Preclinical safety study of a combined therapeutic bone wound dressing for osteoarticular regeneration

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The extended life expectancy and the raise of accidental trauma call for an increase of osteoarticular surgical procedures. Arthroplasty, the main clinical option to treat osteoarticular lesions, has limitations and drawbacks. In this manuscript, we test the preclinical safety of the innovative implant ARTiCAR for the treatment of osteoarticular lesions. Thanks to the combination of two advanced therapy medicinal products, a polymeric nanofibrous bone wound dressing and bone marrow-derived mesenchymal stem cells, the ARTiCAR promotes both subchondral bone and cartilage regeneration. In this work, the ARTiCAR shows 1) the feasibility in treating osteochondral defects in a large animal model, 2) the possibility to monitor non-invasively the healing process and 3) the overall safety in two animal models under GLP preclinical standards. Our data indicate the preclinical safety of ARTiCAR according to the international regulatory guidelines; the ARTiCAR could therefore undergo phase I clinical trial.
Regeneration of osteochondral defects represents a major challenge, especially considering the aging of the population.

The surgical procedures currently applied (bone graft, mosaicplasty, micro-fracture, articular prosthesis, therapeutic implant), are invasive and/or painful for the patient, with limited efficacy and side effects. Lesions of the femoral condyles, for example, are a common side effect that could have serious consequences. A 2002 study found that ≥60% of patients undergoing arthroscopy showed osteochondral defects, in more than half of the cases, such a lesion was classified as grade 3 or higher, according to the International Cartilage Repair Society (ICRS) scale. Osteochondral defects do not heal properly and, even when treated (e.g. by Pridie’s marrow stimulation or by mosaicplasty treatment) consistently led to osteoarthritis (OA). This inevitably has a high impact on the public health system, with the direct costs of the treatment, but it also has repercussions on the general economy (social costs and loss of economic production), setting the overall costs of the disease between 0.25 and 1% of a country’s GDP.

The unique properties of the cartilage (multilayered cell structure, different extracellular matrix composition and fibril orientation) make it difficult to repair. Surgical techniques like micro-fracture, mosaicplasty, osteoarticular transplantation or autologous chondrocytes implant may allow a partial functional recovery, but are mostly aimed to relieve the pain and prevent the lesion to spread.

Besides their variable outcome and intrinsic limitations, none of the aforementioned techniques was shown to restore the hyaline articular surface, justifying the search for alternatives to promote osteoarticular regeneration (OAR). Recently, autologous chondrocytes pre-cultured on a membrane of mammalian collagen were used to fill articular focal lesions and promote cartilage regeneration. However, when performed on subchondral bone, they showed site morbidity and fibrocartilage formation, leading to a dysfunctional repair. To overcome these limitations, mesenchymal stem cell (MSC)-based therapies emerged, which employ autologous bone marrow-derived MSCs to increase the efficiency of OAR. A combination of biomaterials, stem cells and active molecules are therefore needed to promote an effective tissue repair and to achieve a functional recovery of the articulation.

Recently, we proposed the ARTiCAR (ARTicular Cartilage and subchondral bone implant) combined Advanced Therapy Medicinal Products (ATMPs) for personalized OAR (Fig. 1a, b). The implant is made of a nanofibrous wound dressing (FDA-approved resorbable polymeric Poly-e-caprolactone) and autologous bone marrow-derived MSCs (Fig. 1a). The wound dressing is nano-functionalized with nanoreservoirs, for cell contact-dependent delivery of physiological concentrations of bone morphogenetic factor 2 (BMP2). The nanoreservoirs technology enabled to reduce the dose of BMP2 to physiological levels, making it locally and sustainably available, and reducing the adverse effects of its massive release, e.g. from soaked collagen sponges currently used in the clinic.

In this work, we test the safety of the ARTiCAR combined ATMPs in two different animal models. Moreover, we assess the feasibility of non-invasive monitoring of the healing process in sheep, via MRI. The results of the toxicity and biodistribution tests, run accordingly to the international regulatory guidelines for cell therapies and medical devices and Good Laboratory Practice (GLP), prove the biosafety of the ARTiCAR combined ATMPs, which can therefore be used in phase 1 clinical trials as a ready-to-use, flexible implant to address both cartilage and subchondral bone regeneration in OA patients.

Results

Cytotoxicity of the NanoM1-BMP2 wound dressing in vitro. The nanofibrous PCL wound dressing component (NanoM1-BMP2; Fig. 1a) of the ARTiCAR combined ATMPs was tested for cytotoxicity on MRC-5 fetal lung fibroblasts in vitro. Cells were seeded in the presence of the NanoM1-BMP2 wound dressing and compared to positive (polyurethane film; RM-A) and negative (high density polyethylene film; RM-C) controls. Different sizes of both the NanoM1-BMP2 membrane and the control films were tested in the range of 1-20 mm². Cell density and morphology were qualitatively evaluated by bright field microscopy.

Cells cultured in the presence of RM-A started to detach already after 24 h (Fig. 2a–e). One the contrary, cells cultured in the presence of the NanoM1-BMP2 scaffold did not show any morphological abnormalities (Fig. 2f–j), as they did those cultured in the presence of RM-C films (Fig. 2k–o). Next, we assessed the viability of the MRC-5 cells in the 3 conditions tested, using the WST-1 live/dead cell assay. Both RM-A and NanoM1-BMP2 showed a decrease in cell viability over 24 h that was directly proportional to the size of the membrane used, as the interpolated trend lines indicated (solid black lines in Fig. 2p–r). However, in the presence of 20 mm² RM-A, the cell viability reduced to 72 ± 5% compared to t0 (Fig. 2p, p ≤ 0.05), while in the presence of a fragment of NanoM1-BMP2 of the same size, the cell viability reduced to 97 ± 5% (Fig. 2q). No significant reduction of the cell number was also observed in the presence of the negative control film (Fig. 2r). These results indicate that the NanoM1-BMP2 is not toxic to MRC-5 cells in vitro.

Acute toxicity after the implant of the ARTiCAR in nude rats. The acute toxicity of the ARTiCAR was evaluated in vivo in nude rats, and compared to the non active part of the implant (hydrogel without hMSCs) as a vehicle. Clinical, hematological and biochemical parameters were evaluated. The biodistribution and the persistence of the transplanted cells were also assessed.

Brieﬂy, ARTiCAR combined ATMPs (group 1) or vehicles (group 2) were implanted into femoral defects in nude rats. Ventricular blood was taken before the animals were euthanised, 7 days post implant, and femurs were collected for histopathology analysis. Both the ARTiCAR combined ATMPs, nor the vehicle triggered any signiﬁcant effect on the body weight, either in female or male rats, over a period on 90 days following the implant (Fig. 3a). Hematological parameters (Fig. 3b) showed no signiﬁcant differences among the 4 groups of animals (group 1 male, group 1 female, group 2 male, group 2 female). Biochemical parameters were also assessed (Fig. 3c). Female rats in Group 1 showed signiﬁcantly higher plasmatic concentrations of both Alanine aminotransferase (ALAT; 17.0 ± 2.0 U/l vs. 13.2 ± 1.6 U/l for group 1 and 2, respectively; p ≤ 0.05) and Calcium (91.74 ± 1.02 mg/l vs. 88.46 ± 0.91 mg/l for group 1 and 2, respectively; p ≤ 0.05) than those in group 2. These differences were not associated with any additional symptoms and, altogether, the analysis of the hematological and biochemical parameters considered did not show any clinically relevant differences between ARTiCAR-treated animals and the control group. The femur-tibia joints were also collected and subject to histological analysis. Both the ARTiCAR and the vehicle induced comparable levels of inflammatory response at the implant site (delimited by asterisks in 3d, e), compatible with the bone healing process of the induced bone defect (Fig. 3d, e). Eventually, the biodistribution of the human MSCs at day 90 post implant was also assessed, using qPCR for detecting human DNA. Signal from hMSCs DNA was never detected above the threshold level, except in the testis of one male rat in group 1. The migration of the PCR product on 2.5% agarose gel confirmed the speciﬁcity of the ampliﬁcation product. Taken together, clinical, hematological and biochemical data suggest that the ARTiCAR implant did not induce any clinically relevant symptoms; the inﬂammatory response detected from the histological analysis of the implant site revealed no differences.
with the control group, strongly indicating the safety of the ARTiCAR implant for the treatment of bone defects.

**Intra-articular implant of the ARTiCAR in sheep.** To further confirm the safety of the ARTiCAR combined ATMPs, and to assess the feasibility of its usage in large animals, osteoarticular defects were induced in femoral condyles of adult sheep and were either left unfilled (no-treatment control: NT) or filled with the ARTiCAR implant or with an autograft (AG), as for supplemental table 1. The healing process was monitored non-invasively by

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**Fig. 1** Composite advanced therapy medicinal product (ATMP) developed for the osteoarticular regeneration (OAR) of cartilage focal lesions. **a** The ARTiCAR is a composite ATMP that combines an FDA-approved synthetic wound dressing and a living therapeutic made of autologous mesenchymal stem cells (MSCs), embedded in alginate/hyaluronic acid hydrogel. The wound dressing, named NanoM1-BMP2 is made of nanofibrous poly-ε-caprolactone nano-functionalized with Bone morphogenetic protein 2 and aims at subchondral bone regeneration. The cellular hydrogel aims at articular cartilage regeneration. The NanoM1-BMP2 was tested for cytotoxicity in vitro (step 1); the whole ARTiCAR was tested for acute toxicity, biodistribution and persistence in an osteochondral defect nude rat model (step 2) and for feasibility, safety, and non-invasive monitoring of the procedure (step 3) in a sheep intra-articular model. **b** The ARTiCAR aims at the simultaneous regeneration of both the articular cartilage and the subchondral bone in a one-step surgical procedure. After harvesting MSCs from patient’s bone marrow, the NanoM1-BMP2 is applied in contact with the injured subchondral bone; afterwards, the MSCs mixed with the hydrogel is applied to fill the defect.
means of magnetic resonance imaging (MRI) at 0, 12 and 26 weeks (Fig. 4a–c). After either 12 or 26 weeks from implant, sheep were euthanized and the femur-tibia joints were explanted and scanned via micro-computing tomography (micro-CT; Fig. 4d); a 3D surface rendering of the joint was also built from 2D section images (Fig. 4e). The explanted joints were macroscopically scored according to the ICRS score system as follows: grade I = normal cartilage, grade II = nearly normal, grade III = abnormal cartilage and grade IV = severely abnormal cartilage (Fig. 4f). Eventually, the explants were stained in a solution of safranin o–fast green and examined histologically (Fig. 4g). The following parameters were taken into consideration within the repaired tissue and scored according to ICRS II score system39: subchondral bone abnormalities/marrow fibrosis, tissue
These results suggest that the ARTiCAR safety in an osteochondral defect nude rat model. In most cases, revisions were required within 2-5 years after the primary implant. Regenerative nanomedicine combines the use of biomaterials, nanotechnologies and cells to offer better solutions to issues like OAR, where a complex infection, fibrinous exudates and fatty infiltrates were detected in the tissue adjacent to the defect in both the ARTiCAR and the AG groups (supplemental table 3), indicating that the ARTiCAR combined ATMPs treatment has a safety over the long term comparable to that of an autograft.

Discussion

The global cartilage repair/regeneration market is valued at USD 4.2 billion in 2016 and is expected to grow at a CAGR of 5.4% during the 2014-2025 period, owing to the increased life expectancy. Currently, the international standard treatment for OA is total knee arthroplasty (TKA). Despite its rapidly increasing utilization (77,000 patients/year younger than 55 years, in the US), TKA is prone to complications, like a higher risk of infection, persistent knee pain, patellar resurfacing problems and prosthetic fracture. Once complications developed, the consequences for the patients are severe, which is why 20% of the patients who underwent TKA were unsatisfied about the surgery outcome. In most cases, revisions were required within 2-5 years after the primary implant. Regenerative nanomedicine combines the use of biomaterials, nanotechnologies and cells to offer better solutions to issues like OAR, where a complex interface regeneration is required. In this work, we assessed the feasibility, non-invasive monitoring and safety of the ARTiCAR combined ATMPs. Similarly to other smart implantable scaffolds that promote osteochondral differentiation, the ARTiCAR releases a bone promoting factor. However, thanks to our
Fig. 4 Feasibility, non-invasive monitoring and safety evaluation of the ARTiCAR combined ATMPs implanted in sheep intra-articular defect model. **a-e** the OAR process after ARTiCAR implant was monitored non-invasively by means of MRI immediately after surgery (**a**) and at 12 (**b**) and 26 (**c**) weeks post implant. The extension of the bone defect (white asterisks) and the ongoing OAR (white arrowheads) are visible. Micro CT-scan (**d**) followed by 3D surface rendering (**e**) were performed on freshly explanted joints. **f** The level of cartilage regeneration was macroscopically assessed based on ICSR score system. **g, h** Whole explants were sectioned and stained with safranin o - fast green (blue: bone; red: cartilage), imaged at ×4 and stitched together (**g**) to evaluate the OAR according to the ICRS II score system (**g**). Scale bar: 1 mm. ICRS II parameters were examined for ARTiCAR and the control groups considered and analyzed by mean of two-way ANOVA followed by Bonferroni post hoc test (**h**). * = \( p \leq 0.1 \); ** = \( p \leq 0.05 \). Dots represent individual scores, bars represent mean scores and error bars represent standard errors. **i-n** Zones of osteochondral remodeling within the treated defects in animals from AG (**I, L**), NT (**j, m**) and ARTiCAR (**k, n**) groups, either at 12 or 26 weeks post implant. Bone (white asterisks), cartilage (black asterisk), fibrocartilage (black arrow) and subchondral bone (yellow arrow) are shown. Scale bar: 500 µm
patented nanoreservoir technology that provide cell contact-
dependent gradual release, the total amount of BMP2 used in the
ARTiCAR is 10.000 times lower than that of BMP2-soaked col-
lagen membranes used in the clinic36, reducing both potential
inflammatory side effects (Fig. 3d; Fig. 4g, h, k, n) and the overall
costs of the procedure. Differently to other approaches where a
poor subchondral bone regeneration was achieved47,48, the
ARTiCAR address simultaneous regeneration of both the sub-
chondral bone and the cartilage24,27,29 (Fig. 4g, k, n), repre-
senting an innovative technology for promoting OAR in a
localized osteochondral defect. For cartilage regeneration, the
ARTiCAR incorporates MSCs. Human MSCs are currently used
in clinical trials for promoting OAR49-51, because of their trans-
differentiation potential coupled to immunomodulatory effect52,53.
In future clinical trials, the MSCs could be directly
harvested from patients, allowing for autologous transplantation.
In order to be transplanted in human, MSCs will undergo an
implanted quality control (supplemental table 4), and will be
used only if the minimum release criteria for this type of ATMP
are satisfied54. However, since tumorigenicity of MSCs is still
defeated55,56, the biodistribution of hMSCs is a critical concern of
preclinical safety57,58. After the implant of ARTiCAR, traces of
hMSC DNA were found in the testes of one male nude rat, out of
40 implanted animals (Fig. 3c). Also, no tumor formation
was observed in the transplanted rats and sheep, neither at the
implant site, nor in the rest of the body. Altogether, these data
pinpoint the safety of the ARTiCAR implant in respect to the
tumorigenicity of the transplanted MSCs.

In summary, we showed the feasibility of the ARTiCAR
implant in a large animal model and the possibility to follow OAR
non-invasively, by mean of MRI (Fig. 4a-c). More importantly, we
showed the safety of the ARTiCAR, as no acute or long term
toxicity was detected, neither in nude rats (Fig. 3d), nor in sheep
(Fig. 4g, h, k, n). Therefore, the ARTiCAR can enter phase
I clinical trials as a treatment for osteochondral defects, with the
potential to be used also for other conditions, like tendon
degeneration and age-related degenerative musculoskeletal issues.
As such, the ARTiCAR could replace more invasive current
treatments, with the potential to impact 300.000 to 450.000
patients/year only in the US ($4.5 billion global market).

Methods

Study design. All the experiments in this study were planned and performed
according to the international regulatory guidelines33-35 for cell therapies
and medical devices. Good Laboratory Practice38 and Standard Operating Procedures
(SOPs) for all protocols were used. In vitro cytotoxicity assay was done according
to ISO 10993-5 (2009 and 2012) guidelines. Assessment of the OAR was done
according to the ICRS II score system39.

Production of the nanofibrous compartment of the ARTiCAR. The nanofibrous
component of ARTiCAR was obtained via electrospinning of PCL, as previously
described44. Briefly, PCL (PURASORB®; PURAC, Corbion, Amsterdam, Nether-
lands) was dissolved in a 25% (wt/vol) dimethylformamide/dichloromethane
solution (3/2, v/v) and delivered at a constant rate of 1 ml/h to the EC-DIG
electrospinning device (IME Technologies, Eindhoven, Netherlands), set to a high
voltage (20 ± 3 kV). Following electrospinning, PCL membranes were kept in a
desiccator at 45 °C, to remove residual solvents, and sterilized by gamma irradia-
tions (25 kGy). Membranes were then dipped alternately in 200 µg/ml
BMP2 solution (rh-BMP2, Inductos, Medtronic, France) in 40 mM 4-
Morpholineethanesulfonic acid (Sigma-Aldrich, Saint-Quentin Fallavier, France),
150 mM Sodium Chloride (Sigma-Aldrich), pH 5.5 (MES buffer) and 0.5 mg/ml
Chitosan (Protasan UP CL 113, Novamatrix, Sandvika, Norway), for 12 times. Each
bath was followed by three washes in MES buffer.

Production of the hydrogel compartment of the ARTiCAR. Twelve mg/ml
sodium alginate (Sigma-Aldrich) and 3 mg/ml hyaluronic acid (LifeFect Biome-
dical, Chaska, USA) were dissolved in 9 mg/ml Sodium Chloride (Sigma-Aldrich).
Prior to implant, the hydrogel was mixed with either human or sheep MSCs. After
the MSC/hydrogel compartment was applied to fill the defect, gelation was
achieved using 102 mM calcium chloride (Sigma-Aldrich).

Cell culture. Human lung fetal fibroblast MRC-5 cell line (ECAC, Sigma-
Aldrich) was cultured in 75 cm2-flasks with EMEM (Lonza, Levallois-Perret,
France) containing 10% Fetal Bovine Serum (Lonza), 2 mM Glutamine (Lonza)
and 1% Non Essential Amino Acids (Lonza), under 5% CO2 humidified atmos-
phere at 37 °C. Cell culture was performed accordingly to ISO 10993-5:2009
guidelines. The presence of mycoplasma in culture media was tested according to
internal SOPs.

Cytotoxicity assessment in vitro. MRC5 cells were plated into 24-well plates.
The NanoM1-BMP2 wound dressing was tested side-by-side with polyurethane film
containing 0.1% zinc diethyldithiocarbamate (known for inducing cytotoxic effects;
Hatano Research Institute/Food and Drug Safety Center, Japan) and high density
polyethylene film (negative control; Hatano Research Institute). To assess cyto-
toxicity, pieces of different size (20, 16, 9, 4, 1 mm2) were plated in to the
culture (n = 10). After 72-80% confluence was reached, the pieces were cultured in
the presence of the membranes for 3 days before being examined microscopically for
changes in the general morphology, presence of vacuolization, detachment, lysis
and membrane integrity, following the criteria for the qualitative evaluation of
cytotoxicity according to ISO 10993 guidelines, part 5 (2009) and part 12 (2012):
Class 0, no reactivity (no effects around or below sample); Class 1, slight reactivity
(few malformed or degenerated cells); Class 2, mild reactivity (small area of mal-
formed or degenerated cells below the sample); Class 3, moderate reactivity
(malformed or degenerated cells in an area larger than the size of the sample but
<1 cm2); Class 4, severe reactivity (malformed or degenerated cells in an area larger
than the size of the sample but >1 cm2). A grade higher than 2 was considered as
cytotoxic.

Quantitative cell viability assay. As a quantitative measure of cytotoxicity, cell
viability was evaluated. At day 3, membranes were discarded, cells were washed
twice with PBS, fed with 1 ml culture medium and 100 µl/well of Cell Viability
Reagent (CellTiter96® Aqueous One Solution, Promocell, Heidelberg, Germany)
were added twice a week. Differently to other approaches where a
poor subchondral bone regeneration was achieved47,48, the
ARTiCAR incorporates MSCs. Human MSCs are currently used
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localized osteochondral defect.
DNA from the different tissues of the implanted rats was amplified with DNA from control rats, spiked-in with variable amount of DNA from U87-MG human cells (22, 7, 0.7 ng; 70, 7, 2.2, 0.7, 0.07 pg or no DNA), to build a mix (BioRad, Marnes-la-Coquette, France) was used to quantify human Alu sequences. Four weeks (28 ± 2 days) prior to harvest site was disinfected and a needle was introduced into the defect. In the NT group (=n=13) the defect was neither treated, filled. Up to 5 mL 0.25% bupivacaine were instilled. Histopathology analysis of nude rats implants. Histopathology analysis was conducted on the fasted animals used for blood test. Briefly, a macroscopic autopsy was performed on freshly euthanized rats. Organs (treated knee, spleen, mesenteric lymph nodes, liver, lungs with bronchi and bronchiolo, kidneys and heart) were macroscopically observed, explanted and collected. The right hind paw was sectioned at the epiphyses of both femur and tibia to recover knee joints subject to implant. Spleen, liver, kidneys and heart were weighed and preserved with the other organs at room temperature in 4% formalin (Sigma-Aldrich) until histological analysis. Organs were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, sectioned and examined for histopathology.

Tissue harvesting for human DNA qPCR. Ninety days post implant, five male and five female rats/group (=n=20) were euthanized by exsanguination under anesthesia. The knee joints were homogenized with a GentleMACS Dissociator (Miltenyi Biotec, Paris, France). Knee joints were homogenized using Ultra-Turrax® dissociator instrument in buffer G2. Following extraction, the DNA pellet was dissolved in molecular biology grade water, and stored at 20 °C. Quantitative PCR (qPCR) with the iTaq Universal Probes Supermix (BioRad, Marnes-la-Coquette, France) was used to quantitate human Alu sequences with the TaqMan AluYB8 Probe (Thermo Fischer Scientific). Genomic DNA from the different tissues of the implanted rats was amplified side-by-side with DNA from control rats, spiked-in with variable amount of DNA from U87-MG human cells (22, 7, 0.7 ng; 70, 7, 2.2, 0.7, 0.07 pg or no DNA), to build a standard curve. Samples were run in triplicate for 35 cycles on a CFX System (Bio-Rad). The limit of detection corresponded to the average signal from control rat DNA not spiked-in with human DNA.

Harvesting of MSCs from sheep bone marrow. Four weeks (=28 ± 2 days) prior to interarticular surgery, adult sheep females (Rideau Arcott Hybrids strain) were subject to bone marrow aspiration procedure. Briefly, animals were placed in ventral recumbency, anesthetized with ketamine and xylazine, administered intramuscularly (IM). An IV catheter was placed in the appropriate vein. The larynx was sprayed with lidocaine and the animals were intubated with an appropriate sized cuffed orotracheal tube. If intubation was not possible under IM anesthesia, induction was performed using isoflurane in O2 (1–5%). The animals were then mechanically ventilated with isoflurane in O2. The harvest site was disinfected and a needle was introduced in the iliac crest. A sterile 10 mL syringe was filled with 1 mL of 5000 IU/mL heparin and filled with ~8 mL of bone marrow. The syringe containing bone marrow sample and heparin was sealed with an appropriate sterile cap for transportation.

Isolation and expansion of bone marrow-derived sheep MSCs. The MSCs harvested from sheep iliac crest were isolated according to their adherence to cell culture plastic. Bone marrow aspirates were first washed by addition of an equal volume of phosphate buffer saline (PBS; Sigma-Aldrich, France) and centrifuged at 220 x g for 5 min. The cell pellets were suspended in Dulbecco’s Modified Eagle Medium (DMEM; Lonza, Germany) containing 10% heat-inactivated fetal bovine serum (Gibco, Thermo Fisher Scientific, France), 50 U/mL of penicillin (Lonza, Germany), 50 µg/mL of streptomycin (Lonza, Germany), 2.5 µg/mL Fungizone (Lonza, Germany), and seeded in a T75 culture flasks, under standard cell culture conditions. The non-adherent cells were discarded and attached cells were gently washed up several times with PBS to remove non-adherent cells. Flasks were then incubated for several days in DMEM, replaced every 72 h to promote emergence of colonies from adherent cells. When cells finally reached sub-confluence, they were sub-cultured until passage 2, when they were expanded for stemness characterization.

Characterization of sheep MSCs. The MSCs were characterized according to their ability to form colonies and to their multipotency. Colony-forming unit-fibroblast (CFU-F) assays were performed in triplicates (=n=3) with two different ranges of serial dilutions. After 14 days of culture, MSCs were rinsed with PBS, and fixed with 4% (w/v) paraformaldehyde. The colonies were stained using hema-toxylin and eosin (H&E, Sigma-Aldrich, Germany), observed under light microscope, counted, and the number of colonies was expressed as colony forming units (CFUs) per mL. The cell pellets were then re-suspended in a medium containing 10% FBS, 1% Pen/Strep in PBS. The suspension was transferred to a 6-well plate and placed into the defect. In the NT group (=n=13) the defect was neither treated, nor filled. Up to 5 mL 0.25% bupivacaine were infiltrated into the surgical site to achieve local anesthesia and manage pain after surgery. The tissues were repositioned and closed layer-by-layer using appropriate sutures. Postoperative analgesia and antibiotic therapy were performed, 5 mg/kg Excide (IM) was administered during recovery from anesthesia, and 4 mg/kg Carprofen (IM) was administered 3 days after surgery.

Non-invasive monitoring of ARTiCAR via MRI. For the longitudinal analysis of the knee repair, sheep were examined three times via MRI, immediately after surgery, at 15 and 26 weeks, using a Magnetom Verio 3 T (Siemens). For the induction of osteochondral defect in sheep. A total of 16 adult sheep underwent surgical induction of osteochondral defect into the medial femoral condyle. Three groups of sheep (ARTiCAR, AG, control, NT control) were considered; each group was implanted on either the proximal or distal part of the right or left condyle of posterior legs (supplemental table 1). For surgery, the hind limb was flexed to a position at which the medial condyle could be palpated under the skin. A 15 cm medial parapatellar skin incision was performed. After blunt dissection of the subcutaneous tissues, the fascia overlying the vastus medialis muscle was incised just distal to the belly muscle with a small incision parallel to the muscle fibers and the vastus was retracted proximally. Blunt dissection was used to expose the peristeum down to the medial condyle of the femur. The joint capsule and periosteum were incised just proximal to the origin of the medial collateral liga- ment. Overlying soft tissues were removed from the bone only in the vicinity of the drill holes. Holes were prodded using a 6-mm drill bit to a depth of 3 mm, except for the AG group, where the hole had a depth of 6 mm.

Intra-articular implant of ARTiCAR in sheep. Following the induction of the defect, the NanoM1-BMP2 was placed, and the defect was filled with MSCs/hydrogel mix (ARTiCAR combined ATMPs, n = 9). In the AG group (=n=10), a bone sample of 6 mm of diameter and 6 mm deep was taken out from the condyle and placed into the defect. In the NT group (=n=13) the defect was neither treated, nor filled. Up to 5 mL 0.25% bupivacaine were infiltrated into the surgical site to achieve local anesthesia and manage pain after surgery. The tissues were repositioned and closed layer-by-layer using appropriate sutures. Postoperative analgesia and antibiotic therapy were performed, 5 mg/kg Excide (IM) was administered during recovery from anesthesia, and 4 mg/kg Carprofen (IM) was administered 3 days after surgery.
procedure, sheep were anesthetized with an intravenous injection of 0.05 mg/kg xylazine and 5 mg/kg ketamine and placed in dorsal decubitus. A total of six sites of surgery were imaged for each group. Proton density-weighted, fat-saturated sagittal sections of the acquisitions were analyzed using the Osirix open-source software.

Three-dimensional micro-CT of explanted femoral condyles. For analysis of the bone mineralization, sheep were anesthetized, weighed and euthanized by a lethal injection of 540 mg/ml Euthanyl rapid IV bolus 26 weeks after surgery. Death was confirmed and recorded by observation of asystole or ventilricular fibrillation, either on the electrocardiogram or by auscultation. Femoral condyle from were explanted from euthanized sheep and imaged via 3D micro-CT (Quantum Fx mCT, Julien Becker, ICS, IGBMC, Strasbourg, France). A total of six sites of surgery were imaged for each group. Three-dimensional surface rendering was obtained from micro-CT 2D images using the Osirix open-source software.

Histopathology analysis of implants in sheep. Treated femurs were removed from euthanized animals and subject to macroscopic inspection of the articular surface. The distal femoral epiphysis (with condyles) were individually identified and collected in 10% neutral buffered formalin, after macroscopic examination. Bone blocks were cut in two halves, by sawing in the middle of the sample along its longitudinal axis. Sections were cut through the defect along its deeper axis, from the bone surface to the end of the drill hole producing rectangular-shaped defect half sections. Full-thickness femoral bone-cartilage defect sites underwent undetracted collagen type I/III membrane (MACiR) implant improves cartilage healing in the equine patellofemoral joint model. Osteoarthr. Cartil. 23, 648–660 (2015).

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Author contributions
L.K., L.P. and Y.L.-G. prepared and characterized the active implants, contributed to the experiments and to the analyses of data; L.P. and F.B. performed and analyzed MRI; L.B. and M.T. prepared MSCs and performed qPCR; L.K., L.P. and L.G. contributed to the analyses of the data and drafted the manuscript. P.A. designed acute cytotoxicity and biodistribution experiments, and acquired data; R.M.G.-D. and E.G.B. contributed to the study design and supervision; N.B.-J. conducted the hypothesis, designed the experiments, directed the work and contributed to draft the manuscript.

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