Review

Gene activated matrices for bone and cartilage regeneration in arthritis[1)]

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Abstract

The GAMBA Consortium is developing a novel gene-activated matrix platform for bone and cartilage repair with a focus on osteoarthritis-related tissue damage. The scientific and technological objectives of this project are complemented with an innovative program of public outreach, actively linking patients and society to the evolvement of this project. The GAMBA platform will implement a concept of spatiotemporal control of regenerative bioactivity on command and demand. A gene activated matrix is a biomaterial with embedded gene vectors that will genetically modify cells embedded in or colonising the matrix. The platform comprises modules that self-adapt to the biological environment and that can be

independently addressed with endogenous biological and exogenous physical or pharmacological stimuli, resulting in a temporally and spatially coordinated growth factor gene expression pattern. This reproduces, within the matrix, key elements of natural tissue formation. The modules are a biomimetic hyaluronan gel, a ceramic matrix, growth factor-encoding gene vector nanoparticles, magnetic nanoparticles and mesenchymal stem cells. Anatomical adaptivity is achieved with engineered thermal properties of the polymer matrix, which embeds other modules, selected according to functional requirements. Mechanical support is provided by Micro Macroporous Biphasic Calcium Phosphate (MBCP™), a resorbable material approved for clinical use. Spatiotemporal control of bioactivity and responsiveness to physiological conditions is represented, firstly, in the spatial distribution and release profiles of gene vectors within the composite matrix and, secondly, by letting local and external biological or physical stimuli activate the promoters driving the expression of vector-encoded growth factor transgenes. This concept is implemented by a multidisciplinary team from leading European institutions. Here, we report on the concepts, objectives and some preliminary results of the GAMBA project which is funded in 7th Framework Programme of the European Union THEME [NMP-2009-2.3-1], Biomimetic gels and polymers for tissue repair.

Keywords: citizen participation in science; gene activated matrices; gene vectors; hyaluronan hydrogel; mesenchymal stem cells; micro macroporous biphasic calcium phosphate; spatiotemporal control.

Introduction

Due to demographic and life style changes, degenerative diseases are an enormous medical and socio-economic challenge in industrialised nations. Among them, the musculoskeletal diseases osteoarthritis, rheumatoid arthritis and osteoporosis are the most prevalent. Osteoarthritis (OA) is a degenerative disease of the joints affecting, above the age of 45, more women than men, with an incidence increasing with age (1). The cartilage of the affected joint becomes rough and degenerates. With disease progression, the cartilage disappears and bone rubs on bone. The etiology of OA is still unknown. The current management of osteoarthritis is not regenerative but merely symptomatic, aimed at reduction of pain, controlling inflammation with non-steroidal anti-inflammatory drugs with an ultimate option of total joint replacement. Implantation of autologous chondrocytes cultivated on biomaterial scaffolds is a more recent approach, it

does not, however, it account for subchondral bone that supports the cartilage and can not avert the destructive inflammatory processes (2).

It is estimated that 80% of the population will have radiographic evidence of OA by age 65 and that every fifth individual in industrialised countries is actually affected by clinical symptoms of the disease. An estimated 103 million Europeans (3), 3.85 million Australians (4), 6 million Canadians and 46 million citizens of the USA (3, 5, 6) suffer from OA. The population burden of OA is increasing dramatically (3) due to the ageing population and the rising prevalence of obesity, being the principal non-genetic risk factor for OA. This leads to a tremendous economic burden, being evident by an escalation of costs. The direct medical expenses for the treatment of musculoskeletal diseases amounted to 26.6 billion Euro per year in Germany alone (7, 8), which constituted 11% of the total direct medical costs for all diseases, of which 7.5 billion Euro were spent for treatment of OA (equivalent to 3.6% of total direct costs) in 2006. An estimation of indirect expenses (lost wages, lost productivity) adds another 3–5 billion Euro for arthritis and about 20 billion Euro for all musculoskeletal diseases.

Summarising, there is currently no gold standard for the repair or prevention of onset of osteoarthritis and the disease represents a tremendous burden in terms of suffering and costs. There are three major challenges: reducing inflammation, cartilage repair and subchondral bone repair. Biomimetic approaches for tissue repair in osteoarthritis require tight spatiotemporal control of bioactivity in order to address these challenges in a coordinated fashion.

Innovative approaches for treatments of cartilage and bone defects are the subject of research worldwide. Recombinant growth factors have been used with success to improve bone healing and first products are in clinical studies or are approved for clinical use (9–13). In order to achieve high local bioavailability, implantable biomaterials and biodegradable surface coatings with sustained and controlled release technologies have been developed (14, 15). A viable alternative to applying growth factors is transfecting or transducing their respective cDNAs under the control of suitable promoters in target cells ex vivo or in vivo. The gene activated matrix (GAM) concept (16–18) is particularly appealing in this context as it combines gene therapeutic approaches to tissue regeneration with a sustained release concept which in addition provides target cells with a matrix to grow on. GAMs are biomaterial scaffolds comprising gene vectors. Cells growing on or into the matrix will become transfected/transduced by the immobilized or released vector and will consequently express the transfected (growth factor) gene, resulting in local autocrine and paracrine stimulation of a desired differentiation process. This has yielded promising results in preclinical models of tissue regeneration (16–18). Several gene therapy trials for arthritis (not involving the GAM concept) have been conducted, with only two addressing osteoarthritis (transplantation of allogeneic chondrocytes expressing TGF-β1) while the others addressed rheumatoid arthritis, notably with anti-inflammatory approaches [rev. in (19)]. Summarising, a variety of promising innovative approaches are underway. None of these approaches so far has comprehensively implemented

the concept of spatio temporal control of bioactivity on demand and command. In agreement with leading experts in the field (14), we think that systems for spatial and temporal control of drug action will be essential elements in future therapies, notably for treatments involving growth factors.

Concept and objective

Healthy tissue features unique plasticity characterised by continuous remodelling in response to physiological and external stimuli, resulting in a controlled balance of anabolic and catabolic processes. Tissue damage is characterised by imbalance, loss of control and in the case of osteoarthritis a dominance of catabolic processes. No single established treatment of tissue defects implements the features of natural tissue formation, namely cell differentiation in response to spatiotemporally controlled growth factor gene expression patterns on command and demand. Conventional treatments of tissue defects are mostly conservative in nature, in other words consolidating a status quo or delaying disease progression. Innovative concepts, in contrast, are regenerative in nature, thriving on the regenerative potential imprinted in our genetic background. The challenge is seizing this potential. This can be achieved, in principle, with growth factors and stem cells. While the latter are inherently multipotent, the former can be used to reawaken silenced endogenous programmes of tissue formation which are active during natural morphogenesis. These programs consist initially of temporally and spatially coordinated gene expression patterns, leading ultimately to a highly regulated concerted action of growth factors. In the context of tissue regeneration with biomimetic implants, such concerted action is exceedingly challenging to reproduce with *recombinant* growth factors as they have no inherent elements for responding to physiological conditions or external stimuli. In contrast, gene vectors can be engineered to have these elements and thus can reproduce spatiotemporally controlled gene expression patterns in situ.

Therefore, the main objective of the GAMBA project is developing a biomimetic implant system that delivers regenerative bioactivity in a temporally and spatially controlled fashion in response to endogenous and external stimuli. In this manner, the system shall respond to and control inflammation and induce cartilage and subchondral bone repair in OA-related tissue defects. The implant system shall alter the phenotype of cells in a physiologically meaningful way to elicit the desired therapeutic outcome.

Gene activated matrices shall serve as scaffolds for cells and provide a temporary genetic environment by virtue of signal-driven gene promoters controlling the expression of genes of interest. This will deliver spatiotemporally controlled stimuli for bone and cartilage repair and inflammation modulation that the body cannot deliver for age and/or disease-related reasons. Having fulfilled their function, the implant materials will be resorbed, becoming part of and giving way to natural tissue. In this manner we envisage the reawakening of tissue regeneration processes also in cases where such processes are difficult to induce otherwise.

This concept is to be established and evaluated with model systems in vitro and in vivo. Spatial control is to be achieved by positioning gene vectors encoding different growth factors in different phases of composite implant materials that consist of a thermo-responsive hyaluronan-based hydrogel and a biphasic calcium phosphate ceramic which is embedded in the hydrogel (Figure 1). Spatial and temporal control can be obtained by heat triggered vector release. Temporal control of bioactivity is to be achieved with inducible promoters that control the expression of vector encoded growth factor genes, once they were taken up by cells. This concept allows in principle for an enormous number of possible combinations of inducible promoters, growth factor genes, vector positioning and biomaterials. The consortium will realise only a few combinations that are useful in OA tissue defect repair to provide proof of principle of spatiotemporal control using gene activated matrices. In a first step, a "construction kit" or "toolbox" of elements (inducible vectors, biomaterials)
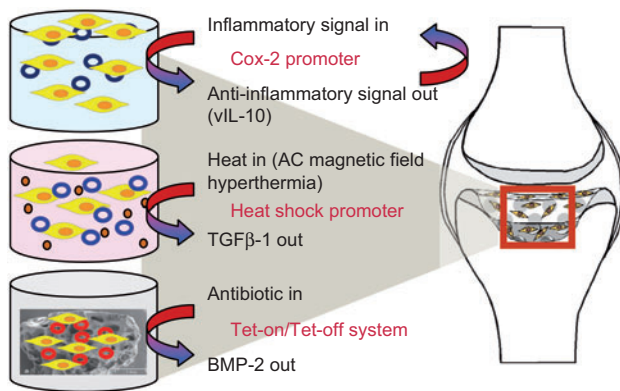

Figure 1   The concept of the GAMBA platform.
A model tissue defect due to arthritis related tissue degeneration, for example, in the knee joint (right) is envisaged to be filled with a composite gene-activated matrix colonized with mesenchymal stem cells. The composite matrix is envisaged to consist of three compartments, symbolized on the left side. A compartment facing the synovium comprises a vector construct which will respond to inflammatory signals. Cells being transduced while growing in this compartment should to respond to inflammatory signals from the synovium by producing anti-inflammatory IL10. The second compartment comprises a vector construct with the TGFβ gene driven by a heat-inducible promoter. Upon heat induction by AC magnetic field hyperthermia mediated by magnetic nanoparticles which are comprised in this compartment, transduced cells will express TGFβ to promote cartilage regeneration. The third and lower compartment comprises a beta tricalcium phosphate ceramic with a vector construct having the BMP-2 gene under the control of the tet-on/tet-off system. Upon pharmacological induction, transduced cells growing in this compartment will produce BMP-2 to promote bone regeneration. A bioactive, temperature-sensitive hyaluronan-based polymer serves as embedding matrix for the other modules, chosen according to the desired functionality. The polymer itself is supposed to provide controlled release properties for gene vectors and/or factors expressed by transduced cells. Spatial and temporal control of bioactivity is to be achieved by differential positioning of the vectors within the composite matrix and by differential expression of the trans-genes upon induction by internal and/or external stimuli.

will be developed and a proof of principle for spatiotemporal control of growth factor gene expression will be generated. In a second step, elements of this construction kit will be chosen for tissue repair in OA tissue defect models in vitro, ex vivo and in vivo. The factors that will be implemented in this system have a proven track record in the field of musculoskeletal regenerative medicine. They are BMP-2 to induce bone formation under spatial control; TGF-β for induction of chondrogenic differentiation under spatial control; IL-10 for reduction of inflammation. Combinations of these genes with inducible promoters in defined scaffold areas will provide a powerful tool for controlled tissue repair.

The modules of the GAMBA platform

A polymer hydrogel based on hyaluronan is used to embed vectors, mesenchymal stem cells and a calcium phosphate ceramic in a model osteo-chondral defect. Hyaluronan has important roles in organ development and cell signalling (20). Commonly, hyaluronan hydrogels are obtained by cross-linking reactions involving chemicals and/or UV light (21), which are potentially detrimental to the biologicals embedded in the gels. The AO Foundation has developed proprietary novel physical cross-linked hyaluronan hydrogels exploiting "gentle" and efficient click chemistry allowing for cells to be injected and encapsulated (22). Covalently grafting a synthetic, thermo-reversible polymer, such as poly(N-isopropylacrylamide) onto the backbone regulates the gelation characteristics, the mechanical properties and the structure of the hydrogels formed. The thermo-responsiveness of the hydrogels can be modulated from room temperature to above body temperature depending on the thermo-responsive segment and the composition, hence providing temperature-dependent spatiotemporal control. Remote thermal switching of functional states and release properties shall be achieved via AC magnetic field induction of embedded superparamagnetic nanoparticles.

MBCP™ (micro macroporous biphasic calcium phosphate) is a 100% synthetic resorbable bone substitute composed of hydroxyapatite (HA) and beta tricalcium phosphate (βTCP) (23, 24) being comprised in several product lines of Biomatlante approved for clinical use (see www.biomatlante.com) (25). Such scaffolds are efficient in chondroblast phenotype expression (26) and have great potential in tissue engineering via the endochondral bone formation mechanism. Preliminary work has shown the utility of the material to serve as a gene-activated matrix. Hence, MBCP as carrier of gene vector encoding a factor A embedded in a hydrogel carrying gene vector B will be an element of spatiotemporal control in this project.

The gene vectors: Non-viral and adenoviral PEG-shielded vectors are used in this project. They are based on our Copolymer Protected Gene Vector (*COPROG*) approach described previously (27–31). *COPROG*s provide steric stabilisation, protection from opsonisation, allow freeze drying of the vector with little loss of activity and even resuspension

in organic solvent. Their application to adenoviral vectors is intended to shield from immune responses, similar to approaches published by others (32). The consortium will construct vectors having the cDNAs of interest (IL-10, TGFβ and BMP-2) under the controls of inducible promoters [Cox-2 responding to an inflammatory environment (33), Hsp70 responding to heat (34), tet system responding to doxycycline (35)]. Vector pairs encoding different factors will be embedded in different phases of the composite matrix (e.g., BMP-2 on MBCP/TGFβ1 in polymer hydrogel) yielding a system with multiple levels of spatiotemporal bioactivity control for cells colonising the matrix.

Magnetic nanoparticles are widely used in biomedical research and some clinical applications. Iron oxide magnetic nanoparticles are biodegradable and are an integral element in magnetofection technology for nucleic acid delivery (36). Furthermore, iron oxide nanoparticles can locally transmit heat via external AC magnetic field induction, an approach which is approved for cancer thermotherapy (hyperthermia) (37). In our approach, AC magnetic field induction is used to activate the HSP70 promoter. Non-cancerous cells can easily cope with short-term heating to 42°C. The feasibility of this approach has been shown recently (38).

Adult mesenchymal stem cells (MSCs) are multipotent cells in the bone marrow and can, under appropriate conditions, differentiate into mesenchyme-derived cell types [osteoblasts, chondrocytes, and adipocytes (39, 40)]. MSCs are harvested and expanded in large quantities without causing donor site morbidity. They represent a rich source for tissue engineered constructs for treating of musculoskeletal defects (41, 42). The MSCs retain their multi-lineage differentiation capacity (43) until stimulated by specific cytokines and growth factors (44–46). The use of MSCs for the treatment of osteoarthritis has been described in a large animal model (47) and in humans (48). MSCs will be used in this project to regenerate cartilage to compensate for the low intrinsic regenerative capacity of damaged cartilage. The thermo-sensitive polymer will serve as a scaffold for MSCs, and spatiotemporal control of growth factor release will be achieved with genes triggered by exogenous signals (temperature, doxycycline) to direct cartilage and subchondral bone regeneration (49, 50). Alternatively, MSC can be transduced ex vivo by magnetofection (51, 52) and then injected within the thermo-sensitive polymer into the damaged cartilage and activated as above.

Implementation plan

The GAMBA project proposes combing materials science and biophysics with gene vector development and cell biology in order to achieve bone and cartilage repair with a focus on osteoarthritis-related tissue damage. The project comprises eight scientific work packages including one work package dedicated to public outreach, and second management work packages:

Work Package 1 is planned to deliver the required gene expression constructs and gene vectors which are a central element of this project. A "tool box" of gene vectors for regulated expression of reporter or growth factor genes will be created. Regulation will be by heat (physical), doxycycline (pharmacological) as well as regulation by inflammatory signals (biological), providing a feedback response element for the biological milieu. Growth factors, representative of the different constituents involved in OA will be expressed as model genes. An in vitro model for combining three genes that can be independently addressed by virtue of three different inducible promoters will be established. This may be used ultimately to address the three important aspects of OA: cartilage repair, bone formation and modulation of immune response. Different stimuli will be given to demonstrate in proof-of-principle experiments spatiotemporal control of gene expression.

Materials science and biophysical analyses are represented by Work Packages (WP) 2 and 3, mainly representing a thermo-responsive injectable biomimetic hydrogel and a biphasic ceramic/calcium phosphate based matrix. Materials from WP 1, 2 and 3 will be combined and their efficiency of transduction of cells in situ by non-viral or viral gene vectors will be analysed in WPs 2 and 3, respectively.

WPs 4–6 focus on the proof of concept of the efficacy of these tools for the modulation of response in inflammation and cartilage and bone repair.

WP 4 deals with the modulation of inflammation as one of the components of osteoarthritis using gene vectors that allow for expression of the anti-inflammatory cytokine IL-10. Expression of this cytokine will be driven by the Cox-2 promoter, known to be up-regulated in response to inflammation. The novelty of our approach will be that gene vectors will additionally be released slowly from a matrix, either continuously or on demand. Implementing this approach means a close interaction with WP 1 and 2 in order to define the best possible choice of gene vectors and matrices for optimal anti-inflammatory response. A combination of cells and vectors in the WP 2 biomaterial will be employed to assess the anti-inflammatory properties of this module in vitro, ex vivo (synovial explants) and finally in a small animal model of joint inflammation.

WP 5 focuses on cartilage regeneration. Initially within this WP optimal dosage and timeframe of TGF-β will be titrated to determine the required extent and duration of vector expression. This will allow for choosing the most suitable external control (heat/doxycycline). This information will feed back into WP 1, and vectors embedded in biomimetic materials provided by WP 2 will be analysed for transduction efficiency of co-embedded cells and regulation of growth factor expression. Following these preliminary experiments, the efficacy of this combination on chondrogenesis and cartilage repair will be assessed in 3D culture in vitro and eventually in an ex vivo osteochondral plug model.

WP 6 focuses on bone regeneration. Similarly as outlined for WP 5, inducible gene expression, here of the BMP-2 gene will be characterised and results will feed back into vector design and the setup of combinations with biomaterials from

WP 2 and WP 3. Following these preliminary experiments the efficacy of this combination on osteogenesis and bone repair will be assessed in small animal models.

Only if small animal experiments are successful, a large animal study in goats will be carried out in WP 7. Small animal models, such as small rodent and rabbit models, have been well described, and the former has the advantage, through genetic or surgical manipulation, to allow disease models to be included in the study of osteochondral defects. The small animal models have the advantage of convenience and cost, and are excellent choices for screening treatment options. However, differences between the propensity for healing between small animals and humans make them less favoured for comparative research. In addition, the expected biological response to healing in these clinical situations depends heavily on the size of the defect due to issues relating to vascularisation and nutrition. Therefore, the successful proof of the efficacy of regenerative medicine/tissue engineered solutions requires an in vivo model with a defect of a similar size to the human clinical situation, to be maintained for at least 6 months, to fully evaluate the potential for healing. For translational research in cartilage the goat is commonly used as a large animal model due to similarities to humans with regard to cartilage thickness and structural properties, and as they are well adapted to a research environment. Depending on the outcomes of WPs 4, 5 and 6 the most optimal modules will be combined and the most appropriate defect model will be decided upon.

Public outreach Work Package

The societal implications of innovative therapies have hardly ever been discussed in depth with members of the general public and/or patients, often leading to rejection or lack of interest. Likewise health technology assessment is usually done by experts only and is mainly performed when a new application is at least in clinical studies or after marketing approval. In order to overcome these shortcomings, WP 8 is designed as an active tool to start a dialogue with the public. In this WP, elements of WPs 1–7 that are of interest to patients and the general public are presented to and discussed with affected patients and interested citizens in the patients' and citizens' panels. Researchers of the consortium are involved in the panels as experts and dialogue partners and results of the dialogues will feed back to the members of the consortium. During the panels, the scientists give presentations or perform in a hearing with several experts and discuss with patients or citizens their needs, expectations and reservations. The panels are moderated by neutral facilitators to ensure that information is presented in a way understandable for lay people so that active discussion can take place. Neutral facilitators are trained in understanding the main facts of the scientific subject, but without the "tunnel view" of the scientists. They act as "mentors" to the panel members and help the scientists "translate" their views and interests to lay people. Therefore, they mediate the information and interests from the scientists to the panel members and vice versa.

Although input of the patient and citizen panels may not have an immediate impact on everyday experimental work, it will have a general influence on how research will/should be performed. Nowadays basic science even in the biomedical disciplines is often carried out mainly for scientific purposes and is not perceived from the point of view of the "end user" (the patient). The thought patterns and scientific strategies are dominated by the way scientists have learned to think, or in the case of knowledge that can be commercialised by economic aspects. We therefore propose to complement the scientific and economic approach with the needs that patients and the general public express in the context of novel technological developments. In brief, we would like to have the scientists participating in this project be aware of the needs of patients and members of society and incorporate this knowledge in their strategies of conducting science. The direct contact of the scientists with patients and interested citizens can therefore sharpen the attitude of the scientists towards societal needs and expectations.

Preliminary results

Gene vectors and constructs for inducible gene expression

Due to their non-integrative and non-immunogenic character non-viral vectors are among the safest vector types available. However, viral vectors generally show higher transduction efficiencies. Adenoviruses are among the most efficient viral vectors for in vitro and in vivo gene therapy. They do not integrate their DNA into the host genome either. For this reason and despite their high immunogenicity they are considered in the GAMBA approach for proof of principle experiments. To avoid elimination by interaction with neutralising antibodies or erythrocytes, shielding with a polymer can be achieved (53, 54).

Non-viral and adenoviral vectors for regulated gene expression have been produced and initially characterised. To allow for proof-of-principle analyses of spatio-temporal control in combinatorial approaches, different reporter genes were combined with the different regulatory units: eGFP or *firefly* luciferase (FfLuc) were combined with the Tet-on-system, the secreted *Metridia longa* luciferase gene (MetLuc) was set under the control of the inflammation inducible Cox-2 promoter and red fluorescent protein dsRed2 was fused to the human heat-inducible promoter (Hsp70B).

Heat induction

Heat induction has been achieved in a non-viral vector system using the human Hsp-70B promoter to control the expression of fluorescent protein dsRed2-N1. The 436 bp fragment of pGL3hsp70Bp Sma/Hind (55) carrying the heat-inducible promoter was cloned into a dsRed2-N1 expressing plasmid. Non-viral vectors for magnetofection were prepared in serum-free medium using the inducible plasmid construct, the lipofection reagent DOGTOR (OZ Biosciences) and the PEI-coated magnetic nanoparticles SO-MAG6-125 (56). Magnetofection of

murine fibrosarcoma CMS-5 cells was carried out in 24-well format. Gene expression was induced 24 h post transfection in a water bath at the indicated times and temperatures. Reporter gene expression was analyzed 24 h post induction of by flow cytometry. Red fluorescent protein expression was indeed inducible by 42°C treatment and depended on duration of heat shock: significant induction was seen at heat shock times of 30 min and longer (Figure 2A,B). Although overall gene expression of Hsp-70-dsRed transduced cells was only 30% of CMV promoter (Figure 2 A), up to 14-fold induction of dsRed gene expression was achieved (Figure 2B). Additionally, the dependence of gene expression on temperature was evaluated over a range from 39°C to 43°C (Figure 2C). Induction of gene expression was only seen above 41°C.

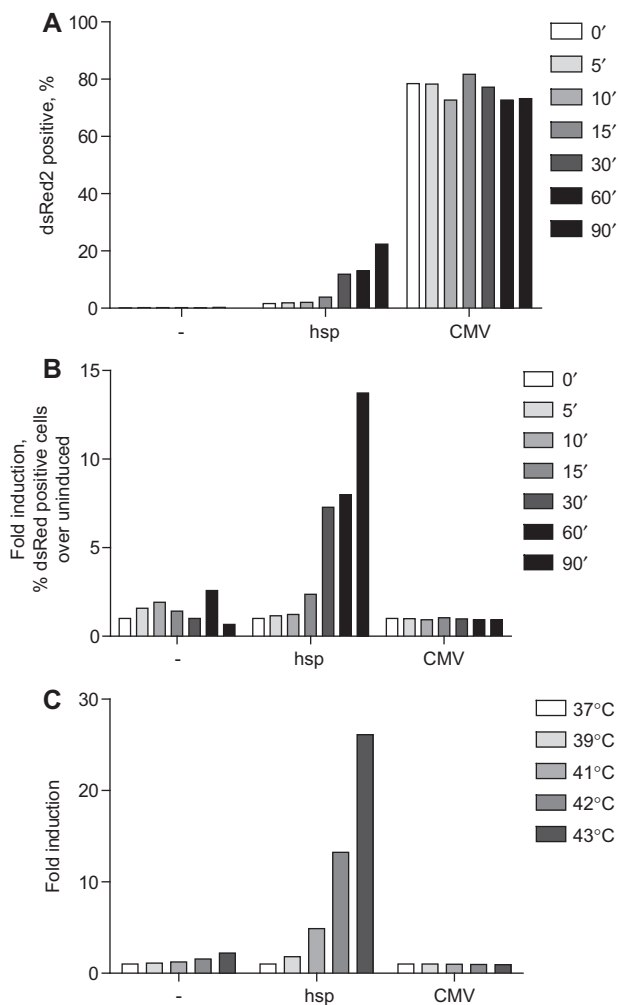In conclusion, these results demonstrate the functionality of the HSP-70B promoter constructs.

Induction by inflammation

As human prostaglandin endoperoxide synthase 2 cDNA is induced by inflammatory cues (57, 58), the regulating sequence of this gene, also known as Cox-2 was chosen to drive the expression of a secreted form of luciferase (MetLuc).

For the Cox-2 promoter two different lengths of promoters (–1432/+59 and –327/+59) (57) were cloned to drive the *Metridia longa* luciferase cDNA in a vector backbone comparable to the above-mentioned plasmid. First analyses exhibited no major differences for both promoter constructs (data not shown). Transfection of CMS-5 tumour cells was as described above and treatment started 24 h later with 100 nM 12-O-tetradecanoylphorbol-13-acetate (TPA; Sigma, Taufkirchen, Germany) or 10 µg/mL lipopolysaccharide from *Escherichia coli* serotype 055, B5 (LPS; Sigma, Taufkirchen, Germany) or TPA+LPS for 5 h. Contrary to the literature (58) no effect was seen after induction with LPS alone (Figure 3A), likewise no additive or synergistic effect of induction of MetLuc expression was seen when transfected cells were treated with both inducers. Best induction levels of MetLuc secretion was
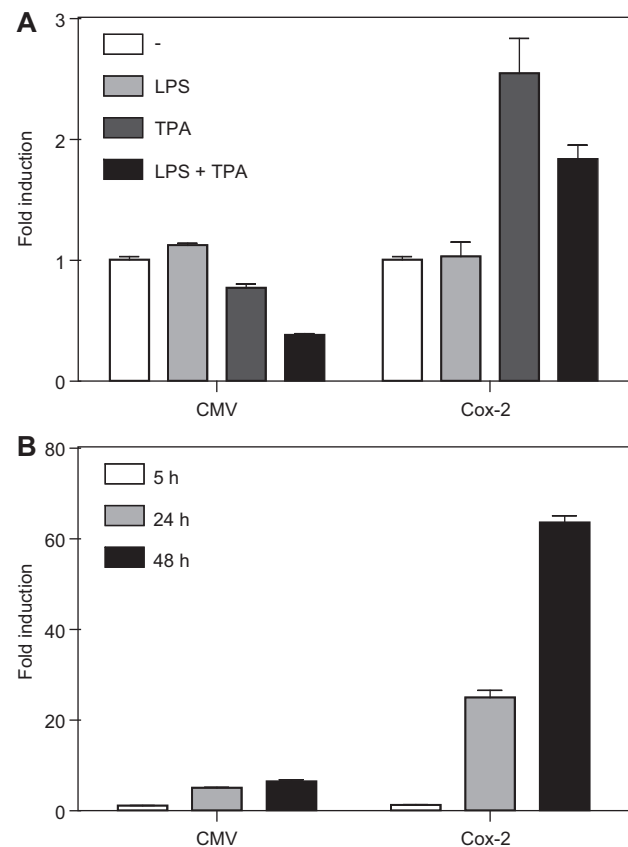

Figure 2   Regulation of gene expression by heat.
CMS-5 tumour cells were transfected with plasmids driving dsRed expression from HSP70B promoter (hsp) or constitutive CMV promoter (CMV) and 24 h later induced at the indicated temperatures for the indicated times. Cells were analyzed 24 h post induction by flow cytometry. (A) Analysis of red fluorescence after induction at 42°C for the indicated times (min). (B) Fold induction calculate from A. (C) Fold induction as function of temperature. Induction time: 90 min.

Figure 3   Functionality of Cox-2 promoter constructs.
CMS-5 cells were transfected with the Cox-2 promoter plasmid (Cox-2), covering sequences from –327 to +59 or the constitutive CMV promoter (CMV) and 24 h later induced with TPA, LPS or TPA+LPS for 5 h (A) or TPA+LPS for the indicated times (B). Media were collected and bioluminescence of secreted *Metridia* luciferase activity was determined after addition of the substrate coelenterazin.

seen when cells were treated with TPA (Figure 3A). Extended treatment with TPA and LPS resulted in increase MetLuc production (Figure 3B).

No such induction was seen in the NIH3T3 cell line, indicating that not all cells might express the necessary transcription factors for Cox-2 promoter-driven expression. Unfortunately, we did not observe the desired induction of Cox-2 promoter-driven expression upon adenoviral transduction either (see below).

As part of the GAMBA project, control of inflammation in the joint was to be achieved by overexpression of IL-10 in a population of human MSCs. The first step in achieving this was to establish a highly efficient protocol for large scale adenoviral transduction of cells. A lanthanide based approach (59) was used to achieve over 90% transduction efficiency as assessed by FACS analysis using an adenovirus expressing GFP; Figure 4).

Alternatively an approach using magnetically enhanced adenoviral gene transfer to human MSC in monolayer culture has been successfully established using AdenoMag and the Viro-MICST infection method on a magnetic cell separation column (51, 52).

When human MSC were infected with an adenoviral vector expressing vIL10 under the control of the –327/+59 Cox-2 promoter, no increase of vIL10 secretion into cell culture supernatant was observed after stimulation with TNF-α and IL-1β (Figure 5).

In order to induce Cox-2 promoter activity, MSCs were stimulated with TNF-α (50 ng/mL) and IL-1β (100 ng/mL). This cocktail has previously been shown to induce expression of IL-4 under the control of a Cox-2 promoter, following transfection of canine chondrocytes with a Cox-2 promoter driven IL-4 expressing construct (33). Furthermore, Yang et al. showed inducible Cox-2 activity in MSCs using the same cytokine combination (60). Human MSCs were transduced with AdCox2(–327/+59)vIL10 at MOI 100 in the presence of lanthanum chloride. Control cultures were exposed to lanthanum chloride without virus (59). MSCs were stimulated with
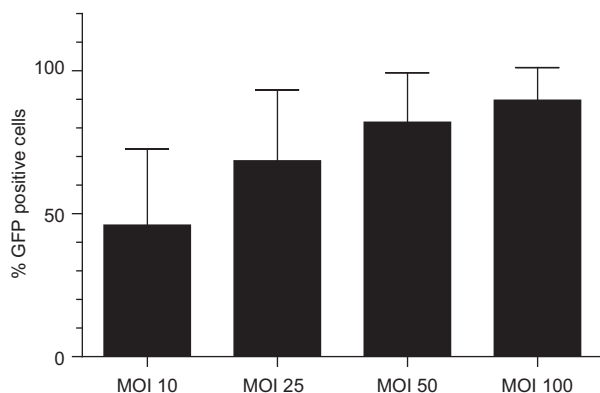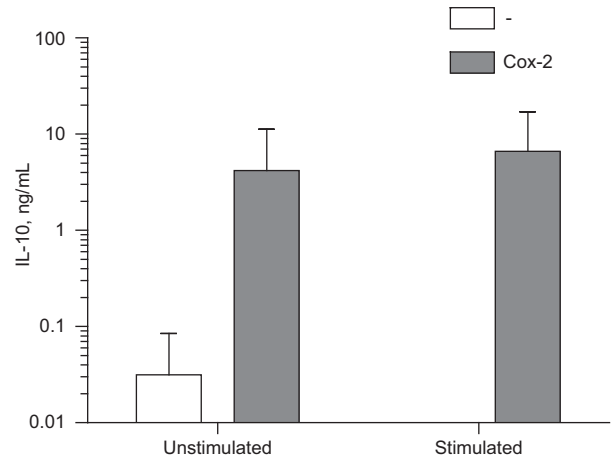

Figure 5 Effect of pro-inflammatory cytokines on induction of IL-10 under the control of the Cox-2 promoter.
Human MSCs were transduced with AdCOX2(–327/+59)vIL10 at MOI 100. 50 ng/mL TNF-α and 100 ng/mL IL-1β were added 72 h later with secreted IL-10 determined after a further 72 h.

TNF-α (50 ng/mL) and IL-1β (100 ng/mL) 72 h post transduction. Cell culture supernatants were analysed for IL-10 secretion by ELISA 72 h post cytokine stimulation. Negligible levels of IL-10 were observed in the untransduced controls. Transduced samples showed increased IL-10 expression in both unstimulated and stimulated samples. A similar effect was observed in *Metridia* luciferase expressing MSCs also under the control of the Cox-2 promoter with luciferase activity observed in both unstimulated and stimulated samples (data not shown).

We also briefly assessed the effects of a lower MOI on secretion of IL-10, however, we did not observe an appreciable level of IL-10 secretion above unstimulated controls (data not shown).

These data indicate that although the Cox-2 promoter constructs chosen show functionality in some cell types [CMS-5, above and bovine aorta endothelial cells (58)], they were not functional in the present MSC setting. The reasons for this are currently not understood and require further research.

Pharmacological induction

For pharmacological regulation of gene expression the tetracyline inducible Tet-on system was chosen: it consists of a constitutively expressed reverse Tet transactivator (rtTA2S-M2) (TA) (61) and the response vector carrying the Tet response element (TRE-Tight) (Clontech) upstream of eGFP (TREeGFP) or firefly luciferase (TRELuc). In the presence of the pharmacological inducer – the antibiotic doxycycline – the conformation of TA is changed allowing binding to the TRE, which in turn leads to reporter gene expression.

Bone marrow derived MSC of the rat was transfected with non-viral vectors as described above or infected with adenoviral vectors carrying TA and TRE reporter, respectively. The non-viral system resulted in a 12-fold induction of luciferase expression upon addition of doxycycline (1 μg/mL) (data

Figure 4 Selection of optimal transduction conditions.
Human MSCs were transduced with AdGFP at MOI 10, 25, 50 and 200 in the presence of lanthanum chloride. The percentage of GFP positive cells was assessed by flow cytometry at 72 h.

not shown). When MSC were infected with magnetic nano-particle complexes consisting of 1000VP/cell (of each viral vector) and 5 fg Fe/VP of SO-Mag6-125 in presence of a magnetic field strong induction of gene expression was achieved after addition of doxycycline (Figure 6A and B). Expression of eGFP was induced approximately 50-fold, luciferase was induced more than 1000-fold. In both cases reporter gene expression resulted in higher values than the CMV-promoter driven constructs, which were not doxycycline inducible.

Gene-activated matrices

Synthesis and characterisation of a temperature sensitive hydrogel carrier   The desired properties of a polymeric matrix to meet the objectives of the GAMBA concept are challenging to accomplish. The matrix ought to be injectable, responsive to heat and ought to be combined
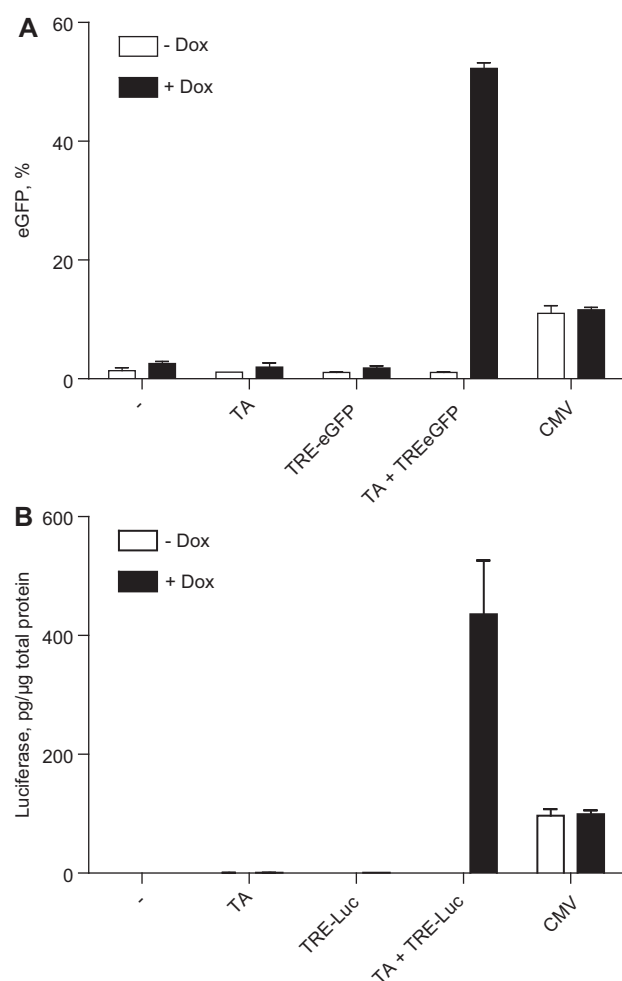
with transfection agents, nanoparticles, and MBCP™ while providing an adequate environment for encapsulated cells. Thus, the ability to generate highly controlled macromolecular structures and precise functionalization of polymers was mandatory. This was achieved by developing controlled radical polymerisation as well as very efficient reactions called "click" reactions (62, 63). The precision and versatility offered by these methods have simplified and expanded the synthesis of hydrogels and nanogels with complex architectures. Hyaluronic acid was selected as the backbone of the gene activated matrix as it is a major component of the extracellular matrix in connective, epithelial, and neural tissues and is known to play an important role in organ development, cell proliferation and migration. First, a thermoreversible semi-synthetic poly(N-isopropylacrylamide)-hyaluronan composition, designed to be a stable or a flowing deformable liquid at room temperature and to form a stiff physical gel when exposed to body temperature has been developed using these advanced synthesis techniques (64) (Figure 7). Thermoresponsive poly(N-isopropylacrylamide) (PNIPAM) represented an attractive candidate to introduce physical crosslinks via association of hydrophobic domains because it has a gelling temperature below body temperature (~32°C) and good biocompatibility. It has also been commonly used as a carrier in thermoresponsive drug delivery systems (65). Second, a chemically crosslinked hyaluronan hydrogel has been developed by grafting N-acrylate groups to the backbone of hyaluronic acid and then reacting thiolated poly(ethylene glycol) and peptide crosslinkers under mild conditions suitable for cells encapsulation. The development and characterisation of the two hyaluronic acid hydrogel platforms aim at improving an understanding on transfection mechanism and efficiency in a 3D hydrated matrix with different architectures (66). Polymeric materials are currently evaluated with partners as carriers loaded with biologics, transfecting agents and calcium phosphate particles. For example, calcium phosphate particles disperse easily in thermoresponsive compositions at room temperature and significantly increase the viscosity of the polymeric solution without modification of its thermal transition (Figure 7). These early works should lead to the assessment of the in vivo efficiency of selected injectable carrier systems for the delivery of the complex GAMBA therapeutic solution and the ability to induce transfection on-demand (67).

Figure 6   Tet-regulated, MNP enhanced reporter gene expression in rat MSC.
Infection was at MOI of 1000 VP AdV vector/cell with PEI coated MNP SO-MAG6-125 at 5 fg Fe/VP and magnetic field. After infection doxycyclin was added as indicated at a final concentration of 1 µg/mL and incubated for 48 h at 37°C.

3DFect-IN™ in atelo-collagen

Apart from the temperature-sensitive hyaluronan matrix – the composition of which is refined and the synthesis of which is up-scaled as part of the GAMBA project – various other hydrogel matrices can be suitable to embed vector-loaded MBCP™ granules, mesenchymal stem cells and vectors. Project partner OZ Biosciences has a proprietary and commercially available cationic lipid transfection reagent called 3DFect-IN™ which was developed especially for use with gene-activated matrices.
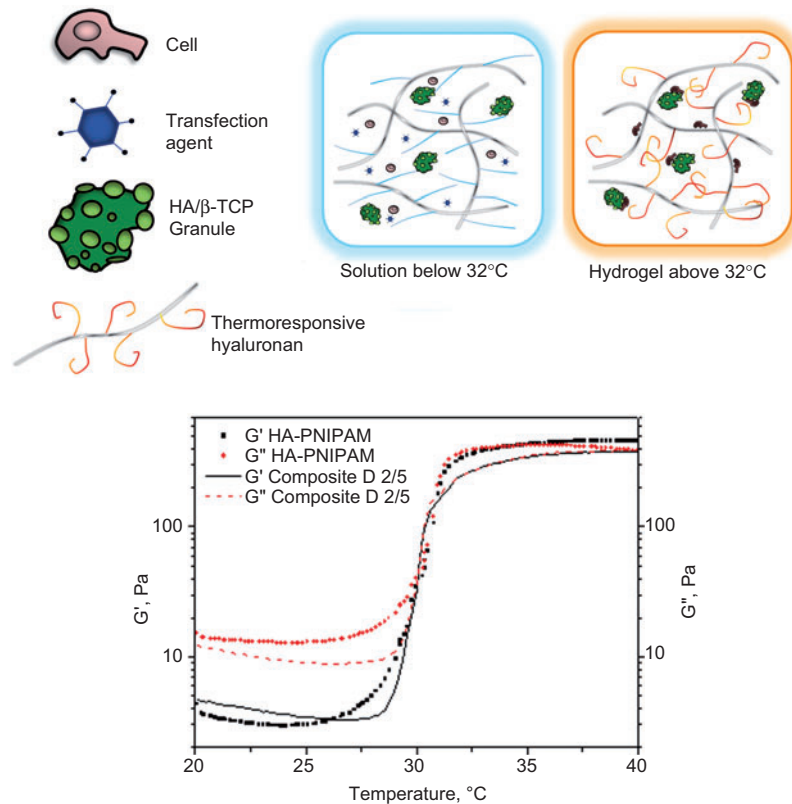
Figure 7 Schematic of thermoreversible semi-synthetic poly(N-isopropylacrylamide)-hyaluronan composition for delivery of calcium phosphate particles (HA/β-TCP granules), transfection agents and cells, and comparison between viscoelasticity of HA-PNIPAM hydrogel and a composite thermoresponsive hyaluronan (HA-PNIPAM)/calcium phosphate particles in weight ratio 2/5 as a function of the temperature.

3DFect-IN™/DNA complexes encoding the eGFP reporter protein were prepared in DMEM as described by the manufacturer protocol (OZ Biosciences) and mixed with atelocollagen solution (Koken Co. Ltd., Japan). After gelation of the matrix, a suspension of human mesenchymal stem cells provided by project partner Dr. Murphy (REMEDI, National University of Ireland, Galway, Ireland) was added. After 48 h, transfection was assessed by fluorescence microscopy. As shown in Figure 8, the cells become transfected at reasonably high efficiency.

COPROGs on MBCP™

As mentioned above, COPROGs are PEG-shielded vectors. In contrast to other PEG-shielded vectors, the PEG is applied as a strictly alternating copolymer with amino acid derivatives to which functional groups are coupled covalently. For shielding polyethylenimine-DNA polyplexes, we have used protective copolymers derivatized in their side chains with the branched anionic peptide "YE5C" (sequence: [Ac-YE$_5$]$_2$K-ahx-C). The copolymer is attached to pre-formed PEI-DNA polyplexes via electrostatic interaction (68). This way of shielding reduces complement activation (27) and the acute toxicity of PEI polyplexes in vitro and in vivo. We have used COPROGs in the past to prepare gene-activated matrices with collagen sponges (29, 30), fibrin glue (31, 69) and

with PDLLA coatings on titanium (28). Hence, it can be assumed that COPROGs are suited to be embedded both in the gel phase and the MBCP™ solid phase of the GAMBA composite matrix (Figure 1). As described previously (31) COPROGs are assembled in aqueous suspension. Hence, we have determined in a first step the maximum volume of water per weight of MBCP™ that the granules can soak. Granules without gene vector are shown in Figure 9. The granules were incubated with exactly this volume of aqueous suspensions of increasing amounts of COPROGs, followed by drying under high vacuum. The release of vector from the granules immersed in cell culture medium was determined using 125-iodine-labelled plasmid DNA in COPROG formulation as described previously (28) (Figure 10). Surprisingly, major amounts of DNA were released within 1 h of incubation in medium and the initial burst of release was essentially complete within 4 h.

In contrast, when NIH 3T3 cells were cultivated on MBCP™ granules loaded with COPROGs comprising a *Metridia* luciferase reporter plasmid, reporter gene expression peaked at 5 days followed by a drastic decline of expression (Figure 11A). The *Metridia* luciferase is secreted from the transfected cells and is measured in cell culture supernatants. The location of transfected cells was determined with COPROG-loaded MBCP™ comprising the eGFP reporter gene. Transfected cells were found almost exclusively on
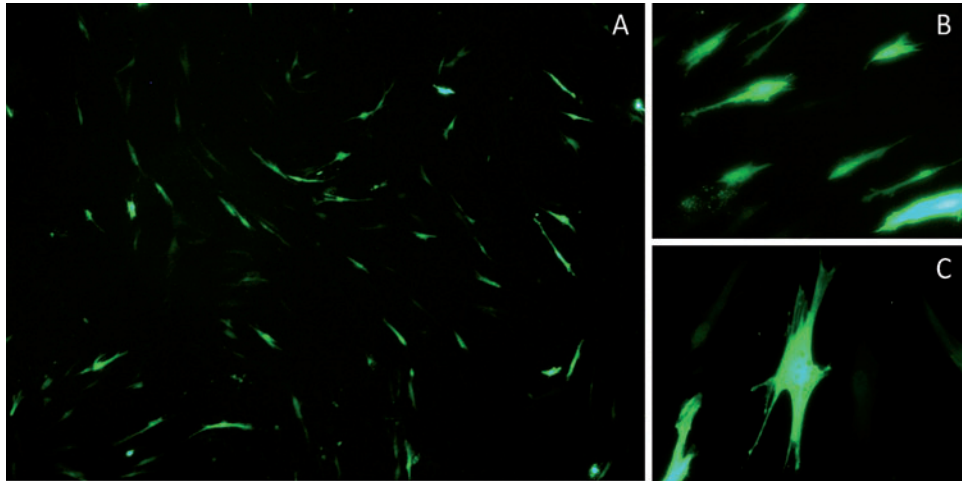
Figure 8   Transfection of cells colonising a hydrogel.
Images of human mesenchymal stem cells acquired 48 h after in gel transfection with 3D-FectIN™/GFP plasmid at a 2:1 ratio (A). Differences in fluorescence intensity renders the depth of the transfected cells within the gel as illustrated by 20× (B) and 40× magnification (C).
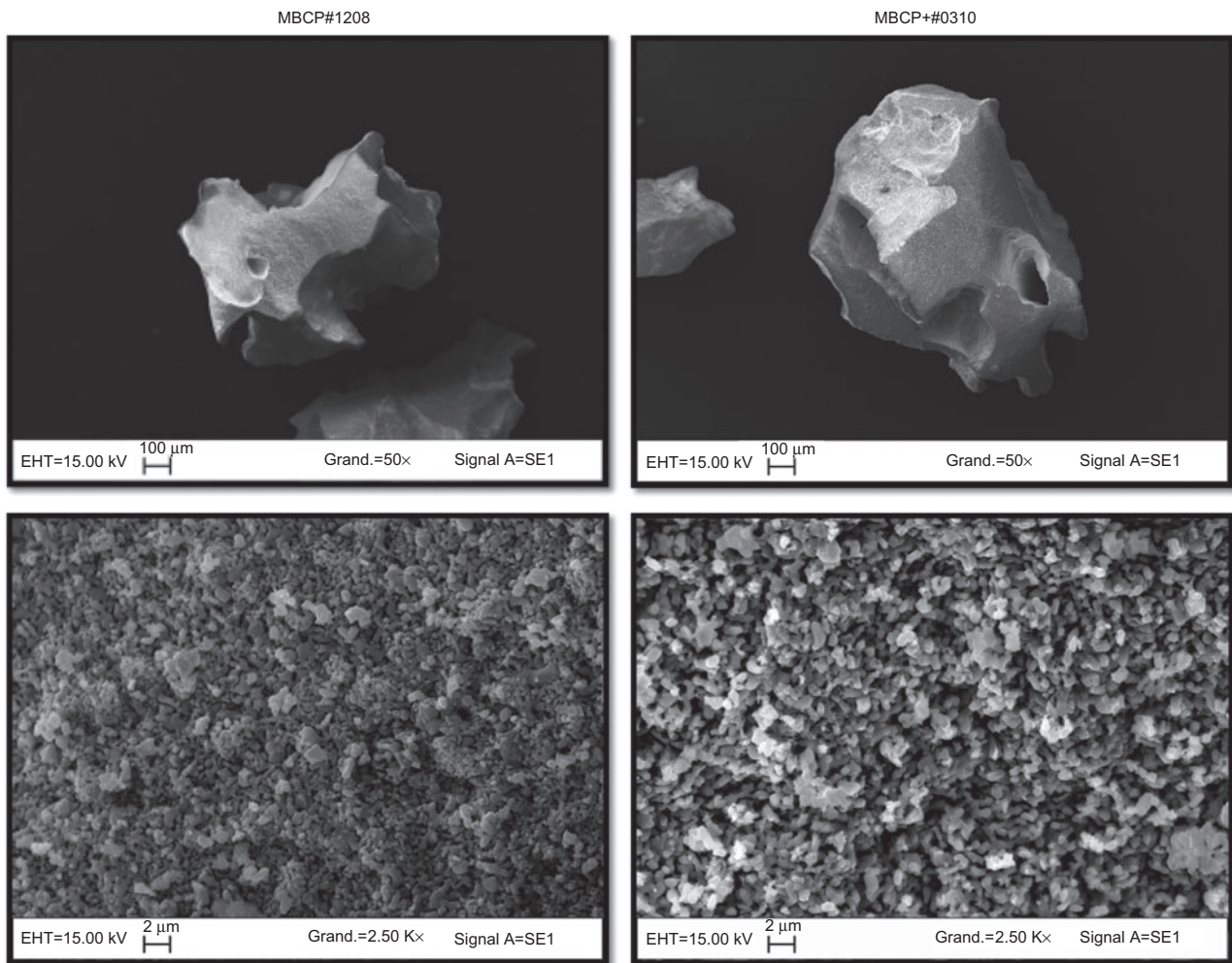

Figure 9   Surface morphology of native MBCP granules by SEM. The microporosity and size of the crystals provides part of the bioactivity of the material. For both samples, the crystal size is lower than 1 µm and the porosity <0.5 µm.
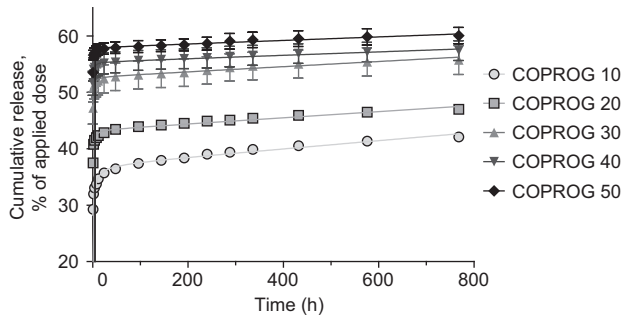
Figure 10   Release of DNA from COPROG-loaded MBCP™ granules upon incubation in cell culture medium.

Preparatory experiments for the implementation of the GAMBA concept

As becomes obvious from the above preliminary results, there are multiple materials, vectors and ways for assembling composite gene-activated matrices for achieving spatially and temporally controlled gene expression. Ongoing work focuses on assembling the modules for achieving spatially and temporally controlled expression of therapeutically relevant genes. This is technological task. At the same time it is essential to better understand how the biomaterials alone and the factors envisaged to be expressed in a composite gene-activated matrix according to Figure 1 affect chondrogenic and osteogenic differentiation of MSCs and other cell types.

For this purpose, we have set up an in-vitro model to test eventually gene-activated matrices using Cox-2 inducible IL10 expression. First we have confirmed that hMSC upregulate endogenous Cox-2 gene expression in response to inflammation, thus demonstrating, that in principle hMSC have an indigenous functioning Cox-2 promoter and thus the capability the to respond to inflammatory cues. Furthermore, we have cultured hMSCs in a hydrogel in chondrogenic differentiation medium. Addition of medium that was previously conditioned by factors secreted by human osteoarthritic synovium inhibited chondrogenic differentiation of hMSCs. This demonstrates that osteoarthritic synovium indeed secretes factors that inhibit cartilage repair by MSCs. We are currently investigating the effect of IL-10 on the human osteoarthritic synovium.
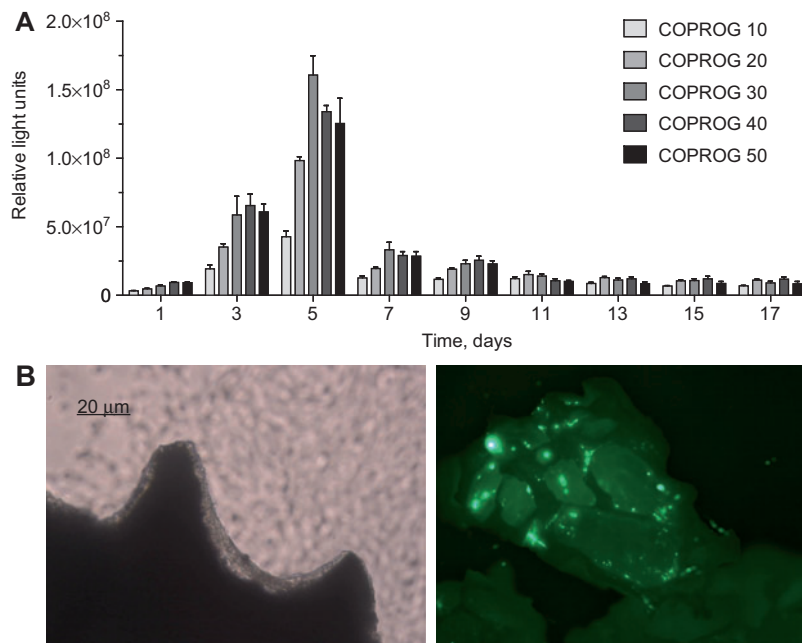
the granules and not amongst cells having colonised the cell culture plate in which the granules were incubated (Figure 11B). Therefore, it is likely that not the initially released vector fraction accounts for the successful transfection but rather the fraction of the vector which is retained on the MBCP™ granules. Of note, the release experiment specifies the amounts of DNA which are released from the carrier but discloses nothing of the state of the DNA, whether DNA naked or still in COPROG formulation. Given the late reporter gene expression peak we speculate that MBCP™ granules can dissociate COPROGs and that the released DNA is in naked state. Preliminary experiments indicate that the released material is transfection-incompetent and that it is in fact naked DNA.

Figure 11   Reporter gene expression by NIH 3T3 mouse fibroblasts upon transfection on COPROG-loaded MBCP™ granules.
(A) *Metridia* luciferase expression over a 17 day time period. (B) eGFP expression after 5 days of cell cultivation on COPROG-loaded MBCP™. Left: The bright field micrograph shows a seam of multiple cell layers covering the surface of a granule while other cells have colonized the bottom of the cell culture plate. Right: eGFP expression is exclusively found associated with the granules.
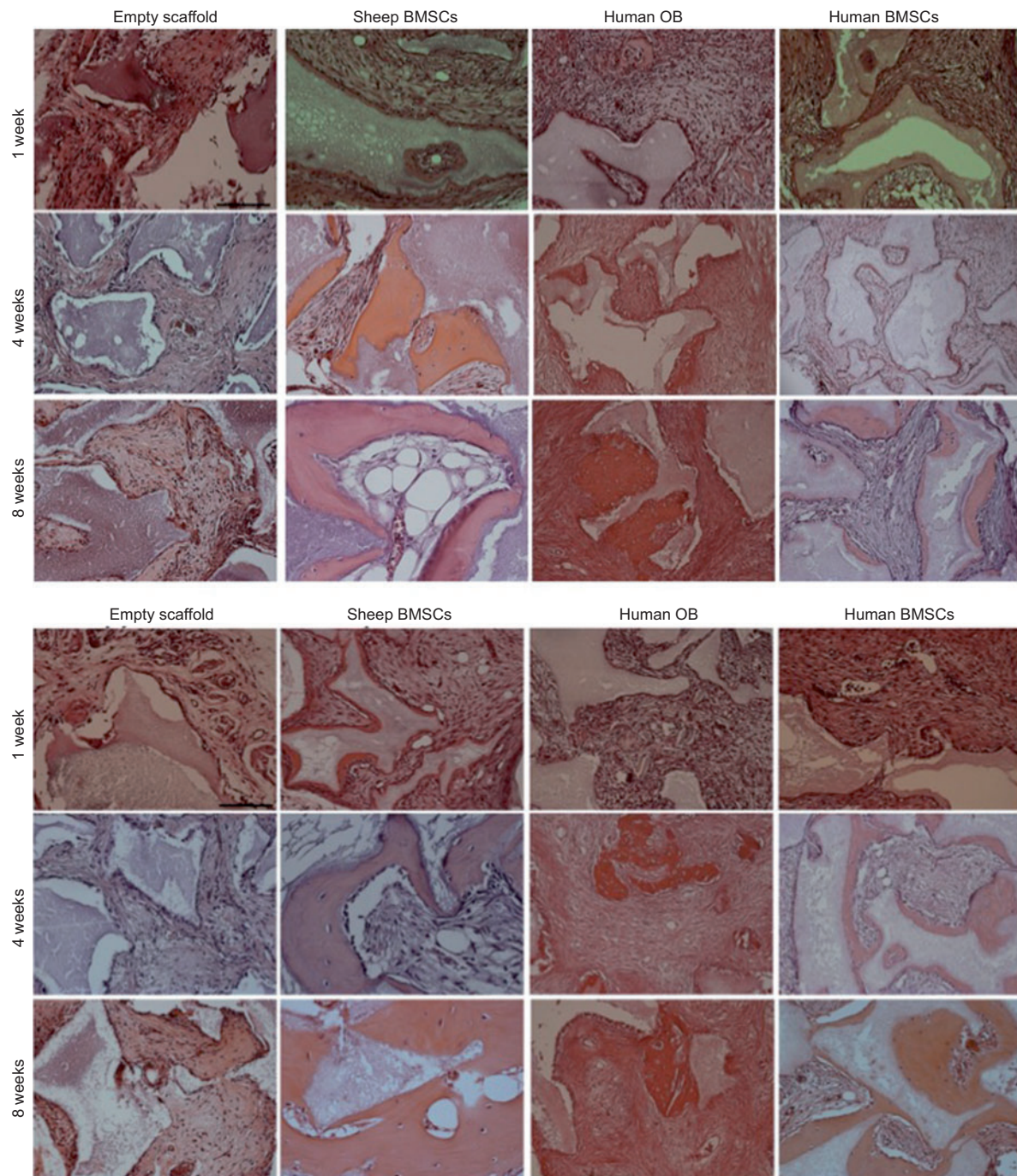
Figure 12   Bone formation of sheep BMSCs (positive control), human BMSCs and human OB on ceramics of different composition after nu/nu mice implantation.

(A) Paraffin histological sections of MBCP A stained with haematoxylin & eosin (H&E). No osteoconductive properties of this scaffold were observed after implantation without cells proved by the absence of bone formation. A fibrous tissue presenting some parallel orientation was observed (left column of images). Sheep BMSCs and human OB formed large amounts of bone in 8 weeks explants (second and third column). No bone or just a small amount of bone deposition was observed after 1 and 4 weeks of MBCP A seeded with human BMSCs (fist two pictures of the last column) while 8 weeks explants showed some bone formation (last picture of the column). (B) Histological sections of MBCP+ scaffold explants. Scaffold in absence of seeded cells was not able to induce bone deposition by athymic mice recruited cells (first column of images). Sheep BMSCs seeded within MBCP+ scaffold were able after 1 week of implant to deposit new bone (first image of the second column). Upon 4 and 8 weeks areas of newly bone populated almost the total cellular area of the scaffold (third image of the column). Human OB formed new bone areas at 4 and 8 weeks explants (second and third images of the third column). Human BMSC within MBCP+ scaffold deposited new bone areas at 4 weeks. These newly formed bone zones were bigger in size at 8 weeks. Haematoxylin & eosin (H&E) stains the newly formed bone in orange, the scaffold is shown in smooth grew. Scale bar=100 µm.

Based on experiments we have decided to use TGFβ-1 for chondrogenic induction in (70). In a culture system with renewal of the medium two or three times per week, 10 ng/mL TGFβ-1 is preferred for stimulation of chondrogenic differentiation. In our standard culture procedure for 5 weeks, cartilage formation was best if TGFβ was added during the entire culture period. Addition of TGFβ for a shorter period resulted in less cartilage. However, this standard culture procedure is not representing the situation in the patient. The hMSCs eventually will have to form cartilage to repair a defect in cartilage and bone where it will be exposed to environmental effects. Therefore, we have set-up a model to study the performance of MSCs in a more realistic environment by using an osteochondral biopsy where a defect can be created (71). In the defect, exposed to factors secreted by cartilage and bone, MSCs appear to respond different than in the conventional culture. The concentration of TGFβ secreted by the osteochondral biopsy is much lower than 10 ng/mL. This model is now being used to investigate the effect of cartilage and bone tissue on the fate of MSCs and to evaluate the use of the hyaluronan gel and later on the gene-activated matrix.

To prepare for testing endochondral-bone formation in response to constructs/vectors developed in the GAMBA consortium, we evaluated the capacity of human bone marrow stromal cells (BMSCs) and human osteoblasts (OB) to form bone in vivo after their seeding on two different types of MBCP™ granules obtained from project partner Biomatlante. For this purpose, colonized and empty granules were compared upon subcutaneous implantation in mice.

Human and sheep BMSCs were isolated from adult bone marrow and cultured as previously described (72–74). Human OB were harvested from bioptic pieces of large bones by several mechanical and enzymatic digestions and cultured according to Tortelli et al. (75). We used two micro/macroporous biphasic calcium phosphate ceramics (MBCP) with different compositions; MBCP A, composed of 60% of hydroxyapatite and 40% of beta tricalcium phosphate and MBCP-plus, composed of 20% of hydroxyapatite and 80% of beta tricalcium phosphate. To evaluate the effect of the different ceramics on ectopic bone formation by hMSCs and hOB, we seeded 2×10^6 cells at passage 1 per three MBCP (A and +) scaffolds. Twelve immunodeficient mice (CD-1 nu/nu; Charles River, Calco, Italy) were anesthetized and four subcutaneous pockets were created. Scaffolds of each of the calcium phosphate ceramics were implanted for 1, 4 and 8 weeks. At the end time points, animals were sacrificed and implants were harvested for histological analysis to assess bone formation. As required by the Italian Ministry of Health, all animals were maintained in accordance with the standards of the Federation of European Laboratory Animal Science Associations (FELASA).

The preliminary results show that good bone formation occurs when implanting different MBCP scaffolds loaded with non-transfected BMSC. An analysis of histological sections shows that hBMSCs deposit more bone extracellular matrix in the scaffolds made with MBCP-plus granules (Figure 12).

Patient and citizen panels

The patient and citizen panels (Figure 13) are conducted in three partner countries: Germany, Ireland and Switzerland. Goal of the panels is a qualified discussion of the GAMBA topics with patients and interested citizens. The patient panel in Munich, Germany took place in May 2011 with 16 patients aged between 42 and 72 years. In the panels, the participants are in a first step introduced to the field of innovative basic research into gene and stem cell therapies, for example, through a "manual" in easily comprehensible language, expert presentations, their own research on the subject ("mini-expertises") and a hearing with experts selected by the citizens.

Figure 13 The facilitation team from ScienceDialogue working with the patient panel (courtesy of ScienceDialogue, 2011).

With the support of independent facilitators from GAMBA partner ScienceDialogue who does not have a stake in the research process itself, the participants in a second step negotiate about the benefits, risks and ethical/social aspects in the field. As a result, the patients and citizens evaluate the GAMBA field from their special point of view as patients and interested citizens with relevance to societal sights and values. They draw up recommendations for the scientific world as well as for other sectors of society, such as industry and politics. These recommendations are recorded in a so-called citizen opinion which will be presented to the researchers, a representative of the EU commission and representatives of industry and politics in a concluding ceremony.

In their patient opinion, the German patients state that "GAMBA offers the chance to one day heal arthritis. We approve GAMBA under the condition of a careful risk assessment. A thorough ethical evaluation has to be performed once GAMBA is in the state of being applied to humans (clinical trials)".

What do the participants think about the panel? "Our views and opinions were taken seriously. We were informed in a very competent and comprehensive manner", says one participant. Another one states: "I liked the open discussion and the fair, respectful contact with each other. Although taking in all this information was demanding and we had a tight time schedule, the good organization and the nice atmosphere helped us draw the patient opinion". Three quarters of the participants stated after the panel that they now feel able to assess the ethical and social aspects of GAMBA "well" or "very well".

Therefore, in the panels both "parties", researchers and the public can learn from one another. Furthermore, decision makers in politics, administration, business and other fields will profit from the ideas and assessments of the participants. Their views and associations give an early indication of the level of acceptance of the new technologies. The citizen panel in Munich/Germany and the panels in Ireland and Switzerland, take place in 2012.

Concluding remarks

As is evident from the preliminary results presented here, GAMBA is an ongoing project and its main objective – establishing an implant system that delivers regenerative bioactivity in a temporally and spatially controlled fashion in response to endogenous and external stimuli – has yet to be achieved. With millions of patients urgently awaiting technological progress towards efficient therapies, and given the fact that a composite gene-activated matrix comprising multiple therapeutic genes is unlikely to obtain regulatory approval in the near future, the question is whether this objective is reasonable. In this context it needs to be emphasised that the goal of GAMBA is not to deliver a novel therapy, at least not within the scope of this project. GAMBA is a project funded under the European Union's nanotechnology programme as opposed to a regenerative medicine programme. The research in GAMBA is basic as opposed to applied research. The

question is whether a composite bioactive matrix that delivers therapeutic activity on command and demand can be implemented at all. GAMBA's task is to go the first exploratory steps. We, the GAMBA consortium believe that the project's main objective is reasonable. Nature itself has implemented morphogenesis and healing processes as a concerted action of multiple factors under tight spatial and temporal control. And a lesson learned from the patient panel. Every patient suffers his/her own personalised history and manifestation of arthritis. Future medical therapies will require a choice of therapeutic modules selected according to the individual patient's needs and, if feasible, responding at a molecular level to the individual patient's disease. It is worthwhile to explore such systems.

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