EXTENDED REPORT

Genome-wide association and functional studies identify a role for *IGFBP3* in hip osteoarthritis

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ABSTRACT

Objectives To identify genetic associations with hip osteoarthritis (HOA), we performed a meta-analysis of genome-wide association studies (GWAS) of HOA.

Methods The GWAS meta-analysis included approximately 2.5 million imputed HapMap single nucleotide polymorphisms (SNPs). HOA cases and controls defined radiographically and by total hip replacement were selected from the Osteoporotic Fractures in Men (MrOS) Study and the Study of Osteoporotic Fractures (SOF) (654 cases and 4697 controls, combined). Replication of genome-wide significant SNP associations ($p \leq 5\times10^{-8}$) was examined in five studies (3243 cases and 6891 controls, combined). Functional studies were performed using in vitro models of chondrogenesis and osteogenesis.

Results The A allele of rs788748, located 65 kb upstream of the *IGFBP3* gene, was associated with lower HOA odds at the genome-wide significance level in the discovery stage (OR 0.71, $p=2\times10^{-8}$). The association replicated in five studies (OR 0.92, p=0.020), but the joint analysis of discovery and replication results was not genome-wide significant ($p=1\times10^{-6}$). In separate study populations, the rs788748 A allele was also associated with lower circulating IGFBP3 protein levels ($p=4\times10^{-13}$), suggesting that this SNP or a variant in linkage disequilibrium could be an *IGFBP3* regulatory variant. Results from functional studies were consistent with association results. Chondrocyte hypertrophy, a deleterious event in OA pathogenesis, was largely prevented upon *IGFBP3* knockdown in chondrocytes. Furthermore, *IGFBP3* overexpression induced cartilage catabolism and osteogenic differentiation.

Conclusions Results from GWAS and functional studies provided suggestive links between IGFBP3 and HOA.

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INTRODUCTION

Hip osteoarthritis (HOA) is one of the most common forms of arthritis, with nearly 10% of individuals over the age of 70 having this condition.[1] The degeneration of the hip joint that occurs with OA is characterised by a loss of articular cartilage, an increase in new bone formation at the margins of the bones, and remodelling of the bone that is adjacent to the joint.[2] HOA is associated with pain and disability and often requires total joint replacement.[3] Currently, there are no available treatments that can alter the course of this disease.

Genetic and environmental factors contribute to HOA pathogenesis, and the heritability of HOA in women has been reported to be as high as 60%.[4] A number of genome-wide significant (GWS; $p \leq 5\times10^{-8}$) single nucleotide polymorphism (SNP) associations with HOA and related traits have been identified. Variants at the *GDF5* locus and *MCF2L* locus have been associated with OA of the hip and knee.[5–8] *DOT1L* SNPs have been associated with cartilage thickness and HOA,[9 10] and SNPs near the gene *NCOA3* have been associated with HOA.[11] A recent genome-wide association study (GWAS) of OA that included 24 sub-phenotypes identified five GWS associations.[12]

It has been previously noted that the heterogeneity of radiographic HOA classification could hinder the identification of genetic risk factors.[13] OA is typically defined using a summary grade score, such as the Kellgren and Lawrence (K/L) score[14] or the Croft score,[15] but for a given summary grade score, the radiographic features can vary between studies.[13] In order to minimise phenotype heterogeneity, we selected 654 HOA cases and 4697 HOA controls from two studies, the Osteoporotic Fractures in Men (MrOS) Study and the Study of Osteoporotic Fractures (SOF), in which the same radiographic scoring methods were applied by a common set of radiograph readers, and we performed a GWAS meta-analysis of HOA. GWS SNP associations were examined for replication in 3243 cases and 6891 controls from five case–control studies. To link HOA-associated SNPs with a potential target gene, we examined whether significantly associated SNPs near *IGFBP3* were associated with circulating IGFBP3 through a look-up of results from a GWAS meta-analysis of circulating IGFBP3.[16] The biological role of the candidate gene *IGFBP3* was evaluated through functional studies using model systems of chondrogenesis and osteogenesis.

METHODS

Study populations and case and control definitions

In the MrOS and SOF studies, HOA cases were defined using a modified Croft grade score ≥2 on either hip or total hip replacement (THR).[2] HOA controls were those without THR and meeting the following three criteria on both hips: Croft grade ≤1, joint space narrowing ≤1 and osteophytes ≤1. GWS SNP associations were examined for replication using case–control status defined by K/L scores or THR in the Rotterdam study (RS-I and RS-II) and Chingford study, and by THR in the Genetics of Osteoarthritis and Lifestyle (GOAL) study and the Nottingham Study. See online supplementary text S1 for details regarding study descriptions and case–control definitions. All studies were approved by their respective institutional review boards and informed consent was obtained from all participants involved.

Genotyping and statistical analysis

Genome-wide SNPs were genotyped in samples from the MrOS and SOF studies using the Illumina HumanOmni1-Quad array. Quality control procedures for SNPs and samples are described in online supplementary table S1. Principal component analysis was performed to detect evidence for population structure and to generate principal components used to adjust for genetic ancestry.[17] Genotypes were imputed using the HapMap phase II release 22 reference panel using MACH.[18] SNP associations were estimated using logistic regression models adjusted for the effects of age, sex (if applicable), study site (if applicable) and principal components. Genomic control was applied to results from MrOS and SOF, and fixed-effect meta-analysis with inverse variance weights was performed using METAL.[19] The two discovery studies and five replication studies were included in the joint meta-analysis as individual studies. Details regarding genotyping and statistical analysis in discovery and replication studies are presented in online supplementary table S1 and text S1.

Cell culture experiments

Functional studies of chondrogenesis and osteogenesis were performed using the ATDC5 and MC3T3 cell lines, respectively. The ATDC5 cell line exhibits multi-stage chondrogenic differentiation similar to the process that is observed during chondrogenesis and endochondral bone formation.[20] Human articular chondrocytes, isolated from OA patients as previously described,[21] were also used in functional studies. Gene knockdown was performed by stable transfection with plasmid overexpressing shmiRNA directed against *IGFBP3*. Further details are presented in online supplementary text S1.

RESULTS

Characteristics of the study samples

Average age was significantly higher in MrOS participants (100% male) than SOF participants (100% female). Age and minimum joint space width differed by case–control status in MrOS and SOF, but height and body mass index (BMI) did not (see online supplementary table S2).

Locus associated with HOA

A GWAS of HOA was performed with 2 459 845 genotyped and imputed SNPs that passed quality control. There was little to no evidence of p value inflation in the individual studies (MrOS λ=1.01, SOF λ=1.01) or the meta-analysis (λ=1.00) (see online supplementary figures S1–S3). GWS SNPs were located at a single locus on chromosome 7 (see online supplementary figure S4). The two GWS SNPs, rs788748 and rs879966, were directly genotyped in both cohorts, and were in moderate linkage disequilibrium (LD) (HapMap CEU r^2=0.54), and the effect alleles were associated with decreased odds of HOA (table 1 and figure 1). The nearest gene was *IGFBP3* (figure 1). In conditional analysis, the HOA association p values were not significant for rs788748 and rs879966 (p≥0.05), indicating a strong degree of dependence.

Our discovery stage had sufficient power (≥0.8) at $\alpha=5\times10^{-8}$ to detect an OR of 0.68 for SNPs with a minor allele frequency (MAF) of 0.5. As sufficient power was nearly achieved to detect the observed rs788748 OR, the winner's curse[22] adjusted[23] rs788748 OR of 0.75 was similar to the observed OR of 0.71.

Genetic variants in components of the growth hormone/insulin-like growth factor (GH/IGF) axis, which includes *IGFBP3*, have been associated with human height,[24] and genetic

Table 1 SNP association results in discovery and replication studies

		rs788748		rs879966	
		Chr 7: 45 992 706*		Chr 7: 46 015 992*	
		A (0.49)†		G (0.39)†	
	Cases/controls	OR (95% CI)	p Value	OR (95% CI)	p Value
MrOS	411/2512	0.68 (0.58 to 0.79)	7×10^{-7}	0.67 (0.57 to 0.78)	8×10^{-7}
SOF	243/2185	0.76 (0.63 to 0.92)	5×10^{-3}	0.74 (0.61 to 0.91)	4×10^{-3}
Meta-analysis	654/4697	0.71 (0.63 to 0.80)	2×10^{-8}	0.70 (0.61 to 0.79)	1×10^{-8}
Replication					
GOAL	1291/783	0.85 (0.75 to 0.97)	0.01	ND	ND
Nottingham	1258/758	1.01 (0.89 to 1.16)	0.83	ND	ND
RS-I	462/3428	0.86 (0.74 to 0.99)	0.03	0.92 (0.79 to 1.06)	0.24
RS-II	149/1430	0.98 (0.75 to 1.28)	0.88	1.09 (0.83 to 1.44)	0.54
Chingford	83/492	1.11 (0.79 to 1.56)	0.54	1.00 (0.72 to 1.40)	0.99
Meta-analysis replication	3243/6891	0.92 (0.86 to 0.99)	0.02	0.96 (0.85 to 1.08)	0.48

*NCBI build 36 chromosome and position.
†Effect allele (effect allele frequency).
GOAL, Genetics of Osteoarthritis and Lifestyle Study; MrOS, Osteoporotic Fractures in Men Study; ND, not determined; RS, Rotterdam Study; SNP, single nucleotide polymorphism; SOF, Study of Osteoporotic Fractures.

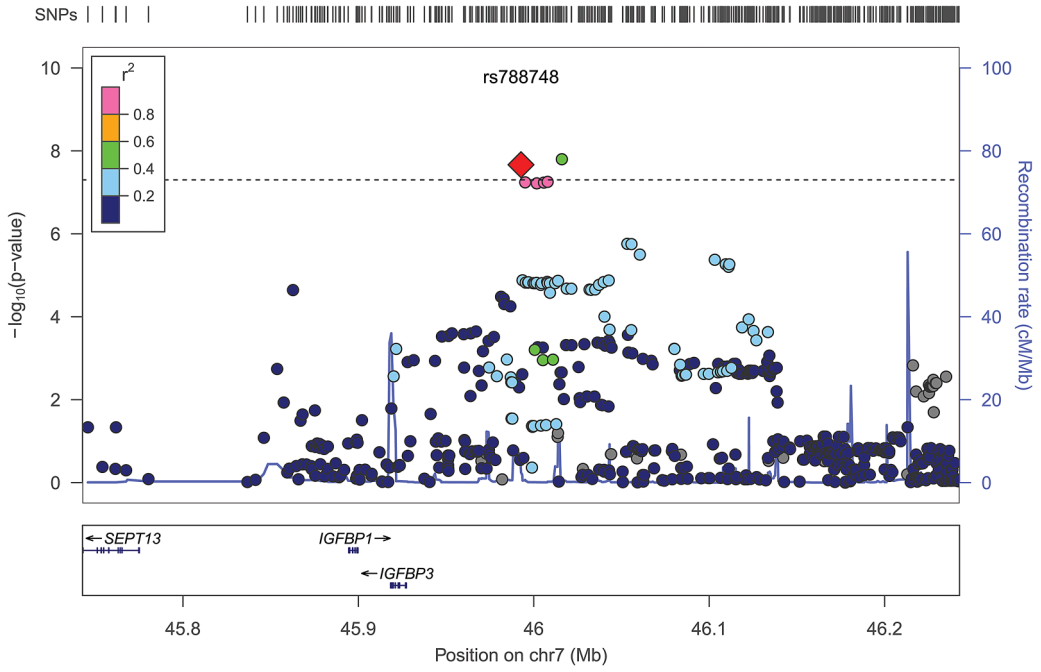

Figure 1 Regional hip osteoarthritis (HOA) association plot of IGFBP3 locus. Association p values are plotted against genomic location. Negative log base 10 of the association p value is shown on the left-hand y-axis. The HapMap-based recombination rate is shown on the right-hand y-axis and is depicted using light blue peaks. Genomic location on chromosome 7 in megabases is shown on the x-axis. The dashed line marks the genome-wide significance threshold. HapMap CEU linkage disequilibrium (r^2) relative to the index single nucleotide polymorphism (SNP), rs788748, is shown using filled colours according to the legend. RefSeq genes are shown in the bottom panel.

associations are shared between OA and height.[25] Rs788748 and rs879966 were in low LD (1000 Genomes phase 1 CEU r^2=0.09 and 0.16, respectively) with a functional *IGFBP3* promoter polymorphism (−202 A/C, rs2854744)[26] associated with GH-dependent growth,[27] and adjustment for height or BMI had a minimal effect on rs788748 and rs879966 HOA associations (see online supplementary table S3), indicating that height is unlikely to mediate the SNP associations.

The two GWS SNP associations were examined for replication in five HOA case–control studies (see online supplementary tables S4 and S5). In the meta-analysis of replication results, the rs788748 HOA association was significant and in the same direction as the discovery stage (p=0.020), with little evidence for heterogeneity (Q test p=0.20, I^2=36.67) (table 1, figure 2). The rs879966 genotyping assay failed in the two largest replication studies, GOAL and Nottingham, and not surprisingly, it was not significantly associated with HOA among the replication studies (table 1). In a joint meta-analysis of discovery and replication stage results, the rs788748 OR (95% CI) was 0.86 (0.81 to 0.91) and the p value was 1×10^{-6}.

Previously reported SNP associations with OA at rs11177/rs6976 near *GNL3/GLT8D1*,[12] rs10948172 near *SUPT3H* and *RUNX2*,[12] rs11842874 in *MCF2L*,[8] rs12982744 in *DOT1L*,[9 10] and rs6094710 near *NCOA3*[11] were significantly associated (p≤0.05) with HOA in study-specific or meta-analysis results, but the direction of effect did not replicate for rs11842874 or rs6094710 (see online supplementary table S6).

Characterisation of associated SNPs

ENCODE project data indicated that histone modifications typically associated with enhancers (histone-3 lysine-4 monomethylation and histone-3 lysine-27 acetylation)[28] were located near rs788748 and rs879966. In addition, DNase I hypersensitivity sites and transcription factor binding sites identified using ChIP-Seq were located near rs788748 (see online supplementary figure S5).

A GWAS meta-analysis of circulating IGFBP3 and IGF-I levels reported an association region that spanned rs788748 and rs879966, but the association estimates for these two SNPs were not reported.[16] A look-up of the associations for these SNPs revealed that the rs788748 and rs879966 alleles associated with lower odds of HOA were significantly associated with lower

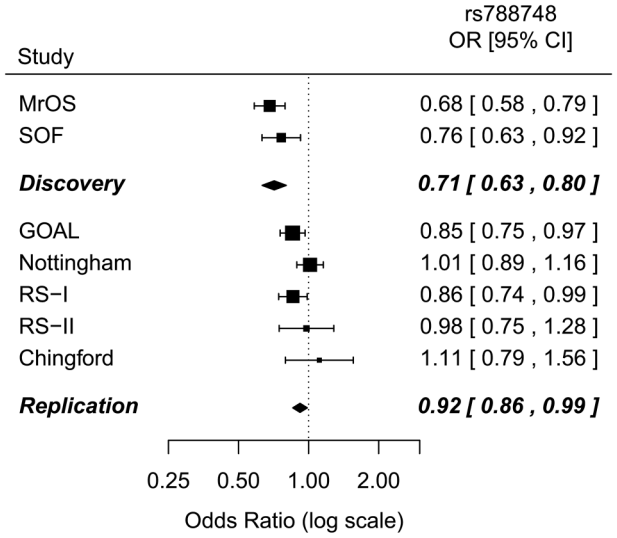

Figure 2 Forest plot of rs788748 hip osteoarthritis association. Point sizes are proportional to the inverse variance weights, and 95% CIs are shown. Within discovery and replication stages, studies are ordered by the number of cases. GOAL, Genetics of Osteoarthritis and Lifestyle Study; MrOS, Osteoporotic Fractures in Men Study; RS, Rotterdam Study; SOF, Study of Osteoporotic Fractures.

circulating IGFBP3 but not circulating IGF-I (R Kaplan, personal communication) (table 2). The two reported independent SNPs most significantly associated with circulating IGFBP3 (rs11977526 and rs700752)[16] were not significantly associated with HOA (see online supplementary table S7). Based on HapMap CEU genotypes, rs788748 was in low LD with rs11977526 (r^2=0.05), rs700752 (r^2=0.04) and a potentially causal SNP related to IGFBP3 levels, rs2854746 (r^2=0.05).[29]

Functional studies of *IGFBP3*

The function of *IGFBP3* was explored in the ATDC5 chondrogenesis model system. Hypertrophic differentiation of chondrocytes, a process related to cartilage loss, is associated with decreased expression of type II collagen and aggrecan, matrix calcification, and increased expression of the *Runx2* transcription factor.[30] *Runx2*, together with *MEF2C*, increases the expression of *type X collagen*, matrix metalloproteinases (MMPs) and *Indian Hedgehog* (*Ihh*).[30]

Efficient knockdown of *IGFBP3* was achieved in ATDC5 cells (figure 3A). By day 21, matrix calcification (Alizarin Red staining), sulfated proteoglycan synthesis (Alcian Blue and Safranin O staining) and collagen content (Sirius red staining) were reduced in *IGFBP3*$^-$ cells compared to controls (all $p<0.05$) (see online supplementary figure S6), indicating that *IGFBP3* knockdown impairs mineralisation and the early phase of chondrogenesis.

IGFBP3 knockdown in ATDC5 cells resulted in a lack of expression of *type X collagen* and lower expression levels of *Runx2* and *Ihh*, indicating that hypertrophy was largely prevented (figure 3C, D, F). Expression of *type II collagen* was also reduced, but to a lesser extent (figure 3B). WNT signalling is known to activate *Runx2* expression and stimulate hypertrophy.[30] Consistent with prevention of hypertrophy, *IGFBP3* knockdown resulted in lower levels of *Tcf1*, a marker of active WNT signalling (figure 3E).

The effect of *IGFBP3* overexpression was examined in articular cartilage isolated from patients who underwent knee replacement for OA (figure 3G, H). Overexpression of *IGFBP3* in human articular cartilage pellets resulted in lower expression levels of *aggrecan* (figure 3G) and increased expression of *MMP-13* (figure 3H), consistent with increased cartilage catabolism.

HOA is a disease of both cartilage and bone, with a significant component involving bone remodelling.[31] The role of *IGFBP3* in osteogenic differentiation was examined using the MC3T3-E1 cell-based model, a cell line that exhibits temporal gene expression comparable to the in vivo differentiation process.[32] Knockdown experiments were not performed because *IGFBP3* is not expressed in the MC3T3-E1 cell line.[33] *IGFBP3* overexpression strongly enhanced extracellular matrix mineralisation assayed by Alizarin Red staining and alkaline phosphatase activity (see online supplementary figure S7a, b). Expression levels of *Osteocalcin* and *Osteopontin* were 2.5- and 22.3-fold higher, respectively, in *IGFBP3*-overexpressing cells at day 21 (see online supplementary figure S7c, d). At the first day of induced *IGFBP3* overexpression, before osteoblastogenesis was induced, *Osterix* mRNA levels were already 50-fold higher, and they remained 14-fold more expressed at day 21 (see online supplementary figure S7e).

DISCUSSION

In this study, we identified a novel GWS HOA locus near the gene *IGFBP3*. The genetic variant allele associated with lower odds of HOA was also associated with lower levels of circulating IGFBP3, and experimental knockdown of *IGFBP3* prevented chondrocyte hypertrophic differentiation, a deleterious event in OA pathogenesis that results in cartilage loss. Moreover, *IGFBP3* overexpression in articular cartilage from OA patients increased expression of genes associated with cartilage catabolism, and *IGFBP3* overexpression in a cellular model of osteogenic differentiation resulted in an increase in matrix mineralisation, consistent with an activation of osteoblastic differentiation. Our results from GWAS and functional studies indicate that *IGFBP3* levels in cartilage are related to cartilage maintenance and HOA.

Understanding molecular mechanisms underlying genetic associations requires the identification of genes affected by associated genetic variants.[34] Our integration of a GWAS meta-analysis of circulating IGFBP3[16] with our GWAS of HOA revealed that the HOA-associated SNPs rs788748 and rs879966 were significantly associated with circulating IGFBP3, which indicated that these SNPs or variants in LD could be regulatory variants associated with *IGFBP3* expression. Circulating IGFBP3 reflects *IGFBP3* gene activity from multiple tissues, and associated SNPs could be regulatory variants associated with *IGFBP3* expression in at least one tissue, which is then reflected in circulating protein levels. These results cannot be used to determine the tissue in which associated variants might regulate *IGFBP3*, but the direction of effect for SNP alleles can be inferred, namely, that the rs788748 A allele associated with lower odds of HOA is also associated with lower levels of *IGFBP3* expression in at least one tissue. It is also important to note that even though the SNPs near *IGFBP3* previously reported to be the most significantly associated with circulating IGFBP3[16] are not significantly associated with HOA, these SNPs explain a small percentage of the variance in IGFBP3 levels, thus preventing meaningful interpretation of the relationship between circulating IGFBP3 and HOA.

IGFBP3 is one of six insulin-like growth factor binding proteins (IGFBPs) in humans.[35] IGFBPs modulate IGF signalling by

Table 2 SNP association with circulating IGFBP3

		IGFBP3			IGF-I		
SNP	EA (freq)	p Value$_{GC}$	Effect direction*	I^2	p Value$_{GC}$	Effect direction*	I^2
rs788748	A (0.49)	6×10^{-13}	−−−−	61.3	0.82	−−+−	0.0
rs879966	G (0.40)	4×10^{-5}	−−−−	56.2	0.80	−++−	0.0

rs788748 was genotyped in CHS, KORA and SHIP and imputed in FHS (imputation accuracy=1.0). rs879966 was genotyped in CHS and imputed in FHS, KORA and SHIP (imputation accuracy=0.98, 0.99 and 0.99, respectively).
*Study order: KORA, FHS, SHIP and CHS.
EA, effect allele; SNP, single nucleotide polymorphism.

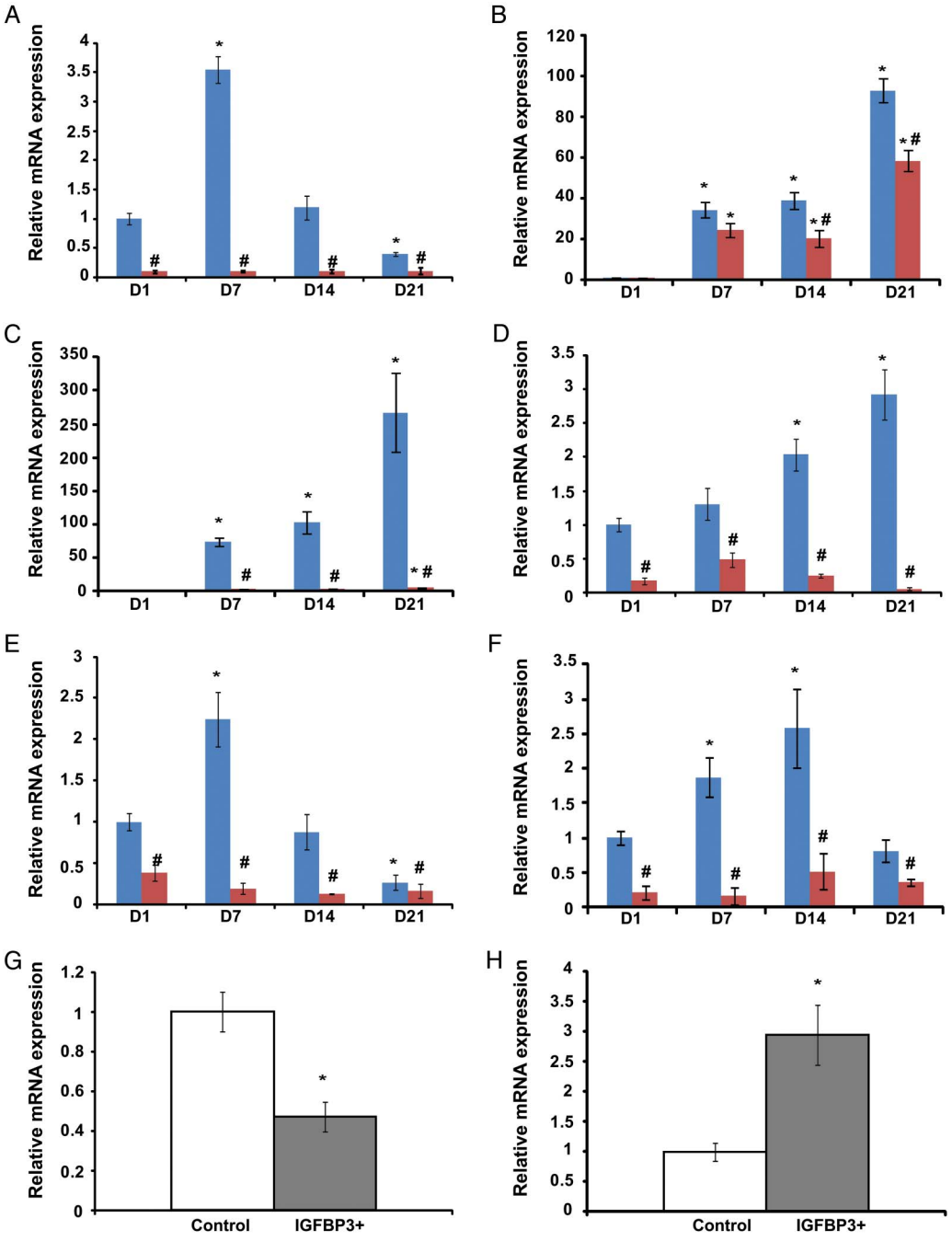

Figure 3 Impact of IGFBP3 knockdown and overexpression on markers of cartilage homeostasis. ATDC5 cells stably transfected with a transgene expressing siRNA directed against *IGFBP3* are marked in red, and control cells in blue (A–F). *IGFBP3* overexpression (grey bars) and control cells (white bars) in articular cartilage (G and H). Gene expression levels quantified by RT-PCR were *IGFBP3* (A), *type II collagen/Col2A1* (B), *type X collagen/Col10A1* (C), *Runx2* (D), *Tcf1* (E), *Ihh* (F), *aggrecan* (G) and *MMP13* (H). *$p \leq 0.05$ difference with day 1 levels. #$p \leq 0.05$ difference with control at each day.

binding to the two ligands (IGF-I and IGF-II) that primarily signal through the type I IGF-I receptor (IGF-IR).[35] In articular cartilage from individuals without OA, IGF-I activates anabolic processes and inhibits catabolism of cartilage.[36–38] In OA cartilage, IGF-I's anabolic activity is greatly diminished, despite the presence of its receptor (IGF-IR).[39 40] Higher levels of IGFBPs, in particular IGFBP3, have been observed in OA cartilage, leading to the notion that IGFBPs could decrease IGF-I's bioavailability and could at least be partially responsible for the reduced responsiveness of OA cartilage to IGF-I.[40–45] Consistent with this notion, an IGFBP-insensitive IGF-I analogue exhibited a stronger anabolic effect on cartilage than IGF-I alone,[42] and a small molecule that inhibits the binding of IGFBPs to IGF-I restored the anabolic effect of IGF-I on OA chondrocytes.[46] Our experimental results also suggest that lower levels of IGFBP3 could promote cartilage maintenance. Further studies are needed to determine whether the HOA association with rs788748 is mediated through a potential alteration in the role of IGFBP3 in cartilage homeostasis during developmental growth to determine joint morphology or perhaps through a subtle impact on IGFBP3 throughout life to affect the threshold for developing HOA. Furthermore, whether IGFBP3's

effect on cartilage homeostasis depends on IGF-I will need to be explored in future studies, as IGFBP3 possesses a nuclear localisation sequence, it has been found to localise to the nucleus in cartilage, and it has been proposed to have IGF-independent effects in other biological processes.[35 47]

The nearly complete elimination of *IGFBP3* expression in our knockdown experiments had strong effects on the prevention of chondrocyte hypertrophy, but subtle effects on cartilage maintenance were also observed, arguing for a minimum required level of IGFBP3. The HOA-associated variants rs788748 and rs879966 were associated with a reduction, but not elimination, of circulating IGFBP3. We also observed that chondrocyte hypertrophy was stimulated by overexpression of *IGFBP3* in OA cartilage, which is already in a diseased state, leaving it unknown whether this same effect would be observed in healthy cartilage. With this caveat in mind, higher levels of IGFBP3 could contribute to cartilage loss in diseased cartilage. Taken together, our cell-based experiments argue for a certain range of IGFBP3 in cartilage that supports healthy cartilage homeostasis. In order for IGFBP3-targeted HOA treatments to potentially be effective, this proposed healthy IGFBP3 range will need to be carefully characterised. Furthermore, IGFBP3-targeted therapies must consider the potential impacts on cancer and other IGFBP3-associated diseases.[35]

A number of factors that regulate *IGFBP3* expression have been identified, but *IGFBP3* transcriptional enhancers that function in chondrocytes are not well characterised.[35] HMGA1 binds to several sites 2 kb upstream of the *IGFBP3* promoter and is required for *IGFBP3* expression in chondrocytes,[48] but DNA near rs788748 and rs879966 has not been experimentally tested for enhancer activity. HMGA1 binding data was not included in the ENCODE project, but there is evidence that C/EBPβ, HMGA1's binding partner, binds DNA near rs788748 (see online supplementary figure S5). Functional studies of this genomic region could help to elucidate the dynamic role *IGFBP3* plays in OA development.

Despite a directionally consistent and significant association in the replication studies, the joint analysis p value (combined discovery and replication) was less significant than the discovery stage p value, which is not surprising given that the replication stage sample size was larger and the replication stage effect size was closer to the null, indicating a possible winner's curse effect[22] even though a correction for this effect had little impact on the discovery stage OR.[23] While joint analysis provides greater power in two-stage designs than replication analysis to identify GWS associations, even greater power is achieved in one-stage designs where genome-wide SNPs are genotyped in all samples.[49] We performed a one-stage design in the MrOS and SOF cohorts to identify GWS SNP associations, followed by a standard replication analysis of the two GWS associations in independent studies. As genome-wide SNPs were tested in the discovery stage, the significance threshold accounting for the multiple testing burden should only be applied to discovery stage results. Furthermore, given our allocation of studies in discovery and replication, a replication stage two-tailed p value of 1×10^{-3} is required to achieve genome-wide significance in a joint analysis (see online supplementary text S1), a threshold that exceeds typical significance thresholds for replication. Moreover, the practice of combining discovery and replication stage results to achieve genome-wide significance has been noted to present multiple testing problems.[50]

In summary, our results from GWAS and functional studies support the role of *IGFBP3* in HOA. Further work is needed to determine whether IGFBP3's effect on cartilage homeostasis requires its interaction with IGF-I. If the IGFBP3 and IGF-I interaction plays a role in HOA, consideration of this interaction could enhance HOA therapies that target the anabolic effect of IGF-I.

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Competing interests None.

Ethics approval All studies participating in the GWAS meta-analysis were approved by their respective institutional review boards.

Provenance and peer review Not commissioned; externally peer reviewed.

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