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Temporal and spatial patterning of transgene expression by near-infrared irradiation

Francisco M. Martin-Saavedra [a, b, 1], Virginia Cebrian [a, b, 1], Leyre Gomez [c], Daniel Lopez [d], Manuel Arruebo [a, c], Christopher G. Wilson [e], Renny T. Franceschi [e], Richard Voellmy [f, g], Jesus Santamaria [a, c], Nuria Vilaboa [a, b, *]

[a] CIBER de Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), 28029 Madrid, Spain
[b] Hospital Universitario La Paz-IdiPAZ, 28046 Madrid, Spain
[c] Department of Chemical Engineering, Nanoscience Institute of Aragon (INA), University of Zaragoza, 50018 Zaragoza, Spain
[d] Institute of Polymer Science and Technology (ICTP-CSIC), 28006 Madrid, Spain
[e] Center for Craniofacial Regeneration and Department of Periodontics and Oral Medicine, University of Michigan School of Dentistry, Ann Arbor, MI 48109, USA
[f] Department of Physiological Sciences, University of Florida, Gainesville, FL 32611, USA
[g] HSF Pharmaceuticals S.A., 1814 La Tour-de-Peilz, Switzerland

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ABSTRACT

We investigated whether near-infrared (NIR) light could be employed for patterning transgene expression in plasmonic cell constructs. Hollow gold nanoparticles with a plasmon surface band absorption peaking at ~750 nm, a wavelength within the so called "tissue optical window", were used as fillers in fibrin-based hydrogels. These composites, which efficiently transduce NIR photon energy into heat, were loaded with genetically-modified cells that harbor a heat-activated and ligand-dependent gene switch for regulating transgene expression. NIR laser irradiation in the presence of ligand triggered 3-dimensional patterns of transgene expression faithfully matching the illuminated areas of plasmonic cell constructs. This non-invasive technology was proven useful for remotely controlling *in vivo* the spatiotemporal bioavailability of transgenic vascular endothelial growth factor. The combination of spatial control by means of NIR irradiation along with safe and timed transgene induction presents a high application potential for engineering tissues in regenerative medicine scenarios.

1. Introduction

Engineered functional tissues must achieve a high level of cellular organization in structures that resemble those intended to be replaced. To accomplish this, major research efforts have been undertaken to develop scaffolds that mimic the geometry of the replaced tissue and provide a 3-dimensional environment that supports specific cell function. A multitude of signaling factors, many of which have well established roles in tissue development and homeostasis, regulates interactions and behavior of cells seeded in scaffolds. However, recapitulating the production of control factors responsible for native tissue formation over appropriate spatial and time scales remains a central challenge in regenerative medicine.

Scaffolds may instruct surrounding environments by releasing bioactive agents. Most porous scaffolds currently used in tissue engineering deliver cargos passively, through mechanisms of molecular diffusive transport that provide limited control on release kinetics and hamper the effectiveness of the approach. Recently, the implementation of nanotechnology-enabled strategies in the design of porous scaffolds has made possible triggered delivery of growth factors and signaling molecules using external stimuli. Examples of these strategies are porous ferrogels intended to control locally the cellular microenvironment through the release of recombinant regenerative factors such as SDF1-α [1] or FGF-2 [2] subsequent to magnetic stimulation. Such approaches usually involve a burst release of therapeutic agent after stimulus application that precludes the re-induction of the system and limits its long-term functionality.

* Corresponding author. Hospital Universitario La Paz-IdiPAZ, 28046 Madrid, Spain. Tel.: +34 912071034; fax: +34 917277524.
E-mail address: nuria.vilaboa@salud.madrid.org (N. Vilaboa).
[1] These authors contributed equally.

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Alternatively, precise control over the production and the subsequent release of growth factors and signaling molecules from scaffolds can be achieved by seeding these substrates with cells that are genetically engineered to express the latter bioactive factors. In this case, external activation is also a desirable feature to achieve control over the release profile of targeted factors. In this regard, gene therapy systems that employ promoters sensitive to physical stimuli such as light, ionizing radiation or heat [3,4] are promising tools for remotely controlling the spatiotemporal bioavailability of therapeutic proteins. The promoter of the *HSP70B* gene (*HSPA7*), one of the most highly heat-inducible genes [5], has been successfully used for local control of transgene expression in combination with non-invasive methods for focused heating such as ultrasound [6,7]. A key to realizing the full potential of this approach is the development of reliable, non-invasive methods capable of heating deep-seated areas without causing tissue harm. It is noted that excessive acoustic pressure and high temperature from focused ultrasound may generate cavitation leading to tissue damage [6,8].

Plasmonic photothermia, in which photon energy is converted into heat by photothermal nanotransducers, is a nanotechnology resource that can be adapted for inducing *HSP*-based gene expression systems [9,10]. On account of the phenomenon of localized surface plasmon resonance (LSPR), gold nanoparticles (GNPs) exhibit strong visible and near-infrared light absorption that depends on the size, shape and surrounding medium of the nanomaterial. The ability to control GNP dimensions makes it possible to prepare nanostructures tailored for absorption between 650 and 950 nm. This wavelength range that does not include the major absorption peaks of blood and water provides a therapeutic window that maximizes light penetrance in biological tissues [11,12].

In this study, we explore the feasibility of providing plasmonic properties to fibrin hydrogels by the incorporation of hollow gold nanoparticles (HGNPs) [10] and the suitability of the resulting scaffolds to encapsulate cells harboring a transgene expression system triggered by heat and dependent on a dimerizer-controlled chimeric transactivator [13]. We tested whether the combination of NIR irradiation and dimerizer administration induces transgene expression in plasmonic cell constructs implanted in mice, with spatial patterns that match the NIR-illuminated region.

2. Materials and methods

2.1. Vector construction

Coding sequence of enhanced green fluorescent protein (EGFP) was PCR-amplified from pEGFP-C1 (Clontech, Mountain View, CA, USA) using primers 5′-TAGCGCTACTAGTCGCCAC and 5′-GGCTGATATCGATCAGTTATC, digested with SpeI and ClaI and ligated to SpeI/ClaI-cut pZ12I-PL-2 (ARIAD Gene Therapeutics Inc, Cambridge, MA, USA). The resulting plasmid was named pZ12-EGFP. To prepare pSV40-Hyg, pGL4.16 (Promega, Madison, WI, USA) was digested with BamHI and BglII followed by religation of the resulting larger fragment.

2.2. Cell culture, transfection, and isolation of cell lines

C3H/10T1/2-fLuc and C3H/10T1/2-VEGF cells, derived from C3H/10T1/2 cells (ATCC CCL-226) as described elsewhere [13], stably incorporate a heat-activated and dimerizer-dependent gene switch that controls a firefly luciferase (*fluc*) gene or a human vascular endothelial growth factor isoform 165 (*VEGF165*) gene, respectively. Transfection of HeLa cells (ATCC CCL-2) was carried out using Lipofectamine 2000 (Invitrogen, Barcelona, Spain) following the manufacturer's recommendations. HeLa cells stably harboring the Hsp70/12xZFHD1-TA transactivator construct [13] were isolated using 600 μg mL^{-1} G418 (InvivoGen, San Diego, CA, USA). Stable clones were screened for heat and rapamycin responsiveness after transient transfection with pZ12-fLuc [13]. One day after lipofection, cells were treated for 1 h with rapamycin (InvivoGen) or vehicle and then subjected or not to heat treatment at 45 °C for 30 min in a thermostatically-controlled water bath. A highly inducible clone was isolated and then co-transfected with pZ12-EGFP and pSV40-Hyg at a 5:1 M ratio. Clones were isolated subsequent to selection using 600 μg mL^{-1} G418 and 200 μg mL^{-1} hygromycin B (InvivoGen). For EGFP induction assays, cells were seeded in 24-wells plates (Sigma–Aldrich, Madrid, Spain) at a density of 2.5×10^4 cells cm^{-2}. One day later, cells were or were not subjected to heat treatment in the presence of rapamycin or vehicle and then cultured further for 24 h. Cells were visualized using a fluorescence microscope Leica AF6000 (Leica Microsystems, Heidelberg, Germany). The average fluorescence of 10 individual fields for each condition was measured using Fiji, an open source image processing package based on ImageJ (http://fiji.sc). A cell line, termed HeLa-EGFP, was identified that exhibited strong transgene expression exclusively after heat treatment in the presence of rapamycin. Clonal C3H/10T1/2- and HeLa-derived cells were maintained in a humidified 5% CO_2 atmosphere at 37 °C in Dulbecco's modified Eagle's medium (DMEM; Lonza, Madrid, Spain) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U mL^{-1} penicillin and 0.1 mg mL^{-1} streptomycin, under continuous selection with G418 and hygromycin B.

2.3. Rapamycin

For *in vitro* experiments, rapamycin was dissolved in DMSO and used at a final concentration of 10 nM. For *in vivo* injections, rapamycin was dissolved in N, N-dimethylacetamide (DMA) to prepare a stock solution (3 mg mL^{-1}) which was then diluted in a mixture of 50% DMA, 45% polyoxythylene glycol (average molecular weight of 400 Da) and 5% polyoxyethylene sorbitan monooleate (both from Sigma–Aldrich). Rapamycin was injected intraperitoneally at a dose of 1 mg kg^{-1} in a volume of 50 μL.

2.4. Preparation of fibrin-based plasmonic hydrogels

To prepare plasmonic scaffolds, bovine fibrinogen (fbg; Sigma–Aldrich) was dissolved in ice-cold DMEM at a concentration of 20 mg mL^{-1} of clottable protein. HGNPs, synthetized as described elsewhere [10], were added to the fbg solution at 0.02–0.1 mg mL^{-1}. Next, 0.8 volumes of DMEM alone or DMEM containing C3H/10T1/2-fLuc, C3H/10T1/2-VEGF or HeLa-EGFP cells at 2.5×10^6 mL^{-1} were added to the mixture. Finally, 0.2 volumes of ice–cold 20 μ mL^{-1} bovine thrombin (Sigma–Aldrich) in DMEM were added. After pipetting briefly to ensure uniform dispersion of components, the suspension was distributed to multiwell culture plates or polystyrene cuvettes (all from Sigma–Aldrich). Final volumes of suspensions were 0.5, 1 or 2 mL for 48-, 24- or 12-well plates, respectively, and 3 mL for polystyrene cuvettes. Suspensions were allowed to clot in a humidified 5% CO_2 atmosphere at 37 °C for 30 min. After clotting, 1 volume of DMEM containing 20% FBS was incubated with the hydrogel for 1 h to equilibrate its serum content and then replaced with culture medium. In some experiments the suspensions were supplemented with 30 μg mL^{-1} fbg-Alexa Fluor 546 (fbg-AF546, Invitrogen). To determine cell-mediated biodegradation of scaffolds, release of fbg-AF546 into culture medium was measured after excitation at 558 nm and quantification of the emitted fluorescence at 573 nm, using a Synergy4 spectrofluorimeter (BioTek Instruments, Vermont, USA).

2.5. Rheology

Small deformation oscillatory measurements were carried out in an AR-G2 controlled stress rheometer (TA Instruments, New Castle, DE, USA). The linear viscoelastic region (LVR) was determined through stress sweeps performed at 37 °C and an oscillatory frequency of 1 Hz. Frequency sweeps were performed at 37 °C between 0.1 and 100 Hz and an oscillatory strain of 0.5% within the LVR. Finally, temperature sweeps between 10 and 80 °C were recorded at an oscillatory frequency of 1 Hz and an oscillatory strain of 0.5%.

2.6. Electron microscopy

Scanning transmission electron microscopy (STEM) images of HGNPs were obtained with a Tecnai T30 electron microscope (FEI, Hillsboro, OR, USA) operated in the bright field mode at an accelerating voltage of 300 kV. To characterize plasmonic matrices using transmission electron microscopy (TEM), the above-described components for preparing plasmonic scaffolds were mixed and then diluted tenfold in phosphate-buffered saline (PBS). A drop of this dilution was carefully placed on a 400-mesh carbon-coated copper grid and allowed to polymerize for 5 min at 37 °C. After removing the excess solution with a filter paper, the sample was visualized using a JEM 1010 electron microscope (JEOL, Peabody, MA, USA). The internal morphology of plasmonic scaffolds, lacking or containing cells, was visualized by scanning electron microscopy (SEM). Hydrogels were rinsed gently with PBS, fixed with 4% paraformaldehyde (PFA) and sliced using a scalpel blade. Slices were lyophilized at −80 °C and then mounted on aluminum stubs, sputter-coated with carbon and imaged with a FEI Quanta 200 electron microscope.

2.7. Cell viability assays

Viability of cells entrapped in hydrogels was investigated using the alamarBlue-based assay (Biosource, Nivelles, Belgium). After washing with PBS, samples were incubated in DMEM-10% FBS containing 10% (v/v) alamarBlue dye for 2 h at 37 °C. After excitation at 530 nm, emitted fluorescence at 590 nm was quantified using a Synergy4 spectrofluorimeter. Cell viability was additionally assessed by staining with 50 μM calcein violet-acetomethyl ester (Calcein violet AM, Invitrogen). Samples