GREM1, FRZB and DKK1 are key regulators of human articular cartilage homeostasis


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Running head: Natural brakes of articular cartilage hypertrophy

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Abstract

Objective: The development of osteoarthritis may be caused by activation of hypertrophic differentiation of articular chondrocytes. Healthy articular cartilage is highly resistant to hypertrophic differentiation, in contrast to other hyaline cartilage subtypes such as growth-plate cartilage. In this study, we set out to elucidate the molecular mechanism responsible for the difference in propensity to undergo hypertrophic differentiation between human articular and growth plate cartilage.

Methods: Whole genome gene expression micro-array analysis of healthy human growth-plate and articular cartilage derived from the same adolescent donors was performed. Candidate genes, enriched in articular cartilage, were validated at the mRNA and protein level and examined for their potential to inhibit hypertrophic differentiation in two models. In addition, we studied a possible genetic association with osteoarthritis.

Results: Pathway analysis demonstrated decreased Wnt-signaling in articular cartilage compared to growth-plate cartilage. This was at least partially due to increased expression of BMP- and Wnt-antagonists GREM1, FRZB and DKK1 at mRNA and protein level in articular cartilage. Supplementation of these proteins diminished terminal hypertrophic differentiation without affecting chondrogenesis in long-bone explant cultures and chondrogenically differentiating human mesenchymal stem cells. Additionally, we show that SNP-rs12593365, located in a genomic control region of GREM1, significantly associates with a 20% reduced risk for radiographic hip-osteoarthritis in two population-based cohorts.

Conclusion: Taken together, our data identifies GREM1, FRZB and DKK1 as natural brakes of hypertrophic differentiation in articular cartilage. As hypertrophic differentiation of articular cartilage may contribute to the development of osteoarthritis, our findings may open new avenues for therapeutic intervention.
Healthy articular cartilage is largely resistant to undergo hypertrophic differentiation. In contrast, other forms of hyaline cartilage such as growth plate cartilage, which is responsible for bone elongation via endochondral ossification, naturally undergoes hypertrophic differentiation (1). The molecular mechanism underlying this discrepancy between these two types of hyaline cartilage is largely unknown. It has been postulated that articular cartilage secretes (a) factor(s) that inhibit(s) chondrocyte hypertrophy. This is based on the observation that articular cartilage explants efficiently block hypertrophic differentiation of growth plate cartilage explants in transwell co-culture experiments (2). Interestingly, recent data obtained in transgenic mouse models has provided evidence that deregulated hypertrophic differentiation of articular chondrocytes may be a driving factor in the onset and progression in subsets of osteoarthritis (OA). Several signaling networks have been identified that can stimulate hypertrophic differentiation of chondrocytes in the growth plate. These networks include the Wnt/β-catenin (3-5), Bone Morphogenetic Protein (BMP) (6), Indian Hedgehog (IHH) (7) and hypoxia induced signaling (8) pathways. Interestingly, forced activation or conditional inactivation of these pathways in articular chondrocytes results in a relief of the brake of hypertrophic differentiation and induces features of OA in mice (9-12). Whether the same pathways are also involved in the pathogenesis of OA in man is still largely unclear.

In this study, we set out to elucidate the underlying molecular mechanism that is responsible for the difference in the propensity to undergo hypertrophic differentiation between human articular and growth plate cartilage by making a detailed comparison of gene expression profiles of both types of cartilage. We hypothesized that this approach could reveal the identity of the articular cartilage secreted factor(s) that inhibit chondrocyte hypertrophy (2). We expected that this factor(s) should act up-stream of known pathways involved in the regulation of chondrocyte hypertrophy. Furthermore,
since such (a) factor(s) would be of paramount importance for joint cartilage homeostasis, we anticipated that this approach might reveal new candidate genes that are possibly correlated with OA.

Materials and Methods

Human material

Use of patient material was approved by local hospital-ethical-committees and for all samples informed written consent was obtained. Samples were directly frozen in Cryomatrix (Shandon) or fixated, decalcified and embedded in paraffin. Paired growth plate and articular cartilage samples were obtained from 9 to 14 year old patients undergoing amputation surgery with etiologies unrelated to cartilage. Fetal femurs were obtained from fetuses undergoing elective termination of pregnancy with 20 weeks of gestation. Healthy nature of all obtained samples was validated macroscopically and histologically.

Cell isolation and cultivation

After written consent was obtained, MSCs were isolated from fresh bone-marrow samples and cultured as described previously (13). MSCs pellets of $2.5 \times 10^5$ cells were cultured up to 35 days in a previously described TGFβ containing chondrogenic culture medium (14). Medium was supplemented with 0, 20 or 200 ng/ml recombinant human GREM1, FRZB or DKK1. On day 0, 11, 21 and 35 eight pellets were pooled for RNA isolation and two pellets were fixated for histological analysis.

Organ cultures

All animal procedures were approved by University Utrecht’s animal-care-committee. Tibiae were isolated from E17.5 fetal FVB mice (Harlan) and cultured in medium consisting of $\alpha$-MEM
supplemented with 10% heat inactivated fetal bovine serum (Biowhittaker) and 100 U/ml penicillin with 100 mg/ml streptomycin (Gibco) as previously described (15). Medium was supplemented with 0, 50 or 500 ng/ml of murine Grem1, Frzb or Dkk1 recombinant proteins. After 7 days of culture, tibiae were fixated for histological analysis.

**Histology and immunohistochemistry**

Paraffin embedded samples were cut into 5 µm sections and stained with haematoxylin and eosin, toluidine blue, toluidine blue combined with Von Kossa or Alcian blue combined with Nuclear fast red according to standard procedures. For each staining, all samples were processed identically and simultaneously. For immunohistochemistry, sections were incubated with 1:100 diluted primary antibodies against GREM1, FRZB or DKK1 (scw28873, scw13941 and scw25516 respectively; Santa Cruz) and developed using rabbit ABC staining (scw2018; Santa Cruz) according to manufacturer’s protocol. For image analysis ImageJ software was used. Length of the cartilaginous zones was determined in midsagittal sections as the shortest possible length between the indentations present at the articulating surface in between the future lateral condyles.

**Total RNA extraction**

After removal of the bone, growth plate and articular snap frozen cartilage-samples were cut into 8 µm sections. Every fifth slide was stained with haematoxylin to ensure inclusion of the majority of the hypertrophic zone without the presence of bone contamination. Subsequently, from each sample approximately 200 sections were collected and homogenized in a 4M guanidine thiocyanate solution (Sigma). Total RNA isolation was performed as previously described (16).
Microarray processing and quantitative real-time reverse transcriptase-polymerase chain reaction

Biotinylated antisense cRNA was prepared from isolated RNA of eight articular and five growth plate specimens of four patients, according to Affymetrix’s protocol. Hybridization was performed on a GeneChip Hybridization oven 640, dyed in the GeneChip Fluidics Station 450 and scanned with a GeneChip Scanner3000 (Affymetrix). Gene expression profiling of donor paired AC and GP specimen was performed using Affymetrix HG-U133_Plus_2-type microarrays (Affymetrix). Each donor pair was analysed on the same microarray batch (supplemental table 2). Raw fluorescence intensity values were normalized applying quantile normalization. Differential gene expression was analysed based on loglinear mixed-model ANOVA (17, 18), using SAS JMP7 Genomics, version3.1 (SAS Institute). A false positive rate of α=0.05 with Holm correction was taken as the level of significance. Principal Component Analysis (PCA) with Pearson product-moment correlation was performed to compute correlations between gene expression of growth plates and articular cartilages. Changes of gene expression in pathways were visualized using Genmapp2.0 (19). Changes in gene ontology were investigated using PANTHER (20). Raw and normalized data are deposited in the Gene Expression Omnibus (GSE-32398).

For single gene expression analysis, cDNA was synthesized using 1 µg non-amplified total RNA isolated from independent tissue specimens from the same donor in accordance with manufacturer’s instructions (BioRad). 20 ng cDNA of all samples was amplified using iQ SYBR-Green Supermix (BioRad) on a MyIQ RT-qPCR system (BioRad) and analyzed using iQ5 software (BioRad). Primers sequences are available upon request. Normalization using GAPDH, ACTB or B2M rendered consistent data-trends. Presented data is based on GAPDH normalization.

Genetic Study population
The Rotterdam Study is a population-based prospective cohort study that is comprised of men and women aged 55 years and older. A detailed description of the study design has been described previously (21). The medical ethics committee of Erasmus University Medical School approved the study and written informed consent was obtained from each participant. The Rotterdam Study I (RSI) (N=5193) and RSII (N=1949) were investigated on associations between single nucleotide polymorphisms (SNPs) of GREM1 and DKK1 and radiographic OA. All SNPs in a genomic region in between 100Kb upstream and 100Kb downstream of the two selected genes were used. In total 116 SNPs were analyzed. Associations with OA phenotypes were tested using an allelic chi-square test (1 df) assuming an additive effect for each SNP tested. Analyses were carried out using MACH and GRIMP (22) and were adjusted for age, gender and BMI.

Results

Differential gene expression between articular and growth plate cartilage

Macroscopical and histological examination of all cartilage specimens did not reveal indications for cartilage disease (supplemental figure 1A). From 4 donors, pairs of growth plate and articular cartilage tissue specimens were collected and RNA was isolated. Samples were subjected to whole-genome gene expression profiling. In total, 2915 genes were found to be significantly differentially expressed of which 1321 were articular cartilage-enriched and 1594 growth plate cartilage-enriched. Of these, the fold change of only 418 genes was higher than two-fold and as few as 35 genes were expressed with a more than five-fold difference (figure 1A). Differential gene expression of 10 genes of interest was verified using RT-qPCR (supplemental figure 1B). Examination of the list of differentially expressed genes identified several BMP-related molecules including GREM1, CHRDL2, LTBP1, LTBP2, ID2, ID3, GDF10, SMAD3, and TGFB3 that were significantly
higher expressed in articular chondrocytes. Furthermore, expression of several extracellular matrix molecules was as well dissimilar with COL3A1, COL5A2, KAL1, COL6A3, FBN1 and COL14A1 being higher expressed in articular chondrocytes and MATN1, COL12A1, COL4A5, MFAP3, FBN3 and COL4A6 being higher expressed in growth plate chondrocytes. Growth plate and articular cartilage are both hyaline cartilage subtypes; as expected traditional hyaline cartilage markers such as COL2A1, COL9A1, and ACAN were not found to be differentially expressed at the mRNA level.

*Wnt signaling and cell cycle pathways genes are differentially expressed between growth plate and articular cartilage*

Pathway analysis, based on gene ontology, identified differential expression of the cell cycle (p≤0.001) and Wnt pathway (p=0.04). Detailed analysis showed that expression of the vast majority of cell cycle related genes was higher in growth plate cartilage (supplemental figure 1C). Only four cell cycle related genes were more expressed in articular cartilage, of which three have an inhibitory role in cell proliferation. The higher activity of the cell cycle pathway is in line with the growth plate’s role cartilage in bone elongation. The changes in Wnt signaling suggested a higher activity in growth plate cartilage than in articular cartilage. This is based on i) higher expression of Wnt agonists WNT4, WNT5A and WNT11 in growth plate cartilage, ii) increased expression of established Wnt target genes CCND1, MMP2, PTTG1, LEF1, MYC, RUNX2, TIAM1, FST and WISP3 in growth plate cartilage, iii) enriched expression of TLE2, an established transcriptional inhibitor of canonical Wnt-signaling in articular cartilage, and iv) higher expression of direct and indirect antagonists of Wnt-signaling FRZB, DKK1 and GREM1 in articular cartilage (figure 1B). Furthermore, these three proteins were the three most differentially expressed genes between growth plate and articular cartilage with an average fold change in mRNA expression of 539, 205 and 97 respectively as determined by RT-qPCR. Notably,
BMP antagonist GREM1 inhibits Wnt indirectly in contrast to FRZB and DKK1 which are direct Wnt inhibitors (23).

Protein expression of GREM1, FRZB and DKK1 is predominantly present in articular cartilage and the resting zone of growth plate cartilage

We next performed immunohistochemistry to study protein expression of GREM1, FRZB and DKK1 in donor pairs of articular and growth plate cartilage specimens. All three proteins stained more intense in articular cartilage compared to growth plate cartilage, corroborating the mRNA expression patterns (figure 1C). The proteins were detected in all zones of articular cartilage in contrast to growth plate cartilage. In the latter tissue, GREM1, FRZB and DKK1 staining was only witnessed in the resting zone and to a lesser extent in the late hypertrophic zone of growth plate cartilage, albeit at lower levels than in articular cartilage.

To determine whether differential expression of GREM1, FRZB and DKK1 was already present before formation of the secondary centre of ossification, we investigated human fetal femurs. The healthy nature of all fetal femurs was confirmed by macroscopical and histological analysis (figure 2A). Immunohistochemistry revealed that GREM1 and DKK1 expression predominantly resided at the proximal resting zone, which is the region destined to become articular cartilage (figure 2B). Although some GREM1 and DKK1 could still be detected in the distal resting zone, the staining was less intense. Moreover, the weakest staining was observed in the proliferative and hypertrophic zones. FRZB was readily detected in the proximal resting zone and became progressively less intense in more distal zones.

RNA isolated from seven different regions of the fetal femur was subjected to RT-qPCR analysis (figure 2C). FRZB mRNA levels steadily declined between the proximal resting zone and the
hypertrophic zone. Although GREM1 and DKK1 mRNA levels also progressively declined, there was a stronger regional decline around the mid-resting zone and the proliferative zone. Immunohistochemical analysis corroborated the gene expression analysis. Both analyses suggested a gradient-wise expression of GREM1, FZRB and DKK1, with highest expression near the future articular cartilage that became progressively lower towards the subchondral bone.

**GREM1, FRZB and DKK1 inhibit longitudinal growth of explanted fetal tibiae dose dependently**

To investigate whether GREM1, FRZB and DKK1 play a role in the hypertrophic differentiation and endochondral ossification of long bones, we cultured explanted mouse fetal tibiae in the presence or absence of 50 or 500 ng/ml of recombinant Grem1, Frzb and Dkk1. Histological analysis showed no gross abnormalities in the metaphysis and diaphysis (figure 3A) nor in the epiphyseal cartilaginous heads (figure 3B) of all tibiae.

Tibiae cultured in the presence of a single protein or a combination of all three proteins grew more slowly than untreated controls in a dose-dependent manner (supplemental figure 2), resulting in a lower total tibiae length. This was due to a decrease in length of the metaphysis and diaphysis with increasing dosages of Grem1, Frzb and Dkk1. In contrast, the length of epiphyseal cartilaginous heads showed no difference (figure 3C). Detailed zonal analysis of the cartilage showed a significant decrease in length of chondrocyte columns (proliferative zone), a modest but significant decrease in height of hypertrophic zone and a significant strong increase in length of non-columnar (resting) zone (p<0.001, p=0.013, p=0.010 respectively when comparing non-treated with treated explants (500 ng/ml)) (figure 3D). Taken together, these results suggest that addition of recombinant Grem1, Frzb and Dkk1 inhibits endochondral ossification of explanted tibiae dose-dependently by inhibiting one of
the earlier stages of hypertrophic differentiation in chondrocytes and retaining chondrocytes in the resting zone.

**Chondrogenically differentiating MSCs resemble growth plate chondrocytes**

We used the list of differentially expressed genes between growth plate and articular cartilage to determine whether chondrogenically differentiating MSCs acquire a gene expression fingerprint more characteristic of growth plate than articular chondrocytes. To investigate this, we performed a 3-dimensional PCA, which involved expression profiles of growth plate and articular cartilage specimen as well as a previously established profile of chondrogenically differentiating fetal bone marrow derived MSCs (Van Gool et al, in preparation) (figure 4A). Growth plate and articular cartilage grouped distinctly from each other as well as from undifferentiated MSCs. Over time, chondrogenically differentiating MSCs gradually, but progressively, acquired a gene expression fingerprint more closely resembling growth plate than articular chondrocytes.

We then selected a panel of differentially expressed genes and used this panel to assess cartilage lineage formation in chondrogenically differentiating adult bone marrow MSCs in pellet cultures in the presence of TGFβ3 and BMP6 (24, 25). None of the investigated AC enriched genes were induced during 5 weeks of differentiation despite histological evidence of cartilage formation. More strikingly, both GREM1 and ABI3BP were significantly down-regulated to levels normally found in growth plate-cartilage (figure 4B). In sharp contrast, growth plate enriched genes, such as LEFT1 and PANX3, were up-regulated almost matching growth plate expression levels (figure 4C). Similar findings were made in MSCs cultured in pellets, discs or encapsulated in hydrogels. This indicated that bone marrow-derived MSCs in the presence of TGFβ3 and BMP6 differentiate into growth plate-like hyaline cartilage irrespective of the culture method.
Addition of GREM1, FRZB or DKK1 during chondrogenic differentiation of MSCs inhibits hypertrophic differentiation

Since chondrogenically differentiated MSCs did not express GREM1, FRZB or DKK1, we reasoned that their addition during the differentiation period might steer the rather growth plate-like phenotype towards a more articular cartilage phenotype. To investigate this, chondrogenically differentiating MSCs were treated with 20 or 200 ng/ml recombinant human GREM1, FRZB and DKK1 from day 7 onwards. Histological analysis showed that after at least 3 weeks of culture all pellets stained positive for glycosaminoglycans (figure 5A). Mineralization was first observed in the control cultures after week three, progressed over time and was almost exclusively present in the pellets’ periphery.

Addition of 20 ng/ml of protein resulted in slightly less intense toluidine blue staining after 5 weeks of differentiation compared to control (figure 5A). Addition of 200 ng/ml of FRZB or DKK1 showed increased glycosaminoglycan staining, predominantly in the pellets’ periphery. This was not observed in GREM1 treated pellets. Importantly, addition of all three proteins reduced matrix mineralization to undetectable levels. In line with this, treatment with the proteins significantly downregulated the expression of hypertrophy-related genes such as ALPL and COL10A1 (figure 5B). In contrast, addition of GREM1, FRZB and DKK1 did not affect COL2A1, ACAN and SOX9 mRNA levels (supplemental figure 3).

Supplementation of 20 or 200 ng/ml recombinant of GREM1, FRZB or DKK1 did not result in an increase in expression of articular cartilage-enriched genes GREM1, FRZB, DKK1 and ABI3BP and only modestly decrease in expression of growth plate-enriched genes such as WNT5A, WNT11, EPYC and PANX3, but this did not reach significance (figure 5C). Absence of a strong effect was not due to
loss of biological activity as the Wnt-target gene \textit{AXIN2} mRNA level was significantly down-regulated. Taken together, these results demonstrated that exogenous GREM1, FRZB and DKK1 were able to inhibit hypertrophic differentiation and mineralization of chondrogenically differentiating MSCs without affecting chondrogenesis per se, but were not able to steer the differentiation towards an articular cartilage phenotype.

SNP rs12593365 is associated with radiographic osteoarthritis

We next reasoned that articular cartilage enriched genes that prevent hypertrophic differentiation are prime candidates for association with the development and progression of OA. To test this hypothesis, we selected 2 genes of interest, namely: \textit{GREM1} and \textit{DKK1} for which most common genetic variants were tagged by a total of 116 SNPs located within the annotated gene or within 100Kb up-stream or down-stream of the genes. These SNPs were analyzed for an association with radiographic knee or hip OA in RSI, a large population cohort study. The study population comprised of 3243 controls, 771 hip OA cases, and 1492 knee OA cases (supplemental table 1). We found 12 SNPs nominally significant associated with hip OA, and 3 SNPs associated with knee OA (Table 1).

We then tried to replicate these initial findings in RSII, which contains 1466 controls, 158 hip OA cases and 369 knee OA cases. We observed that 11 of the 12 SNPs originally associated with hip OA in RSI were also associated with hip OA in RSII in the same direction (Table 1). However, only 2 SNPs reached nominal significance in RSII. A combined analysis of RSI and RSII for the top SNP rs12593365 showed that the C-allele was associated with 20% decreased risk for hip OA (OR:0.80 (95%CI: 0.71-0.92; p=0.001). SNP rs12593365 is located 80 KB downstream of \textit{GREM1} within a region that is known to regulate GREM1 expression (26).

Discussion
In this study, we have identified i) transcriptional markers distinctly present in growth plate or articular cartilage of adolescents, ii) three secreted antagonists: GREM1, FRZB and DKK1, which are enriched in articular cartilage and showed that iii) they are able to mitigate hypertrophic differentiation and endochondral ossification and iv) revealed that SNP rs12593365 near GREM1 is associated with radiographic hip OA.

Whole-genome gene expressions analysis comparing donor-paired articular cartilage and growth plate cartilage showed remarkably few genes expressed with more than 2-fold difference. Only 35 genes were expressed with more than five-fold difference, which represents just 1.6% of all genes that were detected to be significantly differently expressed. This underlines the fact that both tissues, despite their distinct functions and fates, are both hyaline cartilage. Genes typically over-expressed in the hypertrophic zone of the growth plate such as COL10A1 and ALPL were not differentially expressed in our data set. This can be explained by the fact that the hypertrophic zone in pre-adolescent humans encompasses only approximately 3% of the entire growth plate. Indeed, established differentially expressed genes with widespread expression in either growth plate or articular cartilage e.g. MMP2 were present in our dataset. It should be noted that our initial findings were made in hyaline cartilage specimens from preadolescent donors without macroscopic evidence of disease. Our major findings were reproduced in healthy fetal cartilage of long-bones. We cannot exclude that gene expression patterns in articular cartilage of growing individuals are different from articular cartilage of adults. This is subject of further study.

In contrast to osteoarthritic cartilage, healthy articular cartilage is largely resistant to hypertrophic differentiation. It has been postulated that articular cartilage expresses secreted factors that can prevent hypertrophic differentiation of growth plate cartilage or chondrogenically differentiating MSCs (2). Therefore, it is intuitive to assume that these secreted molecules might
control the fate of hyaline cartilage by acting as natural brakes that prevent hypertrophic differentiation of normal articular cartilage via pathways known to be involved in hypertrophic differentiation. Indeed, the three most up-regulated genes in articular cartilage in comparison to growth plate cartilage were the secreted BMP- and Wnt-antagonists GREM1, FRZB and DKK1. The Wnt- and BMP-signaling pathways play a stimulatory role in the tightly orchestrated hypertrophic differentiation process (3, 27). Inhibition of these pathways via supplementation of culture medium with recombinant GREM1, FRZB and DKK1 led to an inhibition of hypertrophic chondrocyte differentiation and matrix mineralization in chondrogenically differentiated MSCs. In cultured mouse fetal explanted tibiae, they mitigated endochondral ossification by retaining the chondrocytes in the resting zone. Therefore, GREM1, FRZB and DKK1 are strong candidates for the above-mentioned secreted factors.

It is well established that in vitro generated MSC-derived cartilage is prone to undergo endochondral ossification when implanted subcutaneously in suitable animal models (14). Using our dataset, we confirmed and at least partly explained this observation at the genetic level. We demonstrate that chondrogenically differentiating MSCs acquire a gene expression fingerprint more characteristic of growth plate cartilage than articular cartilage. In chondrogenically differentiating MSCs, the expression of GREM1, FRZB and DKK1 is invariably low, which may at least partly explain the propensity to undergo hypertrophic differentiation. We furthermore provide evidence that measuring the gene expression of four markers enriched in articular cartilage (GREM1, FRZB, DKK1 and ABI3BP) and four markers enriched in growth plate cartilage (PANX3, MATN1, EPYC and LEF1) are sufficient to identify the hyaline cartilage subtype. This marker set can be used to develop and optimize differentiation protocols to help in obtaining articular-like cartilage from chondrogenically differentiating MSCs.
We hypothesize that the articular cartilage enriched secreted factors GREM1, FRZB and DKK1 act as a natural brake on terminal hypertrophic differentiation in healthy joint cartilage. Accumulating evidence in animal models suggests that activation of hypertrophic differentiation of articular chondrocytes is one of the underlying pathophysiological mechanisms of OA (9, 28). Whether hypertrophic differentiation also plays a role in human osteoarthritis is still unclear (29). Furthermore, it remains to be established whether histological signs of hypertrophic differentiation in human OA fully resembles hypertrophic differentiation in endochondral ossification. In animal models, hypertrophic differentiation of articular chondrocytes is mediated amongst others via a shift in balance between hypoxia inducible factor (HIF)1α and HIF2α, favoring the latter. Not only does this lead to up-regulation of catabolic genes such as MMP3, MMP9 and MMP13, but also of genes that are involved in stimulation of chondrocyte hypertrophy such as IHH and RUNX2 (10, 30). Other evidence has shown that dysregulation of Wnt/β-catenin leads to an osteoarthritic phenotype via hypertrophic differentiation (31, 32). This cartilage degradation can occur both via the canonical and non-canonical pathway (33). Wnt/β-catenin signaling is able to alter HIF and IHH signaling (34, 35). Based on our data in human donors we postulate that GREM1, FRZB and DKK1 act as the natural brakes on hypertrophic differentiation of articular cartilage by directly or indirectly antagonizing Wnt-signaling at least in growing long bones. Following this reasoning, it is not unreasonable to envision a correlation between these factors and the development and/or progression of OA. Indeed, it has been previously shown that DKK1 expression decreases in OA (11, 36). FRZB has also been associated with OA. FRZB<sup>−/−</sup> mice have a mild phenotype, but show accelerated cartilage destruction following exposure to OA-inducing triggers (37). Given the compensatory potential of other Wnt antagonists in articular cartilage, in particularly DKK1 and to a lesser extent GREM1, it seems feasible that they can
compensate for the lack of FRZB in knockout mice under normal conditions. The above mentioned lines of evidence are consistent with our hypothesis.

A possible important role for the identified genes in OA etiology is further corroborated by the results of our genetic-association study. We demonstrated a significant association for a polymorphism near the GREM1 gene with radiographic hip OA. Interestingly, this SNP is located downstream of the GREM1 gene in a region that is known to regulate GREM1 expression (26). However, confirmation in additional study cohorts is necessary. FRZB polymorphisms have previously been described to be associated with osteoarthritis in some studies, but have not been replicated in others. A recent meta-analysis found no overall effect of FRZB genetic variants on hip or knee OA (38). Taken together, multiple lines of evidence support a role for GREM1, FRZB and DKK1 in OA, and based on our data we postulate that a decrease in their expression may favor OA development by diminishing a break on hypertrophic differentiation.

In summary, we used whole-genome gene expression micro-array analysis to discriminate between two subtypes of hyaline cartilage, which are either resistant or prone to undergoing endochondral ossification. Using this dataset, we designed a panel of genetic expression markers able to distinguish between these two hyaline cartilages. We showed that the BMP antagonist GREM1 and the Wnt antagonists FRZB and DKK1 are highly enriched in articular cartilage and are likely to act as a natural mechanism in preventing hypertrophic differentiation of articular cartilage of growing long bones. Deregulation of this mechanism in adults may disturb articular cartilage homeostasis by enabling hypertrophic chondrocyte differentiation, which may ultimately contribute to the pathogenesis of OA.
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Figure 1. Gene expression analysis of healthy human growth plate and articular cartilage. (A) Volcano-plot depicting all 2915 differentially expressed genes between growth-plate and articular cartilage of 4 donor-pairs as measured with whole-genome gene expression analysis. Y-axis indicates the significance level, while the fold change after log2 transformation is plotted on the x-axis. The 3 most differentially expressed genes, GREM1, FRZB and DKK1, are boxed (B) Immunohistochemistry of articular and growth plate cartilage sections derived from the same patients with antibodies against GREM, FRZB and DKK1. More staining was witnessed for all three proteins in articular cartilage compared to growth plate cartilage. (n=4 pairs of articular and growth plate cartilage specimens of adolescent donors). Lower panel denotes a semi-quantitative scoring of the staining ranging from strong positive (+++) to undetectable levels (-). AC, articular cartilage. GP, growth plate cartilage. SZ, superficial zone. MZ, mid zone. DZ, deep zone. RZ, resting zone. PZ, proliferating zone. PHZ, prehypertrophic zone. HZ, hypertrophic zone. Scale bar, 200 µm.

Figure 2. Expression of GREM1, FRZB and DKK1 in fetal cartilage. (A) Representative haematoxylin and eosin stained section of 20 weeks-old human fetal distal femoral cartilage. Black boxes indicate respective locations of depicted immunohistochemical stainings shown in B. Lines denote the borders of the manually dissected zones of the primary growth plate from the proximal resting zone (RZ) to the distal hypertrophic zone (HZ) that were included in the mRNA analysis (B) Immunohistochemistry of fetal distal femoral cartilage sections with antibodies against GREM, FRZB and DKK1 of the boxed regions in A. All stainings showed positive cells near the joint-side. Staining became gradually less intense towards the subchondral bone. (C) Quantitative real-time PCR on mRNA isolated from the seven manually dissected zones of the primary fetal growth plate as shown in A demonstrated a gradual decrease in GREM1, FRZB and DKK1 mRNA expression from the
proximal resting zone towards the hypertrophic zone. Data are mean of n=3 donors +/- SEM. Abbreviations: PRZ, proximal resting zone. MRZ, middle resting zone. DRZ, distal resting zone. PZ, proliferative zone. HZ, hypertrophic zone. All pictures in panel B are taken using the same magnification. Scale bar, 200 µm.

**Figure 3. Grem1, Frzb and Dkk1 slow down longitudinal growth by retaining chondrocytes in the resting zone.** Explanted E17.5 mouse tibiae were cultured in the presence or absence of 50 or 500 ng/ml of each Grem1, Frzb and Dkk1. After 7 days of culture, midsagittal 5 µm paraffin sections were stained with (A) alcian blue and nuclear fast red or (B) haematoxylin and eosin. Using image analysis software the lengths of (C) the whole organ, the cartilaginous primary growth plates and bone shaft, and (D) the resting zone, proliferative zone and hypertrophic zone of the cartilage were determined. Yellow bars indicate the resting zone. Purple bars represent the proliferative zone and blue bars indicate the hypertrophic zone. Data represent the mean of 6 tibiae +/- SEM. Scale bar, 200 µm.

**Figure 4. Chondrogenically differentiated MSCs resemble growth plate chondrocytes.** (A) Three dimensional principal component analysis based on whole genome gene expression analysis of articular cartilage (red spheres), growth plate cartilage (green spheres) and weekly time points of chondrogenically differentiating human fetal bone marrow MSCs up to five weeks (blue spheres). MSCs were differentiated in pellet cultures in the presence of TGFβ3 and BMP6. Over time the gene expression profile of MSC derived cartilage progressively resembled more growth plate than articular cartilage. (B) Quantitative real-time PCR analysis of chondrogenically differentiating adult bone marrow MSCs (n=3 donors) on articular enriched genes. Data are expressed as mean +/- SEM. Expression of none of these genes was induced during chondrogenic differentiation of the MSCs. (C)
In marked contrast, gene expression of two growth plate chondrocyte enriched genes was induced. AC, articular cartilage. GP, growth plate cartilage.

**Figure 5. GREM1, FRZB and DKK1 inhibit matrix mineralization of chondrogenically differentiating MSCs.** (A) Representative histological sections (n=6) of chondrogenically differentiated adult bone marrow MSCs stained with toluidine blue and Von Kossa cultured for either 3 weeks (i) or 5 weeks (a-h, j-l). Cells were supplemented with PBS (a,e,i), 20 ng/ml (b,f) or 200 ng/ml (j) of GREM1, 20 ng/ml (c,g) or 200 ng/ml (k) of FRZB or 20 ng/ml (d,h) or 200 ng/ml (l) of DKK1. After three weeks of chondrogenic differentiation low levels of mineralization are visible, while after five weeks mineralization is readily detected. Addition of GREM1, FRZB or DKK1 did not inhibit chondrogenesis, but did prevent mineralization. Arrows indicate nodules of mineralization. (B) and (C) Quantitative real-time PCR analysis was performed on MSCs chondrogenically differentiated for 5 weeks in the presence or absence of GREM1, FRZB or DKK1 (20 ng/ml). Investigated genes are enriched in hypertrophic differentiation (B) or the growth plate (C). Data are expressed as mean (n=3 donors) +/- SEM. * = P<0.05. Large scale, 100 µm; short scale bar, 200 µm.
Supplemental figure 1. Gene expression analysis of healthy human growth plate and articular cartilage. (A) Representative histological sections of growth plate and articular cartilage from the same patient stained with either toluidine blue or haematoxylin and eosin confirm the healthy nature of both specimens. (B) 10 differentially expressed genes were selected for validation of the microarray data by using quantitative real-time PCR in all donor pairs. Data are expressed as mean +/- SEM. (C) Pathway analysis using the significantly differentially expressed genes between growth plate and articular cartilage demonstrated higher expression of many cell cycle genes in growth plate cartilage (red) compared to articular cartilage (green). (D) Detailed Wnt pathway analysis of significantly higher expression in either growth plate (red) and articular cartilage (green). *GREM1 indirectly inhibits Wnt signaling.

Supplemental figure 2. Explanted E17.5 tibiae cultured in the presence or absence of Greml, Frzb, Dkk1 or a combination of all three antagonists. (A) Lengths of tibiae were measured over a period of 7 days. (B) Length of the resting, proliferative and hypertrophic zone were determined after either 7 or 14 days of culture in the presence or absence of 0, 50 or 500 ng/ml of each Greml (G), Frzb (F) and Dkk1 (D). All data represent the mean of 6 observations +/- SEM.

Supplemental figure 3. Addition of different concentration of GREM1, FRZB, or DKK1 to chondrogenically differentiating MSCs did not affect chondrogenesis. (A) Micromasses of 2.5x10^5 MSCs were chondrogenically differentiated for 35 days in the presence of GREM1, FRZB or DKK1. SOX9, ACAN and COL2A1 mRNA levels were investigated in the presence of (A) 20 ng/ml and (B) 200 ng/ml GREM1, FRZB or DKK1 using RT-qPCR. The addition of the antagonists did not affect the mRNA levels of SOX9, ACAN and COL2A1 mRNA showing that they did not interfere with
chondrogenesis per se. All data are expressed as mean fold change (n=3 donors) +/- SEM relative to day 0. * = P<0.05.
Figure 1. Gene expression analysis of healthy human growth plate and articular cartilage. (A) Volcano-plot depicting all 2915 differentially expressed genes between growth-plate and articular cartilage of 4 donor-pairs as measured with whole-genome gene expression analysis. Y-axis indicates the significance level, while the fold change after log2 transformation is plotted on the x-axis. The 3 most differentially expressed genes, GREM1, FRZB and DKK1, are boxed (B) Immunohistochemistry of articular and growth plate cartilage sections derived from the same patients with antibodies against GREM, FRZB and DKK1. More staining was witnessed for all three proteins in articular cartilage compared to growth plate cartilage. (n=4 pairs of articular and growth plate cartilage specimens of adolescent donors). Lower panel denotes a semi-quantitative scoring of the staining ranging from strong positive (+++) to undetectable levels (-). AC, articular cartilage. GP, growth plate cartilage. SZ, superficial zone. MZ, mid zone. DZ, deep zone. RZ, resting zone. PZ, proliferating zone. PHZ, prehypertrophic zone. HZ, hypertrophic zone. Scale bar, 200 µm.
Figure 2. Expression of GREM1, FRZB and DKK1 in fetal cartilage. (A) Representative haematoxylin and eosin stained section of 20 weeks-old human fetal distal femoral cartilage. Black boxes indicate respective locations of depicted immunohistochemical stainings shown in B. Lines denote the borders of the manually dissected zones of the primary growth plate from the proximal resting zone (RZ) to the distal hypertrophic zone (HZ) that were included in the mRNA analysis. (B) Immunohistochemistry of fetal distal femoral cartilage sections with antibodies against GREM, FRZB and DKK1 of the boxed regions in A. All stainings showed positive cells near the joint-side. Staining became gradually less intense towards the subchondral bone. (C) Quantitative real-time PCR on mRNA isolated from the seven manually dissected zones of the primary fetal growth plate as shown in A demonstrated a gradual decrease in GREM1, FRZB and DKK1 mRNA expression from the proximal resting zone to the hypertrophic zone. Data are mean of n=3 donors +/- SEM. Abbreviations: PRZ, proximal resting zone. MRZ, middle resting zone. DRZ, distal resting zone. PZ, proliferative zone. HZ, hypertrophic zone. All pictures in panel B are taken using the same magnification. Scale bar, 200 µm.
Figure 3. Grem1, Frzb and Dkk1 slow down longitudinal growth by retaining chondrocytes in the resting zone. Explanted E17.5 mouse tibiae were cultured in the presence or absence of 50 or 500 ng/ml of each Grem1, Frzb and Dkk1. After 7 days of culture, midsagittal 5 µm paraffin sections were stained with (A) alcian blue and nuclear fast red or (B) haematoxylin and eosin. Using image analysis software the lengths of (C) the whole organ, the cartilaginous primary growth plates and bone shaft, and (D) the resting zone, proliferative zone and hypertrophic zone of the cartilage were determined. Yellow bars indicate the resting zone. Purple bars represent the proliferative zone and blue bars indicate the hypertrophic zone. Data represent the mean of 6 tibiae +/- SEM. Scale bar, 200 µm.

1677x661mm (72 x 72 DPI)
Figure 1. Gene expression analysis of healthy human growth plate and articular cartilage. (A) Volcano-plot depicting all 2915 differentially expressed genes between growth-plate and articular cartilage of 4 donor pairs as measured with whole-genome gene expression analysis. Y-axis indicates the significance level, while the fold change after log2 transformation is plotted on the x-axis. The 3 most differentially expressed genes, GREM1, FRZB and DKK1, are boxed. (B) Immunohistochemistry of articular and growth plate cartilage sections derived from the same patients with antibodies against GREM, FRZB and DKK1. More staining was witnessed for all three proteins in articular cartilage compared to growth plate cartilage. (n=4 pairs of articular and growth plate cartilage specimens of adolescent donors). Lower panel denotes a semi-quantitative scoring of the staining ranging from strong positive (+++ to undetectable levels (-). AC, articular cartilage. GP, growth plate cartilage. SZ, superficial zone. MZ, mid zone. DZ, deep zone. RZ, resting zone. PZ, proliferating zone. PHZ, prehypertrophic zone. HZ, hypertrophic zone. Scale bar, 200 µm.

1604x865mm (72 x 72 DPI)
Figure 5. GREM1, FRZB and DKK1 inhibit matrix mineralization of chondrogenically differentiating MSCs. (A) Representative histologic sections (n=6) of chondrogenically differentiated adult bone marrow MSCs stained with toluidine blue and Von Kossa cultured for either 3 weeks (i) or 5 weeks (a-h, j-l). Cells were supplemented with PBS (a,e,i), 20 ng/ml (b,f) or 200 ng/ml (j) of GREM1, 20 ng/ml (c,g) or 200 ng/ml (k) of FRZB or 20 ng/ml (d,h) or 200 ng/ml (l) of DKK1. After three weeks of chondrogenic differentiation low levels of mineralization are visible, while after five weeks mineralization is readily detected. Addition of GREM1, FRZB or DKK1 did not inhibit chondrogenesis, but did prevent mineralization. Arrows indicate nodules of mineralization. (B) and (C) Quantitative real-time PCR analysis was performed on MSCs chondrogenically differentiated for 5 weeks in the presence or absence of GREM1, FRZB or DKK1 (20 ng/ml). Investigated genes are enriched in hypertrophic differentiation (B) or the growth plate (C). Data are expressed as mean (n=3 donors) +/- SEM. * = P<0.05. Large scale, 100 µm; short scale bar, 200 µm.
Table 1. Genetic association study for hip osteoarthritis in RSI and RSII

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<tr>
<th>SNP</th>
<th>minor allele</th>
<th>major allele</th>
<th>minor allele freq</th>
<th>RSI OR (95%CI)</th>
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RSI and RSII: Rotterdam Study I and II, SNP: Single nucleotide polymorphisms, OR: odds ratio, CI: confidence interval.