Type II Collagen Expression Is Regulated by Tissue-specific miR-675 in Human Articular Chondrocytes[*]

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miRNAs have been shown to be essential for normal cartilage development in the mouse. However, the role of specific miRNAs in cartilage function is unknown. Using rarely available healthy human chondrocytes (obtained from 8 to 50 year old patients), we detected a most highly abundant primary miRNA *H19*, whose expression was heavily dependent on cartilage master regulator SOX9. Across a range of murine tissues, expression of both *H19*- and *H19*-derived miR-675 mirrored that of cartilage-specific SOX9. miR-675 was shown to up-regulate the essential cartilage matrix component COL2A1, and overexpression of miR-675 rescued COL2A1 levels in *H19*- or SOX9-depleted cells. We thus provide evidence that SOX9 positively regulates COL2A1 in human articular chondrocytes via a previously unreported miR-675-dependent mechanism. This represents a novel pathway regulating cartilage matrix production and identifies miR-675 as a promising new target for cartilage repair.

MicroRNAs (miRNAs)[3] are a recently discovered class of endogenous regulators of gene expression and, together with transcription factors, are the most numerous regulators defining cell-specific gene expression (1). Specifically, miRNAs are small non-protein coding RNA species that bind to complementary sequences in the 3′-UTR of target mRNA and inhibit their translation (by blocking translation or inducing transcript decay/degradation) (2). Thus, miRNAs affect cellular phenotype in a direct manner. Deletion studies in mice have shown that individual miRNAs can strongly influence specific mammalian differentiation processes, *e.g.* B cell differentiation (3), cardiomyocyte hypertrophy (4). *In vitro* studies using human preadipocytes showed that a single miRNA was critical for adipogenesis (5). However, at present, almost nothing is known about the function of individual miRNAs in cartilage or chondrocytes, with only one published study reporting detection of a tissue-specific microRNA (miR-140) in murine embryonic cartilage (6). However, the importance of miRNAs in normal skeletal development has been demonstrated recently by Kobayashi and colleagues (7), who generated cartilage-specific Dicer-null mice. (Dicer is a critical enzyme in the miRNA biogenesis path-

way.) Dicer-null growth plates showed both greatly decreased chondrocyte proliferation and accelerated terminal differentiation (hypertrophy), leading to severe growth defects. Hence, miRNAs are essential for normal chondrocyte differentiation in the murine growth plate.

The chondrocyte is the sole cell type in cartilage, and it alone produces and maintains the tough extracellular matrix that gives the tissue its load-bearing function. The chondrocyte phenotype is unstable *in vitro* (8) and also is altered in disease states (9). Uncovering mechanisms that promote the differentiated phenotype has implications for cartilage repair and restoration of tissue function as it enables identification of potential therapeutic intervention points.

In the current study, from genome-wide profiling experiments using healthy human chondrocytes, we unexpectedly detected a most highly abundant noncoding RNA transcript, *H19*, whose expression correlates highly with the differentiated phenotype. Recent work has shown that *H19* is a primary miRNA transcript, which is processed to the mature form, which is miR-675 (10). The role of miR-675 itself is completely unexplored; however, the pre-miR-675 region of *H19* is highly conserved in therian mammals implying this miRNA plays a key role in *H19* function (11). All miRNAs are processed initially from much longer transcripts known as primary miRNA to short stem-loop structures called pre-miRNA and finally to mature, functional miRNA, which are only 21–23 nucleotides in length (12). Although little is known about their regulation, it is believed that miRNA levels are dependent heavily on transcription (*i.e.* of the primary transcript) (12). Here, we report that expression of primary miRNA *H19*, like the main cartilage matrix genes, is dependent on master chondrocyte regulator transcription factor SOX9. In addition, inhibition of *H19* and/or miR-675 significantly down-regulates COL2A1, the most abundant and functionally important cartilage matrix protein; whereas overexpression increases COL2A1 levels. Finally, overexpression of miR-675 rescues COL2A1 levels in *H19*- or SOX9-depleted cells, suggesting that regulation of COL2A1 by SOX9/*H19* is mediated specifically by the mature microRNA. Hence, we propose that miR-675 lies at the core of a new mechanism promoting the differentiated chondrocyte phenotype and matrix production and thus represents a potential new target for cartilage repair.

EXPERIMENTAL PROCEDURES

Cell Culture—Healthy articular cartilage was obtained from patients after informed consent and following local ethics committee guidelines. Cartilage was harvested from the femoral condyle and tibial plateau following amputation due to sarco-

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[3] The abbreviations used are: miRNA, microRNA; HAC, human articular chondrocyte; siRNA, small interfering RNA; OA, osteoarthritis.
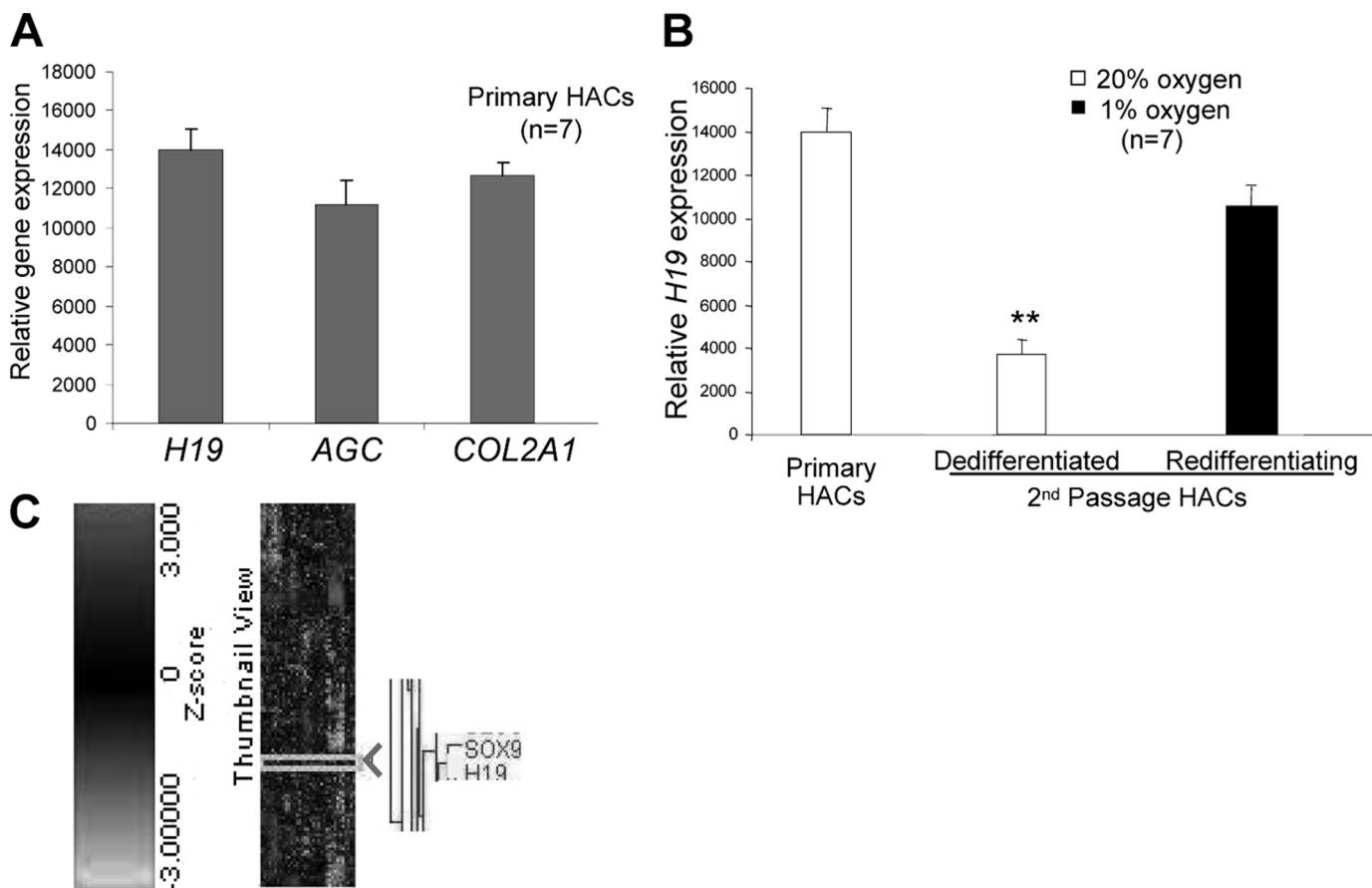
FIGURE 1. **Noncoding RNA *H19* is highly expressed in normal HACs and correlates with the differentiated phenotype.** *A, H19* gene expression levels were comparable to the most abundant cartilage matrix genes aggrecan (*AGC*) and *COL2A1* as measured in primary HACs. ($n = 7$, *i.e.* cultures from seven different patients). *B, H19* expression levels in HACs significantly decreased with passage and subsequently increased upon redifferentiation in hypoxia (**, $p < 0.01$; $n = 7$). *C, H19* clusters directly with SOX9 in HACs. Unbiased, hierarchical clustering analysis of genome-wide array data were performed on an entire data set of 35 arrays from HACs ($n = 7$, *i.e.* separate cultures derived from seven individual donors) undergoing dedifferentiation and redifferentiation in culture. The *scale* refers to Z-scores as it reduces the multiplicative error effect of the expression intensity measurements. Gene expression is relative to that of ribosomal protein RPLP0.

mas not involving the joint. Tissue was obtained from nine donors (six male and three female; age range of 8–50 years, average age of 30 years). Cartilage specimens were collected on the day of surgery and cut into small pieces ($1–2$ mm^3). Diced cartilage was placed in 1.5 mg/ml collagenase type 2 (Worthington®) with Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Biosera) and incubated at 37 °C for 18 h with shaking. Isolated human articular chondrocytes (HACs)[3] were then passed through a cell strainer, pelleted, and washed twice with medium. Cells were seeded at a density of 8×10^3 cells/cm^2 in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Cultures were passaged at confluency (~ 7 days) and subsequently seeded at 5×10^3 cells/cm^2. Both primary (unpassaged) and passaged cells (up to passage 2) were used in experiments.

Transient Transfections—HACs were transfected as described previously (13). Briefly, HACs were seeded at 15×10^4 cells/well in 6-cm dishes. The following day, transfection with siRNA was carried out at a final concentration of 10 nM using Lipofectamine 2000 (Invitrogen) for 4 h in serum-free Opti-MEM I. Gene-specific siRNAs against *H19* and SOX9 were used (Eurofins MWG Operon, Ebersberg, Germany). As a negative control, siRNA against luciferase (Dharmacon) was transfected in parallel. Following transfection, medium was changed

with pre-equilibrated Dulbecco's modified Eagle's medium (in 20% or 1% O_2) containing 10% fetal calf serum, and HACs were incubated in each oxygen environment in a Galaxy® triple gas incubator for 3 days. The same procedure was used to transfect pre-miR-675 (Ambion, Inc./Applied Biosystems, Warrington, UK), antisense oligonucleotides (anti-miR-675) (Exiqon, Vedbaek, Denmark) or a negative control anti-miR microRNA inhibitor (catalog no. 199002-00, Exiqon).

RNA Extraction, Reverse Transcription, and Real-time PCR— Total RNA, including small RNA, was extracted and prepared using the Qiagen miRNeasy kit for both HACs and mouse tissue. RNA from mouse tissue was pooled from 10 mice (Balb/B, male, 5–6-weeks-old), and the procedure was repeated for a further 10 mice.

COL2A1, SOX9, or *H19* and the housekeeping gene *RPLP0* were analyzed using TaqMan technology. cDNA was generated using the Promega kit and random hexamers from 0.5 μg of total RNA. For these genes, predeveloped primer/probe sets were used (Applied Biosystems).

miR-675 PCR amplifications were performed using SYBR Green. 0.1 μg of total RNA (including small RNA) were first polyadenylated and then reverse-transcribed following the manufacturer's instructions (NCode miRNA kit, Invitrogen). miR-675 also was measured using miRNA-specific TaqMan-
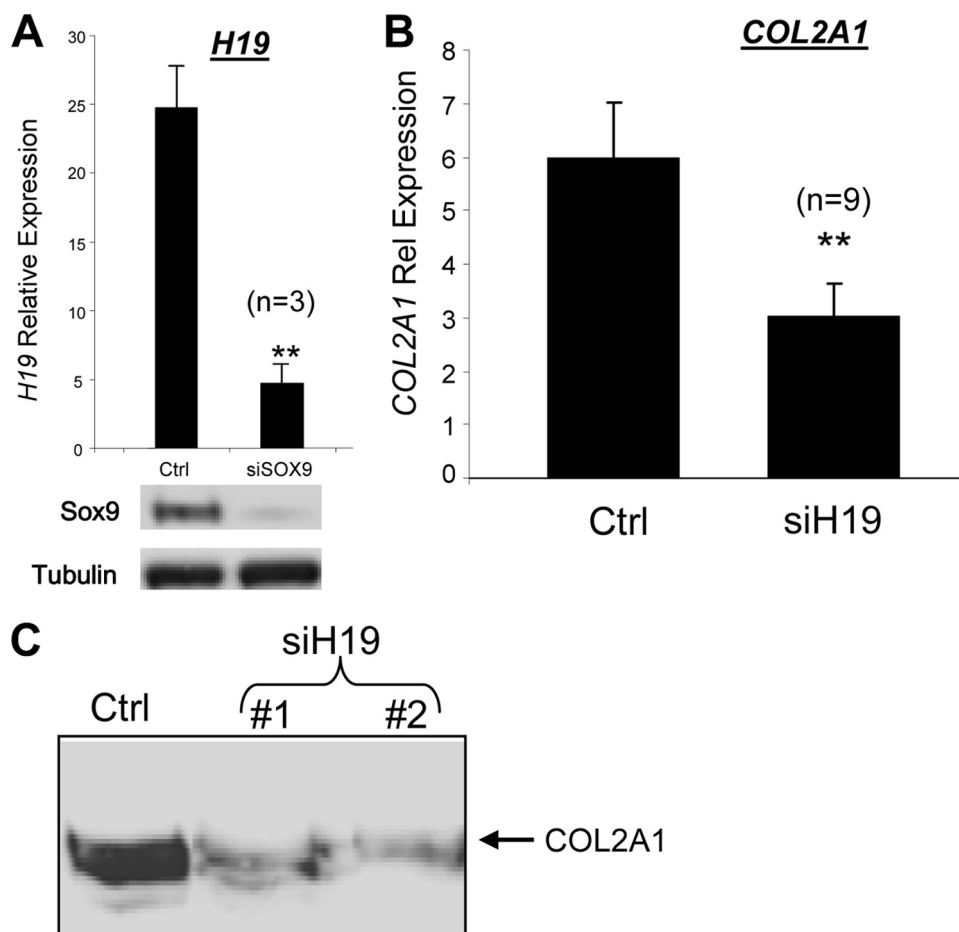
FIGURE 2. **H19 is SOX9-dependent and positively regulates COL2A1 in HACs.** *A*, *H19* levels are significantly reduced by RNA interference-mediated depletion of SOX9 in HACs. (TaqMan PCR analysis, **, $p < 0.01$). *B*, depletion of *H19* in HACs significantly reduced *COL2A1* mRNA levels (**, $p < 0.01$). *C*, depletion of *H19* greatly reduced levels of COL2A1 detectable in the medium of cultured HACs. All experiments were performed in 1% oxygen over a period of 3 days. Gene expression is relative to that of ribosomal protein RPLP0; *Ctrl*, negative control siRNA-targeting luciferase; *si*, small interfering.
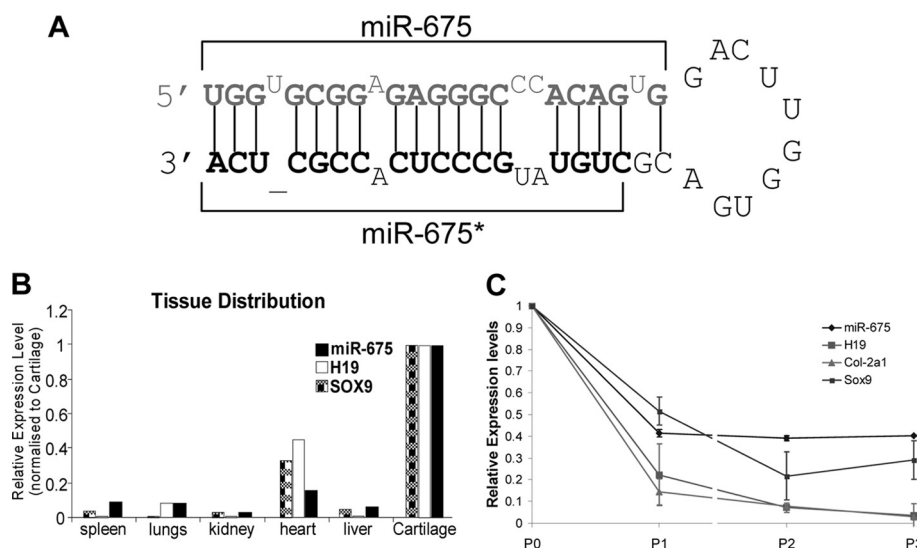

FIGURE 3. **H19 and H19-derived miR-675 are, like SOX9, differentially expressed in articular cartilage.** *A*, miR-675 containing hairpin structure of human *H19*. *B*, real-time PCR data of miR-675, *H19* and *Sox9* levels in mouse spleen, lungs, kidney, heart, liver, and articular cartilage. Data represents two pooled RNA samples, each obtained from ten BalbB mice. Note: Expression levels were arbitrarily set to a value of 1 in cartilage. *C*, during serial passage of primary HACs (which causes dedifferentiation) expression of miR-675 and *H19* followed that of *SOX9* and *COL2A1*, which all showed decreased expression. (Results are the average from three independent experiments.) *P*, passage number. Gene expression is relative to that of ribosomal protein RPLP0.

based stem loop primers. In both cases, the ΔΔCt method of relative quantitation was used to calculate relative transcript levels.

Protein Extraction and Western Blotting—For type II collagen-secreted protein analysis, HACs were serum-starved for 2 days, 24 h after transfection. Approximately 1 ml of conditioned medium was precipitated with trichloroacetic acid, loaded on a 6% PAGE gel and blotted with anti COL2A1 antibody (Chemicon MAB8887, 1:1000). The exact volume of medium used was adjusted after standardization according to total protein content (measured using the Bradford assay) from the cell layer.

Statistical Analysis—Data were compared using one-way analysis of variance with Bonferroni's post hoc test using Prism 4 software (GraphPad). Results are presented as mean ± S.E. $p < 0.05$ was considered statistically significant. *n* values refer to data obtained from individual donor chondrocyte cultures.

RESULTS

Noncoding RNA H19 Is Highly Expressed in Human Articular Chondrocytes, Correlates with the Differentiated Phenotype, Is SOX9-dependent, and Regulates COL2A1—*H19* gene expression levels were measured in primary HACs and were found to be as great as the most abundant cartilage matrix genes, *COL2A1* and aggrecan (Fig. 1*A*, *AGC*). *H19* levels correlated with expression of the differentiated HAC phenotype, *i.e.* levels were high in primary cells, significantly decreased with passage of the cells, which leads to their dedifferentiation, and subsequently increased with hypoxia-induced redifferentiation of the chondrocytes ($p < 0.01$) (Fig. 1*B*) (14).

Unbiased, hierarchical clustering analysis of genome-wide microarray data obtained previously (15) revealed *H19* clusters with the master chondrocyte regulator, transcription factor SOX9 (Fig. 1*C*). Such an extremely tight clustering of both genes through the dedifferentia-

A

Polarised light | Fluorescence

Ctrl

Glo-Anti-miR675

B

C

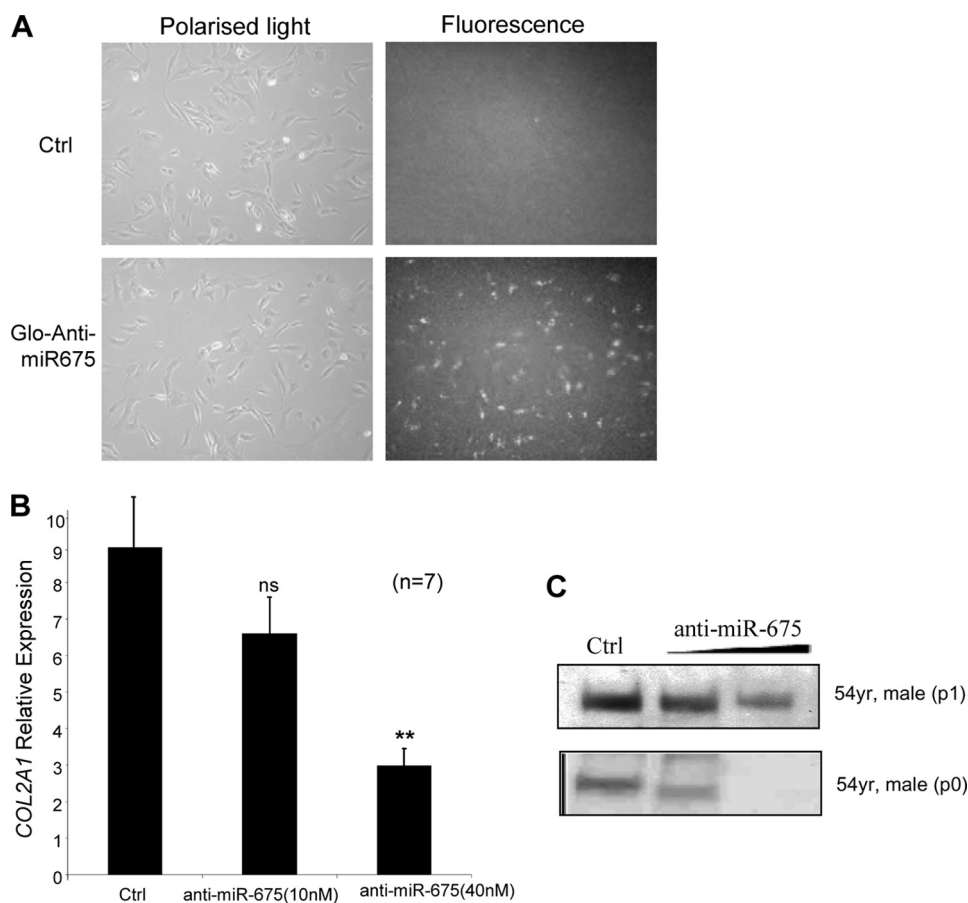
Ctrl | anti-miR-675

54yr, male (p1)

54yr, male (p0)

FIGURE 4. **Inhibition of miR-675 decreases COL2A1 mRNA and secreted protein levels in HACs.** *A*, HACs were successfully transfected with fluorescently labeled anti-miR-675 at 40 nM. *B*, Lipofectamine-mediated transient transfection of anti-miR-675 (40 nM) significantly reduced *COL2A1* mRNA levels in HACs (**, $p < 0.01$), $n = 7$, *i.e.* data were obtained from cultures derived from seven different donors and expressed relative to ribosomal protein RPLP0. *C*, levels of COL2A1 secreted into the culture medium were similarly reduced upon inhibition of miR-675. *p*, passage number. In all experiments, HACs were incubated in 1% oxygen for 3 days before analysis. *Ctrl*, negative control siRNA-targeting luciferase; *ns*, not significant.

To investigate the tissue distribution of miRNA-675, its expression was assessed across a range of murine tissues (male BalbB, aged 5–6 weeks). Its primary transcript *H19* and cartilage-specific *Sox9* also were measured. As can be seen in Fig. 3*B*, miR-675 and *H19* are highly expressed in articular cartilage compared with all other tissue investigated. Their expression pattern also closely follows Sox9, which complements our data from human chondrocytes showing a SOX9 dependence on *H19* expression (Fig. 2, *B* and *C*). In addition, expression of miR-675 and *H19* in HACs mirrored that of *SOX9* and *COL2A1* during dedifferentiation of the cells, all showing decreased expression upon serial passage (Fig. 3*C*).

miR-675 Positively Regulates COL2A1 Expression in Human Articular Chondrocytes—The next step was to assess whether *H19*-derived miR-675 itself was regulating COL2A1. To investigate this, the mature 23-nucleotide miRNA was inhibited (with antisense oligonucleotides) or overexpressed in transient transfection experiments. Transfection efficiency in HACs was high, with no negative effect on cell viability as shown by the use of fluorescently labeled anti-miR-675 sequences (Fig. 4*A*). Like *H19* depletion/redifferentiation process suggested their possible co-regulation. We therefore investigated whether *H19* is regulated by SOX9 by depletion of the latter using RNA interference. As shown in Fig. 2*A*, depletion of SOX9 in HACs greatly decreased *H19* levels ($p < 0.01$). Depletion of *H19* had no effect on SOX9 levels (data not shown).

Having established its SOX9 dependence, we next investigated the effect of *H19* on the chondrocyte phenotype. *H19* depletion in HACs significantly reduced *COL2A1* mRNA levels ($p < 0.01$) (Fig. 2*B*). In addition, levels of COL2A1 secreted into the culture medium were reduced greatly in *H19*-depleted cells (Fig. 2*C*). Two different siRNA oligonucleotides targeting different sequences in the *H19* transcript were used, and both showed a similar effect on COL2A1, suggesting the results are not due to any possible off-target effects of the siRNAs (Fig. 2*C*).

H19 and H19-derived miR-675, Like SOX9, Are Differentially Expressed in Murine Articular Cartilage—The noncoding RNA *H19* is a primary miRNA giving rise to a 23-nucleotide mature miRNA, miR-675, which, before final Dicer cleavage, forms one arm of a hairpin structure (Fig. 3*A*). The so-called "passenger" strand, designated miR-675*, is three nucleotides shorter than miR-675 and contains four mismatches.

tion, miR-675 inhibition significantly reduced *COL2A1* gene expression ($p < 0.01$) (Fig. 4*B*) and secretion of COL2A1 protein into the culture medium (Fig. 4*C*). These effects were detected in both primary and passaged HACs. To minimize the number of cultures needed, the negative control used in these experiments was an siRNA-targeting luciferase because preliminary experiments showed similar results to the negative control anti-miR microRNA inhibitor (Exiqon).

Conversely, overexpression of miR-675 significantly increased COL2A1 mRNA and secreted protein in HACs compared with negative control levels (Fig. 5, *A* and *B*). SOX9 depletion resulted in greatly reduced levels of secreted COL2A1 levels, whereas *H19* depletion similarly reduced COL2A1 levels without altering endogenous SOX9 levels (Fig. 5*C*). This reduction in COL2A1 levels by either SOX9 or *H19* depletion could be rescued completely by concurrent overexpression of miR-675 (Fig. 5*D*). This suggests that miR-675 is mediating the effect of SOX9 and SOX9-regulated *H19* on COL2A1 levels in HACs. Unlike, COL2A1, expression of SOX9-dependent COL9A1 was not affected by depletion of *H19* or overexpression of miR-675 (Fig. 5*E*).
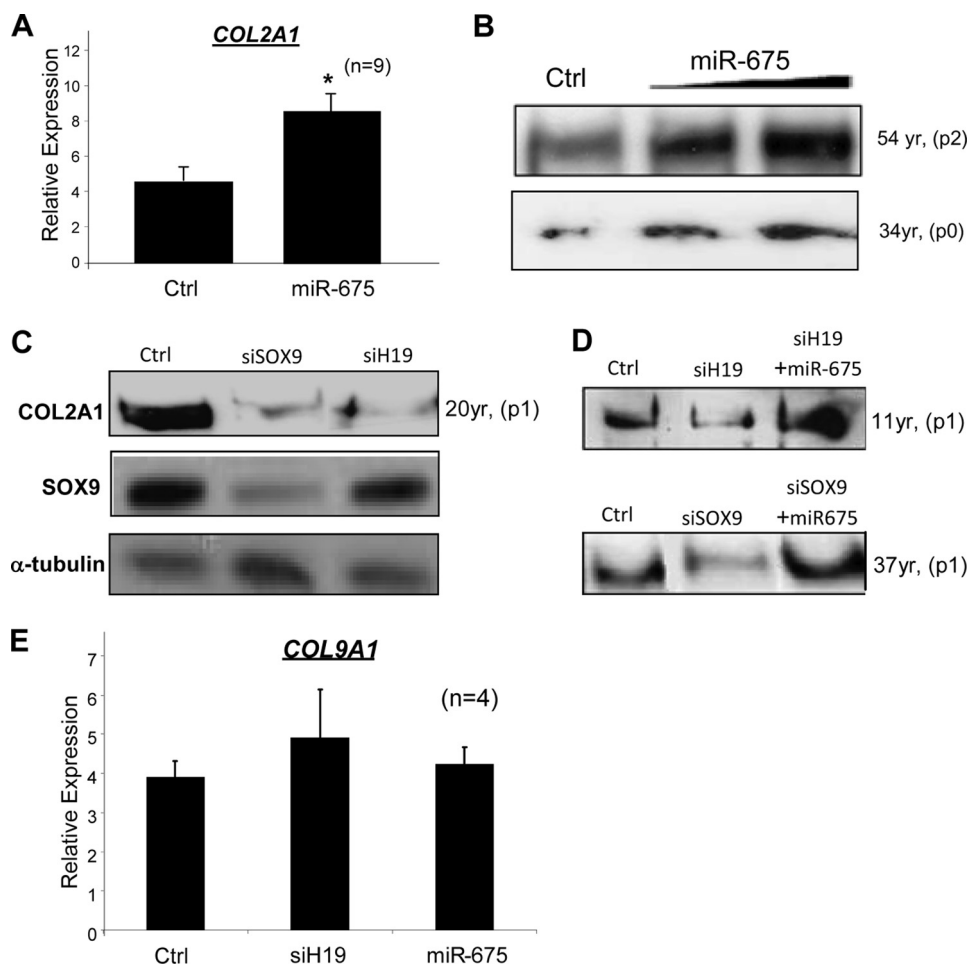
FIGURE 5. **Overexpression of miR-675 up-regulates COL2A1 expression and rescues COL2A1 levels in *H19*- and SOX9-depleted HACs.** Overexpression of miR-675 (10 nM) significantly up-regulates *COL2A1* mRNA (*A*) (*, $p < 0.05$) and secreted protein levels (*B*) in HACs (1 nM and 10 nM of miR-675 used). *H19* depletion resulted in reduced levels of secreted COL2A1, whereas SOX9 was unaffected (*C*). In addition, overexpression of miR-675 (10 nM) rescues COL2A1 levels in *H19*- and SOX9-depleted HACs (*D*). Expression of COL9A1 was not affected by depletion of *H19* or by overexpression of *H19*-derived miR-675 (*E*). In all experiments, HACs were incubated in 1% oxygen for 3 days before analysis. *p*, passage number. Gene expression is relative to that of ribosomal protein RPLP0. *Ctrl*, negative control siRNA-targeting luciferase; *si*, small interfering.

is highly conserved, and *H19* is reported to have been a primary miRNA gene for 148 million years (11), suggesting the mature miRNA may play a fundamental role in *H19* function. In comparing a range of tissues, our data show *H19* is, in fact, differentially expressed in cartilage in young adult mice. Indeed, we also found *H19* was extremely highly expressed in healthy primary human articular chondrocytes (average age of 30 years), surprisingly at comparable levels to that of the most abundant matrix genes *COL2A1* and aggrecan core protein. It is possible that *H19* has a function in chondrocytes in addition to generation of miR-675. However, the rescue of COL2A1 levels in *H19*-depleted HACs by overexpression of miR-675 suggests that regulation of COL2A1 by *H19* is mediated by the mature microRNA, rather than the full-length *H19*.

Transcription factor SOX9 is essential for cartilage formation (20). Here, we provide the first evidence of the SOX9 regulation of a primary microRNA transcript, *H19*. We hypothesize that *H19* is targeted directly by SOX9 because analysis of the human *H19* promoter has revealed two putative SOX9 binding sites in the proximal promoter. Most interestingly, the two sites are paired, *i.e.* staggered by two nucleotides, and arranged in opposite orientation to each other; such paired SOX9 binding sites have been shown in SOX9 target genes (21). Future work will investigate SOX9 recruitment to these putative binding sites in the *H19* promoter in HACs.

In mouse fetal cartilage, Sox9 is thought to directly regulate *Col2a1* through binding to specific enhancer elements in the first intron because mutation of these sites abolishes expression of a COL2A1-driven reporter gene in transgenic mice (20, 22). Binding of SOX9 to similar *COL2A1* enhancer elements *in vitro* also has been demonstrated in human fetal chondrocytes (23). More recently, endogenous binding of Sox9 to *Col2a1* was demonstrated in mouse growth plate chondrocytes (24). We have shown previously that *COL2A1* expression is highly dependent on SOX9 in normal HACs (13). While not discounting the possibility of direct binding, in the present study, we provide evidence (from juvenile and adult HAC experiments) that SOX9 can activate *COL2A1* indirectly via a miR-675 mediated mechanism, because COL2A1 levels can be rescued in SOX9-depleted cells specifically by overexpression of miR-675. Our data in human articular chondrocytes raise the possibility

DISCUSSION

In the present study, we have identified *H19*-derived miR-675 as a highly expressed, cartilage-specific, SOX9-dependent positive regulator of COL2A1, the most abundant and functionally important cartilage matrix protein. Thus, this represents a new mechanism regulating cartilage matrix expression and identifies miR-675 as a potential new target for cartilage repair. *H19* is a non-protein coding, imprinted, maternally expressed gene whose expression is abundant in embryonic tissue of endodermal and mesodermal origin but is thought to be repressed after birth (16, 17). Germ line deletion in mice is not lethal to embryos, suggesting it does not play a key role in development (18). Sequence analysis of H19 suggests this gene is functional, since it appears the RNA is subject to a stabilizing selection (19). Nevertheless, despite extensive study, its role remains unclear. However, important recent work by Cullen and colleagues (10) has shed new light on *H19* and has shown it can function as a primary miRNA transcript, giving rise to the mature miRNA, miR-675. Although the function of miR-675 is unknown, the miRNA-containing region of *H19*

of differences in transcriptional control of cartilage genes between developing cartilage and the permanent articular cartilage. However, it remains to be seen whether miR-675 is specific to articular cartilage or whether it plays a similar role in *COL2A1* regulation in growth plate chondrocytes.

Expression profiling studies by ourselves and others have recently identified altered expression of specific microRNAs in cartilage pathologies such as osteoarthritis (OA) (25, 26). However, the microRNA expression profile is likely to change with disease progression. For example, expression of miR-146 was found to be down-regulated in late stage human OA cartilage compared with normal controls (25) but increased in low-grade (presumably early stage) OA cartilage (27). The specific role and phenotypic effects of these differentially expressed miRNAs remain to be elucidated. Due to its relatively recent discovery (10), miR-675 was not analyzed in these profiling studies; however, it would be most interesting to investigate whether its expression is altered in OA. For example, could aberrant expression of miR-675 explain the reported lack of correlation between SOX9 and *COL2A1* expression in human OA cartilage (28)?

Our results suggest that, in addition to the action of transcription factors and of growth factors and their downstream signaling pathways, miRNAs also exert significant control over the chondrocyte phenotype. In addition, there are most likely complex interactions between these different classes of regulators. For example, miR-199a has been shown to inhibit transcription factor Smad1 by directly targeting it, and growth factor BMP2 can transiently decrease miR-199a levels in chondrocytic cell lines leading to the release of Smad1 inhibition, which, because Smad1 is a key mediator of BMP signaling, allows BMP2-induced chondrocytic differentiation to occur (29). In the present study, we have shown that the master cartilage-specific transcription SOX9, which is thought to drive cartilage matrix gene expression (20), also drives expression of *H19*, a highly abundant primary microRNA transcript, which gives rise to mature miR-675. This miRNA, in turn, positively regulates COL2A1 expression in human articular chondrocytes. Interestingly, transcription of both *H19* and *Col2a1* were reported to be coordinately regulated in an immortalized hamster cell line study (30). Similarly, as HACs are dedifferentiated through serial passage, we observed that *H19* and *COL2A1* levels decrease in an almost identical manner. From our data, we propose a new miRNA-dependent mechanism by which key transcription factor SOX9 can activate *COL2A1* transcription. *In silico* analysis reveals COL2A1 is not a predicted target of miR-675, and because miRNAs most commonly inhibit expression of their direct targets, it may be that *H19*-derived miR-675 is up-regulating COL2A1 by a derepression mechanism, *i.e.* by targeting a COL2A1 transcriptional repressor (Fig. 6). In fact, it is thought that derepression may be an important mechanism by which miRNAs influence cell-specific gene expression (1). Further work is now needed to test this hypothesis, *i.e.* to identify the direct miR-675 targets, and in particular those regulating COL2A1. Interestingly, we have found that miR-675 does not appear to regulate the SOX9-dependent gene COL9A1, indicating that miR-675 does not mediate all of the effects of SOX9. The full phenotypic effects of miR-675 in HACs remain
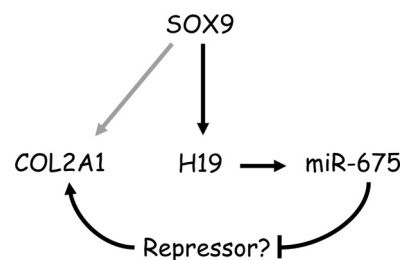

FIGURE 6. **Proposed new mechanism of SOX9 activation of COL2A1 in HACs.** Although evidence exists for direct activation of *Col2a1* by Sox9 in murine growth plate chondrocytes (*gray arrow*), in the present study, we provide evidence for an additional pathway where master cartilage regulator transcription factor SOX9 acts through *H19*-derived miR-675, which up-regulates COL2A1 in human articular chondrocytes. We postulate this may occur by a derepression mechanism, *i.e.* by miR-675 targeting a COL2A1 transcriptional repressor.

to be elucidated. A growing body of evidence suggests that miRNAs are essential for both normal development and tissue and cellular homeostasis (31). In a recent study, miRNA delivery conferred dramatic tumor suppression without toxicity in a hepatic tumor mouse model, thus highlighting the potential therapeutic value of miRNAs (32). Based on our findings, miR-675, by up-regulating the most important cartilage matrix protein COL2A1, offers a means to induce cartilage repair. This exciting possibility first must be tested in an appropriate animal model.

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