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Spatiotemporally-controlled transgene expression in hydroxyapatite-fibrin composite scaffolds using high intensity focused ultrasound.

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Abstract

Conventional tissue engineering approaches rely on scaffold-based delivery of exogenous proteins, genes, and/or cells to stimulate regeneration via growth factor signaling. However, scaffold-based approaches do not allow active control of dose, timing, or spatial localization of a delivered growth factor once the scaffold is implanted, yet these are all crucial parameters in promoting tissue regeneration. To address this limitation, we developed a stable cell line containing a heat-activated and rapamycin dependent gene expression system. In this study, we investigate how high intensity focused ultrasound (HIFU) can spatiotemporally control firefly luciferase (fLuc) transgene activity both in vitro and in vivo by the tightly controlled generation of hyperthermia. Cells were incorporated into composite scaffolds containing fibrin and hydroxyapatite particles, which yielded significant increases in acoustic attenuation and heating in response to HIFU compared to fibrin alone. Using 2.5 MHz HIFU, transgene activation was observed at acoustic intensities of 201 W/cm² and higher. Transgene activation was spatially patterned in the scaffolds by rastering HIFU at speeds up to 0.15 mm/s. In an in vivo study, a 67-fold increase in fLuc activity was

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6. Data Availability

The data obtained in this study are available on reasonable request from the corresponding author.

observed in scaffolds exposed to HIFU and rapamycin versus rapamycin only at 2 days post implantation. Repeated activation of transgene expression was also demonstrated 8 days after implantation. No differences in in vivo scaffold degradation or compaction were observed between +HIFU and -HIFU groups. These results highlight the potential utility of using this heat-activated and rapamycin-dependent gene expression system in combination with HIFU for the controlled stimulation of tissue regeneration.

Graphical Abstract

Keywords

High intensity focused ultrasound; hyperthermia; gene therapy; fibrin; hydrogel; hydroxyapatite

1. Introduction

Growth factors play a critical role in tissue regeneration by stimulating multiple cellular responses including proliferation, migration, and differentiation. The regenerative activity of a growth factor is controlled by four major parameters: concentration, spatial profile (i.e., gradient), temporal profile, and sequence of delivery (i.e., for processes regulated by multiple factors)[1, 2]. In cases where the body's natural ability to promote tissue regeneration is impaired or insufficient, exogenous administration of growth factors is being explored as a therapeutic option. In most regenerative applications, such as angiogenesis and osteogenesis, bolus delivery of growth factors is ineffective and can cause side effects. Therefore, scaffold-based delivery systems are utilized in an attempt to control the release parameters mentioned above. Controlled release is achieved by typically manipulating properties of the growth factor, such as covalently linking it to the polymer matrix of the scaffold [3, 4] or by manipulating the scaffold itself, such as by altering porosity or mesh size [5, 6].

Using conventional scaffolds to deliver growth factors has two inherent limitations. First, controlled release of growth factors is typically achieved by modifying material properties, which cannot be actively or non-invasively modulated after a scaffold is implanted. Second, a scaffold can only contain a finite amount of growth factor. Thus, if a target dose of growth factor is needed to elicit tissue regeneration, the dose must either be delivered in one scaffold or in multiple scaffolds that are periodically implanted over the course of therapy. With the former scenario, a supraphysiological dose of growth factor could elicit undesired effects. In the latter case, periodic implantation of scaffolds may hinder regeneration.

As an alternative to scaffold-based delivery, cell and gene-based systems have been developed where expression of the regenerative factor of interest is actively controlled by small molecule ligands, light, or heat shock [7]. These approaches provide a route for long-

term, regulated protein delivery that is superior to scaffold-based approaches. With a ligand-dependent system, gene expression is temporally controlled based on the timing of ligand administration, while heat shock inducible switches can be activated by hyperthermic water baths. In both cases, good temporal control of factor delivery is achieved, but spatial control is not possible [8–11]. Optogenetic [12] and photothermal [13] techniques have also been shown to provide tight spatiotemporal control of gene expression regulated by light or heat shock, respectively; although activation is restricted to superficial tissues due to the relatively limited penetration of light. The use of heat-shock inducible promoters has the additional restriction of being subject to undesirable activation by local inflammation or fever.

To overcome problems related to the uncontrolled activation of a heat shock response, we previously developed gene switches that require both a hyperthermic stimulus and small molecule ligand such as rapamycin or mifepristone for activation [14, 15]. Cells containing these gene switches were suspended in a fibrin scaffold and exposed to local hyperthermia in the presence of ligand. The hyperthermic stimulus was achieved using a water bath [16], or by exposure to near infrared radiation [17] or high intensity focused ultrasound (HIFU) [18]. In all cases, strong induction of a firefly luciferase reporter or the regenerative factors - vascular endothelial growth factor (VEGF) or bone morphogenetic protein 2 (BMP2) - was achieved. In addition, the localized hyperthermia generated by both near infrared radiation or HIFU provided tight spatial control of transgene expression. While both approaches are being actively pursued, in the present study, we further refine the HIFU-based approach.

HIFU is being explored in many medical applications and is currently approved by the United States Food and Drug Administration for the treatment of bone metastases [19], essential tremor [20], and uterine fibroids [21]. HIFU-generated heating can be used for therapies based on mild hyperthermia (i.e., 43–45°C) or thermal ablation (i.e., >55°C). In both cases, the heated region - which can be in superficial or deeply located tissue - is spatially and temporally restricted based on the application of the HIFU [22]. HIFU has been used in vivo to activate reporter genes - regulated by heat shock protein 70B (hsp70B) - using plasmids [23, 24], adenovirus [25, 26], and cell mediated delivery approaches [27], as well as in transgenic mice [28].

HIFU-activated gene switches have a number of advantages for gene therapy applications including the ability to spatially and temporally control gene expression in an on-demand manner as well as reactivate expression and protein production. This could enable personalization of a growth factor therapy based on the actual progress of regeneration and the recapitulation of growth factor release patterns seen during normal tissue healing. This inherent tunability differs from scaffold-based delivery where the spatiotemporal profiles of growth factor release are designed *a priori* (e.g., scaffolds with patterned spatial gradients).

One challenge with using HIFU to induce thermally-activatable heat switches in cells contained in hydrogels is that these matrices possess a very low acoustic attenuation (e.g., 0.05 dB·cm⁻¹·MHz⁻¹ for 1% w/v fibrin [29]), which is due to their high water content. Comparatively, the attenuation of muscle and cortical bone is 1.09 dB·cm⁻¹·MHz⁻¹ [30] and 3.2–4.4 dB·cm⁻¹·MHz⁻¹ [31], respectively. Thus, high acoustic intensities and/or long

exposure durations are required to generate hyperthermia within the scaffold since the rate of heating is proportional to the acoustic attenuation [32]. This could be problematic if the scaffold is implanted within or adjacent to soft tissue or bone since these tissues would preferentially heat in response to HIFU, thus reducing inducible transgene expression and increasing the likelihood for thermal damage.

In this study, we overcome these problems by developing and characterizing a composite scaffold consisting of fibrin and hydroxyapatite particles, which enables fLuc transgene activation at lower HIFU intensities and exposures than in our previous study [18]. Material properties of the composite scaffolds were characterized, including acoustic attenuation and heating profiles, as well as cell viability in the scaffolds. We evaluated transgene activation in vitro, including spatial patterning, and in vivo with subcutaneous implants. Overall, our results establish that HIFU induces spatiotemporally-controlled gene expression within the composite scaffolds at lower acoustic intensities relative to fibrin only scaffolds.

2. Materials and Methods

2.1 Cell culture

C3H/10T1/2 cells carrying a heat-activated and rapamycin-dependent gene switch controlling fLuc expression (i.e., C3H-fLuc) are described in detail elsewhere [14, 15, 18]. Cells were cultured in complete media consisting of high glucose Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Grand Island, NY, USA) containing L-glutamine and sodium pyruvate, 10% fetal bovine serum (Denville Scientific, Holliston, MA, USA), 100 U/mL penicillin, 100 µg/mL streptomycin, and 5 µg/mL Plasmocin (InvivoGen, San Diego, CA, USA). Cells were cultured under continuous selection by supplementing the media with 600 µg/mL hygromycin B (Thermo Fisher Scientific, Waltham, MA, USA) and 1.2 mg/mL G418 (Thermo Fisher Scientific). For incorporation into scaffolds, cells were trypsinized and suspended in DMEM.

2.2 Scaffold fabrication

Hydroxyapatite (HA)-doped fibrin scaffolds (HAFib) were prepared by modifying a previous method [29]. Briefly, scaffolds were prepared with 5 mg/mL clottable protein by first dissolving bovine fibrinogen (Sigma-Aldrich, St. Louis, MO, USA) in degassed (36% O₂ saturation) DMEM. HA powder (Ø: < 200 nm or <2.5 µm, Sigma-Aldrich) was added to a 1:1.25 (v/v) solution of serum and DMEM. Fetal bovine serum and mouse serum (heat inactivated, Atlanta Biologics, Flowery Branch, GA, USA) were used for in vitro and in vivo experiments, respectively. The resulting HA mixture was sonicated with a probe sonicator (model 450, Branson, Danbury, CT, USA) for 60 s to disperse and wet the HA powder and then degassed (2 mmHg) in a vacuum oven at ambient temperature (i.e., 23–25°C). Scaffolds were generated by combining the fibrinogen solution and HA mixture with bovine thrombin (Thrombin-JMI, King Pharmaceuticals, Bristol, TN, USA), bovine lung aprotinin (Sigma-Aldrich), rapamycin, and cell suspension. The final concentrations of serum, thrombin, aprotinin, rapamycin, and cells in the scaffold were 20% (v/v), 2 U/mL, 0.025 U/mL, 10 nM, and 10⁶ cells/mL, respectively. Scaffolds were prepared with final HA concentrations of 5–50 mg/mL.

2.3 HIFU localization and exposure

The acoustic exposures used to induce hyperthermia were conducted as follows. A calibrated, single element ultrasound transducer (2.5 MHz, H108, f-number = 0.83, focal length = 50 mm, Sonic Concepts, Inc., Bothell, WA, USA) was driven by a continuous waveform generated using a function generator (33500B, Agilent Technologies, Santa Clara, CA, USA), amplified by a radiofrequency amplifier (A300, Electronics & Innovation, Rochester, NY, USA), and passed through a matching circuit (H108_3MN, Sonic Concepts) to reduce the impedance mismatch between the transducer and amplifier. Radiofrequency signals were monitored with an oscilloscope (HDO4034, Teledyne LeCroy, Chestnut Ridge, NY, USA). Acoustic exposures were done at the following spatial peak temporal average (SPTA) intensities unless otherwise stated: 145, 201, 258, and 314 W/cm².

In all experiments, the focus of the HIFU transducer was spatially localized in the axial direction using a pulse echo technique. Briefly, the single element transducer was driven by a pulser-receiver (5077PR, Olympus, Center Valley, PA, US) that generated a low energy signal. The reflected signal was visualized on an oscilloscope, and maximized in amplitude by modifying the distance between the transducer and scaffold.

2.4 Acoustic attenuation of acellular scaffolds

The acoustic attenuation of acellular scaffolds, polymerized inside custom sample chambers made of polyvinyl chloride pipe, was measured as previously described [29]. Each chamber consisted of a segment of pipe (inner diameter: 20 mm, height: ~10 mm or ~20 mm) sealed on both ends with a Tegaderm membrane (3M Health Care, St. Paul, MN, USA). The attenuation of each scaffold formulation was measured using a broadband pulse technique with a single-element ultrasound transducer (3.5 MHz, diameter: 19.1 mm, f-number: 2; Olympus, Waltham, MA, USA) at 24–25°C.

2.5 Temperature profiles in acellular scaffolds exposed to HIFU

Measurements were performed to determine the temperature elevation in each scaffold formulation as a function of ultrasound intensity. As described above, acellular scaffolds were prepared in custom sample chambers (inner diameter: 34 mm, height: 12 mm) sealed with Tegaderm. Prior to polymerization, a needle-type thermocouple (diameter: 0.3 mm, type K; Omega Engineering Inc., Stamford, CT, USA) was inserted through the sidewall of the chamber and into the center of the scaffold. The sample chamber was immersed and mounted in a tank of degassed water (30–36% O₂ saturation) at 37°C. Hyperthermia was generated inside the scaffold using HIFU, as described in section 2.3, by positioning the ultrasound focus 1–3 mm from the tip of the thermocouple. Temperature profiles were recorded using a thermocouple data logger (TC-08, Pico Technology, Tyler, TX, USA) and associated data acquisition software.

2.6 Viability of C3H-fLuc cells in scaffolds

Scaffolds containing C3H-fLuc cells, each 0.06 mL in volume, were polymerized in a 96 well plate. Each scaffold was covered with 0.2 mL complete media and the plate was placed in a standard tissue culture incubator (37°C, 5% carbon dioxide). At 1–7 days after polymerization, cell viability was quantified using the CellTiter 96 AQueous One Solution

Cell Proliferation Assay (Promega, Madison, WI, USA), according to the manufacturer's instructions. Percent cell viability was calculated relative to scaffolds whose viability was measured on the same day of polymerization (i.e., day 0).

2.7 In vitro activation of C3H-fLuc cells using HIFU

Two different in vitro experiments were conducted to demonstrate HIFU-mediated activation of fLuc activity. In the first experiment, a custom 24-well plate was made by machining 9.5 mm diameter holes in a sheet of poly(methyl methacrylate) (PMMA) (85 mm × 125 mm × 5.5 mm). A Tegaderm membrane was applied to the sheet to create the well bottom. To facilitate the eventual removal of the scaffolds, each well was blocked with 1% (w/v) bovine serum albumin (Sigma-Aldrich) in phosphate buffered saline. Scaffolds containing C3H-fLuc cells, each 0.4 mL in volume, were polymerized in the wells and the scaffolds were then sealed with another Tegaderm membrane to prevent water intrusion during the ultrasound exposure. The plate was immersed and fixtured in a tank of degassed water (30–36% O₂ saturation) at 37°C. HIFU was applied to the center of each scaffold, as described in section 2.3, to generate hyperthermia. Following HIFU exposure, the scaffolds were removed from the plate, transferred to a conventional 24-well plate containing complete media with 10 mM rapamycin, and placed in a tissue culture incubator overnight. The following day, fLuc activity was measured using the Luciferase Assay System (Promega), according to the manufacturer's instructions.

In a second experiment, we investigated the ability of HIFU to produce a spatially patterned region of fLuc activity within the scaffolds. Oblong wells, each 9.5 mm × 20 mm, were machined in a sheet of PMMA as described above. Scaffolds containing C3H-fLuc cells, each 1.0 mL in volume, were polymerized in the wells using identical procedures as in the first experiment. Using a computer-controlled positioning system, the HIFU transducer used to generate hyperthermia was moved in a 10 mm line across the long axis of each scaffold. Following exposure, the scaffolds were transferred to a 6well plate and cultured as described above. The following day, D-luciferin substrate (Promega) was added to each well at a final concentration of 80 µg/mL. After incubation for 30 min, the bioluminescence signal from the scaffolds was measured on an IVIS Spectrum imaging system (Perkin Elmer, Waltham, MA, USA) at the University of Michigan Center for Molecular Imaging. Quantitative image analysis of fLuc expression was performed using Living Image software (version 4.0, Perkin Elmer).

2.8 In vivo activation of C3H-fLuc cells using HIFU

This in vivo research was conducted with approval of the Institutional Animal Care & Use Committee at the University of Michigan. Female C3H/HeNCrl mice (n = 10, 4–5 weeks old, 22.9 ± 1.6 g, Charles River Laboratories, Wilmington, MA, USA) were anesthetized with isoflurane (5% for induction and 1.5% for maintenance). The lower dorsal hair was removed by shaving and applying depilatory cream (Nair, Church & Dwight Co, Ewing, NJ USA); the skin was disinfected with povidone-iodine (Betadine, Purdue Products L.P., Stamford, CT USA). The scaffold mixture (0.4 mL per implant), which contained 0.15 mg/mL Alexa Fluor 647-labeled fibrinogen (Thermo Fisher Scientific), was injected subcutaneously using a 20 gauge needle (Becton Dickinson, Franklin Lakes, NJ, USA) at

two locations within the lower, dorsal region and allowed to polymerize for 2 minutes prior to removal of the needle.

The following day (i.e., day 1), 50 μ L of 100 nM rapamycin was injected subcutaneously adjacent to each implanted scaffold. The injectable solution of rapamycin was generated by initially preparing a 10 μ M rapamycin stock solution in dimethyl sulfoxide and then diluting 100x with phosphate buffered saline. Approximately 90 min later, HIFU was applied using the following method. Each mouse was anesthetized with isoflurane and secured to a platform in a prone position. The platform was partially submerged in a degassed water tank (30–36% O₂ saturation) at 37°C such that the implanted scaffolds were completely submerged. A coupling cone (C106, Sonic Concepts) was placed on the ultrasound transducer, filled with degassed water (30–36% O₂ saturation), and the water was sealed in with a Tegaderm membrane. The focus of the transducer was positioned 1 mm below the skin surface and HIFU (2 min continuous wave at 258 W/cm²) was applied to generate hyperthermia. The window on the coupling cone was not in contact with the scaffold during the HIFU exposure. For each mouse, HIFU was applied to only one scaffold on day 1 and the scaffolds exposed to HIFU (i.e., left or right implant) were randomized. To demonstrate reactivation of fLuc expression, HIFU was applied on day 7 following the administration of rapamycin. Hyperthermia was generated in the same scaffolds on days 1 and 7.

fLuc activity was measured on days 2–5 and 8–11 by subcutaneously injecting 50 μ L of 40 mg/mL D-luciferin substrate adjacent to each implanted scaffold. fLuc activity and fibrin degradation (i.e., Alexa Fluor 647-labeled fibrinogen) were measured using an IVIS Spectrum imaging system. Fibrin degradation was quantified using identical procedures as described previously [33, 34]. Scaffold structure was monitored on days 1, 3, 7, and 10 using a ZS3 ultrasound system (Mindray, Mahwah, NJ, USA) and an L25–8 linear probe operating at 25 MHz. B-mode ultrasound images were taken at the thickest cross-section of the scaffold.

2.9 Retention of C3H-fLuc cells in implanted scaffolds

To determine the in vivo localization of implanted cells, C3H-fLuc cells were transduced with lentivirus expressing green fluorescent protein (GFP) and a puromycin resistance gene under control of a CMV promoter (University of Michigan Vector Core, Ann Arbor, MI, USA). Cells were selected for 3 weeks with 1 μ g/mL puromycin and then suspended in the composite scaffolds, as described in section 2.2. Cells were then subcutaneously implanted into female C3H/HeNcrI mice (n = 5, 2 scaffolds/mouse), as described in section 2.8. Mice were euthanized at 1, 3, 7, and 10 days post implantation. The scaffolds were harvested together with attached host tissue, fixed in paraformaldehyde, and whole implant GFP fluorescence was visualized on an IVIS imaging system as in section 2.8.

For histology and analysis of GFP⁺ cell distribution, implants were harvested 1 day post implantation, fixed with paraformaldehyde, and demineralized for 2 weeks in ethylenediaminetetraacetic acid (EDTA). Frozen sections were either stained with hematoxylin and eosin (H&E) or with 4',6'-diamidino-2-phenylindole (DAPI) and examined by brightfield or fluorescence microscopy using a Nikon 50i microscope (Melville, NY, USA).

2.10 Statistics

All statistical analyses were performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA USA). All data is expressed as the mean $\pm$ standard error of the mean of measured quantities. All n-values are listed below each corresponding figure. Statistically significant differences of all data sets were determined with a Student's t-test corrected for multiple comparisons using the Holm-Šídák method, with differences deemed significant for $p < 0.05$.

3. Results

3.1 US Attenuation

Figure 1A shows the ultrasound attenuation coefficient for composites containing < 200 nm and < 2.5 μ m HA particles, denoted as 200 nm and 2.5 μ m HAFib scaffolds, as a function of HA concentration. The lowest concentration of HA tested was 5 mg/mL, which yielded attenuation coefficients of 0.06 ± 0.01 dB $\cdot$ cm $^{-1}$ $\cdot$ MHz $^{-1}$ and 0.11 ± 0.01 dB $\cdot$ cm $^{-1}$ $\cdot$ MHz $^{-1}$ for 200 nm and 2.5 μ m HAFib scaffolds, respectively. The attenuation coefficient increased linearly with HA concentration, with attenuation coefficients at 50 mg/mL HA of 0.44 ± 0.04 dB $\cdot$ cm $^{-1}$ $\cdot$ MHz $^{-1}$ and 0.92 ± 0.05 dB $\cdot$ cm $^{-1}$ $\cdot$ MHz $^{-1}$ for 200 nm and 2.5 μ m HAFib, respectively. At each HA concentration, there was a significant difference between 200 nm and 2.5 μ m HAFib scaffolds.

3.2 HIFU induced heating of HAFib scaffolds

HIFU based heating of HAFib scaffolds after 2 min of ultrasound exposure are shown in Figures 1(B–D). Figure 1B shows the temperature profile for a 200 nm HAFib, containing 50 mg/mL HA, as a function of time. The temperature change (ΔT) correlated inversely with the distance between the focus of the transducer and the embedded thermocouple. Additionally, ΔT consistently increased during HIFU exposure for all distances measured due to the heating effects caused by HIFU, and a subsequent switch off of the HIFU resulted in cooling of the HAFib scaffold and thus a drop in ΔT with time. The temperature profile for 2.5 μ m HAFib containing 25 mg/mL HA (not shown) showed a similar trend to that observed with 200 nm HAFib (Figure 1B).

Figure 1(C, D) show the maximum ΔT measured in 200 nm and 2.5 μ m HAFib scaffolds as a function of HIFU intensity. The maximum ΔT observed for 200 nm HAFib, prepared with 50 mg/mL HA, and 2.5 μ m HAFib, prepared with 25 mg/mL HA, were not statistically different. This is consistent with the measured attenuation coefficients in Figure 1A, where there were no significant differences between these two scaffold compositions. The max ΔT correlated directly with HIFU intensity and inversely with distance between the transducer focus and thermocouple. It is important to note that since activation of the heat shock-mediated transgene is a threshold-based phenomenon, there exists combinations of distances and intensities that yield a max ΔT that exceed the activation threshold.

For both types of HAFib scaffolds, the maximum ΔT resulted from the highest HIFU exposure intensity (314 W/cm 2) and measured at the smallest distance (1 mm) from the focal point of the transducer ($8.8 \pm 1.5^\circ\text{C}$ and $8.7 \pm 0.5^\circ\text{C}$ for 200 nm and 2.5 μ m HAFib,

respectively). As expected, the lowest T resulted from the lowest US exposure intensity (145.0 W/cm^2) and furthest distance (3 mm) from the focal point ($1.0 \pm 0.1^\circ\text{C}$ and $1.2 \pm 0.1^\circ\text{C}$ for 200 nm and 2.5 μm HAFib, respectively). Figure 1(C, D) also reinforce the data shown in Figure 1B showing that locations in the HAFib scaffolds that are closer to the focal spot have a higher rate of heating. In Figure 1(C, D) this is supported by the inverse relationship between slope and distance from the focal spot.

3.3 Viability of C3H-fLuc in HAFib scaffolds

Figure 2A compares the metabolic activity of C3H-fLuc cells in fibrin-alone, 200 nm HAFib containing 50 mg/mL HA, and 2.5 μm HAFib scaffolds containing 25 mg/mL HA. One day after polymerization, the metabolic activity was $96.6 \pm 1.4\%$, $87.8 \pm 0.9\%$, and $68.1 \pm 0.6\%$ for fibrin, 200 nm HAFib, and 2.5 μm HAFib scaffolds, respectively. On day 7, cells entrapped in fibrin alone showed greater metabolic activity than cells in 200 nm HAFib scaffolds, with $111.4 \pm 4.9\%$ relative to the initial cell loading. There were no statistically significant differences between fibrin alone and 2.5 μm HAFib scaffolds on day 7. Cell cultured in fibrin alone and 2.5 μm HAFib scaffolds had significantly greater metabolic activity on day 7 ($111.4 \pm 4.9\%$ and $101.6 \pm 6.5\%$, respectively) than on day 1. There was no difference in metabolic activity between day 1 and day 7 in 200 nm HAFib scaffolds.

3.4 In vitro HIFU induced transgene activation at a single spot

The in vitro activation of C3H-fLuc cells in fibrin-alone, 200 nm HAFib containing 50 mg/mL HA, and 2.5 μm HAFib scaffolds containing 25 mg/mL HA as a function of HIFU intensity is displayed in Figure 2B. The greatest activation in HAFib scaffolds was achieved at the highest intensity of HIFU tested (314 W/cm^2), which resulted in 149-fold and 45-fold increases in fLuc activity, relative to each -HIFU control (i.e., 0 W/cm^2), for 200 nm and 2.5 μm HAFib, respectively. Among the levels of HIFU tested, no significant increases in fLuc activity were observed in the fibrin-only scaffolds when compared to the corresponding -HIFU control. fLuc activity in both 200 nm and 2.5 μm HAFib scaffolds were statistically different from fibrin-only hydrogels at intensities of 201 W/cm^2 or higher.

3.5 Patterned gene activation in vitro using HIFU

HIFU can induce spatially patterned transgene activation by limiting the subvolume of the scaffold that is heated. Regions of fLuc activity induced within the scaffold, obtained by rastering the HIFU transducer at 0.02, 0.05, and 0.1 mm/s, are shown in Figure 3A for 200 nm HAFib scaffolds containing 50 mg/mL HA and C3H-fLuc cells. A larger and more intense region of fLuc activity was achieved at the slower raster velocity (0.02 mm/s) than at the faster velocity (0.1 mm/s). Cross-sectional views of the scaffolds reveal that the depth of transgene activation correlated inversely with velocity. Transverse (i.e., short axis) and longitudinal (i.e., long axis) profiles of the region of fLuc activity obtained at 0.02 mm/s are illustrated in Figure 3B. The activation profiles were characterized by the full width half maximum (FWHM) and are shown in Figure 3(C,D). In general, the FWHM correlated inversely with transducer velocity and directly with US intensity. No activation was observed at a HIFU intensity of 258 W/cm^2 and a raster velocity of 0.15 mm/s.

3.6 In vivo HIFU induced gene activation

The components of the composite hydrogel, including C3H-fLuc cells, were subcutaneously injected and polymerized in situ in C3H/HeN mice. Figure 4 qualitatively shows the bioluminescence signal obtained one day after the initial HIFU exposure at 258 W/cm² (i.e., day 2) for mice containing 200 nm HAFib scaffolds with 50 mg/mL HA. fLuc activity was selectively observed in scaffolds exposed to HIFU. The time profile of fLuc activity, based on a region-of-interest analysis of the acquired bioluminescence data, is displayed in Figure 5. On days 1 and 7, rapamycin was injected and then HIFU was subsequently applied to one of the implants. Overall, the highest levels of fLuc activity were detected in the group of +HIFU implants at every time point. On day 2, there was 67-fold greater activation in the +HIFU implants compared to -HIFU implants. fLuc activity decreased from day 2 to day 6. The transgene was reactivated on day 8, with 57-fold greater fLuc activity in the +HIFU implants compared to -HIFU implants. Transgene activity decreased from day 9 to day 11. The highest average radiance for the -HIFU condition was also observed one day after each rapamycin administration and HIFU exposure. This residual level of activation may be due to thermal conductance from the contralateral +HIFU scaffold. Overall, the focal nature of the HIFU used for these experiments yielded a well confined activation region.

3.7 In vivo characterization of HAFib scaffolds

Figure 4 also shows the fluorescence signal from Alexa-Fluor 647 labeled fibrinogen that was incorporated into the implanted 200 nm HAFib scaffolds containing C3H-fLuc cells. The shape of the implants varied, though the injected volume was constant, since the scaffolds were injected and polymerized in situ. Similar to the bioluminescence data, the fluorescence data was quantified using a region-of-interest analysis. The quantified data, shown in Figure 6A, demonstrates that there are no differences in fibrin degradation between +HIFU and -HIFU groups of implants. By day 11, there was $16.3 \pm 5.6\%$ and $14.2 \pm 5.9\%$ of the scaffold remaining for the +HIFU and -HIFU condition, respectively. Thus, the rate of fibrin degradation appears to be independent of HIFU exposure.

Brightness mode (B-mode) ultrasound was used to characterize the morphology of the implanted scaffolds. Figures 6B displays a sagittal B-mode image of the scaffold, with a clear distinction between the scaffold and the surrounding tissue due to the higher echogenicity (i.e., pixel brightness) of the HAFib scaffold. The echogenicity of the scaffolds was longitudinally quantified, based on a region-of-interest analysis, and is shown in Figure 6C. On day 0, the average pixel intensity was 94.1 ± 2.6 and 91.0 ± 3.4 for +HIFU and -HIFU groups, respectively. By day 10 the average pixel intensity was 104.8 ± 3.1 and 99.3 ± 2.9 for +HIFU and -HIFU, respectively. There were no differences between +HIFU and -HIFU groups at any time point, except on day 1.

The measured implant height, based on the B-mode images, is shown in Figure 6D. A rapid decrease occurs from day 0 to day 1. On day 0 (i.e., immediately after polymerization in situ), the average height was 3.4 ± 0.1 mm and 3.6 ± 0.2 mm for +HIFU and -HIFU groups, respectively. On day 10 the average height was 2.0 ± 0.1 mm and 2.3 ± 0.1 mm for +HIFU and -HIFU groups, respectively. There were no differences between +HIFU and -HIFU at any timepoint.

3.8 Retention of cells within HAFib scaffolds

C3H-fLuc cells were labelled by transduction with a lentiviral vector encoding GFP, suspended in HAFib scaffolds and implanted. Macroscopic (whole-implant imaging) and microscopic fluorescence imaging techniques were used to measure retention and distribution of implanted cells (Figure 7). Macroscopic imaging of duplicate implants showed good retention of the GFP signal over a 10 day period with a possible gradual reduction over time. Microscopic examination of implants revealed that the GFP+ implanted cells were present inside the HAFib scaffold and had not migrated into the adjacent host tissue. Although considerable variability was observed in the GFP signal, no strongly GFP+ cells were detected outside the margins of the implant. Taken together, these results indicate that most or all C3H-fLuc cells were contained within the HAFib scaffold at the time of initial HIFU exposure and that cells remain localized in the area of implantation for at least 1 week.

4. Discussion

Here we investigate a modified HAFib scaffold containing cells that stably harbor a rapamycin-dependent, heat shock activatable gene switch and demonstrate exquisite spatial and temporal control of gene expression in vitro and in vivo using HIFU. Composites containing fibrin and HA, the mineral component of bone, have been explored in regenerative applications such as lentiviral gene delivery [35], bone engineering [36], and vasculogenesis [37]. In the present work, incorporation of HA particles in fibrin hydrogels was used to increase acoustic attenuation such that transgene activation was observed at HIFU intensities which did not yield activation in fibrin only scaffolds. This is consistent with our goal of generating a biocompatible, composite scaffold that heats more effectively than a conventional hydrogel (e.g., fibrin).

The rate of heating in a material is proportional to the acoustic intensity and attenuation coefficient, while inversely proportional to the density and specific heat [38]. The inclusion of HA within fibrin increased the attenuation significantly in a manner that was dependent on HA concentration and particle size. The observed trends shown in Figure 1A are consistent with theoretical models [39–41] and earlier experimental data [42, 43] regarding the attenuation of dilute suspensions. In scenarios where the product of the ultrasound wavenumber (k) and the particle radius (r) are significantly less than 1 (i.e., $kr \ll 1$), the attenuation of a dilute suspension scales linearly with the concentration and/or volume fraction of particles. In this linear range, the ultrasound scattering is predicted to be single in occurrence. As the particle concentration increases, multiple US scattering occurs, which causes a deviation from linearity. However those elevated concentrations were not explored in the work presented. In this study, ultrasound with a fundamental frequency of 2.5 MHz was used, which has a wavelength of 0.6 mm at 37°C. Composite scaffolds were generated using HA particles with diameters less than 200 nm or 2.5 μ m, which yields kr less than 0.001 and 0.01, respectively. The highest tested HA concentration was 50 mg/mL, which corresponds to an estimated solid volume fraction of 0.02. Additionally, although HA has a higher density than fibrin, the smaller specific heat of HA facilitates heating in response to ultrasound.

The in vitro cytotoxicity of the HAFib scaffolds was evaluated over 7 days by measuring the viability of C3H-fLuc cells encapsulated within the scaffolds (Figure 2A). Lower metabolic activity was observed in both HA-containing scaffolds on day 1 compared to fibrin only, with 2.5 μm HAFib displaying the lowest activity. The decreased levels of in vitro transgene activation in 2.5 μm HAFib shown in Figure 2B could be attributed to the lower cellular metabolic activity. In a previous study, primary osteoblasts displayed higher attachment, proliferation, and mineralization when cultured on nanostructured HA versus microstructured HA [44]. In addition to size, the shape of HA particles can impact viability, with needle-shaped particles displaying the highest cytotoxicity [45]. Furthermore, the cytotoxic effects of HA can be cell type dependent, with epithelial cells displaying greater effects than macrophages [46].

Significant transgene activation was observed at HIFU intensities of 201 W/cm^2 or greater in both types of HAFib scaffolds, whereas only residual fLuc activity was observed in the fibrin only scaffolds. The lack of activation in fibrin-only scaffolds is consistent with our prior study where a 5 min HIFU exposure at 658 W/cm^2 was required for significant activation [18]. Based on the heating profile in Figure 1C, exposure at 201 W/cm^2 was expected to generate a temperature of 41°C within the scaffold, which has been previously shown to induce transgene expression under control of hsp70B [47]. It is important to note the differences in sample dimensions used in the temperature profile and in vitro activation studies. A thicker scaffold (i.e., 12 mm vs. 5.5 mm), with a thermocouple embedded in the center, was used to generate the temperature profiles. Thus, greater signal attenuation is expected as ultrasound propagates through the thicker sample, which would decrease the achievable heating at the ultrasound focus. Furthermore, higher harmonics generated by the ultrasound transducer are more attenuated in the thicker sample due the frequency dependence of attenuation. These higher harmonics can also contribute to the overall observed heating.

fLuc activity was spatially patterned in vitro by laterally moving the HIFU transducer in a 10 mm line across the scaffold. At the highest velocity (i.e., 0.15 mm/s), this pattern of HIFU exposure was completed in 67 s. For both the short and long axes, the largest difference in FWHM was observed at the slowest raster speed (i.e., 0.02 mm/s). The inverse trend between transducer velocity and FWHM was observed at 314 W/cm^2 , but not at 258 W/cm^2 . An example of the exquisite spatial control that can be achieved using this approach is shown in Figure 8. The ability to control the spatial distribution of gene expression may be particularly beneficial for regeneration applications. For example, during wound healing, variations in endogenous angiogenic growth factor expression can affect the directionality and density of blood vessel growth [48, 49]. Being able to control this process by actively controlling the spatial distribution of growth factor expression could greatly improve the efficiency and effectiveness of wound healing.

The incorporation of fluorescently-labeled fibrinogen within the implanted scaffolds enabled longitudinal monitoring of fibrin degradation in vivo. The correlation between fibrin degradation and fluorescence signal has been previously correlated using fluorophore-conjugated fibrinogen [50]. In vivo fibrin degradation did not differ between -HIFU and +HIFU groups (Figure 6A). In both cases, rapid degradation was seen between days 2–4 and

then slower degradation over days 4–11. Overall, fibrin degradation was more rapid in this study with cell-loaded scaffolds compared to our previous studies with acellular fibrin scaffolds [33, 34]. Since fibrin degradation is mediated by proteases secreted by cells, it is expected that faster degradation would occur when cells are initially seeded in the scaffolds versus a scenario where acellular scaffolds are implanted. Cell-mediated compaction of the implanted scaffolds was clearly evident in vivo as measured using B-mode US (Figure 6D). Rapid compaction occurred within one day of implantation, with decreases in height of 45% and 41% for HIFU and +HIFU groups, respectively. After day 1, scaffold height remained statistically unchanged. Fibrin hydrogels seeded with cells can undergo significant compaction within one day, with smaller reductions occurring at 3 and 7 days post polymerization [51]. The inclusion of HA within composite fibrin-collagen scaffolds has been shown to reduce compaction in vitro in a dose dependent manner [52]. Based on our results, HA seems to impede further scaffold compaction in vivo after day 1. Despite significant compaction, the echogenicity (i.e., contrast) within the scaffold did not correlate with the dimensional change (Figure 6C). Echogenicity occurs because of ultrasound reflecting at a boundary where there is a mismatch in acoustic impedance. Significant changes in density and/or speed of sound within the scaffold could generate changes in contrast. Thus, the relatively constant contrast within the scaffold suggests retention of HA over the course of the experiment.

The most significant contribution from this work is the demonstration of in vivo non-invasive gene activation (Figure 5) at modest HIFU intensities (up to 258 W/cm²). Comparatively, clinical HIFU systems can generate intensities up to 18 kW/cm² [53]. The ability to activate a HAFib construct paves the way for customizability, providing a modifiable hydrogel (i.e., fibrin concentration, HA concentration, HIFU intensity, etc.) that can be adapted to reach a desired treatment goal (i.e., control of concentration and timing of regenerative factor expression). The ability to reactivate the cells in the HAFib construct 7 days after implantation shows that repeated induction of gene expression can be achieved using HIFU. In addition, the ability to reactivate the construct meets one of the requirements of wound healing, which is to have the ability to tune regenerative factor expression to achieve the desired outcome [54]. This can be achieved by properly dosing the rapamycin added prior to activation as well as timing ultrasound exposure.

With any HIFU therapy, image guidance is critical to ensure the safety and efficacy of the intervention. A common approach is the use of a magnetic resonance imaging (MRI) guided HIFU system in hyperthermic [55] and ablative [56] applications. MRI enables high resolution mapping of the intended treatment site as well as real-time monitoring of the temperature increase generated by the HIFU. Thus, MRI-guided HIFU could be used to ensure that sufficient heating is obtained with the scaffold, thereby activating gene expression, while also avoiding overheating that could cause cell death and off-target effects.

In this study, the cells containing the heat-activated and ligand-dependent gene switch were C3H/10T1/2 cells, an immortalized murine mesenchymal cell line derived from C3H mice. For translatability and eventual clinical application, non-immortalized, host-derived mesenchymal stem cells containing the gene switches could be developed. Mesenchymal stem cells have several advantages for tissue regeneration including: an immunoprivileged

status, thereby enabling evasion of the immune system [57]; the potential to differentiate to mesenchymal cell types including osteoblasts and chondrocytes [58]; and the ability to migrate to sites of tissue damage and/or inflammation [59, 60].

5. Conclusion

We have demonstrated that HIFU can be used to non-invasively, thermally activate fLuc-expressing C3H/10T1/2 cells *in vitro* and *in vivo*. The thermal activation process involved continuous HIFU exposure for 2 minutes at various acoustic intensities, resulting in sufficient localized heating to activate the two-component gene switch. The rate of the temperature increase, as well as the change in temperature, was proportional to HIFU intensity and decreasing distance from the ultrasound focal spot. A single *in vivo* HIFU exposure was shown to stimulate fLuc activity 67-fold or 57-fold higher than those observed in non-exposed controls 2 or 8 days after implantation, respectively. Furthermore, the system could be reactivated with a second exposure to HIFU after 7 days. fLuc activity was spatially patterned *in vitro* to generate regions and complex shapes with millimeter precision. These results show that HAFib scaffolds, in conjunction with HIFU, can be used to achieve millimeter resolution of transgene activation. Future work will focus on activating VEGF- and BMP2-expressing cells for angiogenic- and osteogenic tissue engineering applications, respectively.

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Figure 1.

The inclusion of HA particles within fibrin scaffolds increased the acoustic attenuation, which enabled more efficient heating by ultrasound. A) The acoustic attenuation of HAFib gels ($n = 9$) as a function of HA particle size and concentration. B) Time course of HIFU-induced heating within a 200 nm HAFib scaffold containing 50 mg/mL HA, where HIFU was applied for 2 min at 201 W/cm². The distance between the focus of the ultrasound transducer and the thermocouple, embedded within the scaffold, was varied. The maximum temperature change (ΔT) measured in 200 nm HAFib with 50 mg/mL HA (C) and 2.5 μm HAFib with 25 mg/mL HA (D) scaffolds ($n = 3$) as a function of acoustic intensity and distance between the transducer focus and thermocouple. Statistically significant differences ($p < 0.05$) are denoted as follows A) α: 200 nm HAFib vs 2.5 μm HAFib, (C, D); α: 1 mm vs 2 mm, β: 1 mm vs 3 mm, and γ: 2 mm vs. 3 mm. In (A), (C), and (D), the data are represented as mean $\pm$ standard error of the mean.

Figure 2.

Transgene activation occurred at lower HIFU intensities in HAFib scaffolds compared to fibrin only (i.e., Fibrin) scaffolds. A) The *in vitro* metabolic activity of C3HfLuc cells was longitudinally quantified in fibrin and HAFib scaffolds. B) At ultrasound intensities 201 W/cm², transgene activation was observed in 200 nm HAFib with 50 mg/mL HA and 2.5 μm HAFib with 25 mg/mL HA scaffolds containing C3H-fLuc cells. No enhancement in transgene activation was observed in fibrin scaffolds for any intensity of HIFU tested. Statistically significant differences ($p < 0.05$) are denoted as follows: α: Fibrin vs. 200 nm HAFib, β: Fibrin vs 2.5 μm HAFib, and γ: 200 nm HAFib vs. 2.5 μm HAFib. All data are represented as mean ± standard error of the mean for n=5 scaffolds.

Figure 3.

fLuc transgene activation was spatially patterned by rastering the focus of the ultrasound transducer across 200 nm HAFib scaffolds containing 50 mg/mL HA and C3H-fLuc cells. A) The ultrasound transducer was linearly moved 10 mm at 0.02, 0.05, and 0.1 mm/s across 200 nm HAFib scaffolds while operating at 258 W/cm². Patterned transgene activation is observed in the bioluminescence image. The bottom row of images display a cross-sectional view of each scaffold. The black arrow denotes the incident direction of the HIFU. Scale bar: 10 mm. B) Profiles of transgene activation for the region exposed at 0.02 mm/s. Inset: The red and blue lines denote the short (i.e., lateral) and long (i.e., longitudinal) axes encompassing the region of transgene activation. The full width half maximum (FWHM) was used to characterize the length of the activated region in the short (C) and long (D) axes. No transgene activation was observed at 258 W/cm² and 0.15 mm/s, which is denoted by an 'x' in the figure. In (C) and (D), the data are represented as mean $\pm$ standard error of the mean for n=3 scaffolds. Statistically significant differences ($p < 0.05$) are denoted as follows: α : vs. 314 W/cm² (0.02 mm/s), β : vs. 258 W/cm² (0.1 mm/s).

Figure 4.

In vivo transgene activation was demonstrated in C3H mice using subcutaneously-implanted 200 nm HAFib scaffolds 50 mg/mL HA and C3H-fLuc cells (n=2 scaffolds/mouse). The bioluminescence image (top) shows significantly greater transgene activation in the scaffolds exposed to HIFU (continuous wave at 258 W/cm² for 2 min, denoted by the red arrows) than the contralateral scaffolds not exposed to HIFU. The locations of the scaffolds can be visualized using fluorescence imaging (bottom) due to the presence of fluorescently-labeled fibrinogen in the scaffolds. Scale bar: 10 mm.

In Vivo fLuc Gene Activation

Figure 5.

Quantification of the bioluminescence data shows the time course of fLuc transgene activation, with significantly greater transgene activation in the +HIFU group. On day 0, 200 nm HAFib scaffolds containing C3H-fLuc cells were subcutaneously implanted in C3H mice (n=2 scaffolds/mouse). On day 1, rapamycin was administered to all scaffolds followed by HIFU exposure to only one scaffold in each mouse. Using bioluminescence imaging, transgene activation was monitored on days 2–5. To demonstrate reactivation of the transgene, rapamycin and HIFU were administered on day 7 as on day 1, followed by bioluminescence imaging on days 8–11. The data are represented as mean + standard error of the mean for n=10 scaffolds/group. Statistically significant differences ($p < 0.05$) are denoted as follows: α : -HIFU vs. +HIFU

Figure 6.

Fluorescence and B-mode ultrasound imaging reveal the *in vivo* time course of fibrin degradation and scaffold compaction in the 200 nm HAFib scaffolds containing 50 mg/mL HA and C3H-fLuc cells. A) Fluorescently-labeled fibrinogen was incorporated into the scaffolds, which enabled longitudinal monitoring of fibrin degradation via fluorescence imaging. B) B-mode imaging shows the *in situ* morphology of a subcutaneously-implanted scaffold. Scale bar: 10 mm. The scaffold echogenicity (C) and height (D) were measured from the B-mode images. Rapamycin and ultrasound were administered on days 1 and 7, which are denoted by red arrows. In (A), (C), and (D), the data are represented as mean $\pm$ standard error of the mean for $n=10$ scaffolds/group. Statistically significant differences ($p < 0.05$) are denoted as follows: (6A) α : +HIFU days 4–11 vs. +HIFU day 2, β : –HIFU days 4–11 vs –HIFU day 2, (6C) –HIFU vs. +HIFU, (6D) α : +HIFU days 1–10 vs. +HIFU day 0, β : –HIFU days 1–10 vs HIFU day 0. All data are represented as mean $\pm$ standard error of the mean for $n=10$ scaffolds/group.

Figure 7.

In vivo retention and distribution of implanted C3H-fLuc-GFP cells. C3H-fLuc cells were transduced with lenti-GFP, subcutaneously implanted, and harvested at the times indicated. The time course of GFP signal was visualized in ex vivo samples using whole-implant fluorescence imaging (A) and quantified using a region-of-interest analysis (B). The dotted line in (B) denotes the signal obtained from an HAFib scaffold without cells. For histology and fluorescence microscopy, 24 h post implantation scaffolds and adjacent tissue were removed and processed for histology as described in the Methods. (C) H&E staining of a scaffold (lower left) and adjacent host tissue. (D-F) Adjacent section to C was stained with DAPI and examined by fluorescence microscopy for DAPI (D) and GFP (E). Panel F shows a merged image of DAPI and GFP. Scale bar: (A) 1 cm, (C-F) 50 μm.

Figure 8:

Bioluminescence image of fLuc transgene activation that was spatially patterned into a block “M” by rastering the focus of the HIFU transducer across a scaffold containing C3H-fLuc cells. The ultrasound transducer was moved at 0.02 mm/s, using a computer controlled positioning system, across a 200 nm HAFib scaffold with 50 mg/mL HA while operating at 314 W/cm². Scale bar: 10 mm.