In vivo potency assay for the screening of bioactive molecules on cartilage formation

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Abstract

Cartilage regeneration is a priority in medicine for the treatment of osteoarthritis and isolated cartilage defects. Several molecules with potential for cartilage regeneration are under investigation. Unfortunately, in-vitro chondrogenesis assays do not always predict the stability of the newly formed cartilage *in vivo*. Therefore, there is a need for a stringent, quantifiable assay to assess in vivo the capacity of molecules to promote the stable formation of cartilage that is resistant to calcification and endochondral bone formation. We developed an ectopic cartilage formation assay (ECFA) that enables to assess the capacity of bioactive molecules to support cartilage formation *in vivo* using cartilage organoids.In this assay, articular chondrocytes from human donors or animals are injected either intramuscularly or subcutaneouslyin nude mice. As early as two weeks later, cartilage organoids can be retrieved. The size of the implants and their degree of differentiation can be assessed by histomorphometry, immunostainings of molecular markers and real-time PCR. Mineralization can be assessed by micro CT or by staining. The effects of molecules on cartilage formation can be tested following the systemic administration of the molecule in mice previously injected with chondrocytes, or after co-injection of chondrocytes with cell lines overexpressing and secreting the molecule of interestHere we describe the ECFA procedure, including steps for harvesting human and bovine articular cartilage, isolating primary chondrocytes, preparing overexpression cell lines, intramuscular injection of the cells and retrieval of the implants. This assay can be performed by technicians and researchers with appropriate animal-training, including PhD students, within 3 weeks.

Introduction

Cartilage loss is the main cause of chronic disability in arthritis and after joint trauma. Osteoarthritis alone, the most common form of arthritis, affects the majority of people over the age of 65 and costs between 1 and 2.5% of the gross domestic product (GDP) in developed countries[1]. In spite of the high prevalence and impact on patients' quality of life, no effective treatments for osteoarthritis are available and patients ultimately require prosthetic joint replacement. Although a low degree of inflammation and bone changes are also present, cartilage loss is the main feature of osteoarthritis [2.]

Cartilage loss can be caused by a number of different factors, including inflammation and acute trauma, which can further lead to chronic degeneration[3]. In spite of the heterogeneity of causes, the mechanisms that result in cartilage loss are surprisingly uniform. These events include ectopic hypertrophic differentiation[4] (similar to that taking place during endochondral bone formation) mineralization[5], apoptosis[6], and activation of proteolytic enzymes including metalloproteinases (which cleave collagens and aggrecan) and aggrecanases (which specifically cleave aggrecan)[7].

Once the joint is severely compromised, the only way to reduce pain and improve mobility is prosthetic joint replacement [8,9]. One exception is localized joint defects in otherwise healthy knees. These lesions can be treated with either bone marrow-stimulating techniques (e.g. microfracture) [10], or Autologous Chondrocyte Implantation (ACI)[11], in which chondrocytes obtained from a low weight bearing area of the joint are expanded *in vitro* and re-implanted into the lesion. Although both techniques are in general efficacious, the outcomes are variable and around 20% of cases fail[12]. The repaired cartilage generated after bone marrow stimulating techniques is often short-lasting[13], and although ACI has demonstrated very good long-term outcomes, the procedure is costly and laborious. Therefore, the identification of other pharmacological or non-pharmacological approaches for cartilage repair is desirable.

Development of the protocol

Here we describe a cartilage formation assay which combines the clinical relevance of using human cells and the biological relevance of *in vivo* settings. This assay is simple to perform and analyze, while providing abundant qualitative and quantitative readouts. Importantly, this assay allows to distinguish between the formation of the transient epiphyseal cartilage (cartilage destined to undergo chondrocyte hypertrophy, calcification and to be eventually replaced by bone), from the formation of healthy, stable articular cartilage (non-calcified, aneural, avascular cartilage that enables frictionless motion) (Fig. 1)[14].

We initially developed a model of cartilage formation in immunodeficient nude mice with the purpose of generating a potency assay for cell products to be used in ACI. The rationale was that we were aware that culture expansion, necessary for obtaining enough chondrocytes from a biopsy, is associated with a rapid downregulation of chondrocyte differentiation markers[15]. We argued that this progressive de-differentiation could be associated with a loss of the capacity to form stable cartilage once implanted *in vivo*. Therefore, we predicted that culture expansion might be associated with a progressive loss of potency/efficacy of chondrocyte preparations for ACI.

To test this hypothesis, we took advantage of the unique capacity of articular chondrocytes to retain a remarkable phenotypic stability and form cartilage organoids when injected in suspension, in the absence

of a scaffold, within the muscle of immunodeficient nude mice[15]. The newly-formed cartilage (retrievable as early as 2 weeks post-injection) remained stable for at least 6 months without evidence of vascular invasion and endochondral bone formation, and expressed markers and histological features of hyaline-like articular cartilage[15]. The phenotypic stability of the cells injected in the absence of a scaffold was exclusive to primary chondrocytes. Chondrogenic cell lines and mesenchymal stem cells (MSCs) failed to produce any detectable cartilage implant, even when pre-differentiated *in vitro* in pellet culture[16].

The site of injection was important. When injected without a scaffold, implants were retrieved after intramuscular injection, but not after subcutaneous injection. In those early experiments, we chose to inject the cells in the absence of a scaffold because the composition of the scaffold itself influenced cellular differentiation. For instance, when primary human articular chondrocytes were implanted subcutaneously within Collagraft© scaffolds (collagen type I, hydroxyapatite and tricalcium phosphate) they differentiated towards the osteogenic lineage and formed ossicles[17]. The cell type was also important. Articular or tracheal chondrocytes resulted in stable hyaline, articular-type cartilage, whereas no implant could be retrieved after injection of human costal chondrocytes (unpublished data F Dell'Accio). Epiphyseal-like cartilage with evidence of vascular invasion and bone formation was retrieved after the injection of primary porcine epiphyseal chondrocytes of a pig fetus (Fig. 1). The cartilage forming capacity was shared by human[15], porcine[18], bovine[14] and goat[19] primary articular chondrocytes. The number of cells to inject was chosen as the minimum number that consistently resulted in the retrieval of an implant where duplicate injections of primary chondrocytes were performed.

Importantly, in this assay, both human and porcine chondrocytes rapidly lost their capacity to form cartilage with passaging. After only 4 population doublings, implants were still retrieved after cell injection but they displayed inferior differentiation potential; and after 8-10 population doublings, no implant could be detected[15]. We therefore identified molecular markers that, when measured before implantation, predicted the outcome of the ECFA[15]. These markers were used to generate the ChondroCelect® (CC) score[13] which was utilized as a quality control for efficacy of chondrocyte preparations before implantation in patients with cartilage defects. A subgroup analysis revealed a correlation between the CC score before implantation and the Knee injury and Osteoarthritis Score (KOOS) in patients [20]. Therefore, the ECFA predicted good clinical outcome in a prospective multicenter study[20].

Subsequently, we set out to use the ECFA as a screening and potency assay for bioactive molecules to inducing cartilage repair/regeneration. Whereas chemical compounds would easily be administered to the mice to study their effects on cartilage formation, the delivery of proteins within the cartilage implant in a sustained manner and at sufficient doses was challenging. Direct injection of proteins into avascular cartilage organoids would not be uniformly retained within the organoid and would largely diffuse into the surrounding muscle. Adenoviral transduction only achieves a burst of expression that is too short for the duration of the assay[21]. Lentiviral transduction, if performed directly on the chondrocytes, would require in vitro expansion and sometimes selection of stable transfectants which may cause loss of the cartilage formation capacity. We circumvented these problems by injecting non-manipulated chondrocytes together with a cell line that had been engineered to express the ligand of interest and subsequently growth-arrested with mitomycin C[14,18]. This approach has several advantages: first, the

chondrocytes do not undergo any manipulation that might affect their biology; second the mitomycin C treatment efficiently arrests growth but preserves the secretion of the transgene in a biologically active form for at least 3 weeks[14,18]; finally, the growth-arrest prevents plasmids from being diluted through cell division and therefore the cell lines constantly produce the protein under test, even when the cells were transiently transfected[14]. This method allowed us to study the effect of WNT3A and Agrin on the capacity of human, porcine and bovine chondrocytes to form stable cartilage *in* vivo.

Applications of the method

The ECFA can be used for the following applications:

- As a quality control/potency assay for cell preparation for cartilage repair (ACI), including the effect of passaging and in-vitro manipulation on the capacity of cell preparations to form cartilage *in vivo*.
- To screen/validate the capacity of bioactive molecules to induce cartilage formation *in vivo.*
- To study the effects of different scaffolds on cartilage formation/differentiation/homeostasis.
- Gain and loss of function of intracellular molecular pathway investigations can be performed using transfection/transduction, RNA interference and CRISPR technology prior to implantation of the articular chondrocytes

Advantages and limitations

Advantages

The ECFA enables to perform efficacy studies, pharmacodynamics and pharmacokinetics studies on human cartilage organoids in vivo. Furthermore, the ECFA allows to distinguish stable cartilage formation from the formation of transient cartilage destined to be replaced by bone such as that induced by bone morphogenetic proteins[22,23]. Given its reproducibility and the possibility to implant multiple samples on the same animal, the ECFA does not require a high number of experimental animals.

Limitations

This assay does not enable the study of the interaction between cartilage and other joint tissues such as the subchondral bone and the synovial membrane, for these reasons, joint biomechanics cannot play a role in this system. A large number of primary chondrocytes are required, which can be a restriction, particularly when using human cells or multiple treatment groups. A certain degree of donor variability must be expected, particularly when using human chondrocytes. Therefore, it is recommended to pool chondrocytes from multiple donors or to repeat the experiment at least twice, with at least 6 replicates per group. Chondrocytic cell lines or MSCs do not form a cartilage implant in this assay, but this limitation

could be used as a tool to test whether growth factors/molecules/culture conditions can induce cartilage formation in MSCs.

Alternative methods

Preclinical models are needed to identify and optimize strategies to preserve and restore cartilage integrity These models are used essentially for three purposes: firstly, the screening of molecules that promote cartilage formation; secondly, the validation of such molecules in more complex systems and in disease models; and thirdly to assess the capacity of cell preparations to form stable cartilage *in vivo* and their suitability for cartilage tissue engineering.

In vitro models

Available in vitro models include:

- Monolayers of primary cells: Cell lines and MSCs of human or murine origin are a convenient system for high and medium-throughput studies to identify molecules/targets/drugs/conditions that either enhance the capacity of chondrocytes to produce cartilage-specific extracellular matrix (ECM) or to prevent the catabolic effect of inflammatory cytokines[24,25].
- Three-dimensional cultures: Pellets, micromasses, hydrogel or scaffold cultures of chondrocytes/MSCs models are characterized by a much higher degree of differentiation of the cells and result in the in vitro formation of cartilage tissue[26,27].
- Cartilage explants: Ex-vivo cartilage explants have the advantage of retaining the original architecture of the tissue and allow for measuring effects of molecules and treatments on cartilage ECM turnover[28].

Readouts of the *in vitro* methods include histological analysis using strongly cationic dyes[29,30], which at low pH specifically stain cartilage glycosaminoglycans (GAGs), gene expression analysis and reporter assays[18].

In vitro models present several limitations. The results obtained from any *in vitro* system are highly affected by culture conditions. For instance, the presence of serum is required for the cellular response to some growth factors such as WNT proteins[31], but serum masks the effect of other growth factors, such as bone morphogenetic proteins (BMPs)[32]. Cells lines and primary cells differ in their biological response to stimuli. Primary cell types can vary in their response to stimuli depending on passage number or due to donor-related differences including age, genetic factors and degree of osteoarthritis[15,33].

In vitro systems cannot fully predict whether any compound/molecule will result in terminal differentiation of chondrocytes and whether they will promote vascular invasion and mineralization. For instance, Serafini et al.[34] demonstrated that although bone marrow MSCs can form non-mineralized, hyaline-like cartilage *in vitro* in presence of transforming growth factor (TGF)-β, when the cartilage pellets are implanted *in vivo* in nude mice they undergo vascular invasion, mineralization and are ultimately replaced by bone[34].

Finally, the use of *in vitro* systems separates the chondrocytes from the other tissues of the joint. Consequently, the contribution of the synovial membrane and subchondral bone on the effects of the stimulation cannot be tested. In vitro models also do not take into account joint biomechanics.

In vivo models

Once novel potential pharmacological targets have been identified *in vitro*, they need to be validated *in vivo*, typically in animal models of acute and chronic cartilage breakdown. Several inflammatory and non-inflammatory models of arthritis have been developed:

- Osteoarthritis can be induced surgically in animal models by destabilizing the joint through resection (meniscus ligament injury model[35]) or destabilizing the medial meniscus and/or ligaments[36], by injecting proteolytic enzymes such as collagenase[37] or toxic substances that kill chondrocytes (e.g. monoiodoacetate)[38]. Alternative methods to study osteoarthritis include spontaneous ageing[39] and high fat diet models [40].
- Inflammatory arthritis is typically induced by triggering autoimmunity[41].
- Acute cartilage defects can also be surgically generated to study either spontaneous healing or the efficacy of tissue engineering approaches for cartilage regeneration[42].

The undisputed advantage of such *in vivo* models is that cartilage biology can be studied in the context of the whole joint, which may produce further-reaching effects in other areas of the body. These *in vivo* models also allow the quantification of cartilage damage, bone changes and inflammation in the synovial membrane using standardized, reproducible and universally accepted scoring systems[43].

However, these in vivo models present several limitations. The use of animal models assumes a high degree of conservation of molecular mechanisms across species. It is therefore essential to verify that the affinity/specificity/activity of tested ligands/compounds in the animal are comparable to those assessed in human before starting the experiment. By contrast, the ECFA allows to study efficacy in human cartilage tissue *in vivo.* Most of these *In vivo* experiments are costly, labor intensive and time consuming (a typical experiment often requires up to 6 months for a complete analysis). Compared to disease models of osteoarthritis or cartilage defects, the ECFA is rapid, simple and relatively inexpensive.

Experimental design

Here, we describe the main variant of the ECFA assay, in which chondrocytes are delivered in the mice via intramuscular injection into the posterior compartment of the thigh, in the absence of scaffolds. Molecular markers identified through the use of this variant of the assay predicted the clinical outcome of patients treated with ACI[13,20,44], providing strong evidence that ECFA can be used as a surrogate potency assay. We are also describing the delivery of bioactive proteins to the injected chondrocytes by co-injection of a growth-arrested transfected cell line. We believe that this technology will be particularly useful to scientists who want to assess the function of growth factors and matrix molecules *in vivo* on human chondrocytes. For simplicity, a flow chart summarizing the main steps of the protocol is described in Fig. 2. Briefly, cartilage is harvested from a donor (human/bovine/porcine origin) and the chondrocytes are isolated. Meanwhile, a cell-line over-expressing a protein of interest is growth arrested. The

chondrocytes and the protein-delivering cells are co-injected into the muscle of a nude mouse. Two weeks later, the organoids can be retrieved for analysis.

Choice of mice

It is essential to use CD1-Foxn1^{nu} mice because, in our experience, this nude mouse strain gives the best and most consistent results. Although, in our experience, the sensitivity of the assay is not significantly affected by the age and sex of the animals, female mice are preferred because they are less likely to fight. For the intramuscular injection, the age of the mice does not affect the outcome and therefore younger mice may be used to reduce maintenance of genetically modified mice in line with the 3R principles. For the subcutaneous injection on the back of the mice, instead, 8 week-old mice are preferred to younger mice because their larger size allows for more space between implants, thereby avoiding that the implants merge.

Experimental unit

The individual implant should be considered as an experimental unit. Cage, individual mice and the donor (when chondrocytes are obtained from different donors) can be used as a blocking factor or co-variates in the statistical analysis.

Management of possible confounders

Treatments should be randomized in each cage and for each cell donor. If the treatment is a genetic modification, such as a gene being deleted using CRISPR directly in the chondrocytes, and cannot diffuse systemically (not an over-expressed protein), it is advisable to randomize treatments also within each mouse. If the ligand/treatment is likely to diffuse, each mouse should host injections with the same treatment.

Blinding is essential at the stage of injection, health scoring, dissection, sample processing and data collection.

Inclusion and exclusion criteria

Mice displaying signs of suffering either locally (e.g. skin lesions, erythema or ulcers) over the site of injection or systemically (e.g. weight loss >20% body weight) must be excluded from analysis. Samples where dissection or processing, including sectioning or staining, is not optimal should be excluded *a priori* from the analysis.

Statistical analysis

The statistical approach will vary depending on the readout and the experimental design. In the simplest case, when the readout is interval and meets the necessary assumptions, a t-test (two groups) or analysis of variance (ANOVA) (multiple groups) are recommended. If the readout is not interval or the data do not fulfil the requirements for an ANOVA, a non-parametric test is necessary. Under such circumstances, building a generalized linear model (glm function in R language) followed by comparison of the estimated

marginal means (R package emmeans) is often useful as it allows dealing with data distributions other than normal and to build covariates in the model.

Consistency and power calculations

When comparing the percent of the implant area positively stained with a metachromatic staining (safranin O or toluidine blue)[14,45] and using a Student t-test, the detection of a 1.5 fold change in the means with a power of 0.8 and a significance level of 0.05 will require 8 implants (4 mice) using the direct injection of 5 million chondrocytes into the muscle of the thigh or 6 implants (1 mouse) when using the implantation of 1 million chondrocytes in a collagen gel subcutaneously on the back (Table 1).

Importantly, to reduce variability and bias due to the host (individual mouse) if the treatment is a genetic modification and cannot diffuse systemically, it is advisable to randomize treatments within each mouse. If the treatment is likely to diffuse this will not be possible, and, when using the miniaturized system with pellets implanted subcutaneously[45], it is advisable to have at least 2 mice per treatment with 4-6 implants each, ideally around 8 weeks of age to allow more space between the implants.

Experimental controls

The choice of the experimental controls will vary depending on the purpose of the experiment. If the scope of the experiment is to assess the chondrogenicity of a certain cell preparation, it is useful to include dermal fibroblasts as a negative control and primary (freshly isolated or P0) articular chondrocytes as a positive control. If the scope of the experiment is to assess the effect of a certain signaling molecule -using mitomycin C growth-arrested transfected cells, then GFP transfection is recommended as a negative control for the co-injected cell line. If viral delivery is used, then GFP, empty vector or a scrambled transgene could be used as negative controls.

De-differentiation of chondrocytes in culture

Articular chondrocytes progressively lose their phenotype when cultured in standard conditions[15]. De-differentiated cells do not form cartilage implants upon injection in suspension[15]. Regardless of the species of origin, chondrocytes exceeding 4-6 population doublings will not form a cartilage implant upon injection in suspension.

Choice of cell-line to co-inject

To achieve constant stimulation of the chondrocytes *in vivo*, chondrocytes can be co-injected with a cell-line over-expressing and secreting a molecule of interest. The ideal cell line will express low endogenous levels of the molecule being tested to maximize the difference with control cells, which is the same cell line transfected with a control vector. The control vector is usually a GFP or other fluorescent marker expressing plasmid, which will also allow the determination of the transfection efficiency of the cells prior to the injection. High transfection efficiency (90-100%) is required, particularly when using transient transfection. We have successfully used COS7 cells for this purpose[14]. We have also used commercially available, stably transfected L-cells[18] and obtained comparable results.

Growth inhibition of cell lines

To avoid the formation of a tumor alongside the cartilage implants, we growth-arrest the cell line overexpressing the protein of interest prior to co-injection *in vivo* using mitomycin C, a commercially available proliferation inhibitor. The concentration of mitomycin C needs to be optimized for different cell lines. For COS7 cells and L-cells, a 2 hour treatment with 7.5 µg/ml mitomycin C was sufficient[14,46]. Some cell death upon growth inhibition is unavoidable and usually 10-20% of the cells die upon mitomycin C treatment. This cell loss should be taken in account when culturing the cells, to ensure the appropriate numbers of viable cells are available for the co-injection with the chondrocytes. If the cell death is >20%, the concentration of mitomycin C must be further optimized. In parallel to the ECFA assay, an aliquot of growth-arrested cells should be kept in culture to monitor the inhibition of their growth over time. Finally, given that mitomycin C can compromise the production and secretion of the molecule under investigation, in our experiments, we used reporter assays[18] and immunofluorescence[14] to test the bioactivity of the molecule of interest following growth inhibition.

Materials

Biological materials

- Human cartilage: joint tissue removed during total knee replacement surgery**. !CAUTION** Informed consent must be obtained from all subjects' prior collection of the tissue for research purposes. Procedures must be performed in accordance with institutional review board policies for obtaining human tissue, including informed consent by personnel certified and trained to work with blood-borne pathogens. **!CAUTION** human samples must be treated with maximum caution as they have the potential to carry blood-borne infections. All procedures involving human tissue should be performed at biosafety level 2 with appropriate personal protection.
- Bovine lower limbs cut through the carpal/tarsal bones (including metatarsophalangeal/metacarpophalangeal joints) of freshly slaughtered 6-24 month old steers (sourced from local abattoirs).
- Three/four week old female CD1-Foxn1^{nu} mice (Charles River, strain 086) for the intramuscular assay. Mice are usually injected one week following their arrival to the animal facility. **CAUTION!** All animal studies must be reviewed and approved by the relevant institutional animal care and use committees and must conform to all relevant ethics regulations. All the animal work described in this manuscript was done in accordance with relevant guidelines and regulations/ Local Ethics committee and the UK Home Office. Our research was executed under UK Home Office Project License awarded to FDA and Ethics committee approval.

Reagents

- DMEM/F12 with GlutaMAX™ supplement (Thermo Fisher Scientific, cat. no. 31331-028)
- Fetal bovine serum (FBS) (Thermo Fisher Scientific, cat. no. 10270-106)
- Antibiotic-antimycotic 100X (Thermo Fisher Scientific, cat. no. 15240-062)
- Sterile phosphate buffered saline (PBS, Lonza, cat.no. LZBE17-516F12)
- Trypsin (Thermo Fisher Scientific, cat. no. 25200-056)
- Pronase (Roche, cat. no. 11459643001)
- Collagenase P (Roche, cat. no. 11213873001)

- Mitomycin C (Sigma-Aldrich, cat. no. M4287) **CAUTION!** Mitomycin C inhibits DNA replication. Use appropriate personal protective equipment to avoid exposure. **Δ CRITICAL** Mitomycin C is light sensitive and is rapidly degraded in acidic solutions (pH <6). The reagent progressively loses activity with time. Store filter sterilized stock solution at -20C° for up to 6 months.
- Industrial methylated spirits (IMS, Thermo Fisher Scientific, cat. no. 10552904)
- Paraformaldehyde (PFA, Sigma-Aldrich, cat. no. 76240) **CAUTION!** Highly toxic by inhalation. Wear a mask and work in a fume cabinet when handling PFA.
- Trypan blue (Sigma T8154-20ML)

- NaOH
- HCl
- Distilled water
- Acetic acid (BDH, cat.no.100012K)
- Sodium acetate (Sigma, S-2889)
- Ethanol

Equipment

- Laminar flow hood
- Tissue culture incubator (37 °C, 5% CO2, 95% humidity)
- 10 cm petri dishes (Sigma, cat. no. CL430591-500EA)
- Tissue culture flasks and plates appropriate for your experiment
- Centrifuge tubes, 15 ml and 50 ml (Starlab, cat. no. E1415-0200 and E1450-0200)
- Orbital shaker
- Benchtop centrifuge
- Hemocytometer
- Cell strainer, 40 µm (Falcon, cat. no. 352340)
- Glass Pasteur pipettes
- Pipetboy
- Gilson pipettes and sterile filter tips
- 1.5 ml microcentrifuge tubes
- Sterilizing syringe filters, 0.22 µm and 0.45 µm (Sartorius Stedim UK Limited, cat. no. 16534K and 16555K)
- Vacuum filter, 250 ml (Appleton Woods, cat. no. BC502)
- Filter paper
- Syringes, 1 ml (Fisher Scientific, cat. no. 10142104) and 20 ml (BD, cat.no. 300613)
- Needles, 26G and 30G (BD, cat. no. 300300 and 304000)
- Surgical equipment (Regular and fine-tipped surgical forceps; Regular and fine surgical scissors)
- Isolator cages
- Scale
- Scalpel handle, 4G/S (Swann-Morton, cat.no. 0914)
- Scalpel blades, 23 (Swann-Morton, cat.no. 0510)
- Parafilm (SciMart, cat.no. HS-234526C)
- Tissue paper

Reagent Setup

Isolation and culture of primary chondrocytes: complete medium

Prepare complete medium that contains DMEM/F-12 Glutamax, 10% fetal bovine serum, 1% antibiotic-antimycotic solution (final concentrations: 100 units/ml of penicillin, 100 µg/ml of streptomycin and 0.25µg/ml of amphotericin B). Prepare under sterile conditions and store at 4°C in the dark.

Isolation and culture of primary chondrocytes: 2XAA complete medium

Prepare the medium as above but with 2% antibiotic-antimycotic solution.

Isolation and culture of primary chondrocytes: pronase digestion medium

Dissolve pronase (7U/mg) at a final concentration of 1 mg/ml in complete medium. Five milliliters of digestion medium are required per gram of articular cartilage. Sterilize by filtration through a 0.22 µm syringe filter before use.

Isolation and culture of primary chondrocytes: collagenase P digestion medium

Dissolve lyophilized collagenase P (1.7 U/mg) at a final concentration of 1 mg/ml in complete medium. Five ml of digestion medium are required per 1 gram of articular cartilage. Sterilize by filtration through a 0.22 µm syringe filter before use.

Preparation of donor cell line: *Mitomycin C –stock solution*

Dissolve the reagent in distilled water to a final concentration of 0.5 mg/ml and sterilize by using a 0.22 µm syringe filter. Store at -20°C.

Preparation of implants for histological characterization*: 4% PFA*

Dissolve PFA in distilled water to a final concentration of 4% (w/v). **Δ CRITICAL** PFA is only soluble in alkaline conditions: to facilitate the complete dissolution of the PFA add 100 µl 10N NaOH/100ml of water and heat to 60 °C. Cool down to room temperature and neutralize the solution with 1 ml 1N HCl /100ml solution. Filter the solution using a vacuum filter to avoid the presence of residues. **!CAUTION** PFA is highly toxic by inhalation: weigh, solubilize and filter the solution in a fume cabinet. Store at 4C°.

Procedure

Harvesting of articular cartilage Timing 3 h

1. Source human or animal joints from hospital, abattoir, butchers or animal facility.

Δ CRITICAL STEP Always use fresh samples and not frozen material.

!CAUTION Appropriate national laws and institutional regulatory board guidelines must be followed for the procurement of human cartilage, including informed consent obtained from human subjects, use and storage of human biologic material. Our research was conducted in compliance with our local Ethics Committee approval.

2. To harvest primary chondrocytes, follow option A for bovine chondrocytes and option B for human chondrocytes. Other species can be utilized in this assay if required.

(A) Harvesting of bovine articular cartilage

i. Place the limb in lukewarm water and soak for 1 h before scrubbing thoroughly to remove dirt.
ii. Immerse the limb in a bucket filled with 70% IMS for 15 min.
iii. Remove the limb carefully from the bucket to avoid spilling IMS, dab dry.
iv. Spray the limb thoroughly using 70% ethanol.
Δ CRITICAL STEP The following steps should be carried out in a sterile laminar flow hood.
!CAUTION A pair of autoclaved chainmail gloves are recommended for safety.
v. Cut longitudinally along the skin covering the anterior part of the limb along the metatarsal/metacarpal bone using a scalpel (Fig. 3a-b).
vi. Make a perpendicular horizontal cut along the metacarpo/metatarsophalangeal joint line (Fig. 3c-e).
vii. Peel the skin away from the joint to expose the joint capsule (Fig. 3f-i).
viii. Spray the exposed intact joint capsule with 70% ethanol to remove any hair from the capsule surface.
Δ CRITICAL STEP If the capsule has been opened accidentally do not spray ethanol inside.
ix. Spray the intact capsule three times thoroughly with PBS.
Δ CRITICAL STEP Change scalpel blade now to ensure sterility within the joint.
x. Using the joint line as a guide, cut the capsule perpendicularly (Fig. 3j-k).
? TROUBLESHOOTING
xi. Cut the ligaments connecting the joint surfaces to completely open the joint (Fig. 3l-n).
Δ CRITICAL STEP Change scalpel blade now to ensure sterility of the cartilage surface.
xii. Slice full thickness cartilage from the exposed proximal metacarpal/metatarsal surface (Fig. 3o-p).
xiii. Using sterile forceps, place the sliced cartilage in a 10 cm petri dish containing 2XAA complete medium.
Δ CRITICAL STEP each joint yields approximately 50-100x10^6 cells. Repeat the steps for additional joints if more chondrocytes are required.
PAUSE POINT Cartilage slices can be maintained for up to 1 week in normal tissue culture incubator conditions providing that the 2XAA complete medium is changed regularly.

? TROUBLESHOOTING

(B) Harvesting of human cartilage

i. Place human tissue discarded from total knee replacement surgery in a sterile laminar flow hood (Fig. 4 a).
ii. Wash the sample twice with sterile PBS. Discard any bone and synovial tissue (Fig. 4 b).
iii. Carefully dissect the cartilage from the femoral condyles using a scalpel (Fig. 4 c-d).

Δ CRITICAL STEP Upon dissection, keep the cartilage wet with medium at all times to avoid chondrocyte death (See Fig 4b)

iv. Using sterile forceps, place the sliced cartilage in a 10 cm Petri dish containing 2XAA complete medium (Fig. 4 e-f).

PAUSE POINT Cartilage slices can be maintained for up to 1 week in normal tissue culture incubator conditions providing that the 2XAA complete medium is changed regularly.

Isolation of primary chondrocytes Timing 18 h

3. Place the Petri dish containing the cartilage in a sterile laminar flow hood. Carefully cut the tissue into 1x1mm pieces (See Fig. 4f).
4. Remove the 2XAA complete medium, transfer the cartilage to a 50 ml centrifuge tube and add Pronase digestion medium (5 ml/1 g of cartilage) in the tube to start the dissociation of the tissue.
5. Incubate the cartilage on an orbital shaker at ~ 80 RPM at 37°C for 30 min.
6. Remove the Pronase digestion medium and wash the cartilage with PBS.
7. Replace the PBS with collagenase P digestion medium (5 ml/1 gr of cartilage).
8. Incubate the cartilage on an orbital shaker at ~ 80 RPM at 37°C for 16 h.

Δ CRITICAL STEP Shaking too vigorously can compromise cell viability. Orbital shaking ensures that all the tissue is in constant contact with digestion buffer and allows its complete dissociation.

9. Following digestion, filter the cell suspension through a sterile 45 µm cell strainer set over a 50 ml centrifuge tube.
10. Centrifuge for 10 min at 500 g, 21°C.
11. While carefully avoiding the cell pellet, remove the collagenase digestion medium and replace with complete medium.
12. Count the chondrocytes using a hemocytometer. To test the viability of extracted cells, perform a trypan blue exclusion test.
    Δ CRITICAL STEP Yields vary from donor to donor and are age dependent[47] . Typical yields are >1.8×10^6 cells/gram of cartilage can be considered satisfactory.
13. Use chondrocytes immediately, or seed on a culture dish at density of 1×10^4 cells/cm^2.
    !CAUTION The cells should not be frozen.
    Δ CRITICAL STEP Chondrocytes de-differentiate in monolayer culture. Human chondrocytes progressively lose the ability to form ectopic cartilage *in vivo* with passaging. It is therefore recommended to use freshly isolated chondrocytes to perform the experiment.
    Δ CRITICAL STEP Five million chondrocytes are required per intramuscular injection. Cells from more than one donor can be pooled together to achieve a sufficient amount of chondrocytes to perform all the injections required by the experiment.
    PAUSE POINT Chondrocyte cultures can be maintained in standard culture conditions, without passaging, if required, to collect enough chondrocytes to perform the assay.

Overexpression of gene of interest Timing up to 48 h

14. Overexpression of a gene by a cell line can be achieved by transient (option A) or stable (option B) transfection.

    Δ CRITICAL STEP High transfection efficiency (95-100%) is required, particularly when using transient transfection. **Transient transfection**

    i. Culture and transfect a suitable cell line. We have previously optimized the use of COS7 cells using the transfection reagent JetPrime© (BOX 1)

A) **Stably transfected cell line**

    i. Culture stably transfected cell lines[18].

Growth-arrest Timing 2 h

15. When the transfected cells are 80-90% confluent, remove the complete medium and replace with 0.25 ml/cm^2 culture area of complete medium supplemented with 7.5 µg/ml mitomycin C.

    Δ CRITICAL STEP The concentration of mitomycin has been optimized for COS7 cells and L-cells. Optimization may be required for alternative cell lines[14,18].

16. Incubate the cells with mitomycin C at 37°C for 2 h in standard culture conditions.
17. Remove the medium supplemented with mitomycin C, wash the cells twice with PBS and replace with complete medium.

Confirming growth arrest Timing 48 h+

18. Monitor the mitomycin C-treated cells throughout the duration of the *in vivo* assay to ensure cells remain growth-arrested.

    Δ CRITICAL STEP Following mitomycin C treatment it is important to change the medium regularly to remove any dead cells.

    Δ CRITICAL STEP Sacrifice the animals if the growth-arrest is discovered to be inefficient as the cell lines can promote tumor formation alongside the cartilage implant, especially if overexpressing a growth factor.

    Δ CRITICAL STEP Growth-arrest by mitomycin C treatment may result in changes in protein expression/bioactivity of the overexpressed molecule. This can be assessed by reporter assays, immunocytochemistry, functional assays or western blotting[14,18] (Fig. 5).

    Δ CRITICAL STEP If more than 20% cell death is observed, the concentration of mitomycin C should be re-optimized.

    ? TROUBLESHOOTING

Preparation of cells for injection Timing 1 h

Δ CRITICAL Prepare the cells immediately before injection of the mice.

19. Count the chondrocytes (either freshly isolated or trypsinized upon passaging) using a hemocytometer and aliquot 5 million chondrocytes into individual microcentrifuge tubes.

Trypsinize the protein-overexpressing, growth-arrested cell line and count the cells by using a hemocytometer. Add 500,000 cells to each tube of chondrocytes (final ratio of growth-arrested cells:chondrocytes, 1:10).

20. Centrifuge the microcentrifuge tubes at 500 g for 10 min and replace the medium with 70 µl of sterile PBS.

Cell injection Timing 1 h

!CAUTION Appropriate national laws and institutional regulatory board guidelines must be followed. Our research was executed under UK Home Office Project License awarded to FDA and local Ethics committee approval.

21. Place the mice under a laminar flow hood.

    Δ CRITICAL STEP The mice are immunodeficient: the use of the hood is required to avoid possible contact with pathogen/contaminants.

22. Load a 1 ml syringe with a 26 or 30 gauge needle with the cell suspension.

    Δ CRITICAL STEP It is very important not to aspirate any air. Only aspirate the cell suspension, which will stay in the void volume of the needle and will not be visible in the barrel of the syringe.

23. Hold the mouse with one hand and gently extend one lower limb with the other hand. Inject the cell suspension (from step 21) into the middle of the posterior compartment of the thigh while avoiding the bone (Fig. 6).

    Δ CRITICAL STEP This is a delicate procedure. Do the injection in a single, smooth movement to avoid the penetration of the leg from side to side and any spillage from the syringe. It is preferable that two people work on this step together: one steadily holding the animal while the other injects the cell suspension.

24. Complete injections for all conditions.
25. Monitor the mice for the duration of the assay. Mice with severe signs of suffering should be sacrificed and excluded from analysis.

    Δ CRITICAL STEP: Two weeks is the minimum time required to retrieve cartilage implants. This can be extended for up to 6 months if desired. Sacrifice the animals if any adverse events occur.

Retrieval of the implants Timing 2 h

26. Sacrifice the mice. Refer to local ethical and regulatory guidelines.
27. Remove the skin from the lower limbs of the mice. The implant retrieval site will be in the posterior compartment of the thigh (hamstrings) and is usually palpable although often not visually recognizable. Using a scalpel, identify the margins of the implant, which will be firmer than the surrounding muscle, and remove as much muscle fiber as possible while ensuring not to include any bone in the dissection. Use tweezers to secure the muscle and gentle tether away the muscle fibers using a scalpel.

    Δ CRITICAL STEP The implants may be different shapes and sizes so care must be taken to avoid cutting through the muscle with the blade; rather lightly tether the muscle away to avoid any damage to the implant. The implant will appear translucent in comparison to the muscle fibers.

28. Once the implant has been retrieved, various forms of analysis can be carried out. Basic analysis of the wet weight as well as histomorphometrical analysis (see BOX 2) can be utilized as simple and quick readouts. However, additional informative data can be obtained by real time qPCR and/or immunostaining etc. If both histological and gene expression readouts are desired, cut each implant along its longest axis **to ensure the largest area of each implant can be represented when measuring total area? TROUBLESHOOTING**

Anticipated Results

Two weeks post-injection, hyaline-like cartilage implants can be retrieved from the muscles of the mice[14,15,18]. Once retrieved, the implants may have irregular shape, are usually translucent and may vary in size (between 2-5 mm). We have shown that the co-injection of cells overexpressing anabolic factors can boost the size and weight of the retrieved implants in comparison with controls[14,18]. Sometimes implants fail to form as cells disperse between the muscle fibers upon injection instead of aggregating. This phenomenon is rare (1 non-retrievable implant in every 10-12 successfully retrieved) and unpredictable. It is therefore important to perform an adequate number of injections to gain statistically relevant data.

Articular chondrocytes de-differentiate upon passaging *in vitro* and progressively lose their ability to form cartilage *in vivo*. This typically happens after 3-4 passages for human and porcine cells, whereas bovine chondrocytes retain their phenotypic stability for longer periods of time (unpublished data F Dell'Accio). De-differentiated chondrocytes do persist in the mice at the site of injection, but mostly form fibrous tissue and only a portion of the cells integrate in the murine muscle[17]. This limitation can be capitalized upon to evaluate whether molecules/culture conditions can promote the maintenance of or re-establish the chondrocyte phenotype.

After being excised, the implants can be analyzed macroscopically (e.g. weight and volume) and histologically. The implants do not require de-calcification unless the stimulation is expected to induce ossification. Standard proteoglycan staining such as toluidine blue and safranin O (Box 2) can be performed after sectioning, as well as alizarin red or Masson's trichrome staining, to assess the degree of calcification and vascular invasion respectively[14,15,18]. Sequential sectioning can be used to perform

histomorphometrical analysis[18] with dedicated software (e.g. ImageJ) and immunodetection of relevant targets. If the number of injections that can be performed is limited, the implants can be halved: one part can be dedicated to histological examination whereas the other can be used for gene expression analysis. To ensure sufficient yield and purity, a phenol-based RNA extraction protocol is recommended, as this improves extraction in implants with high proteoglycan content. We successfully retrieved between 500 ng-1µg RNA per half implant. Q-PCR data can be used to assess the level of differentiation by measuring the expression levels of cartilage phenotypic markers such as *COL2A1*, *ACAN* and *SOX9*; of markers of hypertrophy (*COL10A1*), matrix remodeling (*MMP*s and *ADAMTS*) or target genes related to the molecule/conditions tested. Q-PCR analysis can be performed using primers specific to the species of the chondrocytes injected, thereby excluding any contaminating host (mouse) cells from the analysis. To generate species-specific primers it is sufficient to ensure that the last 3 bases at the 3' end of each primer mismatch with the mouse gene.

In conclusion this protocol describes a cost and time-effective cartilage formation assay *in vivo*. The results of this assay are easily analyzed in a quantitative and qualitative manner. The ECFA is a clinically relevant tool to investigate the effect of bioactive molecules on the regenerative capacity of chondrocytes *in vivo.*

Troubleshooting

Troubleshooting advice can be found in Table 2.

Timing

Step*s* 1-2, harvesting of articular cartilage: 3 h

Steps 3-13, isolation of primary chondrocytes: 18 h

Step 14, overexpression of gene of interest: 48 h

Steps 15-17, growth-arrest: 2 h

Step 18, confirming growth arrest: 48 h+

Steps 19-21, preparation of cells for injection: 1 h

Steps 22-26, cell injection: 1 h

Steps 27-28, retrieval of the implants: 2 h

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Author contributions

A.S.T, S.C, G.N. and S.E.E. designed and performed the co-injection experiments, analyzed the data and wrote the manuscript; B.L.T. performed experiments and contributed to writing the manuscript; Y.S. performed experiments; F.P.L. designed the ECFA and contributed to writing the manuscript; F.D.A. designed and developed the ECFA, supervised the co-injection experiments and contributed to writing the manuscript.

Conflict of interest

F.P.L and F.D.A. are co-inventors on patent WO2008061804 (A2): "*In vivo* assay and molecular markers for testing the phenotypic stability of cell populations, and selecting cell populations for autologous transplantation". All the other authors declare not to have any conflict of interests in the publication of this manuscript.

Data availability

There is no big-data shared in this manuscript. The data that support the findings of this study are available from the corresponding author upon request.

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BOX 1 Transfection of cell lines

Additional reagents

- Suitable donor cell lines. Published examples include: Monkey kidney COS7 cells (American Type Culture Collection, cat. CRL-1651) and Murine fibroblasts L-cells (American Type Culture Collection, cat. CRL-2648) **!CAUTION** Cells used should be tested for mycoplasma contamination using a suitable method (ELISA/PCR)
- JetPrime© transfection buffer and transfection reagent (PolyPlus transfection, cat. no. 114-15)
- Mammalian expression vector containing gene of interest (including a control plasmid pcDNA3.1 or pmaxGFP (Amaxa))

Additional equipment

- Fluorescent inverted microscope (Olympus IX81 and CellSens/ImageJ software)

Procedure

1. Culture COS7 under standard conditions until 60-70% confluency is reached.
   Δ CRITICAL STEP Mitomycin C can induce cell death Ensure that a surplus amount of cells are prepared to account for any reduction in cell number after induction of cell growth arrest.
   ?TROUBLESHOOTING
2. Mix 1 ml of JetPrime© buffer, 10 µl of JetPrime© reagents and plasmid DNA (10 µg per million cells) in a microcentrifuge tube. Repeat this step for each expression vector.
   Δ CRITICAL STEP Include in the experimental design a GFP-expression vector (or other fluorescent marker) as a control to evaluate transfection efficiency.
3. Vortex and incubate for 15 min at room temperature.
4. Remove the medium from the cells and replace with 10 ml of fresh complete medium.
5. Add the mixture from step 2 to the cells.
6. Culture the cells with the transfection reagents for 24 h under standard culture conditions.
7. Remove the transfection medium and replace with normal complete medium.
8. Check the transfection efficiency by microscopy on the GFP-transfected cells.
   Δ CRITICAL STEP Do not continue with the experiment if the transfection efficiency is lower than 90%.
   ?TROUBLESHOOTING
9. Maintain the transfected cells in culture for an additional 24 h to monitor transfection efficiency and cell viability.
   Δ CRITICAL STEP Do not continue with the experiment if the cells are no longer viable.

BOX 2 Safranin O staining

Safranin O is a cationic dye that metachromatically stains the negatively charged highly sulfated proteoglycans orange-red. Samples can be either paraffin or cryo-embedded and sectioned at a thickness of 5µm. This protocol describes the Safranin O staining of sections after paraffin embedding.

Additional equipment

- Upright microscope (Olympus BX61 and CellSens/ImageJ software)
- Cover glass for slides (WVR International, cat. no. 631-0880)

Additional reagents

- 100% ethanol
- 100% xylene
- DPX
- Safranin O (Fisher Scientific, cat. No. 12663927)-

Additional reagent set up

Safranin O 0.2% (w/v)

To make 50 ml of 0.2% Safranin O, mix together 41 ml of 0.2M acetic acid with 9 ml of 0.2M sodium acetate buffer and dissolve 0.1 g of Safranin O. Following complete dissolution of the powder, filter the solution with filter paper to remove any residue.

Procedure

1. To facilitate the removal of paraffin, heat the sections lying face up to 40-50°C for 30 min.
2. Transfer the slides to xylene for 5 min, then replace with fresh xylene for a further 5 min.
3. Transfer the slides to ethanol for 5 min, then replace with fresh ethanol for a further 5 min.
4. Rehydrate the slides in distilled water for 10 min, and then air-dry face up for approximately 40 min.
5. Once dry, cover the implant sections in Safranin O for 1 min.
   Δ CRITICAL STEP: The incubation time might require optimization
6. Remove the excess Safranin O by washing twice gently for 5 min in distilled water.
7. Serially dehydrate the sample: fully submerge the slides for 2X 5 min in 100% ethanol followed by 2X 5 min in xylene.

8. Mount the slides with DPX
9. Allow the DPX to set overnight.
10. Images can be taken in brightfield setting using an upright microscope, keeping exposure constant (Fig. 7.

    Δ CRITICAL STEP Digital images must be taken using identical parameters and turning off all the auto-enhancing features of the camera and of the acquisition program. Automatic auto-enhancing settings will alter the measurements. Discard sections with cutting or staining artefacts.
11. Open the image in ImageJ or similar analytical software such as CellSens.
12. Using the manual free-hand tool, demarcate the cartilage area from the host muscle tissue which can be readily identified morphologically[18] (Fig. 7).
13. Calculate the total area within the cartilage mask drawn in step 12.
14. To measure the differentiated area, the Safranin O metachromatic staining can be isolated from the background by thresholding[14,18]. Repeat step 13 after thresholding.

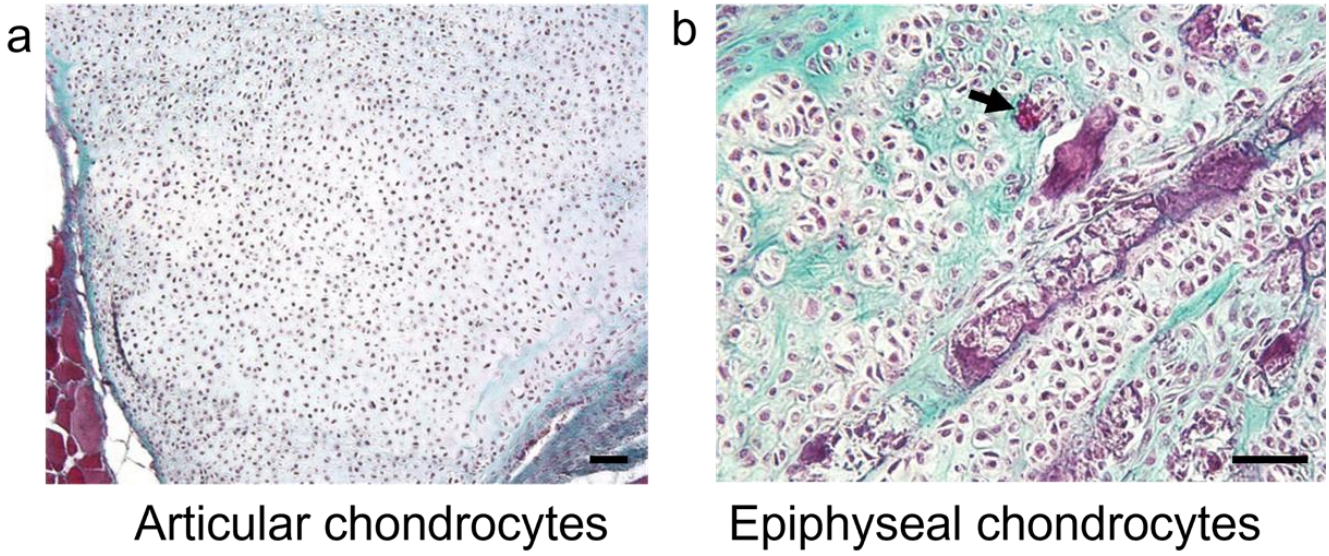

Fig. 1| The ECFA distinguishes between articular versus epiphyseal cartilage formation. a-b Masson's trichrome staining of cartilage implants retrieved from a nude mouse post injection of articular (**a**) or epiphyseal (**b**) chondrocytes isolated from a pig fetus. Injection of epiphyseal chondrocytes results in cartilage implants that undergo vascular invasion and endochondral bone formation (scale bar: 100µm). Arrow indicates blood vessel.

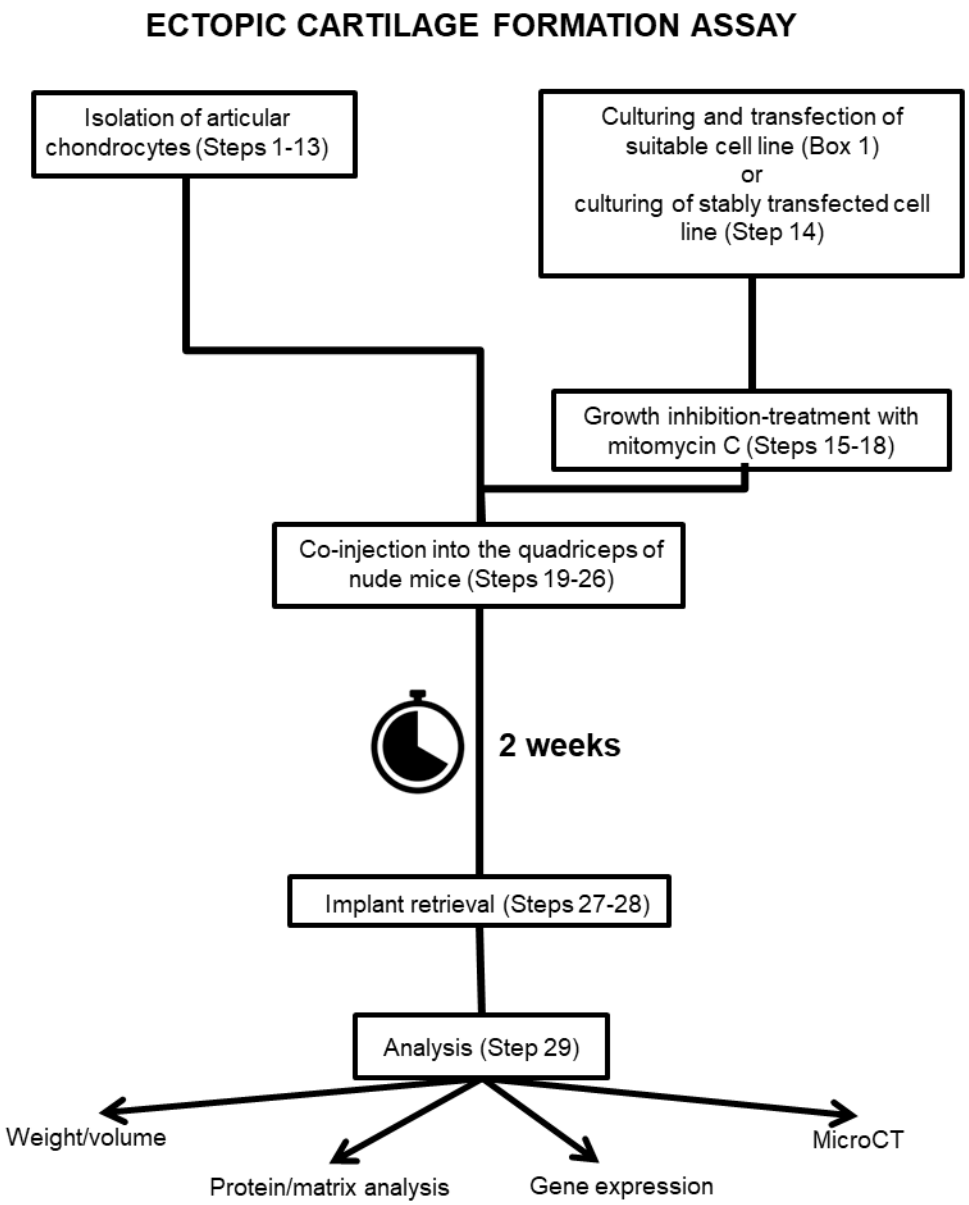

Fig. 2| Flow chart describing the ECFA. Diagram showing the entire ECFA procedure in one glance.

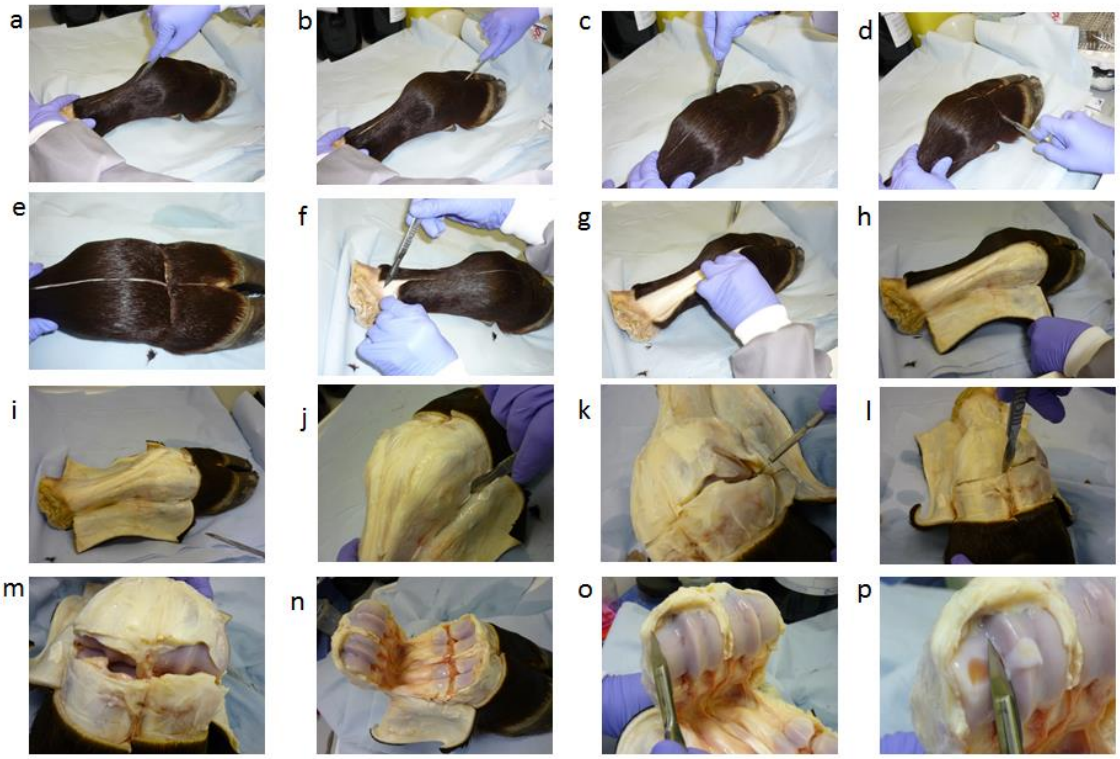

Fig. 3| Isolation of bovine chondrocytes. Dissection of a bovine limb to isolate articular chondrocytes for injection into the nude mouse ECFA. A & b vertical incision parallel to the length of the bone; b, incision stretches down to the hooves; c-e, with the hoof bent, make a horizonal incision reaching from the far right to the far left; g, pull the hide taut and cut the connective tissues to separate the hide; h -i, remove the hide from all four quadrants; j, cut through the connective tissue into the joint capsule along the joint interzone; k, cut through the medial and lateral ligaments at either side of the joint; l, cut through the anterior cruciate ligament; m, bend the joint to reach and cut the posterior cruciate ligament; n, open the joint to expose the cartilage; o & p, slice the cartilage off the bone.. Appropriate institutional regulatory board permission was obtained.

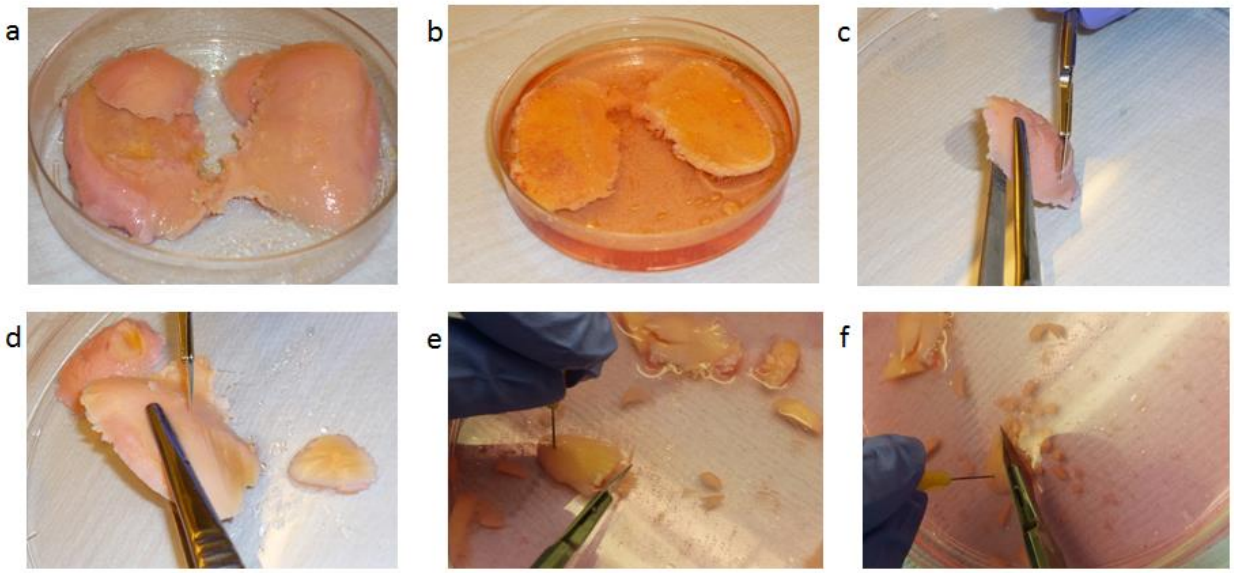

Fig. 4| Isolation of human articular chondrocytes. Processing of human cartilage for the isolation of chondrocytes for the use in the nude mouse ECFA model. Only cartilage from areas that at visual inspection have an Outerbridge score =<3 are removed and used for chondrocyte isolation[48]. A, human femoral condyles; b, femoral condyles facing cartilage down in complete DMEM; c &d, holding cartilage with tweezers, slice the cartilage off with a scalpel; e &f, finely slice the cartilage into smaller (2-10mmx2-10mm) pieces.

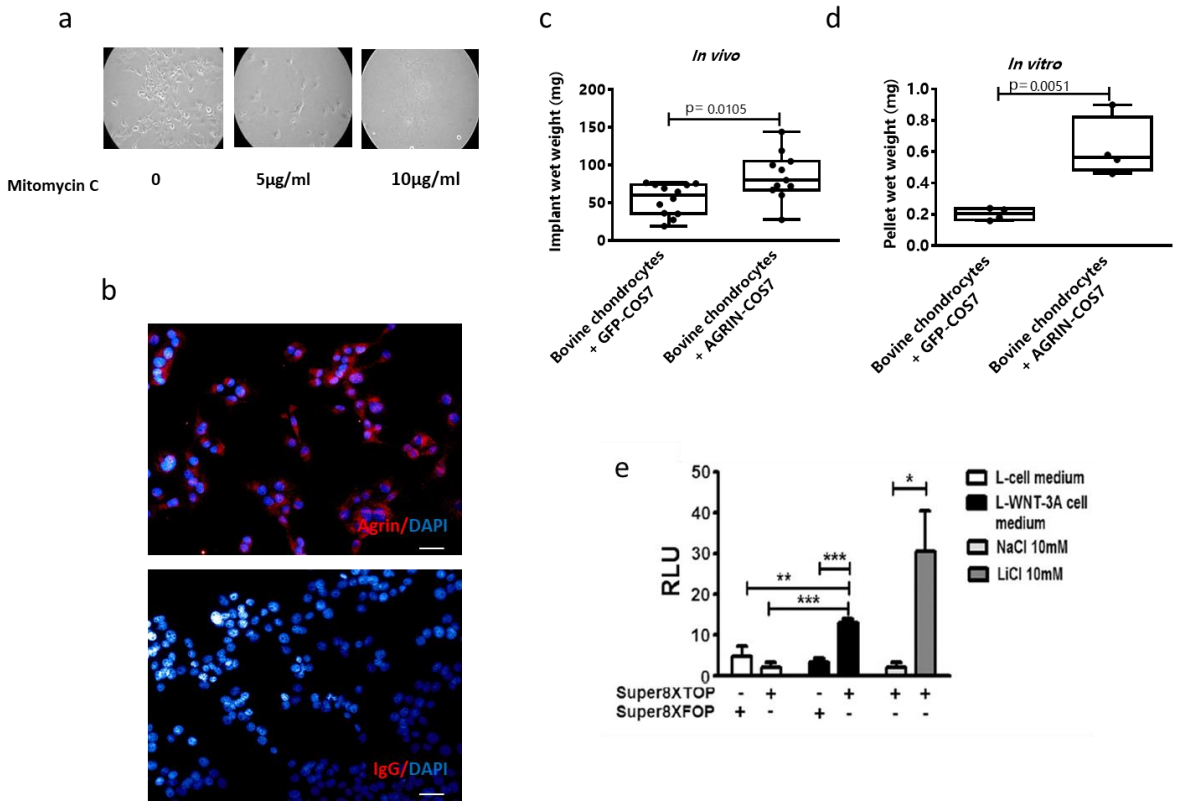

Fig. 5| *In vitro* validation of secretion and bioactivity of the overexpressed gene in cell lines following growth inhibition with mitomycin C. **a,** Optimization of growth inhibition of COS7 cells with mitomycin C. **b,** Immunofluorescence staining of Agrin (using anti-agrin antibody - red) of COS7 cells successfully growth arrested with 7.5µg/ml of mitomycin C after transient transfection with an Agrin plasmid (scale bar: 50µm). **c,** Wet weight of pellet cultures of chondrocytes co-cultured with growth-arrested COS7 cells overexpressing Agrin or GFP. **d,** Wet weight of *in vitro* pellet cultures of bovine chondrocytes directly transfected either with an Agrin- or a GFP-expression vector. The anabolic effect of Agrin on the chondrocytes was comparable in c and d. **e,** Reporter assay of chondrocytes measuring the activation of the Wnt-beta catenin signalling. Conditioned medium obtained by growth-arrested L-cells overexpressing WNT3A was still capable of inducing the activation of the reporter in porcine articular chondrocytes. These results further show that growth inhibition did not affect the biological activity of WNT3A on the cells. Panel e is reproduced without modifications from[18].

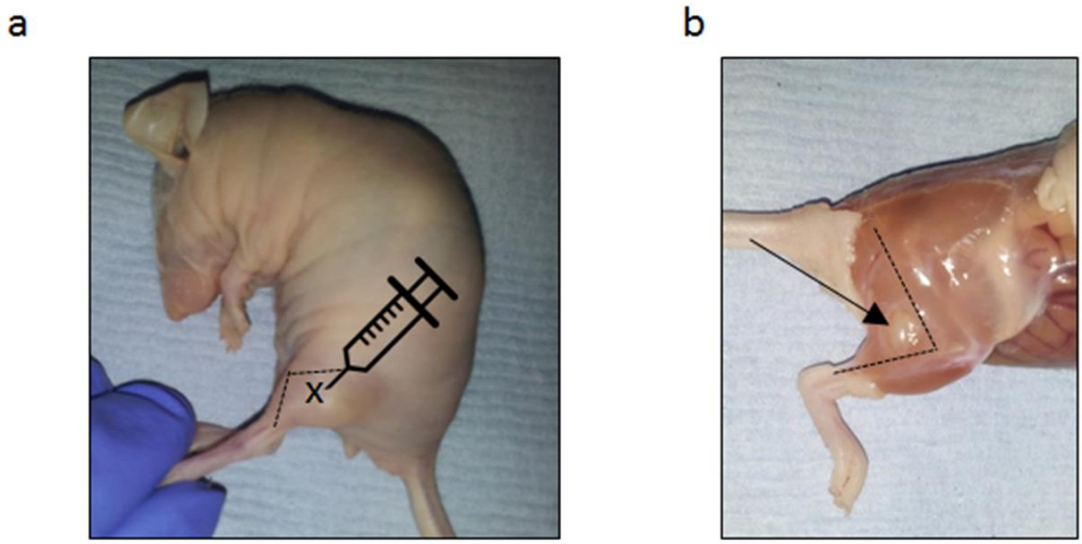

Fig. 6| Intramuscular injection and retrieval site. a, Image showing the injection site in the posterior compartment of the thigh of the nude mouse **b,** Image showing a cartilage implant before retrieval, 2 weeks post bovine chondrocyte injection. Appropriate institutional regulatory board permission was obtained.

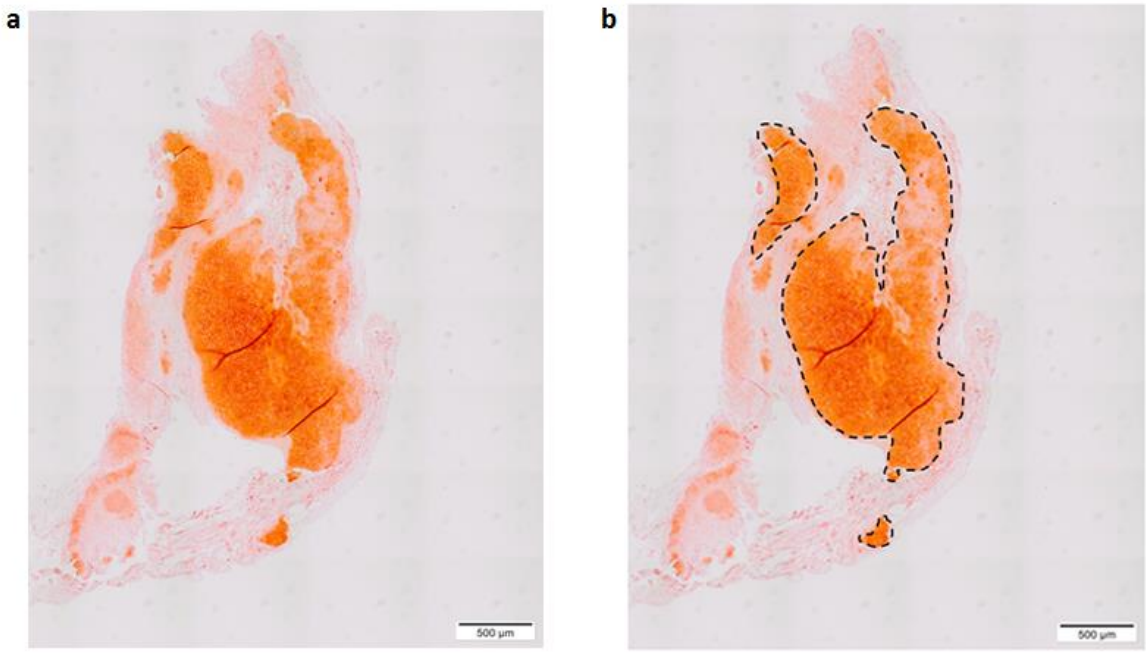

Fig. 7| Histological expected outcome. **a,** Example of Safranin O staining of a cartilage implant. **b,** Dotted line defining the boundaries of differentiated (safranin O positive) cartilage from the surrounding muscle tissue (scale bar: 500µm).

Table 1| Power calculations for intramuscular and subcutaneous assays

Model	Mean % differentiated area in control group	Standard deviation % differentiated area in control group	Number of implants required to detect with 0.8 power and significance level of 0.05 a 1.5% fold change
5 million chondrocytes intramuscularly[14]	34.086	11.705	8.481 per group (4 mice for 8 implants)
1 million chondrocytes in a type I collagen gel implanted subcutaneously [46]	37.956	10.785	6.194 per group (1 mouse for 6 implants)

Table 2|Troubleshooting table

Step	Problem	Possible reason	Solution
2	Difficulty identifying the joint capsule and cutting it/accessing the joint	Orientation of the limb is not optimal to access the joint	The joint line can be identified more easily by bending the joint to 45°. Cut tendons and ligaments at the medial and lateral side of the joint to allow access to the ligaments connecting the two joint surfaces (Fig. 3k)
2	Too few chondrocytes	The number of chondrocytes retrieved from bovine joints will vary depending on age/size of the bovine. Less chondrocytes will be	Source more porcine or bovine limbs in preparation to carry out the ECFA model. Animal age, strain and sourcing will contribute to variation in size of joints sourced

		obtained from porcine joints	
18	Too few protein-overexpressed, growth-arrested cells	Mitomycin C treatment will result in the loss of approximately 10-20% of the transfected cells	This should be considered when calculating the number of cells required for each injection. If more than 20% cell death is observed, the concentration of mitomycin C should be re-optimized
29	Results show a clear trend towards differences, but the statistical analysis does not reveal significant differences	The study is under-powered. Power analysis should be performed to predict the number of injections needed to gain statistically significant data	A minimum of 10 injections per condition is recommended[14,18]. Both hind limbs can be injected, although it is recommended that both injections in a single mouse represent the same condition, as the secreted molecule being tested can circulate and could potentially influence the development of the contralateral implant
Box 1, step 1	Transfected cells dying	Cell death could be due to transfection technique	Testing different transfection techniques for the cell line being used
Box 1, step 7	Transfection efficiency is <90%	The protocol was optimized for COS7 cells. Different transfection protocols may be required for different cell lines	Optimizing cell number, media, transfection reagents and expression vectors may be required