditions, and to dilate at least 0.2 nm upon stimulation with the TRPA1 agonist mustard oil (MO) (Karashima et al. 2010).
The role of the N-terminal ankyrin repeat domains remains uncertain, but some functions have been suggested. The ankyrin repeats are likely to be involved in protein-protein interactions (Gaudet 2008), they may also be involved in the transition of the channel to the plasma membrane, as this function is impaired when the domain is truncated (Nilius et al. 2011). Deletions in the ARD area have rendered the channel nonfunctional, probably due to impaired trafficking and insertion of the truncated channel to the plasma membrane (Nilius et al. 2011). It has also been reported that Ca\(^{2+}\) binding to the ARD causes changes in the structure of the N-terminus by increasing its stiffness and altering its end-to-end distance. Thereby affecting the open state probability of the channel (Zayats et al. 2013).
Within the N-terminus is a putative EF hand motive which is thought to be involved in intracellular Ca\(^{2+}\)-dependent activation of TRPA1 (Doerner et al. 2007; Zurborg et al. 2007). The role of the EF hand-like domain is controversial, however, since point mutations have only been shown to have a modest effect on Ca\(^{2+}\)-dependent activation (Nilius et al. 2011; Wang, YY. et al. 2008). In the intracellular S4-S5 linker region of TRPA1 situates an important amino acid residue N855S, in which a gain-of-function mutation causes familial episodic pain syndrome, which is characterized by debilitating upper body pain episodes triggered by cold, fasting and physical stress. The mutation does not seem to alter ligand binding but causes an increase in the inward current through the activated channel (Kremeyer et al. 2010; Zayats et al. 2013; Zima et al. 2015).

Located in the N-terminal end of TRPA1 are multiple cysteine residues, which are involved in electrophilic activation. The cysteine residues are predicted to locate at the interface of adjacent subunits, where covalent modification of cysteines may alter interactions between the subunits and promote conformational changes leading to channel activation. In addition to activation, modification of cysteine residues in TRPA1 has also been shown to have a role in desensitization of the channel (Cvetkov et al. 2011; Ibarra and Blair 2013; Macpherson et al. 2007; Wang, L. et al. 2012).

**Electrophysiology**

TRPA1 is classified as a nonselective cation channel, which is permeable to both monovalent and bivalent cations such as Ca\(^{2+}\), Na\(^{+}\), and K\(^{+}\). Despite being nonselective, TRPA1 has a rather high Ca\(^{2+}\) permeability (P\(_{Ca}\)), with P\(_{Ca}\)/P\(_{Na}\) ranging from 0.84 to 7.9 and the fractional Ca\(^{2+}\) current being up to 23 %. TRPA1 can be activated by agonists, or to a lesser extent be open constitutively. Agonist activated TRPA1 shows a higher P\(_{Ca}\)/P\(_{Na}\) compared to constitutively open TRPA1, with respective P\(_{Ca}\)/P\(_{Na}\) reported to be 7.9 and 5.7 (Karashima et al. 2010; Nilius et al. 2011; Story et al. 2003). TRPA1 pore, which is estimated to be 1.10 nm in size, can undergo dilation upon stimulation, and thereby increase Ca\(^{2+}\)
permeability and allow passage of larger molecules through the channel (Karashima et al. 2010). TRPA1 has a single-channel conductance of 40 – 180 pS, which is dependent on the experimental conditions and used stimuli (Zygmunt and Högestätt 2014).

Extracellular and intracellular Ca\(^{2+}\) is a significant regulator of TRPA1 activity. It has been shown to both potentiate and inactivate TRPA1 (Jordt et al. 2004; Nagata et al. 2005). The exact mechanisms behind these effects are not yet clear. Proposed mechanisms include the entry of calcium through TRPA1 and the subsequent elevation of intracellular calcium (Wang, YY. et al. 2008), the release of calcium from intracellular stores (Jordt et al. 2004), and binding of permeating calcium ions to a site within or close to the TRPA1 pore (Nagata et al. 2005). Ca\(^{2+}\) has been shown to have a bimodal effect on TRPA1, activating TRPA1 at low concentrations and inhibiting it at high concentrations. The presence of extracellular Ca\(^{2+}\) also causes activated TRPA1 currents to decay rapidly, whereas in its absence both the current activation and the decay are delayed. (Nilius et al. 2011; Wang, YY. et al. 2008; Zygmunt and Högestätt 2014).

2.3.2 TRPA1 expression

TRPA1 is abundantly expressed by a subpopulation of primary sensory neurons of the dorsal root ganglia (DRG), trigeminal ganglia (TG) and vagal ganglia (VG). TRPA1 is primarily expressed in neurons with unmyelinated C- and thinly myelinated A\(\delta\)-fibres, and rarely in large myelinated fibres (Bhattacharya et al. 2008; Nassini et al. 2014; Story et al. 2003). TRPA1 is expressed in both peptidergic and non-peptidergic neurons, and mostly in a subpopulation of TRPV1-positive neurons. Although some non-TRPV-expressing neurons are also known to express TRPA1.

More recently, TRPA1 has also been found to be expressed in some nonneuronal cells, which are typically located in lining-tissues. Nonneuronal cells expressing TRPA1 include lung fibroblasts and alveolar epithelial cells (Jaquemar et al. 1999; Mukhopadhyay et al. 2011), skin keratinocytes, fibroblasts
and melanocytes (Atoyan et al. 2009; Jain et al. 2011), mast cells (Oh et al. 2013), rodent β-cells (Cao et al. 2012), urothelial cells (Streng et al. 2008a), enterochromaffin cells (Nozawa et al. 2009), endothelial cells (Earley et al. 2009), oligodendrocytes (Hamilton et al. 2016), astrocytes (Shigetomi et al. 2011), Schwann cells (De Logu et al. 2017), odontoblasts and periodontal ligament cells (El Karim et al. 2015; Tsutsumi et al. 2013), and interestingly with regard to OA pathogenesis, fibroblast-like synoviocytes (Hatano et al. 2012; Kochukov et al. 2006). The roles of TRPA1 in these cells under physiological and pathological conditions require further research. Some functions have however been identified, and putative roles of TRPA1 in these cells include the production of inflammatory mediators, vasodilation, and insulin secretion. The regulation of TRPA1 expression is reviewed in the discussion.

2.3.3 Activators and blockers of TRPA1

TRPA1 activators

TRPA1 is activated by a wide array of electrophilic and non-electrophilic factors that cause irritation, pain and inflammation. Many TRPA1 agonists are electrophilic compounds, which activate TRPA1 by covalently modifying N-terminal reactive cysteine and lysine residues (Eberhardt et al. 2012; Hinman et al. 2006; Macpherson et al. 2007). Electrophiles occur naturally in pungent or spicy plants and are found in many industrial products (Viana 2016). Exogenous electrophilic TRPA1 activators include isothiocyanates in wasabi, mustard oil and horseradish, cinnamaldehyde in cinnamon, allicin and diallyl disulphide in garlic, methyl salicylate in wintergreen oil, and acrolein in tobacco smoke, exhaust fumes and tear gas (Bandell et al. 2004; Bautista et al. 2005; Bautista et al. 2006; Jordt et al. 2004; Macpherson et al. 2007). In research, a commonly used TRPA1 agonist is allyl isothiocyanate (AITC), a pungent compound found in mustard oil. AITC has long been used in pain studies to induce neurogenic inflammation by triggering the release of neuropeptides SP and CGRP, and to promote thermal and
mechanical hypersensitivity (Bautista et al. 2013; Louis et al. 1989). The mechanism of action of AITC remained unknown for years, and it was later discovered to be a robust activator of TRPA1 (Jordt et al. 2004). AITC is also used in the current study as a TRPA1 agonist. Some important TRPA1 activators are listed in Table 1.
Table 1. Significant exogenous, endogenous and indirect activators/sensitizers of TRPA1.
Modified from Gouin et al. 2017.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Pathways of activation</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td><strong>Exogenous activators</strong></td>
<td></td>
<td></td>
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<tr>
<td>Allyl isothiocyanate</td>
<td>Direct activation: Inward current (i\text{Ca}^{2+}) elevation</td>
<td>Jordt et al. 2004</td>
</tr>
<tr>
<td>Cinnamaldehyde</td>
<td></td>
<td>Bandell et al. 2004</td>
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<tr>
<td>Allicin</td>
<td></td>
<td>Macpherson et al. 2005</td>
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<tr>
<td>Acetaldehyde</td>
<td>Direct activation: Inward current (i\text{Ca}^{2+}) elevation</td>
<td>Bautista et al. 2006</td>
</tr>
<tr>
<td>(\Delta^9)-tetrahydrocannabinol</td>
<td>Release of neuropeptides</td>
<td>Jordt et al. 2004</td>
</tr>
<tr>
<td>Acrolein</td>
<td></td>
<td>Bautista et al. 2006</td>
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<tr>
<td>Formalin</td>
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<td>McNamara et al. 2007</td>
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<tr>
<td>Menthol</td>
<td></td>
<td>Story et al. 2003</td>
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<tr>
<td><strong>Endogenous activators</strong></td>
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<tr>
<td>Reactive oxygen species:</td>
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<tr>
<td>Hydrogen peroxide</td>
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<td>Andersson et al. 2008,</td>
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<tr>
<td>Hydroxyl radical</td>
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<td>Bessac et al. 2008,</td>
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<tr>
<td>Superoxide</td>
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<td>Sawada et al. 2008</td>
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<tr>
<td>Reactive nitrogen species:</td>
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<tr>
<td>Nitric oxide</td>
<td>Direct activation: Inward current (i\text{Ca}^{2+}) elevation</td>
<td>Takahashi et al. 2008,</td>
</tr>
<tr>
<td>Peroxynitrite</td>
<td>Inward current (i\text{Ca}^{2+}) elevation</td>
<td>Sawada et al. 2008</td>
</tr>
<tr>
<td>Hydrogen sulfide</td>
<td>Release of neuropeptides</td>
<td>Streng et al. 2008</td>
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<td><strong>Lipid oxidation-derived</strong></td>
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<tr>
<td>Prostaglandin A(_2)</td>
<td>Release of inflammatory mediators</td>
<td></td>
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<tr>
<td>15dPGJ(_2)</td>
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<td><strong>Lipid oxidation products</strong></td>
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<tr>
<td>4-hydroxynonenal</td>
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<td>4-oxo-nonenal</td>
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<td>Nitro-oleic acid</td>
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<td>Diacylglycerol</td>
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<td>Methylglyoxal</td>
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<tr>
<td><strong>Indirect activators/sensitizers</strong></td>
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<tr>
<td>Bradykinin</td>
<td>Bradykinin receptor B2 (GPCR)</td>
<td>Bandell et al. 2004</td>
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<tr>
<td>Growth factors (eg. NGF)</td>
<td>Receptor tyrosine kinase</td>
<td>Malin et al. 2011</td>
</tr>
<tr>
<td>Proteases</td>
<td>Protease activated receptors (GPCR)</td>
<td>Dai et al. 2007</td>
</tr>
<tr>
<td>Low (i\text{Ca}) concentration</td>
<td>EF-hand (i\text{Ca}^{2+})-binding domain</td>
<td>Akopian et al. 2007</td>
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<tr>
<td>Cytokines (eg. TSLP)</td>
<td>TSLP receptor</td>
<td>Wilson et al. 2013</td>
</tr>
<tr>
<td>TRPV1</td>
<td>Physical interaction or (i\text{Ca}^{2+}) second messenger</td>
<td>Honda et al. 2014</td>
</tr>
</tbody>
</table>
15dPGJ$_2$ - 15-deoxy-δ12,14-prostaglandin J$_2$, GPCR - G protein-coupled receptor, iCa$^{2+}$ - intracellular calcium, NGF - nerve growth factor, TRPV1 - transient receptor potential vanilloid 1, TSLP - thymic stromal lymphopoietin.

TRPA1 is also activated by endogenous electrophiles, many of which are produced in inflammatory and hypoxic conditions. Important endogenous activators of TRPA1 are reactive oxygen and nitrogen species (ROS and RNS). ROS, such as hydrogen peroxide (H$_2$O$_2$), hydroxyl radical, and superoxide activate TRPA1 by cysteine oxidation and/or disulfide formation (Andersson et al. 2008; Bessac and Jordt 2008; Sawada et al. 2008). RNS, which include nitric oxide (NO) and peroxynitrite (ONOO$^-$), have been suggested to regulate TRPA1 through S-nitrosylation (Sawada et al. 2008; Shimizu et al. 2014; Streng et al. 2008b; Takahashi et al. 2008; Takahashi and Mori 2011). At moderate concentrations, ROS and RNS are important regulatory mediators in various signaling processes, such as signal transduction from membrane receptors, and oxidative stress responses. However, tissue damage and inflammation can lead to detrimental accumulation of ROS and RNS in pathological conditions including osteoarthritis, in which oxidative stress and the upregulation of reactive species have been linked to chondrocyte apoptosis and cartilage degradation (Droge 2002; Lepetsos and Papavassiliou 2016; Ziskoven et al. 2010). In addition to directly activating TRPA1, reactive species can cause the peroxidation and nitration of membrane phospholipids resulting in the formation of highly reactive substances 4-hydroxynonenal, 4-oxononenal, 4-hydroxyhexenal and nitrooleic acid, which are also potent TRPA1 activators (Andersson et al. 2008; Taylor-Clark et al. 2008a; Taylor-Clark et al. 2009; Trevisani et al. 2007). Interestingly, some prostaglandins have also been found to activate TRPA1. 15-deoxy-δ12,14-prostaglandin J$_2$ and prostaglandin A$_2$, which are electrophilic carbon-containing metabolites of prostaglandins D$_2$ and E$_2$, have been shown to directly activate the TRPA1 channel (Cruz-Orengo et al. 2008; Taylor-Clark et al. 2008b).

In addition to electrophilic compounds, TRPA1 is also activated by some non-electrophiles. These include nicotine (Talavera et al. 2009), some general and local anesthetics including propofol (Lee, SP. et al. 2008; Matta et al. 2008) and lidocaine (Leffler et al. 2008), non-electrophilic NSAIDs such as ketoprofen and diclofenac (Hu et al. 2010), and menthol, which has a bimodal effect on TRPA1,
activating at low concentrations and blocking at high concentrations (Karashima et al. 2007). It is currently not known how these non-electrophilic compounds activate TRPA1. However, it is not thought to be through covalent modifications, but instead by direct interaction with the TRPA1 protein, or some other hitherto unknown mechanism (Nilius et al. 2012; Zygmunt and Högestätt 2014).

Sensitization of TRPA1 by endogenous signaling pathways

Endogenous signaling pathways can also activate and sensitize TRPA1 indirectly. Downstream signaling of G protein-coupled receptors (GPCRs), such as the bradykinin receptor B2 (B2R) and protease-activated receptor 2 (PAR2), and receptor tyrosine kinases (RTKs), have been shown to modulate TRPA1 function (Dai et al. 2007; Gouin et al. 2017; Wang, S. et al. 2008). Secondary mediators shown to be involved in GPCR-mediated TRPA1 modulation include phospholipase C (PLC) and protein kinase A (PKA), which have been suggested to mediate phosphorylation of TRPA1. These endogenous mediators sensitize TRPA1 towards other ligands such as AITC and possibly endogenous agonists, as well as induce the trafficking of TRPA1 to the cell membrane (Dai et al. 2007; Meents et al. 2017; Schmidt et al. 2009; Wang, S. et al. 2008).

TRPA1 blockers

TRPA1 has become an attractive drug target due to its role in neuropathic pain and inflammation. A promising attribute of TRPA1 as a drug target is its peripheral expression pattern allowing for selective targeting of drugs by inhalation or topical application, thus reducing possible side effects of systemic administration. In addition, genetic deletion or pharmacological blockade of TRPA1 have generally not been found to significantly alter protective basal tactile and pain detection. The increasing knowledge of endogenous modulators of TRPA1 may also be utilized to selectively target TRPA1. Difficulties in targeting TRPA1 and developing novel TRPA1 antagonists are variations in the effects of TRPA1 antagonists between different species, as well as the widespread expression of TRPA1 in many cells and tissues, which may cause unwanted side effects (Bautista et al. 2013; Nassini et al. 2014; Viana 2016). Despite the
complexities involved, several promising TRPA1 antagonists have been developed. Most are currently used as research tools, but some have already been investigated in clinical trials.

Among the first commercial TRPA1 antagonists are HC-030031 (Eid et al. 2008; McNamara et al. 2007) and its derivative TCS 5861528 (also called Chembridge-5861528) (Wei et al. 2009; Wei et al. 2010). HC-030031 was developed by Hydra Biosciences in 2007, and it remains one of the most utilized TRPA1 antagonists to this day. Both HC-030031 and TCS 5861528 are routinely used as pharmacological research tools in both in vitro and in vivo models, and they were used in in vitro experiments in this thesis. HC-030031, as well as most other existing TRPA1 antagonists, is a small molecule TRPA1 antagonist that functions as a reversible negative allosteric modulator, binding to a site distinct from the endogenous TRPA1 agonist binding sites (Koivisto et al. 2014). The exact site of action of HC-030031 is however still unclear. According to a recent study by Gupta et al. (2016), a single amino acid residue N855 in the linker region of S4 and S5 of the TRPA1 protein is important for the antagonistic function of HC-030031 in humans. The amino acid potentially binds to HC-030031 through hydrogen bonding and interacts with the C-terminal region of TRPA1 to increase inhibition (Gupta et al. 2016). Interestingly, the amino acid N855 is also the location of the gain-of-function mutation causing familial episodic pain syndrome (Kremeyer et al. 2010). Other commonly used TRPA1 antagonists in in vitro and in vivo studies include the oxime derivative AP-18 from Novartis (Petrus et al. 2007) and the later developed, structurally related compound A-967079 from Abbott (Chen, J. et al. 2011). Unlike HC-030031, which has not been shown to bind inside the TRPA1 pore, A-967079 inhibits TRPA1 by interacting with amino acids inside the TRPA1 pore vestibule (Klement et al. 2013; Paulsen et al. 2015). The search and development of novel TRPA1 antagonists is ongoing and has yielded some novel compounds in recent years (Chen, H. et al. 2018; Lehto et al. 2016; Nyman et al. 2013; Mäki-Opas et al. 2019; Rooney et al. 2014).

One of the most promising advances in the development of TRPA1 antagonists for clinical use occurred in 2011, when Glenmark Pharmaceuticals Ltd. announced that their TRPA1 antagonist GRC 17536 was entering clinical trials.
for pain and respiratory conditions (*Glenmark's novel molecule 'GRC 17536' for pain and respiratory entering human trials*, 2011). Shortly after, in 2012 and 2013, phase 2a double-blind placebo-controlled proof of concept studies in patients with painful diabetic peripheral neuropathy and refractory chronic cough commenced. Glenmark reported to have received positive results from their proof of concept study in patients with painful diabetic neuropathy (*Glenmark's TRPA1 Antagonist 'GRC 17536' Shows Positive Data in a Proof of Concept Study*, 2014), and according to their research pipeline (updated January 2019), Glenmark aims to initiate a Phase 2b dose-range finding study in 2019-2020 in patients with neuropathic pain (http://www.glenmarkpharma.com/novel-molecular-entities/research-pipeline).

### 2.3.4 Functions of TRPA1

Many of the physiological and pathological functions of TRPA1 have been studied in sensory neurons and the peripheral nervous system. Currently, the functional roles of non-neuronal TRPA1 are also under increasing investigation. The physiological function of TRPA1 is considered to be mainly chemo- and mechanosensation, through which TRPA1 can act as a sensor for noxious compounds, tissue damage, cellular stress, and inflammation. Chemosensation is the most widely studied function of TRPA1. As discussed earlier, TRPA1 is primarily located in the peripheral ends of small myelinated Aδ- and unmyelinated C-fibers, where it is activated by numerous noxious compounds such as AITC in mustard oil and acrolein in tear gas (Nassini et al. 2014). Upon activation, TRPA1 transmits a painful sensation to the central nervous system in order to avoid contact with the potentially harmful substance (Nilius et al. 2012).

The role of TRPA1 in mechanosensation is less clear. Soon after the discovery of TRPA1 it was suggested to act as a mechanosensor due to its long ankyrin repeat structure being able to form a spring-like structure that can potentially sense mechanical forces (Howard and Bechstedt 2004; Sotomayor et al. 2005). Since then, multiple *in vitro* and *in vivo* models have supported the role of TRPA1 as a mechanosensor in physiological and pathophysiological
mechanotransduction pathways (Brierley et al. 2009; Brierley et al. 2011; Kerstein et al. 2009; Kwan et al. 2009; Vilceanu and Stucky 2010; Zhang, XF. et al. 2008). However, heterologous TRPA1 gene expression has rarely been able to confer mechanosensitivity to the recipient cells, and more research is needed to confer whether TRPA1 is intrinsically mechanosensitive (Nilius et al. 2012; Zygmunt and Högestätt 2014).

TRPA1 may also be activated by cold temperatures, although data on the subject is controversial (Caspani and Heppenstall 2009; Nilius et al. 2012; Viana 2016). Species-dependent differences in the cold activation of TRPA1 have been observed, and it is possible that TRPA1 is involved in cold transduction in rodents but not humans (Chen, J. et al. 2013). It is also possible that instead of being involved in physiological cold sensation, TRPA1 has a role in cold hyperalgesia and might be sensitized by cold temperature towards other ligands (del Camino et al. 2010; Fernandes et al. 2016; Obata et al. 2005).

Additionally, TRPA1 has been reported to have a role in vasodilation, insulin secretion, bladder function, and gastrointestinal tract function (Aubdool et al. 2016; Earley 2012; Nilius et al. 2012; Yu et al. 2016). The wide expression of TRPA1 in many barrier tissues such as skin, lungs, gastrointestinal tract and vascular endothelium suggests that TRPA1 may have a broader role in tissue homeostasis than is currently known.

TRPA1 has a well-established role in the mediation of pain. TRPA1 has been reported to mediate spontaneous pain, hyperalgesia, and allodynia via direct and indirect activation. In hyperalgesia, there is an increased pain response to stimuli that normally cause pain, whereas in allodynia pain is elicited by an innocuous stimulus (Jensen and Finnerup 2014). TRPA1 has been shown to mediate acute nociception as well as chronic pain such as inflammatory and neuropathic pain experienced in diseases like arthritis, diabetes and fibromyalgia. This has been extensively shown in experimental models, where activation of TRPA1 results in painful states, which are attenuated or prevented in TRPA1 KO animals or by TRPA1 blockers (Galindo et al. 2018; Laing and Dhaka 2016). TRPA1 activator-evoked pain responses are also seen in humans; healthy human volunteers have experienced spontaneous pain and hyperalgesia after being subjected to
cinnamaldehyde (Namer et al. 2005; Olsen et al. 2014). Interestingly, TRPA1 also contributes to guarding pain (representing ongoing postoperative pain) in a rat model of postoperative pain (Wei et al. 2012). In addition to neurons, nonneuronal cells can contribute to TRPA1-related pain by producing inflammatory pain mediators, such as prostaglandins, bradykinin, ROS/RNS and nerve growth factor (NGF), which can stimulate the expression and/or activation of TRPA1 in neurons (Diogenes et al. 2007; Dray 1995; Ronchetti et al. 2017; Taylor-Clark et al. 2008b; Wang, S. et al. 2008). TRPA1 is also implicated with itch, a sensation closely related to pain. Itch sensation can lead to the avoidance of noxious endogenous stimuli, such as allergens, parasites or plant particles. Itch may also be chronic resulting from conditions including atopic dermatitis and neuronal lesions. TRPA1 has been shown to mediate multiple forms of acute and chronic itch, such as serotonin-induced itch, thymic stromal lymphopoeitin (TSLP)-induced itch, and itch associated with chronic atopic dermatitis (Kittaka and Tominaga 2017; Oh et al. 2013; Wilson et al. 2013; Xie and Hu 2018).

In addition to pain, TRPA1 has a significant role in neurogenic inflammation. In neurogenic inflammation, noxious stimuli activate TRPA1 channels in peripheral sensory nerve terminals, triggering signal transmission as well as the release of inflammatory neuropeptides from the activated nerve. Commonly released neuropeptides CGRP and SP contribute to rubor, calor and tumor by eliciting vasodilation and increasing capillary permeability (Chiu et al. 2012; Koivisto et al. 2014). In addition to CGRP and SP, nociceptors release many other neuropeptides and molecular mediators, including neurokinins, nitric oxide and cytokines (Chiu et al. 2012). Release of neuropeptides to the site of inflammation potentiates the inflammatory reaction and causes pain hypersensitivity, sometimes referred to as neurogenic pain. Furthermore, recent evidence indicates a role for TRPA1 also in inflammatory responses of some nonneuronal cells, where TRPA1 activation can trigger the production of inflammatory mediators (Bautista et al. 2013; Gouin et al. 2017).
TRPA1 in osteoarthritis

The pathogenesis of osteoarthritis can be investigated in experimental models including spontaneous and post-traumatic models of OA as well as chemically and mechanically induced OA (Kuyinu et al. 2016). Chemically induced OA models are commonly used to study the effects of drugs on the inflammation or pain associated with OA and are performed by injecting a toxic or inflammatory compound into the joint of the experimental animal (Kuyinu et al. 2016).

So far, there are very few reports on the role of TRPA1 in the pathogenesis of OA. In most of them, the role of TRPA1 has been investigated by inducing OA with the chemical monosodium iodoacetate (MIA). In MIA-induced osteoarthritis, an injection of MIA into the articular cavity of an experimental animal causes OA-like histopathological changes in the cartilage, subchondral bone and synovium, along with increased hyperalgesia and allodynia (Bove et al. 2003; Fernihough et al. 2004; van der Kraan et al. 1989). The degradative effects of MIA on cartilage include inhibition of proteoglycan synthesis and induction of chondrocyte death by inhibiting glyceraldehyde-3-phosphate dehydrogenase and increasing caspase-3 activation (Dumond et al. 2004; Dunham et al. 1992; Grossin et al. 2006; Jiang, L. et al. 2013). MIA also induces inflammation and oxidative stress, as shown by increased production of ROS and multiple inflammatory factors including IL-1β, IL-6, MMP-13 and inducible nitric oxide synthase (iNOS) (Dumond et al. 2004; Jiang, L. et al. 2013; Lee, J. et al. 2013).

At the time of this thesis, four studies had investigated the role of TRPA1 in MIA-induced experimental OA in the mouse or rat. These studies concentrated either on the aspect of pain (McGaraughty et al. 2010; Okun et al. 2012) or on both pain and inflammation (Horvath et al. 2016; Moilanen, Hämäläinen, Nummenmaa et al. 2015b). McGaraughty et al. (2010) showed that systemic administration of a TRPA1 blocker attenuated the responses of wide dynamic range neurons, i.e. sensory neurons which sense a wide array of stimuli, to mechanical stimulation, when measured 21 days after MIA injection (McGaraughty et al. 2010). In a study conducted by Okun et al. (2012), it was reported that both systemic and intra-articular TRPA1 blocker failed to block
MIA-induced decrease in weight bearing or ongoing pain, as investigated 14 days post MIA-injection (Okun et al. 2012).

Unlike in the previous studies, Moilanen et al. (2015b) investigated the role of TRPA1 in MIA-induced inflammation and cartilage degradation in addition to joint pain (Moilanen, Hämäläinen, Nummenmaa et al. 2015b). The effect of intra-articular injection of MIA on nociceptive behavior and cartilage damage was assessed by spontaneous weight bearing tests up to 28 days and histological analysis at day 28 after MIA injection. The results showed that MIA-induced histopathological changes in the cartilage, as well as MIA-induced joint pain, were attenuated in TRPA1 KO mice as compared to WT mice. MIA was also injected into the mouse paw, where it caused an acute inflammatory edema, which was attenuated with a TRPA1 antagonist and significantly reduced in TRPA1 KO mice compared to WT mice (Moilanen, Hämäläinen, Nummenmaa et al. 2015b). In a study by Hórváth et al. (2016) the effects of intra-articular MIA injection on knee swelling, histopathological changes and pain behavior were investigated during a 21-day experiment. The study found that hypersensitivity and decreased weight bearing on the osteoarthritic limb was significantly attenuated in TRPA1 KO mice, while MIA-evoked knee swelling and histopathological destruction were not altered compared to WT mice (Horvath et al. 2016).

A small number of studies have also indicated a role for TRPA1 in other forms of inflammatory arthritides using experimental models such as complete Freund’s adjuvant (CFA)-induced inflammatory arthritis (Fernandes et al. 2011; Fernandes et al. 2016) and monosodium urate (MSU) crystal-induced gout (Moilanen et al. 2015a; Trevisan et al. 2014). In the CFA model, TRPA1 was found to mediate mechanical hyperalgesia and ongoing nociception (Fernandes et al. 2011; Fernandes et al. 2016). TRPA1 was also shown to disrupt the transmission of mechanical stimulation of neurons, and mediate chronic mechanical hypersensitivity, tibiotarsal joint swelling, histopathological alterations as well as myeloperoxidase activity and vascular leakage in CFA-induced arthritis (Horvath et al. 2016; McGaraughty et al. 2010). TRPA1 has additionally been linked to the pathogenesis of gout, which is one of the most common forms of inflammatory arthritis (Richette and Bardin 2010). Experimental gouty arthritis can be induced
by intra-articular injections of MSU crystals. Using this model, TRPA1 has been shown to mediate pain and hyperalgesia (Moilanen et al. 2015a; Trevisan et al. 2014) as well as increase inflammatory cell infiltration and production of inflammatory mediators (Moilanen et al. 2015a).

Taken together, it is seen that the role of TRPA1 in osteoarthritis has not yet been extensively studied, but some encouraging results on the role of TRPA1 in OA-associated pain, inflammation and histopathology have been reported (Galindo et al. 2018). In addition to the in vivo data discussed above, some in vitro findings also support the concept of TRPA1 as a mediator in OA. Especially the discoveries that TRPA1 is activated by inflammatory and hypoxic mediators (Zygmunt and Högestätt 2014), such as those found in OA joints, and that TRPA1 activation regulates the production of inflammatory agents in human synoviocytes (Hatano et al. 2012; Yin et al. 2018), encourage further studies investigating the expression of TRPA1 in chondrocytes and the potential role of TRPA1 in OA pathogenesis.
Cartilage degradation, which is the hallmark of the pathogenesis of OA, is driven by an imbalance between the production of catabolic, anabolic and inflammatory mediators within the joint. There is currently no disease-modifying drug treatment available, and novel drug targets are urgently needed.

The aim of the present study was to examine the expression of TRPA1 in human OA chondrocytes and to investigate its potential role in regulating the expression of OA-related catabolic and inflammatory factors, in order to assess the potential role of TRPA1 as a novel mediator and drug target in OA.

The detailed hypotheses of this study were:

1. TRPA1 is expressed in human OA chondrocytes (I)
2. TRPA1 expression is enhanced by OA-related inflammatory factors (I)
3. The expression of TRPA1 in chondrocytes is inhibited by anti-inflammatory drugs (II)
4. TRPA1 has an effect on OA-associated catabolic and/or inflammatory responses in chondrocytes and cartilage (I, III)
5. FGF-2, which was found to be enhanced by TRPA1, has destructive effects in human OA chondrocytes/cartilage (IV)
4 MATERIALS AND METHODS

4.1 Patients (I-IV)

Cartilage samples were collected from patients undergoing knee-joint replacement surgery in Coxa Hospital for Joint Replacement, Tampere, Finland. All patients in this study fulfilled the American College of Rheumatology classification criteria for OA (Altman et al. 1986), and written informed consent was obtained from the patients. The studies were approved by the Ethics Committee of Tampere University Hospital, Tampere, Finland, and carried out in accordance with the Declaration of Helsinki. Cartilage samples were used for tissue explant and chondrocyte cultures as described below. In the fourth study (IV) cartilage tissue samples were collected from 97 patients with OA (60 females and 37 males, BMI 30.9 ± 0.6 kg/m², age 69.8 ± 1.0 years; mean ± SEM).

4.2 Mice (I,III)

Wild type and TRPA1 knock-out mice (B6;129P-Trpa1<sup>tm1Kykw</sup>/J mice, Charles River Laboratories, Sulzfeld, Germany) aged 19-22 days were used in mouse cartilage culture experiments (studies I, III), and aged 6-12 days in chondrocyte culture experiments (study III) as described below. The mice were housed under standard conditions (12-12 h light-dark cycle, temperature 22±1 °C) with food and water provided <i>ad libitum</i>. Animal experiments were carried out in accordance with the legislation for the protection of animals used for scientific purposes (Directive 2010/63/EU) and the experiments were approved by The National Animal Experiment Board. Animals were sacrificed by carbon monoxide followed by cranial dislocation.
4.3  Cell and cartilage culture

4.3.1  Human osteoarthritic cartilage (IV)

Leftover pieces of OA cartilage from knee-joint replacement surgery were used. Full-thickness pieces of articular cartilage from femoral condyles, tibial plateaus and patellar surfaces showing macroscopic features of early OA were removed aseptically from subchondral bone with a scalpel, cut into small pieces and cultured in DMEM with GIBCO GlutaMAX-I supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (250 ng/ml) (all from Invitrogen/Life Technologies, Carlsbad, CA, USA) at 37°C in a humidified 5% carbon dioxide atmosphere. Cartilage samples were incubated for 42 hours without stimulation. After incubation the culture media were collected and kept at -20°C until analyzed, the cartilage explants were weighed, and the results were expressed per 100 milligrams of cartilage.

4.3.2  Human osteoarthritic chondrocytes (I-IV)

Cartilage pieces obtained from OA patients undergoing knee-joint replacement surgery were processed as described in section 4.3.1. The cartilage pieces were then washed with PBS, and chondrocytes were isolated by enzymatic digestion for 16 hours at 37°C in a shaker by using a collagenase enzyme blend (0.25 mg/ml Liberase™ Research Grade medium; Roche, Mannheim, Germany). Isolated chondrocytes were washed and plated on 6, 24, or 96-well plates in culture medium [DMEM High Glucose (Sigma-Aldrich, St. Louis, MO, USA) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (250 ng/ml), all from Gibco/Life Technologies, Carlsbad, CA, USA] containing fetal bovine serum (10 %) (Lonza, Verviers, Belgium)].

During experiments in studies I-III, the cells were treated with IL-1β (R&D Systems Europe Ltd, Abingdon, UK, studies I-III), IL-17 (R&D Systems Europe Ltd, study I), resistin (BioVision Inc., Milpitas, CA, USA, study I), LPS (Sigma-
Aldrich, study I), dexamethasone (Sigma-Aldrich, study II), aurothiomalate (Sigma-Aldrich, study II), the NF-κB inhibitor ammonium pyrrolidinedithiocarbamate (PDTC, Sigma-Aldrich, study II), the TRPA1 antagonist HC-030031 (Sigma-Aldrich, studies I, III), the TRPA1 antagonist TCS 5861528 (Tocris, Bio-Techne Ltd, Abingdon, UK, study III), or with combinations of these compounds as indicated.

In study IV, the cells were subjected to serum starvation for 24 h before experiments. During the experiments, the cells were treated with FGF-2 (recombinant human FGF-basic; Peprotech, NJ, USA) or with IL-1β (R&D Systems Europe Ltd), which was used as a control compound. In FGF receptor antagonist experiments, the cells were treated with the selective FGF receptor antagonists AZD4547 and NVP-BGJ398 (both from Selleckchem, Munich, Germany) with and without FGF-2 stimulation. The cells were pre-treated with AZD4547 and NVP-BGJ398 1 h prior to the addition of FGF-2.

4.3.3 Human T/C28a2 chondrocytes (I,II)

Immortalized human T/C28a2 chondrocytes (Goldring et al. 1994) were cultured in DMEM (Sigma-Aldrich) and Ham’s F-12 medium (Lonza) (1:1, v/v) containing heat-inactivated fetal bovine serum (10 %) (Lonza), penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (250 ng/ml) (all from Gibco/Life Technologies). Cells were grown on 24-well plates for mRNA measurements, on 6-well plates for Western Blot measurements, and on 96-well plates for Ca$^{2+}$ assays. During experiments the cells were treated with IL-1β (R&D Systems Europe Ltd, studies I,II), IL-17 (R&D Systems Europe Ltd, study I), LPS (Sigma-Aldrich, study I), dexamethasone (Sigma-Aldrich, study II), aurothiomalate (Sigma-Aldrich, study II), methotrexate (Orion Pharma, Espoo, Finland, study II), sulfasalazine (Sigma-Aldrich, study II), hydroxychloroquine (Orion Pharma, study II), ibuprofen (Alexis Biochemicals, San Diego, CA, USA, study II), ammonium pyrrolidinedithiocarbamate (PDTC, Sigma-Aldrich, study II), the TRPA1 antagonist HC-030031 (Sigma-Aldrich, study I) or with combinations of these compounds as indicated.
4.3.4 HEK 293 cells (I)

HEK 293 human embryonic kidney cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Eagle’s Minimum Essential Medium (EMEM) supplemented with heat-inactivated fetal bovine serum (10 %), sodium bicarbonate (1.5%), sodium pyruvate (1 mM), non-essential amino acids (1 mM each) (all from Lonza), penicillin (100 U/ml), streptomycin (100 mg/ml) and amphotericin B (250 ng/ml) (all from Gibco/Life Technologies) at 37 °C in 5 % CO2. The cells were transfected using 0.42 mg/cm² of human TRPA1 plasmid DNA (pCMV6-XL4 from Origene Rockville, MD, USA) with lipofectamine 2000 (Invitrogen/Life Technologies) according to the manufacturer’s directions. Cells were grown on 6-well plates for Western Blot analysis and on 96-well plates for Ca²⁺ assays.

4.3.5 Mouse cartilage culture (I,III)

After mice were euthanized, full-thickness articular cartilage from the femoral heads was removed and incubated at 37°C in 5% CO₂ in DMEM supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (250 ng/ml) (all from Gibco/Life Technologies) containing fetal bovine serum (10 %) (Lonza). The cartilage pieces were exposed to IL-1β (R&D Systems Europe Ltd.) for 42 h and thereafter culture media were collected and MMP-3, IL-6 and PGE₂ concentrations were measured by immunoassay.

4.3.6 Primary murine chondrocytes (III)

After mice were euthanized, full-thickness articular cartilage from the femoral heads was removed, and chondrocytes were isolated by enzymatic digestion o/n without agitation at 37°C in 5% CO₂ with Collagenase D enzyme (3 mg/ml, Sigma-Aldrich), according to the protocol by Jonason et al. (Jonason et al. 2015). Isolated chondrocytes were plated on 24-well plates (2.0 x 10⁵ cells/ml) in culture
medium [DMEM High Glucose (Sigma-Aldrich) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (250 ng/ml) (all from Gibco/Life Technologies) containing fetal bovine serum (10 %) (Lonza)] and cultured for seven days before conducting the experiments. During experiments, the cells were treated for 24 h with IL-1β (R&D Systems Europe Ltd), the TRPA1 antagonists HC-030031 (Sigma-Aldrich) or TCS 5861528 (Tocris, Bio-Techne Ltd) or with combinations of these compounds as indicated.

4.4 RNA extraction and quantitative RT-PCR (I-IV)

At the indicated time points, culture medium was collected, and total RNA extraction was carried out with GenElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich) or RNeasy Mini Kit (Qiagen Inc., Hilden, Germany). The amount of RNA was measured with a spectrophotometer, and total RNA was reverse-transcribed to complementary DNA (cDNA) using Maxima First Strand cDNA synthesis kit (Fermentas UAB, Vilnius, Lithuania) or TaqMan Reverse Transcription reagents and random hexamers (Applied Biosystems, Foster City, CA, USA). After the transcription reaction, the obtained cDNA was diluted 1:5 (for Maxima First Strand cDNA synthesis kit) or 1:20 (for TaqMan Reverse Transcription reagents and random hexamers) with RNase-free water. Quantitative PCR was performed using TaqMan Universal PCR Master Mix and ABI Prism 7000 or 7500 sequence detection systems (Applied Biosystems).

The primer and probe sequences and concentrations were optimized according to the manufacturer’s guidelines in TaqMan® Universal PCR Master Mix Protocol part number 4304449 revision C (Applied Biosystems) (Table 2). TaqMan Gene Expression assays (Table 3) were purchased from Life Technologies (Life Technologies Europe BV, Bleiswijk, the Netherlands). PCR cycling parameters were: incubation at 50 °C for 2 min, incubation at 95 °C for 10 min, and thereafter 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. The relative mRNA levels were quantified using a standard curve method as described in the Applied Biosystems User Bulletin. The ΔΔCt method was used for the TaqMan Gene Expression assays. When
calculating results, mRNA expression levels were first normalized against
glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels.

### Table 2. Primer and probe sequences for quantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotide</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>Human GAPDH</td>
<td>Forward primer</td>
<td>5'-AAGGTCGGAGTCAACGGATTT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>5'-GCAACAATATCCACTTTACCAGATTAA-3'</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>5'-CGCCTGGTCACCAGGGCTGC-3'</td>
</tr>
<tr>
<td>Human AGC1</td>
<td>Forward primer</td>
<td>5'-GCCTGCACCTCAATGACT-3'</td>
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<td>Reverse primer</td>
<td>5'-TAATGGAAACACGATGCCTTTCA-3'</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>5'-CCATGCATCACCTCGACAGGGTA-3'</td>
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<tr>
<td>Human COL2A1</td>
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<td></td>
<td>Reverse primer</td>
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<td></td>
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<td></td>
<td>Probe</td>
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<tr>
<td>Mouse GAPDH</td>
<td>Forward primer</td>
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<tr>
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<td>Reverse primer</td>
<td>5'-CAAGTGCATCAGTTTTCTAC-3'</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>5'-CAGAATTGGCACTTGGCACAACACTCTCATA-3'</td>
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</tbody>
</table>

AGC - aggrecan, COL - collagen, GAPDH - glyceraldehyde-3-phosphate dehydrogenase, IL - interleukin, RT-PCR - reverse transcription polymerase chain reaction, MMP - matrix metalloproteinase
<table>
<thead>
<tr>
<th>Gene</th>
<th>TaqMan Gene Expression Assay</th>
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<td>Human IL-11</td>
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<td>Mouse LIF</td>
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</tbody>
</table>

FGF - fibroblast growth factor, FGFR - fibroblast growth factor receptor, IL - interleukin, LIF - leukemia inhibitory factor, TRPA1 - transient receptor potential ankyrin

### 4.5 Protein extraction and western blotting (I,II)

After the cell culture experiments, cells were rapidly washed with ice-cold PBS and solubilized in cold lysis buffer containing Tris-HCl (50 mM, pH 8), NaCl (150 mM), EDTA (5 mM), Nonidet P-40 (1%), Na deoxyrate (0.5%), SDS (0.1%), phenylmethylsulfonyl fluoride (0.5 mM), sodiumorthovanadate (1 mM), leupeptin (20 μg/ml), aprotinin (50 μg/ml), NaF (5 mM) and sodiumpyrophosphate (2 mM). The cells were incubated in the lysis buffer at 4 °C for 30 min, vortexing every 5 min. Thereafter the samples were centrifuged (12 000 g, 4 °C, 15 min) and supernatants were collected and stored at -70 °C. An aliquot of the supernatant was used to determine protein concentration by the Coomassie blue method (Bradford 1976).

Prior to Western Blot, TRPA1 proteins were immunoprecipitated with TRPA1 antibody NB110-40763 (2 μg, NovusBiologicals, LCC, Littleton, CO, USA) or SAB2105082 (2 μg, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) using protein A/G PLUS-Agarose (sc-2003, Santa Cruz Biotechnology, Inc.), according to the manufacturer’s instructions. The immunoprecipitated TRPA1 protein samples were loaded onto an 8 % SDS-polyacrylamide electrophoresis
gel and electrophoresed for 5 h at 100 V in a buffer containing Tris–HCl (95 mM), glycine (960 mM), and SDS (0.5%). After electrophoresis, the proteins were transferred to a nitrocellulose membrane (Novex, Life Technologies). After transfer, the membrane was blocked in TBS/T (20 mM Tris–base pH 7.6, 150 mM NaCl, 0.1% Tween-20) containing 5% nonfat milk for 1 h at room temperature and incubated in the blocking solution at 4 °C overnight with the primary antibody for TRPA1 (NB110-40763, 1:1000, NovusBiologicals). On the next day, the membrane was incubated in the blocking solution for 1 h at room temperature with the respective secondary antibody for TRPA1 (goat anti-rabbit HRP-conjugate sc-2004, 1:10 000, Santa Cruz Biotechnology, Inc.). Bound antibody was detected using Super Signal® West Dura chemiluminescent substrate (Pierce, Rockford, IL, USA) and Image Quant LAS 4000 mini imaging system (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

4.6 Enzyme-linked immunosorbent assay (ELISA) (I,III,IV)

Culture medium samples were stored at -20 °C until assayed. Concentrations of IL-6, MMP-1, MMP-3, MMP-13, PGE₂ and FGF-2 in medium samples were determined by ELISA with commercial reagents (PGE₂: Cayman Chemical Co., Ann Arbor, MI, USA; human IL-6: eBioscience Inc. San Diego, CA, USA; MMP-1, MMP-3, MMP-13, FGF-2 and mouse IL-6: R&D Systems Europe Ltd).

4.7 Fluo-3-AM measurements (I,II)

TRPA1 mediated Ca²⁺ influx was measured in HEK 293 cells transfected with human TRPA1 plasmid, in human T/C28a2 chondrocytes and in primary human OA chondrocytes. The cells were loaded with 4 μM fluo-3-acetoxymethyl ester (Fluo-3-AM, Sigma-Aldrich) and 0.08 % Pluronic F-127® (Sigma-Aldrich) in Hanks’ balanced salt solution (HBSS, Lonza) containing 1 mg/ml of bovine serum albumin, 2.5 mM probenecid and 25 mM HEPES pH 7.2 (all from Sigma-Aldrich) for 30 min at room temperature. The intracellular free Ca²⁺ levels were
assessed by Victor3 1420 multilabel counter (Perkin Elmer, Waltham, MA, USA) at excitation/emission wavelengths of 485/535 nm. In the experiments, the cells were first pre-incubated with the TRPA1 antagonist HC-030031 (100 μM, Sigma-Aldrich) or the vehicle for 30 min at +37 °C. Thereafter, the TRPA1 agonist allyl isothiocyanate (AITC, 50 μM, Sigma-Aldrich) was added and the measurements were continued for 30 s after which a robust Ca^{2+} influx was induced by application of the control ionophore compound ionomycin (1 μM, Sigma-Aldrich).

4.8 Next-Generation RNA-sequencing (NGS) (III)

Samples for next-generation RNA sequencing were prepared by pooling chondrocytes from 11 TRPA1 KO mice and 12 corresponding WT mice. Five samples obtained from the pool of TRPA1 KO mice and four samples from the pool of WT mice were sequenced. Sequencing of the samples was performed at the Finnish Institute of Molecular Medicine (FIMM) sequencing core, Helsinki, Finland, using the Illumina HiSeq 2500 sequencing platform. Sequencing depth was 20 million paired-end reads 100 bp in length. Initial analysis of the data was performed in the FIMM sequencing core (up to the preparation of the count matrices). Read quality was first assessed using FastQC (Andrews 2010), and the reads were trimmed using Trimmomatic (Bolger and Giorgi 2014). Trimmed reads were aligned to reference mouse genome with STAR (Dobin et al. 2013). Count matrices were prepared with the featureCounts program (Liao et al. 2014). Gene expression levels were calculated as Reads per Kilobase per Million (RPKM) (Mortazavi et al. 2008). Differential gene expression was assessed with DESeq2 (Love et al. 2014) using the Chipster software (Chipster software, CSC - IT Center for Science, Espoo, Finland). For the purposes of further analysis, genes with a minimum of 2.0 fold change (FC) and False Discovery Rate (FDR)-corrected p-value < 0.05 were deemed biologically and statistically significant. Functional analysis was performed using the DAVID database (Huang da et al. 2009).
Data were analyzed using SPSS version 20.0 for Windows software (SPSS Inc, Chicago, IL, USA) and Graph-Pad InStat version 3.00 software (GraphPad Software, San Diego, CA, USA). The results are presented as mean ± SEM unless otherwise indicated.

Unpaired t-test, paired t-test, ordinary or repeated measures one-way analysis of variance (ANOVA) followed by Dunnett or Bonferroni correction for multiple comparisons were used in the statistical analysis as indicated. Differences were considered significant at * = p < 0.05, ** = p < 0.01 and *** = p < 0.001.

In study IV, the data used for correlation studies were natural logarithm (LN) transformed in order to obtain a normal distribution. Pearson’s correlation analysis was carried out, with r values over +0.3 and under -0.3 considered to indicate a correlation.
5 SUMMARY OF THE RESULTS

5.1 TRPA1 is functionally expressed in human osteoarthritic chondrocytes (I)

5.1.1 TRPA1 mRNA is expressed in human chondrocytes

The first step of this study was to examine if TRPA1 mRNA is expressed in human chondrocytes. By stimulating primary human osteoarthritic chondrocytes and immortalized human T/C28a2 chondrocytes with the inflammatory cytokine IL-1β, we were able to show that TRPA1 mRNA is indeed expressed in both of these cell types. The TRPA1 mRNA expression was found to peak at 48h in primary human OA chondrocytes, and at 6h in human T/C28a2 chondrocytes after adding IL-1β, and to decline thereafter (Figure 5).
Figure 5. Primary human OA chondrocytes (A) and human T/C28a2 chondrocyte cell line (B) express TRPA1 mRNA and its expression is enhanced by IL-1 in a time-dependent manner. Cultures of primary human OA chondrocytes (A) and human T/C28a2 chondrocytes (B) were stimulated with IL-1β (100 pg/ml) for 0–96 h and 0–24 h, respectively, and thereafter total RNA was extracted. TRPA1 mRNA levels were measured by RT-qPCR, and the results were normalized against GAPDH mRNA. The mRNA levels are expressed as arbitrary units with the levels measured at 24 h (A; primary OA chondrocytes) or 6 h (B; T/C28a2 chondrocytes) set as 100%; and the values at the other time points are related to those values. Primary chondrocyte samples were obtained from three to five different donors and the experiments were carried out in duplicate. Human T/C28a2 chondrocyte experiments were carried out in quadruplicate. Results are expressed as mean ± SEM. One-way ANOVA followed by Dunnett’s post-test was used in the statistical analysis; **indicates p < 0.01 compared to the control (0 h) sample. (Modified from Nummenmaa et al. Arthritis Res Ther. 2016, 18:185)
5.1.2 TRPA1 mRNA expression is increased by OA-related inflammatory factors

We next investigated whether known OA-related inflammatory mediators IL-1β, IL-17, LPS and resistin increased the expression of TRPA1 mRNA in human chondrocytes. Primary human OA chondrocytes were stimulated with increasing doses of IL-1β, IL-17 and resistin, and a single dose of LPS. Human T/C28a2 chondrocytes were treated with increasing doses of IL-1β, and single doses of IL-17 and LPS. All of these factors upregulated the expression of TRPA1 mRNA; IL-1β, IL-17 and resistin in a concentration dependent manner in primary chondrocytes and IL-1β in T/C28a2 chondrocytes (Figure 6).
Figure 6. TRPA1 mRNA expression is increased following stimulation with inflammatory factors IL-1β, IL-17, LPS, and resistin in primary human OA chondrocytes (A-D) and in human T/C28a2 chondrocyte cell line (E). Isolated primary human OA chondrocytes were stimulated with IL-1β (1–100 pg/ml) (A), IL-17 (10–100 ng/ml) (B), LPS (10 ng/ml) (C) and resistin (0.3–3 μg/ml) (D); and human T/C28a2 chondrocytes with IL-1β (1–100 pg/ml), IL-17 (100 ng/ml) and LPS (10 ng/ml) (E) for 24 h; and thereafter total RNA was extracted. TRPA1 mRNA levels...
were measured by RT-qPCR, and the results were normalized against GAPDH mRNA levels. The results are expressed as a percentage in comparison to untreated control samples, which were set as 100%. Primary chondrocyte samples were obtained from four different donors and the experiments were performed in duplicate. Human T/C28a2 chondrocyte experiments were carried out in quadruplicate. Results are expressed as mean ± SEM. Repeated measures ANOVA (A, B, D) and one-way ANOVA (E) followed by Dunnett’s post-test or paired t test (C) was used in the statistical analysis; *p < 0.05 and **p < 0.01, compared to the untreated control samples. (Modified from Nummenmaa et al. Arthritis Res Ther. 2016, 18:185)

5.1.3 Human chondrocytes express a functional TRPA1 channel

After determining the expression of TRPA1 mRNA in human chondrocytes, we set out to show that the mRNA is translated into protein. We stimulated primary human OA chondrocytes and immortalized human T/C28a2 chondrocytes with IL-1β, extracted total protein, and performed Western Blot analysis. Remarkably, we were able to show the production of TRPA1 protein in primary human OA chondrocytes as well as immortalized T/C28a2 chondrocytes (Figure 7). HEK 293 cells transiently transfected with hTRPA1 plasmid were used as positive control cells in the Western Blot analysis.

In order to show that TRPA1 proteins produced by primary human OA chondrocytes and immortalized T/C28a2 chondrocytes form a functional TRPA1 channel, Ca^{2+} measurements were performed. The activation of the TRPA1 channel causes a substantial influx of Ca^{2+} into the activated cell (Nilius 2007). Therefore, to establish the presence of a functional TRPA1 channel, we stimulated the chondrocytes with the selective TRPA1 agonist AITC and measured the change in intracellular calcium with the Fluo-3-AM assay. We found that AITC stimulation elicited an increase in intracellular calcium. Furthermore, it was seen that treatment with the selective TRPA1 antagonist HC-030031 prevented this reaction. HEK 293 cells transiently transfected with hTRPA1 plasmid were used as positive control cells (Figure 8).
Figure 7. **TRPA1 protein is expressed in primary human OA chondrocytes and human T/C28a2 chondrocyte cell line.** Chondrocyte cultures were stimulated with IL-1β (100 pg/ml) for 24 h. Extracted proteins were immunoprecipitated and TRPA1 was detected with Western blot analysis. HEK 293 cells transiently transfected with human TRPA1 plasmid were used as a positive control. Representative blot of three independent experiments with similar results. (Reprinted with permission from Nummenmaa et al. Arthritis Res Ther. 2016, 18:185)
Figure 8. The TRPA1 ion channel is functional in primary human OA chondrocytes (A, B) and human T/C28a2 chondrocyte cell line (C) as shown by TRPA1-mediated Ca\textsuperscript{2+} influx. Primary human chondrocytes (A, B) and human T/C28a2 chondrocytes (C) were cultured with or without IL-1β (100 pg/ml) for 24 h. HEK 293 cells transfected with a plasmid encoding human TRPA1 were used as positive control cells (D). The cells were loaded with Fluo-3-AM and the TRPA1-mediated Ca\textsuperscript{2+} influx was measured by Victor3 multilabel counter at excitation/emission wavelengths of 485/535 nm at 1/s frequency. The cells were first preincubated with the TRPA1 antagonist HC-030031 (100 μM) or the vehicle for 30 min at +37 °C. In the measurements, basal fluorescence was first recorded for 15 s and thereafter the selective TRPA1 agonist allyl isothiocyanate (AITC; 50 μM) was added and the measurement was continued for 30 s after which the control ionophore compound ionomycin (1 μM) was introduced to the cells. IL-1β stimulation resulted in an elevation in AITC-induced Ca\textsuperscript{2+} influx compared to unstimulated control cells, and it was attenuated by the selective TRPA1 antagonist HC-030031. The results were normalized against the background and expressed as mean of eight simultaneous measurements. Curves in A, C and D express results from one representative experiment of four independent experiments with similar results. In (B) area under the curve (AUC) from 15 to 45 s was calculated from measurements of primary chondrocytes from four donors (each with eight repeats). Results are expressed as mean ± SEM.
Repeated measures ANOVA followed by Dunnett’s post-test was used in the statistical analysis; *p < 0.01 compared to the IL-1β-treated samples. (Modified from Nummenmaa et al. Arthritis Res Ther. 2016, 18:185)

5.2 TRPA1 expression is downregulated by dexamethasone and aurothiomalate in human chondrocytes (II)

Since TRPA1 is an inducible channel, we wanted to investigate whether clinically used drugs affect the expression of TRPA1. We tested the effects of disease modifying antirheumatic drugs (DMARDs) methotrexate, sulfasalazine, hydroxychloroquine and aurothiomalate, the glucocorticoid dexamethasone and the non-steroidal anti-inflammatory drug ibuprofen on the expression of TRPA1 in human T/C28a2 chondrocytes. Dexamethasone and aurothiomalate were found to inhibit TRPA1 mRNA expression in a dose-dependent manner (Figure 9A), whereas methotrexate, sulfasalazine, hydroxychloroquine and ibuprofen had no effect when tested at a 10 μM concentration. Importantly, dexamethasone and aurothiomalate were also found to inhibit TRPA1 protein expression in these cells (Figure 9C).

We next confirmed that dexamethasone and aurothiomalate inhibited TRPA1-dependent Ca2+ influx, indicating that the formation of a functional TRPA1 channel was also inhibited. Human chondrocytes that were cultured in the presence of IL-1β showed an increase in TRPA1-dependent Ca2+ influx. This effect was prevented in cells, which were treated with dexamethasone or aurothiomalate, confirming the downregulation of a functional TRPA1 channel by these two drugs (Figure 10).

To verify the effects of dexamethasone and aurothiomalate in primary chondrocytes, we stimulated primary human OA chondrocytes with IL-1β in the presence and absence of dexamethasone and aurothiomalate. We discovered that dexamethasone and aurothiomalate also inhibited IL-1β-induced TRPA1 expression in these cells (Figure 9B).
Glucocorticoids and aurothiomalate have previously been shown to inhibit activation of the transcription factor NF-κB (Hartmann et al. 2016; Vuolteenaho et al. 2005). Furthermore, the NF-κB signaling pathway has been proposed to be involved in TRPA1 induction (Hatano et al. 2012). Due to this, we investigated the effect of NF-κB inhibitor PDTC on the expression of TRPA1. Interestingly, also PDTC downregulated TRPA1 expression in primary human OA and immortalized T/C28a2 chondrocytes (Figure 9A-B).

**Figure 9.** Dexamethasone and aurothiomalate inhibit TRPA1 expression in human chondrocytes. Human chondrocytes (primary human chondrocytes or T/C28a2 chondrocyte cell line) were cultured with IL-1β alone or in combination with the anti-inflammatory compounds dexamethasone or aurothiomalate, or with the selective NF-κB inhibitor PDTC at concentrations...
given in the figure. (A, B) For TRPA1 mRNA expression analysis, human T/C28a2 chondrocytes (A) were incubated for 6 hours and the experiments were carried out in quadruplicate; primary chondrocytes (B) were incubated for 24 hours and the experiments were carried out in duplicate and repeated with cells from six different donors. TRPA1 mRNA levels were measured by RT-qPCR, and the results were normalised against GAPDH mRNA levels. (C) For TRPA1 protein analysis human T/C28a2 chondrocytes were incubated for 24 hours after which proteins were extracted and immunoprecipitated. TRPA1 protein was detected with Western blot. The figure shows one representative blot of six independent experiments with similar results. Results are expressed as a percentage compared to IL-1β-treated samples which are set as 100%, and shown as mean + SEM. Ordinary (A, C) or repeated measures one-way ANOVA (B) followed by Bonferroni post-test was used in the statistical analysis, ***p < 0.001 compared to the IL-1β-treated samples. (Modified from Nummenmaa et al. RMD Open 2017, 3(2):e000556)

**Figure 10.** Dexamethasone and aurothiomalate inhibit the functional TRPA1 ion channel. Human T/C28a2 chondrocytes were incubated with IL-1β alone or in combination with dexamethasone or aurothiomalate for 24 hours. Thereafter, the cells were loaded with Fluo-3-AM and TRPA1-mediated Ca²⁺ influx was measured by Victor3 multilabel counter at excitation/emission wavelengths of 485/535 nm at 1/s frequency. In the measurements, basal fluorescence was first recorded for 15 s and thereafter the selective TRPA1 agonist allyl isothiocyanate (AITC; 50 μM) was added and the measurements were continued for 30 s. The results were normalized against the background and expressed as a mean of eight measurements. Results in (A) are expressed as relative fluorescence units, and in (B) AUC from 15 to 45 s was calculated. Results in (B) are expressed as mean + SEM. One-way ANOVA followed by Bonferroni post-test was used in the statistical analysis, ***p < 0.001 compared to the IL-1β-treated samples. (Modified from Nummenmaa et al. RMD Open 2017, 3(2):e000556)
5.3 Effects of TRPA1 in chondrocytes and cartilage (I,III)

Not only was the expression of TRPA1 in chondrocytes and cartilage shown for the first time in this study, but it was also the first time for the functions of TRPA1 in this tissue to be examined. The effects of TRPA1 on the expression of OA-related inflammatory and catabolic factors in chondrocytes and cartilage were investigated by using pharmacological inhibitors, as well as genetic deletion of TRPA1.

5.3.1 TRPA1 mediates IL-6 expression in human OA chondrocytes, and murine cartilage and chondrocytes

We examined the effect of TRPA1 on the OA-related cytokine IL-6 by using cartilage and chondrocytes from TRPA1 KO and corresponding WT mice, as well as primary human OA chondrocytes. In next-generation RNA sequencing (RNA-Seq) analysis, IL-6 was found to be highly expressed in IL-1β-treated chondrocytes from WT mice, with the expression level being significantly lower in chondrocytes from TRPA1 KO mice (Table 4). Due to this finding, we examined the RNA-seq data for other members of the IL-6 cytokine family and found that LIF and IL-11 were also significantly downregulated in chondrocytes from TRPA1 KO mice (Table 4).
Table 4. Members of IL-6 cytokine family with attenuated expression in chondrocytes from TRPA1 KO mice compared to cells from corresponding WT mice. Next-generation RNA sequencing was performed on IL-1β-treated chondrocytes obtained from TRPA1 deficient (knockout, KO) and corresponding wild-type (WT) mice. Mean expression is given as reads per kilobase per million (RPKM). Ratios of gene expression levels are given as fold change (FC) values determined with DESeq2, with negative values denoting downregulated genes in chondrocytes from TRPA1 KO mice. RNA-seq results were verified by qRT-PCR, where p-values are given as Bonferroni-adjusted.

<table>
<thead>
<tr>
<th>Gene</th>
<th>RPKM (WT)</th>
<th>RPKM (TRPA1 KO)</th>
<th>FC</th>
<th>Adj. p-value</th>
<th>FC</th>
<th>Adj. p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>202.37</td>
<td>48.26</td>
<td>-3.86</td>
<td>&lt; 0.0001</td>
<td>-8.93</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Lif</td>
<td>12.03</td>
<td>5.19</td>
<td>-2.16</td>
<td>&lt; 0.0001</td>
<td>-3.95</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>IL-11</td>
<td>1.11</td>
<td>0.21</td>
<td>-4.14</td>
<td>&lt; 0.0001</td>
<td>-4.56</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Next, we verified the results from the RNA-Seq data with qRT-PCR (Table 4) and examined the findings further with a larger population of chondrocytes from TRPA1 KO and corresponding WT mice. Chondrocytes from both genotypes were treated with IL-1β alone, and chondrocytes from WT mice were additionally treated with IL-1β together with two selective TRPA1 antagonists: HC-030031 and TCS 5861528. IL-1β upregulated the expression of IL-6 in both genotypes, however this response was significantly attenuated in chondrocytes from TRPA1 KO mice. Further, the TRPA1 antagonists HC-030031 and TCS 5861528 significantly inhibited the IL-1β-enhanced expression of IL-6 in WT chondrocytes (Figure 11A). The expression of IL-11 was also upregulated by IL-1β and attenuated by the TRPA1 antagonists HC-030031 and TCS 5861528, in chondrocytes from WT mice. IL-1β had no effect on IL-11 expression in chondrocytes from TRPA1 KO mice (Figure 11B).

The effect of TRPA1 on the production of IL-6 was also examined by using articular cartilage explants from TRPA1 KO and corresponding WT mice. IL-1β treatment upregulated IL-6 production in cartilage from WT mice. This response
was attenuated in cartilage from TRPA1 KO mice, where IL-1β did not cause a significant increase in the production of IL-6 (Figure 12).

**Figure 11.** IL-1β-induced IL-6 (A) and IL-11 (B) expression is attenuated by genetic deletion and pharmacological inhibition of TRPA1 in murine chondrocytes. Chondrocytes were obtained from TRPA1 deficient (knockout, KO) mice and corresponding wild-type (WT) mice. The chondrocytes were cultured with IL-1β (100 pg/ml) alone, or together with the selective TRPA1 antagonists HC-030031 (100 μM) or TCS 5861528 (100 μM) for 24 h and thereafter total RNA was extracted. IL-6 and IL-11 mRNA levels were measured with qRT-PCR and normalized against GAPDH mRNA levels. The results are expressed as fold change in comparison to the control samples of each genotype. WT n = 13 (TCS treatment n = 10), KO n = 15. Results are expressed as mean ± SEM. One-way ANOVA followed by Bonferroni post-test was used in the statistical analysis; *** p < 0.001 compared to the IL-1β-treated WT samples.
Figure 12. IL-1β-induced production of IL-6 in cartilage is attenuated by genetic deletion of TRPA1. Cartilage samples were obtained from TRPA1 deficient (knockout, KO) mice and corresponding wild-type (WT) mice. The cartilage pieces were cultured in the presence of IL-1β (100 pg/ml) or without stimulation for 42 h, after which the culture medium was collected and IL-6 was measured by immunoassay. The results are expressed as mean ± SEM, n = 6. One-way ANOVA followed by Bonferroni post-test was used in the statistical analysis; ** p < 0.01, *** p < 0.001.

Finally, we wanted to verify that the results seen in mouse cartilage and chondrocytes are also found in human chondrocytes. Primary human OA chondrocytes were treated with IL-1β and the selective TRPA1 antagonist HC-030031. The results showed that the TRPA1 antagonist attenuated the expression of IL-6, LIF and IL-11 in these cells, suggesting that TRPA1 is involved in the expression of these factors also in human OA cartilage (Figure 13).
Figure 13. IL-1β-enhanced expression of IL-6 (A,B), LIF (C) and IL-11 (D) in primary human OA chondrocytes is attenuated by pharmacological inhibition of TRPA1. Cultures of primary human OA chondrocytes were stimulated with IL-1β (100 pg/ml) in the presence and absence of selective TRPA1 antagonist HC-030031 (100 μM) for 24 h, after which the medium was collected, and total mRNA was extracted. IL-6 was measured from the cell culture medium with immunoassay (B), and IL-6, Lif and IL-11 mRNA was measured by qRT-PCR and normalized against GAPDH mRNA levels (A, C-D). Results are expressed as a percentage compared to IL-1β-treated samples, which are set as 100 % (A, C-D) and shown as mean + SEM. Samples were obtained from seven different donors and the experiments were carried out in duplicate. Paired t-test was used in the statistical analysis; *p < 0.05 and ** p < 0.01 compared to IL-1β-treated samples.

5.3.2 FGF-2 expression is attenuated by pharmacological inhibition and genetic deletion of TRPA1 in human OA and murine chondrocytes

FGF-2 is an intriguing inflammatory and catabolic factor that is found at elevated concentrations in the synovial fluid of OA patients (Ellman et al. 2013). We set out to investigate whether TRPA1 is involved in the regulation of FGF-2 expression. By utilizing next-generation RNA sequencing analysis, we found that
the expression of FGF-2 was attenuated in IL-1β-treated chondrocytes from TRPA1 KO mice compared to corresponding WT mice (RPKM 9.74 and 31.48, respectively). These results were verified with qRT-PCR using a larger set of analogous samples. Treatment with IL-1β resulted in the upregulation of FGF-2 expression in chondrocytes from WT mice, but not in chondrocytes from TRPA1 KO mice (Figure 14).

The results were then further investigated with primary human OA chondrocytes by stimulating the cells with IL-1β alone and together with the selective TRPA1 antagonist HC-030031. In these cells, IL-1β treatment increased the expression of FGF-2, and this effect was significantly attenuated by the TRPA1 antagonist HC-030031 (Figure 15). Together these results suggest that TRPA1 is involved in the regulation of FGF-2 expression in chondrocytes.

Figure 14. IL-1β-enhanced expression of FGF-2 is attenuated by genetic deletion of TRPA1 in mouse chondrocytes. Chondrocytes were obtained from TRPA1 deficient (knockout, KO) mice and corresponding wild-type (WT) mice. The chondrocytes were cultured without stimulation or with IL-1β (100 pg/ml) for 24 h and thereafter total RNA was extracted. FGF-2 mRNA levels were measured with qRT-PCR and normalized against GAPDH mRNA levels. The results are expressed as fold change in comparison to the control samples of each genotype. WT n = 13-14, KO n = 16. Results are expressed as a percentage compared to IL-1β-treated samples which are set as 100 %, and shown as mean ± SEM. One-way ANOVA followed by Bonferroni post-test was used in the statistical analysis; *** p < 0.001 compared to the IL-1β-treated WT samples.
Figure 15. IL-1β-enhanced expression of FGF-2 is attenuated by pharmacological inhibition of TRPA1 in primary human OA chondrocytes. Cultures of primary human OA chondrocytes were stimulated with IL-1β (100 pg/ml) in the presence and absence of the selective TRPA1 antagonist HC-030031 (100 μM) for 24 h, after which total mRNA was extracted. FGF-2 mRNA was measured by qRT-PCR and normalized against GAPDH mRNA levels. Results are expressed as a percentage compared to IL-1β-treated samples, which are set as 100 % and shown as mean + SEM. Samples were obtained from nine different donors and the experiments were carried out in duplicate or quadruplicate. Paired t-test was used in the statistical analysis; *p < 0.05 compared to IL-1β-treated samples.

5.3.3 MMP-enzyme and PGE2 production is attenuated by genetic deletion and pharmacological inhibition of TRPA1

MMP-enzymes are considered key mediators of cartilage ECM degradation in OA (Glyn-Jones et al. 2015; Troeberg and Nagase 2012). In addition, the prostaglandin E2 is an important mediator of pain in arthritic conditions (Lee, AS. et al. 2013). Due to the important roles these factors play in OA, we aimed to investigate whether TRPA1 affects their production. This was examined by utilizing cartilage explants from TRPA1 KO and corresponding WT mice as well as pharmacological inhibition of TRPA1 in primary human OA chondrocytes.

We treated cartilage explants from TRPA1 KO and corresponding WT mice with IL-1β, which expectedly upregulated the production of MMP-3 and PGE2. Remarkably, this effect was significantly attenuated in the cartilage explants from TRPA1 KO mice as compared to the corresponding WT mice (Figure 16). We
then further investigated these findings in primary human OA chondrocytes by stimulating the cells with IL-1β alone and together with the selective TRPA1 antagonist HC-030031. We found that IL-1β-enhanced production of MMP-1, MMP-3, MMP-13 and PGE₂ was attenuated with HC-030031 in these cells (Figure 17). The results suggest that TRPA1 has a role in the upregulation of these catabolic factors in OA cartilage.

Figure 16. **IL-1β-induced production of MMP-3** (A) and **PGE₂** (B) in the cartilage is attenuated by **genetic deletion of TRPA1**. Cartilage samples were obtained from TRPA1-deficient (knockout, KO) and corresponding wild-type (WT) mice. The samples were cultured with and without IL-1β (100 pg/ml) for 42 h and thereafter the culture medium was collected and analyzed for concentrations of MMP-3 and PGE₂ by ELISA. The results are expressed as a percentage compared to untreated control samples, which are set as 100 % and shown as mean + SEM, n = 6–9. Unpaired t-test was used in the statistical analysis; *p < 0.05, **p < 0.01, and ***p < 0.001 compared to the WT mice. (Modified from Nummenmaa et al. Arthritis Res Ther. 2016, 18:185)
Figure 17. IL-1β-enhanced expression of MMP-1 (A), MMP-3 (B), MMP-13 (C) and PGE₂ (D) in primary human OA chondrocytes is attenuated by pharmacological inhibition of TRPA1.

Cultures of primary human OA chondrocytes were stimulated with IL-1β (100 pg/ml) in the presence and absence of the selective TRPA1 antagonist HC-030031 (100 μM) for 24 h. MMP-1, MMP-3, MMP-13, and PGE₂ concentrations in the culture media were measured by ELISA and the results are expressed as mean ± SEM. Samples were obtained from eight patients and the experiments were carried out in duplicate. Paired t-test was used in the statistical analysis; *p < 0.05, **p < 0.01, and ***p < 0.001 compared to the IL-1β-treated samples. (Modified from Nummenmaa et al. Arthritis Res Ther. 2016, 18:185)
5.4 Effects of FGF-2 and FGF receptor antagonists in human OA cartilage and chondrocytes (IV)

During this thesis, we observed that TRPA1 is involved in regulating the expression of FGF-2 in chondrocytes. Due to this result and the intriguing roles fibroblast growth factors have been proposed to play in chondrocyte metabolism (Ellman et al. 2013), we wanted to investigate the effects of FGF-2 and commercial FGF receptor antagonists in OA cartilage and chondrocytes.

5.4.1 FGF-2 correlates with MMP-enzymes in human OA cartilage explants

The correlations between FGF-2 and catabolic MMP-enzymes were investigated using cartilage samples obtained from 97 OA patients undergoing knee replacement surgery. The concentrations of FGF-2, MMP-1 and MMP-13 released from the cartilage explants into the culture media during a 42 h incubation period were measured. OA cartilage was found to release all of the measured factors: FGF-2 28.4 ± 1.5 pg/100 mg cartilage, MMP-1 12.7 ± 1.4 ng/100 mg cartilage, and MMP-13 1.0 ± 0.1 ng/100 mg cartilage. Notably, the levels of FGF-2 correlated positively with the levels of both MMP-1 (r = 0.414, p < 0.001) and MMP-13 (r = 0.362, p < 0.001) (Figure 18).
Figure 18. Levels of FGF-2 released by human OA cartilage correlate positively with the levels of (A) MMP-1 and (B) MMP-13. Cartilage explants from OA patients (n = 97) undergoing joint replacement surgery were incubated for 42 h and the levels of FGF-2, MMP-1, and MMP-13 released into the culture medium were measured by immunoassay. Results were natural log (LN) transformed to obtain a normal distribution and analyzed using Pearson correlation. (Reprinted with permission from Nummenmaa et al. Scand J Rheumatol. 2015, 44(4):321)
5.4.2 FGF-2 stimulation exerts catabolic and anti-anabolic responses in human OA chondrocytes

FGF-2 has been shown to signal through four tyrosine kinase FGF receptors: FGFR1-4 (Ornitz and Itoh 2015). We wanted to confirm that the human OA chondrocytes express these receptors and found that the chondrocytes expressed all four receptors, with the expression of the catabolism-associated receptor FGFR1 being the highest and FGFR4 being extremely low (Figure 19).

![Primary human OA chondrocytes](image)

**Figure 19. FGF receptor expression profiles of primary human OA chondrocytes.** Chondrocytes were cultured for 24 h and the mRNA expression of FGFR1–4 was detected by qRT-PCR. The results from three patients were combined. Results are expressed as a percentage compared to FGFR1, which is set as 100% and are shown as mean ± SEM. (Modified from Nummenmaa et al. Scand J Rheumatol. 2015, 44(4):321)

Primary human OA chondrocytes were then cultured in the presence and absence of FGF-2 for 24h and the expression of catabolic MMP-enzymes as well as cartilage matrix components aggrecan and collagen II were determined. FGF-2 increased the production of the cartilage matrix degrading enzymes MMP-1 and MMP-13. Conversely, the expression of aggrecan and collagen II were decreased by FGF-2 stimulation compared to untreated control cells (Figure 20).
Figure 20. FGF-2 enhances the production of MMP-1 (A, B) and MMP-13 (C, D) and down-regulates the expression of aggrecan (E) and collagen II (F) in primary human OA chondrocytes. Chondrocyte cultures were stimulated with FGF-2 (200 ng/ml) for 24 h. MMP, aggrecan and collagen II mRNA expression was detected by qRT-PCR, and MMP production was measured by immunoassay. The mRNA expression levels were normalized against GAPDH mRNA levels. The results from nine patients were combined for protein production analysis (B, D) and from 10 patients for mRNA expression analysis (A, C, E, F). Results are expressed as a percentage compared to untreated control samples, which are set as 100% and shown as mean ± SEM. Paired t-test was used in the statistical analysis, *p < 0.05, **p < 0.01, and ***p < 0.001 compared to untreated control samples. (Modified from Nummenmaa et al. Scand J Rheumatol. 2015, 44(4):321)
5.4.3 FGF-2 receptor antagonists induce anabolic and anti-catabolic responses in human OA chondrocytes

Primary human OA chondrocytes were treated with increasing concentrations of the FGF-2 receptor antagonists AZD4547 and NVP-BGJ398 in the presence and absence of FGF-2. Both antagonists inhibited the mRNA and protein production of MMP-1 and MMP-13 in a dose-dependent manner. This effect was seen with the exogenous FGF-2 stimulation, and surprisingly, also without FGF-2 stimulation (Figures 21-22). Due to the results seen without exogenous FGF-2 treatment, we investigated whether the primary human OA chondrocytes produced FGF-2. We found that the cells did indeed produce FGF-2 at a level of 1.8 ± 1.0 ng/10^6 cells (n = 5).

Figure 21. FGF-2 antagonists AZD4547 and NVP-BGJ398 downregulate the expression of MMP-1 (A, B) and MMP-13 (C, D) in the presence and absence of exogenous FGF-2. Isolated primary human OA chondrocytes were treated with increasing concentrations of AZD4547 and BGJ398 (1–300 nM) in the presence and absence of exogenous FGF-2 (200 ng/ml) for 24 h. MMP-1 and
MMP-13 mRNA levels were measured by qRT-PCR, and the results were normalized against GAPDH mRNA levels. The results from two or three patients were combined. The results are expressed as a percentage in comparison to untreated control samples, which are set as 100% and shown as mean ± SEM. Repeated-measures ANOVA with Bonferroni’s post-test was used in the statistical analysis, *p < 0.05, **p < 0.01, and ***p < 0.001 compared to untreated control or FGF-2 treated samples. (Modified from Nummenmaa et al. Scand J Rheumatol. 2015, 44(4):321)

**Primary human OA chondrocytes**

Figure 22. FGF-2 antagonists AZD4547 and NVP-BGJ398 down-regulated the production of MMP-1 (A, B) and MMP-13 (C, D) in the presence and absence of exogenous FGF-2. Isolated primary human OA chondrocytes were treated with increasing concentrations of AZD4547 and BGJ398 (1–300 nM) in the presence and absence of exogenous FGF-2 (200 ng/ml) for 24 h. MMP-1 and MMP-13 protein levels in the culture media were measured by immunoassay. The results from two or three patients were combined. The results are expressed as a percentage in comparison to untreated control samples, which are set as 100% and shown as mean ± SEM. Repeated-measures ANOVA with Bonferroni’s post-test was used in the statistical analysis, *p <0.05, **p <0.01, and ***p < 0.001 compared to untreated control or FGF-2 treated samples. (Modified from Nummenmaa et al. Scand J Rheumatol. 2015, 44(4):321)
We also examined the effects of the FGF-2 receptor antagonists AZD4547 and NVP-BGJ398 on the major cartilage matrix components aggrecan and collagen II. Both compounds dose-dependently increased the expression of aggrecan and collagen II in the presence and absence of FGF-2 stimulation (Figure 23). Taken together, these FGF receptor antagonists showed a beneficial effect on the balance between catabolic and anabolic factors in cartilage by shifting the balance towards anabolism.

Figure 23. FGF-2 antagonists AZD4547 and NVP-BGJ398 up-regulated the expression of aggrecan (A, B) and collagen II (C, D) in the presence and absence of exogenous FGF-2. Isolated primary human OA chondrocytes were treated with increasing concentrations of AZD4547 and BGJ398 (1–300 nM) in the presence and absence of exogenous FGF-2 (200 ng/ml) for 24 h. Aggrecan and collagen II mRNA levels were measured by qRT-PCR, and the results were normalized against GAPDH mRNA levels. The results from three patients were combined. The results are expressed as a percentage in comparison to untreated control samples, which are set as 100 % and shown as mean + SEM. Repeated-measures ANOVA with Bonferroni’s post-test was used in the statistical analysis, *p < 0.05, **p < 0.01, and ***p < 0.001 compared to untreated control or FGF-2 treated samples. (Modified from Nummenmaa et al. Scand J Rheumatol. 2015, 44(4):321)
6 DISCUSSION

6.1 Methodology

At commencement of this study, the expression and function of the TRPA1 cation channel had been widely studied in neurons, but its expression and functions in nonneuronal cells were less understood. The aim of the present study was to examine the possible expression of TRPA1 in primary human OA chondrocytes and to investigate its functions in OA cartilage. In the experiments, cartilage explants and primary chondrocytes isolated from leftover pieces of OA cartilage from knee-joint replacement surgery were used, along with an immortalized human chondrocyte cell line. In addition, cartilage tissue and primary chondrocytes obtained from TRPA1 deficient (knock-out, KO) and corresponding wild type (WT) mice were used.

All the primary human chondrocytes and cartilage explants used in this study were obtained from OA patients. This is advantageous, as the metabolism and gene expression profiles of OA chondrocytes are altered compared to healthy cells. Therefore, studying them gives a more accurate depiction of the responses seen in cartilage during osteoarthritis. It would be valuable to compare non-osteoarthritic primary chondrocytes and cartilage with osteoarthritic ones; however, the availability of such human tissue is extremely limited.

Many of the experiments in this study were performed with cultures of primary chondrocytes. The use of primary cells is beneficial as they better resemble the in vivo situation compared to immortalized cells. Cultured primary cells may undergo transcriptional alterations and phenotypic changes during culture. Due to this, primary chondrocytes in this study were cultured for short periods and never passaged during experiments, as a long culture period and passaging of chondrocytes have been shown to increase dedifferentiation (Cope et al. 2019;
Darling and Athanasiou 2005; Salvat et al. 2005). Other disadvantages include the relatively small number of cells obtained from each donor, limiting the size of individual experiments, and the heterogeneity of samples due to different disease stages and phenotypes, and patient-related variables. To overcome the limitations of primary cultures, an immortalized human chondrocyte cell line T/C28a2 (Goldring et al. 1994) was used. Immortalized cell lines have several advantages, such as the abundant availability of cells for each experiment, the uniformity of the cells, and the stability of the cell line for multiple passages. A notable disadvantage is that immortalization often leads to some phenotypic changes and loss of typical chondrocyte characteristics. In addition to the cell culture methods used in this study, the induced pluripotent stem cell (iPSC) technology offers a potential new tool for OA research. With iPSC technology, adult somatic cells can be reprogrammed into self-renewing induced pluripotent stem cells, which can then be differentiated into chondrocyte-like cells. This is an interesting possibility, as it provides an abundant source of cells and can be utilized to investigate hereditary risk genes when using patient-derived iPSCs. However, the current techniques to differentiate iPSCs to chondrocyte-like cells are still suboptimal, and research to improve them is ongoing. In addition, the iPSC-derived chondrocytes do not resemble osteoarthritic chondrocytes in all aspects (Murphy et al. 2017; Suchorska et al. 2017).

In addition to chondrocytes, cartilage tissue explants were also used. This method has the advantage of chondrocytes remaining in their natural micro-environment; in contact with their extracellular matrix in a three-dimensional setting, with the cells remaining non-dividing. This experimental setup better resembles the natural in vivo environment and maintains the typical chondrocyte phenotype. However, it is harder to control the differences between each sample, as the number of cells varies between tissue pieces. Also, cell death occurs at the cut tissue edges, and physical attributes of cartilage may change during culture. Additional complexities with using cartilage explants include limited penetration of stimulants and drugs into the cartilage, difficulty of RNA extraction and small number of cells per sample (Cope et al. 2019). As discussed in the review of literature, chondrocytes residing in different layers and locations in cartilage have slightly different attributes. In order to obtain representative samples with all
types of chondrocytes present, multiple full thickness cartilage pieces (i.e. from the cartilage surface to the bone edge) from numerous parts of the joint were used.

In the present study, multiple models (cartilage explants, primary cells and immortalized cell lines) were used in parallel, and they produced consistent and mutually confirmative results.

Cartilage and chondrocytes from TRPA1 KO and corresponding WT mice were used in this thesis in addition to human samples. Commercially available B6;129P-Trpa1^{tm1Kykw/J} mice and their corresponding WT mice were obtained from Charles River laboratories. These KO mice have been developed by deleting the pore-forming region of TRPA1. Essentially, the exons encoding for S5 and S6 transmembrane domains as well as the pore loop of mouse TRPA1 were disrupted. This may still result in the production of a truncated form of TRPA1, and to avoid any unwanted effects, an endoplasmic reticulum retention signal and stop codon were inserted in frame with the altered exons (Kwan et al. 2006).

The mRNA and protein levels of the genes of interest were investigated with standard methods of molecular and cell biology. Quantitative reverse transcription PCR (qRT-PCR) was used for the detection of mRNA, and Western blot and ELISA were used for protein measurements. In addition, next-generation RNA sequencing (RNA-seq) was used for genome-wide expression analysis comparing chondrocytes from TRPA1 KO and corresponding WT mice. RNA-seq is a widely used method to study the transcriptome, i.e. the set of mRNA transcripts expressed by a cell at a given time. RNA-seq is considered very reliable, and in this study, the expression of the genes of interest were also verified with qRT-PCR. A limitation of RNA-seq is its high cost, due to which only a small amount of samples can be analyzed for a given experiment. In this study, the findings from the RNA-seq data have been verified with a larger set of analogous samples with qRT-PCR and by examining the observed effects in further cartilage and chondrocyte culture experiments.

The functionality of the TRPA1 channel in chondrocytes was assessed by performing the Fluo-3-AM assay, which is a widely used method to assess changes in intracellular calcium concentration. HEK 293 cells transiently
transfected with a hTRPA1 plasmid were used as positive controls and nontransfected HEK 293 cells as negative controls in the experiments. During the experiments, the cells were exposed to the potent TRPA1 agonist AITC to selectively activate TRPA1 channels. AITC treatment induced a potent Ca\textsuperscript{2+} influx in the TRPA1 expressing cells but not in the nontransfected negative controls, thus indicating that the AITC-mediated Ca\textsuperscript{2+} influx was specific for TRPA1. This was further validated by the result that pre-treatment with HC-030031, a widely used selective TRPA1 antagonist (Eid et al. 2008), prevented AITC-induced Ca\textsuperscript{2+} influx.

The present study focused on the presence of TRPA1 in cartilage/chondrocytes and their potential roles in the pathogenesis of osteoarthritis. Tissue and chondrocytes from OA patients were used, and in addition to this, an OA-like environment was mimicked by treating cultures with the pro-inflammatory cytokine IL-1\textbeta. IL-1\textbeta is found in OA joints and is considered to be a key cytokine in the pathogenesis of OA. The presence of IL-1\textbeta in culture creates an environment closer to the inflammatory state of OA joints and is a widely used \textit{in vitro} model to investigate the pathogenesis of OA. The concentration used in experiments (100 pg/ml) is similar to the levels that have been measured in the synovial fluid of OA patients (Tsuchida et al. 2014; Westacott et al. 1990).

6.2 TRPA1 expression in human chondrocytes

A major finding of this study was that the TRPA1 cation channel is functionally expressed in human chondrocytes. This was shown in primary human osteoarthritic chondrocytes as well as in immortalized human chondrocyte cell line T/C28a2. The expression of TRPA1 was shown at the mRNA level with qRT-PCR and at the protein level by Western blot. We also showed the functionality of the channel by measuring TRPA1-mediated Ca\textsuperscript{2+} influx with the Fluo-3-AM assay. The expression and activation of TRPA1 in human chondrocytes was therefore comprehensively shown, to satisfy the criteria set by Fernandes et al. (Fernandes et al. 2012).
TRPA1 was first cloned from lung fibroblasts in 1999 by Jaquemar et al. (Jaquemar et al. 1999). Since then it has primarily been studied in cells of the nervous system, where it is widely expressed by a subpopulation of sensory neurons of the dorsal root ganglia (DRG), trigeminal ganglia (TG) and vagal ganglia (VG) (Nassini et al. 2014). The function of TRPA1 in these cells has also been extensively studied and includes nociception, itch, and neurogenic inflammation (Nassini et al. 2014; Nilius et al. 2012). More recently, the expression of TRPA1 in nonneuronal cells has also started to gain interest. Before the beginning of this thesis, the expression of TRPA1 had been conclusively shown in a very small number of nonneuronal cells, and further studies are needed to elaborate the functional significance of TRPA1 expression in these cells. Nonneuronal cells shown to express the TRPA1 channel include lung epithelial cells, keratinocytes, lung and skin fibroblasts as well as synoviocytes (Atoyan et al. 2009; Hatano et al. 2012; Jaquemar et al. 1999; Kochukov et al. 2006). The present study added primary human OA chondrocytes to the list of nonneuronal cells identified to express the TRPA1 channel.

The expression and functionality of TRPA1 in primary human OA chondrocytes is a major finding that may explain some of the responses seen in osteoarthritic chondrocytes and cartilage. Within the OA joint, there is a hypoxic and inflammatory state, which is characterized by excessive production of reactive species such as ROS and RNS as well as a number of inflammatory factors including IL-1, TNF, IL-6, IL-11, IL-17 and LIF (Franz et al. 2018; Rahmati et al. 2016; Robinson et al. 2016). The expression and activation of TRPA1 has previously been shown to be increased by factors associated with hypoxia and inflammation (Andersson et al. 2008; Hatano et al. 2012; Yoshida et al. 2006), which led us to hypothesize that the environment within the OA joint is conducive to stimulate TRPA1 expression. Therefore, we investigated the effects of multiple OA-related factors on the expression of TRPA1 in chondrocyte cultures and found that IL-1β, IL-17, LPS and the adipocytokine resistin upregulated TRPA1 expression compared to untreated controls. The OA- or hypoxia-related factors IL-6, NGF, YKL-40 (chitinase-3-like protein 1) and CoCl2 (hypoxia-mimetic agent cobalt chloride) showed no or a very small effect on TRPA1 expression (data not shown). The upregulation of TRPA1 by multiple
OA-related and inflammatory factors suggests that the expression of the TRPA1 channel may be upregulated within the joint during OA.

As IL-1β had the strongest effect on TRPA1 mRNA expression, we also investigated its effect on TRPA1 protein production and TRPA1-mediated Ca\(^{2+}\) influx. As a positive control, we used HEK 293 cells, which were transiently transfected with hTRPA1 plasmid to attain a stable and high expression of the channel. We used nontransfected HEK 293 cells as negative controls. The human TRPA1 protein is 128 kDa in size. Using specific TRPA1 antibodies and the Western blot method, we saw a protein band of this size in the primary human OA chondrocytes and immortalized human T/C28a2 chondrocytes, as well as in the TRPA1-transfected HEK 293 cells, but not in the nontransfected HEK 293 cells. Similarly, we observed TRPA1-dependent Ca\(^{2+}\) influx in both types of human chondrocytes, as well as the TRPA1-transfected HEK 293 cells, but not in the nontransfected control cells.

Chondrocytes have also been shown to express other members of the TRP ion channel family. By using PCR analysis, articular chondrocytes from OA patients were shown to express TRPC1, TRPC3, TRPC6, TRPM5, TRPM7, TRPV1 and TRPV2 (Gavenis et al. 2009). In addition, Western blot and immunohistochemical analysis demonstrated that equine articular chondrocytes express TRPV4, TRPV5 and TRPV6 (Hdud et al. 2012).

During the course of this project, additional TRPA1-expressing nonneuronal cells have been identified. In an interesting study by Pereira et al., peripheral blood leukocytes from RA patients were found to express TRPA1. Furthermore, the expression of TRPA1 correlated positively with pain severity and disability (Pereira et al. 2017). In addition, mouse CD4\(^+\) T cells were recently identified to express TRPA1 (Bertin et al. 2017). These results demonstrate the ongoing interest in investigating the involvement of nonneuronal TRPA1 in rheumatic diseases as well as inflammation.
6.3 Effects of dexamethasone and aurothiomalate on TRPA1 expression

We showed in the current study that synthetic glucocorticoid dexamethasone and DMARD aurothiomalate inhibit the production of a functional TRPA1 channel in human chondrocytes. This thesis has therefore produced novel data on the pharmacological regulation of TRPA1 expression, an area that is still largely unknown, especially in nonneuronal cells. Concurrently, this study produced new data on the pharmacological effects of two clinically used anti-inflammatory drugs, dexamethasone and aurothiomalate. These drugs are effective in the treatment of arthritis by retarding cartilage degradation and attenuating the inflammatory reaction.

This is the first study showing that clinically used anti-inflammatory and antirheumatic drugs inhibit the expression of TRPA1 in human chondrocytes, or to my knowledge in any other cell type. There are, however, some previous reports on the effects of anti-inflammatory and antirheumatic drugs on the activation of the TRPA1 channel. A metabolite of the NSAID ibuprofen, namely ibuprofen-acyl glucuronide, was reported to selectively antagonize the human and rodent TRPA1 channel in nociceptors, which may contribute to the anti-inflammatory and analgesic properties of ibuprofen (De Logu et al. 2019). Additionally, the NSAID etodolac, which is a semi-selective COX-2 inhibitor, was reported to selectively activate, and subsequently desensitize TRPA1 channels in TRPA1-expressing HEK 293 cells and murine DRG neurons. Notably, stimulating the TRPA1-expressing HEK 293 cells with etodolac at concentrations found in plasma during clinical use showed poor activation but significant desensitization of the TRPA1 channel (Inoue et al. 2012; Wang, S. et al. 2013). Furthermore, Hatano et al. have shown the antirheumatic drug auranofin to be a potent stimulator of TRPA1 channels in a human neuroblastoma cell line and TRPA1-transfected HEK 293 cells (Hatano et al. 2013).

Glucocorticoids are commonly used for their potent anti-inflammatory effects and, in the treatment of knee OA, glucocorticoids are used as intra-articular injections (Hochberg et al. 2012; McAlindon et al. 2014). Glucocorticoids have a
wide effect on cellular functions through regulation of innumerable inflammatory and metabolic genes. Generally, glucocorticoids function by entering the cell and binding to cytoplasmic glucocorticoid receptors (GRs). The ligand-bound GRs then dimerize, translocate to the nucleus, and interact with specific DNA sequences called glucocorticoid response elements (GREs) to initiate the expression of target genes. The suppression of gene transcription is largely attributed to the direct or indirect inhibition of transcription factor activation. By these mechanisms, glucocorticoids enhance the expression of many anti-inflammatory genes and suppress the activity of a wide range of pro-inflammatory genes and those encoding cartilage matrix degrading enzymes, and the present results add TRPA1 to the inflammatory genes down-regulated by glucocorticoids (Hartmann et al. 2016; Rang et al. 2019).

Important anti-inflammatory actions of dexamethasone include inhibition of pro-inflammatory transcription factors, particularly NF-κB and AP-1, leading to a reduction in the transcription of pro-inflammatory genes, including multiple cytokines COX-2 and iNOS (Hartmann et al. 2016; Hayashi et al. 2004; Rang et al. 2019). Some of the actions of glucocorticoids may also be mediated in the cytosol through interactions between liganded GRs and regulatory factors such as kinases and phosphatases (Hartmann et al. 2016). Dexamethasone also functions by increasing the expression of certain anti-inflammatory factors such as annexin-1 and mitogen-activated protein kinase phosphatase 1 (MKP-1) (Janka-Juntila et al. 2012; Kassel et al. 2001; Tuure et al. 2017). Some anti-inflammatory effects are also thought to be caused by post-transcriptional regulation, as dexamethasone has been reported to destabilize iNOS and COX-2 mRNA, thereby reducing NO and PGE₂ synthesis (Korhonen et al. 2002; Lasa et al. 2001; Newton et al. 1998).

The DMARD aurothiomalate is a gold compound that is administered as intramuscular injections (Kean and Kean 2008; Rang et al. 2019). Aurothiomalate reduces pain and retards the progression of rheumatoid arthritis by reducing cartilage degradation and bone erosion, and relieving inflammation. Despite having been used for many decades, its molecular mechanisms of action remain poorly understood. Currently known effects of aurothiomalate include reducing serum levels of IL-6 in rheumatoid arthritis patients (Lacki et al. 1995) and
inhibiting the production of nitric oxide as well as prostaglandins such as PGE$_2$ (Niemenen et al. 2010; Tuure et al. 2015; Vuolteenaho et al. 2005). Aurothiomalate has also been shown to inhibit the expression of other inflammatory and catabolic factors COX-2, IL-6, IL-1$\beta$, TNF-$$\alpha$$ and MMP-3 (Niemenen et al. 2008; Niemenen et al. 2010; Yanni et al. 1994). Suggested mechanisms for the inhibition of inflammatory factors include suppressing NF-$$\kappa$$B activation, increasing the expression of anti-inflammatory phosphatase MKP-1, and reducing COX-2 mRNA stability (Niemenen et al. 2008; Niemenen et al. 2010; Vuolteenaho et al. 2005).

Exactly how these drugs regulate the expression of TRPA1 in chondrocytes remains unknown. We showed in this study that inhibition of the transcription factor NF-$$\kappa$$B downregulated TRPA1 mRNA expression in chondrocytes. Accordingly, it has been reported by Hatano et al. that the TRPA1 promoter region contains at least six putative binding sites for NF-$$\kappa$$B, and that NF-$$\kappa$$B is involved in TRPA1 induction in human synoviocytes (Hatano et al. 2012). Additionally, the NF-$$\kappa$$B signaling pathway was shown to be essential for TRPA1 mRNA expression in an experimental model of allergic contact dermatitis, where inhibiting NF-$$\kappa$$B activation also inhibited TRPA1 expression (Kang, J. et al. 2017). These results, together with the previous findings that dexamethasone and aurothiomalate inhibit NF-$$\kappa$$B activation (Hartmann et al. 2016; Vuolteenaho et al. 2005), suggest that their effects on TRPA1 expression in chondrocytes may be, at least partly, via NF-$$\kappa$$B inhibition. It is also possible that dexamethasone and/or aurothiomalate regulate TRPA1 by post-transcriptional mechanisms, as both drugs have previously been shown to destabilize mRNA of pro-inflammatory factors (Korhonen et al. 2002; Lasa et al. 2001; Niemenen et al. 2010). Overall, the regulation of TRPA1 expression has not been extensively elucidated, and current knowledge of the field is reviewed in Paragraph 6.3.1.

Treatment with glucocorticoids and aurothiomalate can cause various unwanted effects. Single intra-articular injections or short-term treatment with low doses of glucocorticoids do not usually cause adverse effects. Although, despite the common conception that intra-articular glucocorticoid injections in the treatment of OA do not cause adverse events, concerns have been raised about
potential long-term effects (Baschant et al. 2013; Juni et al. 2015). There are some indications of deleterious effects of glucocorticoids in chondrocytes and cartilage. In a clinical trial with OA patients, intra-articular glucocorticoid treatment was shown to cause greater cartilage volume loss measured by MRI compared to placebo treatment (McAlindon et al. 2017). Furthermore, the synthesis of cartilage ECM components aggrecan and type II collagen were shown to be downregulated in cultured chondrocytes following glucocorticoid stimulation (Song et al. 2012). Glucocorticoids have also been suggested to reduce chondrocyte viability by increasing oxidative stress and apoptosis (Song et al. 2012; Suntiparpluacha et al. 2016).

In the treatment of conditions requiring prolonged systemic administration or high doses, glucocorticoids are associated with multiple serious unwanted effects, including osteoporosis, suppression of the body’s response to infection and injury, weakened production of endogenous glucocorticoids as well as metabolic disorders such as hyperglycemia, muscle wasting and Cushing’s syndrome (Rang et al. 2019; van der Goes et al. 2014). Aurothiomalate on the other hand slowly accumulates in the synovial cells of joints, along with other cells and tissues including kidney tubules, the adrenal cortex, liver cells and macrophages, and can cause potentially severe skin rashes, stomatitis, flu-like symptoms, proteinuria, thrombocytopenia and other blood dyscrasias (Rang et al. 2019). Therefore, a better understanding of the specific molecular mechanisms of both of these drugs is needed for the development of more specific drugs with potentially fewer side effects. Accordingly, this study has presented a novel mechanism of action for these drugs in the downregulation of an inducible cation channel that mediates inflammatory and analgesic responses.

6.3.1 Current knowledge on the regulation of TRPA1 expression

Considering that TRPA1 has predominantly been studied in sensory neuronal cells, it is not surprising that the regulation of its expression is best known in DRG neurons. Especially the upregulation of TRPA1 expression has been reported in multiple studies. In DRG neurons, the expression of TRPA1 has been reported to

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be increased by IL-6 cytokine family signal transducer glycoprotein 130 (gp130), the cytokine IL-13, neuronal survival factor artemin, the TRPA1 agonist mustard oil (MO), and nerve growth factor (NGF), which also increases TRPA1 expression in TG neurons (Diogenes et al. 2007; Elitt et al. 2006; Ikeda-Miyagawa et al. 2015; Malsch et al. 2014; Obata et al. 2005; Oh et al. 2013; Schmidt et al. 2009). Of these stimulants, we also investigated the effect of NGF on TRPA1 expression in chondrocytes, due to the growing interest in NGF as an important mediator in OA and OA pain. According to our preliminary results, NGF alone did not affect TRPA1 expression (data not shown). However, the effects of NGF inhibition are not known. Notably, the anti-NGF antibodies tanezumab and fasinumab are currently being investigated in Phase 3 clinical trials in OA patients, where they were shown to increase the risk of rapidly progressing OA (RPOA) (Dakin et al. 2019; Karsdal et al. 2018). It is interesting to speculate whether blocking TRPA1 in these patients could affect the incidence of RPOA during tanezumab treatment.

Most of the aforementioned studies do not offer a molecular mechanism for the upregulation of TRPA1 expression. However, Obata et al. suggested NGF to upregulate TRPA1 via p38 MAPK activation, and Malsch et al. proposed the upregulation of TRPA1 expression by gp130 to be mediated by the JAK-STAT pathway (Malsch et al. 2014; Obata et al. 2005). Conversely, the AMP activated kinase (AMPK), which is an intracellular sensor and modulator of energy expenditure, was shown to decrease plasma membrane expression of TRPA1 in cultured DRG neurons (Wang, S. et al. 2018).

There are also fragmentary data on the regulation of TRPA1 expression in nonneuronal cells. In human fibroblast-like synoviocytes TRPA1, expression was reported to be increased by inflammatory factors IL-1α and TNF-α through NF-κB signaling and the activation of the transcription factor hypoxia-inducible factor-1α (HIF1α) (Hatano et al. 2012). The expression of TRPA1 is also potently increased by IL-13 and to a lesser extent by interferon γ (IFN-γ) and reactive oxygen species H2O2, in murine bone marrow-derived mast cells (Oh et al. 2013). Additionally, in cultured human bronchial epithelial cells, cigarette smoke extract, which has many compounds that agonize the TRPA1 channel, increased
TRPA1 mRNA expression (Lin et al. 2015). In the same study, it was seen that exposing mice to cigarette smoke increased lung epithelial TRPA1 expression, when compared to air-exposure control mice. The mechanisms behind the increase of TRPA1 expression in these studies are however not clear.

Overall, the regulation of TRPA1 expression in nonneuronal cells is poorly understood and warrants further investigation. The current study contributed to this data by identifying factors that induce or inhibit TRPA1 expression in human chondrocytes. TRPA1 expression was shown to be upregulated by IL-1β, IL-17, LPS and resistin. Notably, the IL-1β-induced upregulation of TRPA1 was reduced by the NF-κB inhibitor PDTC. Further, two novel factors, dexamethasone and aurothiomalate, were found to downregulate TRPA1 expression, potentially through inhibition of the NF-κB pathway.

According to literature, IL-1β, IL-17, LPS and resistin (i.e. the factors shown to upregulate TRPA1 expression in chondrocytes in this study) all activate the NF-κB signaling pathway. IL-1β and IL-17 can do so through IL-1 and IL-17 receptors, respectively, while LPS and resistin have been shown to signal through the receptor TLR4. LPS is an exogenous TLR4 activator, whereas resistin has been reported to function as an endogenous activator of TLR4. Signaling through TLR4 leads to the activation of the NF-κB pathway, ultimately resulting in the expression of NF-κB-dependent genes (Liu, T. et al. 2017; Tarkowski et al. 2010). In addition to NF-κB, various other putative transcription factor binding sites have been identified in the promoter region of TRPA1 (Table 5), but the functional significance of most of these sites remains largely unknown.

Table 5. Transcription factors of the human TRPA1 gene. Transcription factors identified to have a binding site in the human TRPA1 promoter region. (GeneCards human gene database, www.genecards.com, Hatano et al, 2012)

<table>
<thead>
<tr>
<th>Transcription factors of the human TRPA1 gene</th>
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<tbody>
<tr>
<td>ER-α, FOXI1, HFH-3, HIF1α, ITF-2, NF-AT, NF-AT1, NF-AT2, NF-AT3, NF-AT4, NF-κB, Tal-1β</td>
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The TRPA1 channel has long been known to mediate neurogenic inflammation and has more recently also been linked to inflammatory responses in some nonneuronal cells. In inflammatory reactions, neuronal TRPA1 channels are involved in the excitation of neurons, release of inflammatory neuropeptides, and consequent inflammation-associated pain. Also, in some nonneuronal cells, inflammatory agents activate TRPA1, which feeds forward the inflammatory reaction by increasing the production of inflammatory factors, which can then further activate nearby cells and cause cell and tissue damage (Bautista et al. 2013; Viana 2016). Therefore, after we had discovered that a functional TRPA1 channel is expressed in human OA chondrocytes, and that its expression is upregulated by the key OA-related factor IL-1β, we aimed to investigate whether TRPA1 regulates the expression of inflammatory and/or catabolic factors in chondrocytes and cartilage. Remarkably, we found TRPA1 to be involved in the regulation of the IL-6 family cytokines IL-6, LIF and IL-11 as well as the growth factor FGF-2, catabolic factors MMP-1, MMP-3, MMP-13, and inflammatory and analgesic factor PGE2, which are significant factors in the pathogenesis and pathology of OA.

A limitation of this study is that the mechanisms by which TRPA1 affects the production of inflammatory and catabolic factors in chondrocytes have not been determined and warrant further studies. This is not uncommon, as some other studies have reported a link between TRPA1 and the up- or downregulated expression of inflammatory agents but have not reported the exact mechanisms behind these effects (Gouin et al. 2017; Viana 2016). However, in an interesting study by Lin et al., an elaborate mechanism for TRPA1-mediated production of IL-8 was suggested. In the study, cultured lung epithelial cells were stimulated with cigarette smoke extract. This increased the level of extracellular ROS leading to the activation of the TRPA1 channel. This resulted in substantial Ca2+ influx into the activated cells, promoting the activation of NADPH oxidase, which in turn led to an increase in intracellular ROS levels, subsequently activating MAP kinases and the NF-κB signaling cascade, ultimately leading to the upregulation of IL-8 (Lin et al. 2015).
In general, the effects of TRPA1 are largely attributed to the substantial Ca\textsuperscript{2+} influx and subsequent elevation of intracellular Ca\textsuperscript{2+} following TRPA1 activation (Zygmunt and Högestätt 2014). In addition to Ca\textsuperscript{2+} influx from the extracellular environment, Ca\textsuperscript{2+} can also be liberated from intracellular stores such as the endoplasmic reticulum, mitochondria and lysosomes (Gees et al. 2010; Raffaello et al. 2016). TRPA1 is hypothesized to be involved in this mechanism, as it is also intracellularly active and expressed in secretory vesicles and granules. There is also data to support a role for TRPA1 in Ca\textsuperscript{2+} release from these secretory vesicles (Dong et al. 2010; Gees et al. 2010). Regulation of inflammatory gene expression is one of the effects of intracellular calcium increase (Berridge et al. 2000; Korhonen et al. 2001). This regulation of gene expression can occur for example through the regulation of cyclic AMP (cAMP), activation of PLC and the MAPK signaling pathway as well as activation of transcription factors such as NF-κB and NF-AT (Berridge et al. 2000; Racioppi and Means 2008; Vuong et al. 2015). TRPA1 has the highest permeability towards Ca\textsuperscript{2+} but is also permeable to other monovalent and bivalent ions such as Zn\textsuperscript{2+}, Mg\textsuperscript{2+}, Na\textsuperscript{+} and K\textsuperscript{+} (Zygmunt and Högestätt 2014). Other ions besides Ca\textsuperscript{2+} therefore likely also account for some of the actions of TRPA1. However, their significance in TRPA1-induced changes in cellular functions have been far less researched.

The following paragraphs discuss in more detail our current results on the effects of TRPA1 on the production of proinflammatory and catabolic factors, placing them in the context of current knowledge of TRPA1-mediated inflammatory gene expression, and discussing their relevance with regards to OA pathogenesis.

6.4.1 IL-6

According to the present results, TRPA1 regulates IL-6 expression in articular chondrocytes. TRPA1 was also found to be involved in the regulation of other IL-6 cytokine family members, LIF and IL-11. These are significant findings, as IL-6 is currently considered to be one of the key inflammatory mediators driving the pathogenesis of OA (Kapoor et al. 2011; Rahmati et al. 2016). Elevated levels of
IL-6 are found in the serum and synovial fluid of OA patients, with the levels correlating positively with cartilage loss as well as the incidence, or severity, of radiographic knee OA (Kaneko et al. 2000; Laavola et al. 2018; Larsson et al. 2015; Livshits et al. 2009; Stannus et al. 2010). In addition, LIF and IL-11 are reported to be involved in the inflammatory reaction of the OA joint (Rahmati et al. 2016).

The current results showing TRPA1-dependent upregulation of IL-6 expression are supported by other in vivo and in vitro studies. In experimental models of allergic contact dermatitis and MSU crystal-induced inflammation, genetic deletion and pharmacological inhibition of TRPA1 reduced the production of IL-6 (Kang, J. et al. 2017; Liu, B. et al. 2013; Moilanen et al. 2015a). Additionally, TRPA1 was recently shown to mediate peritoneal IL-6 synthesis in response to peritoneal dialysis fluid administration in the rat (Nilsson et al. 2017). In cultured primary human periodontal ligament cells, activation of TRPA1 increased the expression of IL-6. This was repressed by a Ca\(^{2+}\) chelator, indicating that the increase in IL-6 expression was due to the TRPA1-mediated increase in intracellular Ca\(^{2+}\) (Son et al. 2015). Unlike in the aforementioned studies, in cultured human synoviocytes the activation of TRPA1 was shown to downregulate IL-1\(\alpha\)-induced IL-6 production (Hatano et al. 2012), suggesting that the effect of TRPA1 on IL-6 production may be cell-type specific. However, in a more recent study, LPS-stimulated primary human OA synoviocytes showed decreased production of IL-6 following pharmacological inhibition or gene silencing of TRPA1 (Yin et al. 2018). These findings in synoviocytes suggest that the regulation of IL-6 production was influenced by the different stimulants, or that the phenotype and gene expression patterns of OA synoviocytes differed from that of non-OA synoviocytes.

IL-6 has recently raised increasing interest as one of the key inflammatory mediators in OA pathogenesis. It is crucially involved in the cartilage degradation as well as subchondral bone remodeling in OA (Haseeb and Haqqi 2013; Kapoor et al. 2011; Kwan Tat et al. 2004). Considering the effects of IL-6 in joint tissues, it is not surprising that it is being investigated as a drug target in OA. The IL-6 receptor antagonist tocilizumab is currently in a phase 3 clinical trial with
indications for pain and function in patients with refractory hand OA (NCT02477059). In addition to antagonizing the IL-6 receptor, another approach could be to target the expression of IL-6. Based on the findings of this thesis, and previous reports on the effects of TRPA1 on IL-6 production, it can be hypothesized that selectively inhibiting the expression and/or activation of TRPA1 in OA cartilage could downregulate the expression of IL-6. Notably, the inhibition of TRPA1 in cartilage could simultaneously downregulate the production of other deleterious agents, such as LIF and IL-11.

6.4.2 FGF-2

We investigated the effect of TRPA1 on the growth factor FGF-2 and found that in chondrocytes, TRPA1 was involved in regulating its expression. This is a novel finding, linking TRPA1 to the expression of FGF-2 for the first time. As FGF-2 is an interesting factor in the pathogenesis of cartilage degradation in OA, we decided to investigate the effects of FGF-2 in human OA cartilage and chondrocytes further.

We first examined the possible correlations between the levels of FGF-2 and cartilage matrix degrading enzymes MMP-1 and MMP-13 in cartilage explant cultures from 97 OA patients undergoing knee-joint replacement surgery. The levels of FGF-2 correlated positively with both enzymes MMP-1 and MMP-13, which encouraged us to continue the study using primary human OA chondrocyte cultures.

FGF-2 exerts its effects by signaling through four receptors: FGFR1-4 (Ornitz and Itoh 2015; Zhang, X. et al. 2006). Before investigating the effects of FGF-2 further, we examined the receptor expression profiles of the primary human OA chondrocytes and found that they expressed all four receptors, with the expression of FGFR1 being highest. In support of this finding, human chondrocytes have previously been reported to express all four receptors with the expression of FGFR1 and FGFR3 being the most prominent (Yan et al. 2011). Further, it has been reported that in OA cartilage the expression of FGFR1 is increased while the expression of FGFR3 is decreased (Weng et al. 2012; Yan et al. 2011). This
is significant as the catabolic effects of FGF-2 are mediated through FGFR1, whereas signaling through FGFR3 is thought to elicit anabolic effects (Ellman et al. 2013; Muddasani et al. 2007; Yan et al. 2011; Yan et al. 2012). Notably, another member of the FGF growth factor family, FGF-18, has been shown to signal through receptor FGFR3 and induce anabolic responses in chondrocytes, and is being investigated as a novel drug in OA (Davidson et al. 2005; Gigout et al. 2017; Reker et al. 2017). The recombinant human FGF-18 drug sprifermin is currently undergoing clinical trials for osteoarthritis and cartilage injury of the knee (Lohmander et al. 2014; Roemer et al. 2016).

The effects of FGF-2 and FGF receptor antagonists AZD4547 (Gavine et al. 2012) and NVP-BGJ398 (Guagnano et al. 2011) were then investigated in cultured primary human OA chondrocytes. FGF-2 treatment increased the production of catabolic enzymes MMP-1 and MMP-13, whereas the expression of major cartilage matrix components aggrecan and type II collagen was decreased. This catabolic and anti-anabolic function of FGF-2 in human chondrocytes is supported by previous findings, showing that FGF-2 stimulation increased MMP-13 and ADAMTS-5 expression, and inhibited aggrecan expression in adult human articular chondrocytes (Im et al. 2007; Wang, X. et al. 2004; Yan et al. 2012), and that in alginate-cultured human articular chondrocytes and cartilage explants FGF-2 inhibited proteoglycan synthesis (Loeser et al. 2005; Posever et al. 1995). As discussed in the review of literature, FGF-2 has been found to have anabolic effects in murine cartilage. This is hypothesized to result from differing receptor expression profiles between different species. Unlike in human cartilage, the expression of receptors FGFR2 and FGFR4 are predominant in murine articular cartilage, and the expression of the catabolism-associated receptor FGFR1 is low. Further, in murine articular cartilage the expression of anabolism-associated receptor FGFR3 is upregulated in response to FGF-2 (Li et al. 2012).

Treating cultured human OA chondrocytes with FGF-2 together with the FGFR antagonists AZD4547 and NVP-BGJ398 counteracted the catabolic and anti-anabolic effects of FGF-2: the expression of MMP-1 and MMP-13 was decreased, while the expression of aggrecan and collagen II was increased.
Surprisingly, this beneficial effect was also seen without exogenous FGF-2 stimulation, which lead us to hypothesize that the cells produced endogenous FGF-2. We had already shown in this study that FGF-2 was released from cartilage explants of OA patients, and we wanted to investigate whether FGF-2 is produced by chondrocytes or perhaps had diffused into the cartilage through the synovial fluid. We found that the chondrocytes did indeed produce FGF-2, which is a significant finding considering the potential in vivo activity of FGFR antagonists in the treatment of OA. We also measured the concentrations of FGF-2 in the synovial fluid and plasma of OA patients and found FGF-2 to be present at concentrations of $25.0 \pm 25.7$ pg/ml ($n = 33$) and $33.3 \pm 22.0$ pg/ml ($n = 93$), respectively (data not shown).

AZD4547 and NVP-BGJ398 are selective FGFR antagonists, which target the catabolism-associated receptor FGFR1 with the highest selectivity. The current results are supported by a study published the following year, where a novel FGFR1 antagonist G141 inhibited FGF-2-induced upregulation of MMP-13 and ADAMTS-5 expression as well as downregulation of aggrecan and collagen II expression in human articular chondrocytes, and reduced proteoglycan degradation in FGF-2-stimulated human cartilage explants (Xu et al. 2016). The use of FGFR1 antagonists as DMOADs is also supported by studies in mouse models, showing that $fgfr1$ deletion or pharmacological inhibition reduced articular cartilage degradation (Weng et al. 2012; Xu et al. 2016).

Our results, together with those of others, have shown FGF-2 to be a catabolic and anti-anabolic mediator in human cartilage and chondrocytes. Inhibition of FGF-2 with specific receptor antagonists has shown promising effects on the balance of catabolic and anabolic factors in in vitro and ex vivo studies as well as experimental mouse models, but none have reached clinical trials yet. Another approach to preventing the effects of FGF-2 could be to decrease the expression of FGF-2 in chondrocytes. The results of this thesis indicate that TRPA1 is involved in the upregulation of FGF-2 expression in chondrocytes. Therefore, the inhibition of TRPA1 and the subsequent downregulation of FGF-2 could have beneficial effects on the balance of catabolic and anabolic mediators within OA cartilage.
Finally, we wanted to investigate the potential effects of TRPA1 on major catabolic factors associated with cartilage ECM breakdown in osteoarthritis. As discussed in the review of literature, the matrix metalloproteinases MMP-1, MMP-3 and MMP-13 are among the key cartilage matrix degrading enzymes in OA (Troberg and Nagase 2012). Additionally, we wanted to investigate the effect of TRPA1 on the eicosanoid PGE\textsubscript{2}, which is an important mediator of pain and inflammation in OA (Lee, AS, et al. 2013; Sokolove and Lepus 2013). Furthermore, PGE\textsubscript{2} has been reported to increase the production and activation of MMP-enzymes and inhibit proteoglycan synthesis in osteoarthritic cartilage (Attur et al. 2008). In this study, genetically or pharmacologically inhibiting TRPA1 reduced the production of MMP-1, MMP-3, MMP-13 and PGE\textsubscript{2} in cultured primary human OA chondrocytes and mouse cartilage explants, suggesting a role for TRPA1 in the regulation of their production.

The effect of TRPA1 on MMP-enzyme production in chondrocytes and cartilage is a novel finding. Similar results have however been obtained from cultured primary human fibroblast-like synoviocytes and synovial fibroblasts. A study by Yin et al. showed that in LPS-stimulated human OA fibroblast-like synoviocytes pharmacological inhibition with a specific TRPA1 antagonist, and gene silencing by small interfering RNA (siRNA), downregulated the production of MMP-1 and MMP-3, along with the inflammatory cytokines IL-1\textbeta, TNF-\alpha and IL-6 (Yin et al. 2018). In cultured human RA synovial fibroblasts, TNF-induced production of MMP-3, as well as IL-6 and IL-8, were downregulated following treatment with the cannabinoid receptor antagonist anandamide in a TRPA1-dependent manner (Lowin et al. 2015).

The inhibition of MMP expression and activity would be an important aspect in the treatment of OA as well as other pathologies such as cancer and periodontal disease. Accordingly, research into MMP inhibitor design has been active for decades. Most commonly, the development of MMP inhibitors has focused on active site inhibition, but unfortunately, these inhibitors have lacked specificity and caused unwanted off-target effects. Recently, utilizing secondary binding sites in addition to the active site has been investigated to improve specificity of
the inhibitors (Fields 2015). Some factors regulating the expression of MMPs have been identified, and one approach to MMP inhibition could be to target their expression instead of just their activity. In cartilage, TRPA1 inhibition could potentially downregulate the expression of key OA-related MMP-enzymes.

The effect of TRPA1 on the production of PGE₂ in cartilage and chondrocytes is also a novel finding and is supported by previous in vivo and in vitro data. In experimental models of hyperalgesia and cough, TRPA1 was identified as a key mediator of PGE₂-induced nociceptive and tussive effects (Dall'Acqua et al. 2014; Grace et al. 2012). Additionally, there is in vitro data showing that the activation of TRPA1 in cultured human dermal keratinocytes and fibroblasts increases the secretion of PGE₂ (Jain et al. 2011). TRPA1 activation has also been reported to increase expression of the inducible prostaglandin synthase COX-2 (Moilanen et al. 2012; Moilanen, Hämäläinen, Nummenmaa et al. 2015b). PGE₂ is a significant mediator of pain in OA (Lee, AS. et al. 2013). Upregulated production of PGE₂ due to TRPA1 activation is significant in both neuronal and nonneuronal cells: in nonneuronal cells of the joint, such as chondrocytes or fibroblasts, the increased production of PGE₂ can stimulate nearby neurons leading to increased OA-related pain.

6.5 Clinical aspects

At present, there is no effective treatment to prevent or retard the structural changes occurring in the joint during osteoarthritis. Therefore, there is an urgent need for novel drug targets for the development of disease-modifying OA drugs. In addition to the prevention of structural changes in the joint tissues, effective analgesic treatment is required.

Osteoarthritis is a disease that affects the entire joint structure. Cartilage is the most severely affected tissue, but pathognomonic features are also found in the subchondral bone, synovial membrane, ligaments and periarticular muscles (Glyn-Jones et al. 2015). What initiates the inflammatory and catabolic process in the OA joint is unkown, and drug development is complicated by the lack of early diagnosis as well as the fact that OA is a heterogeneous disease (Karsdal et
OA is commonly divided into multiple phenotypes, which can be classified as metabolic, subchondral bone, synovitis-driven, cartilage-driven, ageing-driven and trauma-driven phenotypes (Mobasher et al. 2017; van der Kraan et al. 2016). Ultimately, in all forms of OA an inflammatory and hypoxic state develops within the OA joint, characterized by the presence of pro-inflammatory mediators, reactive species, and cartilage degrading enzymes (Robinson et al. 2016; Wang, X. et al. 2018). These mediators exert many deleterious effects in the joint tissues, one of the most important being the acceleration of cartilage degradation. Inhibiting their production and/or activity is therefore a promising prospect in the development of novel drugs.

In the present study, we investigated the cation channel TRPA1 as a possible mediator and novel drug target in OA. We discovered TRPA1 to be expressed in human chondrocytes, where its expression is enhanced by IL-1β, a key cytokine in OA pathogenesis. In addition to IL-1β, also IL-17, LPS and resistin were found to increase TRPA1 expression. According to literature, some other OA-associated cytokines, as well as reactive oxygen and nitrogen species, also increase TRPA1 expression and activation in other cell types (Bautista et al. 2013; Viana 2016). We also showed that the anti-inflammatory drugs dexamethasone and aurothioma late inhibited the formation of a functional TRPA1 channel in chondrocytes, suggesting that some of the effects of these drugs may be elicited through TRPA1 inhibition. Finally, we showed TRPA1 to be involved in regulating the production of multiple OA-related inflammatory and catabolic factors in chondrocytes, namely IL-6, IL-11, LIF, FGF-2, MMP-1, MMP-3, MMP-13 and PGE2. The major findings of this thesis on the expression and functions of TRPA1 in chondrocytes/cartilage are summarized in Figure 24.

Due to the multiple inflammatory and catabolic factors regulated by TRPA1 in chondrocytes, as well as other cell types, it presents as an intriguing novel drug target. TRPA1 antagonists have previously been investigated in experimental disease models, including atopic dermatitis, allergic asthma, acute allergic inflammation, colitis, acute inflammation, gouty inflammation and arthritis (Caceres et al. 2009; Engel et al. 2011; Liu, B. et al. 2013; Moilanen et al. 2012; Moilanen et al. 2015a; Moilanen, Hämäläinen, Nummenmaa et al. 2015b;
Moilanen et al. 2019). In addition, Glenmarcks TRPA1 antagonist GRC 17536 has been investigated in clinical trials for painful diabetic neuropathy. Results from these in vivo models and clinical trials seem promising, and importantly, the TRPA1 antagonists do not seem to cause significant systemic side-effects. The notion of TRPA1 as a drug target in OA has been proposed due to its functions in sensory neurons and the potential to diminish neuropathic and inflammatory pain (Galindo et al. 2018; Koivisto et al. 2018). The present study extends this concept also to nonneuronal cells involved in OA. Since TRPA1 is expressed in chondrocytes and involved in the upregulation of inflammatory and catabolic mediators, it serves as potential drug target in OA. Intriguingly, according to the present results, showing the inhibition of TRPA1 by the drugs dexamethasone and aurothiomalate, TRPA1 may already be a previously unidentified target of anti-inflammatory drug treatment.

Locally inhibiting TRPA1 in cartilage and in the knee joint could simultaneously exert analgesic and disease modifying effects. Analgesic effects could be obtained with TRPA1 antagonists by blocking the activity of TRPA1 in innervating neurons, and additionally, by downregulating the production of inflammatory pain mediators in nonneuronal TRPA1-expressing cells of the joint. Based on the results of this thesis, the inhibition of TRPA1 in osteoarthritic cartilage and chondrocytes could potentially decrease the production of multiple inflammatory and catabolic mediators, thus reducing the deleterious inflammation and cartilage degradation of OA. Although as mentioned earlier, the research on TRPA1 antagonists is mostly reliant on experimental disease models, and additional clinical trials are needed to evaluate the potential of TRPA1 antagonists as drug treatment.
Figure 24. Schematic summary of the main results on the expression and function of TRPA1 in chondrocytes in the present study. TRPA1 was discovered to be functionally expressed in human OA chondrocytes and immortalized human T/C28a2 chondrocytes. The expression of TRPA1 is enhanced by IL-1β, IL-17, LPS and resistin, and the activation of TRPA1 results in an elevation of intracellular Ca^{2+}. TRPA1 is involved in regulating the expression of multiple mediators of inflammation, catabolism and inflammatory pain: IL-6-family cytokines (IL-6, LIF, IL-11), FGF-2, MMP-1, MMP-3, MMP-13 and PGE_2 in chondrocytes. The expression of TRPA1 is downregulated by anti-inflammatory drugs dexamethasone and aurothiomalate, as well as the NF-κB inhibitor PDTC. [Ca^{2+}] - intracellular calcium, FGF - fibroblast growth factor, IL - interleukin, LIF - leukemia inhibitory factor, LPS - lipopolysaccharide, MMP - matrix metalloproteinase, NF-κB - nuclear factor κB, PDTC - ammonium pyrrolidinedithiocarbamate, PGE_2 - prostaglandin E_2, TRPA - transient receptor potential ankyrin.
The aim of the present study was to investigate the expression and effects of the TRPA1 cation channel in human OA chondrocytes and cartilage. Primary human OA chondrocytes and cartilage, immortalized human T/C28a2 chondrocytes as well as chondrocytes and cartilage from TRPA1 KO and corresponding WT mice were used in the studies.

The major findings and conclusions are:

1. TRPA1 channel was shown to be functionally expressed in primary human OA chondrocytes and immortalized T/C28a2 chondrocytes. This is a novel finding, adding chondrocytes to the few nonneuronal cells demonstrated to express TRPA1.

2. TRPA1 expression is upregulated by OA-related inflammatory factors IL-1β, IL-17 and resistin, and by the TLR4 activating bacterial product lipopolysaccharide (LPS). Most notably the cytokine IL-1β is considered one of the important inflammatory mediators driving cartilage degradation in OA. Inflammatory factors IL-6, NGF, YKL-40 and the hypoxia-mimetic agent CoCl₂ showed no or a very small effect on TRPA1 expression in human chondrocytes.

3. TRPA1 expression is downregulated by the anti-inflammatory drugs dexamethasone and aurothiomalate in human chondrocytes, potentially through inhibition of the transcription factor NF-κB, while methotrexate, sulfasalazine, hydroxychloroquine and ibuprofen had no effect.
4. TRPA1 is involved in the upregulation of cytokines IL-6, IL-11 and LIF, growth factor FGF-2, catabolic enzymes MMP-1, MMP-3, MMP-13, and inflammatory and analgesic factor PGE2 in chondrocytes/cartilage.

5. The expression of FGF-2 is enhanced by TRPA1 in human OA and murine chondrocytes. FGF-2 exerts catabolic and anti-anabolic effects in human OA chondrocytes. The effects of FGF-2 are counteracted by FGF receptor antagonists, which cause the downregulation of catabolic enzymes MMP-1 and MMP-13 and upregulate the expression of major cartilage matrix components aggrecan and type II collagen.

In summary, this study has shown that the TRPA1 ion channel is functionally expressed in primary human osteoarthritic chondrocytes, where its expression is upregulated by OA-related inflammatory factors and downregulated by clinically used anti-inflammatory drugs dexamethasone and aurothiomalate. The inhibition of TRPA1 by these drugs suggests that TRPA1 may already be a previously unidentified target of anti-inflammatory drug treatment. TRPA1 was also shown to be involved in mediating the expression of pro-inflammatory and catabolic factors significant in the pathogenesis of OA. These results present TRPA1 as a novel factor and a potential drug target in osteoarthritis. Additionally, this thesis has produced valuable information on the nonneuronal expression of TRPA1, expanding our knowledge of this multifunctional ion channel.
Tämä tutkimus on tehty Immunofarmakologian tutkimusryhmässä Tampereen yliopiston Lääketieteen ja terveysteknologian tiedekunnassa yhteistyössä Tekonivelsairaala Coxan kanssa. Olen myös ollut Tuki- ja liikuntelinsairauksien ja biomateriaalien tohtorionohjelman (TBDP) opiskelija.


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Elina Nummenmaa
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10 ORIGINAL COMMUNICATIONS
Transient Receptor Potential Ankyrin 1 (TRPA1) is Functionally Expressed in Primary Human Osteoarthritic Chondrocytes

Elina Nummenmaa, Mari Hämäläinen, Lauri J. Moilanen, Erja-Leena Paukkeri, Riina M. Nieminen, Teemu Moilanen, Katriina Vuolteenaho and Eeva Moilanen

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Transient receptor potential ankyrin 1 (TRPA1) is functionally expressed in primary human osteoarthritic chondrocytes

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Abstract

Background: Transient receptor potential ankyrin 1 (TRPA1) is a membrane-associated cation channel, widely expressed in neuronal cells and involved in nociception and neurogenic inflammation. We showed recently that TRPA1 mediates cartilage degradation and joint pain in the MIA-model of osteoarthritis (OA) suggesting a hitherto unknown role for TRPA1 in OA. Therefore, we aimed to investigate whether TRPA1 is expressed and functional in human OA chondrocytes.

Methods: Expression of TRPA1 in primary human OA chondrocytes was assessed by qRT-PCR and Western blot. The functionality of the TRPA1 channel was assessed by Ca2+-influx measurements. Production of MMP-1, MMP-3, MMP-13, IL-6, and PGE2 subsequent to TRPA1 activation was measured by immunoassay.

Results: We show here for the first time that TRPA1 is expressed in primary human OA chondrocytes and its expression is increased following stimulation with inflammatory factors IL-1β, IL-17, LPS, and resistin. Further, the TRPA1 channel was found to be functional, as stimulation with the TRPA1 agonist AITC caused an increase in Ca2+ influx, which was attenuated by the TRPA1 antagonist HC-030031. Genetic depletion and pharmacological inhibition of TRPA1 downregulated the production of MMP-1, MMP-3, MMP-13, IL-6, and PGE2 in osteoarthritic chondrocytes and murine cartilage, respectively.

Conclusions: The TRPA1 cation channel was found to be functionally expressed in primary human OA chondrocytes, which is an original finding. The presence and inflammatory and catabolic effects of TRPA1 in human OA chondrocytes propose a highly intriguing role for TRPA1 as a pathogenic factor and drug target in OA.

Keywords: Osteoarthritis, Chondrocyte, TRPA1, Inflammation, Matrix metalloproteinase

Background

Transient receptor potential ankyrin 1 (TRPA1) is a membrane-associated cation channel which mediates pain and hyperalgesia [1, 2] and functions as a chemosensor of noxious compounds [3–5]. TRPA1 was first discovered in 1999 [6] and has since then been found to be widely expressed in afferent sensory neurons, especially in Aδ and C fibers of nociceptors [7, 8]. In addition to pain, TRPA1 also has a role in mediating neurogenic inflammation [9, 10]. More recently, TRPA1 has been found to be expressed also in some nonneuronal cells such as keratinocytes [11] and synoviocytes [12] but the functional roles of nonneuronal expression remain to be studied.

TRPA1 is activated by numerous exogenous pungent compounds such as allyl isothiocyanate (AITC) from mustard oil [5], acrolein from exhaust fumes and tobacco smoke [9], and allicin from garlic [3]. Interestingly, TRPA1 is also activated and sensitized by agents formed endogenously in inflammatory reactions, such as nitric oxide [13], hydrogen peroxide [14] and nitro-oleic acid [15]. The activation of TRPA1 causes an influx of cation ions, particularly Ca2+, into the activated cells [16] and this elevation of intracellular Ca2+ has been
shown to trigger an action potential in neuronal cells [16, 17]. Interestingly, among the many regulatory effects of the alterations of intracellular Ca\(^{2+}\) concentration, its increase has also been shown to affect the gene expression of inflammatory mediators [18–20].

Recent evidence suggests TRPA1 to have a role in inflammation through exogenous activation by TRPA1 agonists and also through endogenous mechanisms. TRPA1 has been shown to mediate carrageenan-induced inflammatory edema [21], tumor necrosis factor (TNF)-triggered hyperalgesia [22], airway hyperreactivity and inflammation [23, 24], and to relate to acute gouty arthritis [25, 26]. Very recently we found that TRPA1 has a role in mediating acute inflammation, cartilage destruction, and joint pain in monosodium iodoacetate (MIA)-induced inflammation and osteoarthritis in the mouse [27].

Osteoarthritis (OA) is the most common cause of musculoskeletal disability and pain worldwide and its prevalence is constantly increasing as the population ages. OA is a degenerative disease of the joints, which is characterized by inflammation and hypoxia within the joint, leading to cartilage degradation, joint deformity, disability, and pain [28, 29]. OA-related cartilage degradation is caused by a growing imbalance between the production of catabolic, anabolic, and inflammatory mediators within the joint driven by the increased expression of matrix-degrading metalloproteinases and proinflammatory mediators such as interleukin (IL)-6 and prostaglandin E\(_2\) (PGE\(_2\)) [28].

TRPA1 has not previously been investigated in chondrocytes. However, factors involved in hypoxia and inflammation, such as hydrogen peroxide (H\(_2\)O\(_2\)), nitric oxide (NO), and IL-6 have been shown to upregulate the expression or activation of TRPA1 in some other cells [12–14]. Furthermore, the activation of TRPA1 has been reported to enhance the production of inflammatory factors [12, 21, 26, 30]. Since there is a hypoxic and inflammatory state in OA joints [28, 31], and TRPA1 has been shown to be involved in the mediation of acute inflammation and cartilage degradation in MIA-induced osteoarthritis [27], we hypothesized that TRPA1 is expressed in the chondrocytes in osteoarthritic joints, where its activation could play a vital part in the inflammation and pathogenesis of OA. In the present study, we tested that hypothesis by measuring the expression and function of TRPA1 in primary human OA chondrocytes.

**Methods**

**Cell culture**

Primary chondrocyte cultures were carried out as previously described [32]. Leftover pieces of OA cartilage from knee joint replacement surgery were used under full patient consent. The patients in this study fulfilled the American College of Rheumatology classification criteria for OA [33] and the study was approved by the Ethics Committee of Tampere University Hospital, Tampere, Finland (reference number R09116), and carried out in accordance with the Declaration of Helsinki. The procedures to isolate and culture the primary chondrocytes are described in the supplementary data (Additional file 1). During experiments the cells were treated with IL-1β (R&D Systems Europe Ltd, Abingdon, UK), IL-17 (R&D Systems Europe Ltd.), lipopolysaccharide (LPS) (Millipore Sigma, St. Louis, MO, USA), resistin (BioVision Inc., Milpitas, CA, USA), the TRPA1 antagonist HC-030031 (Millipore Sigma) or with combinations of these compounds as indicated.

Immortalized human T/C28a2 chondrocytes [34] were cultured as described in the supplementary data (Additional file 1). During the experiments T/C28a2 chondrocytes were treated with IL-1β (R&D Systems Europe Ltd), IL-17 (R&D Systems Europe Ltd.), LPS (Millipore Sigma), HC-030031 (Millipore Sigma) or with combinations of these compounds as indicated.

HEK 293 human embryonic kidney cells (American Type Culture Collection, Manassas, VA, USA) were cultured as described in the supplementary data (Additional file 1). The cells were transfected using 0.42 mg/cm\(^2\) of human TRPA1 plasmid DNA (pCMV6-XL4 by Origene, Rockville, MD, USA) with lipofectamine 2000 (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions.

**Animals**

Wild-type (WT) and TRPA1 knockout (KO) male B6;129P-Trpa1(tm1Kykw)/J mice (Charles River Laboratories, Sulzfeld, Germany) aged 19–22 days were used in mouse cartilage culture experiments. Mice were housed under standard conditions (12–12 h light–dark cycle, 22 ± 1 °C) with food and water provided ad libitum. Animal experiments were carried out in accordance with the legislation for the protection of animals used for scientific purposes (Directive 2010/63/EU) and the experiments were approved by The National Animal Experiment Board (reference number UTA 845/712-86). Animals were sacrificed by carbon monoxide followed by cranial dislocation.

**Mouse cartilage culture**

After mice were euthanized, full-thickness articular cartilage from femoral heads were removed and cultured as described in the supplementary data (Additional file 1). The cartilage pieces were exposed to IL-1β (R&D Systems Europe Ltd.) or its vehicle for 42 h and thereafter culture media were collected and matrix metalloproteinase (MMP)-3, IL-6, and PGE\(_2\) concentrations were measured by immunoassay.
Western blot measurements

After the cell culture experiments, total protein was extracted, and TRPA1 was immunoprecipitated and analyzed with Western blot as described in the supplementary data (Additional file 1). TRPA1 antibody NB110-40763 (Novus Biologicals, LCC, Littleton, CO, USA) was used as the primary antibody and goat anti-rabbit HRP-conjugate (sc-2004, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) as the secondary antibody in the Western blot analysis.

Immuoassay

Concentrations of IL-6, MMP-1, MMP-3, MMP-13 and PGE2 in medium samples were determined by enzyme-linked immunosorbent assay (ELISA) with commercial reagents (PGE2: Cayman Chemical Co., Ann Arbor, MI, USA; human IL-6: eBioscience Inc. San Diego, CA, USA; MMP-1, MMP-3, MMP-13 and mouse IL-6: R&D Systems Europe Ltd).

RNA extraction and quantitative RT-PCR

At the indicated time points, total RNA was extracted and analyzed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) for the expression of TRPA1 mRNA as described in the supplementary data (Additional file 1).

Ca2+-influx measurements

TRPA1-mediated Ca2+ influx was measured in HEK293 cells [35] transfected with human TRPA1 plasmid, in human T/C28a2 chondrocytes, and in primary human OA chondrocytes as described previously [36]. Briefly, the cells were loaded with 4 μM fluo-3-acetoxymethyl ester (Fluo-3-AM, Millipore Sigma) and 0.08 % Pluronic F-127® (Millipore Sigma in Hanks’ balanced salt solution (HBSS, Lonza, Verviers, Belgium) containing 1 mg/ml of bovine serum albumin, 2.5 mM probenecid and 25 mM F-127® (Millipore Sigma) was used as the secondary antibody in the Western blot analysis. HEK293 cells transiently transfected with TRPA1 plasmid were used as positive control and the protein was detected with a specific TRPA1 antibody. Remarkably, both cell types were found to express TRPA1 protein as seen in Fig. 3.

Results

TRPA1 is expressed in primary human OA chondrocytes and in immortalized human T/C28a2 chondrocyte cell line

Primary human OA chondrocytes and immortalized human T/C28a2 chondrocyte cell line expressed TRPA1. The expression was measured by quantitative RT-PCR on isolated total mRNA using a specific TaqMan assay. The proinflammatory cytokine IL-1β was found to increase TRPA1 expression in a time-dependent manner: in primary chondrocytes the expression of TRPA1 increased up to 48 hours and declined thereafter (Fig. 1a), whereas in the human T/C28a2 chondrocytes the expression maximum was at 6 hours (Fig. 1b). In addition, TRPA1 expression was also enhanced by inflammatory factors IL-17, LPS, and resistin (Fig. 2).

To verify the translation of TRPA1 mRNA into protein, we extracted total protein from primary human OA chondrocytes and human T/C28a2 chondrocytes and performed Western blot analysis. HEK293 cells transiently transfected with TRPA1 plasmid were used as positive control and the protein was detected with a specific human TRPA1 antibody. Remarkably, both cell types were found to express TRPA1 protein as seen in Fig. 3.

Human chondrocytes express a functional TRPA1 channel

To confirm that TRPA1 mRNA and the subsequent protein expressed by human chondrocytes produces a functional channel, Ca2+-influx measurements were carried out. Primary human chondrocytes and T/C28a2 chondrocytes were cultured with IL-1β, which was found to stimulate TRPA1 expression, or with its vehicle for 24 h, and thereafter TRPA1 was activated with the TRPA1 agonist AITC. IL-1β stimulation resulted in an increased responsiveness to AITC as seen as an enhanced Ca2+ influx, and the selective TRPA1 antagonist HC-030031 was shown to prevent this effect (Fig. 4).

MMP, IL-6 and PGE2 production is downregulated by genetic depletion and pharmacological inhibition of TRPA1

After finding that functional TRPA1 was indeed expressed in chondrocytes, we aimed to further examine the possible arthritogenic role of the TRPA1 channel. We investigated the effect of genetic depletion of TRPA1 on the production of OA-related factors MMP-3, IL-6, and PGE2 by using articular cartilage samples from TRPA1-deficient (knockout, KO) and corresponding wild-type (WT) mice. IL-1β treatment increased MMP-

The results are presented as mean ± standard error of the mean (SEM) unless otherwise indicated. Unpaired t test, paired t test, one-way analysis of variance (ANOVA) or repeated-measures ANOVA, followed by Dunnett’s test were used in the statistical analysis. Differences were considered significant at p < 0.05, p < 0.01, and p < 0.001.
3, IL-6, and PGE2 production in cartilage as expected. Remarkably, this response was significantly attenuated in the cartilage from the TRPA1 KO mice as compared to the corresponding WT mice (Fig. 5). Further, we treated primary human chondrocytes with IL-1β alone and together with the selective TRPA1 antagonist HC-030031 for 24 h. Interestingly, the selective TRPA1 antagonist HC-030031 downregulated IL-1β-enhanced MMP-1, MMP-3, MMP-13, IL-6, and PGE2 production by 25–45% (Fig. 6), suggesting that TRPA1 plays a role in the upregulation of these catabolic and inflammatory factors in OA cartilage.

**Discussion**

The findings of the present study suggest a hitherto unknown role for TRPA1 in the pathogenesis of OA. We have shown for the first time the expression of the TRPA1 channel in primary human OA chondrocytes and in the human T/C28a2 chondrocyte cell line. We showed the expression of TRPA1 mRNA and protein by...
We were also able to show that the expressed TRPA1 was functional, as evidenced by Ca²⁺-influx measurements. Further, we found TRPA1 to have a role in mediating the production of OA-related factors MMP-1, MMP-3, MMP-13, IL-6, and PGE₂ as evidenced by pharmacological inhibition and genetic depletion of TRPA1.

TRPA1 was first discovered in 1999 in fetal lung fibroblasts [6]. Since then it has been mainly studied in different afferent sensory neurons such as Aδ and C fibers of...
nociceptors [7, 8]. More recently, however, TRPA1 has also been found to be expressed in some nonneuronal cells such as keratinocytes [11, 37, 38], synoviocytes [12, 39] and airway epithelial and smooth muscle cells [30]. It is noteworthy, that not all of these studies have shown functionality of the TRPA1 ion channel and some have only reported the expression of TRPA1 at the mRNA level. In the present study, we have comprehensively shown the expression and activation of TRPA1 in human chondrocytes, to support the criteria set by Fernandes et al. [40]. We were able to show for the first time the expression of both TRPA1 mRNA and protein and the functionality of the TRPA1 channel in primary human OA chondrocytes and in human T/C28a2 chondrocyte cell line. This finding is particularly interesting as in OA joints there is a hypoxic [31] and inflammatory [28, 41] state and related factors, H2O2, NO, and IL-6, have previously been shown to upregulate the expression and activation of TRPA1 [12–14]. According to Hatano et al. [12] the human TRPA1 promoter has at least six putative nuclear factor kappa B (NF-kB) binding sites and ten core hypoxia response elements (HREs), which are binding sites for hypoxia-inducible factor (HIF) transcription factors. HIFs are known to mediate adaptive responses to hypoxia as well as to be activated by inflammation [42, 43] and the binding of HIFs to consensus HREs on their target genes regulates gene transcription.

After discovering TRPA1 expression in chondrocytes, we aimed to investigate whether inflammatory factors/mechanisms related to the pathogenesis of OA [28, 29] regulate expression of TRPA1, which would indicate a role for TRPA1 as a mediator in OA. IL-1β is considered as a major player in OA associated with cartilage destruction. IL-1β is elevated in OA joints and it suppresses type II collagen and aggrecan expression, stimulates the release of MMP-1, MMP-3, and MMP-13, and induces the production of IL-6 and some other cytokines as well as PGE2 [28]. In part IL-17 feeds forward these mechanisms as it further induces IL-1β, TNF, and IL-6 production, upregulates NO and MMPs and down-regulates proteoglycan levels related to the pathogenesis of OA [28]. Based on our results, IL-1β and IL-17 both also induce TRPA1 expression and intriguingly, some of the IL-1β-induced inflammatory and catabolic effects are partly mediated by TRPA1. In OA the innate immune system and in particular toll-like receptors (TLRs) activated by cartilage matrix degradation products, also play a significant part in disease progression. Chondrocytes express TLRs, which trigger major inflammatory pathways and are activated by bacterial lipopolysaccharide (LPS) and damage-associated molecular patterns [29], and also the adipocytokine resistin known to be expressed in OA joints [44] has been shown to transduce its effects through toll-like receptor 4 [45]. In the present study, we found that both LPS and resistin increased expression of TRPA1 in human chondrocytes, suggesting a TLR-mediated mechanism to enhance TRPA1 expression in OA cartilage. In support of the present results, Hatano et al. showed that TRPA1 gene expression was enhanced in synovocytes by inflammatory factors TNF-α and IL-1 [12], and the present study together with that of Hatano et al. [12] suggests a previously unrecognized mechanism that links TRPA1 as an inducible factor to joint inflammation. Activation of TRPA1 results in a substantial influx of Ca2+ into the stimulated cells [46]. Here we verified the functionality and activation of the TRPA1 channel in human chondrocytes by measuring Ca2+ influx using the TRPA1 agonist AITC as well as the TRPA1 antagonist HC-030031. As shown previously, elevated intracellular Ca2+ concentration may affect the expression of inflammatory genes both in a direct or indirect manner [20]. In the present study, we found that TRPA1 regulated the production of inflammatory and catabolic factors, namely MMP enzymes, IL-6, and PGE2 in chondrocytes. IL-1-induced MMP-3, IL-6, and PGE2 production in the cartilage from TRPA1-deficient mice was less than half.
of that found in the cartilage from wild-type mice. Accordingly, the selective TRPA1 antagonist HC-030031 reduced IL-1β-induced MMP-1, MMP-3, MMP-13, IL-6, and PGE2 production by 25–45% in primary human OA chondrocytes. In the latter experiment, the cells were incubated in the presence of IL-1 and HC-030031 for 24 h; therefore the result may be an underestimate of the effect of total inhibition of TRPA1 in OA chondrocytes because HC-030031 is a reversible TRPA1 antagonist with a relatively short half-life [47]. These findings...
are supported by previous studies indicating that TRPA1 activation regulates the production of IL-1 in keratinocytes [38], IL-6 and IL-8 in synoviocytes [12], and PGE\textsubscript{2} along with leukotriene B\textsubscript{4} in fibroblasts and keratinocytes [48]. We have recently found that TRPA1 also regulates the expression of cyclooxygenase-2 (COX-2) [21, 27] and the production of monocyte chemotactic protein-1 (MCP-1), IL-6, IL-1\textbeta, myeloperoxidase (MPO), MIP-1\alpha and MIP-2 in inflammatory conditions [26]. The detailed molecular mechanisms of this regulation remain, however, to be studied.

TRPA1 is shown to be involved in pain, hyperalgesia, and neurogenic inflammation [10, 16, 49, 50]. In OA-related pain, the role of TRPA1 has been investigated in studies by Moilanen et al. [27] McGaraughty et al. [51] and Okun et al. [52] using the MIA-model of OA. The two first-mentioned studies [27, 51] concluded TRPA1 to contribute to joint pain in experimental OA. In addition, Moilanen et al. [27] reported that TRPA1-deficient mice developed less severe cartilage changes following MIA injections. Accordingly, we showed here that TRPA1 is functionally expressed in chondrocytes. We also examined the possible functions of the channel by treating primary chondrocyte cultures with IL-1\textbeta and the selective antagonist HC-030031 [2, 53, 54]. Our results suggest an inflammatory and catabolic role for TRPA1 in human chondrocytes, as we found inhibition of TRPA1 to suppress the production of OA-related factors MMP-1, MMP-3, MMP-13, IL-6, and PGE\textsubscript{2}. These results were supported by experiments with cartilage from WT and TRPA1-deficient mice: following stimulation with IL-1\textbeta, MMP-3, IL-6, and PGE\textsubscript{2} production was lower in the cartilage from TRPA1-deficient mice than from WT animals. These results together suggest that TRPA1-activating
factors are present in OA joints, and that TRPA1 mediates, at least partly, OA-related pain, inflammation, and cartilage destruction in neuronal and nonneuronal cells in the joint.

Conclusions
In conclusion, we found the TRPA1 cation channel to be functionally expressed in primary human OA chondrocytes and in part to mediate inflammatory and catabolic effects, which are both original findings. The inflammatory and hypoxic environment in the OA joint is conducive to enhance the expression and activation of TRPA1. The presence and effects of TRPA1 in human OA cartilage as found in the present study, together with the previous findings on TRPA1 in experimentally induced OA [27, 51] propose an intriguing role for TRPA1 as a mediator and drug target in OA.

Additional file

Additional file 1: Supplementary data. Supplementary information to the “Methods”. (DOCX 23 kb)

Abbreviations
AIFC, allyl isothiocyanate; ANOVA, analysis of variance; COX-2, cyclooxygenase-2; EUSA, enzyme-linked immunosorbent assay; H2O2, hydrogen peroxide; HF, hypoxia-inducible factor; HRE, hypoxia response element; IL, interleukin; KO, knockout; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein-1; MIA, monosodium iodoacetate; MIP, macrophage inflammatory protein; MMP, matrix metalloproteinase; MPO, myeloperoxidase; NF-kB, nuclear factor-kappa B; NO, nitric oxide; OA, osteoarthritis; PGE2, prostaglandin E2; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SEM, standard error of the mean; TLR, toll-like receptor; TNF, tumor necrosis factor; TRPA1, transient receptor potential ankyrin 1; WT, wild-type
Acknowledgements

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Availability of supporting data

All the data is reported in the manuscript.

Authors’ contributions

EN, IWH, LJM, E-LP, RMN, TM, KV, and EM contributed to the design of the study and to the acquisition, analysis and interpretation of the data. EM conceived and supervised the study. EN drafted the manuscript and all authors revised the manuscript critically for important intellectual content and have approved the final version of the manuscript for submission.

Authors’ information

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The patients in this study fulfilled the American College of Rheumatology classification criteria for OA [33] and the study was approved by the Ethics Committee of Tampere University Hospital, Tampere, Finland (reference number R09116), and carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients.

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Not applicable.

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TRPA1 Expression is Downregulated by Dexamethasone and Aurothiomalate in Human Chondrocytes: TRPA1 as a Novel Factor and Drug Target in Arthritis

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Transient receptor potential ankyrin 1 (TRPA1) is a ligand-gated membrane-bound cation channel. TRPA1 has been largely studied in neurons, where it mediates pain and neurogenic inflammation and acts as a chemosensor for harmful exogenous compounds.\(^1\)\(^2\) More recently, TRPA1 has been found to be activated also by endogenous compounds formed in inflammatory conditions characteristic to arthritis, such as reactive oxygen and nitrogen species.\(^3\)

We have recently discovered that TRPA1 is functionally expressed in primary human osteoarthritic chondrocytes,\(^4\) where it upregulated the production of mediators related to arthritis: interleukin (IL)-6, prostaglandin E\(_2\) and matrix metalloproteinases 1, 3 and 13.\(^4\) Furthermore, we showed in monosodium iodoacetate-induced experimental arthritis that TRPA1 activation mediates inflammation, cartilage degradation and pain.\(^5\) TRPA1 thus emerges as a novel factor associated with arthritis. Therefore, we investigated the effects of disease-modifying antirheumatic drugs methotrexate, sulfasalazine, hydroxychloroquine and aurothiomalate, glucocorticoid dexamethasone and non-steroidal anti-inflammatory drug ibuprofen on the expression of TRPA1 in human chondrocytes.

Human T/C28a2 chondrocytes\(^6\) were cultured with the aforementioned drugs, along with IL-1β, which is recently found to upregulate TRPA1 expression in chondrocytes.\(^4\) Dexamethasone and aurothiomalate inhibited TRPA1 mRNA expression in a dose-dependent manner (figure 1A), while methotrexate (10 μM), sulfasalazine (10 μM), hydroxychloroquine (10 μM) and ibuprofen (10 μM) had no effect. Dexamethasone and aurothiomalate also decreased TRPA1 protein levels (figure 1C).

Next, we examined if the observed drug effects on TRPA1 expression levels are functional, that is, if they are translated to changes in TRPA1-mediated calcium influx. In chondrocytes, which had been cultured in the presence of IL-1β, TRPA1-mediated calcium influx was increased; while that effect was reversed in cells which had been cultured with a combination of IL-1β and dexamethasone or aurothiomalate (figure 1D,E), confirming

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**Key messages**

**What is already known about this subject?**

- Transient receptor potential ankyrin 1 (TRPA1) is a membrane-associated cation channel primarily studied in sensory neurons, where it acts as a chemosensor of pungent compounds and mediates pain.
- TRPA1 has recently emerged as a potential factor/mediator in arthritis; we have shown TRPA1 to mediate inflammatory and catabolic responses in primary human chondrocytes, as well as cartilage degradation, inflammation and pain in an experimental model of arthritis.

**What does this study add?**

- This study shows that anti-inflammatory drugs dexamethasone and aurothiomalate downregulate the expression of functional TRPA1 in human chondrocytes.

**How might this impact on clinical practice?**

- These results show a previously unknown mechanism of action for dexamethasone and aurothiomalate via downregulation of TRPA1, and thus provides a novel concept for the development of drugs for arthritis with analgesic and disease modifying properties.
Figure 1  Dexamethasone and aurothiomalate inhibit TRPA1 expression in human chondrocytes. Human chondrocytes (primary human chondrocytes or T/C28a2 chondrocyte cell line) were cultured with IL-1β alone or in combination with the anti-inflammatory compound dexamethasone or aurothiomalate, or with the selective NF-κB inhibitor PDTC at concentrations given in the figure. (A, B) For TRPA1 mRNA expression analysis, human T/C28a2 chondrocytes (A) were incubated for 6 hours and the experiments were carried out in quadruplicate; primary chondrocytes (B) were incubated for 24 hours and the experiments were carried out in duplicate and repeated with cells from six donor patients. Total RNA was extracted and TRPA1 mRNA levels were measured by quantitative RT-PCR (TaqMan Gene Expression Assay for human TRPA1, Hs00175798_m1), and the results were normalised against GAPDH mRNA levels. (C) For TRPA1 protein analysis human T/C28a2 chondrocytes were incubated for 24 hours after which proteins were extracted and immunoprecipitated with TRPA1 antibody (SAB2105082, Sigma-Aldrich), and TRPA1 was detected with Western blot (with primary antibody: NB110-40763, NovusBiologicals). The figure shows one representative blot of six independent experiments with similar results. (D, E) For Ca²⁺ influx analysis human T/C28a2 chondrocytes were incubated with IL-1β alone or in combination with dexamethasone or aurothiomalate for 24 hours. Thereafter, the cells were loaded with Fluo-3-AM and the TRPA1-mediated Ca²⁺ influx was measured by Victor3 multilabel counter at excitation/emission wavelengths of 485/535 nm at 1/s frequency. In the measurements, basal fluorescence was first recorded for 15 s and thereafter the selective TRPA1 agonist AITC was added and the measurements were continued for 30 s. The results were normalised against the background and expressed as a mean of eight measurements. In (E), AUC from 15 to 45 s was calculated. Results are expressed as mean±SEM, results in (A–C) are expressed as a percentage in comparison to IL-1β-treated samples which were set as 100%. Statistical significance of the results was calculated with one-way or repeated measures ANOVA followed by Bonferroni post-test. Data were analysed using GraphPad InStat V.3.0.

AITC, allyl isothiocyanate; ANOVA, analysis of variance; AUC, area under the curve; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; NF-κB, nuclear factor kappa B; OA, osteoarthritis; PDTC, ammonium pyrrolidinedithiocarbamate; RT-PCR, reverse transcription PCR; TRPA1, transient receptor potential ankyrin 1.
the functional downregulation of TRPA1 expression by these two drugs.

To confirm the effects of dexamethasone and aurothiomalate in primary human chondrocytes, cells were isolated from cartilage samples obtained from joint replacement surgery and cultured as described previously. Dexamethasone and aurothiomalate downregulated IL-1β-induced TRPA1 expression also in these primary chondrocytes (figure 1B).

The current results show for the first time that two anti-inflammatory drugs which are effective in the treatment of arthritis and retard cartilage degradation, namely dexamethasone and aurothiomalate, downregulate the expression of TRPA1 in human chondrocytes. Notably, inhibition of the transcription factor nuclear factor kappa B (NF-κB) also downregulated TRPA1 expression (figure 1A,B). Accordingly, Hatano et al reported recently that the TRPA1 promoter has at least six putative NF-κB binding sites; they also showed NF-κB to be involved in the induction of TRPA1 in synoviocytes. Glucocorticoids as well as aurothiomalate have been shown to inhibit NF-κB activation. Therefore, the downregulation of TRPA1 expression by these drugs may occur, at least in part, via inhibition of NF-κB.

The present findings, together with previous results, strongly suggest TRPA1 as a novel factor and drug target in arthritis.

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Competing interests None declared.

Patient consent Written informed consent was obtained from all patients.

Ethics approval Ethics Committee of the Pirkanmaa Hospital District, Finland.

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Data sharing statement All the data is reported in the manuscript.

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REFERENCES
TRPA1 as a Factor and Drug Target in Osteoarthritis: TRPA1 (Transient Receptor Potential Ankyrin 1) Mediates Interleukin-6 Expression in Chondrocytes

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Effects of FGF-2 and FGF Receptor Antagonists on MMP Enzymes, Aggrecan and Type II Collagen in Primary Human OA Chondrocytes

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Original research article

Effects of FGF-2 and FGF receptor antagonists on MMP enzymes, aggrecan and type II collagen in primary human OA chondrocytes

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Abstract

Objectives: FGF-2 belongs to the family of fibroblast growth factors and is found in the synovial fluid from patients with osteoarthritis (OA). The purpose of the present study was to investigate the effects of FGF-2 on human OA cartilage/chondrocytes by assessing the associations of FGF-2 with cartilage degrading enzymes MMP-1 and MMP-13 and the major cartilage matrix components aggrecan and collagen II.

Methods: Cartilage samples were obtained from 97 OA patients undergoing total knee replacement surgery. Cartilage tissue cultures were conducted and levels of FGF-2, MMP-1 and MMP-13 released into the culture medium were measured by immunoassay. The effects of FGF-2 on the expression of MMP-1, MMP-13, aggrecan and collagen II were further investigated in cultures of primary human OA chondrocytes.

Results: FGF-2, MMP-1 and MMP-13 were released into the culture medium from cartilage samples obtained from patients with OA. FGF-2 concentrations correlated positively with the concentrations of MMP-1 (r=0.414, p<0.001) and MMP-13 (r=0.362, p<0.001). FGF-2 also up-regulated the production of MMP-1 and MMP-13, and down-regulated the expression of aggrecan and collagen II, in human OA chondrocyte cultures. Further, FGF receptor antagonists AZD4547 and NVP-BGJ398 down-regulated the expression of MMP-1 and MMP-13 and up-regulated aggrecan and collagen II both in the absence and in the presence of exogenous FGF-2.

Conclusions: The present results suggest that, in contrast to its growth factor like effects in some other tissues, FGF-2 induces catabolic effects in human OA cartilage. Moreover, FGF-receptor antagonists showed promising beneficial effects on the balance of catabolic and anabolic factors within OA cartilage.
Introduction

The fibroblast growth factor (FGF) family represents an interesting group of molecules that are involved in the regulation of connective tissue development and metabolism. Recently, two members of the FGF family, FGF-2 and FGF-18, have been suggested to have a regulatory role also in cartilage (1). According to literature, these effects are mediated primarily through receptors FGFR1 and FGFR3 (2). Activation of FGFR3 induces cartilage matrix synthesis (3). FGF-18 is regarded as an anabolic growth factor which signals primarily through FGFR3 and has a role in articular cartilage repair and chondrogenesis (3-5). The effects of recombinant FGF-18, sprifermin, on the progression of osteoarthritis (OA) have been investigated in a Phase II clinical trial and the results were recently published (6). Interestingly, sprifermin treatment had a dose dependent reducing effect on the loss of total and lateral femorotibial cartilage thickness and volume and joint space narrowing in the lateral femorotibial compartment (which were secondary end points in the study). In contrast, FGF-2 has been suggested to signal also through FGFR1 and the role of FGF-2 in cartilage metabolism is controversial. Some studies have indicated a catabolic or anti-anabolic role (7-11), while others have reported chondroprotective effects (12-15).

OA is the most common joint disease worldwide. It is a slowly progressing inflammatory disease of the joints, which involves cartilage degradation, synovial inflammation and subchondral bone remodeling (16,17). Cartilage degradation, which is the hallmark of pathogenesis in OA, is caused by an imbalance between the production of catabolic and anabolic mediators within the joint. This imbalance increases as OA progresses and degradation exceeds regeneration, shifting the balance towards catabolism. Currently OA remains an incurable disease with an unmet need for disease modifying OA drugs (DMOADs), which could enable the prevention, retardation or repair of cartilage destruction.
When the OA process has reached the catabolic phase, the production of matrix degrading enzymes is increased and the *de novo* production of extracellular matrix components, such as aggrecan and collagen II, is decreased (17). MMP-1 (collagenase 1) and MMP-13 (collagenase 3) are enzymes that efficiently degrade collagen II, which is the main collagen component in articular cartilage. These MMPs are secreted in response to different stimuli, such as cytokines and growth factors from arthritic joints (18). Recently, MMP-13 has gained special attention as a cartilage matrix degrading enzyme and is thought to be the main collagen degrading enzyme in OA. Both MMP-1 and MMP-13 are present in normal and OA cartilage and the expression of MMP-13 has been shown to be significantly increased in late stage human OA chondrocytes (19,20).

In OA patients FGF-2 is found in the synovial fluid (9,21) and the expression of its type 1 receptor is up-regulated. FGF-2 may therefore expedite the progression of OA as FGFR1 mediates catabolic responses (11). That assumption is supported by studies in *fgfr1* KO animals (22). We hypothesized that despite having growth factor like functions in some other cell types and animal models (13,15), in human OA chondrocytes FGF-2 may function as a catabolic mediator and a potential drug target. The objective of the current study was to test the hypothesis by investigating the effects of FGF-2 and FGF receptor antagonists on the expression of catabolic enzymes MMP-1 and MMP-13, and the cartilage matrix components aggrecan and collagen II in human OA chondrocytes.
Materials and Methods

Patients and clinical studies

The patients in this study fulfilled the American College of Rheumatology classification criteria for OA (23). The clinical studies were performed as previously described by Koskinen et al. (24). In brief, cartilage tissue samples were collected from 97 patients (60 females and 37 males, body mass index (BMI) 30.9 ± 0.6 kg/m², age 69.8 ± 1.0 years; mean ± SEM) with OA undergoing total knee replacement surgery at Coxa Hospital for Joint Replacement, Tampere, Finland. Cartilage samples were processed as described below, and the amounts of FGF-2, MMP-1 and MMP-13 released by the cartilage ex vivo during a 42-hour incubation were measured as described below. The study was approved by the Ethics Committee of Tampere University Hospital, Tampere, Finland, and carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from the patients.

Cartilage cultures

Cartilage cultures were carried out as previously described by Koskinen et al. (25). Leftover pieces of OA cartilage from knee joint replacement surgery were used. Full-thickness pieces of articular cartilage from femoral condyles, tibial plateaus and patellar surfaces showing macroscopic features of early OA were removed aseptically from subchondral bone with a scalpel, cut into small pieces and cultured in DMEM with GIBCO GlutaMAX-I supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (250 ng/ml) (all from Invitrogen/Life Technologies, Carlsbad, CA, USA) at 37°C in a humidified 5% carbon dioxide atmosphere. Cartilage samples were incubated for 42 hours. Thereafter, the culture media were collected and stored at -20°C until
analyzed; the cartilage explants were weighed and the results were expressed per 100 milligrams of cartilage.

**Primary chondrocyte experiments**

Primary chondrocyte experiments were carried out as previously described (25). The obtained cartilage was processed the same way as for cartilage cultures (see above). Pieces of cartilage were washed with PBS, and chondrocytes were isolated by enzymatic digestion for 16 hours at 37°C in a shaker by using a collagenase enzyme blend (1 mg/ml Liberase TM Research Grade medium; Roche, Mannheim, Germany). Isolated chondrocytes were washed and plated on 24-well plates (2.0 × 10^5 cells/ml) in culture medium (DMEM U1, Lonza, Basel, Switzerland, supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (250 ng/ml) all from Gibco/Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum. Before experiments the cells were subjected to serum starvation for 24 h. During the experiments the cells were treated with FGF-2 (200 ng/ml recombinant human FGF-basic; Peprotech, NJ, USA) and IL-1β (100 pg/ml recombinant human IL-1β; R&D Systems Europe Ltd, Abingdon, UK) which was used as a control compound, for 24 hours. In FGFR antagonist experiments we used selective FGFR receptor antagonists AZD4547 and NVP-BGJ398 (26,27). AZD4547 targets FGFR1/2/3 with IC_{50} values of 0.2 nM/2.5 nM/1.8 nM and shows weaker activity against FGFR4 (IC_{50} of 165 nM) (26). NVP-BGJ398 inhibits FGFR1/2/3 with IC_{50} values of 0.9 nM/1.4 nM/1 nM, respectively, and shows weaker activity against FGFR4 (IC_{50} of 60 nM) (27). The cells were treated with increasing concentrations of AZD4547 or NVP-BGJ398 (1-300 nM; both from Selleckchem, Munich, Germany) with and without exogenous FGF-2 added into the culture medium (200 ng/ml) for 24 h. The cells were pre-treated with AZD4547 and NVP-BGJ398 for 1 h prior to the addition of FGF-2, and then further incubated for 24 h.
Concentrations of MMP-1 and MMP-13 in culture media were determined by immunoassay as described below. To investigate mRNA expression total RNA was extracted and quantitative reverse transcription PCR was carried out as described below.

**Immunoassay**

Concentrations of FGF-2, MMP-1 and MMP-13 in medium samples were determined by ELISA with commercial reagents (R&D Systems Europe Ltd, Abingdon, UK).

**RNA extraction and quantitative RT-PCR**

RNA extraction and quantitative RT-PCR was performed as previously described (28). At the indicated time points, culture medium was collected and total RNA extraction was carried out with GenElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich, St. Louis, MO, USA). The amount of RNA was measured with a spectrophotometer. Total RNA was then reverse-transcribed to cDNA using TaqMan Reverse Transcription reagents and random hexamers (Applied Biosystems, Foster City, CA, USA) in 10 μL reaction volume. After the transcription reaction, the obtained cDNA was diluted 1:20 with RNase-free water. Quantitative PCR was performed using TaqMan Universal PCR Master Mix and ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). The primer and probe sequences and concentrations were optimized according to the manufacturer's guidelines in TaqMan® Universal PCR Master Mix Protocol part number 4304449 revision C (Applied Biosystems, Foster City, CA, USA) and are listed in Table 1. The probes contained 6-FAM as 5’-reporter dye and TAMRA as 3’-quencher. Primers and probes were obtained from Metabion (Martinsried, Germany). TaqMan Gene Expression assays for human FGFR1
(Hs00915142_m1), human FGFR2 (Hs01552926_m1), human FGFR3 (Hs00179829_m1) and human FGFR4 (Hs01106908_m1) were purchased from Life Technologies (Life Technologies Europe BV, Bleiswijk, the Netherlands). PCR cycling parameters were: incubation at 50 ºC for 2 min, incubation at 95 ºC for 10 min, and thereafter 40 cycles of denaturation at 95 ºC for 15 s and annealing and extension at 60 ºC for 1 min. The relative mRNA levels were quantified using a standard curve method as described in the Applied Biosystems User Bulletin. For the TaqMan Gene Expression assays the ΔΔCt method was used. When calculating results, mRNA expression levels were first normalized against GAPDH mRNA levels.

Statistical analysis

Data were analyzed using SPSS version 20.0 for Windows software (SPSS Inc, Chicago, IL, USA) and Graph-Pad InStat version 3.00 software (GraphPad Software, San Diego, CA, USA). The results are presented as means ± SEM unless otherwise indicated. The data were natural log (LN) transformed in order to obtain a normal distribution and Pearson’s correlation analysis was carried out, with r values over +0.3 and under -0.3 considered to indicate a correlation. Differences between groups were tested by one-way analysis of variance (ANOVA) or repeated-measures ANOVA, followed by Bonferroni correction for multiple comparisons when appropriate. Differences were considered significant at *p < 0.05, **p < 0.01 and ***p < 0.001.
Results

The correlations between FGF-2 and MMP-1 and MMP-13 released from human OA cartilage

Ninety-seven OA patients undergoing total knee replacement surgery were included in the study. Cartilage samples from these patients were cultured for 42 h and the concentrations of FGF-2, MMP-1 and MMP-13 released from the cartilage were determined in the culture medium. OA cartilage released all of the measured factors: FGF-2 28.4 ± 1.5 pg/100mg cartilage, MMP-1 12.7 ± 1.4 ng/100mg cartilage and MMP-13 1.0 ± 0.1 ng/100mg cartilage. Interestingly, the levels of released FGF-2 correlated positively with those of MMP-1 (r = 0.414, p < 0.001) and MMP-13 (r = 0.362, p < 0.001) (Fig. 1).

The effects of FGF-2 on MMP-1, MMP-13, aggrecan and collagen II expression in human OA chondrocytes

FGF-2 has been shown to signal through receptors FGFR1-4. To confirm that the OA chondrocytes expressed these receptors, we determined the mRNA levels of FGFR1-4. The chondrocytes expressed all of the receptors, with the expression of FGFR1 being highest and expression of FGFR4 being very low (Fig. 2).

Human OA chondrocytes were cultured in the presence or absence of FGF-2 for 24 hours. The expression of MMP-1 and MMP-13 was increased when FGF-2 was added into the culture, compared to untreated control cells (Fig. 3). The expression of cartilage matrix components aggregan and collagen II, on the other hand, was significantly reduced when FGF-2 was added into the culture (Fig. 3). IL-1 was used as a control compound, and FGF-2 had qualitatively similar but smaller effects than
IL-1, which also increased the expression of MMP-1 and MMP-13 and decreased the expression of aggrecan and collagen II in human OA chondrocytes (Table 2).

The effects of FGF receptor antagonists AZD4547 and NVP-BGJ398

Human OA chondrocytes were treated with increasing concentrations of FGFR antagonists AZD4547 and NVP-BGJ398 in the presence or absence of FGF-2 for 24 h. MMP-1 and MMP-13 protein levels in control samples following 24 h incubation were 221.5 ± 77.0 ng per 10^6 cells, n=5 and 11.0 ± 2.0 ng per 10^6 cells, n=5 (Fig. 5), respectively. Both AZD4547 and NVP-BGJ398 decreased the production of catabolic factors MMP-1 and MMP-13 in a dose dependent manner (Fig. 4-5). This effect was observed at the mRNA (Fig. 4) and protein level (Fig. 5), and interestingly, in the presence and absence of exogenous FGF-2.

Due to the interesting effects of these FGFR antagonists in chondrocytes also when exogenous FGF-2 was not added into the culture, we decided to examine the FGF-2 production of these cells in primary human OA chondrocyte cultures. The cells released FGF-2 1.8 ± 1.0 ng per 10^6 cells, n=5, during 24 h incubation.

Next, we investigated the effects of AZD4547 and NVP-BGJ398 on the expression of major extracellular matrix components aggrecan and collagen II. Both antagonists had a beneficial effect on the expression of these extracellular matrix components by up-regulating the production of aggrecan and collagen II in a dose dependent manner, in the presence and absence of exogenous FGF-2 (Fig. 6). Taken together, the FGFR antagonists shifted the balance between catabolic and anabolic mediators towards anabolism, showing a beneficial effect on the metabolism of cartilage.
Discussion

FGF-2 belongs to the family of fibroblast growth factors. In the present study we investigated the effects of FGF-2 and two commercially available FGFR antagonists on human OA cartilage and chondrocytes with the hypothesis that FGF-2 functions as a catabolic mediator and a potential drug target. The present results suggest that, in contrast to its growth factor role in some other tissues, FGF-2 induces catabolic effects in human OA cartilage and primary human OA chondrocytes by up-regulating the production of collagenses MMP-1 and MMP-13 and down-regulating the expression of cartilage matrix components aggrecan and collagen II.

Four FGF receptors have been described (29). We investigated the receptor expression profiles in OA chondrocytes used in this study and found the expression of FGFR1 to be the highest of the four receptors and significantly higher than that of FGFR3. Previously FGFR1 and FGFR3 have been found to be the most prominent FGF receptors in normal human articular cartilage (11). In cartilage, activation of FGFR1 has been reported to induce mainly catabolic effects (11), whereas activation of FGFR3 has been associated principally with anabolic effects (3). Further, FGFR1 expression has been reported to be increased and FGFR3 expression decreased in degenerative OA cartilage compared to healthy cartilage (9,11), supporting our present results on the receptor expression profiles in OA chondrocytes. Surprisingly, unlike in human OA cartilage, in murine knee articular cartilage FGFR2 and 4 have been shown to be the most prominent receptors (15). In human OA cartilage the role of FGFR1 in mediating the effects of FGF-2 is supposed to be pronounced, because it is the most widely expressed FGF receptor especially in human OA cartilage, but not in murine cartilage. The predominant expression of FGFR2 and 4, and the up-regulation of FGFR3 in response to FGF-2 treatment in murine articular cartilage may explain why FGF-2 has been reported to have anabolic
effects on murine cartilage (15). However at present the precise roles of FGFR2 and FGFR4 are not
clearly understood.

As the receptor expression profile in the primary human OA chondrocytes was shifted towards
catabolic FGFR1, we wanted to explore if this had functional consequences in OA cartilage. The
results showed that human OA cartilage released FGF-2, MMP-1 and MMP-13 into the culture
medium at detectable levels. The levels of FGF-2 released from the cartilage samples correlated
positively with levels of MMP-1 and MMP-13, suggesting a link between the production of FGF-2
and these catabolic factors in articular cartilage. Next, we aimed to investigate the causality of these
correlations in primary human OA chondrocyte cultures. FGF-2 up-regulated the production of matrix
degrading enzymes MMP-1 and MMP-13 and at the same time down-regulated the expression of
matrix components aggrecan and collagen II. FGF-2 was found to increase MMP-1 and MMP-13
mRNA levels in chondrocytes and protein levels in the culture supernatant, measured by quantitative
RT-PCR and immunoassay, respectively. The latter measures total MMP-1 and MMP-13
concentrations but unfortunately does not distinguish between the pro-form and active protein. These
findings of the effects of FGF-2 in chondrocyte cell cultures are supported by previous studies
(7,9,10,30), but there are also contradictory findings indicating a chondroprotective role for FGF-2 in
human articular cartilage explants (12) and KO mouse models (13-15). These contradicting results
can be suggested to result from interspecies variation as FGF-2 seems to have species-specific
functions (15). This does not, however, explain the differing results obtained from human cartilage
explants (12). One possible explanation for these differing results is that the cartilage samples utilized
by Sawaji et al. (12) were obtained from non-OA patients, with no initial macroscopic degradation of
the cartilage. A likely mechanistic explanation for the discrepancies between FGF-2 results are
different FGF receptor expression profiles between species, cell types and progression stages of OA
(9,11,15) as discussed above.
Since in this study exogenous FGF-2 exerted catabolic and anti-anabolic effects in primary human OA chondrocytes, we decided to investigate whether these effects could be counteracted with FGF receptor antagonists. Recently described selective FGF receptor antagonists AZD4547 (26) and NVP-BGJ398 (27) were used. Both compounds have been reported to antagonize FGF receptors 1-3 at nanomolar concentrations, with highest selectivity towards FGFR1 (26,27). As hypothesized, these antagonists counteracted the effects of FGF-2 in a dose-dependent manner resulting in decreased production of MMP-1 and MMP-13 and increased expression of aggrecan and collagen II in the presence of exogenous FGF-2. Interestingly, this effect was observed also in the absence of added exogenous FGF-2 suggesting that OA chondrocytes produce FGF-2 in amounts involved in the pathogenesis of cartilage degradation in OA joints. Therefore we measured the FGF-2 synthesis in OA chondrocytes and found the cells to produce FGF-2, and FGF-2 was also released from OA cartilage in tissue cultures. FGF-2 has previously been reported to be released also from healthy human articular cartilage, where it has been suggested to be sequestered within the extracellular matrix by the heparan sulfate proteoglycan perlecan (31). However, it has been unclear whether FGF-2 is produced by cartilage, or is it produced by other tissues of the joints and diffused via synovial fluid into the cartilage. In the present study, we showed that primary chondrocytes from OA patients produced FGF-2 in monolayer cell cultures. This result is supported by the findings of Vincent et al. showing that porcine chondrocytes produce FGF-2 in alginate bead culture (31). Further, when we treated OA chondrocytes with FGFR antagonists the balance of catabolic and anabolic factors was beneficially shifted towards anabolism. Therefore, the use of FGFR/FGFR1 antagonists as disease modifying OA drugs (DMOADs) is an interesting prospect. This is also supported by recent studies with \( fgfr1 \) KO mouse models (22). However, as the present promising results are based on \( \textit{in vitro} \) data they should be interpreted accordingly and further studies are needed to validate the clinical significance of these observations in the pathogenesis of OA.
FGF-18 has been shown to signal principally through FGFR3 leading to anabolic effects (3). The pharmaceutical company Merck Serono is investigating the effects of an intra-articular recombinant FGF-18 drug, sprifermin, on osteoarthritis progression in humans in a Phase II clinical trial and the first results were published recently (6). Sprifermin treatment showed dose dependent reductions in the loss of total and lateral femorotibial cartilage thickness and volume and joint space narrowing in the lateral femorotibial compartment, which was a secondary end point in the study. However, sprifermin showed no statistically significant dose response in the primary efficacy end point, which was a change in central medial femorotibial compartment cartilage thickness. Considering the predominant expression of FGF receptor type 1 in OA cartilage, and the catabolic and anti-anabolic functions of FGF-2 observed in the present and previous studies, it could also be beneficial to investigate the use of FGF-2 synthesis inhibitors or FGFR1/FGFR antagonists alone or the two former also in combination with FGF-18 as a novel drug approach in OA.

**Conclusions**

Currently, OA remains an incurable disease and there is a critical need to develop disease modifying drugs for OA (DMOADs) which could shift the balance of catabolic and anabolic responses to favor the latter, and enable the prevention, retardation or repair of cartilage destruction. In the present study, we found that FGF-2 produced catabolic effects in the OA cartilage. FGF-2 was released from OA cartilage in correlation to MMP-1 and MMP-13, and in chondrocyte cultures it enhanced the production of MMPs along with inhibitory effect on the de novo synthesis of cartilage matrix molecules aggrecan and collagen II. Further, FGF receptor antagonists reversed the catabolic and anti-anabolic effects of FGF-2 and, more importantly, had comparable beneficial effects also in the absence of exogenous FGF-2 in human OA chondrocytes.
FGF receptor antagonists showed a favorable effect on the balance of anabolic and catabolic factors produced by human OA chondrocytes supporting the idea that FGF receptor antagonists hold therapeutic potential in the treatment of osteoarthritis.

**Acknowledgements**

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**References**


Table 1. Primer and probe sequences and concentrations used in qPCR experiments

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<tr>
<th></th>
<th>Primer/Probe</th>
<th>Concentration</th>
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<tr>
<td>MMP-1 forward</td>
<td>TTGAAGCTGCTTACGAATTTCG</td>
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<tr>
<td>MMP-1 reverse</td>
<td>GAAGCCAAAAGGAGCTGATAGTGC</td>
<td>900 nM</td>
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<td>MMP-1 probe</td>
<td>CAGAGATGAAGTCCGGTTTTCAAAGGGA</td>
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<tr>
<td>GAPDH probe</td>
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MMP-1; matrix metalloproteinase 1, MMP-13; matrix metalloproteinase 13, AGC1; aggrecan 1, COL2A1; collagen II A1, GAPDH; glyceraldehyde 3-phosphate dehydrogenase

Table 2. IL-1β stimulated the production of MMP-1, MMP-13, aggrecan and collagen II in primary human OA chondrocytes

<table>
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<tr>
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<th>MMP-1</th>
<th>MMP-13</th>
<th>Aggrecan</th>
<th>Collagen II</th>
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<tr>
<td></td>
<td>mRNA</td>
<td>protein</td>
<td>mRNA</td>
<td>protein</td>
</tr>
<tr>
<td>control</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>IL-1β</td>
<td>1042 ± 268 *</td>
<td>344 ± 92X</td>
<td>632 ± 103 **</td>
<td>228 ± 32 *</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>62 ± 9 *</td>
<td>9 ± 1 ***</td>
</tr>
</tbody>
</table>

Primary human OA chondrocyte cultures were stimulated with IL-1β (100 pg/ml) for 24 h. MMP-1, MMP-13, aggrecan and collagen II mRNA expression was measured by quantitative RT-PCR and MMP-1 and MMP-13 production was detected by immunoassay. The mRNA expression levels were normalized against GAPDH mRNA levels. N=5 patients for protein production analysis (b, d) and n=10 patients for mRNA expression analysis (a, c, e, f). Results are represented as mean ± SEM. One-way ANOVA was performed and statistical significance is indicated as *p=0.0573, *p < 0.05, **p < 0.01 and ***p < 0.001.

IL-1β; Interleukin 1β, MMP-1; matrix metalloproteinase 1, MMP-13; matrix metalloproteinase 13
Figure legends

Figure 1.

Levels of FGF-2 released by human OA cartilage correlated positively with the levels of MMP-1 (a) and MMP-13 (b). Cartilage explants from OA patients (n=97) undergoing joint replacement surgery were incubated for 42 h and the levels of FGF-2, MMP-1 and MMP-13 released into the culture medium were measured by immunoassay. Results are LN transformed to obtain a normal distribution and analyzed using Pearson correlation.

Figure 2.

FGF receptor expression profiles of primary human OA chondrocytes. Chondrocytes were cultured for 24 h and the mRNA expression of FGFR1-4 was detected by quantitative RT-PCR. The mRNA expression levels were calculated with the ∆∆Ct method. Results from 3 patients were combined and the results are represented as mean + SEM.

Figure 3.

FGF-2 enhanced the production of MMP-1 (a, b) and MMP-13 (c, d) and down-regulated the expression of aggrecan (e) and collagen II (f) in primary human OA chondrocytes. Chondrocyte cultures were stimulated with FGF-2 (200 ng/ml) for 24 h. MMP, aggrecan and collagen II mRNA expression was detected by quantitative RT-PCR and MMP production was detected by immunoassay. The mRNA expression levels were normalized against GAPDH mRNA levels. Results from 5 patients were combined for protein production analysis (b, d) and 10 patients for mRNA expression analysis (a, c, e, f). Results are represented as mean ± SEM. One-way ANOVA was performed and statistical significance is indicated as *p < 0.05, **p < 0.01 and ***p < 0.001.
Figure 4

FGF-2 antagonists AZD4547 and NVP-BGJ398 down-regulated the expression of MMP-1 (a, b) and MMP-13 (c, d) in the presence and absence of exogenous FGF-2. Isolated primary human OA chondrocytes were treated with increasing concentrations of AZD4547 and BGJ398 (1 - 300 nM) in the presence and absence of exogenous FGF-2 (200 ng/ml) for 24 h. MMP-1 and MMP-13 mRNA levels were measured by quantitative RT-PCR, and the results were normalized against GAPDH mRNA levels. Results from 2-3 patients were combined. Results are expressed as percentage in comparison to untreated control samples. Values are expressed as mean ± SEM. Repeated measures ANOVA with Bonferroni’s post-test was performed and statistical significance is indicated as *p < 0.05, **p < 0.01 and ***p < 0.001 compared to control or FGF-2 treated samples.

Figure 5

FGF-2 antagonists AZD4547 and NVP-BGJ398 down-regulated the production of MMP-1 (a, b) and MMP-13 (c, d) expression in the presence and absence of exogenous FGF-2. Isolated primary human OA chondrocytes were treated with increasing concentrations of AZD4547 and BGJ398 (1 - 300 nM) in the presence and absence of exogenous FGF-2 (200 ng/ml) for 24 h. MMP-1 and MMP-13 protein levels in the culture media were measured by immunoassay. Results from 2-3 patients were combined. Results are expressed as percentage in comparison to untreated control samples. Values are expressed as mean ± SEM. Repeated measures ANOVA with Bonferroni’s post-test was performed and statistical significance is indicated as *p < 0.05, **p < 0.01 and ***p < 0.001 compared to control or FGF-2 treated samples.
Figure 6

FGF-2 antagonists AZD4547 and NVP-BGJ398 up-regulated the expression of aggrecan (a, b) and collagen II (c, d) expression in the presence and absence of exogenous FGF-2. Isolated primary human OA chondrocytes were treated with increasing concentrations of AZD4547 and BGJ398 (1 - 300 nM) in the presence and absence of exogenous FGF-2 (200 ng/ml) for 24 h. Aggrecan and collagen II mRNA levels were measured by quantitative RT-PCR, and the results were normalized against GAPDH mRNA levels. Results from 3 patients were combined. Results are expressed as percentage in comparison to untreated control samples. Values are expressed as mean ± SEM. Repeated measures ANOVA with Bonferroni’s post-test was performed and statistical significance is indicated as *p < 0.05, **p < 0.01 and ***p < 0.001 compared to control or FGF-2 treated samples.
Figure 1

A

LN MMP-1 (pg/mg cartilage)

LN FGF-2 (fg/mg cartilage)

\[ r = 0.414 \]

\[ p < 0.001 \]

B

LN MMP-3 (pg/mg cartilage)

LN FGF-2 (fg/mg cartilage)

\[ r = 0.362 \]

\[ p < 0.001 \]

Figure 2

<table>
<thead>
<tr>
<th>FGFR1</th>
<th>FGFR2</th>
<th>FGFR3</th>
<th>FGFR4</th>
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mRNA (% of FGFR1)