

# Autologous engineering of cartilage

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**Treatment of full-thickness damage to hyaline cartilage is hampered by the limited availability of autologous healthy cartilage and the lengthy, cost-prohibitive cell isolation and expansion steps associated with autologous cartilage implantation (ACI). Here we report a strategy for de novo engineering of ectopic autologous cartilage (EAC) within the subperiosteal space (in vivo bioreactor), through the mere introduction of a biocompatible gel that might promote hypoxia-mediated chondrogenesis, thereby effectively overcoming the aforementioned limitations. The EAC is obtained within 3 wk post injection of the gel, and can be press-fit into an osteochondral defect where it undergoes remodeling with good lateral and subchondral integration. The implanted EAC showed no calcification even after 9 mo and attained an average O'Driscoll score of 11 (versus 4 for controls). An "on demand" autologous source of autologous cartilage with remodeling capacity is expected to significantly impact the clinical options in repair of trauma to articular cartilage.**

agarose | hypoxia | in vivo bioreactor | periosteum | regenerative therapies

Annually, in the United States alone over 1 million individuals are treated for lesions in articular cartilage with over 250,000 patients requiring knee arthroscopy (1). Damage to hyaline cartilage often precedes osteoarthritis (2). Among cartilage lesions, osteochondritis dissecans (OCD), which involves full-thickness damage to the hyaline cartilage and underlying bone (3), is being diagnosed with an increasing frequency. While OCD is most often diagnosed in the knee, it may also affect other joints such as ankle, elbow, and shoulder (3). OCD is clinically challenging to treat as it is multifactorial and, furthermore, treatment is hampered by the limited self-repair potential of hyaline cartilage that is due to the absence of resident pluripotent cells and the lack of vasculature and lymphatics (4, 5). Notwithstanding, hyaline cartilage is key to successful repair as its unique cellular organization and extra cellular matrix composition confers load bearing and lubricious properties to the joint surface. Currently, injury to the articular cartilage surface, including OCD lesions, is treated by either physiotherapy, stimulation of regeneration by arthroscopic drilling, autologous osteochondral plugs from non-weight bearing regions, or injection of cells under a periosteal flap (ACI) (5, 6). Whereas the best treatment for cartilage lesions remains to be defined, ACI, in spite of its suboptimal outcomes (i.e., formation of undesirable fibrocartilage, poor, integration, and calcification), along with engineered cartilage and osteochondral tissue constructs, are promising as treatment options (7, 8) However, the clinical implementation of ACI and engineered constructs is hampered by the costs and logistics involved with isolation and expansion of cells and variability in quality of the engineered tissue. We recently proposed a unique paradigm for de novo engineering of tissues, the in vivo bioreactor (IVB), in which a wound-healing response provoked within a confined subperiosteal space adjacent to a source of pluripotent cells and soluble growth factors (9) using a gel biomaterial, serves as the trigger for the neo-tissue development. We demonstrated that large volumes of bone can be engineered de novo within the IVB without cell implantation and administration of growth factors (9). We further showed

that, through localized delivery of liposomes containing an anti-angiogenic agent suramin, supplemented with transforming growth factor-beta-1 (TGF- $\beta$ 1), the differentiation of the periosteal cells within the IVB towards a chondrogenic lineage can be achieved, thereby yielding hyaline cartilage. Although several studies have confirmed, the chondrogenic potential of periosteum in vivo and in vitro (10–12), local growth factor delivery was necessary to generate cartilaginous tissue (9, 13). The implementation of growth factors in cartilage repair, however, faces severe regulatory hurdles. To overcome this limitation, we posed the question: Can the inherent physicochemical properties of a biomaterial be used to trigger chondrogenesis within the IVB? Towards this end we theorized that a gel that can limit vascularization may favor hypoxia and hence chondrogenesis. In this study, we demonstrate that hyaline cartilage can be engineered de novo in the IVB by simply injecting agarose, a biocompatible (14, 15) polysaccharide biomaterial that has no-inherent angiogenic properties (14, 16, 17) but promotes hypoxia (18). The engineered cartilage stains positive for collagen type-II and proteoglycans, is hypercellular, and is capable of remodeling within a full-thickness osteochondral defect with complete integration and does not undergo calcification even after 9 mo.

## Results

**Hypoxia Enhances Chondrogenesis and Type II Collagen Expression in Periosteal Cells.** Because the central premise of our hypothesis is that "hypoxia induced by agarose-gel can trigger and enhance chondrogenesis," in a preliminary study we evaluated the effect of hypoxia on chondrogenesis in isolated periosteal cells. In comparison to cells not exposed to differentiation media, COL2 (collagen type II) mRNA expression levels in periosteal cells after 3 d in differentiation medium increased under normoxic and hypoxic conditions. However, the increase in mRNA levels under normoxic conditions ranged from 15–260-fold, with an average of 133-fold over undifferentiated condition, whereas the fold increase under hypoxic conditions ranged from 125–1191 with an average of 413-fold over undifferentiated conditions. Based on these results, cells isolated from the same donor rabbit were cultured under hypoxic conditions and normoxic conditions in presence of differentiation signals. Relative to normoxic expression levels, hypoxia induced on an average a 7-fold increase (range 4–17-fold increase) in COL2 mRNA expression levels at the end of 3 d (Fig. 1A). Additionally, a significant increase in mRNA for Aggrecan (ACG) and Sox9 was observed relative to normoxic conditions over the same period (Fig. 1A). This result was further confirmed by western blot analysis that showed a more robust

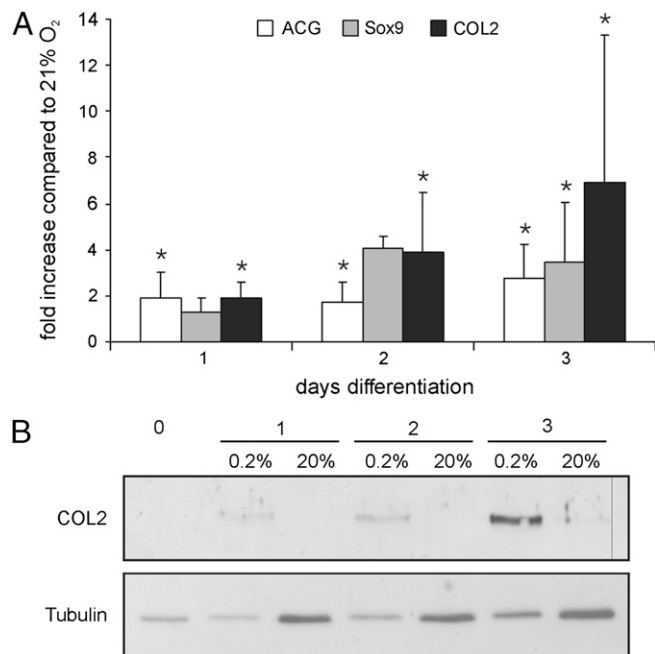
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**Fig. 1.** Chondrogenic differentiation of isolated periosteal cells over 3 d. (A) Compared to normoxic conditions (21% O<sub>2</sub>), after 3 d of differentiation under hypoxic conditions (0.2% O<sub>2</sub>), mRNA expression levels of collagen Type II (COL 2) show an average 7-fold increase (range 3–18) whereas ACG mRNA expression is increased by an average of 2.8-fold (range 1.5–4.7), and Sox9 mRNA levels showed a 3.5-fold increase (range 1.5–6.2). The results of five independent periosteal isolates are shown. All mRNA levels were normalized to 28S rRNA. Standard deviation (SD) is indicated by error bars. (B) Western analysis confirms an increased level of COL2 protein under hypoxic conditions compared to normoxic conditions.

induction of COL2 at the protein level under hypoxic conditions (Fig. 1B).

**Cartilage Formation in the IVB.** In order to verify if the induction of hypoxia within the IVB can result in chondrogenesis, we compared the neo-tissue development within the IVB's filled with HA Gel, HA Gel supplemented with liposomes containing TGF-β1 and suramin, and agarose alone. The neo-tissue formed within the IVB was examined macroscopically, (immuno)histologically, and by chondrogenic marker expression analysis (RT-qPCR). Cartilage formation in the IVB's was visually identifiable and confirmed by palpation (Fig. 2A). Whereas IVB's filled with HA Gel by itself yielded only fibrous scar tissue, in IVB's filled with HA Gel supplemented with the liposomal formulation containing TGF-β1 and suramin induction of hyaline cartilage was observed that was consistent with our past findings (9). However, a surprising result was the formation of hyaline cartilage within IVB's filled with agarose only. This is a remarkable finding as the formation of hyaline cartilage occurred in absence of any local administration of pro-chondrogenic agents or cell implantation. The observation that HA Gel by itself was not capable of inducing chondrogenesis, perhaps, points to an intrinsically different antiangiogenic properties of agarose (14, 16) versus HA Gels in the IVB. In summary, generation of EAC was observed in 63% (12/19) and 65% (13/20) of the IVB filled with agarose and HA-liposome, respectively, and was absent in 100% of the IVB's filled with HA-alone (0/6). The similar success rate in EAC formation within the HA-liposome and agarose groups suggests that surgical technique might be a dominant variable that needs further refining.

**Visual and Histological Assessment of EAC.** At the time of cartilage harvest, based on visual inspection, no residual gel fragments were observed within the IVB in both groups. Additionally, the volume of EAC formation appeared to be comparable in both groups (Fig. 2A), and was large enough to core out a cartilage graft 3-mm in diameter (Fig. 3A) that could be press-fit in an osteochondral defect (Fig. 3A). Tissue sections in both groups stained positive for Safranin-O and COL2 (Fig. 2B). The onset of ectopic cartilage formation (Fig. 2A) was about 7 d slower in the agarose group compared to the HA-liposome group. It is important to mention that the thickness of the normal articular rabbit cartilage is 120–170 μm, and, in comparison the thickness of the cartilage generated within the IVB, which is in the range of 1.5–2.5 mm, is about 10-times thicker.

In line with the histological findings, gene expression analysis at 13 and 21 d in both groups that yielded EAC showed increased expression of early and late chondrogenic markers: COL2, ACG, and collagen type X (COL10) (Fig. 2C). Furthermore, an upregulation of hypoxia inducible factor-1 alpha (HIF-1α) expression and one of its known targets: Vascular Endothelial Growth Factor (VEGF) was observed in agarose and HA-liposome groups, and this is consistent with our earlier findings in this study (Fig. 2C). This confirms a key role for a low-oxygen tension microenvironment within the IVB in EAC formation. The reduced VEGF levels in the HA-liposome group, in comparison to the agarose group, is most likely the direct result of the antiangiogenic effect imposed by suramin, which is a known inhibitor of the TGF-beta super family of proteins (19) (Fig. 2C). In sum, the above data demonstrates that gel-biomaterials with the appropriate physicochemical characteristics are capable of effecting directed differentiation of cells and tissue remodeling within the IVB environment.

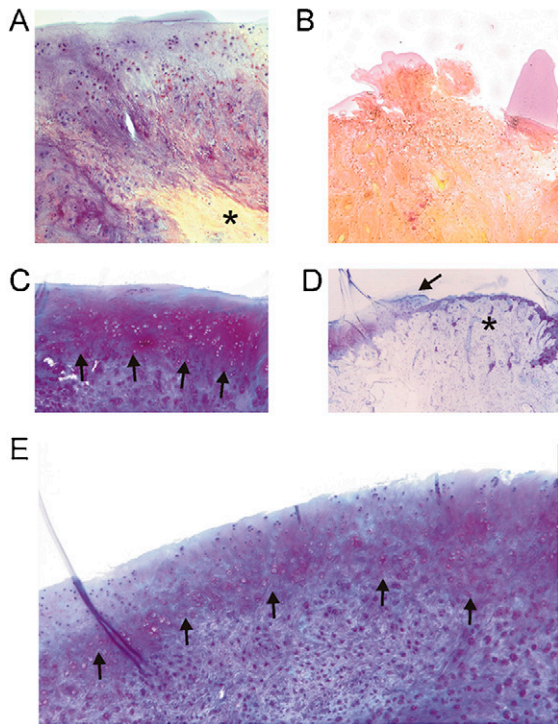
**Osteochondral Repair.** Full-thickness osteochondral defects were created in the medial condyle of New Zealand white rabbits and the defect was filled with EAC plugs that were press-fit or left empty (controls). The size of the defect, that is, 3 mm is significant in comparison to the condyle of a rabbit that is about 4.5–5 mm wide. A 4 mm defect, which would more closely mimic a unicompartamental knee arthroplasty, was not explored as past studies showed that this resulted in cracking and rupturing of the chondyle (20). After 9 mo follow-up, although the empty defects and the defects repaired with cartilage from the IVB were filled with tissue that macroscopically appeared similar, histological analysis revealed significant differences. Whereas evidence of osteophytes was present in 30% (3/10) of the empty defects, only 11% (1/9) of IVB's filled with EAC of the HA-liposome group showed evidence for osteophytes, and no osteophytes were observed in the IVB's filled with EAC of the agarose group. Histologically, in all cases there was integration of the transplanted EAC with the subchondral bone and in 70% of the osteochondral defects filled with EAC of both the agarose group and HA-liposome group there was bonding of the graft to the adjacent cartilage as defined in the O'Driscoll score (12) (Fig. 3B). An important result was the absence of calcification or hypertrophy in the implanted IVB cartilage in the region facing the joint. Penetration of the joint surface by (hypertrophic) subchondral bone was only observed in the empty defect group (Fig. 3B, Fig. 4B and D). The average O'Driscoll scores for cartilage repair were 12.1 and 9.8 for HA-liposome and agarose groups, resp., and were statistically significant ( $p = 0.001$  and  $p = 0.003$ , resp.) in comparison to the empty defect (average repair score 4.5; Fig. 3C). In 44% (four of nine cases) for the HA-liposome group and 38% (three of eight cases) for the agarose group the subchondral bone in the defect area was repaired up to the level of the surrounding subchondral bone. A thick layer of incompletely differentiated/remodeled mesenchyme was observed in 33% (three of nine cases) in the HA-liposome group, and 50% (four of eight cases)











**Fig. 4.** (A–B) Alizarin red staining with hematoxylin counter stain. (A) IVB cartilage graft engineered by using agarose gel, 9 mo post implantation. Note the absence of calcification in the cartilage layer, calcification of the underlying bone is indicated by an Asterisk. Magnification: 200X. (B) An empty defect that shows subchondral bone protrusion with intense alizarin red staining. Magnification: 200X. (C–E) Thionine staining of implanted IVB cartilage graft engineered using (C) HA-TGF- $\beta$ 1/Suramin gel. Magnification: 100X (E) agarose gel (Magnification 100X). Note in C and E the incompletely differentiated mesenchyme and a superficial layer with more pronounced thionine staining can be distinguished (indicated by Arrows). (D) Penetration of hypertrophic subchondral bone in the empty defect and into surrounding cartilage layers indicated by the Arrow marked with an Asterisk, the levels of the original joint cartilage is indicated by an Arrow. Magnification 50X

and TGF $\beta$ 1 (30), would further enhance cartilage formation. On the contrary, the introduction of PRP resulted in complete elimination of cartilage formation in all IVB's studies (0/6) (Table S1 and Fig S1). This is a surprising finding because platelets of a hematoma locally supply chondrogenic/osteogenic growth factors leading to callus formation (30). This implies that the role of agarose in cartilage induction within the IVB is multifactorial and involves subtle changes to biochemistry of the IVB microenvironment. Because agarose gel by itself was never investigated in the osteochondral defect, further studies are required to ascertain if agarose alone can promote chondrogenesis in a non-ectopic site. This could shed more light on the factors impacting agarose-mediated chondrogenesis in the IVB.

Although a robust comparison between cartilage formation in the presence of agarose gel and HA Gel supplemented with TGF- $\beta$ 1/Suramin may be hampered by the delayed chondrogenic response in the IVB when filled with the former, and the sampling strategy for IVB cartilage harvest for transplantation, the volume of cartilage generated, the biochemistry of the cartilage as assessed by the expression of COL2, and its repair potential in an osteochondral defect, appeared very similar. The volume of cartilage generated using the IVB strategy is a variable that needs to be optimized. However, based on the cross-sectional area (Table S1) the rough estimate of the volume of the cartilage engineered within the IVB is in the range of 7.8–19 mm<sup>3</sup>. This is greater than two times the volume of the cartilage covering the entire condyle (~3.5 mm<sup>3</sup>). However, further studies are ne-

cessary to ascertain if such relative cartilage volumes can be generated in a reproducible manner in humans. An interesting aspect of the EAC generated within the IVB is that it is hypercellular and resembles the first chondrogenic phase that precedes endochondral ossification (23, 31). We postulate that the hypercellularity is the key for in vivo remodeling of the EAC. It is reasonable to assume that attaining cellular homeostasis within the IVB cartilage implant might involve cell death, a process that is known to release signaling molecules. However, the extent to which cell death impacts remodeling and integration of the hypercellular IVB cartilage at the site of implementation needs to be further investigated.

Transplanting the EAC into an osteochondral defect appears to trigger differential responses in the graft, that is, the cartilage facing the bone ossifies, whereas ossification of cartilage that is subject to the influence of mechanical forces within the joint and factors in the synovial fluid appears inhibited. Whereas integration with the host bone was found in all cases, bonding to the adjacent cartilage could be further improved, by treatment of the implant site with highly purified collagenase (32). In addition, histological findings indicate signs of transplanted EAC remodeling into the characteristic architecture of native articular cartilage (Fig. 3B). In contrast to other techniques for osteochondral repair, in which biodegradable scaffolds are used, post-implantation remodeling of EAC within the IVB is not influenced nor hindered by the presence scaffold or its degradation products. Such tissue remodeling is expected to be crucial for optimal osteochondral repair.

This study represents a potentially significant advancement in the clinical options for cartilage repair. The IVB can be created as an out-patient procedure and implemented in two steps. The ability to generate ectopic hyaline cartilage that is capable of remodeling in situ ensures adequate quality of tissue, an outcome not attainable with current options. The EAC is easily harvested, is mechanically strong enough to be press-fit into an osteochondral defect, and, more importantly, undergoes remodeling to yield integration with the host cartilage without any calcification. The optimization of technical aspects surrounding injection of the gel into the IVB, development of gels with tailored physicochemical properties (BioGel), and improved understanding of microenvironmental factors regulating cartilage formation within the IVB is expected to positively impact the success rate, the time of harvest; and accelerate the clinical implementation of this procedure.

## Methods

**Periosteal Cell Isolation and Culture.** Dutch laws on animal experimentation were strictly followed throughout the study and the experimental animal protocol was approved by the Maastricht University committee for animal experiments. Periosteum (7 × 15 mm) of five skeletally-mature, female New Zealand white rabbits was harvested from the upper medial site of both tibia from and cells were isolated as described earlier (20). In brief, after rinsing, the periosteum was incubated for 3 h at 37 °C in a shaking water bath in 5 mL DMEM-Hepes medium containing type II collagenase (300 U/mL) (Invitrogen). Collagenase-treated periosteum was transferred into T25 cell culture flasks and cultured in MEM/D-valine (MEM-DV) (Servichem GmbH), supplemented with 10% FBS, L-glutamine (2 mM), and antibiotics (culture medium) to avoid fibroblast overgrowth (33, 34). Cells were allowed to grow out of the tissue. After 7–10 days the periosteum was removed and at 80% confluency, cells were detached from the flask by using a trypsin/EDTA solution (Invitrogen), and subcultures were continued in monolayer in (MEM-DV) (Servichem GmbH), supplemented with 10% FBS, L-glutamine (2 mM), and antibiotics. The medium was refreshed three times a week until sufficient cells were obtained for differentiation experiments.

**Hypoxia and Periosteal Chondrogenesis.** At passage two, expanded cells were transferred to a hypoxic culture chamber (MACS VA500 microaerophilic workstation, Don Whitley Scientific). One-half of the expanded cells of each rabbit were allowed to differentiate in monolayer under normoxic (21% O<sub>2</sub>) and the other half under hypoxic conditions (0.2% O<sub>2</sub>) using DMEM/F12 supplemented with insulin/transferrin/selenium (ITS) and 10ng TGF- $\beta$ 3/mL for up to 3 d. The composition of the atmosphere in the chamber consisted of 0% H<sub>2</sub>, 5% CO<sub>2</sub>, 0.2% or 21% O<sub>2</sub>, and residual N<sub>2</sub>. Oxygen monitoring confirmed

that the desired oxygen levels remained stable during culturing, mRNA, and protein isolation.

**Preparation of Agarose Gels.** A 2-wt percent agarose solution was prepared by dissolving 2 g of ultra pure agarose granules (*SI Text*) in 100 ml of 0.9% NaCl followed by steam sterilization. The preparation was warmed to 45 °C in a water bath to liquefy it prior to use.

**In Vivo Engineering of Cartilage.** As stated earlier, all animal studies were carried out with the approval and in accordance with strict guidelines of the committee for animal care at the University of Maastricht. Surgical procedures were performed under general anesthesia using strictly aseptic techniques. Anesthesia was induced by using a mixture of ketamine hydrochloride (35 mg/kg) and xylazine hydrochloride (5 mg/kg) via intramuscular injection. Rabbits were intubated and anesthesia was maintained using inhalation of 2% isoflurane in oxygen. Postoperative pain was managed by the administration of 0.05 mg/kg/day buprenorphine intramuscularly for 2 d. In order to minimize animal numbers, both legs of the rabbits were operated.

The IVB was created as described in the literature with minor modifications (9, 35). Specifically, the periosteum was exposed by incision of the proximal part of the *pēs ancersinus* while keeping the tendon of the *semitendinosus* muscle untouched. The IVB's were filled with either agarose, HA, or HA Gel supplemented with a liposomal formulation containing Suramin/TGFβ1 (HA-liposome) (*SI Text*). In the case of agarose, because the solution was warm, gelation was accelerated by cooling the IVB location with 5 °C sterile 0.9% NaCl. The number of IVB's per group was 19, 20, and 6, for agarose, HA-liposome, and HA groups, resp. The use of pimonidazol to demonstrate hypoxia during periosteal chondrogenesis was not implemented in this study, as a positive pimonidazol staining shortly after surgery may be an effect of the surgery itself.

In order to simulate a clinically realistic situation, during the creation of the IVB, the knee joint was opened medially, the patellar tendon was incised along its length, and a cartilage-only defect 3-mm in diameter was created on the medial condyle by using a sharp dermal biopsy punch. The rationale for this defect-model is that it resembles the clinical situation where patients

with isolated articular cartilage defects are operated weeks to months after the trauma causing the defect (34). The knee was closed with Polysorb 2.0 (Tyco Healthcare). The formation of cartilage within the IVB was followed by palpation and inspection (Fig. 2A). The time-point for cartilage harvest in the HA-liposome group (13 d post injection) and agarose group (20 d post injection) was determined by using palpation (*SI Text*).

**Autologous Cartilage Transplantation.** The osteochondral defects groups were as follows: (i) untreated (left empty) ( $n = 10$ ), (ii) press-fit filled with autologous cartilage from IVB filled 2% agarose IVB's ( $n = 8$ ), and (iii) press-fit filled with autologous cartilage from IVB filled HA- with TGFβ1/Suramin IVB's ( $n = 9$ ). **Cartilage Harvest:** The autologous IVB cartilage was separated from the periosteum by dissecting with a scalpel and the overlying fibrous tissue was dissected to yield a large mass of cartilage (Fig. 3A). From the center of the harvested IVB cartilage, a plug 3-mm in diameter was cored out by using a sharp dermal biopsy punch and the thickness of the plug was measured to determine the depth of osteochondral defect (Fig. 3A). **Cartilage Transplantation:** The cartilage defect on the medial condyle (created during the IVB creation step) was now extended to an osteochondral defect of the same depth as the thickness of the cartilage plug by using an electric powered drill. The IVB cartilage plug was then press-fit as is done in a clinical situation (Fig. 3A). The remaining adjacent cartilage (Fig. 2B) was processed for immunohistochemistry/histology and RT-qPCR analysis. **Excision of implanted cartilage:** Nine mo after implantation, the rabbits were euthanized with an overdose of Pentobarbital. The femoral condyles and upper medial tibia (at the site where the IVB was located) were harvested and fixed in 4% paraformaldehyde for histomorphometric analysis and scoring. Please see *SI Text* for staining protocols, RT-qPCR, western analysis, and statistical analysis.

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