universität wien

DIPLOMARBEIT / DIPLOMA THESIS

Titel der Diplomarbeit / Title of the Diploma Thesis

„Molecular effects of galectins in human osteoarthritic chondrocytes“

verfasst von / submitted by

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angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Magistra der Pharmazie (Mag.pharm.)

Wien, 2017

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 449
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Diplomstudium Pharmazie
Betreut von / Supervisor:	O. Univ. Prof. Mag. Dr. Helmut Viernstein
Mitbetreut von / Co-Supervisor:	Assoc.-Prof. Mag. Dr. Stefan Tögel

Danksagungen

Zunächst möchte ich mich ganz herzlich bei O. Univ. Prof. Mag. Dr. Helmut Viernstein bedanken, der es mir ermöglicht hat, meine Diplomarbeit im Karl Chiari Lab for Orthopaedic Biology zu verfassen.

Stefan, danke für die einzigartige Möglichkeit, die du mir geboten hast! Ich hatte sehr viel Freude mit meinem Diplomarbeitsthema und der praktischen Arbeit, die damit verbunden war. Danke, für deine Geduld, deine Unterstützung und, dass du jederzeit für mich da warst! Ich hätte mir keinen besseren Betreuer wünschen können!

Dani, bei dir möchte ich mich auch ganz herzlich bedanken! Du warst für das positive Ergebnis meiner Diplomarbeit genauso wichtig und mitverantwortlich! Danke, dass ich dich immer fragen durfte, wenn ich nicht weiterwusste und, dass du soviel Geduld bei der praktischen Einschulung mit mir hattest!

Bettina, Ruth, Meli und Sonja! Danke auch euch, dass ihr mir immer mit Rat und Tat zur Seite gestanden seid und meine Arbeit im Labor so lustig und angenehm gemacht habt.

Ingrid, auch dir bin ich sehr dankbar, dass du mir vor allem im ersten Jahr meines Studiums immer wieder gut zugesprochen hast, und auch für die Unterstützung in den Jahren danach!

Michi, ohne dich hätte ich das Studium wahrscheinlich hingeschmissen! Dir bin ich besonders dankbar, für deine Unterstützung, deine Motivation und eine Freundschaft, die weit über das Studium hinausgeht! Danke, dass du das gemeinsam mit mir durchgezogen hast!

Philipp auch dir möchte ich dafür danken, dass du mir in der letzten Phase meines Studiums die nötige Kraft und Energie gegeben hast, die ich sehr dringend gebraucht habe! Dein positiver Zuspruch ist mir sehr wichtig, und mitverantwortlich für die letzte schwierige Phase!

Andreas mein Bruderherz, danke, dass du mir immer wieder in den Hintern getreten hast, wenn ich es gebraucht habe! Du bist mir immer ein Vorbild und hast mich dadurch angetrieben und motiviert das Studium zu Ende zu bringen!

Mama und Papa, euch gebührt mit Abstand die größte Dankbarkeit! Durch euren Glauben an mich, habt ihr meinen teilweise beschwerlichen Bildungsweg möglich gemacht! Danke, dass ihr mir die Zeit gelassen habt, die ich gebraucht habe! Ich weiß das sehr zu schätzen und habe es nie als selbstverständlich erachtet! Ich bin sehr froh, euch als Eltern zu haben! DANKE!

Abstract

Galectins are β-galactosides-binding proteins that can bind to cell-surface and extracellular matrix glycans. Galectins play an important role in many physiological and pathological processes. Galectins are known to be present in osteoarthritic degenerated cartilage. In the present study, the focus was set on the impact of galectin-1, -3 and -8 (Gal-1, -3 and -8) on chondrocytes of osteoarthritic cartilage. Osteoarthritis (OA) is a widespread degenerative joint disease that results in structural alteration of cartilage matrix and causes a painful breakdown of articular cartilage.

This study aimed to reveal the cellular activity of Gal-1, -3 and -8 in human OA chondrocytes. By the use of quantitative real time reverse transcription polymerase chain reaction (RT-qPCR), mRNA levels of IL1B, TNFA, MMP13 and RELA in Gal-1, -3 and -8 treated chondrocytes, were assessed. Following treatment with Gal-1, -3 and -8 for 30 min up to 32 h, IL1B, MMP13 and RELA mRNA levels increased over time. Moreover, our experiments revealed that the expression of IL1B, MMP13 and TNFA was up-regulated with increasing concentrations of Gal-1, -3 and -8. In agreement, enzyme linked immunosorbant assays (ELISA) showed an elevated secretion of pro-MMP-1, total MMP-3 and pro-MMP-13 after Gal-1 and Gal-3 treatment. Moreover, Western blot experiments indicated the participation of the NFκB pathway in Gal-1-mediated signaling.

Collectively, the findings of this study point to a potential role of Gal-1, -3 and -8 in the processes of cartilage destruction. Therefore, galectins, galectin-counterreceptors or galectin-mediated signaling might represent novel potential drug targets for OA in the future.

Zusammenfassung

Galektine sind β-galaktosidisch-bindende Proteine, die an Glykane von Zelloberflächen und extrazellulärer Matrix binden können. Galektine spielen eine wichtige Rolle in vielen physiologischen und pathologischen Prozessen. Es ist bekannt, dass Galektine in durch Osteoarthrose degeneriertem Knorpel vorkommen. In dieser Studie haben wir den Fokus auf den Einfluss von Galektin-1, -3 und -8 (Gal-1, -3 und -8) auf Chondrozyten von osteoarthritisch degeneriertem Knorpel gelegt. Osteoarthrose ist eine weitverbreitete, degenerative Gelenkskrankheit, die durch Veränderung der Knorpelmatrix in einem schmerzhaften Zusammenbruch des Gelenksknorpels resultiert.

Das Ziel dieser Studie war, die zelluläre Aktivität von Gal-1, -3 und -8 an humanen OA Chondrozyten aufzuzeigen.

Durch die Verwendung von quantitativer Echtzeit-Polymerasen-Kettenreaktion (RT-qPCR) konnte der Anstieg der mRNA Levels von IL1B, TNFA, MMPR13 und RELA in mit Gal-1, -3 und -8 behandelten Chondrozyten gezeigt werden. Zeitabhängige Experimente, die mit Gal-1, -3 und -8 für 30 Minuten bis zu 32 Stunden durchgeführt wurden, zeigten, dass die Genexpressionslevels von IL1B, MMP13 und RELA proportional mit der Zeit ansteigen. Weiters zeigen unsere Experimente, dass die Expression von IL1B, MMP13 und TNFA mit zunehmenden Gal-1, -3 und -8 Konzentrationen steigt. In Übereinstimmung damit, zeigten enzymatischen Immunadsorbtionsverfahren (ELISA) eine erhöhte Sekretion von pro-MMP-1, totalem MMP-3 und pro-MMP-13 nach Behandlungen mit Gal-1 und Gal-3. Dazu kommt, dass Western Blot Experimente darauf hindeuten, dass der NF-ΚB Signalweg an der Wirkung des Gal-1 beteiligt ist.

Zusammenfassend deuten die Ergebnisse dieser Studie darauf hin, dass Gal-1, -3 und -8 eine mögliche Rolle in der Knorpeldegeneration spielen. Daher könnten Galektine, Galektin-Rezeptoren oder Galektin-Signalwege neuartige Ansätze für die Entwicklung von Arzneimitteln in der OA Behandlung liefern.

1 Introduction

1.1 Anatomy of the human knee joint

The knee joint is the largest and most complex joint in the human body (Figure 1). Femur, tibia and patella form the osseous part of the joint. In fact, the knee joint consists of two separate joints. The thigh bone (the femur) and the large shin bone (the tibia) form the main knee joint. The kneecap (the patella) joins the femur forming the second joint, called the patellofemoral joint, to protect the front of the knee joint. The knee joint is embedded in an articular capsule with ligaments strapping the inside and outside of the joint (collateral ligaments) as well as crossing within the joint (cruciate ligaments). For a painless and smooth movement, the ends of articulating bones are covered by a very sleek layer of hyaline cartilage. The femur is a bicondylar joint. The proximal part consists of two cylindrical femoral condyles, and two slightly concave oval-shaped articular surfaces of the tibial condyles form the distal part.

The fibrocartilaginous lateral and medial menisci are located between the femoral condyles and the tibial plateau. The menisci follow rotation and increase the size of the contact surface between tibial plateau and femur (Lippert H, Lehrbuch der Anatomie, 8. Auflage, Elsevier GmbH München 2011).

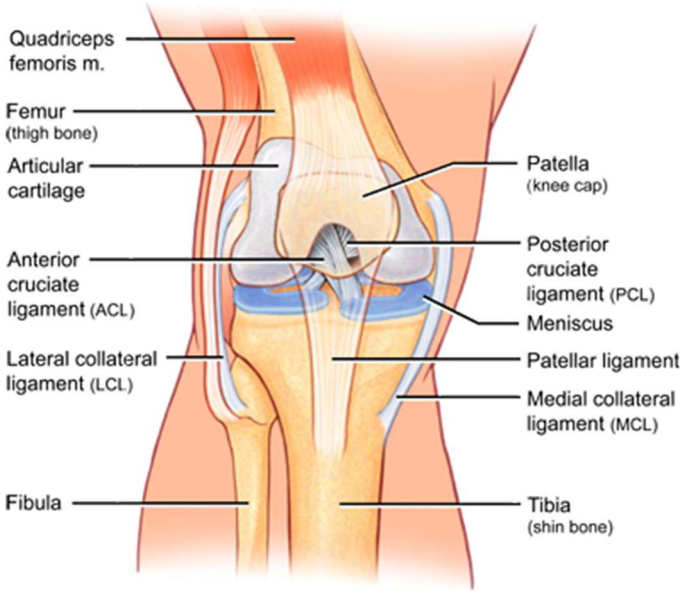

Figure 1 – Frontal view of a human knee joint
Schematically shown in the frontal view of a human knee joint are the articulating bones (tibia, femur and patella), the fibula, the menisci (blue), and ligaments (patellar, medial and lateral collateral ligaments). (Image taken from http://www.theknee.com)

1.2 Cartilage

Cartilage is an aneural and non-vascular connective tissue. There are three types of cartilage: hyaline cartilage, elastic cartilage and fibrocartilage, which differ in composition and the amount of collagen fibers. Articular cartilage is built of hyaline cartilage (Figure 2). The cartilage matrix harbors chondrocytes and chondroblasts. Chondroblasts are progenitor cells of the chondrocytes and originate from mesenchymal stem cells. Furthermore, chondroblasts are the active part of the cartilage cells as they can synthesize all components of the cartilage matrix. When chondroblasts stop synthesizing, they differentiate into chondrocytes, the predominant cartilage cells.

Chondrocytes are spherically formed, have a round cell nucleus and are located in matrix cavities called lacunae. The major amount of the extracellular matrix (ECM) is composed of type II collagen fibers, elastin fibers and a basic substance which is rich in aggrecan, the predominant proteoglycan in cartilage, Aggrecan consists of a core protein and keratin sulfate glycosaminoglycan (GAG) chains and is attached to

hyaluronic acid. Because of the high negative charge of aggrecan, it attracts water into the tissue to resist compression. About 80% of the matrix consists of water.

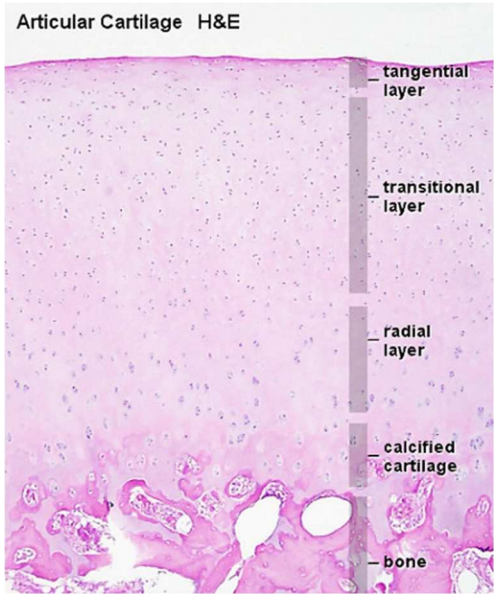

Figure 2 – Articular Cartilage
This image shows a histological section of articular cartilage stained with hematoxilin eosin (H&E).
The articular cartilage is organized in tangential, transitional and radial layers, differing in shape and arrangement of chondrocytes and collagen fibers. The calcified cartilage lies between the subchondral bone and cartilage. (Image taken from Lutz Slomianka, School of Anatomy and Human Biology at the University of Western Australia – http://www.lab.anhb.uwa.edu.au)

1.3 Osteoarthritis

Osteoarthritis (OA) is a very common, progressive and debilitating disease, typically affecting elderly people. There are two types of OA. Primary OA with no obvious predisposing cause, and secondary OA with a clearly defined predisposing pathology.
OA is a degradation of articular cartilage and subchondral bone. A deformed irregular articular surface is the consequence of the loss of cartilage and the collapse of neighboring subchondral bone. The extracellular matrix in OA joints is destroyed and therefore, articular cartilage can no longer serve as a shock absorber.
There are multiple risk factors for cartilage degeneration and OA:

- aging

- female gender
- abnormal loading: malalignment, trauma, heavy manual labor, obesity
- impaired joint
- subluxation
- crystal deposition disease

The dull and ill-defined pain can proceed from mechanical irritation, inflammation and sclerosis. Usually, the pain is exacerbated by joint motion and relieved at rest, but it can also become chronic at rest. This happens especially when the muscular support mechanism around the joint has been lost. Other symptoms are morning stiffness for about an hour and a localized instability along the joint line in knee OA (Figure 3).

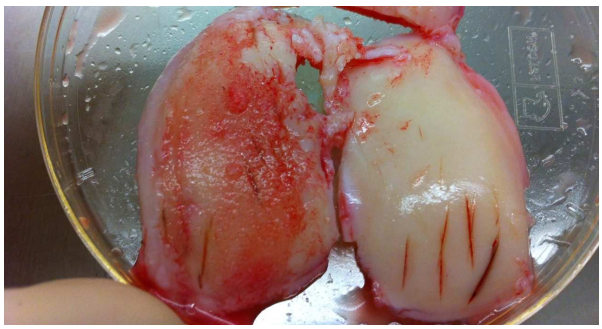

Figure 3 – Osteoarthritic cartilage of various degeneration degrees
This image shows femoral condyles of an OA patient that underwent total knee replacement surgery. The right part is in a relatively good condition in comparison to the very degenerated cartilage on the left side.

If lifestyle modification and NSAIDs (Non-steroidal anti-inflammatory drugs) are insufficient, joint replacement surgery is an option to restore motion. Thereby, the degenerated surfaces of the joint are replaced by metal and plastic prostheses (Figure 4).

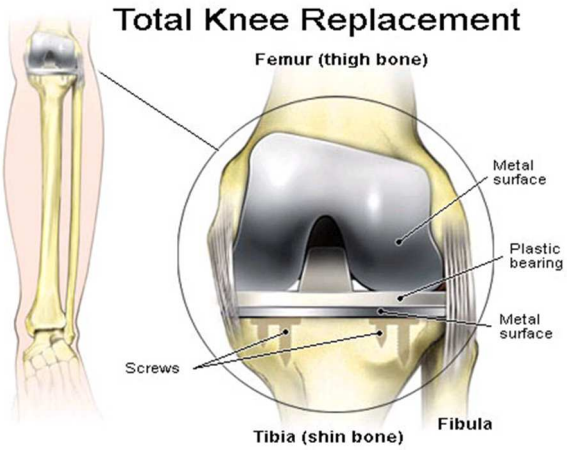

Figure 4 – Knee joint replacement
Metal surfaces and a plastic bearing in between replace the degenerated surfaces of tibia and femur. (Image taken from http://www.hipresurfacingcenter.com)

1.4 Chondrocytes

Chondrocytes have a mean diameter of approximately 13 µm and account for about 5–10% of the total cartilage volume. As mentioned above, they are the only cellular component of articular cartilage, located in matrix cavities called lacunae.

Under physiological conditions, chondrocytes maintain active membrane transport systems for the exchange of cations, including Na^+, K^+, Ca^{2+}, H^+, whose intracellular concentrations fluctuate with load and changes in the composition of cartilage matrix (Wilkins et al., 2000). The transportation of glucose is facilitated by several distinct glucose transporter proteins. Glucose is necessary as a major energy source and is an essential precursor for GAG synthesis (Goldring MB, 2006).

Chondrocytes are responsible for the anabolism and catabolism of ECM macromolecules while the matrix in turn maintains a state of dynamic equilibrium between the cellular environment and the structure of the cartilage (Lin et al., 2006).

Cytokines and transcription factors, such as TGF-β, FGF, IGF-1, are co-responsible in chondrocyte maturation and cartilage formation (Figure 5), whereas the exaggerated production of matrix metalloproteinases (MMP-1, MMP-3, MMP-8, MMP-13) and aggrecanases (A Disintegrin And Metalloproteinase with ThromboSpondin motifs; ADAMTS-4, ADAMTS-5) contributes to cartilage damage. These proteinases are found in synovial fluids of OA patients. The expression of these proteinases is in turn induced by the catabolic cytokines IL-1 and TNF-α. An imbalance between synthesis and degradation of ECM leads to OA.

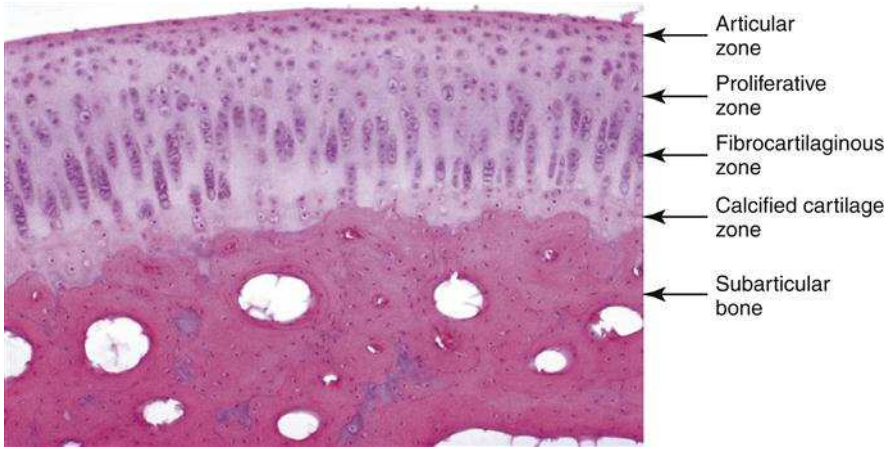

Figure 5 – Histology of articular cartilage
This image shows the various zones of articular cartilage consisting of chondrocytes and intercellular matrix. (Image taken from http://pocketdentistry.com/1-functional-anatomy-and-biomechanics-of-the-masticatory-system/)

1.5 Glycobiology

Glycobiology is the science of structure, biosynthesis and biology of saccharides. All cells and numerous macromolecules in nature carry an array of covalently attached sugars or sugar polymers (glycans). The field of glycobiology comprises a wide range of biological processes. The analysis of the type and extent of glycosylation is an important research approach for the understanding of diseases and disease processes. Glycobiological science is rather complex and many aspects such as the interaction of galectins with certain glycan structures in osteoarthritic cartilage are still largely unexplored (Varki et al., 2009).

1.5.1 Monosaccharides

Monosaccharides are the basic structural units of glycans. A monosaccharide is a carbohydrate that cannot be hydrolyzed into a simpler form. Every monosaccharide can be α- or β-glycosidically linked to another monosaccharide, to build linear or branched glycans. Acyclic monosaccharides form five- or six-membered rings because of their chemical stability. They are aldehydes or rather ketones of polyhydric alcohols and are termed aldoses or ketoses. Several hundred

monosaccharides exist in nature, but only a few of them can be found in animal glycans (Figure 6).

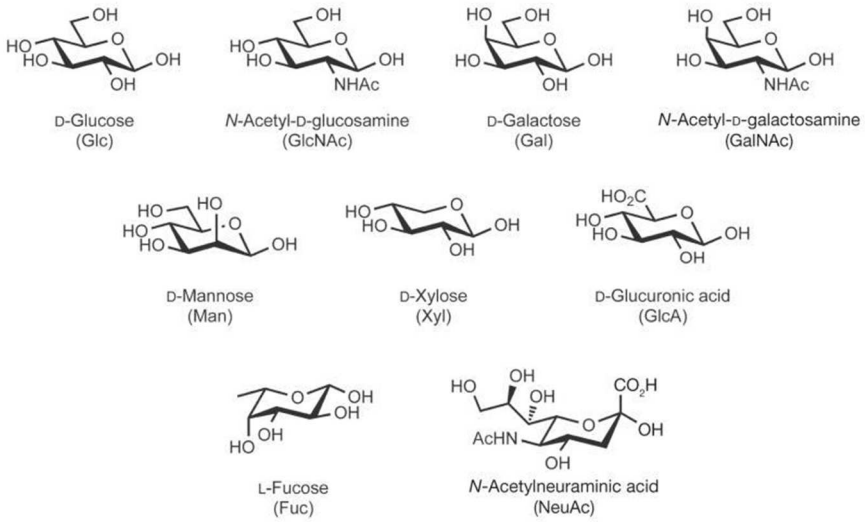

Figure 6 – Common monosaccharides in animal glycans
This images shows common monosaccharides in chair conformation with their widely used abbreviations. (Image taken from Varki et al., 2009)

D-xylose (Xyl) represents a pentose while D-glucose (Glc), D-galactose (Gal) and D-mannose (Man) belong to the hexoses. The hexosamines N-acetyl-D-glucosamine (GlcNAc) and N-acetyl-D-galactosamine (GalNAc) are hexoses with an amino group at the 2´-position. L-fucose (Fuc) is a deoxyhexose with the lack of a hydroxyl group at the 6´-position. Sialic acids are N- or O-substituted derivates of neuraminic acids with a nine-carbon backbone. N-acetylneuraminic acid (Neu5Ac or NeuAc) is one of the most prominent representatives of sialic acids.

1.5.2 Glycoproteins and glycosylation

Glycoproteins are proteins that are bound to covalently attached sugar residues. The chemical characteristics of the protein depend on the hydrophilic and polar characteristics of the attached sugar.
At the surface of cells, glycoproteins function as membrane proteins or as a part of the extracellular matrix. By regulating protein-protein and cell-cell interactions, glycans modulate many biological processes. Glycosylation takes an important part in recognition events involved in development, immunology and cancer biology. The

dysregulation of glycan synthesis causes a growing number of human joint diseases. Glycosylation is the complex post-translational process of adding one ore more sugar molecules to a protein. Usually, glycoproteins are more stable than their corresponding unglycosylated counterparts. The addition of a large glycan structure to the protein backbone can change the structure tremendously, and consequently the function of the polypeptide architecture to which they are attached. The classes of glycoproteins differ by protein linkage. Most commonly, N-linked glycans are attached to asparagines (Asn), whereas serine and threonine (Ser/Thr) residues can be modified with mucin-type O-linked glycans, glycosaminoglycans, or O-linked β-N-acetylglucosamine (O-GlcNAc) (Bond et al., 2007; Imperiali et al., 1999; Varki et al., 2009).

Most of the glycans and glycoconjugates are assembled within the endoplasmatic reticulum (ER)-Golgi pathway but the O-GlcNAc, O-Glc or O-Fuc containing cytoplasmic and nuclear proteins arise in the cytoplasm. For the glycosylation reaction, glycosyltransferases catalyze the transfer of carbohydrate moieties from donor substrates (nucleotide sugars) to acceptor molecules (e.g. proteins) (Varki et al., 2009).

1.5.3 Glycoprotein-binding proteins

Multivalent binding interactions, including cellular adhesion and signal transduction events are involved in a large number of biological processes mediated by glycoproteins. One important example for these biological processes is the binding of lectins with multivalent carbohydrates and glycoconjugates (Sacchettini et al., 2001).

Glycoprotein-binding proteins can be divided into two major groups: lectins and glycosaminoglycan-binding proteins (GAG-binding proteins). Glycosaminoglycan chains are linear polysaccharides and are much larger than other glycans. These chains contain around 80 sugar residues and their disaccharide building blocks are made up of an amino sugar (N-acetylglucosamine or N-acetylgalactosamine) and an uronic acid (glucoronic or iduronic acid) or galactose (Varki et al., 2009).

Lectins can be classified by sequence and structural homology and share their ability to specifically recognize certain carbohydrates. The most common animal lectins can

be classified into galectins, C- (Ca^{2+}), I- (belonging to the immunoglobulin superfamily) and P-type (mannose-6-phosphat) lectins (Varki et al., 2009).

1.5.4 Galectins

Galectins are animal lectins with a binding affinity for β-galactosides. Until now,15 mammalian galectins have been identified. Galectins can induce a variety of cellular processes by binding to cell surface and extracellular matrix glycans. Through protein-protein interactions with other cytoplasmic and nuclear proteins they exert influence on cellular functions such as intracellular signaling pathways. Each member of the galectin family contains conserved carbohydrate-recognition domains (CRDs) of about 130 amino acids, which are responsible for the carbohydrate-binding activity. Galectins can be classified into subfamilies, based on the number and organization of their CRDs. In dependence of their individual carbohydrate-binding preference they are either bivalent or multivalent (Liu et al., 2002; Yang et al., 2008) (Figure 7).

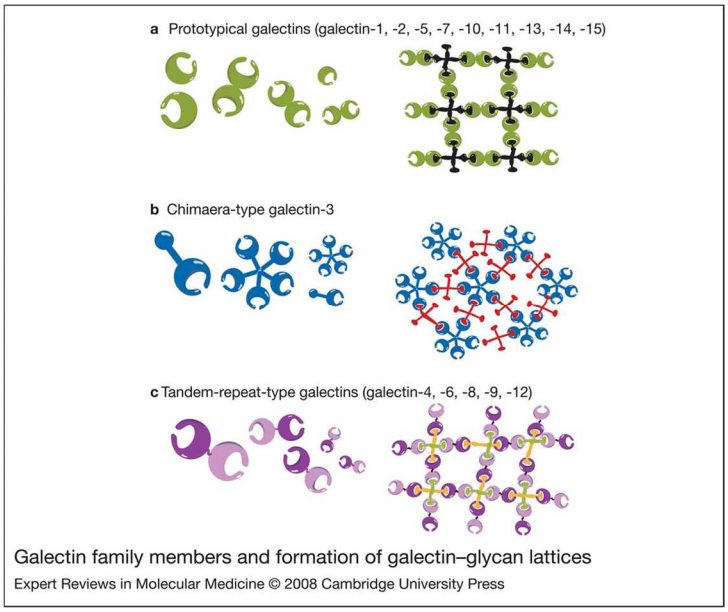

Figure 7 – Galectin family members and formation of galectin-glycan lattices
This image shows the three subtypes of galectins: a) prototypical galectins contain one CRD and often exist as dimers; b) chimaera-type galectin-3 forms pentamers upon binding to multivalent carbohydrates; c) tandem-repeat-type galectins are made of two-CRD galectins with two carbohydrate-binding sites. (Image taken from Yang et al.)

The prototypical galectins (Gal-1, -2, -5, -7, -10, -11, -13, -14, -15) have one CRD and the tandem-repeat-type galectins (Gal-4, -6, -8, -9, -12) contain two homologous

CRDs in a single polypeptide chain, separated by a linker of up to 70 amino acids. The chimaera-type Gal-3 contains a non-lectin N-terminal region connected to a CRD (Yang et al., 2008) (Figure 7). Gal-5, -6, -11, -14, -15 are not found in humans. While Gal-5 and -6 can be found in rodents, Gal-11, -14 and -15 occur in sheep and goats. family members lack any signal sequence for transport into the endoplasmic reticulum (ER) and are therefore not secreted through the classical ER/Golgi pathway. Galectins are not glycosylated, but are capable of forming ordered arrays made of lectin and multivalent glycoconjugates (Hughes, R.C. 1999; Sacchettini et al., 2001). Fine differences in carbohydrate-binding specificities implicate that each galectin can interact with a distinct spectrum of glycoconjugates receptors, inducing individual downstream effects (Hughes, R.C. 1999). Some specific members of the galectin family are involved in metastasis, control of cell growth, activation of inflammatory cells and the regulation of apoptosis (Liu F.T. 2000).

1.6 Gal-1

1.6.1 Structure and properties of Gal-1

Gal-1 is the first protein that was discovered among the family of galectins. The crystal structure of human Gal-1 consists of a six-stranded and a five-stranded β-sheet in an antiparallel arrangement. In solution, Gal-1 exists as a non-covalent dimer (Liu et al., 2002; Yang et al., 2008) (Figure 8).

The LGALS1 (Lectin, Galactoside Binding Soluble 1) gene located on chromosome 22q12 encodes for Gal-1. The result of the splicing of four exons is a 0.6 kb transcript encoding the Gal-1 protein with 135 amino acids (Camby et al., 2006).

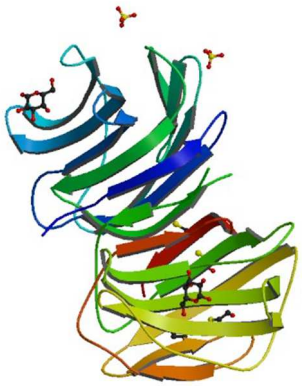

Figure 8 – X-ray crystal structure of human Gal-1 complexed with galactose
This image shows schematically the X-ray structure of human Gal-1 dimer complexed with galactose as a ribbon diagram. (Image from Protein Data Bank – www.pdb.org)

Gal-1 can be found on the inside and the outside of cells and has thereby both intracellular and extracellular functions. Intracellular Gal-1 shows protein-protein interactions while for extracellular Gal-1, a lectin activity can be observed (Camby et al., 2006). The protein-carbohydrate binding is achieved by enthalpy and therefore van der Waals contacts and hydrogen bonds are constituted to be the main forces driving and/or stabilizing complex formations (Lopez-Lucendo et al., 2004). The protein-protein partnering with Gal-1 is a carbohydrate-independent mechanism. The proteins interacting with Gal-1 have no structural similarities nor do they share any common domains or motifs (Camby et al., 2006).

1.6.2 Diverse functions of Gal-1

Gal-1 plays a major role in many biological events such as cell adhesion, cell growth regulation, cell cycle progression, apoptosis, immunomodulation and T-cell receptor counter stimulation. The changes in many cellular processes are caused by interaction of Gal-1 with the ECM (Marsich et al., 2008). Moreover, Gal-1 can regulate innate and adaptive immune responses. It also takes part in chronic, acute and allergic inflammation. Gal-1 is expressed in a variety of different inflammatory cells and regulates the function of theses cells. It is also in charge of cell growth inhibition, obstructs T-cell activation and supports apoptosis of activated T-cells (Liu et al., 2000; Rabinovich et al., 2002). Stromal cells surrounding pre-B cells express Gal-1, which is involved in synapse formation. Gal-1 affects the physiology of antigen-presenting cells, such as monocytes and macrophages, through

mechanisms that are independent of its ability to induce apoptosis (Yang et al., 2008).

Studies from the past few years have shown that Gal-1 is also involved in tumor growth. Gal-1 interacts with oncogenic H-RAS and contributes to membrane anchorage of H-RAS and tumor transformation. Tumor metastasis is a result of cell growth, cell adhesion and cell migration, induced by Gal-1. Moreover, recent evidence implies that tumor cells secrete a considerable level of Gal-1 to avoid T-cell-mediated responses (Rabinovich, 2005) (Figure 9).

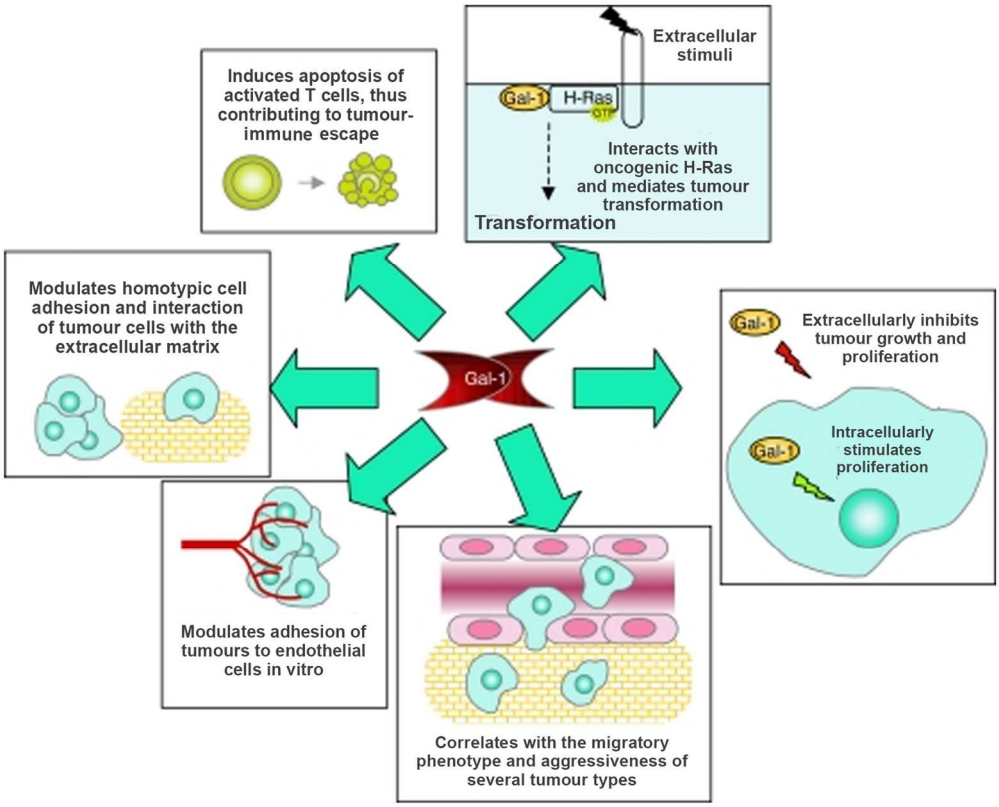

Figure 9 – Contribution of Gal-1 to tumor progression
The multistep process of tumor metastasis includes changes in cell adhesion, increased invasiveness, angiogenesis and evasion of the immune response**.** (Image taken from Rabinovich)

1.6.3 Gal-1 in the context of cartilage

Marcon et al. (2005) were first in publishing experimental evidence of the presence of Gal-1 in porcine chondrocytes. Marsich et al. (2008) found reactivity for Gal-1 in chondrocytes of porcine articular cartilage, and in growth plate chondrocytes. Gal-1 causes changes in many cellular processes, by interacting with the glycoproteins

laminin, fibronectin and elastin of the ECM. Therefore, it can be assumed that Gal-1 plays a significant role in the biology of chondrocytes and cartilage regeneration (Marcon et al., 2005). Marsich and colleagues (2008) examined the effects of Gal-1 in porcine articular and growing type cartilage *in vitro*. They came to the conclusion that Gal-1 has an inhibitory effect on chondrocyte anabolism and can stimulate catabolic processes. They have also observed that Gal-1 stimulates the expression of type-X collagen in articular chondrocytes, together with the up-regulation of MMP13 and the down-regulation of type-II collagen. This indicates a possible role of Gal-1 in the osteoarthritic process (Marsich et al., 2008). Recently Toegel and coworkers (2014) discovered that especially Gal-1 is expressed in OA cartilage. They pointed out that upregulation of Gal-1 is in direct relation to severe cartilage degeneration and mentioned the possibility of additive/antagonistic activities between galectins.

1.7 Gal-3

1.7.1 Structures and properties of Gal-3

The Gal-3 gene (LGALS3) is located on human chromosome 14 and encodes a soluble protein of 30kD (Boileau et al., 2008). Whereas Gal-3 was first detected in macrophages, it was later detected to be more widely distributed in tissues including the gut, brain, kidneys and skeleton (Van den Brule et al., 1997). Gal-3 is the unique member of the galectin family with an extended N-terminal region consisting of tandem repeats of 120 short amino acid segments linked to a C-terminal CRD (Yang et al., 2008). The CRD of Gal-3 preferably attaches to lactose or N-acetyl-lactosamine and is made of a five stranded and a six stranded β-sheet in a β-sandwich arrangement (Figure 10).

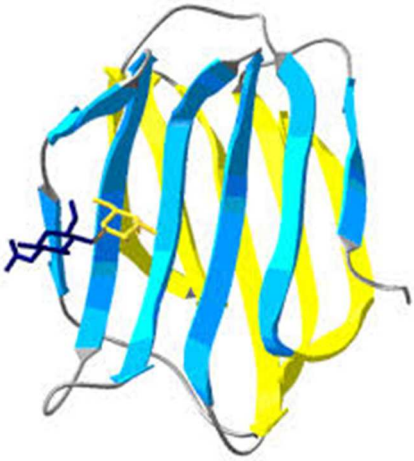

Figure 10 – X-ray of the crystal structure of human Gal-3 bound to LacNAc
This image shows the beta-strands in one sheet (yellow) and the opposing sheet (blue). The galectin moiety of N-Acetyl-D-lactosamin (LacNAc) is shown in yellow and the GlcNAc portion in dark blue. (Image taken from https://www.imperial.ac.uk/animallectins)

In solution Gal-3 exists as a monomer and the N-terminal fragment shows an unfolded, extended structure (Yang et al., 2008). Gal-3 exists in two isoelectric variants, a non-phosphorylated native polypeptide found solely in the nucleus and a phosphorylated derivate located in nucleus and cytoplasm (Liu et al., 2002).

1.7.2 Diverse functions of Gal-3

Gal-3 takes part in extracellular functions such as cell-cell or cell-matrix interactions. RNA-splicing, differentiation and apoptosis are among Gal-3's intracellular functions (Janelle-Montcalm et al., 2007). Gal-3 plays a role in proliferation of activated T-lymphocytes. It inhibits apoptosis and therefore it can be assumed that "Gal-3 regulates cell survival by functioning inside the cell and interacting with some components of the apoptosis-signaling pathways" (Liu et al., 2002). It also fulfills a function in the regulation of the cell cycle (Liu et al., 2002).

1.7.3 Gal-3 in the context of cartilage

Gal-3 exists in osteoblasts, osteoclasts and chondrocytes (Boileau et al., 2008). Boileau et al. (2008) demonstrated a protective role for intracellular Gal-3 and suggested that any downregulation of its expression during the OA process may

induce structural changes in the joint. On the other hand, Janelle-Montcalm et al. (2007) showed that extracellular Gal-3 induced swelling and OA-like lesions in the knee joints of mice. In human OA chondrocytes, Gal-3 stimulates the expression of ADAMTS-5 and MMP-3, the main enzymes leading to proteoglycan degradation in cartilage. Extracellular Gal-3 has harmful effects on chondrocytes and osteoblasts caused by both downregulating the anabolic processes and upregulating the catabolic processes. Therefore, it can be assumed that extracellular Gal-3 takes part in cartilage destruction and subchondral bone erosion, during OA progression (Janelle-Montcalm et al., 2007). Toegel et al. (2014) observed that Gal-3 is present in OA cartilage and also suggested a correlation between the Gal-3 presence and the degree of degeneration.

1.8 Gal-8

1.8.1 Structures and properties of Gal-8

Gal-8 is a 34 kDa, tandem repeat type galectin. Actually, it is a heterodimer of two distinct N- and C-terminal CRDs joined by a linker peptide of variable length (Zick et al., 2004) (Figure 11). Although both CRDs of Gal-8 are comparable in structure, their sugar-binding specificity differs (Cattaneo et al., 2011). Ideo et al. (2003) determined the binding specificity of Gal-8 and its two CRDs for oligosaccharides and glycosphingolipids. A preferred affinity of sialylated and sulfated glycans for the N-CRD, and of blood group antigens and poly-N-acetyl-lactosamine glycans for the C-CRD has been reported (Cattaneo et al., 2011). Gal-8 is a secreted protein. Upon secretion Gal-8 functions as a physiological modulator of cell adhesion (Zick et al., 2004).

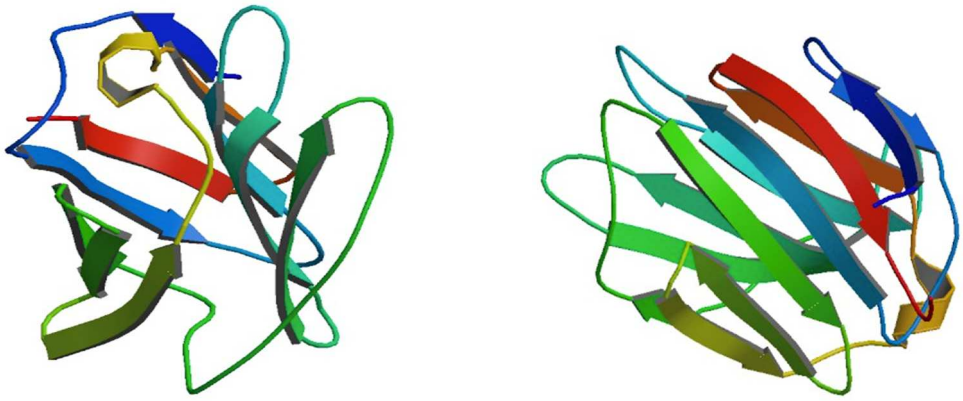

Figure 11 – Crystal structure of C-terminal and N-terminal domain of human Gal-8
This image shows the crystal structure of the C-terminal (left) and the N-terminal (right) domain of human Gal-8. (Image taken from RCSB Protein data bank – 3OJB Structure Summary/ 2YV8 Structure Summary)

Gal-8 can be found in many tissues like lung, liver, kidney, spleen, hind limb, cardiac muscle and in low levels of expression also in intestine, colon, fat and thymus. The expression of Gal-8 can only take place in the cytoplasm (Zick et al., 2004).

1.8.2 Diverse functions of Gal-8

Depending on the extracellular context, Gal-8 can affect the cell adhesion positively or negatively (Figure 12). When immobilized, Gal-8 serves as a matrix protein equipotent to fibronectin in promoting cell adhesion by ligation and clustering of a selective subset of cell surface integrin receptors. But on the other hand, when present in excess as a soluble ligand, Gal-8 builds a complex with integrins that negatively regulates cell adhesion (Zick et al., 2004).

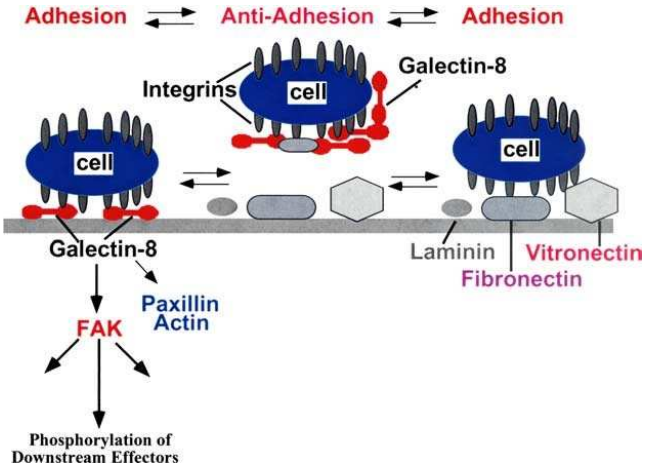

Figure 12 – Role of Gal-8 as a modulator of cell adhesion
This image shows that immobilized Gal-8 functions like fibronectin as a matrix protein, supporting cell adhesion by ligation and clustering sugar moieties of cell surface integrin receptors. The soluble Gal-8 interacts with both, integrins and other soluble matrix proteins and inhibits subsequent cell adhesion. (Image taken from Levy et al.)

Gal-8 causes proliferative and stimulatory activities on T-cells and therefore Cattaneo et al., (2011) assumed a possible involvement of this lectin in inflammatory and autoimmune processes. It also regulates endocytosis of cell-surface receptors and suppresses migration of cancer cells (Yang et al., 2008). Various studies showed a wide expression of Gal-8 in tumor tissues as well as in normal tissues. Although Gal-8 also exists in healthy tissue, the concentration of Gal-8 may correlate with the malignancy of human colon cancers and the degree of severity of lung squamous cell carcinomas and neuro-endocrine tumors (Bidon-Wagner et al., 2004).

1.8.3 Gal-8 in the context of cartilage

Toegel et al. (2014) demonstrated the presence of Gal-8 in chondrocytes of OA cartilage and higher concentrations of Gal-8 in severely damaged areas of cartilage. Therefore, further investigation of Gal-8 for a better understanding of the process of OA is necessary.

1.9 Aims of the study

The main objective of this study was to investigate the influence of Gal-1, -3, -8 on human chondrocytes of OA patients. Toegel et al., (2014) showed that these three galectins are present and part of osteoarthritic proceedings and suggest further experiments with Gal-1, Gal-3 and Gal-8 and their combinations.

Therefore, time and concentration dependent experiments with these galectins were undertaken in human chondrocytes and SW1353 cells. The aim of these experiments was to investigate the cell viability, mRNA levels of OA marker genes and the effects of Gal-1 on the NFκB pathway.

2 Materials and Methods

2.1 Clinical specimens

Knee joint tissue was obtained from patients that underwent total knee replacement surgery at the Department of Orthopaedics, General Hospital Vienna, with informed consent and in accordance with the terms of the ethics committee of the Medical University of Vienna (EK-No.: 1065/2011).

2.2 Isolation of primary human chondrocytes

Orthopedic surgeons provided the knee joint tissue in sterile physiologic salt (NaCl) solution. Articular cartilage from femoral condyles, tibial plateau and patella was processed into small pieces on petri dishes (10 cm in diameter, BD Falcon) by the use of disposable forceps (Ärztebedarf Scherer) and disposable scalpels (Heintel). Then, the small pieces of cartilage were washed with PBS. To release the chondrocytes, cartilage was digested in sterile filtered medium (see "Cultivation and treatment of primary human chondrocytes") containing 2 mg/ml lyophilized Collagenase type II under constant shaking at 37°C until the next day. To separate the chondrocytes from the suspension of digested cartilage, a 40 µm nylon cell strainer (BD Falcon) was used. The cell suspension was then centrifuged at 1,000 rpm for 10 min. After removal of the used medium, the cell pellet was resuspended in fresh medium. Cells were counted (C-Chip Neubauer improved DHC-N01 disposable hemocytometer) and cultured at 20,000 cells/cm^2 in tissue culture flasks (TPP) or well plates (Costar).

2.3 Cultivation and treatment of primary human chondrocytes

Primary human chondrocytes were cultured in "full medium" made of Dulbecco´s Modified Eagle's Medium (DMEM) containing 4.5 g/l glucose and 25 mM HEPES

(Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS, PAA), 1% (v/v) gentamicin (10mg/ml, gibco) and 0.1% (v/v) amphotericin B (250 µg/ml, PAA). In a Labotect C200 incubator (at 37°C, 5% CO_2 and 95% relative humidity) cells were grown in monolayer culture.
For all experiments, 80% confluent chondrocytes in passage 0 were used. Before treatment, chondrocytes were serum starved overnight (o/n). Recombinant human Gal-1, -3 and -8 (provided by Hans-Joachim Gabius) were also dissolved in starvation medium (DMEM containing gentamicin and amphotericin B, without FBS). The chondrocytes were treated with Gal-1, -3, -8 or combinations of these three galectins and compared with an untreated control.

2.4 Cultivation and treatment of SW1353 cell line

SW1353 (ATCC HTB-94TM, Manassas, VA, USA) were thawed and seeded with full medium into a tissue culture flask. When the cells reached confluence, they were ready for splitting. Cell passaging or splitting is necessary to keep cells alive and growing under cultured conditions for a certain amount of time.
Therefore, Trypsin/EDTA solution (Life Technologies) and medium were pre-warmed in a waterbath at 37°C. After removing the old medium, the cells were washed thoroughly with PBS. Then, the Trypsin/EDTA solution was added and incubated for 7 min. Following addition of full medium to inactivate the trypsin, the cells were centrifuged. Afterwards, the pellet was resuspended in fresh full medium. The passaged cells were used for treatments with Gal-1, -3, -8 in the same way chondrocytes have been treated.

2.5 Cell viability assay

To test cell viability, the EASY FOR YOU (EZ4U) assay (Biomedica), which is a non-radioactive cell proliferation and cytotoxicity assay was applied. This assay is based on a simple principle: cells are able to convert the yellow colored tetrazolium compound into its red formazan derivative. For this reduction process, functional

mitochondria are necessary, which would be deactivated within a few minutes if cell death occurs.

For this assay, 3,000 cells per well were seeded in 96-well plates. The chondrocytes were treated with different Gal-1, Gal-3 and Gal-8 concentrations in starvation medium for 6 or 24 h. Afterwards, the culture medium was replaced by 20 µl of the substrate solution combined with 200 µl full medium, as recommended in the instruction manual. After 2 h of incubation, the absorbance was measured at 450 nm using the Wallac 1420 VICTOR 3TM plate reader.

2.6 RNA Isolation

RNA isolation is the purification of RNA from biological samples. Therefore, the RNA isolation kit "NucleoSpin RNA" (Macherey-Nagel) was used. First, primary chondrocytes or SW1353 cells cultured in 6-well plates were washed with PBS. Then, the cells were lysed by adding a combination of 350 µl Buffer RA1 and 3.5 µl β-mercaptoethanol per well and repeated resuspension. The lysis buffer immediately inactivates RNAses. The lysate was purified by filtration through a NucleonSpin Filter placed in a 2 ml Collection Tube under centrifugation for 1 min. All centrifugation steps were carried out as stated in the manual at 11,000 rpm in an Eppendorf Centrifuge 5415D. Then, the homogenized lysate was mixed thoroughly with 350 µl 70% ethanol, loaded onto a NucleoSpin RNA Column and centrifuged for 30 s to bind the RNA onto the membrane. To desalt the silica membrane, 350 µl MDB (Membran Desalting Buffer) were added to the column, which was put in a new collection tube and centrifuged for 1 min. As a next step, DNA was digested for 15 min at room temperature by applying a mixture of 10 µl reconstituted rDNase and 90 µl Reaction Buffer for rDNase directly onto the center of the silica membrane. After that, three washing steps were executed as follows. First, the samples were centrifuged for 30 s with 200 µl Buffer RAW2 to inactivate the rDNase, and secondly – after putting the column into a new Collection Tube – centrifuged with 600 µl Buffer RA3 also for 30 s. The last washing step was performed by adding 250 µl Buffer RA3 and centrifuging for 2 min (for drying the silica membrane). Finally, to elute the RNA, the column was placed into a 1.5 ml nuclease-free tube, 40 µl RNase-free water were added and

centrifuged for another minute. The eluted RNA was stored at -80°C or used immediately.

2.7 RNA quantification

The quantity and purity of the isolated RNA was measured with the NanoDrop 2000 at 260 nm and its associated software (Thermo Scientific). Samples with a ratio of 1.8 - 2.1 of absorbance at 260 nm and 280 nm were generally accepted as pure. NanoDrop is a micro-volume UV-Vis spectrophotometer for the quantification of nucleic acids and proteins. 2 µl RNase-free water were measured first as blank prior to quantification of 2 µl of the isolated RNA.

2.8 cDNA synthesis

The reverse transcription (RT) of total RNA to single-stranded cDNA is necessary to perform quantitative PCR.

Using the results of the RNA quantification, all samples of one experiment were diluted with RNase-free water to the same quantity of RNA per 10 µl in 0.2 ml tubes. Simultaneously, the contents of the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) were slowly thawed on ice. After preparing the adequate amount of 2x reverse transcription Master Mix (Table 1), 10 µl of this Master Mix were mixed with the diluted RNA. All these steps were performed on ice.

Afterwards, the mixture was shortly centrifuged to eliminate air bubbles.

Component	Volume (µl)/Reaction
10X RT Buffer	2.0
25X dNTP Mix (100mM)	0.8
10X RT Random Primers	2.0
MultiScribe Reverse Transcriptase	1.0
Nuclease-free H_2O	4.2
Total per Reaction	10.0

Table 1 – Components of reverse transcription Master Mix

Then, the tubes were placed into the thermal cycler (Mastercycler gradient, Eppendorf) and the predefined reverse transcription program was started (Table 2). The cDNA was either directly used for RT-qPCR or stored at -80°C.

	Step1	Step2	Step3	Step4
Temperature	25°C	37°C	85°C	4°C
Time	10min	120min	5min	∞

Table 2 – Thermal cycler program

2.9 Quantitative PCR

Quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) enables solid detection and measurement of DNA amplification generated during each cycle. To monitor the production of DNA amplification products, fluorescent reporter molecules (SYBR Green) were used.

The gained CT-values of the target genes and a housekeeping gene are necessary to allow a relative quantification of mRNA levels in the samples.

First, the cDNA was diluted with RNase-free H_2O at a ratio of 1:5 to the amount required for the RT-qPCR. Then, the Master Mix was prepared (Table 3 and 4).

Forward Primer (Metabion)	0.5 µl
Reverse Primer (Metabion)	0.5 µl
SYBR Green (Bioline)	12.5 µl
RNase-free H_2O	10.5 µl
Total per Reaction	24 µl

Table 3 – Composition of the qPCR Master Mix

Gene	Forward Primer (5´to 3)	Reverse Primer (5´to3´)
SDHA	TGGGAACAAGAGGGCATCTG	CCACCACTGCATCAAATTCATG
AGC1	ACTGGCGAGCACTGTAAC	TCTTGGGCATTGTTGTTGAC
COL2A1	TGGTGGAGCAGCAAG	GGGAGGCGTGAGGTCTTCTG
COL1	CACTGGTGATGCTGGTCCTG	CGAGGTCACGGTCACGAAC
TNFA	TCAGCAAGGACAGCAGAGG	CAGTATGTGAGAGGAAGAGAACC
IL1B	CTTATTACAGTGGCAATGAGGATG	AGTGGTGGTCGGAGATTCG
MMP1	CTGGAATTGGCCACAAAGTT	TCCTGCAGTTGAACCAGCTA
MMP3	GGTGTGGAGTTCCTGATGTTG	AGCCTGGAGAATGTGAGTGG
MMP13	TCAGGAAACCAGGTCTGGAG	TGACGCGAACAATACGGTTA
IL8	CAAACTTTCAGAGACAGCAGAG	CAGTGAGATGGTTCCTTCCG
CXCL1	AGGGAATTCACCCCAAGAAC	ACTATGGGGGATGCAGGATT
ADAMTS4	ACCCAAGCATCCGCAATCC	GCCCACATCAGCCATACCC

Table 4 – List of used Forward and Reverse Primers

24 µl of the master mix and 1 µl of the diluted cDNA were pipetted into a Fast Optical 96-Well Reaction Plate (Applied Biosystems). All samples were analyzed in duplicates. The reaction plate was sealed with an adhesive film (Applied Biosystems) and shortly centrifuged to eliminate air bubbles. Then, it was put in the StepOnePlus Real-Time PCR System (Applied Biosystems). The used temperature profile is shown in Table 5.

Holding Stage	Step1	95°C	10min	---
Melting Stage (40 Cycles)	Step1	95ºC	15s	---
	Step2	55ºC	1min	measurement
	Step3	72ºC	1min	measurement
Melt Curve Stage	Step1	95ºC	15s	---
	Step2	55ºC	1min	measurement
	Step3	95ºC	15s	---

T**able 5 – Temperature profile of the qPCR**

As a part of the PCR, double-stranded DNA molecules emerge and the fluorescent dye SYBR Green binds to them. An increase in the amount of PCR product will result in greater intensity of fluorescence, which is measured within each cycle.

The threshold has to be set marginally over the background signal, to detect the DNA-based fluorescence. The cycle threshold (C_T) is the number of cycles where the fluorescence trespasses the threshold. For the relative quantification of gene expression between the samples the ΔC_T-method was used. For that reason, the difference between the C_T of the control sample and the C_T of a treated sample from the relevant gene is normalized using a housekeeping gene. As housekeeping gene, SDHA (= a gene that encodes the succinate dehydrogenase complex in humans) was used.

2.10 Protein harvesting

Proteins were harvested from chondrocytes grown in 25 cm^2 flasks. First, the lysis buffer was prepared of 1 ml RIPA buffer (Santa Cruz Biotechnology) supplemented with 10 µl phenylmethylsulfonyl fluoride (PMSF), 10 µl sodium orthovanadate and 15 µl protease inhibitor cocktail. Then, the medium was removed and the cells were washed with PBS. 167 µl of the RIPA lysis buffer were pipetted into each 25 cm^2 flask, which was then placed on ice. After one hour, the cells were scraped (cell

scraper, BD FalconTM) and the lysate was stored at -80°C. Finally, the lysate was put into the ultrasonic bath with ice for about 10 min.

2.11 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a widely used method to separate proteins depending on their molecular weights. For the separation of the proteins, 10% separating gels and 5% stacking gels were prepared (Table 6).

Separating Gel	**10%**	**Stacking Gel**	**5%**
dH_2O [ml]	4.17	dH_2O [µl]	3400
1.5M Tris [ml]	2.5	0.5M Tris [µl]	830
30% Acrylamide [ml]	3.33	30% Acrylamide [µl]	630
10% SDS [µl]	100	10% SDS [µl]	50
TEMED (tetramethylethylendiamine) [µl]	5	TEMED (tetramethylethylendiamine) [µl]	5
10% APS (ammonium persulfate) [µl]	100	10% APS (ammonium persulfate) [µl]	50
Total amount for 2 gels each			

Table 6 – Instruction for the preparation of SDS-Gel

24 µl of the harvested proteins were resuspended with 8 µl of 4x Laemmli Buffer (15 ml consist of: 4.4 ml 0.5 M Tris HCl pH 6.8 + 2.2ml 20% SDS + 4.4ml glycerol + 0.5ml 1% bromophenol blue + 0.5ml β-mercaptoethanol + 3 ml dH_2O). The anionic detergent SDS denatures secondary and non-disulfide-linked tertiary structures and applies a negative charge to each protein in proportion to its mass. β-mercaptoethanol was used to break the proteins disulfide bonds, which supports denaturation. Proteins were heated at 95°C for 5 min (in Eppendorf Thermomixer Compact) and left to cool down before loading.

Then, 3 µl of Precision Plus ProteinTM All Blue Standards (Biorad) were loaded into one slot as molecular weight marker. The other slots were loaded with 30 µl of the protein samples and Laemmli buffer mixture. Proteins were then separated

electrophoretically (50 mA for 2 gels) in a Mini-PROTEAN Tetra cell (Biorad) using the Running buffer made of 100 ml of 10x Tris-Glycin-Buffer (1,000 ml contain: 30.3 g Tris, 144 g Glycine and filled up with dH_2O), 10 ml of 10% SDS and 890 ml of dH_2O. The electrophoretical separation was completed when the blue front leaked out of the bottom of the gel.

2.12 Western Blot

Western blot was used to transfer the separated proteins to a Protran Nitro-cellulose Membrane (Schleicher & Schuell) and detect them with target specific antibodies. In a plastic tub filled with precooled Transfer Buffer (consists of: 100 ml 10x Tris-Glycin-Buffer, 700 ml dH_2O and 200 ml methanol), the transfer cassette was put together in the following order: black side down, black foam sponge, filter paper (Mini Trans-Blot Cell Filter Paper, Biorad), protein gel, membrane, filter paper, white foam sponge and white side up. To transfer the proteins onto the membrane, a Mini Trans-Blot Cell (Biorad) was used. It was filled with Transfer Buffer and cooled with ice all around. Then, the transfer was performed at 250 mA for 2 h.

Then, the membrane was blocked with 5% (w/v) milk powder (Roth) dispersed in 1x PBS pH 7.4 (Morphisto) o/n at 4°C. After the blocking step, the membrane was carefully washed with 1x PBS-T (100 ml 10x PBS, 900 ml dH_2O, 1 ml Tween20) for 10 min.

The membrane was put into a 50 ml Falcon tube and incubated with either the primary NF-κB rabbit monoclonal antibody (Cell Signaling Technology) or the β-actin mouse monoclonal antibody (Sigma-Aldrich), which functioned as loading control, at room temperature for 2 h on the swivel roller mixer. The NF-κB antibody was diluted 1:1,000 and the β-actin antibody 1:5,000 in Odyssey Blocking Buffer (Licor) mixed 1:1 with PBS-T. Then, the membrane was washed three times with PBS-T for 10 min. Afterwards, the membrane was incubated for 1 h with the respective secondary antibodies (DyLight 800nm labeled anti-rabbit IgG made in goat or IRDye680LT anti-mouse IgG made in goat) diluted 1:15,000 in PBS-T (0.02% (v/v) of 20% SDS was added for the IRDye680LT). The membrane was washed again three times with PBS-T for 10 min.

For the visualization of the protein bands, the Odyssey Imager (Licor) was used.

2.13 ELISA (Enzyme-linked immunosorbent assay)

For these assays, R&D Systems Quantikine ELISA kits were applied according to the manufacturer's protocols. These kits use a quantitative sandwich immunoassay technique to detect sample antigens. In brief, either a polyclonal antibody specific for MMP-3 or a monoclonal antibody specific for pro-MMP-1 or a monoclonal antibody specific for pro-MMP-13 were bound onto a 96-well microplate. Standards and samples were applied to the plate and any MMP-3, MMP-1 or MMP-13 present was bound by the immobilized antibody. After four washing steps – which were performed thoroughly to remove any unbound substances – enzyme-linked antibodies specific for MMP-3, pro-MMP-1 or pro-MMP-13 were added. Any unbound antibody-enzyme complexes were removed again by washing four times. Protected from light, a substrate solution was added to each well and color development occurred in proportion to the quantity of MMP-3, pro-MMP-1 or pro-MMP-13 bound in the primary step. After 30 min, the color development was stopped by adding a stop solution, and the intensity of the color was measured at 450 nm using the Wallac 1420 VICTOR 3TM plate reader.

2.14 Statistics

Microsoft Excel t-test function was used to examine the statistical significance of the cell viability tests, RT-qPCR, ELISA and Western Blot results. It was used to determine if the data from the treated samples were significantly different to the data of the untreated controls. For the MTT results, two-tailed tests were used, for all others one-tailed tests were used.

3 Results

3.1 Cell proliferation – an indicator of cytotoxic effects

MTT-tests were implemented to determine the maximum concentration range of Gal-1, -3, and -8, which exert no cytotoxic effect on chondrocytes and SW1353 cells. Therefore, three experiments with SW1353 cells and with chondrocytes of three different OA patients (n=3) were performed. The SW1353 cells were treated for 24 h with different concentrations of Gal-1, -3, or -8 (0.5 – 50 µg/ml). Primary chondrocytes were also treated for 24 h with different concentrations of Gal-1, -3 and -8 (1 – 500 µg/ml).

The results of the three MTT tests show that neither Gal-1 nor Gal-3 or Gal-8 induced any significant negative effects on the cell viability of SW1353 cells at concentrations of up to 50 µg/ml (Figure 13).

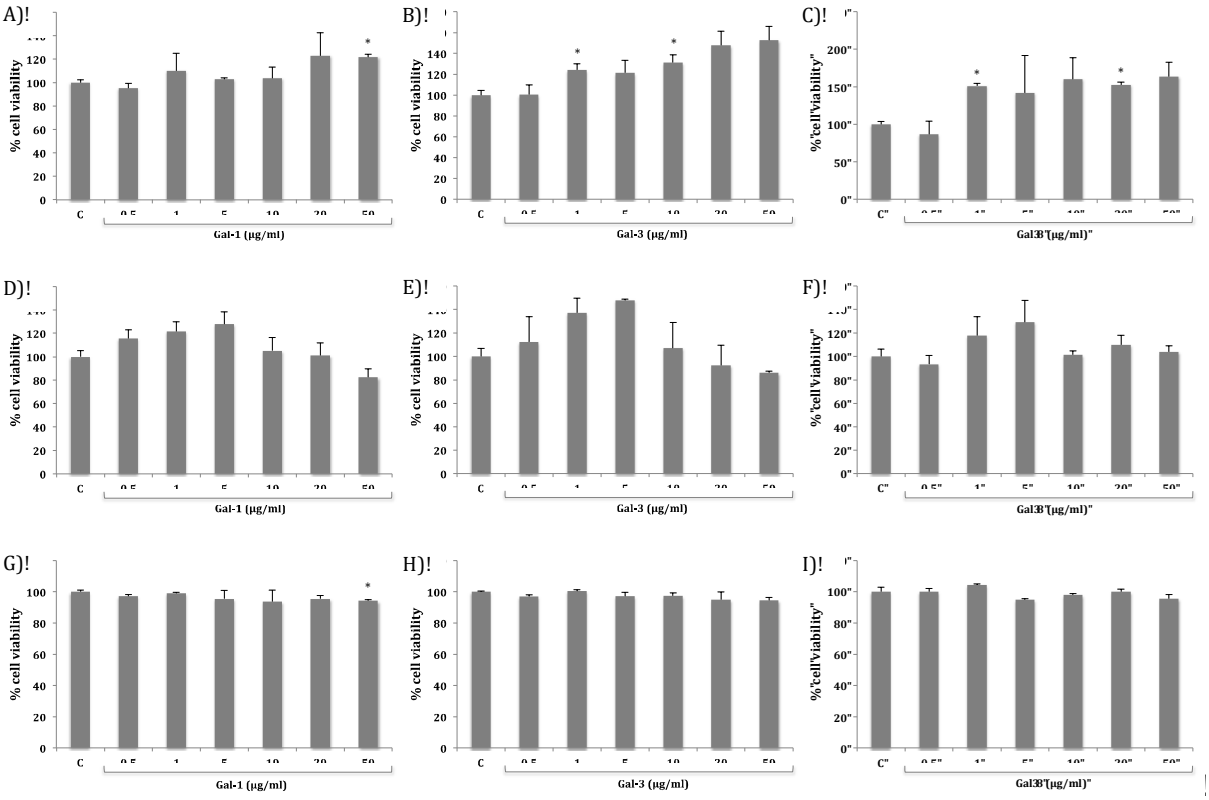

Figure 13 – Proliferation of SW1353 cells treated with different concentrations of Gal-1, -3 and -8

SW1353 cells were treated in three experiments with 0.5 – 50 µg/ml Gal-1 (A, D, G), Gal-3 (B, E, H) and Gal-8 (C, F, I) for 24 h and compared to an untreated control (C) set at 100%. P-values < 0.05 (*) were considered statistically significant (t-test).

Gal-1 (Figure 14 A, D, G) and Gal-8 (Figure 14 C, F, I) did not exhibit any negative influence on the cell viability of primary chondrocytes at concentrations of up to 500 µg/ml, but Gal-3 (Figure 14 B, E, H) treatment induced a significant decline of the cell viability at 500 µg/ml in two out of three patients (Figure 14 E, H).

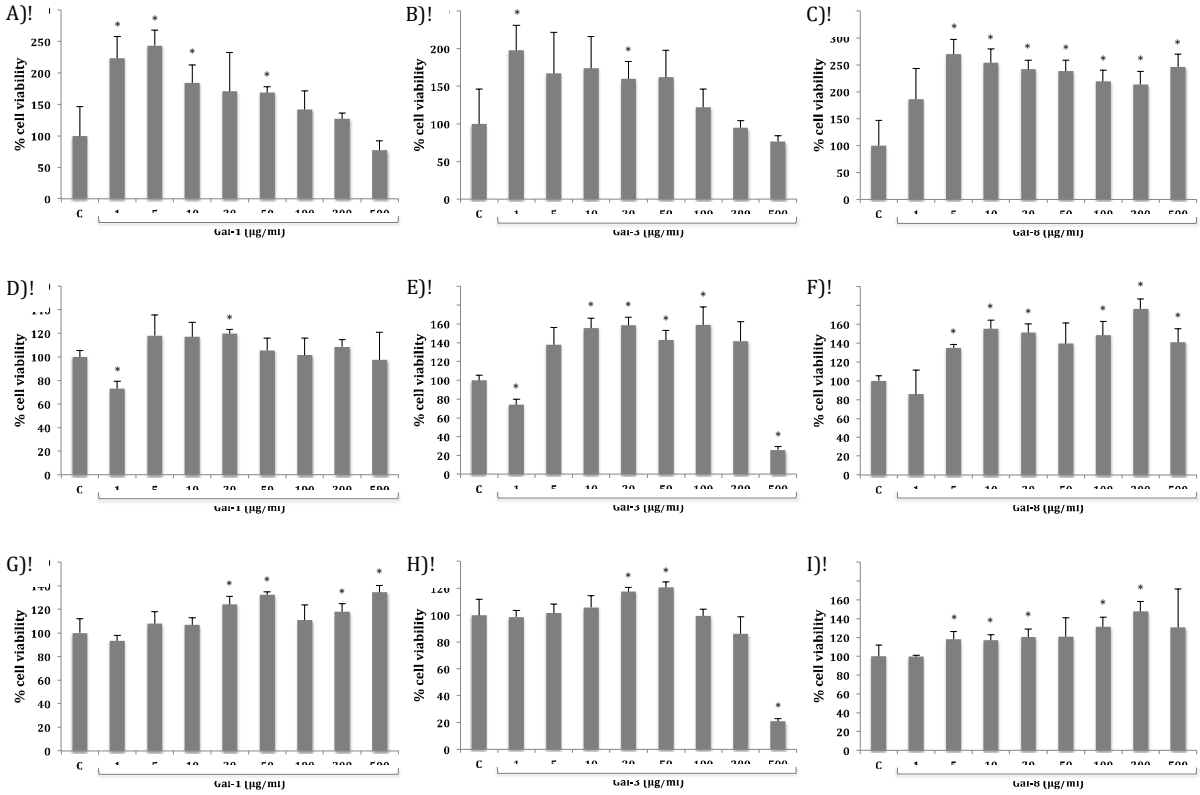

Figure 14 – Proliferation of OA chondrocytes treated with different concentrations of Gal-1, -3 and -8

OA chondrocytes of 3 patients (n=3) were treated with 1 – 500 µg/ml with Gal-1 (A, D, G), Gal-3 (B, E, H) and Gal-8 (C, F, I) for 24 h and compared to an untreated control (C) set at 100%. P-values < 0.05 (*) are considered statistically significant (t-test).

3.2 Effects of Gal-1, -3 and -8 on gene expression

RT-qPCR analysis was performed to examine the alteration of gene expression in OA chondrocytes and SW1353 cells, treated with different concentrations of Gal-1, -3, or -8 or with combinations of Gal-1 and Gal-3 for 24 h or different time intervals as indicated. The alteration in the expression of IL1B, TNFA, RELA, MMP13, ADAMTS4, MMP1, MMP3, COL1, COL2A1, AGC, IL8, CXCL1 levels relative to SDHA was analyzed in RT-qPCR experiments.

3.2.1 Impact of Gal-1, -3 and -8 on gene expression in OA chondrocytes

Time dependency (0.5 – 32 h) of galectin activities is shown in Figure 3. Compared to the maximum increase of MMP13 (46-fold) (Figure 15 D, E, F) and RELA (3.9-fold)

(Figure 15 G, H, I) expression, IL1B (1652-fold) (Figure 15 A, B, C) reached much higher levels of expression. The expression of IL1B started rising at 1 h and reached its peak at 8 h of Gal-1 (Figure 15 A) and at 16 h of Gal-3 and Gal-8 stimulation (Figure 15 B and C, respectively). Overall, Gal-8 (1652-fold) caused a higher mRNA expression of IL1B than Gal-3 (941-fold) and Gal-1 (452-fold). MMP13 expression levels started rising after 4 h with Gal-1 and Gal-3 treatment (Figure 15 D, E) or after 2 h with Gal-8 (Figure 15 F) treatment. The expression of MMP13 reached its peak with Gal-1 at 32 h (Figure 15 D) and with Gal-3 (Figure 15 E) and Gal-8 (Figure 15 F) at 16 h. The increase of gene expression for RELA started at 4 h (Figure 15 G, H, I) and reached the maximum at 32 h with Gal-1 (Figure 15 G) and Gal-8 (Figure 15 I) or 16 h with Gal-3 (Figure 15 H).

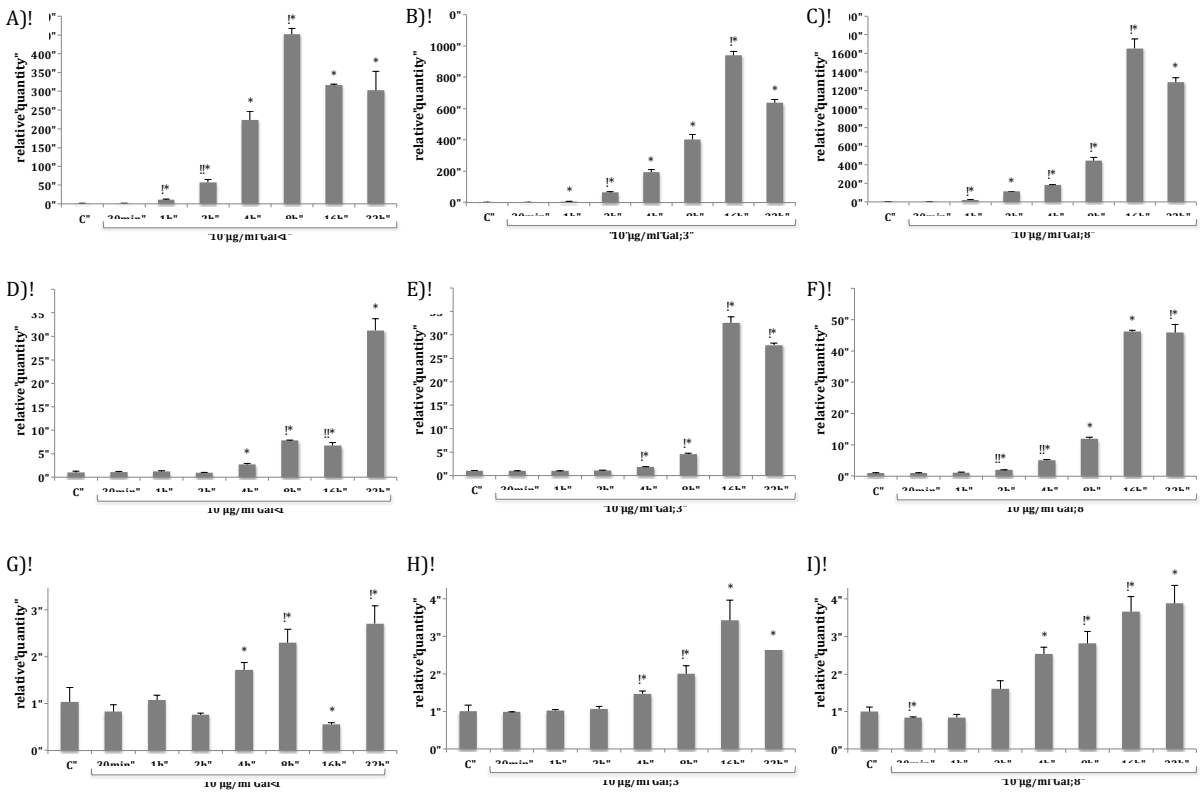

Figure 15 – Time-dependent gene expression in OA chondrocytes under Gal-1, -3 and -8 treatment

Chondrocytes of one patient were treated for 30 min, 1 h, 2 h, 4 h, 8 h, 16 h and 32 h with 10 µg/ml Gal-1 (A, D, G), Gal-3 (B, E, H) and Gal-8 (C, F, I) or were left untreated. RT-qPCR was conducted to quantify the alteration of IL1B (A, B, C), MMP13 (D, E, F) and RELA (G, H, I) expression levels relative to untreated controls. All data were normalized to SDHA as reference gene. P-values < 0.05 (*) are considered statistically significant (t-test).

Concentration dependencies of the galectin effects are shown in Figure 4 and 5. Interindividual differences were noticed for the gene expression of MMP13, TNFA and IL1B in chondrocytes of three patients treated with different concentrations (1 – 100 µg/ml) of Gal-1 (Figure 16) and Gal-3 (Figure 17). The upregulation of MMP13 mRNA expression levels in Gal-1-treated chondrocytes started to reach significance at 1 µg/ml and increased with concentration (Figure 16 A, D, G). In Gal-3-treated chondrocytes (17 A, D, G), the increase of MMP13 expression started to become significant in two out of three cases at 1 µg/ml and reached its peak at 100 µg/ml. TNFA mRNA levels dose-dependently increased in two out of three patients in the Gal-1-treated (Figure 16 B, E, H) as well as in the Gal-3-treated chondrocytes (Figure 17 B, E, H) and reached their peaks at 100 µg/ml. The increase of IL1B expression in Gal-1 treated chondrocytes started at 10 µg/ml and reached its peak in two out of three cases at 100 µg/ml (Figure 16 C, I). Despite a clear tendency towards upregulation of IL1B by Gal-3, no obvious pattern for this increase (Figure 17 C, F, I) could be observed among the 3 donor populations.

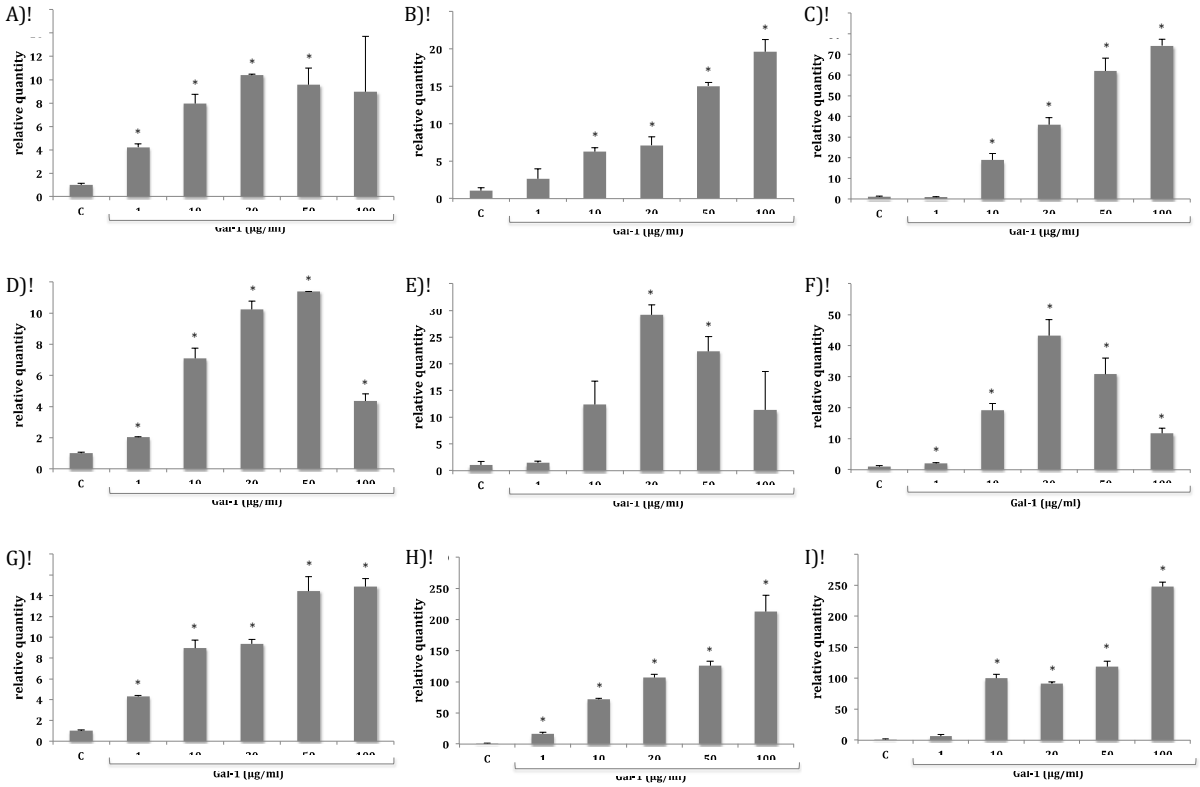

Figure 16 – Gene expression in OA chondrocytes under different concentrations of Gal-1 treatment

OA chondrocytes from three patients were treated with 1 µg/ml, 10 µg/ml, 20 µg/ml, 50 µg/ml and 100 µg/ml Gal-1 for 24 h. Untreated cells served as control (C). RT-qPCR was performed to quantify the increase of MMP13 (A, D, G), TNFA (B, E, H) and IL1B (C, F, I) expression levels relative to untreated controls. All data were normalized to SDHA as reference gene. P-values < 0.05 (*) were considered statistically significant (t-test).

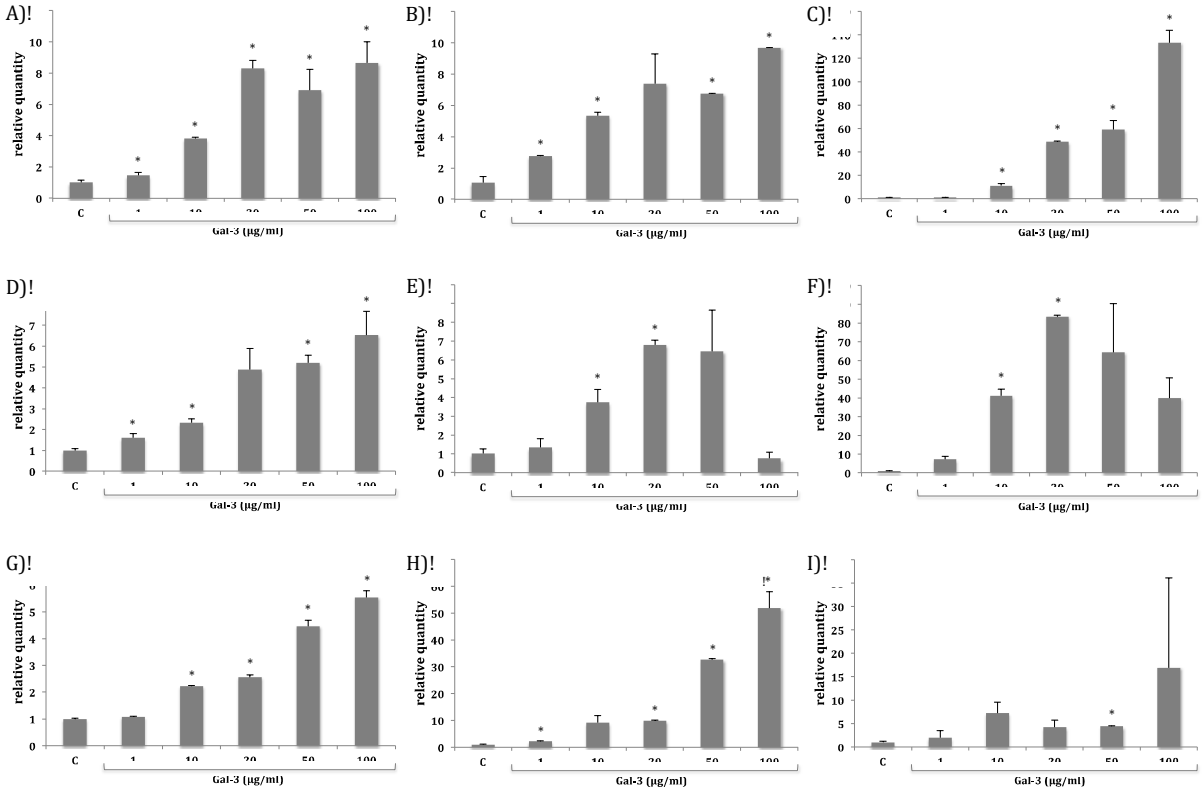

Figure 17 – Gene expression in OA chondrocytes under different concentrations of Gal-3 treatment

OA chondrocytes from three patients were treated with 1 µg/ml, 10 µg/ml, 20 µg/ml, 50 µg/ml and 100 µg/ml Gal-3 for 24 h. Untreated cells served as control (C). RT-qPCR was performed to quantify the increase of MMP13 (A, D, G), TNFA (B, E, H) and IL1B (C, F, I) expression levels relative to untreated controls. All data were normalized to SDHA as reference gene. P-values < 0.05 (*) were considered statistically significant (t-test).

Figure 18 shows that the increase of MMP13, TNFA and IL1B expression started in chondrocytes of one patient at 1 µg/ml treatment with Gal-8. While the expression of MMP13 and IL1B (Figure 18 A, C) rose with concentration of Gal-8, the TNFA (Figure 18 B) expression showed a strong increase with 10 µg/ml (345-fold) and remained rather constant between 250-fold and 345-fold with rising concentration of Gal-8.

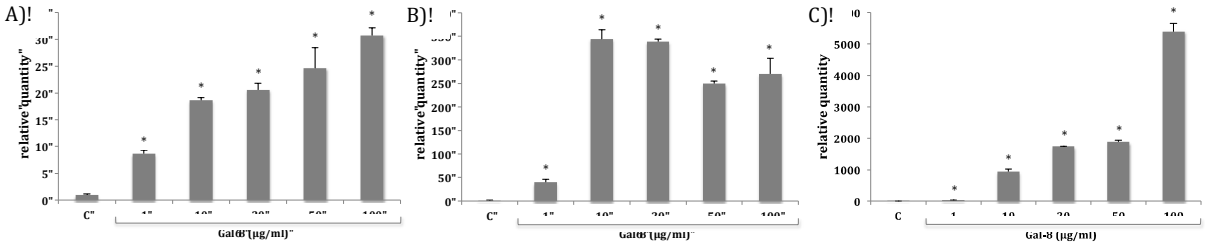

Figure 18 – Gene expression in OA chondrocytes under different concentrations of Gal-8 treatment

OA chondrocytes from one patient were treated with 1 µg/ml, 10 µg/ml, 20 µg/ml, 50 µg/ml and 100 µg/ml Gal-8 for 24 h. Untreated cells served as control (C). RT-qPCR was performed to quantify the increase of MMP13 (A), TNFA (B) and IL1B (C) expression levels relative to untreated controls. All data were normalized to SDHA as reference gene. P-values < 0.05 (*) were considered statistically significant (t-test).

A selection of genes assumed to participate in OA is presented in Figure 19. mRNA expression levels of the target genes were analyzed in chondrocytes from five patients treated with 50 µg/ml Gal-1 for 24 h. The figure shows the fold change of ADAMTS4 (Figure 19 A), MMP1 (Figure 19 B), MMP3 (Figure 19 C), MMP13 (Figure 19 D), COL1 (Figure 19 E), COL2A1 (Figure 19 F) and AGC (Figure 19 G) expression levels induced by Gal-1 across five patients. Gal-1 significantly enhanced the mRNA expression levels of ADAMTS4 (35,8-fold) and MMP3 (5,2-fold) and significantly reduced the mRNA expression levels of COL2A1 (0,51-fold) and AGC (0,76-fold). MMP1 (26,1-fold) and MMP13 (5,6-fold) mRNA expression levels were also upregulated while COL1 (0,72-fold) was reduced.

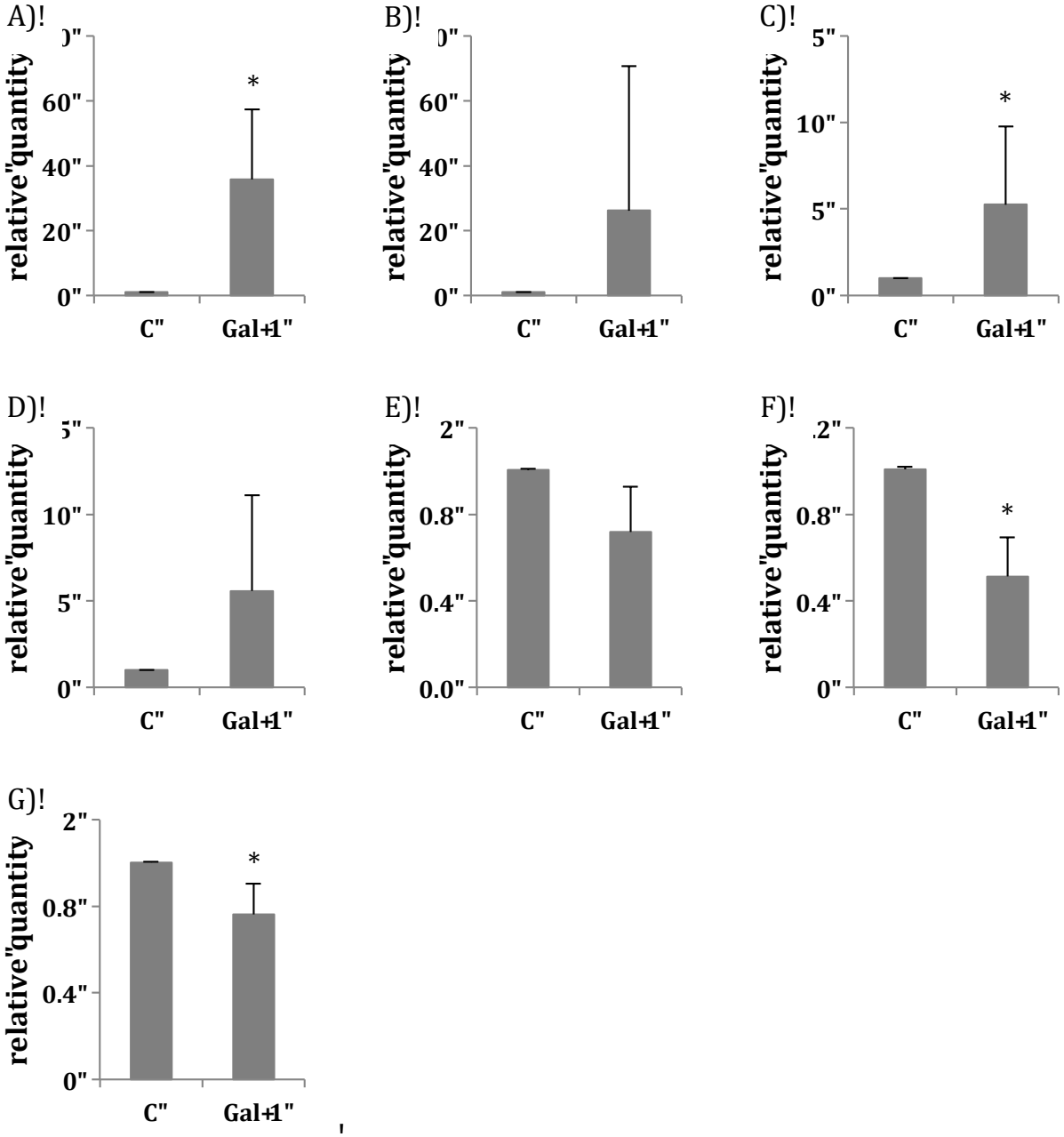

Figure 19 – Gene expression in OA chondrocytes under Gal-1 treatment
OA chondrocytes from five patients were treated with 50 µg/ml Gal-1 for 24h. Untreated cells served as control (C). Using RT-qPCR, the mRNA levels of ADAMTS4 (A), MMP1 (B), MMP3 (C), MMP13 (D), COL1 (E), COL2A1 (F), AGC (G) were quantified relative to the untreated controls. All data were normalized to SDHA as reference gene. Each figure shows the mean value (±SD) of five patients. P-values < 0.05 (*) were considered statistically significant (t-test).

Experiments with co-treatments of Gal-1 and Gal-3 at concentrations of 10 µg/ml, 20 µg/ml and 50 µg/ml are shown in Figure 20. Gal-1 and Gal-3 as well as the combination of them (Figure 20 A, C, E) significantly increased MMP13 expression levels in a concentration-dependent manner in two out of three patients.

Regarding the expression, the extent of the increase differs between the treatments. While Gal-1 (Figure 20 B, D, F) concentration-dependently increased the mRNA expression levels of IL1B to a maximum from 86-fold to 778-fold, differing between the three patients, Gal-3 increased IL1B expression levels to a much lower extent (maximum 43-fold). Interestingly, the combination of Gal-1 and Gal-3 only caused a raise compared to Gal-1 in some cases. Since galectins were dissolved in PBS we also considered an untreated control with a combination of 900 µl full medium + 100 µl PBS to prove that dilution of the medium did not affect mRNA levels. In five PBS controls out of six, there was no increase or decrease of the MMP13 and IL1B expression levels compared to the full medium control.

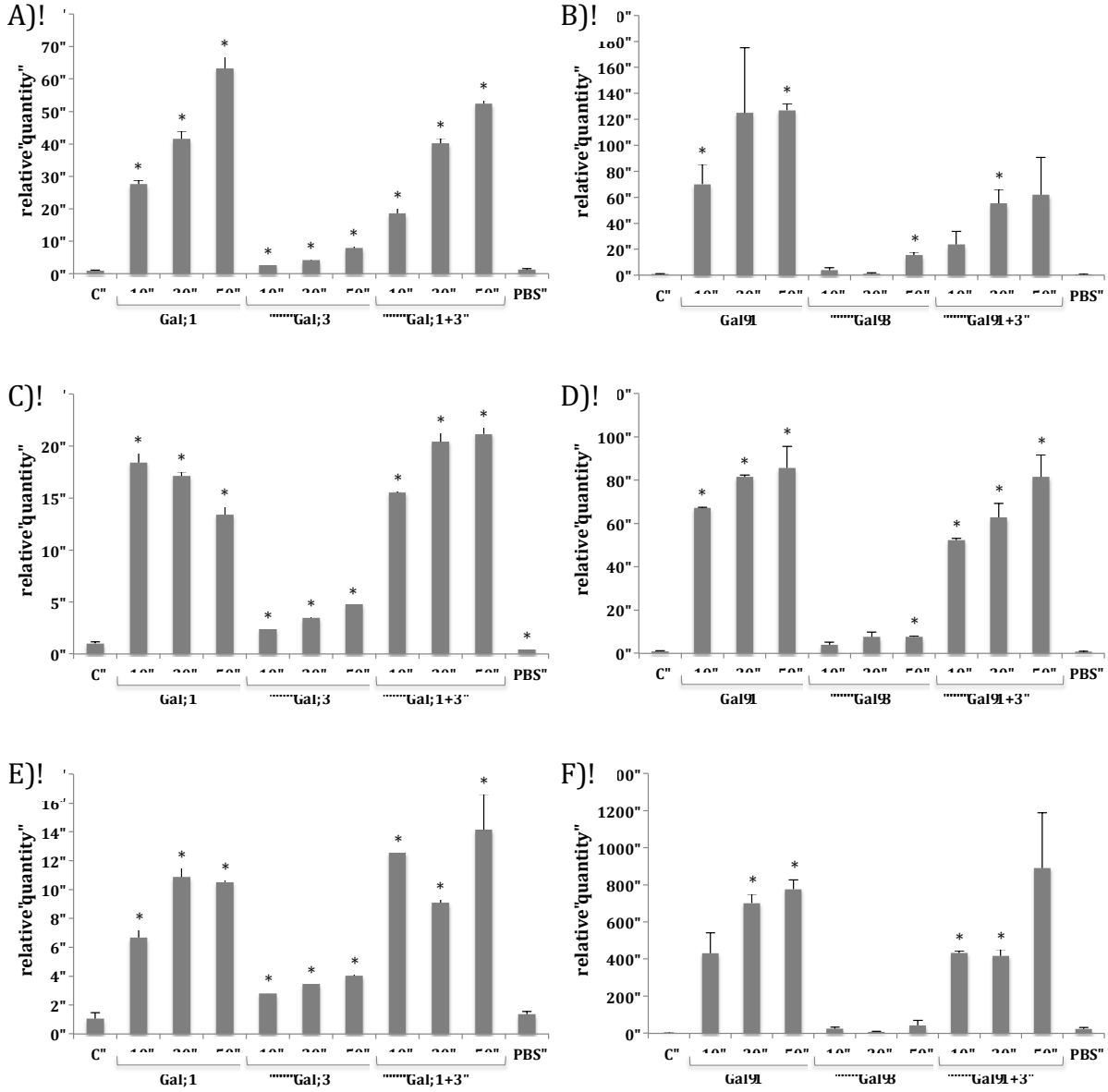

Figure 20 – MMP13 and IL1B mRNA expression in OA chondrocytes treated with Gal-1, -3 or -1+3

OA chondrocytes of three patients were treated with 10 µg/ml, 20 µg/ml and 50 µg/ml Gal-1, Gal-3 and with a combination of Gal-1 and Gal-3 for 24 h. Additionally, a control was prepared with 100 µl PBS (Phosphate buffered saline) combined with 900 µl full medium. Untreated cells served as control (C). RT-qPCR was performed to quantify the gene expression levels of MMP13 (A, C, E) and IL1B (B, D, F) relative to the untreated controls. All data were normalized to SDHA as reference gene. P-values < 0.05 (*) were considered statistically significant (t-test).

3.2.2 Impact of Gal-1, -3 and -8 on gene expression in SW1353 cells

In a first set of experiments, SW1353 cells were treated with 10 µg/ml and 50 µg/ml Gal-1, Gal-3 or a combination of both galectins, i.e. Gal-1+3 (Figure 21).

Interestingly, no significant overall increase of IL1B (Figure 21 A) or TNFA (Figure 21 B) expression could be discovered.

To assess whether the activity of galectins in SW1353 cells might be dependent on concentration, an experiment with a range of different concentrations of Gal-1 (Figure 22 A, B), Gal-3 (Figure 22C, D) and Gal-8 (Figure 22 E, F) was performed. Figure 10 shows the mRNA expression levels of IL1B (A, C, E) and TNFA (B, D, F) of SW1353 cells. Also in these experiments, no significant effect of galectins on mRNA expression levels could be observed.

As the effect of galectins in SW1353 cells might also be time-dependent, experiments for SW1353 cells (Figure 23) treated with Gal-1, Gal-3 and Gal-8 for 1 h, 6 h and 16 h were conducted. Again, no remarkable increases of IL1B (Figure 23 A) and TNFA (Figure 23 B) expression could be detected.

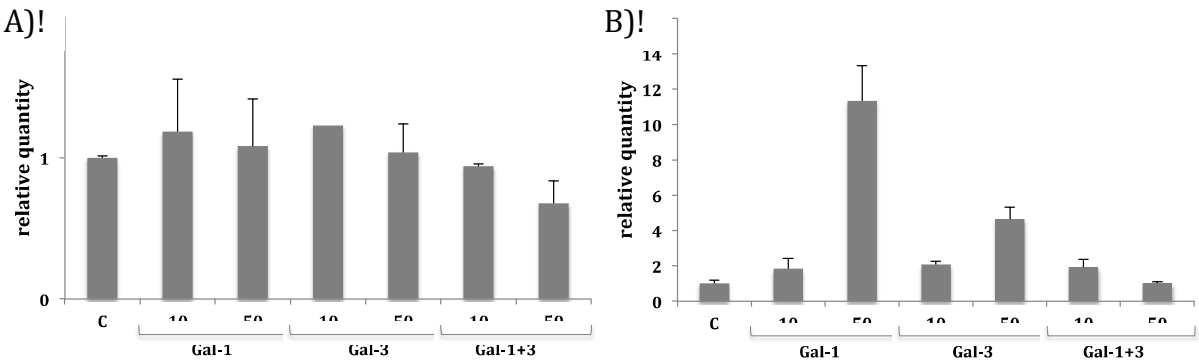

Figure 21 – IL1B and TNFA mRNA expression in SW1353 cells treated with Gal-1, Gal-3 and Gal-1+3

SW1353 cells were treated with 10 µg/ml and 50 µg/ml Gal-1, Gal-3 and with a combination of Gal-1 and Gal-3. Untreated cells served as control (C). To quantify the gene expression levels of IL1B (A) and TNFA (B) relative to the untreated controls, RT-qPCR was accomplished. All data were normalized to SDHA as reference gene. P-values ≤ 0.05 (*) are considered statistically significant (t-test).

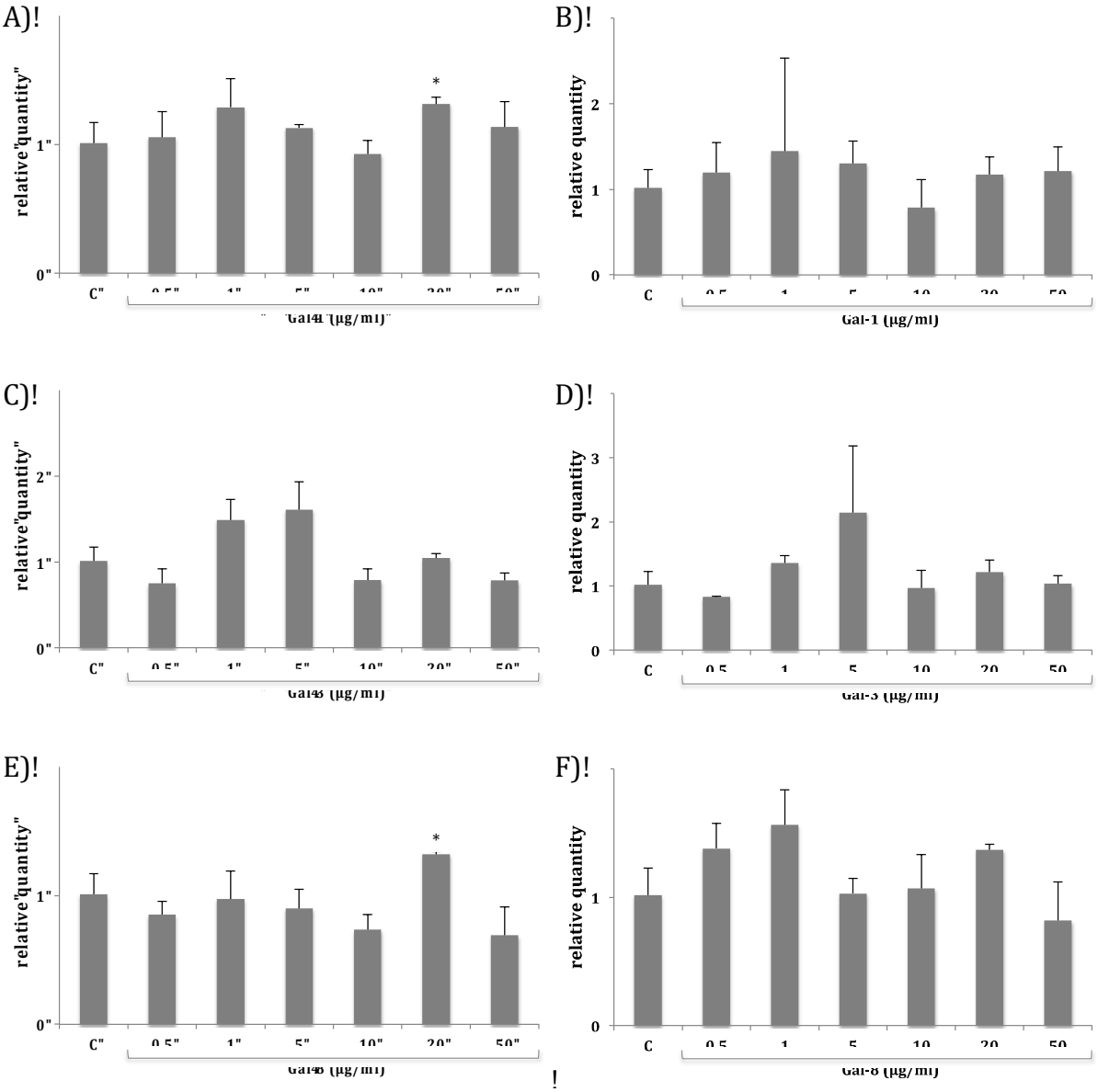

Figure 22 – IL1B and TNFA mRNA expression in SW1353 cells treated with Gal-1, -3, -8
SW1353 cells were treated in one experiment with 0.5 – 50 µg/ml Gal-1 (A, B), Gal-3 (C, D) and Gal-8 (E, F) for 24 h. Untreated cells served as control (C). RT-qPCR was conducted to quantify the IL1B (A, C, E) and TNFA (B, D, F) expression levels relative to the untreated controls. All data were normalized to SDHA as reference gene. P-values < 0.05 (*) were considered statistically significant (t-test).

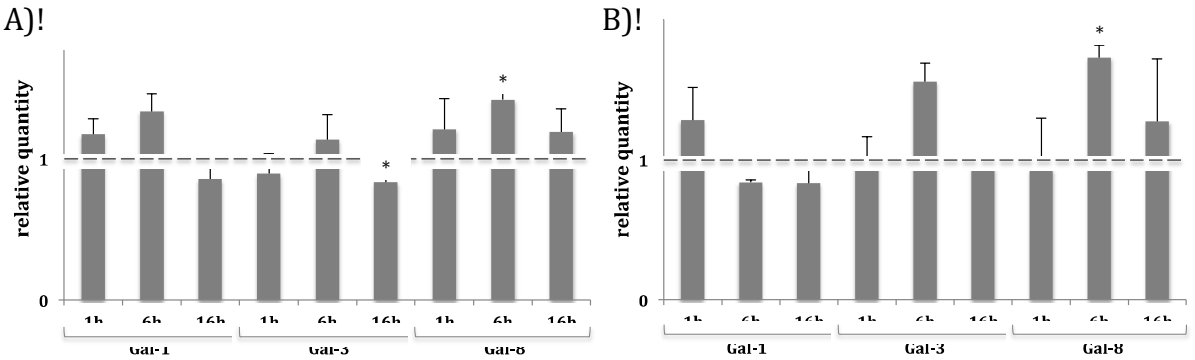

Figure 23 – IL1B and TNFA mRNA expression in SW1353 cells under time-dependent treatments with Gal-1, -3 and -8

SW1353 cells were treated in one experiment for 1 h, 6 h and 16 h with Gal-1, -3 or -8. Untreated cells served as control (C). RT-qPCR was performed to quantify the IL1B (A) and TNFA (B) expression levels relative to an untreated control. All data were normalized to SDHA as reference gene. P-values < 0.05 (*) were considered statistically significant (t-test). The dashed line was set at 1 to illustrate the steady mRNA expression levels.

3.3 Impact of Gal-1 and Gal-3 on the secretion of marker proteins

Cell culture supernatants were used to determine the concentrations of pro-MMP-1, total MMP-3 and pro-MMP-13 using ELISA. Therefore, chondrocytes of three patients (Figure 24 A, D, G) or five patients (Figure 24 B, C, E, F, H, I) were treated with Gal-1 (A, B, D, E, G, H) and Gal-3 (C, F, I) for 24 h.

Untreated chondrocytes secreted 0.18 ng/ml pro-MMP-1 into the culture medium (Figure 24 A). In comparison, the secretion of pro-MMP-1 was increased in Gal-1-treated chondrocytes up to 16 ng/ml at 10 µg/ml Gal-1 ($p > 0.05$), 22 ng/ml at 20 µg/ml Gal-1 ($p > 0.05$) and 24 ng/ml at 50 µg/ml Gal-1 ($p < 0.05$). The total MMP-3 (Figure 24 D) secretion increased significantly up to 60 ng/ml with rising concentrations of Gal-1 treatment. Regarding pro-MMP-13 (Figure 24 G), Gal-1 treated chondrocytes secreted into the culture medium 339 pg/ml at 10 µg/ml Gal-1, 1161 pg/ml at 20 µg/ml Gal-1 and 2902 pg/ml at 50 µg/ml Gal-1 while the untreated control only reached 5 pg/ml ($p > 0.05$ in all cases).

In the culture medium of the 50 µg/ml Gal-1 (Figure 24 B) and Gal-3 (Figure 24 C) treated chondrocytes, the secretion of pro-MMP-1 was significantly higher as compared to the untreated controls. Similarly, the secretion of total MMP-3 was significantly higher in the culture medium of the 50 µg/ml Gal-1 (Figure 24 E) and

Gal-3 (Figure 24 F) treated chondrocytes, compared to the untreated controls. The secretion of total MMP-3 (311 ng/ml) and pro-MMP-1 (111 ng/ml) was much higher than the secretion of the pro-MMP-13 (up to 4884 pg/ml). The secretion of pro-MMP-13 was significantly higher in culture medium of the Gal-1 (Figure 24 H) treated experiments but showed no significant increase in the culture medium of the Gal-3 (Figure 24 I) experiments.

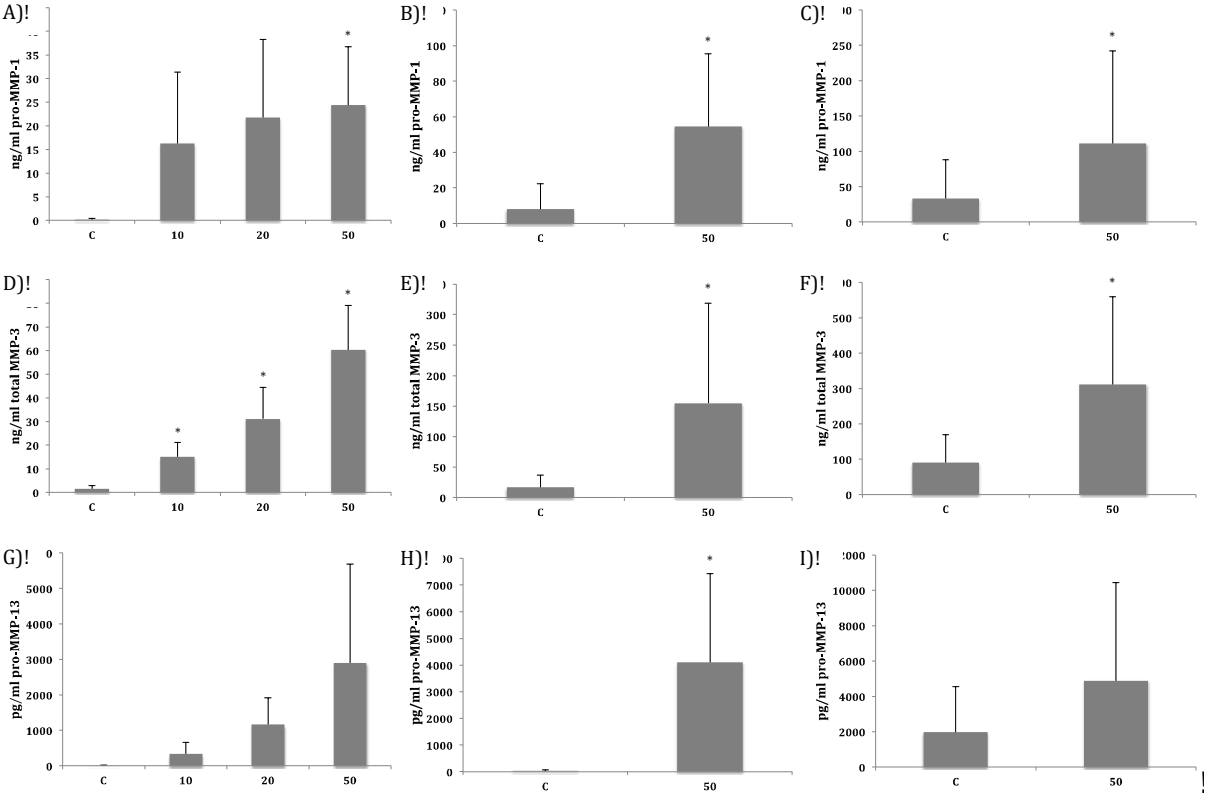

Figure 24 – pro-MMP-1, total MMP-3 and pro-MMP-13 secretion to the cell culture medium

The concentration of pro-MMP-1 (A, B, C), total MMP-3 (D, E, F) and pro-MMP-13 (G, H, I) was assessed in the cell culture supernatants of primary chondrocytes treated with 10 µg/ml, 20 µg/ml and 50 µg/ml Gal-1 (n=3) (A, D, G), with 50 µg/ml Gal-1 (n=5) (B, E, H) and with 50 µg/ml Gal-3 (n=5) (C, F, I) for 24 h. Pro-MMP-1 levels, total MMP-3 levels and pro-MMP-13 levels were compared to the cell culture supernatant of an untreated control, using. P-values < 0.05 (*) were considered statistically significant (t-test).

3.4 Impact of Gal-1 on cell signaling components

Western blot analysis was conducted to get a better understanding of the signaling pathways induced by Gal-1 in chondrocytes. Therefore, chondrocytes from five

patients (n=5) were treated with 50 µg/ml Gal-1 for 24 h. Compared to the untreated controls a strong increase of p50 and p65 proteins could be observed (Figure 25).
NF-κB is a protein complex responsible for the regulation of immune response, cell proliferation and apoptosis. It is in charge of cytokine production and cell survival.

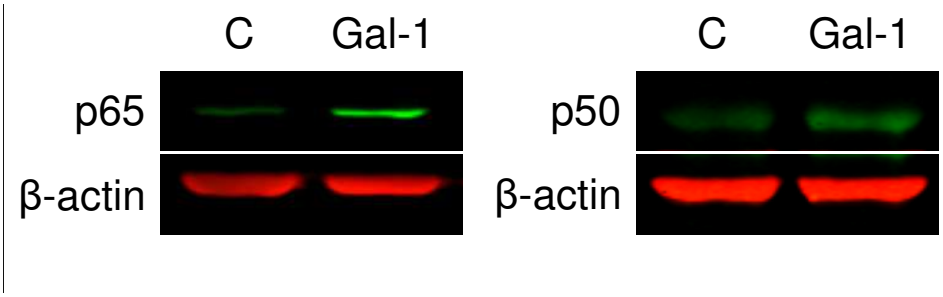

Figure 25 – Gal-1 treatment stimulates components of the NFκB pathway
The p65 and p50 protein amounts (green), isolated from chondrocytes of five patients, compared to the amounts of β-actin (red) used as loading control, is shown in one representative image (B). The chondrocytes were treated with 50 µg/ml Gal-1 for 24h and compared to an untreated control.

The effects of CAPE addition to Gal-1-treated chondrocytes on mRNA expression levels of IL1B, IL8 and CXCL1 are shown in Figure 26. Chondrocytes of one patient were treated with 5 µg/ml, 10 µg/ml and 20 µg/ml of Gal-1 in the presence or absence of 10 µg/ml CAPE. The increase of IL1B (Figure 26 A), IL8 (Figure 26 B) and CXCL1 (Figure 26 C) expression was much higher in the Gal-1-treated chondrocytes than in those cells treated with both CAPE and Gal-1. For instance, at 20 µg/ml Gal-1, the gene expression level of IL1B was reduced significantly by CAPE from 482-fold to 3.2-fold. The addition of CAPE induced a reduction of the gene expression level of IL8 at 20 µg/ml Gal-1 from 821-fold to 36-fold and of CXCL1 from 203-fold to 0.7-fold. This experiment indicates that the activity of Gal-1 in human OA chondrocytes acts, at least partly, via activation of the NF-kB pathway.

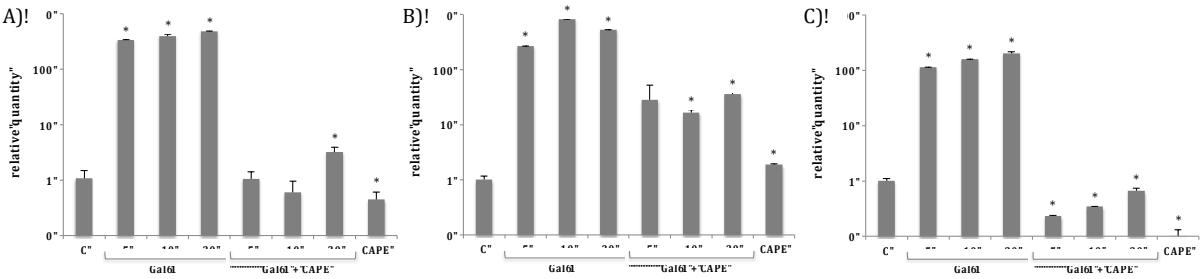

Figure 26 – Gene expression in OA chondrocytes treated with Gal-1 in absence or presence of CAPE

Chondrocytes were either treated with 5 µg/ml, 10 µg/ml and 20 µg/ml Gal-1 or pretreated with CAPE for 1 h and then with Gal-1 for 24 h (n=1 patient). Untreated cells served as control (C). RT-qPCR was performed to quantify IL1B (A), IL8 (B) and CXCL1 (C) expression levels relative to the untreated controls. All data were normalized to SDHA as reference gene. P-values ≤ 0.05 (*) were considered statistically significant (t-test).

4 Discussion

Galectins have a binding affinity for β-galactosides. A range of cellular processes can be caused by galectins binding to cell surface and extracellular matrix glycans. Through protein-protein interactions with other cytoplasmic and nuclear proteins they exert influence on cellular functions such as intracellular signaling pathways. Building on this knowledge and on former studies about the effects of galectins in cartilage degeneration and OA, our aim was to further investigate the role of Gal-1, -3 and -8 in these processes.

It is well known that proinflammatory cytokines play a key role in OA-associated processes. Goldring (2000) investigated the influence of IL-1 and TNFα, on cartilage destruction and the role of chondrocytes in mediating their effects. On the other hand Toegel and coworkers (2010) ascertained the possibility that these OA-relevant cytokines are related to glycans. Considering that galectins can interfere with cellular glycans and the fact that glycans are linked to proinflammatory cytokines it can be assumed that galectins serve as a trigger for OA. In 2014, Toegel and coworkers also observed that a range of galectins is expressed in OA cartilage and encouraged additional studies on galectins, especially on Gal-1, Gal-3 and Gal-8.

Therefore, this study attempted to determine the extent to which these galectins participate in the biological processes of OA and how they correlate with proinflammatory cytokines. We used human chondrocytes and SW1353 cells (a chondrosarcoma cell line) to conduct our galectin experiments. First we wanted to figure out if SW1353 cells could represent a reproducible cell model because they are readily available and easier to cultivate than human chondrocytes. Another reason was to avoid the interindividual differences, encountered in chondrocytes obtained from different patients. Surprisingly, the cell line did not show any significant response in their IL1B and TNFA mRNA expression levels, neither in time-dependent nor in concentration dependent experiments with Gal-1, Gal-3, Gal1+3 and Gal-8. Investigating the mRNA expression levels of IL1B and TNFA in galectin-treated SW1353 cells took us to the same conclusion as the findings of Gebauer and colleagues (2005), who found out that SW1353 cells are no satisfying *in vitro* system for the investigation of chondrocyte anabolism. Hence, it can be assumed that

SW1353 cells cannot be used as a surrogate for human chondrocytes. A reason for this is the sialylation of cell surface glycoproteins, a contributory cause of biological processes such as cell-matrix interactions, which might play a decisive role in the onset of degenerative joint diseases including OA (Toegel et al., 2010). SW1353 cell lines differ considerably from chondrocytes in their molecular phenotype (Gebauer et al., 2005; Toegel et al., 2009). While the characteristic structure observed in primary human chondrocytes are α-2,6-linked sialic acids, the altered phenotype in SW1353 cells is associated with abundant α-2,3-linked sialic acids (Toegel et al., 2010). As a consequence, relevant binding sites for galectins in SW1353 could be missing. Taken all these results together, we came to the conclusion that only primary chondrocytes were useful for our galectin experiments.

Goldring (2000) revealed the influence of MMPs, ADAMTS, IL1β and TNF on cartilage destruction in OA cartilage. In this study, our aim was to evaluate the correlation between galectins and inflammatory factors being responsible for cartilage degradation. To the best of our knowledge, we are first to provide extensive results for primary human chondrocytes treated extracellularly with Gal-1, -3 and -8.

Based on the knowledge that MMPs are part of the degradation of cartilage collagens and proteoglycans in OA, we did some further research for MMP13. Disagreeing with Janelle-Montcalm et al., (2007), who did not provide any data supporting their statement, we proofed that MMP-13 was very well stimulated by Gal-3 in chondrocytes. In accordance with Marsich and colleagues (2008), who used porcine chondrocytes for their experiments, we observed an up-regulation of MMP13 in Gal-1-treated human chondrocytes. All experiments with Gal-1, -3 and Gal-8 showed an up-regulation of MMP13 both in a time and concentration dependent manner. Only in some cases,, the combination of Gal-1 and Gal-3 induced a small additive effect on MMP13 and IL1B expression .

After exploring the increase of mRNA levels of cytokines and MMPs on galectin treated chondrocytes, we wanted to further investigate the impact of Gal-1 and Gal-3 on the secretion of marker proteins. Therefore, using ELISA, we investigated the secretion of pro-MMP-1, total MMP-3 and pro-MMP-13. In all three cases, we observed an increase when cells were treated with Gal-1 and Gal-3.

The transcription factor p65 controls anabolic and catabolic processes during OA development (Kobayashi et al., 2013). In our experiments, the expression levels of RELA (the gene encoding for transcription factor p65) showed a significant increase with all three galectins. With the knowledge that galectins raise the mRNA expression of this transcription factor, we further conducted Western blot experiments to confirm that galectins also have influence on the protein levels. Liu and colleagues (2002) provided information about galectins binding to intracellular ligands and thus regulating a series of signaling pathways. In agreement, our results clearly indicate that Gal-1 has an impact not only on messenger expression levels but also on protein levels in cell signaling of the NF-κB pathway. Hence, we presented evidence that at least Gal-1 acts as an inducer of the NF-κB pathway in human OA chondrocytes.

Ak et al., (2015) recently described that CAPE inhibits many inflammatory factors by blocking NF-κB and oxygen radicals and induces apoptosis in inflammatory cells. In line with this observation, we discovered that CAPE inhibits the Gal-1-induced mRNA expression levels of IL1B, IL8 and CXCL1, suggesting that the activity of Gal-1 in human OA chondrocytes is largely NF-κB-mediated. Further experiments with CAPE and different galectins and its possible coherence with the NF-κB pathway are recommended. Aside from that, the exact gene profile induced by the interaction of galectins with their binding sites, and the possibility of galectins interfering with other pathways than the NF-κB pathway should be examined. Where do galectins come from in OA cartilage and do they occur in other parts (like synovial tissue) of the joint? Are exterior influences like nutrition, living conditions or genetic predisposition inducers of galectin overexpression? Would it be effective to inhibit the secretion of galectins completely or is it sufficient to modify their binding sites and how could it be done? Are other galectins than Gal-1, -3 and -8 acting as participating triggers in OA?

Using glycoproteins as a therapeutic target or as a biomarker would be a desirable development and motivation for further research.

5 Conclusion

In summary, this study suggests that Gal-1, Gal-3 and Gal-8 might play a role in the pathophysiological mechanisms eventually leading to OA. We demonstrated that these galectins induce many factors, known to play a key role in OA progression. RT-qPCR experiments showed that proinflammatory cytokines are upregulated at the mRNA level by the galectins. In agreement, ELISA experiments showed that galectins also enhance catabolic enzymes, which cause degradation of cartilage matrix in OA. Finally, Western blot analysis suggested the participation of Gal-1 in NF-κB signaling.

Comparative experiments with SW1353 cells and primary chondrocytes revealed that SW1353 cells cannot replace chondrocytes as cell culture model when studying galectin activity. Because of this discovery SW1353 should not be used as a surrogate for primary chondrocytes regarding glycobiology-related experiments.

Future studies should further identify the interactions between galectins and their binding sites as well as the resulting effects in OA cartilage. Improved understanding of the role of galectins in OA might lead to the development of novel biomarkers and therapeutic targets for diagnosis and therapy of OA.

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7 Appendix

7.1 Abbreviations

ADAMTS	a disintegrin and metalloproteinase with thrombospontin-like motifs
AGC	Aspartate-Glutamate Carrier
Asn	Asparagine
CAPE	caffeic acid phenetyl ester
cDNA	complementary Deoxyribonucleic acid
COL	Collagen
CRD	carbohydrate-recognition domains
C_T	cycle threshold
DMEM	Dulbecco´s Modified Eagle's Medium
ECM	extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FBS	fetal bovine serum
FGF	fibroblast growth factor
Fuc	L-fucose
GAG	glycosaminoglycan
Gal	D-galactose
Gal-1	Galectin-1
Gal-3	Galectin-3
Gal-8	Galectin-8
GalNAc	N-acetyl-D-galactosamine
Glc	D-glucose
GlcNAc	N-acetyl-D-glucosamine
IGF	Insuline-like growth factor
IL	Interleukin
kDa	kilo dalton
LGALS1	Lectin, Galactoside Binding Soluble 1
LGALS3	Lectin, Galactoside Binding Soluble 3

Man	D-mannose
MDB	Membran Desalting Buffer
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
NeuAc	N-acetylneuraminic acid
NFκB	nuclear factor kappa-light-chain-enhancer of activatet B cells
NSAID	nonsteroidal anti-inflammatory drugs
O-GlcNAc	O-linked β-N-acetylglucosamine
o/n	overnight
OA	Osteoarthritis
PAGE	polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline- Tween20
PMSF	phenylmethylsulfonyl fluoride
MMP	matrix metalloproteinase
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
Rpm	revolutions per minute
RT	reverse transcription/ real time
RT-qPCR	Quantitative real-time reverse transcription polymerase chain reaction
SDHA	succinate dehydrogenase complex, subunit A
SDS	Sodium dodecyl sulfate
Ser	Serin
Thr	Threonin
TNF	tumor necrosis factor
Tris	Trishydroxymethylaminomethane
Xyl	D-xylose

7.2 List of Figures

7.3 List of Tables