The neuropeptide aCGRP impairs the chondrocyte response to mechanical load

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ABSTRACT

Mechanical loading can contribute to articular cartilage homeostasis due to its anabolic effects, for example the stimulation of glycosaminoglycan (GAG) synthesis. During degenerative joint diseases, such as osteoarthritis, the ability to respond adequately to mechanical stimulation may be compromised. The neuropeptide and pain mediator αCGRP has previously been associated with osteoarthritis and is typical for sensory nerve fibres, but chondrocytes also possess the receptors for αCGRP and have been shown to respond to treatment in vitro. Since it was reported that murine macrophages were more sensitive to αCGRP following stretching, we hypothesised that αCGRP may influence the response of chondrocytes to mechanical stimulation. Here, we employed our previously established model system using human articular chondrocytes to generate tissue-engineered cartilage to investigate the effect of the sensory neuropeptide αCGRP on the chondrocyte loading response. Following culture under chondrogenic conditions for 35 days, tissue-engineered cartilage was subjected to a three-hour episode of unconfined dynamic compression in the presence or absence of αCGRP. Addition of αCGRP did not exert obvious anti-chondrogenic effects, and typical mechano-response markers were not altered when mechanical loading was performed in the presence of αCGRP. GAG synthesis rates were stimulated by mechanical loading in the control group as expected, but this anabolic effect was prevented by the presence of αCGRP. Our results suggest that high levels of αCGRP may interfere with the mechano-adaptation of articular cartilage and this may have important implications for osteoarthritis pathology and therapy.

INTRODUCTION

Physiological mechanical loading contributes to articular cartilage homeostasis through its ability to stimulate extracellular matrix synthesis and to promote normal chondrocyte function (1-3). The importance of physiological loads is highlighted by the cartilage degeneration that is observed following both the absence of joint loading, but also excessive load bearing (4-7). Since cartilage itself has a poor intrinsic regenerative capacity, both overuse and underuse, but also injuries, such as torn ligaments, can contribute to cartilage defects, degeneration and ultimately, osteoarthritis (OA) (5,6,8-10). In turn, cartilage that has undergone pathological changes may no longer be able to respond adequately to mechanical load (9,11,12), thus further advancing cartilage degeneration. We and others have previously demonstrated that the proteoglycan content of cartilage or tissue engineered cartilage is crucial for the chondrocyte response to mechanical loading (13,14). Loss of proteoglycans from the cartilage extracellular matrix is an early event in OA (5,15,16) and may contribute to a vicious circle: Following OA initiation for example by mechanical overload, decreased proteoglycan content prevents a functional loading response, further promoting cartilage degeneration. Importantly, preserving a functional loading response may slow degenerative processes during OA progression.

Despite the ever-increasing need for a disease-modifying treatment for OA, none are available to date. Many approaches that appeared promising in preclinical studies have failed to translate into successful clinical trials (17-19). This may at least partly result from heterogenous patient cohorts, lack of established and reliable biomarkers and therefore the insufficient stratification of OA patients into different subgroups. Of note, previous studies have reported elevated levels of the sensory neuropeptide αCGRP in plasma and synovial fluid from patients with painful knee OA (20,21). The neuropeptide αCGRP is well-known for its role in inducing migraines (22,23), and

indeed, successful treatments targeting αCGRP and preventing migraines have been developed (24,25). Although treatment with αCGRP antagonists appeared to deliver promising results in preclinical animal models of OA (26,27), a clinical trial using a monoclonal antibody against αCGRP failed to improve OA-related pain (28).

Nerve fibres within the joint are affected by OA and the increased innervation of joint tissue by sensory nerve fibres has been suggested to correlate with OA severity and play a role in OA-related pain (29,30). Whilst αCGRP-deficiency accelerated cartilage degeneration following surgical OA in the DMM mouse model (31), the absence of αCGRP appeared to have protective effects on knee cartilage in age-related OA (32). In both models, the loss of αCGRP resulted in enhanced subchondral bone sclerosis during OA progression. In general, αCGRP appears to play a more prominent role in bone than cartilage homeostasis by influencing the delicate balance between osteoblasts and osteoclasts (33,34). In addition, αCGRP has also been reported to exert anti-apoptotic properties on human osteoblast-like cells by stabilisation of β-catenin, the central mediator of the canonical WNT signalling cascade (35). Of note, WNT activity is also involved in regulating the chondrocyte response to mechanical loading (14). Still, chondrocytes also express both receptor components and have been shown to respond to αCGRP treatment with an increase in cAMP levels (36). In OA chondrocytes, but not non-OA chondrocytes, treatment with αCGRP appeared to reduce proteoglycan content (36); however, the impact of elevated αCGRP levels on cartilage physiology, such as the chondrocyte loading response has not yet been addressed. We aimed to investigate whether high levels of αCGRP, as they appear to occur at least in a subgroup of patients, could influence mechano-regulated signalling in chondrocytes and whether the metabolic response of chondrocytes to mechanical loading would be impaired by addition of αCGRP. Failure to upregulate glycosaminoglycan synthesis in response to mechanical loading in the presence of

αCGRP could contribute to loss of proteoglycans and ultimately cartilage degeneration. A detailed investigation of the effect of aCGRP on the chondrocyte loading response could improve our understanding of chondrocyte physiology under arthritic conditions, and could contribute to the development of novel therapeutic approaches.

In order to investigate a potential effect of αCGRP on the loading response, tissue engineered neocartilage was generated from human articular chondrocytes and type I/III collagen carriers under standard chondrogenic conditions as described previously (14,37-40). Neocartilage was subjected to dynamic unconfined compression in the presence or absence of αCGRP to examine its effect on the molecular response of chondrocytes to mechanical load.

RESULTS

In order to investigate the effect of αCGRP on the short-term response to mechanical loading, we chose to limit the αCGRP treatment to the last day of culture and the dynamic unconfined compression (a total of 24 hrs), thus avoiding previously reported reductions in proteoglycan deposition following αCGRP treatment for 7 days. At the end of culture, control and αCGRP-treated neocartilage was histologically indistinguishable (Fig. 1A). Neocartilage from both groups exhibited even glycosaminoglycan deposition as evidenced by Safranin-O staining. No significant changes were observed in the DNA content of control or αCGRP-treated neocartilage, indicating that cell numbers per tissue engineered construct were not compromised by αCGRP treatment (Fig. 1B). Although αCGRP-treated neocartilage tended to exhibit slightly lower GAG/DNA ratios, the GAG/DNA ratio was generally maintained in the presence of αCGRP (Fig. 1B). Similar expression levels of the chondrogenic markers and ECM components *COL2A1*, *ACAN* and *SOX9* between

control and αCGRP-treated constructs (Fig. 1C) indicated that αCGRP did not impair chondrogenic re-differentiation.

Next, neocartilage was subjected to dynamic unconfined compression in the presence or absence of αCGRP. In order to assess chondrocyte mechano-transduction, phosphorylation of the MAP kinase ERK1/2 was examined by western blotting (Fig. 2A). As expected, mechanical loading significantly enhanced ERK1/2 phosphorylation in the control group (P=0.027). In the αCGRP-treated group mechanical loading also stimulated ERK1/2 phosphorylation significantly (P=0.018). Mechanical loading of chondrocytes also results in elevated SOX9 levels (14,37,39,41,42), and we observed the expected increase in SOX9 in the control group (P=0.018); however, also in the αCGRP-treated group SOX9 was more abundant following loading in 4 out of 5 donors (Fig. 2B). We then examined the expression of known mechano-regulated genes (Fig. 2C). *FOS* expression was enhanced following mechanical loading in the control group (P=0.005), and in 4 out of 5 donors when mechanical loading was performed in the presence of αCGRP. Similarly, *PTGS2* was induced by mechanical loading in the control group (P=0.003) and in 4 out of 5 donors in the αCGRP-treated group. *SOX9* was significantly upregulated in both control and αCGRP-treated neocartilage following compression (P<0.001 and P=0.026, respectively) as was *BMP2* (P<0.001 and P=0.004). Similarly, *BMP6* expression was significantly enhanced by mechanical loading in the control group (P<0.001) as well as in the αCGRP-treated group (P=0.002). Of note, the same donor exhibited hardly increased ERK-phosphorylation and no upregulation of *FOS* and *PTGS2* following compression in the presence of αCGRP. Since these known mechano-regulated factors generally still responded to compression in the

presence of αCGRP, we concluded that chondrocyte mechano-transduction was not affected by the presence of αCGRP.

We then examined the impact of αCGRP treatment on the functional response of chondrocytes by performing $^{35}SO_4$-isotope labelling of newly synthesised GAGs (Fig. 3A). In the control group, compression resulted in significantly enhanced GAG synthesis as expected from previous studies (P=0.014). Although αCGRP treatment did not appear to result in major alterations in mechano-transduction or mechano-regulated gene expression, it clearly disrupted the stimulation of GAG synthesis following loading (Fig. 3A). This was not due to altered cell numbers, since both the control and αCGRP-treated cartilage-like constructs did not exhibit significant changes in DNA content following dynamic compression (Fig. 3B). GAG/DNA ratio was also maintained in both control and αCGRP-treated neocartilage following mechanical loading (Fig. 3C).

DISCUSSION

OA pathology includes all tissues within the joint and their interaction. Here, we sought to investigate how high levels of αCGRP, a peptide typically associated with sensory nerve fibres, may influence cartilage physiology and contribute to OA progression. Since the application of defined loads in *in vivo* models relies on animal models and is often unsatisfactory, we employed a tissue engineering approach to generate neocartilage to address the role of αCGRP during mechanical loading of human chondrocytes.

Reduced proteoglycan accumulation by isolated chondrocytes in the presence of αCGRP has been reported (36), but our study successfully employed a shorter treatment regimen to prevent indirect effects of lower proteoglycan content on the chondrocyte loading response. In agreement with previous studies that reported

more substantial effects of loss of αCGRP on subchondral bone than cartilage homeostasis (32-34,43), we observed no substantial effects of αCGRP on markers of chondrocyte mechano-transduction. Yet, we observed a clear functional impact of high αCGRP levels. GAG synthesis following loading was no longer stimulated, which may indicate that αCGRP-treated neocartilage was not able to replenish their ECM with GAGs following loading. Over time, this may contribute to cartilage degeneration. In agreement with a role for αCGRP in cartilage degeneration, αCGRP-deficiency in female mice was reported to have chondroprotective effects during age-related knee OA (32). Our findings also suggest that increased sensory innervation of the joint, and thus high αCGRP levels in close proximity to articular chondrocytes may have functional consequences for cartilage homeostasis, in addition to the role of sensory nerve fibres and αCGRP for OA pain that have already been described. Still, several studies have reported increased subchondral bone sclerosis during primary and secondary OA in the absence of αCGRP (31,32), indicating it plays a role in both tissues and highlighting its role during OA. Whether the temporary inhibition of αCGRP could have beneficial effects on cartilage and joint homeostasis for OA patients remains to be investigated.

Of note, the effect of αCGRP on the load-induced stimulation of GAG synthesis was not reflected in SOX9 protein levels or gene expression. During our previous studies, we found that activation of WNT activity by the small molecule CHIR also prevented a stimulation of GAG synthesis rates; however, this was linked to preventing the increase of SOX9 levels (14). Similarly, suppression of GAG synthesis rates following mechanical loading of MSC-derived neocartilage corresponded with inconsistent regulation of SOX9 levels following mechanical loading (39). It remains to be investigated how αCGRP prevents the stimulation of GAG synthesis by mechanical loading independently of SOX9 regulation.

Targeting αCGRP itself did not appear a promising clinical approach for OA (28), despite promising preclinical studies (26,44). This may at least in part result from the used rodent models with injury- or monoidoacetate (MIA)-induced OA. Of note, a previous study also reported that serum αCGRP levels are not elevated following OA induction by DMM surgery (31), whereas another study reported increased αCGRP serum levels in aged mice (32). These findings underline once again the differences between preclinical OA models. It is now very much accepted in the field that preclinical models do not reflect all aspects of OA pathogenesis and progression, and that disease mechanisms – and thus potential treatments – may differ between patients and the specific subtype of OA. Indeed, not all OA patients may exhibit elevated levels of αCGRP. This patient heterogeneity and lack of established biomarkers thereof remain a significant challenge for successful OA treatment development. Indeed, only few potential disease-modifying OA drugs (DMOADs) have progressed into clinical trials (reviewed in 17). Since αCGRP has been detected in synovial fluid and serum samples from OA patients, and has been proposed to be a potential biomarker for OA (20,21), it would be interesting to investigate whether any of the substances currently in clinical trials are able to re-install the stimulation of GAG synthesis following mechanical loading. This could not only reveal the mechanism by which αCGRP negatively impacts GAG synthesis stimulation during mechanical loading, but could also indicate that high αCGRP levels could be used to identify patients responsive to a particular treatment. Importantly, since the loss of αCGRP was associated with subchondral bone sclerosis in both primary and secondary OA models, targeting the functional impact of αCGRP in chondrocytes instead of αCGRP directly may avoid interfering with its role in other tissues.

Our study has several limitations. Since we performed the αCGRP treatment after formation of a cartilage ECM, we used a supra-physiological concentration of αCGRP

to ensure penetration of the dense tissue. Furthermore, since we rely on the generous tissue donations from OA patients undergoing total knee replacement surgery, our chondrocytes are derived from OA patients which may affect their response to αCGRP. Of note, all chondrocytes used in this study were derived from female patients; however, women are also typically more frequently affected by OA. Future studies need to address whether this effect can also be observed in chondrocytes from male donors.

In summary, we show here that the sensory neuropeptide αCGRP can interfere with the physiological response of human chondrocytes to mechanical loading and thus our data suggest high αCGRP levels may directly impact cartilage homeostasis. Whilst this effect was limited to the stimulation of GAG synthesis rates, and did not affect other classical markers of the mechano-response, GAG synthesis rates are a highly relevant, functional marker of the chondrocyte loading response, and so our results add to a growing body of evidence that indicate a functional role of αCGRP in cartilage physiology.

METHODS

Isolation and expansion of articular chondrocytes

Articular cartilage samples were obtained from six donors (female, age range 56-87, mean age 67.5 years) undergoing total knee replacement surgery with informed written consent of the patients. The study was approved by the local ethics committee on human experimentation of the Medical Faculty of Heidelberg and in agreement with the Helsinki Declaration from 1975 (latest version). Articular chondrocytes (AC) were isolated from macroscopically intact cartilage as described previously (45). Cells were expanded in DMEM (1 g/L glucose, 10 % FBS, 1 % penicillin/streptomycin) for two passages.

Generation of tissue-engineered neocartilage

AC were seeded onto type I/III collagen carriers (4 mm diameter, 1.5 mm height, Optimaix, Matricel, Germany) at 500 000 cells/constructs and attached to a glass carrier (ROBU, Germany) to mimic the subchondral bone. Neocartilage was cultured for 35 days as described previously (14,37) using chondrogenic medium (containing DMEM 4.5 g/L glucose, 1 % penicillin/streptomycin, 0.1 mM dexamethasone, 0.17 mM ascorbic acid-2-phosphate, 2 mM sodium pyruvate, 0.35 mM proline, 5 mg/mL transferrin, 5 ng/mL sodium selenite, 1.25 mg/mL bovine serum albumin (all Sigma-Aldrich, Germany), 5 mg/mL insulin (Lantus, Sanofi-Aventis, Germany) and 10 ng/mL TGF-β1 (Peprotech, Germany).

Cyclic unconfined dynamic compression of neocartilage

The dynamic compression protocol was carried out using the software Galil Tools as described previously (14,37). 1 µM αCGRP (Bachem, Germany) was added 21 hrs before start of compression and treatment was continued during loading and subsequent metabolic labelling. In brief, engineered tissue was exposed to a single 3 hr episode of loading in a custom-designed bioreactor. Initially, neocartilage was compressed by 10 % of their thickness to ensure the constant contact with the piston during compression. Starting from this 10 % static offset, neocartilage was compressed by 25 % at 1 Hz during the loading intervals (10 min) whilst maintaining the offset of 10 % during break intervals (10 min). After loading, neocartilage was washed in PBS and snap frozen or used for metabolic labelling analysis.

Radiolabel incorporation, glycosaminoglycan and DNA quantification

Proteoglycan synthesis was analysed after the end of loading as described previously (14).The neocartilage was separated from the bone replacement phase

and placed onto a nylon mesh in a 48-well plate. Engineered tissue was labelled with 4 µCi ^{35}S as Na_2SO_4 (Hartmann Analytic, Germany) for 24 h in 500 µL chondrogenic medium with or without treatments. Neocartilage was then washed 5 times for 20 min with unlabelled Na_2SO_4 (1mM) and digested with 0.5 mg/mL proteinase K at 65°C and 800 rpm overnight. Incorporated radiolabel was measured in tissue lysates by β-scintillation counting (1414 WinSpectral, PerkinElmer, Germany) and normalised to the DNA content of the same lysates. DNA content was determined using Quant iT Pico Green Kit (Invitrogen, Germany). Total glycosaminoglycan content of each sample was determined by 1,9-dimethylmethylene blue (DMMB) assay (46) using a chondroitin-6-sulfate standard.

RNA isolation and mRNA expression analysis

Total RNA was isolated from neocartilage using Trizol (Life Technologies, Germany). 500 ng of total RNA was reverse transcribed using Omniscript RT Kit (Qiagen, Germany) and oligo(dT) primers. Gene expression was analysed by qRT-PCR (Roche Diagnostics, Germany) using SYBR green (ThermoFisher, USA). *CPSF6* and *HNRPH1* were used as housekeeping genes. Primer sequences are listed in supplementary table 1.

Western blotting

Tissue-engineered neocartilage was cut in half using a scalpel and minced in 75 µL PhosphoSafe Extraction Reagent (Merck Millipore, Germany), containing 1 mM Pefabloc SC (Sigma-Aldrich, Germany) in a mixer mill (Retsch, Germany) at 30 Hz for 2 x 2 min, with cooling on ice in between cycles. Samples were centrifuged at 13 000 x g for 20 min at 4°C before cell lysates were mixed with 4 x Laemmli buffer and boiled for 5 min at 95°C. Proteins were separated according to size using standard SDS-PAGE and transferred onto a nitrocellulose membrane (GE Healthcare,

Amersham, Germany). Membranes were blocked in 5 % skimmed milk/TBS-T and probed with primary antibodies (listed in supplementary table 2) at 4°C overnight. Detection was carried out using horseradish peroxidase-conjugated secondary antibodies and enhanced chemoluminescence (Roche Diagnostics, Germany/Advansta, USA). Densitometric analysis was performed using ImageJ.

Histology

Neocartilage was separated from the bone replacement phase and incubated in 4 % PFA for 4 hrs at room temperature. After dehydration in an ascending isopropanol series, samples were embedded in paraffin wax for cutting. After rehydration, proteoglycan deposition was analysed by staining 5 µm sections with 0.2 % w/v) Safranin-O (Fluka, Sigma-Aldrich, Germany) in 1 % acetic acid and counterstaining with 0.04 % Certistain Fast Green (Merck, Germany) in 0.2 % acetic acid.

Statistical analysis

Statistical analysis was performed using SPSS. Fold changes were calculated relative to the respective free-swelling construct. For statistical analysis, fold changes were $\log_2$-transformed before one sample *t*-test was applied to calculate *P*-values, with $P < 0.05$ considered statistically significant.

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Contributions

HFD: Investigation, Conceptualisation, Formal analysis, Data curation, Visualisation, Writing – original draft, Writing – review & editing. NH: Investigation, Conceptualisation, Formal analysis, Data curation, Writing – review & editing. SD: Conceptualisation, Project administration, Supervision, Funding acquisition, Resources, Writing – original draft, Writing – review & editing.

Role of the funding source

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Competing interest statement

The authors declare no competing interests.

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Figure Legends

Fig 1—Treatment of mature cartilage-like constructs with CGRP does not affect cartilage integrity. Human articular chondrocytes were seeded onto a collagen scaffold to generate cartilage-like tissue-engineered constructs, cultured for 35 days and treated with 1 µM CGRP during the last 24 hrs. (A) Safranin-O staining of standard paraffin sections was used to visualise GAG deposition after 35 days of culture, with or without the addition of 1 µM CGRP. N = 3 donors (B) DNA and GAG levels of cartilage-like constructs with or without CGRP treatment were assessed using PicoGreen DNA assay and the DMMB method, and GAG content was normalised to DNA content/construct. N = 6 donors (C) Gene expression of chondrogenic marker genes COL2A1, ACAN and SOX9 was examined in control and CGRP-treated cartilage-like constructs. Gene expression was normalised to the average of two housekeeping genes, CPSF6 and HNRPH1. N= 5 donors. Boxes represent first and third quartiles, medians are depicted as horizontal lines and whiskers show maximum and minimum values. Circles represent extreme values.

Fig 2— Presence of CGRP during mechanical loading of neocartilage does not alter chondrocyte mechanotransduction. Protein lysates from control, compressed, CGRP-

treated or CGRP-treated and compressed neocartilage were subjected to western blotting to examine (A) ERK phosphorylation and (B) SOX9 protein levels. Actin was used as loading control. Quantification was performed using ImageJ. (C) RNA was extracted from neocartilage following dynamic compression in the presence or absence of CGRP and gene expression analysed by qRT-PCR using specific primers for the established mechano-regulated genes FOS and PTGS2, as well as SOX9, BMP2 and BMP6. CPSF6 and HNRPH1 were used as housekeeping genes. Fold changes represent the induction of gene expression relative to the non-loaded construct. A one sample t-test was applied to compare log2-transformed fold changes to 0 (indicating no change/fold change of 1) for each condition. Boxes represent first and third quartiles, medians are depicted as horizontal lines and whiskers show maximum and minimum values. Circles represent extreme values. N = 5 donors.

Fig 3— Presence of CGRP during loading disrupts mechano-adaptation of chondrocytes. (A) GAG synthesis was measured by 35SO4-incorporation over 24 hrs following loading with or without CGRP. Data were normalised to DNA and values of non-loaded controls set to 1. Fold changes are depicted relative to the non-compressed sample. (B) DNA content of cartilage-like constructs following compression, CGRP treatment or compression in the presence of CGRP was measured by PicoGreen assay. (C) GAG content of cartilage-like constructs was measured by DMMB assay and normalised to the DNA content of samples. A one sample t-test was applied to compare log2-transformed fold changes to 0 (indicating no change/fold change of 1) for each condition. Boxes represent first and third quartiles, medians are depicted as horizontal lines and whiskers show maximum and minimum values. Circles represent extreme values. N = 6 donors.

Figure 1

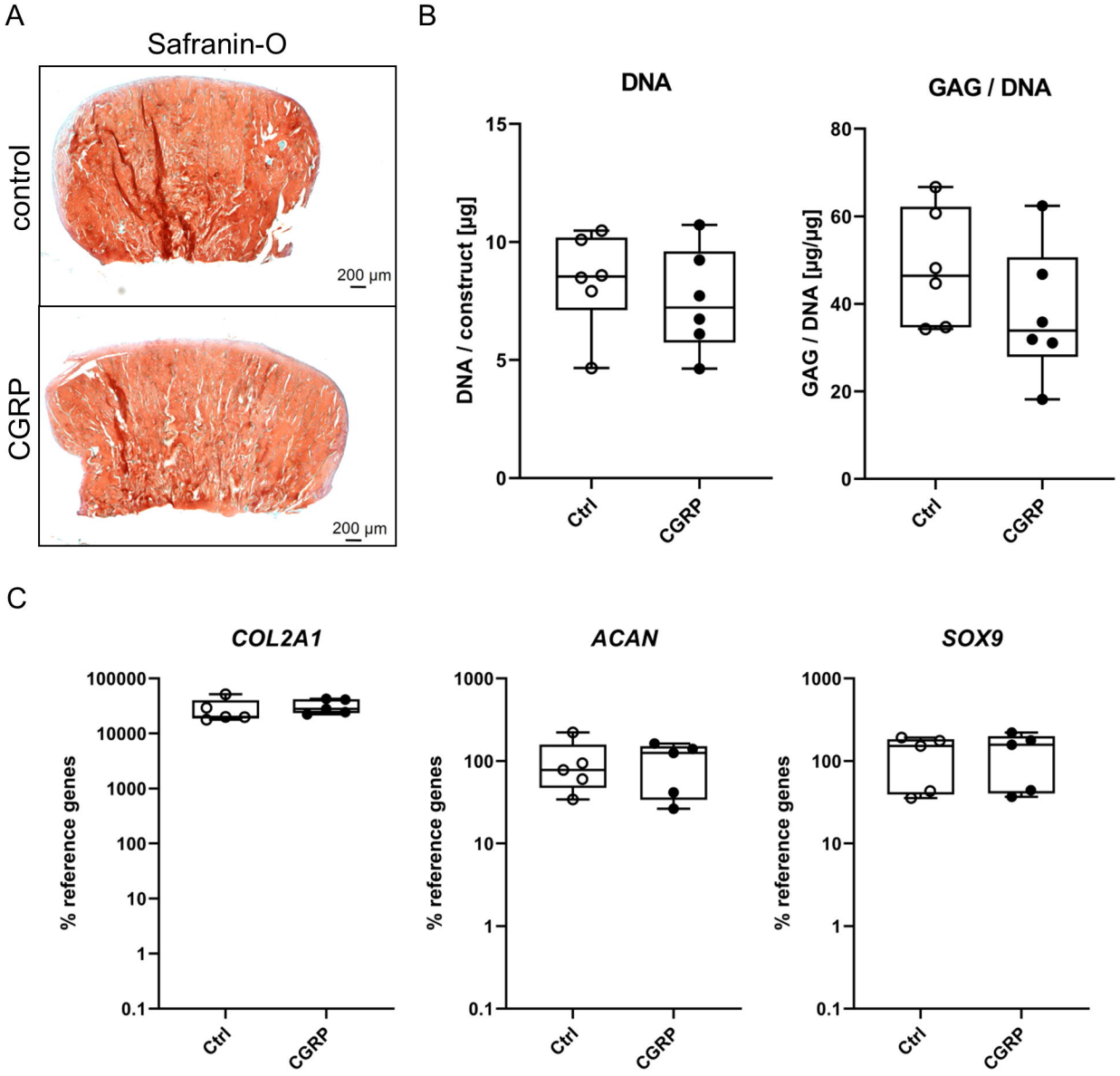
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Figure 2

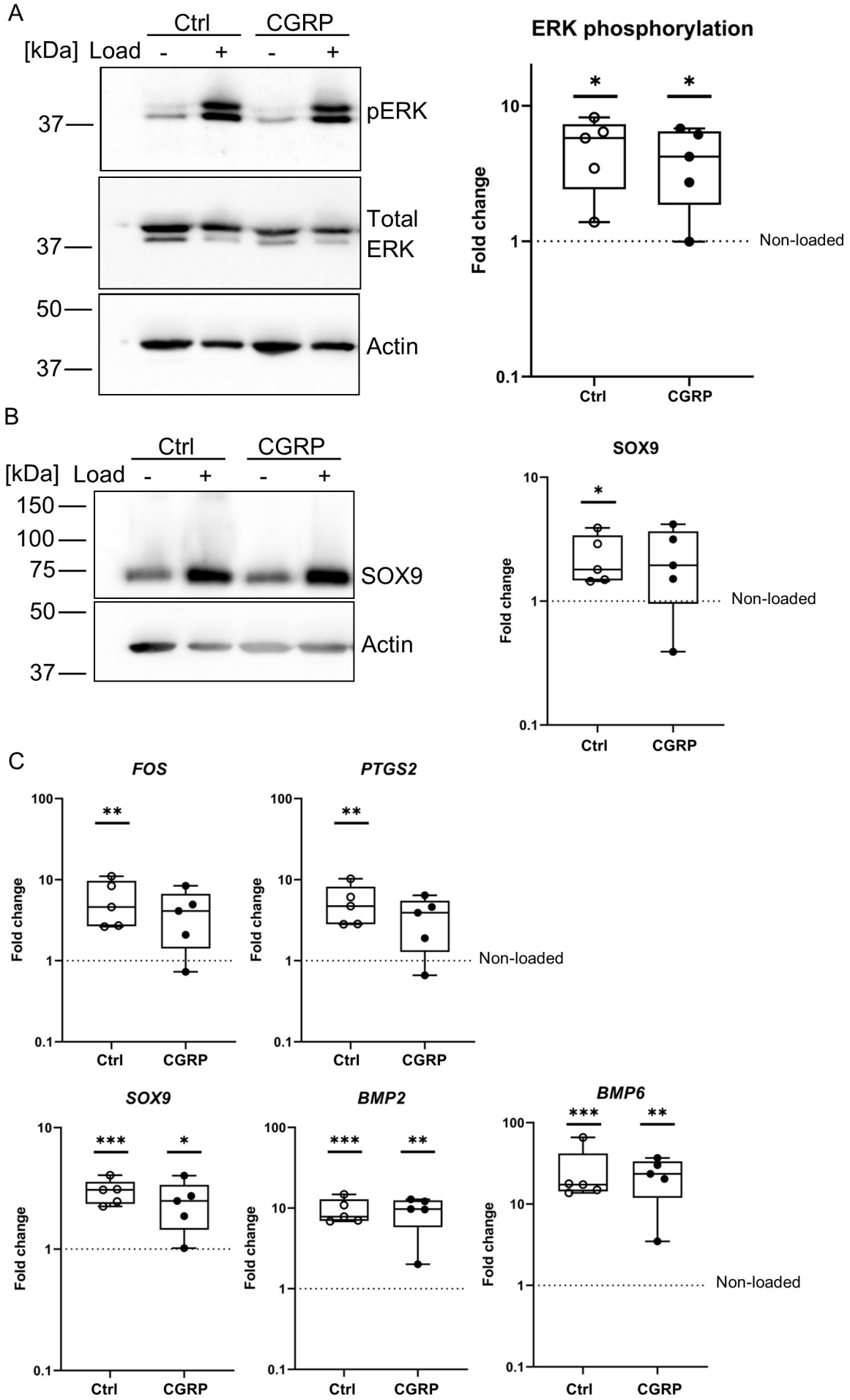
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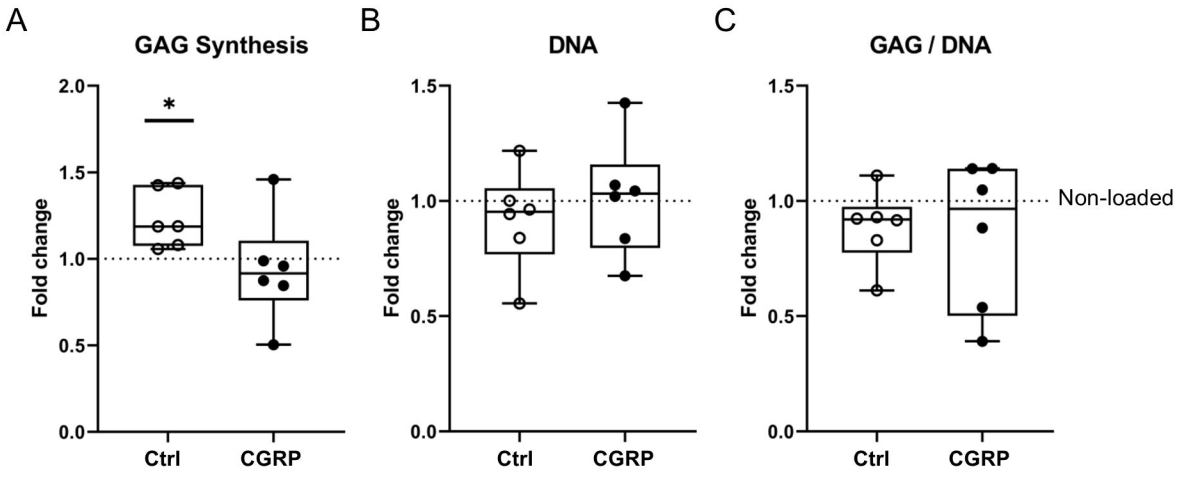
Figure 3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