Comparative transcriptional profiling of regenerating damaged knee joints in two animal models of the newt Notophthalmus viridescens strengthens the role of candidate genes involved in osteoarthritis

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ABSTRACT

Objectives: To compare joint regeneration in adult newts (N. viridescens) upon both newly established surgical removal and previously reported enzymatic destruction of articular cartilage to identify molecular factors and functionally analyze potentially important regulators involved in osteoarthritis (OA).

Methods: Damage of knee cartilage was induced by either intra-articular injection of collagenase or by surgical removal of articular cartilage as a novel additional approach. Changes over time were clinically and histologically analyzed and studied by cDNA microarray analysis, real-time quantitative PCR, immunohistochemistry and functional assays to identify relevant candidate genes and determine their impact on regeneration.

Results: Several genes were found to be up-regulated during regeneration, including extracellular matrix components and mediators of cell-matrix interactions, genes encoding for cellular components, for cell and tissue homeostasis and tissue remodelling, for cellular processes as well as signalling molecules. A high activity and diversity of transcription was detected on days 10 and 20, especially in the surgical model. 10 candidate genes were further analyzed. The matricellular protein tenascin C (TN-C) attracted our particular attention due to its prominent up-regulation during regeneration in both models and at different time points.

Conclusions: Newts are able to regenerate OA-like articular cartilage damage ad integrum both after enzymatic and mechanical injury. Most of the genes involved in amphibians are also known to be operative in humans and other mammals, especially matricellular factors interfering with optimized matrix remodelling. Our results stress the necessity to elucidate mechanistic differences in different species potentially using identical molecules but with different functional results.

1. Introduction

Osteoarthritis (OA) is the most common disorder of the musculoskeletal system [1]. As degenerative joint disease also covering immunological features and inflammation, it is mainly characterized by loss of articular cartilage and alterations in subchondral bone with development of eburnated joint surfaces, subchondral sclerosis and formation of osteophytes, bony erosions and cysts [2]. Due to the altered tissue metabolisms with increasing joint instability, this results in painful loss of function. Since treatment is symptom-restricted with pharmacological and physical options, end-stage joint replacement surgery is often unavoidable [2]. The socio-economic costs due to disability are tremendous [3].

Although several human tissues including bone and muscle are able to mount an efficient healing response after trauma, the avascular and aneural cartilage fails to initiate an appropriate regeneration, so that injury results in fibrous scar formation. However, regeneration of cartilage wounds has been reported in some mouse strains [4].

Compared to mammals, many amphibians, reptiles and fish have the ability to regenerate lost or damaged tissues including limbs, tails, lenses, the heart or even damage in the central nervous system with functionally re-established structures [5-8]. To study the underlying mechanisms, the
regenerating amphibian limb model has been widely used. After amputation in newts, rapid wound closure is first accomplished by migration of epidermal cells from the wound edges into the affected areas. Within the next 10 days a so-called blastema thought to consist of a pool of multipotent or pluripotent cells forms and – dependent on the respective needs – gives rise to multiple tissues including muscle, bone, cartilage, nervous structures or blood vessels [9,10]. In vitro, some progenitor cells such as muscle-derived satellite cells, for instance, are capable to adopt different lineage fates in vitro allowing adipogenic, osteogenic and myogenic differentiation, for instance [11]. Currently, there are different hypotheses on the origin of blastemal cells. They might either arise from de-differentiation of damaged tissues, trans-differentiation of lineage-restricted progenitor cells or proliferation of tissue-resident stem cells [12]. However, it is also conceivable that a combination of these processes is active that are not necessarily mutually exclusive. However, in vivo tracking in axolotl additionally suggested that individual cells keep a memory of their tissue origin and thus are not able to give rise to all cell lineages derived from different germ layers [9]. Furthermore, comparison of limb regeneration in Notophthalmus viridescens and axolotl revealed fundamental differences in these closely related species [10]. While limb regeneration in the axolotl is dependent on Pax7-positive satellite cells, tissue regeneration in N. viridescens apparently relies on de-differentiation of muscle fibers.

As reported, the red-spotted newt N. viridescens retaining its regenerative capacity throughout the whole life is an excellent model organism to study endogenous knee joint regeneration in adult vertebrates [13]. Joint injury is inducible by different approaches. We had established a model based on the intra-articular injection of collagenase. Now, we characterize a second model relying on surgical removal of articular cartilage to carve more severe surface damages seen in chronic mechanical wear and tear injuries. Additionally, in contrast to the remaining uncertainty of the extent of damage induced upon collagenase injection into the joint, optically controlled surgical intervention to monitor lesion induction „as it is“ to the very aimed level proves to be adequately feasible. Unlike in murine OA models, even upon severe mechanical tissue damage joint function is completely restored after 3 months similar to the enzymatic model [13,14].

To investigate our hypothesis that the regenerative capacity in newts might rely on specific biological mechanisms still operative in amphibians but probably silenced upon time during ongoing evolution and the development of novel species and could thus potentially be switched on again in order to promote cartilage and joint regeneration in mammals, we aimed to further evaluate the underlying mechanisms and transcriptional changes in both newt regeneration models using screening approaches and investigated potential candidate genes. The strongly de-regulated tenasin C (TN-C) expression was also analyzed on the protein level, and its effects on migration and adhesion of isolated newt cells and established newt cell lines were examined.

2. Materials and methods

2.1. Animal maintenance and manipulation

Newts were purchased from Charles Sullivan (Nashville, TN, USA), having an age of at least 3 years with completed metamorphosis, thus being sexually mature adults, and were maintained in aerated aquaria at 18–20 °C, fed with gnat larvae and artemia twice a week. Animal care and experiments were in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and with the German Animal Welfare Act and international legislation (Directive 2010/63, European Community) and approved by the local government and ethics authorities.

2.2. Induction of joint damage in newts

To induce osteoarthrit fracture-like knee joint damage in the newts, we used two different experimental models. In the first one, animals received intra-articular injections of collagenase type VII (Sigma Aldrich, Taufkirchen, Germany) as described before [13]. Alternatively, however, articular lesions were now also induced surgically by removal of femoral cartilage after transversal section of the skin and underlying layer and then dissection of the femoral cartilaginous cap with fine surgical instruments (Fig. 1). In contrast to the collagenase model, surgical skin lesions had to be sealed with a tissue glue (Histoacyrl, Braun, Melsungen, Germany). As controls, animals were sham-operated without removal of cartilage (called untreated). The area of the joint was opened but no tissue removed and the wound closed exactly as in treated animals. Before both injection or surgery, animals were anesthetized with 0.1% tricaine (Sigma-Aldrich, Taufkirchen, Germany) for 10–15 min. After the different procedures in both models, newts were kept in a 0.5% sulfamethazine solution (Sigma-Aldrich, Taufkirchen, Germany) for 30–60 min for disinfection and then transferred back into aquarium water.

2.3. Clinical evaluation

Disease symptoms after induction of joint damage were monitored in both models as described previously [13]. For details, also see the supplemental material.

2.4. Histological evaluation

Histology was investigated at days 10, 20 and 40 after treatment and control. Hematoxylin-eosin (HE) and Safranin O stainings were performed. The protocols used are given in the supplemental material section.

2.5. Normalized cDNA libraries for glass microarrays

Detailed background information can be obtained from the supplemental material section.

2.6. RNA isolation of newt knee joints

Total RNA of entire newt knee joints was isolated of arbitrarily grouped pools of 2 or 3 operated or injected animals with both low and high clinical score to facilitate detection of genes expressed at early and later time points. Their respective contralateral controls were also processed. RNA extraction was performed by tissue homogenization in ice-cold Trizol reagent using a swing mill (Retsch MM200, Haan, Germany), following the manufacturer’s instructions (Invitrogen, Karlsruhe, Germany) as described previously. Afterwards, RNA quality was checked on an Agilent 2100 Bioanalyzer with the RNA Nanokit (Agilent Technologies, Santa Clara, CA, USA).

2.7. Microarray hybridization

RNA was amplified with MessageAmp™ II aRNA Kit (Ambion Life Technologies, Darmstadt, Germany). For two-colour microarray hybridization, cDNA labeling was performed with SuperScript™ Plus Direct Labeling Kit (Invitrogen, Karlsruhe, Germany), using Alexa 555 and Alexa 647 dye-coupled nucleotides (Invitrogen, Karlsruhe, Germany). Hybridization experiments of knee-operated newts at days 10, 20 and 40 after surgery and a second set of collagenase-treated newt knee joints at days 10, 20 and 40 after treatment were performed with two pooled samples (n = 3 and n = 2) of each time point against their respective sham controls. The two pooled samples per time point were technically
replicated by dye swap to reach an \( n = 4 \) per experiment. Scanning of microarrays was acquired on a GenePix 4000 B Microarray Scanner with GenePix Pro 6.0 Software and data were statistically analyzed with Acuity 4.0 Software (Axon Instruments/Molecular Devices, San Jose, CA, USA). All spots showing a significant de-regulation together with other robustly detected array spots were selected for Sanger sequencing.

2.8. Sanger sequencing and annotation of de-regulated cDNAs

To identify sequences for de-regulated cDNAs, individual bacterial clones were re-amplified and plasmids were isolated for Sanger sequencing. BLAST homology searches were performed using blastn, blastx and tblastx on NCBI’s NR (protein), NR (Nucleotide), EST and HTGS databases. All hits were sorted according to their taxa. All individual Sanger sequences are available via the newt repository newt-omics (newt-omics.mpi-bn.mpg.de) [15].

2.9. Further data analysis

Annotated high quality spots were separated into up- and down-regulated transcripts at all three different time points in both animal models, wherein expression data of both biological and technical replicates had already been implemented in single MS Excel files per time point and model. Transcripts were then sorted according to their signal log ratio or fold change, respectively. Those genes that were de-regulated at least 2-fold (signal log ratio of 1) in the same direction in at least three of four replicates were filtered and grouped according to their functional role.

Fig. 1. Clinical and histological presentation of newt knee joints after experimentally induced osteoarthritis (OA). During surgery (A), the femoral cartilage cap (shown at the bottom) was removed and the wound was sealed with a tissue glue. Histologically, hemalaun/eosin (HE) staining was performed (B, left panels). Safranin O staining was used to visualize proteoglycan content of articular cartilage (B, right panels). ac – articular cartilage, udt – undifferentiated tissue, nc – newly formed cartilage. Original magnification ×50. Scale bars: 100 μm.
2.10. Cell culture and isolation of newt chondrocytes

Further information on the B1H1 cell line, primarily isolated chondrocytes and the respective protocols with reagents and providers including the associated literature [16,17] can be found within the supplemental material section.

2.11. RNA isolation of cells and real-time PCR

Information on RNA isolation of primary newt chondrocytes and B1H1 cells including kits, suppliers and protocols, can be obtained from the supplemental material section, including a list of primer sequences, referred to as Suppl. I.

2.12. Histology and immunohistochemistry

Tissue samples were embedded in paraffine and 5 μm sections were generated as described previously [13]. Further information is to be found in the supplemental material section.

2.13. Immunofluorescence

After isolation of primary newt chondrocytes, collagen II expression was analyzed by immunofluorescence (IF) staining. For protocol description please see the supplemental material section.

2.14. Assembly of the newt TN-C sequence

Further information on procedures as well as additional data can be obtained from the supplemental material section [18–20].

2.15. Generation of TN-C-specific siRNA pools and nucleofection into newt cells

In subsequent knock-down experiments newt chondrocytes and B1H1 cells were nucleofected with the respective siRNAs for target and control. Further information on that is also given in the supplemental material section.

2.16. Adhesion assays

Cell culture plastic ware was coated with human TN-C protein and rat tail collagen type I to analyze the effect of TN-C cellular adhesion in general as well as to investigate the effects of TN-C knockdown on cellular adhesion. Further information including image analysis [21] is given in the supplements section.

2.17. Spheroid cultures

Spheroid formations were induced to test the influence of TN-C on migration of B1H1 cells out of the spheroids in TN-C-coated, collagen type I-coated or control wells [22]. The protocol is described in the supplements section.

2.18. Statistics

Data are shown as arithmetic mean ± standard error of the mean (SEM). For pairwise comparisons, Student’s unpaired t-test analysis was performed. P values < 0.05 were considered statistically significant. Statistical evaluations were done using Microsoft Excel and the GraphPad Prism 5 software.

3. Results

3.1. Clinical and histological evaluation of regeneration after experimentally induced knee joint damage in newts

Similar to our previous report [13], treated newts showed normal food uptake in both models, but avoided to use the treated leg and reduced active swimming through the aquaria. Surgically treated animals required a slightly longer period of time of 2–6 days to recover from the intervention than enzymatically treated animals. No apparent signs of inflammation such as swelling or redness were observed in surgically treated animals. All newts of both models, however, showed atypical movements and displayed defective positions of the extremity. Two weeks after injury, animals regained higher activity again, although signs of joint defects were still present.

At the morphological level, a part of the collagenase-injected animals showed luxation of the treated joint. In the surgical group, it was also possible to detect a disruption of the anatomical axis of the treated leg. Safranin O staining revealed a prominent decrease of proteoglycan content, preferably seen in injected animals still possessing the entire articular cartilage cap. 20 days after treatment, amorphous tissue surrounding the joint space was visible in both models (Fig. 1), partly along with detachment of muscles from bone and the periarticular zone. After approximately 40 days, joint structure and functionality were usually completely restored in most of the animals. In general, the novel surgical approach proved to be more homogenous than the collagenase model, which is due to the constant mechanical procedure independent of the efficacy of a chemical agent.

3.2. Microarray analysis

Microarray analysis of damaged versus intact knee joints of newts revealed different numbers of de-regulated transcripts at the different time points in both models. In general, more genes differentially expressed were noticed in the surgical model than in enzyme-injected newts, and more genes were found to be up-regulated than down-regulated during regeneration. Thus, in surgically induced joint failure, 110 transcripts for 26 known genes were up-regulated on day 10, 83 transcripts for 25 genes on day 20, and 17 transcripts for 3 genes on day 40. In collagenase-injected animals, in contrast, 37 transcripts for 15 known genes were up-regulated on day 10, no hits fulfilled the criteria on day 20, and 82 transcripts for 17 genes were increased on day 40. When comparing down-regulation, 7 transcripts for 4 genes were decreased in operated animals on day 10, 21 transcripts for 11 genes on day 20, and 9 transcripts for 9 genes on day 40, after subtraction of globin transcripts exclusively noticed at the latter two time points. Interestingly, no hits fulfilled the criteria for down-regulation in collagenase-injected animals.

Among the up-regulated transcripts we then primarily focused on, there were several constituents of the extracellular matrix or for cell–matrix interactions such as collagens, fibronectin, biglycan, decorin, lumican, osteonectin, CSPG-2, tenasin, peristin, ranaspinin, further transcripts encoding for cellular components such as keratins or annexins, genes for cell and tissue homeostasis and tissue remodelling including proteinases such as MMPs and cathepsins, genes for cellular processes including heat shock proteins as well as signalling molecules such as of the vitamin A metabolism. Especially in the surgical model on days 10 and 20, a high activity and diversity of transcription were detectable. These changes over time are illustrated in a grouped manner in Fig. 2.

3.3. Verification of selected candidate genes by real-time PCR

In total, differential expression of 10 selected candidate genes was verified by real-time PCR (Fig. 3). Apart from matricellular proteins and osteoarthritis-associated genes, these also include members of the innate immune system we additionally were interested to study independent of
whether they were detected by microarray.

While the expression pattern of the matricellular proteins osteonectin (OSN), periostin (POSTN) and TN-C showed opposing trends between the two different pools after collagenase treatment, the expression levels of these proteins peaked at day 20 after surgery with the exception of values obtained for TN-C in the 2 knee sample pool. OLFML3 expression was the highest at day 20 after surgery and decreased until day 40. In collagenase-treated animals, no consistent trends in both pools could be observed. TMSB4 up-regulation was present at day 10 after OA induction in both models and ceased afterwards.

Components of the innate immune system including TLR2, TLR5 and MyD88 were up-regulated after surgery, while no consistent changes after collagenase injection were observed. Complement factor B (CFB) was up-regulated in both OA models. However, opposing trends between the two pools were observed for this candidate after surgery and collagenase injection. In contrast to the other selected candidates, HSP27 was found to be down-regulated during the regenerative process, although in both OA models no consistent regulation pattern was observed. Taken together, these findings indicate that induction of OA-like symptoms using surgery yields more consistent results than collagenase injection.

Since TN-C showed the strongest deregulation on the mRNA level and is already known to play an essential role during epimorphic limb regeneration, we selected this candidate for further analyses.

### 3.4. Spatiotemporal expression pattern of TN-C during experimentally induced OA

As already described in the literature [18], TN-C expression in untreated limb tissues was readily detectable in the epidermis, periosteum, tendons and myotendinous junctions (Fig. 4). During knee joint regeneration, strong TN-C protein expression was additionally observed at the site of surgically inflicted damage 10 days after treatment (Fig. 4). Whereas TN-C expression was already seen 10 days after treatment in the surgically induced defect model, TN-C expression was detectable 20 days
after treatment in the collagenase-induced model. In both models, strong expression could be observed in the muscle tissue surrounding the injury site and in newly formed articular cartilage, suggesting that TN-C plays an important role during knee joint regeneration especially in the formation of cartilage.

After assembly of the coding sequence of newt TN-C, the deduced amino acid sequence was aligned with TN-C sequences from human, murine and chicken origin (see Suppl. II in the supplemental material section). Newt-derived TN-C is composed of 13 epidermal growth factor (EGF)-like domains and only 8 fibronectin (FN)-type III domains. While
the number of EGF-like repeats is 15 for human and mouse, and 14 for chicken TN-C, the number of FN-type III varies considerably: 15 are part of human TN-C, while murine TN-C has 14 and chicken TN-C only 11. Using the multiple sequence alignment tool CLUSTAL Omega, the deduced amino acid sequence of newt TN-C shares 45.7% sequence identity with human TN-C, 46.9% with murine TN-C and 58.4% with chicken TN-C.

3.5. Isolation and characterization of primary newt chondrocytes for culture

Due to the small size of the animals, cell numbers after isolation were comparatively low (ranging from 10,000 cells to 52,000 cells per newt). Directly after isolation, cells had a cobblestone-like appearance as it is usual for this cell type (Fig. 5A). Expression of Sox-9, a chondrogenic transcription factor, was also detectable (Fig. 5B). In addition, isolated chondrocytes expressed collagen type II (Fig. 5C). However, during prolonged in vitro culture cells de-differentiated and adopted a fibroblast-like phenotype (further on termed primary fibroblastic cells). In accordance with these phenotypic changes, Sox-9 mRNA expression was lost and collagen type II expression was no longer detectable (data not shown). Interestingly, TN-C mRNA expression levels increased after prolonged in vitro culture.

3.6. Cellular adhesion of newt cells on TN-C substrate and outgrowth of spheroids

Depending on the cell type, TN-C has been described to have anti-adhesive or adhesive properties [23]. Cellular adhesion of both newt-derived primary cells and cell line adhered to TN-C as substrate, but the number of adhering cells was reduced as compared to collagen type I and BSA coating (Fig. 6B). The cellular morphology of the primary cells was altered. They did not spread as compared to wells only coated with BSA as control or gelatine (Fig. 6A). These results indicate that TN-C has anti-adhesive properties on newt-derived cell lines and primary cells.

Regarding the migration of newt primary cells and cell lines out of spheroids, B1H1 cells readily formed spheroids 3–4 days after plating in contrast to primary cells (Fig. 6C). While collagen type I and BSA supported the outgrowth of cells from the spheroids, cells did not migrate on BSA, and only few cells grew out on TN-C and BSA. Stimulation of B1H1 and primary cells with TN-C did not induce the expression of MMPs and ADAMs on the mRNA level (data not shown). In the literature, however, TN-C has been described to stimulate migration of various cell types, especially during metastasis [24]. Addition of soluble TN-C to the supernatants as a possible chemoattractant, however, did not affect cellular migration of primary newt cells and cell lines in wound healing assays (data not shown).

3.7. Knock-down of TN-C in newt cell lines and primary newt cells

Cellular mRNA expression of TN-C was reduced to 60.26 ± 9.07% in primary newt cells and to 57.94 ± 10.13% in B1H1 cells. A time-dependent increase in cell number was observed 48 h after knock-down in collagen type I-coated plates incubated for 15 min, 30 min and 45 min, respectively. While TN-C as substrate interfered with the adhesion of primary cells and cell lines, TN-C knock-down did not affect cellular adhesion. For illustration see Suppl. III.

4. Discussion

The regenerative capacity of adult newts also encompasses articular cartilage as was shown before [13]. Now, we intended to add a mechanical injury model by a surgical approach. Based on the removal of articular knee cartilage, we succeeded in imitating osteoarthritic joint damage with functional impairment. The de novo tissue formation was substantiated again as differentiating from the amorphous beginning to finally intact articular cartilage with completely restored morphology and normal physiological function, indicating that the regeneration upon experimental OA induction rather follows the tissue type of regeneration than epimorphic limb regeneration without simply repeating embryological organogenesis [25]. Moreover, we observed that the novel approach was more homogenous than the collagenase model due to the constant mechanical procedure independent of the efficacy of a chemical agent. To further determine molecular factors involved in both the...
enzyramic model and the novel surgical approach, we performed high-throughput gene expression profiling and conducted validation experiments.

TN-C showed the most predominant differential expression in both models with up-regulation after treatment. It is an extracellular matrix (ECM) constituent assumed to exert major roles during limb and heart regeneration in vertebrates [18,26]. Moreover, it shows a spatiotemporal expression pattern guiding the regenerative process in vertebrates [18,26]. TN-C-positivity areas consecutively displayed higher amounts of proteoglycans and of PCNA activity, a marker of cell proliferation and thus metabolic turnover [32]. Reverse investigations with TN-C-deficient mice substantiated these observations [33]. The positive effects of TN-C on cartilage repair, however, seem to resemble a double-edged sword, since the reactive up-regulation of TN-C expression during cartilage breakdown might lead to the induction of inflammatory and catalytic mediators such as IL-6, PGE-2, nitrate and ADAMTS-4 as was shown in cultured human and bovine chondrocytes, for instance [34]. Thus, when TN-C levels are elevated in the synovial fluid of osteoarthritis patients, this might also reversely trigger inflammation and further enhance matrix degradation and thereby joint destruction. This dual relevance raises the question when and how the positive effects of TN-C expression towards tissue repair overbalance the deleterious way of increasing the damage. Therefore, we were interested in further studying its functional impact on regeneration by in vitro experiments and functional assays.

Our results indicate that fixed TN-C favours anti-adhesive properties of newt-derived cell lines and primary newt cells, even though TN-C knock-down did not interfere with the adhesive capacity. This points to the fact that additional factors are necessary to support regeneration. Thus, matrix metalloproteinases (MMPs) are well known to also serve in remodelling of the ECM and to account for scar-free healing, at least in part [27,35]. This is supported by our findings that MMP-1, -9 and -18 as well as the cathepsins B, D, S and L were up-regulated in our surgery model (data not shown), indicating early repair activity. TN-C might assist in promoting the restoration of intact tissue in a cell-independent but ECM-dependent manner.

Two additional ECM proteins, osteonectin or also SPARC (secreted protein acidic and rich in cysteine) and periostin, were up-regulated in our model. Osteonectin is important for an optimized microenvironment during regeneration and regulates growth and adhesion of cells. Its impact on articular repair is poorly understood. Nevertheless, it was possible to link osteonectin to pseudochondroplasia with early-onset osteoarthritis. Thus, although mutations in the cartilage oligomeric matrix protein (COMP) are responsible for its retention in the rough endoplasmic reticulum of articular chondrocytes, osteonectin is a necessary co-factor for successful COMP trafficking [36]. This might reflect the general importance of osteonectin in regeneration, since osteonectin-deficient mice displayed osteopenia, intervertebral disc degeneration and deteriorated ECM [36]. Periostin has known functions in numerous settings, including cardiovascular and respiratory systems, oncology, inflammatory conditions, and tissue repair [37]. We already demonstrated its up-regulation in osteoarthritic lesions of human articular cartilage [38]. It was said that its implementation in matrix degradation, if so, might act via MMP-13 [39,40]. Novel data point to the fact that periostin could be involved in mechanotransduction [41], an important feature in articular chondrocytes. Moreover, periostin and TN-C expression were coordinately induced by mechanical stress in connective tissue cells via regulation by the actin cytoskeleton, supporting the assumption that periostin-mediated incorporation of TN-C into the ECM is a crucial step in maintaining tissue homeostasis and remodelling upon injury and balancing appropriate versus inadequate repair [41].

Thymosin beta 4 (TMSB4) was also found to be up-regulated in regenerating newt knee joints herein. As was shown with bovine loading-exposed cartilage and chondrocytes stimulated with TMSB4, the induction of MMP-2 and -9 reflected the importance of modulating the chondrocytic cytoskeleton, resulting in downstream mediator elevation prior to targeting ECM remodelling upon functional impairment [42]. This is not contradictory to the fact that TMSB4 was increased in serum and synovial fluid of osteoarthritis patients [43]. An additional role of TMSB4 might consist in prevention of apoptosis as was shown in human intervertebral annulus cells in vitro [44].

Shedding light on further genes retrieved and validated in our study, we found that with heat shock protein 27 (HSP27) there was also a member of the small HSPs group. Their functional role in chondrocytes and cartilage is still unclear with a scarcity of data reporting that small HSPs, especially HSP27, play a role in mediating ECM gene expression.
Thus, proteome analysis of human articular chondrocytes highlighted the importance of HSP27 in maintaining cell homeostasis [45]. Being implemented in protecting cells from thermal, toxic and oxidative stress, wound-healing assays using HSP27-overexpressing NIH3T3 cells demonstrated that HSP27 supports cell adhesion and influences cellular migration by regulating the organization of actin filaments and the expression of MMP-2 [46]. Similar mechanisms might be operative in newt joint regeneration and could be a future therapeutic target in OA.

Further candidates are given by members of the innate immune system. Thus, toll-like receptors (TLR) contribute to inflammation-associated damage in OA [2,47]. Recent evidence together with our results, however, where TLR-2 and -5 were found to be up-regulated during regeneration, suggest that innate immunity-associated mechanisms might act as regulators calibrating tissue renewal and preventing the ECM from excessive over-remodelling. TLR-driven MyD88 adaptor protein signalling, for instance, also up-regulated in our model, was shown to negatively regulate bone regeneration in murine MSC-triggered tissue regeneration [48]. Therein, proliferation, migration and regulation of differentiation of repair-committed cells were controlled by inhibition of the Akt/β-catenin signalling pathway. A similar role might be associated with complement factor B (CFB), additionally verified to be up-regulated in our analysis. Although there are no reports on its impact on articular regeneration, the CFB-dependent alternative complement pathway also gets involved in innate immunity upon TLR activation, as was shown in rat chondrocytes [49].

Finally, olfactomedin-like 3 (OLFML3) belongs to a family of polypeutical glycoproteins. As a matrix-associated molecule, OLFML3 is mainly expressed in placenta during embryonic development, but was also observed in human adult liver and heart as well as in ocular tissues including the endothelial corneal layer, uvea, lens and retina [50]. Its distinction remained elusive so far.

While the newt models with different injury approaches exhibit feasibility for observational purposes and our study revealed several molecular candidates that might prove suitable for future therapy, since they show alteration upon damage of articular cartilage and joint structures and are strongly preserved throughout evolution, this model also contains limitations. Mechanistic intervention at this moment is difficult due to the fact that knock-in or knock-out animals, for instance, are currently not available. Adult animals were evaluated due to their regenerative capacity, but breeding of the animals in captivity is still not possible. Thus, the question of how newt models make the difference between complete (amphibian) regeneration and incomplete (mammal) repair so far remains to be further answered in detail. However, elaborating and establishing procedures in model organisms with full regenerative capacity in adults as presented here allows to identify pathways hitherto unknown to be involved in regeneration despite such a critical scarcity of technical and molecular tools. Although a transfer to the human situation is not easy, known mechanisms could be confirmed in our study, e.g. for tenasin C, showing that these models are promising instruments to unravel the process of adult regeneration.

Author contributions

MG primarily conceived the study together with ThiB, ChS, EN and UML. MG, ChS, CaS, ThiB, SAS, ML and CM performed the different experiments and generated the data. Analysis and interpretation of the data were performed by MG, ThiB, ML, CaS, EN and ThiB which initially wrote the manuscript where then all authors took part in its drafting, and MG, ThiB, EN and UML critically revised it.

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Declaration of competing interests

The authors declare that there are no conflicts of interest related to this work.

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Appendix A. Supplementary data

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