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Lentiviral shRNA knockdown of ADAMTS-5 and -9 restores matrix deposition in 3D chondrocyte culture

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Abstract

Aggrecan is one of the two major constituents of articular cartilage, and during diseases such as osteoarthritis (OA) it is subject to degradation by proteolytic enzymes. The primary proteases responsible for aggrecan cleavage are the aggrecanases, identified as members of the ADAMTS family of proteases, which are upregulated in response to inflammatory stimuli. It is uncertain which of the 6 aggrecanases (ADAMTS-1, -4, -5, -8, -9 and -15) are primarily responsible for the degradation of aggrecan in human cartilage. Here we show that 4 of the 6 aggrecanases are expressed in immortalized chondrocyte cell-lines and can be up-regulated in response to inflammatory cytokines. Using RNA interference, we demonstrate robust knockdown of ADAMTS-5 and -9 expression in these cells, and by culturing them on 3 dimensional scaffolds, show that reduction in expression of ADAMTS-5 enzyme results in an increase in matrix deposition. These data suggest that the quality of tissue-engineered cartilage matrix might be improved by targeted depletion of aggrecanase expression. Moreover, this work also provides further evidence that ADAMTS-5 may be a therapeutic target in the treatment of arthritic disease.

Keywords

ADAMTS; chondrocyte; cartilage; aggrecanase; knock-down; lentivirus; retrovirus; ECM

1. Introduction

Articular cartilage is largely comprised of type II collagen and aggrecan, both of which are secreted by chondrocytes. Collagen, with its fibrillar structure, provides the tissue with its tensile strength, while aggrecan, a large aggregating proteoglycan, is associated with resistance to compression. An appropriate balance of secretion, organization and turnover of matrix molecules by cartilage chondrocytes is crucial to proper maintenance of the tissue. While the rate of turnover is low in normal adult articular cartilage, in arthritic diseases such as osteoarthritis (OA), this is enhanced, under the influence of inflammatory mediators such as interleukin-1 (IL-1) and tumour-necrosis factor (TNF) (Mort *et al.*, 1993; Saklatvala,

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1986; Mohammed-Ali, 1995). In this situation the rate of breakdown exceeds that of synthesis, leading to gradual and irreversible loss of cartilage and thereby joint function (for reviews, see Mort and Billington, 2001; Arner, 2002; Caterson *et al.*, 2000; Nagase and Kashiwagi, 2003).

The most important effectors of cartilage matrix degradation in both homeostasis and pathology are resident proteolytic enzymes whose expression can be upregulated by IL-1 and TNF. Matrix metalloproteinases (MMPs) degrade collagen in an irreversible event (Billinghurst *et al.*, 1997; Ohuchi *et al.*, 1997; Knaüper *et al.*, 1996), while aggrecan is degraded in an earlier and reversible manner, mainly by the aggrecanase enzymes (Arner *et al.*, 1999; Lohmander *et al.*, 1993; Sandy and Verscharen, 2001), at a characteristic cleavage site within the interglobular domain (Sandy *et al.*, 1991). MMP and aggrecanase cleavage products of both collagen and aggrecan have been identified in the synovial fluid of arthritic patients (Lohmander *et al.*, 1993; Sandy *et al.*, 1992; Hollander *et al.*, 1994), as well as in the conditioned media of cartilage explants stimulated with IL-1 (Arner *et al.*, 1999; Dodge *et al.*, 1989)

Aggrecanases are members of the ADAMTS (a-disintegrin and metalloproteinase with thrombospondin type I motifs) family of proteinases (Porter *et al.*, 2005), ADAMTS-4 and ADAMTS-5 being the most studied (aggrecanase-1 and -2 respectively) (Tortorella *et al.*, 1999; Abbaszade *et al.*, 1999). In transgenic mice, ADAMTS-5 appears to be predominantly responsible for aggrecan breakdown in models of arthritis (Stanton *et al.*, 2005; Glasson *et al.*, 2005; Little *et al.*, 2005), whereas in other species, ADAMTS-4 may be more important (Powell *et al.*, 2007; Tortorella *et al.*, 2001). In human cartilage explants, a recent report implicated both ADAMTS-4 and -5 in tissue breakdown (Song *et al.*, 2007), however ADAMTS-1, -8, -9 and -15 also have aggrecanase activity *in vitro* (Rodríguez-Manzaneque *et al.*, 2002; Kuno *et al.*, 2000; Collins-Racie *et al.*, 2004; Somerville *et al.*, 2003; Yamaji *et al.*, 2001), and there has been no study to definitively identify the principle enzyme(s) responsible.

This study describes the use of targeted shRNA-expressing lentiviruses to knock-down the expression of specific aggrecanases in a chondrocyte cell-line. Using C-28/I2 cells, which we have previously shown to produce some rudimentary extracellular matrix (ECM) when grown in 3D culture (Finger *et al.*, 2003), we have undertaken a preliminary investigation of the functional effect of aggrecanase knock-down on matrix deposition.

2. Materials and Methods

2.1. Materials

Tissue culture reagents were from Sigma-Aldrich (Poole, UK), cytokines from Peprotech (London, UK), plasticware from Nunc (Loughborough, UK) and Iwaki (SLS, Nottingham, UK). Molecular biological reagents were from Promega (Southampton, UK). Plasmid extraction kits were the ‘Endofree’ type from Qiagen (Crawley, UK).

2.2. Monolayer cell culture and stimulation

T/C-28a2, C-20/A4 and C-28/I2 chondrocyte cell-lines have been previously described (Goldring *et al.*, 1994; Finger *et al.*, 2003). 293T and GP2-293 cells were obtained from American Type Culture Collection (Teddington, UK). All cells were maintained in DMEM:F12 with ‘Glutamax’ (Invitrogen, Paisley, UK); 10 % FCS; Penicillin (100 U/ml)/ Streptomycin (0.1 mg/ml). For cytokine stimulation, cells were incubated for 6h with a cytokine mix (10ng/ml IL1-β, 500ng/ml TNF-α, 1,000U/ml IFN-γ).

2.3. Lentiviral and retroviral short hairpin RNA constructs

Cloned shRNA-lentiviral construct target sets for the two aggrecanases were from the MISSION™ TRC-Hs 1.0 (Human) library, designed by the RNAi consortium and available via Sigma-Aldrich (Poole, UK) (Moffat *et al.*, 2006). Unique construct designations used were TRCN0000050493 to 50497 (ADAMTS-5) and TRCN0000046633 to 46637 (ADAMTS-9). Controls were the 'scrambled' (non-targeting) shRNA expression construct and the empty cloning vector, also obtained from the RNAi consortium via Sigma.

Packaging vectors were psPAX2 and pMD2.G, obtained from Addgene (http://www.addgene.org/pgvec1). RNAi-Ready pSIREN-RetroQ vector from BD Biosciences (Oxford, UK) was used to create retroviral DNA constructs (Coughlan *et al.*, 2009) and retroviral packaging plasmid (pVSV-G) was obtained from Clontech-Takara Bio Europe (France).

2.4. Transfection and transduction

To make lentivirus, 293T cells were plated into collagen-coated 10 cm dishes, and transfected at 50–80 % confluence using the calcium phosphate precipitation method and 14 μg lentiviral shRNA construct, 12 μg psPAX2, and 4 μg pMD2.G. Retroviruses were made similarly, by transfection of GP2-293 cells with 15 μg of shRNA construct and 15 μg of pVSV-G. Virus-containing supernatant was collected 48 h later and centrifuged for 5 min at 3 000 × g to remove cell debris, before aliquotting and storing at −80 °C. Virus was titered by transduction of serial dilutions onto chondrocytes in the presence of 8 μg/ml polybrene followed by selection in 800 ng/ml puromycin. For knock-down experiments, C-28/I2 chondrocytes were transduced in 10 cm dishes with between 70 000 and 20 000 000 cfu virus, which was sufficient to achieve ~ 90 % knock-down of gene expression without being toxic to cells. After puromycin selection, transduced cells were grown up and cryo-stored to preserve the cell-lines as well as a T75 flask of cells being collected in 4 ml TRI-reagent (Sigma-Aldrich) for RNA preparation.

2.5. RNA extraction and real-time RT-PCR

Total RNA was isolated from cells using TRI-reagent (Sigma-Aldrich) according to the manufacturer's protocol, and subsequently DNAse-treated. Reverse transcription was carried out using 2 μg RNA as template, and incubating with 0.5 μg random hexamer primers for 5 min at 70 °C, before adding 1× buffer, 40 U RNAsin, 200 U MMLV reverse transcriptase (Promega, Southampton, UK) and 2 mM each dNTP, and incubating at 37 °C for 1 h followed by 70 °C for 5 min.

A set of 13 cDNA standards was generated by reverse transcription of known amounts of cytokine-stimulated cellular RNA. cDNA templates including standards were plated in triplicate into separate 386-well optical plates (Greiner, Stonehouse, UK) for analysis of each transcript. Reaction mix added to each well consisted of 1× QPCR buffer (Eurogentec, Southampton, UK); 200 nM each primer, and 100 nM FAM/TAMRA labeled probe (Eurogentec). Primers and probes were designed using 'Primer express' software (ABI) and sequences were as follows:

ADAMTS-1FOR 5'-ggacaggtgcaagctcatctg

ADAMTS-1REV 5'-tctacaaccttgggctgcaaa

ADAMTS-1probe 5'-caagccaaaggcattggctacttcttcg

ADAMTS-4FOR 5'-gtcccatgtgcaacgtcaag

ADAMTS-4REV 5'-tcatctgccaccaccagtgt

ADAMTS-4probe 5’-ttcgcgaaacgaagtgactcatctaaacacc

ADAMTS-5FOR 5’-ggctcacgaaatcggacatt

ADAMTS-5REV 5’-ggaaccaaaggtctcttcacaga

ADAMTS-5probe 5’-tggcctctcccatgacgattccaa

ADAMTS-8FOR 5’-agtgttcgaggccaaggtgat

ADAMTS-8REV 5’-ctggccacggacacagatg

ADAMTS-8probe 5’-caccctgtgtgggccagaaacac

ADAMTS-9FOR 5’-ccctgagaccaagccaacat

ADAMTS-9REV 5’-gctggtatccctgtccacaag

ADAMTS-9probe 5’-tgcatcctggcaggcgggtc

ADAMTS-15FOR 5’-gtgaaataccgatcctgcaatct

ADAMTS-15REV 5’-gtagccgttgaaagcctcaca

ADAMTS-15probe 5’-tcagcctccggaaagagcttccg

Cyclo-EFOR 5’-ggagggagagcccattgc

Cyclo-EREV 5’-ggcttgttcccaatcttgatg

Cyclo-Eprobe 5’-cccgctcaaatcctcaggtgtacatgg

Primer and probe sequences for detection of aggrecan and collagen II transcripts were as follows (Kurth *et al.*, 2007):

Aggrecan_F 5'-ctaccgctgcgaggtgatg

Aggrecan_R 5'-tcgagggtgtagcgtgtagaga

Aggrecan probe 5'-atggaacacgatgcctttcaccacga

ColIIA1_F 5'-ggcaatagcaggttcacgtaca

ColIIA1_R 5'-cgataacagtcttgccccactt

ColIIA1 probe 5'-ccggtatgtttcgtgcagccatcct

Real-time PCR reactions were carried out and analyzed using a Prism 7900 sequence detection system (ABI, Foster City, CA, USA). Relative starting amount of transcripts was interpolated from the standard curves.

2.6. Tissue constructs

For each transduced C-28/I2 cell line, four million cells were seeded onto each of 3 poly-glycolic acid (PGA) scaffolds (5mm diameter × 2mm depth) threaded on stainless steel wires suspended in spinner flasks containing the chondrocyte suspension. After seeding, the resultant cell constructs were transferred to culture dishes and maintained under semi-static conditions in DMEM; 10 % FCS; 10 mM HEPES; penicillin (100 U/ml)/streptomycin (0.1 mg/ml); 1× non-essential amino acids; 1 μg/ml insulin; 50 μg/ml ascorbic acid with 800 ng/ml puromycin on a gently rotating orbital shaker for 40 days. The culture medium was changed every 3–4 days. Both the cell seeding method and subsequent culture methods have been described fully previously (Crawford and Dickinson, 2004). At the end of the culture period, lentivirally transduced tissue constructs were frozen at −20 C for analysis of the proteoglycan content by measurement of the glycosaminoglycan content, while retrovirally transduced tissue constructs were weighed, bisected and one half mounted in the

cryoprotectant OCT, frozen in liquid nitrogen and 8μm frozen sections taken for histology and immunohistochemistry. The remaining construct halves were frozen at −20 °C for glycosaminoglycan assay.

2.7. Functional Assays and controls

The quantity of sulphated glycosaminoglycans (GAGs) in tissue constructs was measured by the standard colorimetric dimethylmethylene blue (DMMB) assay (Farndale *et al.*, 1986). The amount of DNA present in each construct was quantified using the 'DNAQF' kit according to the manufacturer's instructions (Sigma-Aldrich). The rate of formation of the reduced form of the fluorescent vital dye, Alamar Blue™ was used to indicate the cell viability/activity of chondrocyte constructs. Constructs were incubated with Alamar Blue™ according to the manufacturer's instructions. In brief, constructs were incubated with a 10% concentration of the commercial dye solution and the amount of reduced dye determined by measurement of the optical density of the dye solution at 600 nm and 570 nm. Staining with Toluidine Blue was carried out according to standard histological techniques. Cryosections (8 μm) of the engineered constructs were air dried, fixed and stained with 1% Toluidine Blue using routine laboratory protocols to detect GAG. Collagen II was detected immunohistochemically (Kafienah and Sims, 2004) using monoclonal antibodies against collagen type II and type I (Southern Biotechnology, Birmingham, USA).

2.8. Statistical Analysis

Statistical analysis was carried out using either Microsoft Excel 2007 (Washington, USA), or Prism 4.0 (GraphPad Software Inc., San Diego, USA). Pairwise comparisons were made using Student's t test, while multiple comparisons were made using one-way ANOVA and Dunnett's post-hoc test.

3. Results and discussion

3.1. Aggrecanase expression in immortalized chondrocytes

There has not been a comprehensive study of the expression of all 6 aggrecanases in our three well-known immortalized chondrocyte cell-lines C-20A4, C-28/I2 and T/C-28a2 (Finger *et al.*, 2003; Goldring *et al.*, 1994; Koshy *et al.*, 2002). We therefore sought to determine using real-time PCR, which aggrecanases were expressed and were the most highly induced by pro-inflammatory cytokines in these cell lines. Figure 1 shows that 4 of the aggrecanases (ADAMTS-1, -5, -9, and -15) were readily detectable in all three lines (with the exception of C-20/A4, in which ADAMTS-9 was not detected). Neither ADAMTS-4 nor ADAMTS-8 was expressed in any of the three lines, in agreement with previous reports including our own (Kevorkian *et al.*, 2004; Hui *et al.*, 2005; Finger *et al.*, 2003). The transcripts most consistently up-regulated by treatment with a mix of cytokines, IL-1β, TNFα and IFNγ, for 6 h were ADAMTS-5 and -9. Interestingly, in the T/C-28a2 cell-line, ADAMTS-9 was the only transcript to be up-regulated by cytokines. In fact this was due to a relatively higher constitutive level of expression for the 3 other transcripts rather than a simple lack of induction. Since the aggrecanase expression profile of the C-28/I2 cell-line in response to cytokines most closely resembled that of primary human articular chondrocytes (Hui *et al.*, 2005); we concluded that this cell-line could be used as an initial simple chondrocyte model, in which to study the knock-down of ADAMTS gene expression and begin to identify some of the resulting functional consequences.

3.2. Knock-down of ADAMTS-5 and -9 expression

Using lentiviral shRNA construct sets, for which 4 to 5 were designed and supplied per target gene (Moffat *et al.*, 2006), we generated viral particles and transduced C-28/I2

chondrocytes. Cells expressing shRNA were selected using puromycin and maintained as stably transduced populations. Expression of the targeted transcript was assessed by real-time PCR and compared to the level of expression in cells that had been transduced with the non-targeted ('scrambled') shRNA. Figure 2 shows the level of knock-down of ADAMTS-5 expression in cells transduced with each of the 5 target shRNAs designed for this transcript (TRCN0000050493, 50494, 50495, 50496 and 50497), expressed as a percentage of the amount of transcript in control cells. The highest level of knockdown was achieved with shRNA 50496 (96 ± 1.56 %), although knockdown was also seen with constructs 50494 (44 ± 3.87 %) and 50497 (79 ± 1.86 %).

Figure 3 similarly shows the knock-down of ADAMTS-9 expression, where the 5 shRNAs available were TRCN0000046633, 46634, 46635, 46636 and 46637, although 46637-transduced cells did not survive selection. shRNA 46633 provided the highest degree of knock-down (70 ± 2.8 %), while knock-down was also seen with shRNA 46634 (39 ± 3.79 %).

We have presented validation of the lentiviral shRNA target sets provided for these 2 genes by the RNAi consortium, and demonstrate a high degree of mRNA suppression (70–90 %) by comparison to many other RNAi reports.

3.3. Matrix deposition in aggrecanase knock-down tissue constructs

We previously demonstrated that immortalized chondrocyte cell-lines can be grown in a 3D culture (Finger *et al.*, 2003; Goldring, 2004) as pellets, or for a limited time as cultures suspended in alginate gels. In pellet or alginate, the immortalized chondrocytes will secrete ECM components, and although little ECM is formed, the matrix components are similar to that of 3D cultures of differentiated primary cells (Tare *et al.*, 2005; Goldring, 2004). Indeed, we have found that C-28/I2 cells grown as classic pellet cultures produce collagen II and proteoglycans as detected by immunohistochemistry (data not shown). Hence, these cell lines are a useful rudimentary chondrocyte model to enable selection of relevant shRNA sequences in our experiments.

We seeded C-28/I2 cell populations that stably expressed either a lentiviral empty cloning vector (no shRNA), or 'scrambled' shRNA or shRNA targeting either ADAMTS-5 or ADAMTS-9 onto PGA scaffolds and cultured them under standard conditions (Crawford and Dickinson, 2004) with inclusion of puromycin, for 40 days. At the end of the culture period, rudimentary cellular constructs had formed and, although they did not yield an extracellular matrix of the quality of engineered cartilage made from differentiated primary cells, they did produce detectable GAG incorporation. Figure 4 shows the amount of GAG present in each tissue construct, normalized to the amount of DNA present in the constructs. We found that relative to the 'scrambled' shRNA-expressing control constructs, ADAMTS-5 depleted cells formed constructs that incorporated a significantly larger quantity of GAGs (40 % more), implying that greater amounts of matrix are deposited by cells lacking this enzyme. In addition, ADAMTS-9 depleted cells incorporated 24 % more GAGs than the controls, however statistical analysis did not show significance in this comparison. Real-time PCR analysis of aggrecan and collagen II (COL2A1) transcripts confirmed that the observed increase in GAG deposition by knock-down chondrocytes was not due to increased levels of synthesis of these matrix proteins (data not shown). Although there are several conclusive studies demonstrating a role for ADAMTS-5 in matrix turnover (Glasson *et al.*, 2005; Song *et al.*, 2007), our data also suggest a possible role for ADAMTS-9 in human chondrocytes. Expression studies including our own data (Figure 1) do in fact show expression and cytokine-mediated up-regulation of ADAMTS-9 often over and above the levels of the other aggrecanases (Demircan *et al.*, 2005).

We also seeded C-28/I2 cells stably expressing retroviral ADAMTS-5 or control shRNAs for which we have previously shown that ADAMTS-5 expression is knocked down by > 90 % (Coughlan *et al.*, 2009), on PGA scaffolds using the same methodology as that described above. Again, only a limited amount of ECM was formed by the immortalised chondrocytes compared to constructs of primary chondrocytes. However, both control and ADAMTS-5 knock-down constructs formed a rudimentary ECM which stained positively for the hyaline cartilage marker collagen II and showed proteoglycan deposition (Figure 5A) There was a significant difference in size and weight for the control constructs (11.3 mg and 7.7 mg) relative to ADAMTS-5 knockdown constructs (38.4 mg and 47.9 mg), as well as a significantly larger quantity of GAGs incorporated by ADAMTS-5 knock-down constructs (1.8–4 fold increase over the control, Figure 5B). These results suggested that the ADAMTS-5 knock-down chondrocytes formed more extracellular matrix than the control cells. An increase in ECM production would mean that the fibres of the scaffold were covered by ECM components more rapidly than the control constructs. This, in turn, would reduce the rate at which the PGA would dissolve in the ADAMTS-5 knock-down construct, and the slower scaffold dissolution would contribute to the increased construct weight observed with the ADAMTS-5 knock-down construct. Absolute quantification of the cellular activity of 3D constructs is not possible since it is difficult to ensure complete dye penetration into the centre of constructs containing much ECM. However, use of the vital dye Alamar Blue™ does give a reasonable indication of cellular activity in the immortalised chondrocyte constructs, as the limited ECM deposited by these cells would not prevent dye penetration into the constructs. Interestingly, there was no significant difference in the cell activity as determined by Alamar Blue™ (Figure 5B), which suggested that there was no significant difference in the cellular activity or cell number in the control versus ADAMTS-5 knock-down constructs.

It is now well established that the aggrecanases have a pathological role in arthritis, and most studies have focussed on aggrecanases 1 and 2 (ADAMTS-4 and -5 respectively). In a mouse model of OA, Glasson *et al.* (2005) and Stanton *et al.* (2005) showed that ADAMTS-5 gene knock-out was sufficient to prevent aggrecan loss and cartilage erosion, while in mice ADAMTS-4 was not the enzyme involved (Stanton *et al.*, 2005). In a porcine 3D culture model, ADAMTS-4 was found to be the critical aggrecanase responsible for degrading aggrecan upon IL-1 administration (Powell *et al.*, 2007). In bovine cartilage explants treated with cytokines, both ADAMTS-4 and -5 are implicated in aggrecanolysis (Tortorella *et al.*, 2001). In fact a recent study using human cartilage explants transiently transfected with short interfering RNA (siRNA) showed that both genes are also involved in aggrecan loss in IL-1 treated or OA human cartilage (Song *et al.*, 2007). Our study was designed to investigate the effect of stable knock-down of aggrecanase genes on the deposition of ECM by chondrocytes as they form *de novo* rudimentary tissue, since we reasoned that increased quantity of matrix may be observed in the absence of aggrecanase enzymes. Indeed, we show that knock-down of ADAMTS-5 expression results in increased matrix in 3D constructs. Our future aim is to similarly knock-down expression of ADAMTS genes in human primary articular chondrocytes and use them to tissue-engineer transplant quality cartilage. This will reveal whether ablation of aggrecanase expression improves the quality of tissue-engineered cartilage in line with our current observations. As such, our investigations could be of great importance to establishing strategies for future cartilage tissue engineering.

4. Conclusions

The results obtained with immortalised human chondrocyte cell lines suggested that both ADAMTS-5 and ADAMTS-9 may be important in the turnover and formation of cartilage ECM. Hence, depletion of ADAMTS-5 and possibly ADAMTS-9 in primary chondrocytes

may result in better quality ECM formation in tissue-engineered neocartilage constructs. Based on our results, and in agreement with previous studies, ADAMTS-5 is a potential therapeutic target in arthritic disease. We now aim to use our knock-down shRNA constructs to deplete these and the other ADAMTSs, especially ADAMTS-4, in primary human chondrocytes. Further study should help to clarify which of these enzymes play key roles in the aggrecanolysis of native arthritic cartilage.

In summary, we have generated and successfully used a tissue culture model to study the role of proteolytic aggrecanase enzymes in cartilage. This combines RNAi knock-down in a chondrocyte cell-line with growth of rudimentary 3D tissue constructs that produce small amounts of matrix molecules. Using this model we have provided evidence for a role for ADAMTS-5 and possibly also ADAMTS-9 in the turnover of ECM.

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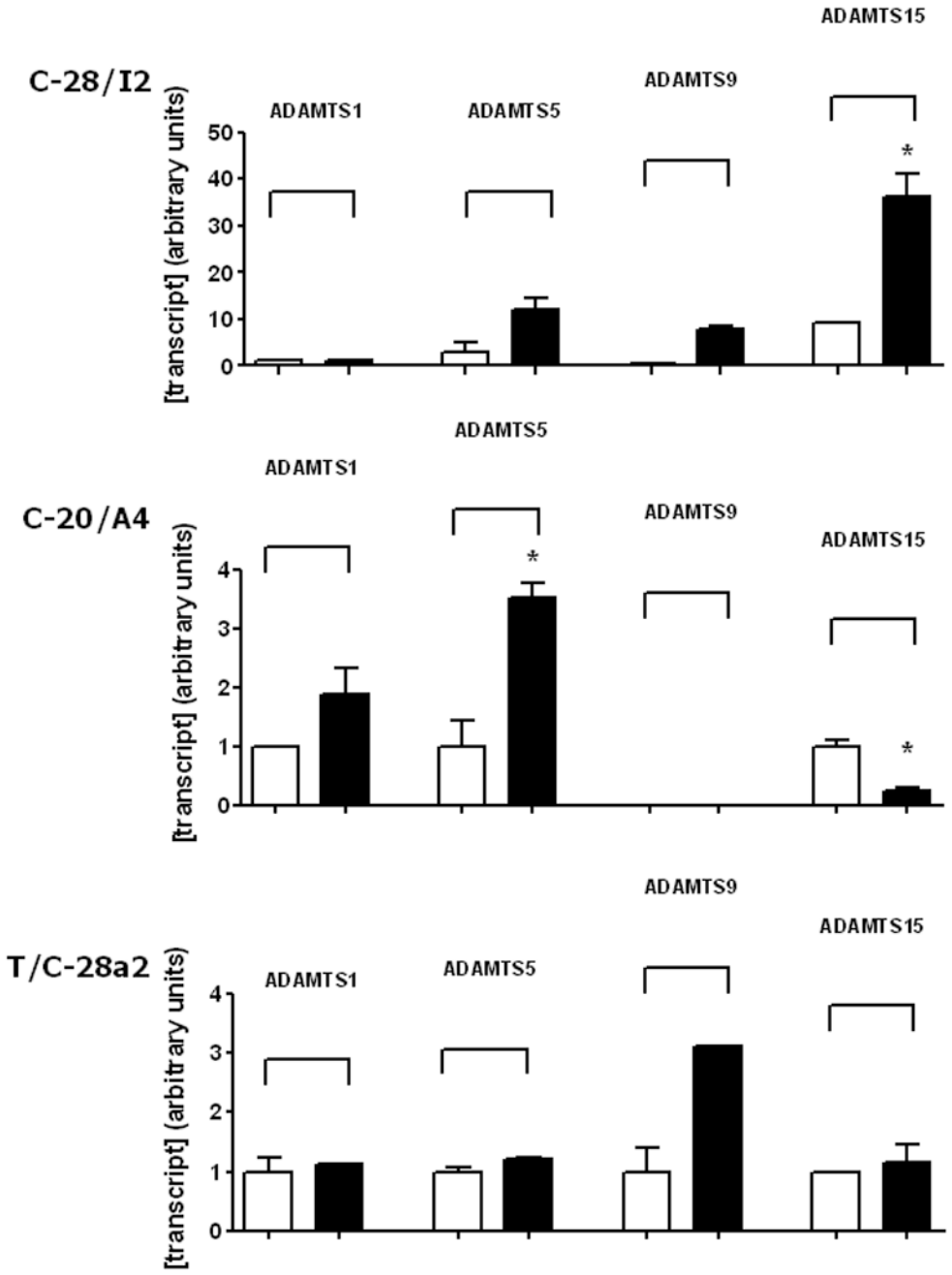

Figure 1. Expression of the 6 aggrecanases in chondrocyte cell-lines

Transcript levels for ADAMTS-1, -4, -5, -8, -9 and -15 in cells with (filled bars) and without (open bars) 6 h stimulation with cytokine mix (10ng/ml IL1-β, 500ng/ml TNF-α, 1,000U/ml IFN-γ) were assessed by real-time PCR. Data are expressed as mean ± S.E.M. mRNA levels were normalized to those of the control cyclophilin-E transcript and n=2. *$p<0.05$ relative to controls using Student's t test. ADAMTS-4 and ADAMTS-8 expression was undetectable in any of the cell-lines.

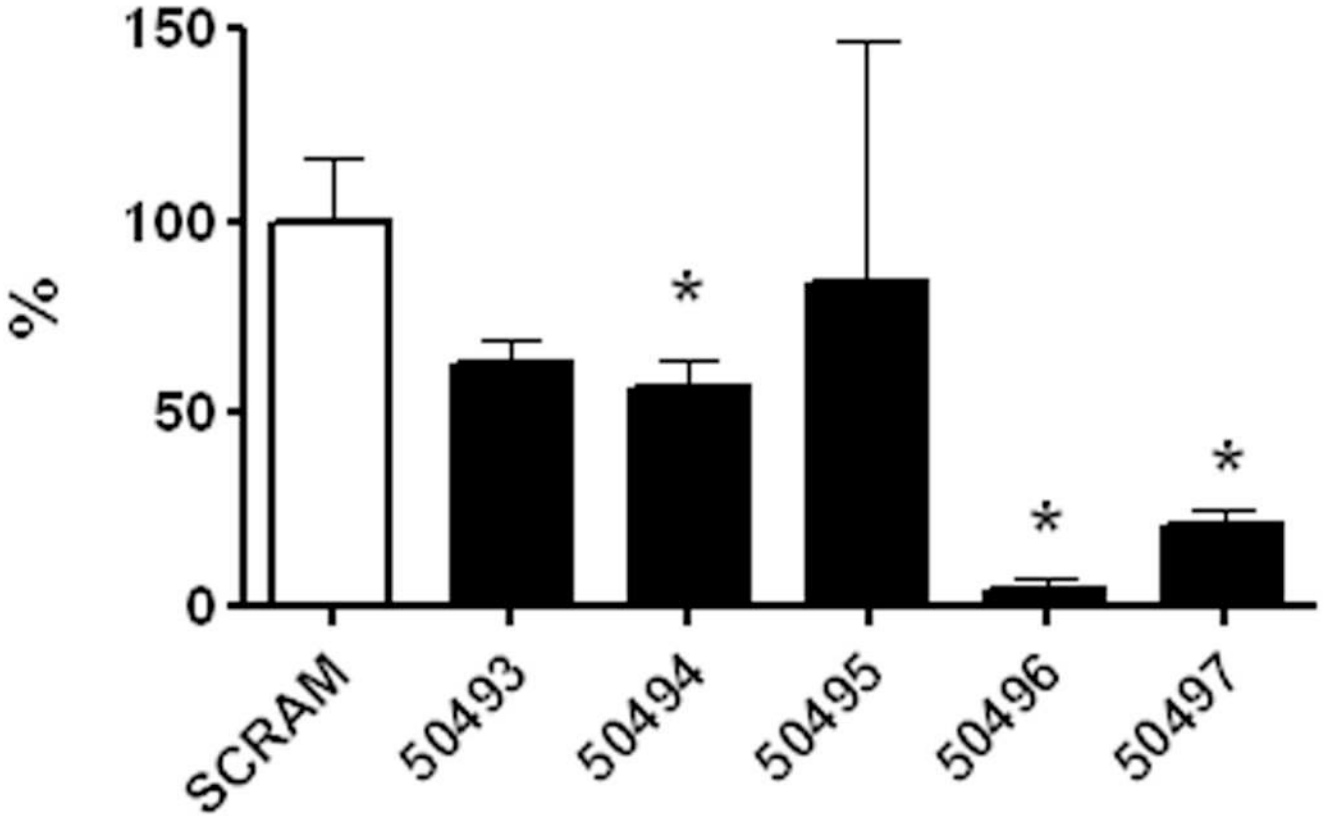

Figure 2. Knock-down of ADAMTS-5 in C-28/I2 chondrocytes

ADAMTS-5 expression was assessed by real-time PCR in cells stably transduced with lentiviral shRNA constructs either targeting the ADAMTS-5 gene (50493-7), or untargeted ('scrambled') as control. Data are expressed as mean ± S.E.M., normalized to cyclophilin-E and calibrated so that mean 'scram'= 100 %. n=4. Representative of 3 independent experiments. *$p<0.05$ relative to 'scram' using Student's t test.

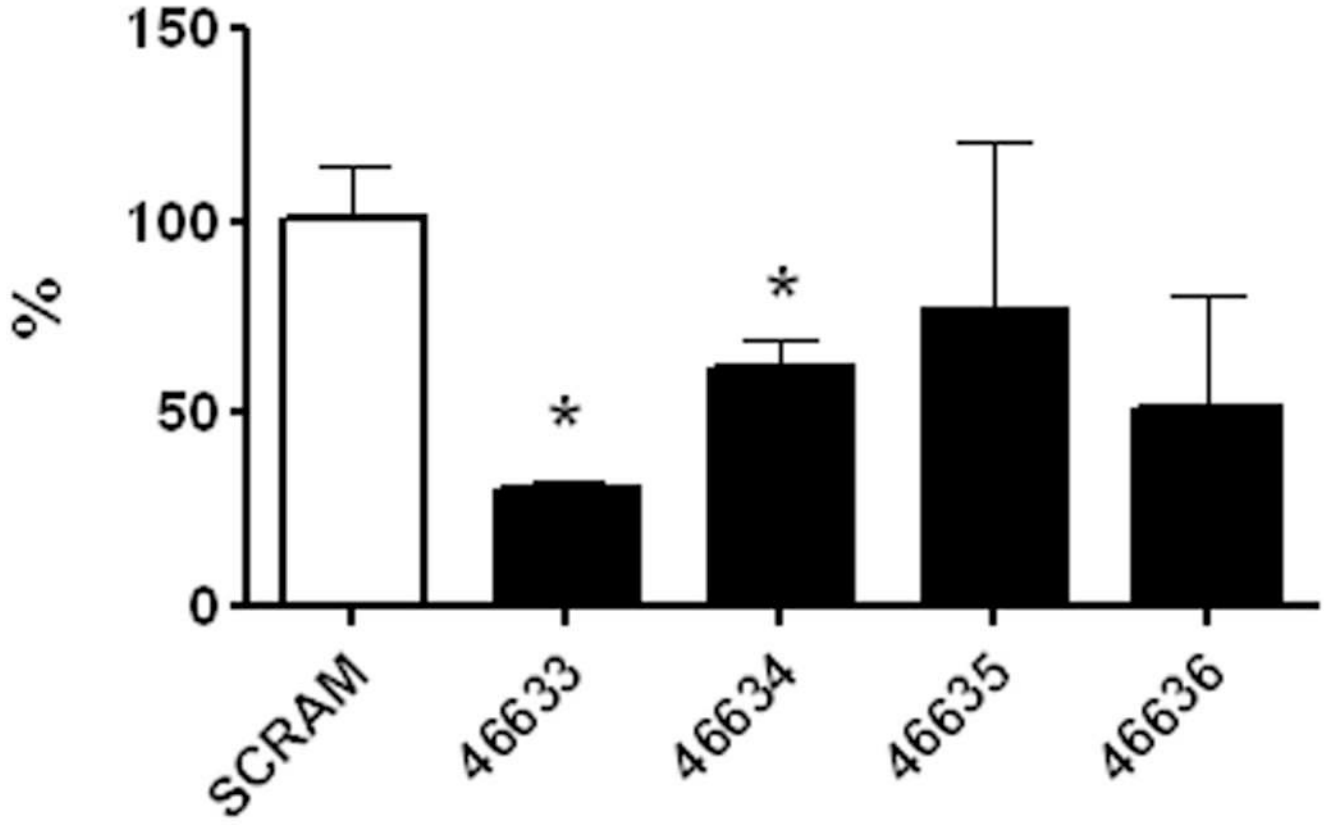

Figure 3. Knock-down of ADAMTS-9 in C-28/I2 chondrocytes

ADAMTS-9 expression was assessed by real-time PCR in cells stably transduced with lentiviral shRNA constructs either targeting the ADAMTS-9 gene (46633-6), or untargeted ('scrambled') as control. Data are expressed as mean ± S.E.M., normalized to cyclophilin-E and calibrated so that mean 'scram'= 100 %. n=4. Representative of 3 independent experiments. *$p<0.05$ relative to 'scrambled' using Student's t test.

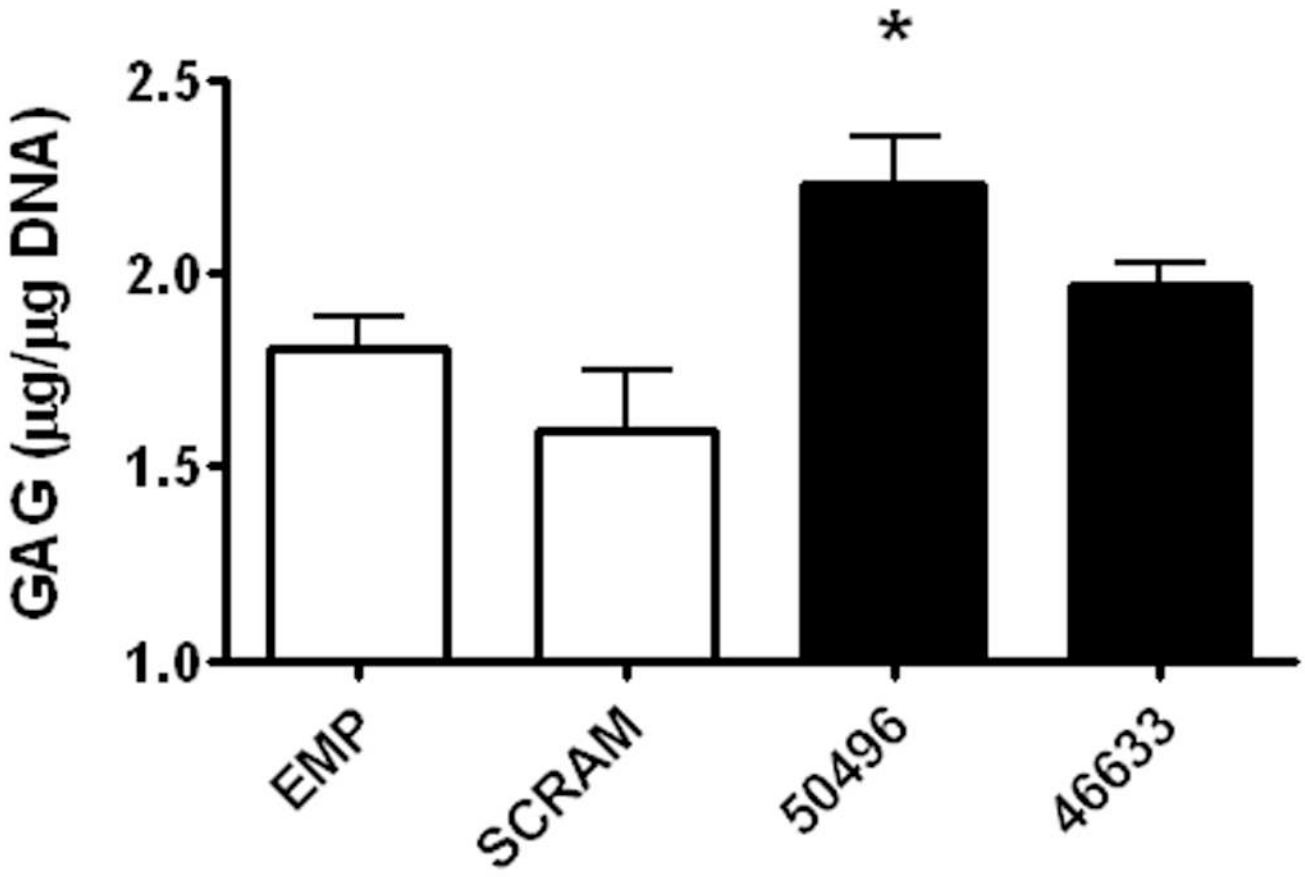

Figure 4. sGAG content of lentiviral knock-down tissue constructs

Lentivirally transduced C-28/I2 cell-lines were seeded and allowed to grow into rudimentary tissue constructs on polyglycolic acid scaffolds. Quantification of GAGs in each construct was carried out using the DMMB assay and repeated twice. Data are expressed as mean ± S.E.M. normalized to amount of DNA per construct. n=3. *$p<0.05$ relative to 'scrambled' using one-way ANOVA and Dunnett's post-hoc test.

Figure 5A

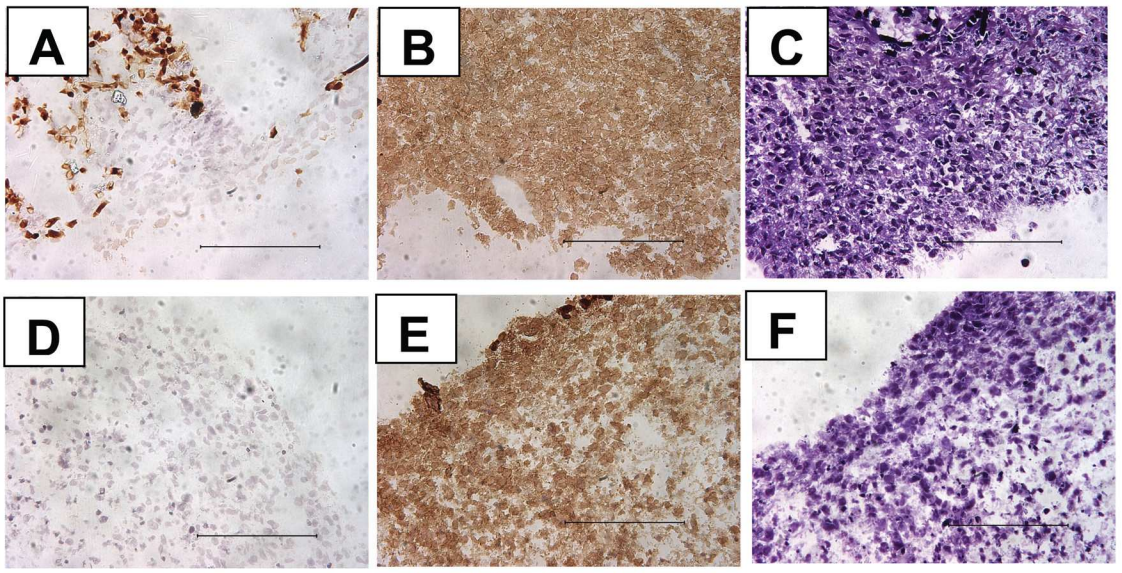

Figure 5B

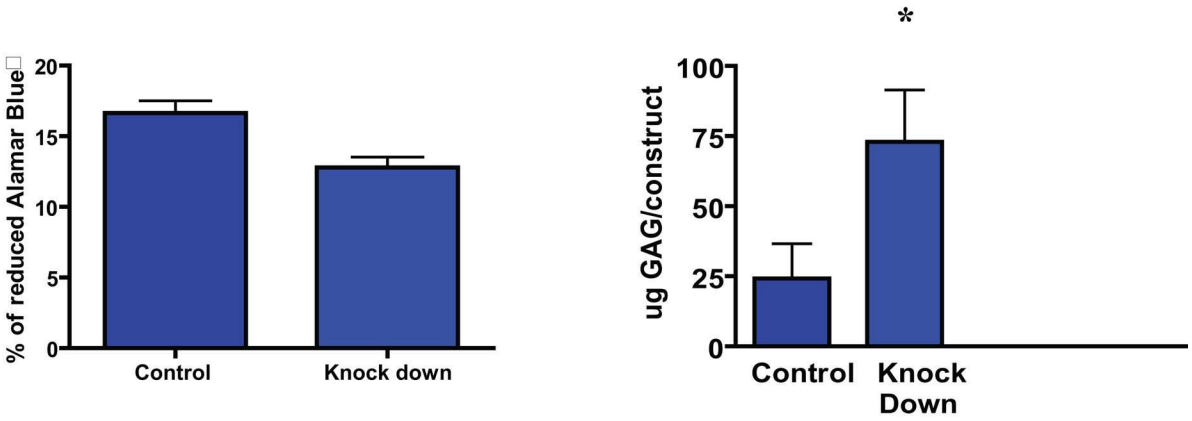

Figure 5. Properties of ADAMTS-5 knock-down C-28/I2 chondrocyte/PGA constructs

ADAMTS-5 retroviral knock-down cell-lines were cloned by limiting dilution. C-28/I2 cell-lines were transduced with retrovirus and chondrocyte/PGA constructs formed as described in the methods section. Figure 5A: Collagen II and proteoglycan deposition in C-28/I2 control and ADAMTS-5 knock-down constructs. Micrographs A, B and C are control constructs and micrographs D, E and F are ADAMTS-5 knock-down constructs. Micrographs A and D show non-specific immunohistochemical staining, as shown in the micrographs some non-specific staining of the PGA fibres was observed. Micrographs B and E show collagen II deposition detected by immunohistochemistry, micrographs C and F show sections stained with Toluidine Blue to detect proteoglycan. Scale bars=200μm. Figure 5B: Cellular activity of C-28/I2 control and ADAMTS-5 knock-down constructs as determined by the reduction of Alamar Blue™ (left-hand panel) and total glycosaminoglycan content of the constructs determined by quantification with 1,9-

dimethylmethylene blue (right-hand panel). Data are expressed as mean ± S.E.M., n=2. *p<0.05, using unpaired t test).