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Silencing of anti-chondrogenic microRNA-221 in human mesenchymal stem cells promotes cartilage repair *in vivo*

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST
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ABSTRACT

There is a growing demand for the development of experimental strategies for efficient articular cartilage repair. Current tissue engineering-based regenerative strategies make use of human mesenchymal stromal cells (hMSCs). However, when implanted in a cartilage defect, control of hMSCs differentiation towards the chondrogenic lineage remains a significant challenge. We have recently demonstrated that silencing the anti-chondrogenic regulator microRNA-221 (miR-221) was highly effective in promoting *in vitro* chondrogenesis of monolayered hMSCs in the absence of the chondrogenic induction factor TGF-β.

Here we investigated the feasibility of this approach first in conventional 3D pellet culture and then in an *in vivo* model. In pellet cultures, we observed that miR-221 silencing was sufficient to drive hMSCs towards chondrogenic differentiation in the absence of TGF-β. *In vivo*, the potential of miR-221 silenced hMSCs was investigated by first encapsulating the cells in alginate and then by filling a cartilage defect in an osteochondral biopsy. After implanting the biopsy subcutaneously in nude mice, we found that silencing of miR-221 strongly enhanced *in vivo* cartilage repair compared to the control conditions (untreated hMSCs or alginate-only). Notably, miR-221 silenced hMSCs generated *in vivo* a cartilaginous tissue with no sign of collagen type X deposition, a marker of undesired hypertrophic maturation. Altogether our data indicate that silencing miR-221 has a pro-chondrogenic role *in vivo*, opening new possibilities for the use of hMSCs in cartilage tissue engineering.

INTRODUCTION

Adult articular cartilage does not heal spontaneously after injury and surgical repair remains a significant clinical challenge with few and sub-optimal therapeutic options [1]. Mesenchymal stromal cells (hMSCs) have been identified as an attractive cell source for cartilage regeneration due to their chondrogenic potential [2-5]. Many studies demonstrating that cartilage tissue can be created from hMSCs have paid special attention to growth factors that are involved in promoting chondrogenesis. These growth factors, particularly the members of the transforming growth factor beta (TGF-β) family, induce hMSCs to acquire a chondrogenic phenotype, and synthesize specific extracellular matrix proteins such as collagen type II and aggrecan [6-8].

The use of TGF-β, however, revealed contradictory findings and undesired off target effects on the synthesis and functionality of cartilage matrix components. In fact, during chondrogenesis release of high levels of TGF-β may drive progenitor cells to become hypertrophic or induce fibrosis [9, 10]. The presence of TGF-β during chondrocyte proliferation may be detrimental for the re-differentiation process and may promote the rapid and undesirable differentiation into fibroblast-like cells [11]. Additionally, recent studies have demonstrated that TGF-β signaling plays a critical role in chondrocytes, MSCs and synovial lining cells during the development and progression of osteoarthritis (OA), one of the most common joint diseases [12]. This emerging body of evidence has stimulated researchers to pay special attention to feasible alternatives, including inhibition of specific TGF-β signaling pathways, to achieve sustained and long-term repair, or reduce degeneration of articular cartilage [9, 13-16].

Alternatively, cartilage regeneration protocols may rely on deploying morphogenetic signals of developmental pathways or removing potentially anti-chondrogenic factors. As part of this effort, we previously demonstrated that silencing of two anti-chondrogenic regulators, Slug transcription factor and miR-221, induced the expression of chondrogenic markers in hMSCs cultured in monolayer without TGF-β [17]. This prompted us to investigate whether these silenced cells, when properly organized in a three-dimensional environment, could be suitable to trigger the repair process of damaged cartilage tissue *in vivo*. The aim of this study was to evaluate the chondrogenic potential of miR-221 silenced hMSCs in a three dimensional environment, without exposure to chondrogenic induction media containing TGF-β. The efficacy of miR-221 depletion to induce neocartilage formation and prevent terminal differentiation was investigated in conventional 3D *in vitro* culture and in an *in vivo* system represented by a well-established osteochondral culture model [18-20]. The experimental approach based on miR-221 silencing is in accordance with recent literature demonstrating the effectiveness of the direct targeting of chondrogenic regulators to

induce cartilage repair or prevent cartilage degeneration [21-26].

We found that silencing of miR-221 promoted chondrogenesis in 3D pellets cultured without TGF-β, a condition that normally does not lead to cartilage formation. Moreover, we demonstrated that miR-221 depleted hMSCs guided the formation of cartilage tissue *in vivo*, and had a reduced tendency to undergo terminal differentiation. This demonstrates that miR-221 silencing can be sufficient to promote chondrogenesis of hMSCs *in vitro* and *in vivo*, thereby having potential for therapeutic applications.

MATERIALS AND METHODS

hMSCs cultures

hMSCs were isolated from two sources, Wharton's jelly of umbilical cords and bone marrow. Human umbilical cords (all from natural deliveries) were collected after mothers' consent and approval of the Ethics Committee of the University of Ferrara and S. Anna Hospital (protocol approved on November 19, 2006). Harvesting procedures of Wharton's jelly from umbilical cord were conducted in full accordance with the Declaration of Helsinki, as adopted by the 18th World Medical Assembly in 1964 and successively revised in Edinburgh (2000), and the Good Clinical Practice guidelines. Cords were processed within 4 hours and stored in sterile saline until use [27]. Typically, the cord was rinsed several times with sterile phosphate-buffered saline (PBS) before processing and cut into pieces (2–4 cm in length). Blood and clots were drained from vessels with PBS to avoid any contamination. Single pieces were dissected, after separating the epithelium of each section along its length, to expose the underlying Wharton's jelly. Subsequently, cord vessels were pulled away and the soft gel tissue was finely chopped. The same tissue (2–3 mm^2 pieces) was placed directly into 75 cm^2 flasks in expansion medium (10% Fetal Calf Serum (Euroclone S.p.A., Milan, Italy), Dulbecco's Modified Eagle's Medium (DMEM) low-glucose supplemented with 100 µg/mL penicillin and 10 µg/mL streptomycin), at 37°C in a humidified atmosphere of 5% CO_2. After 5–7 days, the culture medium was removed and then changed twice a week. At subconfluence, cells were trypsinized, and thereafter expanded and used at passage 3 or 4 for *in vitro* experiments of pellet culture.

hMSCs from bone marrow were obtained from femoral biopsies of donors (age 50–78 years) undergoing total hip replacement, after signed informed consent and with approval of the local ethical committees (Erasmus MC number MEC-2004-142; Albert Schweizer Hospital number 2011.07). Cells from bone marrow aspirates were seeded at a density of approximately 50,000 nucleated cells/cm^2 in expansion medium (10% Fetal Calf Serum, alpha-MEM (GIBCO, Rockville, MD, USA) supplemented with 1 ng/mL FGF2 (AbD Serotec, Oxford, UK), 25 µg/mL ascorbic acid-2-phosphate (Sigma-Aldrich, Saint Louis, MO, USA), 1.5 µg/mL fungizone, and 50 µg/mL gentamicin). Non-adherent cells were washed off after 24 h, and adherent cells were further expanded. At subconfluence, hMSCs were trypsinized and replated at a density of 2,300 cells/cm^2. Medium was refreshed twice a week and expanded cells at passage 3 or 4 were used for the experiments.

Transfections

hMSCs from Wharton's jelly or bone marrow were transfected with 10 nM antagomiR-221 or a non-relevant antagomiR (antagomiR-Scr). For all transfections, Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA) was used as delivering agent (0.43 µL/mL of culture medium), by combination with the oligonucleotides for 20 min at RT. Monolayered hMSCs were transfected twice, the day after the plating and again after 3 days. The transfected cells were cultured in expansion medium without FGF2, at 37°C in a humidified atmosphere of 5% CO_2, then detached and used for *in vitro* or *in vivo* experiments.

Pellet culture of hMSCs

To provide a suitable microenvironment for chondrogenic differentiation, 2.5 x 10^5 hMSCs from the Wharton's jelly of three different donors were transfected with antagomiR-221 or antagomiR-Scr, seeded in 15 ml-polypropylene conical tube, and centrifuged for 5 min at 500g to form a 3D pellet. The supernatant was removed and replaced with DMEM high-glucose supplemented with ITS+Premix: 6.25 µg/mL insulin, 6.25 µg/mL transferrin, 5.33 µg/mL linoleic acid, 1.25 µg/mL bovine serum albumin, 100 nM dexamethasone, 50 µg/ml ascorbate-2 phosphate, 1 mM sodium pyruvate, 100 µg/mL penicillin and 10 µg/mL streptomycin (Sigma-Aldrich), in the absence of conventional chondrogenic inducers (TGF- β). Simultaneously, untransfected hMSCs were cultured as pellets as described above, with or without 10 ng/mL TGF-β3 (Miltenyi, Bergisch, Gladbach, Germany): these conditions were named "TGF-β" and "untreated", respectively. All pellet cultures were maintained at 37°C, 5% CO_2 up to 21 days and the medium was refreshed twice a week. At the chosen time-points (see Fig. 1A), pellets were either disrupted with a pellet pestle and processed for RNA isolation or fixed in 4% PFA, embedded in paraffin, sectioned and processed for immunohistochemistry.

Osteochondral culture model

Osteochondral defects were created in bovine osteochondral biopsies, as previously described by de Vries-van Melle and colleagues [19]. Osteochondral biopsies that were 8 mm in diameter and 5 mm in length were produced using a diamond-coated trephine drill (Synthes, Oberdorf, Switzerland) from the four proximal sesamoid bones of fresh metacarpal phalangeal joints of 3 to 8 month-old calves. Biopsies were incubated overnight in 10% Fetal Calf Serum DMEM high-glucose supplemented with 1.5 µg/mL fungizone and 50 µg/mL gentamicin to verify sterility. Using a 6 mm-diameter dermal biopsy punch (Stiefel Laboratories, Durham, NC, USA) and a scalpel, osteochondral defects were created: the cartilage and calcified cartilage layers were removed completely and parts of the subchondral bone were damaged by scraping the surface with the

scalpel. To prevent outgrowth of cells from the subchondral bone, biopsies were placed in 2% low-gelling agarose (gelling temperature 37-39°C; Eurogentec, Liege, Belgium) in physiological saline solution, in such a way that the subchondral bone was surrounded by the agarose and the cartilage was above the agarose surface.

Culture of hMSCs in the osteochondral model *in vitro*

To combine the use of transfected hMSCs with the osteochondral model, a specific protocol was optimized. Monolayered hMSCs from bone marrow of three different donors were transfected with antagomiR-221 or antagomiR-Scr as described above, then trypsinized at day 7 and resuspended in 1.2% low viscosity alginate (Keltone, San Diego, CA, USA) in physiological saline solution, at a density of 12.5 x 10^6 cells/mL. Simultaneously, 40 µL of alginate cell suspension and 60 µL of 102 mM $CaCl_2$ were added to the simulated osteochondral defects, enabling *in-situ* gelation. To evaluate a possible effect of alginate entrapment and the osteochondral microenvironment on cell viability and efficiency of gene silencing, the hMSCs/alginate constructs were maintained *in vitro* in the presence or absence of the osteochondral biopsies for 4 weeks. The alginate constructs were then harvested and assayed for cell viability. Alternatively, the constructs were dissolved in 450 µL of 55 mM sodium citrate (Sigma-Aldrich) in 20 mM ethylene diamintetraacetate (EDTA, Sigma-Aldrich), incubated at 4°C and subsequently centrifuged for 8 min at 1,200 rpm. The supernatant was removed, the samples were washed twice with PBS and then processed for RNA isolation as reported below.

In vivo implantation of osteochondral biopsies with hMSCs

hMSCs from bone marrow of three donors, either left untreated or transfected with antagomiR-221, were resuspended in 1.2% low viscosity alginate (Keltone) at a density of 25 x 10^6 cells/mL, and the cells suspension was solidified in the osteochondral defects, as described above. Alginate without cells was solidified in osteochondral defects as negative control condition. Biopsies were cultured overnight to allow stabilization of the system. The osteochondral biopsies were implanted subcutaneously on the back of 10 to 14 week old female NMRI nu/nu mouse (Charles River, Wilmington, MA, USA) under isofluorane anesthesia. For each hMSCs donor, three osteochondral biopsies per mice were implanted (duplicate samples, hence two mice per donor) in such a way that the three different conditions (alginate, untreated hMSCs and antagomiR-221 treated hMSCs) were present in the same animal. The osteochondral biopsies were covered using an 8 mm-diameter Neuro-Patch membrane (Braun, Melsungen, Germany) to prevent in-growth of host cell/tissue. Before surgery and 6 h after surgery, mice received 0.05 mg/Kg bodyweight of Temgesic (Reckitt

Benckiser, Slough, UK). During surgery, mice received 9 mg/Kg bodyweight of Ampi-dry (Dopharma, Raamsdonksveer, The Netherlands). After 12 weeks, mice were euthanized by cervical dislocation and the osteochondral biopsies were explanted and fixed in 4% formalin. After 1 week of fixation, biopsies were decalcified using 10% formic acid for 2 weeks and subsequently embedded in paraffin, sectioned and subjected to histological evaluation. Animal experiments were conducted in the animal facility of the Erasmus MC with approval of the local animal ethics committee (under protocol number 116-14-02) according to the national animal act (EMC 2429).

Cell viability

A viability assay was performed by double staining with calcein-AM and propidium iodide (PI) using the LIVE/DEAD® Viability/Cytotoxicity Kit (Invitrogen), according to the manufacturer's instructions. The cells were visualized under a fluorescence microscope: dead cells were stained in red, whereas viable ones appeared in green. For the evaluation of cell viability, calcein-AM- and PI-positive cells in representative hMSCs/alginate and hMSCs/alginate/plug constructs were counted, and viability was expressed as % of living cells in the constructs (five fields per replicate, two replicates).

RNA isolation and Quantitative Real-Time PCR analysis

Total RNA, including miRNAs, was extracted from hMSCs using the RNeasy Micro Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. RNA concentration and quality was measured using a NanoDrop ND1000 UV-VIS spectrophotometer (Isogen Life Science, de Meern, the Netherlands). cDNA was synthesized from total RNA in a 20 µl reaction volume using the TaqMan MicroRNA Reverse Transcription kit (Life Technologies, Carlsbad, CA, USA) for analysis of microRNAs, or the TaqMan High Capacity cDNA Reverse Transcription kit (Life Technologies) for analysis of mRNAs.

Quantification of miR-221-3p and miR-222-3p was performed using the TaqMan MicroRNA Assays (Life Technologies), using U6 snRNA for normalization. For the quantification of collagen type X, alkaline phosphatase (ALP) and matrix metalloproteinase 13 (MMP13) mRNA, the appropriate TaqMan Assays were purchased (Life Technologies); for the quantification of lubricin mRNA, the primers reported in [28] were used. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used for normalization of mRNA abundances. Polymerase chain reactions were performed with the TaqMan Universal PCR MasterMix (Applied Biosystems) or SYBR Green MasterMix (Fermentas), and using the CFX96TM PCR detection system (Bio-Rad, Hercules, CA, USA). Relative gene expression was calculated using the comparative $2^{-\Delta Ct}$ method.

Histology and immunohistochemistry

Histological sections of hMSCs osteochondral constructs (6 μm) were stained with 0.4% thionine solution (Sigma-Aldrich) in demineralized water to detect glycosaminoglycans (GAGs). Sections of hMSCs pellets (5 μm) or osteochondral constructs were subjected to immunohistochemistry. To this aim, non-consecutive sections were immunostained with primary antibodies against the cartilage matrix proteins collagen type II (mouse anti-human, 1:100 dilution, Abcam, Cambridge, UK; mouse anti-human, 1:100 dilution, II-II/II6B3, Developmental Studies Hybridoma Bank, University of Iowa) and collagen type X (mouse anti-human, 1:25 dilution; Quartett, Germany), or the chondroregulatory transcription factors Sox9 (rabbit anti-human, 1:100 dilution; Santa Cruz Biotechnology, CA, USA), TRPS1 (rabbit anti-human, 1:100 dilution; Abcam) and Slug (mouse anti-human, 1:100 dilution; OriGene, Rockville, MD, USA). Histological sections were deparaffinized, rehydrated and enzymatic treated with 1 mg/mL pronase (Sigma-Aldrich) in PBS 1X or 0.1% pepsin (Boehringer Mannheim, Germany) in 0.5 M acetic acid pH 2.0 for collagen type X, followed by treatment with 10 mg/mL hyaluronidase (Sigma-Aldrich) in PBS 1X for antigen retrieval. In the case of the staining for collagen type II of the sections of the osteochondral constructs, the primary antibody was pre-incubated overnight with a goat anti-mouse biotin-conjugated antibody (#115-066-062; Jackson ImmunoResearch Europe, Amsterdam, The Netherlands) to prevent cross-reaction with mouse antigens. Excessive primary antibody was captured by addition of 0.1% normal mouse serum prior to the overnight incubation at 4°C with the sections. Alternatively, slides were incubated overnight with the primary antibodies at 4°C and with the secondary antibody (link; 4plus Universal AP Detection, Biocare Medical, Concord, CA, USA) for 10' at RT. Alkaline phosphatase-labeled antibodies were then used (HK321-UK, Biogenex, Fremont, CA, USA; 4plus Universal AP Detection, Biocare Medical) in combination with the Vulcan Fast Red Chromogen Kit (Biocare Medical) or Neu Fuchsine substrate, resulting in a red staining. An isotype IgG1 monoclonal antibody was used as negative control. The sections were counterstained with hematoxylin and eosin and mounted in glycerol. The stainings were quantified by a computerised video camera-based image analysis system (NIH, USA ImageJ software, public domain available at: *http://rsb.info.nih.gov/nih-image/*) under brightfield microscopy (Nikon Eclipse 50i; Nikon Corporation, Tokyo, Japan). For the analysis of sections obtained from hMSCs pellets, the positive immunostaining was expressed as % of positive area of the pellet (three replicates per donor were acquired; n = 3). For the analysis of sections obtained from the osteochondral samples, thionine positivity and immunostaining for matrix proteins (collagen type II, collagen type X) in the area of the defect was expressed as % of positive area of the osteochondral defect (two replicates per donor; n = 3 for thionine and collagen type II, n = 2 for

collagen type X); for the analysis of transcription factors (Sox9, TRPS1), positive cells in the area of the defect were counted and protein levels were expressed as % of positive nuclei (five fields per replicate, two replicates per donor; n = 3).

Statistical analysis

The normal distribution of data was verified using the Kolmogorov-Smirnov test. In the case of single comparison, statistical significance was determined by paired Student's t-test for normally distributed data and Wilcoxon matched-pairs signed-ranks test for non-normally distributed data. In the case of multiple comparisons, statistical significance was analyzed by one-way analysis of variance (ANOVA) and Bonferroni post hoc test if the values followed a normal distribution, or by Kruskal–Wallis analysis (nonparametric one-way ANOVA) and Dunn's post hoc test if the values were not normally distributed. For all statistical analysis, differences were considered statistically significant for p-values ≤ 0.05.

RESULTS

miR-221 silenced hMSCs Spontaneously Undergo Chondrogenic Differentiation in Pellet Culture

The effect of miR-221 silencing on chondrogenic potential was evaluated in pellets formed by hMSCs from Wharton's jelly in the absence of the chondrogenic inducer TGF-β for 21 days (Fig. 1A). We confirmed here the previous evidence about the downregulation of miR-221 by TGF-β [17] (Fig. 1B). Interestingly, antagomiR-221 treatment was highly effective in miR-221 knockdown up to 14 days of pellet culture, achieving >95% inhibition of miR-221 expression with respect to untreated cells. After 21 days of pellet culture, the effect of antagomiR-221 was still appreciable (~60% silencing), with a residual level of miR-221 comparable to the TGF-β treated cells. This evidence allowed us to compare the effects of two different stimuli that lead to a comparable down-regulation of miR-221 expression. Considering that miR-221 is a paralog of miR-222, the effect of antagomiR-221 on miR-222 levels was also evaluated (Fig. S1). Before pellet formation (day 0), we observed a significant down-regulation of miR-222 by antagomiR-221 treatment. However, the residual levels of miR-222 were higher than those of miR-221, with a ~450-fold difference (4.51% vs 0.01%). At day 7 of pellet culture, the expression of miR-222, unlike miR-221, was strongly recovered (38.41% vs 1.46%), while at day 21 miR-222 was unaffected by antagomiR-221 treatment (Fig. S1). Overall, these data indicate that the effects due to a long exposure to the treatment can be mainly attributed to the silencing of miR-221.

Histological analysis performed on sections from day-21 pellets revealed a comparable positive staining for collagen type II in the antagomiR-221 treated and TGF-β treated cells, (Fig. 2A), and, accordingly, a strong Alcian Blue staining for GAGs (data not shown). Collagen type X was not detectable on immunohistochemistry (Fig. 2A) nor on mRNA expression (data not shown), indicating that these cells in the pellets were not prone to undergo hypertrophic differentiation. Notably, antagomiR-221 treatment was more effective than TGF-β in inducing the expression of the pro-chondrogenic Sox9 and TRPS1 transcription factors (Fig. 2B) [8]. In agreement with previous data [17], antagomiR-221 treatment, unlike TGF-β, maintained Slug protein at very low levels, a favorable condition for the chondrogenic process (Fig. 2B). These results indicate that miR-221 silencing in hMSCs in pellet culture is effective and sufficient to induce chondrogenesis, in the absence of exogenously added growth factors. The effect of antagomiR-221 treatment was also confirmed in hMSCs from bone marrow (Fig. S2 and S3), allowing us to strengthen the starting hypothesis about the effectiveness of silencing miR-221 on chondrogenic induction. Interestingly, in this case the known TGF-β dependent up-regulation of collagen type X was avoided by antagomiR-

221 treatment, as revealed by immunohistochemistry (quantification of the positive area: 24.0% vs 1.8% at day 21, and 64.5% vs 2.8% at day 28) and qRT-PCR analysis. Two additional hypertrophic markers, alkaline phosphatase (ALP) and matrix metalloproteinase 13 (MMP13), were found down-regulated, further confirming the reduced tendency of miR-221 depleted hMSCs to undergo hypertrophy.

miR-221 silenced hMSCs Are Effective in Regenerating Cartilage *in vivo*

To validate the anti-chondrogenic role of miR-221 *in vivo*, hMSCs were cultured in an osteochondral microenvironment represented by a cartilage defect model [18-20] (Fig. 3A). We first evaluated whether the osteochondral model could influence cell viability and silencing efficiency, by culturing hMSCs/alginate and hMSCs/alginate/plug constructs *in vitro* (Fig. 3B). We showed that cell viability was not affected for at least 4 weeks, as demonstrated by the high percentage (>85%) of Calcein-AM-positive cells in the constructs. Furthermore, antagomiR-221 treated cells maintained very low levels of miR-221 expression in both conditions (~75% silencing with respect to untreated cells), thereby demonstrating that our experimental setting was suitable for culturing miR-221 depleted hMSCs *in vivo*. For *in vivo* experiments, hMSCs/alginate/plug constructs with miR-221 depleted or untreated cells were then implanted subcutaneously in nude mice (Fig. 3B). After 12 weeks, miR-221 silenced hMSCs generated a tissue characterized by extensive production of glycosaminoglycans, as evidenced by the presence of a large thionine positive area throughout the entire region of the defect (~37% compared to 12% in control condition; Fig. 4A). This was different from the newly formed tissue localized in small spots observed when untreated hMSCs were used, and the absence of cartilage formation in defects that were filled with alginate without hMSCs (Fig. 4A). Consistent with these observations, immunohistological analysis revealed a significantly stronger staining for the chondrogenic markers collagen type II (matrix protein), Sox9 and TRPS1 (transcription factors) in the osteochondral defects filled with miR-221 silenced hMSCs, compared to the osteochondral defects filled with untreated hMSCs (Fig. 4B, C and Table 1).
The expression of collagen type X was close to detection limit in two out of three hMSCs donors (Fig. 4D and Table 1). These data confirmed the *in vitro* results, showing again that an increased deposition of collagen type II is not correlated with an increased deposition of collagen type X after antagomiR-221 treatment.

DISCUSSION

Repair of damaged cartilage remains a major clinical challenge that may rely upon the development of innovative technologies, including regenerative strategies based on the use of hMSCs. Different hMSCs application modalities have been described, some in combination with bio-inspired smart biomaterials and growth factors, to provide better targeted tissue regeneration. However, the optimal strategy has not yet been identified. Much remains to be investigated, such as which hMSCs molecular signaling supports chondrogenic potential, and which culture methods improve hMSCs chondrogenic differentiation before implantation. In the present study, we demonstrated that miR-221 depleted hMSCs are able to spontaneously undergo chondrogenesis in pellet culture *in vitro* and form cartilage *in vivo*, without requiring growth factor supplementation. We showed that maintaining low levels of a negative factor by silencing is an effective alternative to induce chondrogenic differentiation, compared to standardized procedures primarily based on TGF-β treatment.

In recent years, many researchers are moving to investigate specific sets of culture conditions which attempt to resemble the physiological environment. In the natural milieu, chondrogenesis goes through a complex differentiation program with production of specific matrix components, due to exposure to a combination of factors with a tight spatiotemporal regulation. Previous molecular studies have shown that mesenchymal progenitors can be stimulated to adopt a chondrogenic fate by TGF-βs [5, 6]. We here confirmed our previous observation that one of the effects of TGF-β is the down-regulation of miR-221, but not its complete suppression [17]. Therefore, the pro-chondrogenic activity of TGF-β may be counteracted by anti-chondrogenic factors such as miR-221, that still persist in the TGF-β enriched environment. Conversely, hMSCs cultured with antagomiR-221 are able to undergo chondrogenesis avoiding the expression of undesired proteins such as Slug or collagen type X. Therefore, our findings suggested the silencing of miR-221 as a new tool to repair a cartilage defect.

We used an *in vivo* approach with subcutaneous implantation in mice of a recently developed osteochondral defect model [18-20]. This tool proved the enhanced potential of engineered hMSCs to regenerate articular cartilage in a microenvironment similar to that found in damaged cartilage. Previously, implantation of osteochondral biopsies containing hMSCs in osteochondral defects demonstrated the importance of the subchondral bone for the synthesis of cartilage repair tissue, and the contribution of soluble factors others than those of the TGF-β family [18-20]. In the present work, the results obtained *in vitro* were validated by the *in vivo* experiments. Indeed, implantation of osteochondral biopsies with miR-221 silenced hMSCs resulted in significantly more

cartilaginous repair tissue compared to the use of non-engineered hMSCs. miR-221 depleted hMSCs promoted a differentiation program that led to the expression of genes required for hyaline chondrogenesis, such as collagen type II and the transcription factors Sox9 and TRPS1. Moreover, one of the most interesting aspect of our results was the observation that the newly formed *in vivo* tissue was characterized by an ECM that was negative for collagen type X. It is well known that the expression of collagen type X, a marker for chondrocyte hypertrophy and apoptotic death, is an undesired outcome, and remains an unresolved issue in the cell-based approach for cartilage regeneration [29, 30]. Therefore, the ability of miR-221 depleted hMSCs to downregulate collagen type X, as well as other hypertrophic genes such as ALP and MMP13, represents a crucial event during the formation of cartilaginous repair tissue, and a promising approach for the application of hMSCs in the repair of articular cartilage defects.

An unequivocal characterization of the participation of donor cells in the neoformation/repair tissue is technically challenging, raising the question about the kind of phenomenon that supports the formation of neo-cartilage in our *in vivo* model. Several explanations on the regeneration process we observed *in vivo* may be postulated: i. at an early stage, the silenced hMSCs produce cartilage-like matrix which subsequently guides the resident cells towards chondrogenic differentiation and production of cartilage components; ii. the chondrogenic properties of the silenced hMSCs are maintained over time thanks to a sort of autocrine circuit; iii. our 3D *in vivo* setting provides a favorable microenvironment to promote the survival and maintenance of the chondrogenic phenotype of individual or groups of silenced hMSCs; iv. pro-chondrogenic growth factors are expressed endogenously by the resident cells in response to trophic factors secreted by the implanted cells (paracrine effect). Although the exact mechanism cannot be deduced from our current experiments, the cartilage formation process observed after implantation of miR-221 silenced hMSCs is very promising for further research and applications.

It is important to underline that silencing experiments in pellet cultures were here conducted initially with hMSCs from Wharton's jelly, to validate our hypothesis with the same cell source previously used in monolayer culture [17]. Due to the need for a higher number of cells for the *in vivo* experiments, we then moved to the use of hMSCs from bone marrow. By doing so, we further confirmed the pro-chondrogenic effect of miR-221 silencing, and we also showed that antagomiR-221 treatment was able to induce the expression of the cartilage ECM protein lubricin [31]. In general, the use of hMSCs from different sources allowed us to prove the validity of our hypothesis, concerning the key role of miR-221 as an anti-chondrogenic factor involved in a common regulatory pathway associated with cell fate.

Currently, validated data on the role and mechanism of action of miR-221 during the chondrogenic

process, as well as in diseased cartilage, are limited. Putative targets of miR-221 have been recently proposed by a miRNA signature which regulates the chondrogenic mechanism in unrestricted somatic stem cells [32]. miR-221 has been also postulated to be a mechanically responsive miRNA in chondrocytes, being highly expressed in weight bearing compared with non-weight bearing regions of bovine articular cartilage [33, 34]. Moreover, miR-221 expression has been correlated with age, and its low levels have been associated with an increase in phosphatidylinositol 3-kinase signaling, a pathway shown to be implicated in OA [35].

Within the group of the main predicted and experimentally validated targets of miR-221 and its paralog miR-222, we highlighted the genes known to have a role in cartilage-related pathways, and thus worth pursuing as potential miR-221/222 chondro-targets (Table S1). These two miRNAs are encoded by a gene cluster, have the same seed sequence and thus share common predicted target genes. miR-221, as many other miRNAs, can control cellular differentiation and may function through different mechanisms depending on the tissue microenvironment. Therefore, it is not surprising that the list in Table S1 includes genes such as TIMP, FOXO3, DKK2, DVL2, MEOX2, PGC1α, SEMA3B, STMN1, MDM2, TRPS1, RECK, ICAM1, FOS, DICER1, ETS1, and ESR1 (references reported in Table S1), which are involved in chondrogenesis and cartilage biology. Among these genes, only cyclin-dependent kinase inhibitor 1B (p27) and mouse double-minute 2 homolog (MDM2) have been experimentally validated as relevant miR-221 chondro-targets [36, 37]. In fact, in bovine cartilage and isolated chondrocytes, Yang and coauthors demonstrated that miR-221 mimic suppressed the expression of p27 leading to the stimulation of chondrocyte proliferation [36]. Down-modulation of MDM2 by miR-221 prevented the degradation of Slug protein, which negatively regulates the proliferation of chondroprogenitors during chondrogenesis of chick limb mesenchymal cells [37]. Consistent with this last evidence, we demonstrated here a very low level of Slug protein in antagomiR-221 treated hMSC cultures, supporting the hypothesis that miR-221 may act through MDM2 to prevent cartilage ECM synthesis.

Hypothesized mechanisms for miR-221 action in the chondrogenic process have been illustrated in Fig. 5 and S4. We suggest that antagomiR-221 treatment promotes chondrogenic processes since it leads to a weakening of the negative control that Slug exerts on chondrogenic factors, such as Sox9 and TRPS1, and cartilage ECM proteins, such as collagen type II and aggrecan (Fig. 5). Although Slug is not a direct target of miR-221 [17], it might act as an effector in a circuit that we hypothesize to end with a reciprocal negative regulation between TRPS1 and miR-221. Accordingly, we demonstrated here an increase in TRPS1 expression after miR-221 depletion and, previously, the ability of TRPS1 to strongly inhibit miR-221 expression [17]. Since TRPS1 acts as a repressor of Runx2 function [38], the increased TRPS1 expression may contribute to repress the

Runx2-mediated transactivation of genes associated with cartilage hypertrophy and ECM degradation, such as collagen type X, alkaline phosphatase (ALP) and matrix metalloproteinases (MMPs).

It is also conceivable that Sox9, collagen type II and aggrecan are direct miR-221 targets. These genes do not show sequences homologous to miR-221 seed region in their 3'-UTR. However, increasing evidence demonstrates that targeting can also be mediated through sites others than the 3'-UTR, and that seed region base pairing is not necessarily required [39]. Accordingly, a high-throughput screening of a human 3'-UTR library has recently shown that Sox9 is indeed a candidate target gene of miR-221 [40]. In addition, the roles of TGF and Wnt signaling in regulating cell fate during differentiation [41, 9] suggest other scenarios possibly correlated with miR-221 action (Fig. S4). Specific modulation of those factors lead to the production of a stable cartilage phenotype, where hypertrophy is repressed. In light of the results herein reported, we postulate that molecules involved in the TGF/Wnt pathways and regulators of cell cycle and proliferation might be targets of miR-221, thereby affecting the cell fate and the ECM produced by the chondrocytes (Fig. S4).

Novel insight onto the mechanisms by which miR-221 works and its interplay with regulatory networks may come from microarray analysis and non-invasive technologies, such as *in vivo* molecular imaging, that could be applied to our model [42, 43]. In this perspective, our results demonstrating for the first time the critical role of miR-221 in the control of chondrogenesis *in vivo,* strengthens the need to further investigate miR-221 targets, and offers a new model to apply the aforementioned techniques. We are aware that the demonstration of the effectiveness of silencing anti-chondrogenic factors in MSCs for cartilage repair, does not solve the challenges of creating an environment conducive for long term tissue survival, or restoring functional cartilage. For this purpose, future experiments will also be aimed at assessing the mechanical properties of our constructs, to better define the quality of neo-cartilage that has been formed following the approach herein described. In any case, our results provide a proof of concept for developing experiments which would allow measurement of function restoration, using large animal models of critical size osteochondral defects.

CONCLUSIONS

The work outlined above demonstrates the effectiveness of an innovative approach based on transient transfection of an antagomiRNA as a non-integrative means of direct lineage differentiation. In addition to demonstrating that the expression of miR-221 is a hindrance or major delayer of chondrogenic differentiation, this study showed how knockdown of this molecular regulator is sufficient to enable hMSCs to repair an osteochondral defect.

More generally, the modulation of endogenous molecular cues in directed differentiation strategies will enable greater efficiency and leverage in the generation of target cell types for basic research and regenerative medicine applications.

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FIGURE LEGENDS

Fig. 1 3D pellet culture of antagomiR-221 treated hMSCs. **(a)** Monolayered hMSCs from Wharton's jelly were transfected with antagomiR-221 or a scrambled oligonucleotide (antagomiR-Scr) at the indicated times, and then transferred to 3D pellet culture for 21 days in the absence of TGF-β. Simultaneously, untransfected hMSCs were cultured as pellets, either untreated or treated with TGF-β. hMSCs pellets were harvested for RNA isolation and processed for histology at the indicated times. **(b)** The expression levels of miR-221 were measured at day 7, 14 or 21 in hMSCs pellets by quantitative RT-PCR. Data are presented as percentage variation from U6 expression levels and results represent means ± s.e.m. (three donors). Statistical analysis was performed versus untreated cells (§) and antagomiR-Scr (*) or TGF-β (^) treated hMSCs. $p \leq 0.05$ were considered statistically significant.

Fig. 2 Evaluation of the *in vitro* chondrogenic potential of antagomiR-221 treated hMSCs from Wharton's jelly. At day 21 of culture, hMSCs pellets were immunostained for the matrix proteins collagen type II and collagen type X **(a)** and for the chondroregulatory transcription factors Sox9, TRPS1 and Slug **(b)**. Representative optical photomicrographs are reported. Scale bars correspond to 100 μm and insert bar in **(b)** corresponds to 200 μm. Protein levels were quantified by densitometric analysis of immunohistochemical pictures using ImageJ software and expressed as % of positive area of the pellet (three replicates per three donor). Data are presented as median and interquartile range. Statistical analysis was performed versus untreated cells (§) and antagomiR-Scr (*) or TGF-β (^) treated hMSCs ($p \leq 0.05$).

Fig. 3 Establishment of an *in vivo* experimental model of cartilage defect to test the chondrogenic potential of miR-221 silenced hMSCs. **(a)** Osteochondral plugs were harvested from the sesamoid bones of young calves (3-8 months) after exposure of the metacarpal-phalangeal joint. Osteochondral defects with a diameter of 6 mm were produced. **(b)** hMSCs/alginate and hMSCs/alginate/plug constructs were cultured *in vitro* for 4 weeks in order to evaluate the effect of culture conditions on cell viability and silencing efficiency. Cell viability was assessed by double staining with calcein-AM/propidium iodide and representative merged photomicrographs are reported (scale bars: 50 μm). Viability was expressed as percentage of Calcein-AM-positive (green fluorescence) living cells in the constructs. The levels of miR-221 were measured by quantitative RT-PCR and data are presented as $2^{-\Delta Ct}$ or percentage variation from miR-221 expression in untreated hMSCs, taken as 100%. Statistical analysis was performed versus untreated cells (§) and antagomiR-Scr (*) treated hMSCs ($p \leq 0.05$, three donors). For the *in vivo* experiments,

hMSCs/alginate/plug constructs were implanted subcutaneously in nude mice as duplicate samples (two mice per donor, three donors), as outlined in the implantation scheme. After 12 weeks, the constructs were harvested and processed for histological characterization. In case of donor 3, the constructs could be retrieved from one animal only as the second one had to be sacrificed early due to illness.

Fig. 4 Evaluation of the ability of miR-221 depleted hMSCs to stimulate cartilage repair *in vivo*. Representative sections of hMSCs/alginate constructs in simulated cartilage defects implanted subcutaneously in nude mice for 12 weeks are reported (two replicates per three donors). Cartilage formation was assessed by thionine staining **(a)** and immunostaining for collagen type II matrix protein **(b)**. The organization of the newly formed matrix is shown at different magnifications. Osteochondral defects filled with alginate without cells were used as negative control condition. Scale bars correspond to 1 mm and 70 μm for the lower and higher magnification photomicrographs, respectively. **(c)** The expression of Sox9 and TRPS1 by the cells in the defect area is shown by representative immunohistochemical pictures (positive cells are indicated with arrows). Scale bar corresponds to 30 μm. **(d)** The newly formed matrix was immunostained for the hypertrophic marker collagen type X. The absence of collagen type X positive area is shown at different magnifications. Scale bars correspond to 1 mm and 70 μm for the lower and higher magnification photomicrographs, respectively. Protein levels were quantified by densitometric analysis using ImageJ software and expressed as % of defect for thionine and matrix proteins staining or as % of positive nuclei for transcription factors. Data are presented as median and interquartile range. Statistical analysis was performed versus untreated cells (§) ($p \leq 0.05$).
(NT = newly formed tissue, NC = native cartilage, SB = subchondral bone)

Fig. 5 Schematic representation of miR-221-dependent regulatory interplays potentially mediating its effects on chondrogenesis. miR-221 targets (blue lines) and putative downstream interactions that are hypothesized to be more directly involved in the remodeling of cartilage ECM are outlined. Common targets of miR-221 and miR-222 are circled in yellow. We speculated that miR-221 might block cartilage synthesis mainly by targeting MDM2 and TRPS1, while sustaining cartilage hypertrophy and degradation by promoting the Runx2-mediated transactivation of ALP, MMPs and collagen type X. Moreover, miR-221 might exert further anti-chondrogenic effects via a direct inhibition of Sox9 transcription factor and the cartilage ECM proteins collagen type II and aggrecan (dashed red lines), in a manner to be explored (see Discussion for a detailed explanation).

Fig. S1 Effect of antagomiR-221 treatment on the expression of miR-222 in Wharton's jelly hMSCs pellet cultures. **(a)** Schematic representation of the stem-loop structure of the primary

transcript of the miR-222/221 cluster (pri-miR-222/221) containing miR-221-5p and miR-221-3p (red nucleotides; source: *miRBase.org*). As highlighted in the scheme, mature miR-221-3p and miR-222-3p are highly homologous miRNAs, sharing the same "seed-region" (underlined nucleotides) and consequently several targets. **(b)** The expression levels of miR-221-3p and miR-222-3p were measured before pellet formation (day 0), and at day 7 and 21 of pellet culture, by quantitative RT-PCR. Data are presented as percentage variation of expression in comparison to untreated cells at the same time-point (set as 100% of expression), and results represent means ± s.e.m. Statistical analysis was performed versus untreated cells (§) and antagomiR-Scr (*) treated hMSCs ($p \leq 0.05$).

Fig. S2 Evaluation of the *in vitro* chondrogenic potential of antagomiR-221 treated hMSCs from bone marrow (three replicates per time-point; n = 2). **(a)** hMSCs pellets were immunostained for collagen type II and X at day 21 and 28. The red staining represents positivity for collagen type II or collagen type X. The sections were counterstained with hematoxylin and eosin (scale bar: 200 μm). **(b)** Protein levels were quantified by densitometric analysis of immunohistochemical pictures. GAGs content was measured by quantification of thionine staining (a representative staining is reported). Data were quantified using ImageJ software and expressed as % of positive area of the pellet. Average densitometric values ± s.e.m. are reported. The data demonstrated that collagen type II and GAGs are similarly induced in TGF-β and antagomiR-221 treated cells, while collagen type X expression is markedly reduced following antagomiR-221 treatment. **(c)** mRNA quantification of the cartilage marker lubricin. Results represent means ± s.e.m. and are presented as fold change respect to TGF-β treated cells at day 28. Interestingly, an appreciable level of lubricin mRNA was detected both in TGF-β and antagomiR-221 treated cells. **(d)** Measurement of DNA content in hMSCs pellets at day 28 of culture. Data are presented as median and interquartile range. Since no differences in DNA content were detected, the increase in size of TGF-β and antagomiR-221 treated pellets is to be attributed mainly to matrix production (see collagen type II and thionine staining) rather than variation of the proliferation rates.

Fig. S3 Effect of antagomiR-221 on the expression of hypertrophic markers in hMSCs pellet cultures. The mRNA levels of collagen type X, ALP and MMP13 were measured at day 21 and 28 of pellet culture of bone marrow-derived hMSCs. Results represent means ± s.e.m. and are presented as fold change respect to TGF-β treated cells at day 28.

Fig. S4 General overview of miR-221 targets and downstream effects potentially affecting the synthesis and maintenance of cartilage ECM. Four possible scenarios have been considered: cartilage hypertrophy and ECM degradation, cartilage ECM synthesis, Wnt signaling, cell cycle and

proliferation (see Table S1 for the references). Among the experimentally validated targets of miR-221 (blue lines) are transcription factors and regulators involved in signaling pathways that directly or indirectly affect cartilage ECM remodeling. Common targets of miR-221 and miR-222 are circled in yellow. Based on our experimental evidence, we specifically focused on the two pathways circled in black and detailed in Fig. 5 and Discussion.