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Spatiotemporal Control of Vascular Endothelial Growth Factor Expression Using a Heat-Shock-Activated, Rapamycin-Dependent Gene Switch

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Abstract

A major challenge in regenerative medicine is to develop methods for delivering growth and differentiation factors in specific spatial and temporal patterns, thereby mimicking the natural processes of development and tissue repair. Heat shock (HS)-inducible gene expression systems can respond to spatial information provided by localized heating, but are by themselves incapable of sustained expression. Conversely, gene switches activated by small molecules provide tight temporal control and sustained expression, but lack mechanisms for spatial targeting. Here we combine the advantages of HS and ligand-activated systems by developing a novel rapamycin-regulated, HS-inducible gene switch that provides spatial and temporal control and sustained expression of transgenes such as firefly luciferase and vascular endothelial growth factor (VEGF). This gene circuit exhibits very low background in the uninduced state and can be repeatedly activated up to 1 month. Furthermore, dual regulation of VEGF induction *in vivo* is shown to stimulate localized vascularization, thereby providing a route for temporal and spatial control of angiogenesis.

Introduction

Tissue development and regeneration are highly coordinated events requiring exquisite control over the sequence, concentration, timing, and distribution of regenerative factors. For example, in fracture healing many molecules, including cytokines, bone morphogenetic proteins (BMPs), and angiogenic factors, are expressed in distinct temporal sequences and have unique spatial distributions within the fracture callous (Gerstenfeld *et al*., 2003; Yu *et al*., 2010). In nonhealing fractures and trauma-induced high-volume tissue defects, these spatially and temporally encoded signals are absent or attenuated. A major challenge for regenerative medicine, therefore, is to develop approaches for temporal and spatial control of growth factor delivery that will mimic these natural events to jumpstart and guide tissue regeneration.

While some progress has been made designing protein delivery systems that can release factors in a controlled manner (King and Krebsbach, 2012), gene therapy offers major advantages in this regard. Initial uses of this technology in orthopedic regeneration employed vectors that constitutively expressed growth factors and BMPs under the control of strong viral promoters to stimulate regeneration of cartilage, bone, muscle, and tendons. Much of this work was recently reviewed (Steinert *et al*., 2008; Meloni *et al*., 2010; Kimelman Bleich *et al*., 2012; Nixon *et al*., 2012). Using this approach, successful healing of large segmental bone defects was achieved. More recently, several regulated gene expression systems have been developed, thereby providing the potential to more tightly control the timing and sequence of regenerative factor delivery. These genetic tools typically contain two elements: (1) a transactivator or transinhibitor regulated by an exogenous element (usually a small-molecule ligand), and (2) a gene of interest controlled by a transactivator-responsive or transinhibitor-susceptible promoter. Included in this toolbox are gene switches regulated by tetracycline-responsive elements, transactivators

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derived from steroid receptors, and synthetic transcription factors activated by rapamycin or blue light (Vilaboa *et al*., 2011; Wang *et al*., 2012). For example, tetracycline-/doxycycline-regulated expression of BMPs was shown to induce bone formation at both ectopic and orthopic sites (Moutsatsos *et al*., 2001; Peng *et al*., 2004, 2005). Using this approach, bone regeneration was demonstrated in both spinal fusion and segmental defect animal models (Moutsatsos *et al*., 2001; Hasharoni *et al*., 2005). Rapamycin-responsive gene expression systems represent an additional approach to regulated gene expression that provides lower background and higher inducibility relative to other ligand-activated systems. These systems use a synthetic transactivator composed of separate DNA-binding and activation domains that cannot interact. Only after a dimerizer (rapamycin or related nonimmunosuppressive analogs) is added will the separate components form a functional transcription factor. In this way, very tight control over transgene expression is achieved. Our previous work demonstrated the efficacy of this approach as a means of stringently regulating the magnitude and temporal pattern of BMP-2 expression *in vivo,* which resulted in uniform bone healing of a critical-sized calvarial defect (Koh *et al*., 2006).

While providing good temporal control, small-ligand-regulated systems cannot by themselves regulate the spatial distribution of transgene expression. To achieve this, it is necessary to use promoters capable of responding to targetable, externally directed physical stimuli such as localized heating. The heat shock protein (Hsp) gene promoters are particularly well suited to this purpose (Welch, 1993; Cotto and Morimoto, 1999). Heating can be achieved by a variety of methods, such as direct application of a localized thermal stimulus or by using high-intensity ultrasound, which can be focused to small volumes. The human HSP70B promoter, which exhibits extremely low basal activity and high heat inducibility (Dreano *et al*., 1986), has been successfully used to target several transgenes *in vivo* (Braiden *et al*., 2000; Vekris *et al*., 2000; Smith *et al*., 2002; Deckers *et al*., 2009). However, because it is susceptible to thermal activation associated with local inflammation or fever, it does not provide the necessary stringency for gene therapy applications. This promoter is also rapidly deactivated upon withdrawal of the hyperthermic stimulus, thus limiting the capacity for the heat shock (HS) pathway to drive sustained expression of therapeutic transgenes. To address these issues, we developed a dual-requirement-inducible system wherein the HSP70B promoter is used to drive expression of a ligand-activated transcription factor that subsequently activates a target gene only when the activating ligand is present. To demonstrate the feasibility of this strategy, we constructed a regulatory circuit that used a hybrid promoter containing the human HSP70B promoter and GAL4-responsive elements to drive expression of a mifepristone-activated chimeric transactivator, GLp65, and, as a second component, a luciferase reporter gene linked to a GAL4-responsive promoter. *In vivo* heat activation, combined with administration of mifepristone, induced reporter activity, which was maintained by addition of mifepristone and reversed after withdrawal of the drug (Vilaboa *et al*., 2005).

Here we describe the development of a second gene expression system that combines HS induction with the specificity, low background, and high inducibility of rapamycin-regulated systems to control human vascular endothelial growth factor (hVEGF), a key factor in vasculogenesis and angiogenesis (Semenza, 2007). As will be shown, this system provides stringent control over transgene expression *in vitro* and *in vivo* and induces the synthesis of high levels of biologically active VEGF in experimental animals.

Materials and Methods

Vector construction

A sequence containing a tandem of 12 ZFHD1-binding sites was PCR-amplified from construct pZ12I-PL-2 using primers 5′-TAAGGCGCGCCAGGTACCGAGCTCTTAC and 5′-TATGGCGCGCCTTATGGGGGTGTCAAAATG, digested with *Asc*I and inserted in an *Asc*I-cut Hsp70/GAL4-fLuc plasmid. The resulting construct was named Hsp70/12xZFHD1-flucS2. An Hsp70/12xZFHD1 cassette was excised from Hsp70/12xZFHD1-flucS2 using *Bam*HI and *Hin*dIII and subcloned in pcDNA 3.1(−) (Invitrogen, Barcelona, Spain). The resulting plasmid was digested with *Bgl*II and *Bam*HI to remove CMV promoter sequences, and ligated to prepare Hsp70/12xZFHD1. *Eco*RI and *Bam*HI sites were inserted in Hsp70/12xZFHD1 by the QuickChange procedure (Agilent, Madrid, Spain) using primers 5′-CGACAAGAA GCTTAAGTTGGATCCGCTGATCAGCCTCGAC and 5′-CG CAGCATCCGACAAGAATTCTAAGTTGGATCCGCTGATCA, respectively. The resulting plasmid was digested with *Eco*RI and *Bam*HI and ligated to an *Eco*RI/*Bam*HI fragment from pC4N2-RHS3H/ZF3, a plasmid containing sequences coding for the two-component rapamycin-binding transcription factor (TA). The resulting plasmid was designated pL2N2-pHsp70/12xZFHD1-TA. The fragment containing Hsp70/12xZFHD1 promoter, TA coding, and the adjacent BGH poly(A) sequences was flanked by *Cla*I sites using the QuickChange procedure and primers 5′-GCTGCTGAAGCT GGAGACTAGTTAATATCGATACGGCTGTGGAATGT and 5′-ATTCCAGAAGCCACCATCGATTATCCTGCTGCCAA GAG. The resulting construct was digested with *Cla*I and ligated to the larger fragment of a *Cla*I-cut pL2N2-RHS3H/ZF3 construct in which *Eco*RI and *Bam*HI sites have been previously replaced by *Cla*I sites in a QuickChange reaction. Primers employed were 5′-GGATCTCCCGATCCATCGAT GTCAGGTGGCACTT and 5′-AGTGTAGCGGTATCGAT GCGCGTAACCACCACACCCG. To prepare pZ12-fLuc, pGL-Basic (Promega, Madison, WI) was digested with *Hin*dIII and *Bam*HI to recover firefly luciferase (fLuc) coding sequences and the adjacent SV40 poly(A) sequence and then ligated to the larger *Hin*dIII–*Bam*HI fragment of pZ12-I-PL-2.

Human VEGF 165 (hVEGF165) cDNA was PCR-amplified from pBLAST49-hVEGF v2.0 (Invivogen, San Diego, CA) using primers 5′-CCTACCTAAGCTTACCGGTAGGA and 5′-CTTATCATGTCTAGATAGCTCACC, digested with *Hin*dIII and *Bgl*II and ligated to *Hin*dIII/*Bgl*II-cut pZ12I-PL-2. The resulting plasmid was digested with *Mlu*I and *Bam*HI to recover a fragment containing hVEGF165 coding sequence and minimal hIL-2 promoter fused to a tandem of 12 ZFHD1. This fragment was ligated to the largest fragment of an *Mlu*I/*Bam*HI-cut pLH-Z12I-PL version in which a *Bam*HI site was introduced at the multicloning site using the QuickChange procedure and primer 5′-GCTTGCCCTGCA GCG GGGGATCCACTAGTCGAGATCT. The resulting plasmid was named pLHZ12-hVEGF165.

All subcloning and mutagenesis steps were monitored by restriction analysis and nucleotide sequencing. Plasmids pZ12I-PL-2, pC4N2-RHS3H/ZF3, pL2N2-RHS3H/ZF3, and pLH-Z12I-PL were obtained from ARIAD Gene Therapeutics, Inc. (Cambridge, MA), under the terms of a material transfer agreement.

Drugs

For *in vitro* experiments, rapamycin (LC Laboratories, Woburn, MA) was dissolved in dimethyl sulfoxide and the rapalog AP21967 (kindly provided by ARIAD Gene Therapeutics, Inc.) was dissolved in 100% ethanol. For *in vivo* injections, rapamycin or AP21967 were initially dissolved in N,N-dimethylacetamide (DMA) as a stock solution (3 mg/ml) and then diluted to each specific concentration in a mixture of 50% DMA, 45% polyoxythylene glycol (average molecular weight of 400), and 5% polyoxyethylene sorbitan monooleate. Intraperitoneal (ip) injections were in a total volume of 50 μl. Rapamycin and AP21967 were administered *in vivo* at doses of 1 and 10 mg/kg, respectively.

Cell culture, transfection, and isolation of cell lines

The clonal mouse multipotent cells, C3H/10T1/2, clone 8 (ATCC Catalog Number CCL-226), were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, and 0.1 mg/ml streptomycin in a humidified 5% CO_2 atmosphere at 37°C. Subconfluent cultures were transfected using Lipofectamine 2000 reagent (Invitrogen) following manufacturer's recommendations. C3H/10T1/2 transformants stably expressing pL2N2-Hsp70/12xZFHD1-TA were selected using G418 (1200 μg/ml). Stable clones were screened for HS and rapamycin responsiveness after transient transfection with pZ12-fLuc. One day after lipofection, cells were treated for 1 hr with 10 n*M* rapamycin or vehicle, and then subjected to heat treatment at 45°C for 30 min in a water bath. Cells were lysed and assayed for firefly luciferase activity 24 hr after HS. A highly inducible clone that exhibited negligible levels of fLuc activity after heat treatment or exposure to rapamycin alone was isolated and then transfected with pLHZ12-hVEGF165 or a combination of pZ12-fLuc and Linear Hygromycin Marker (Clontech, Mountain View, CA) at a 20:1 molar ratio. Clones were selected using hygromycin B (600 μg/ml) and screened for VEGF secretion or fLuc activity 1 day after applying the aforementioned stimulation conditions. C3H/10T1/2 cell lines exhibiting low background of fLuc activity or VEGF secretion and strong induction exclusively after combined HS and ligand treatments were termed C3H/10T1/2-fLuc and C3H/10T1/2-VEGF, respectively. Clonal cells were maintained in DMEM containing 10% FBS and 1% penicillin and streptomycin under continuous selection with G418 and hygromycin B.

Pooled human umbilical vein endothelial cells (HUVECs), endothelial basal medium (EBM), endothelial growth medium (EGM), and a solution for coating culture wares for endothelial cells (ECs) growth were purchased from Angioproteomie (Boston, MA). HUVECs (passage number below 5) were plated at a density of 3.2×10^4 cells/cm^2 in EGM. After 24 hr, cells were washed with phosphate-buffered saline (PBS) and cultured with EGM, or EBM-10% FBS conditioned by C3H/10T1/2-VEGF cells. Media were routinely replaced every 2 days. Cell viability was assessed using the alamarBlue reagent (Biosource, Nivelles, Belgium) following manufacturer's recommendations.

Measurement of target gene activity

For *in vitro* experiments, cells were seeded at a density of 1.5×10^4 cells/cm^2. One day after, cells were exposed to the indicated HS treatment in a thermostatically controlled water bath in the presence of the drug or vehicle. Cells were lysed using Passive Lysis Buffer (Promega), and protein extracts were assayed for fLuc activity using the Luciferase Assay System (Promega) according to the manufacturer's recommendations. Luciferase light counts were detected in a Monolight 2010 luminometer (BD Biosciences, San Jose, CA). VEGF secretion was measured using a commercially available ELISA kit (R&D Systems, Inc., Minneapolis, MN). Luciferase activities and VEGF secretion were normalized after measurement of total protein concentration in the cell lysates using Bio-Rad Protein Assay (Bio-Rad Laboratories, Berkeley, CA).

Animal experiments

All procedures were approved by the University Committee on the Use and Care of Animals and were in compliance with state and federal laws. For subcutaneous implants, 1 million of C3H/10T1/2-fLuc or C3H/10T1/2-VEGF cells were suspended in 450 μl of a serum-free DMEM solution containing 10 or 7.5 mg/ml of bovine fibrinogen (Sigma-Aldrich, St. Louis, MO), respectively. About 50 μl of 20 U/ml bovine thrombin diluted in DMEM (Sigma-Aldrich) was added, and the mixture was immediately injected subcutaneously into the backs of 4–5-week-old C3H/HeNCrl mice (Charles River Laboratories, Inc., Wilmington, MA) in the anterior or posterior location. Before injection, hair was removed from injection areas by shaving and depilatory treatment, and animals were anesthetized with isoflurane (2% for induction and 1.5% for maintenance). During implant polymerization, mice were kept stationary for 5 min and subsequently placed in the recovery cage. After 24 hr, animals receiving heat treatment were anesthetized and heat was administered to the lower-back implants by partial immersion of the animal body in a water bath at 44.5°C for the indicated times. Drug or vehicle was administered 90 min before HS treatment. At retrieval, animals were euthanized, injection areas were photographed, and implants including surrounding skin and muscle layers were surgically removed. The erythematous areas in photographs of the injections areas were measured using Fiji, an open-source image-processing package based on ImageJ. For determination of VEGF content in implanted constructs, fibrin explants were excised, swirled in PBS, and homogenized in RIPA buffer (Sigma-Aldrich) before being tested by ELISA.

Bioluminescence imaging

Mice were anesthetized with isoflurane, and 50 μl of a solution of 40 mg/ml D-luciferin (Promega) in PBS was injected subcutaneously near the implant region. Immediately after, animals were placed in the dark chamber for light acquisition in an IVIS charge-coupled device camera system

(Xenogen, Alameda, CA) and analyzed with the Living Image 4.0 software (Xenogen). The region of interest covering the implantation area was defined, and quantification of light emission was measured.

Histology

Formalin-fixed samples were dehydrated through a series of graded ethanols, xylol, and finally xylene, and embedded in paraffin. Five-micrometer sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E) or, alternatively, were subjected to immunohistochemical staining using specific antibodies for von Willebrand factor (vWF) or CD31. Briefly, sections were stained for vWF first underwent antigen retrieval with 0.1 *M* citrate buffer, pH 6.0, at 95°C for 20 min. Endogenous peroxidases were quenched by treatment with 1% H_2O_2 for 20 min, and the slides were then blocked for 1 hr with a buffer containing 2% (w/v) bovine serum albumin (Sigma-Aldrich) and 2% (v/v) normal sheep serum (Sigma-Aldrich). After rinsing with 0.05% (v/v) Tween-20 in PBS (PBST), sections were incubated for 2 hr at room temperature with anti-vWF rabbit polyclonal antibody (AB7356; Millipore, Temecula, CA) or nonimmune rabbit IgG (Vector Labs, Burlingame, CA) diluted to 5 μg/ml in the blocking buffer. The slides were then rinsed in PBST and processed using the Vectastain ABC rabbit peroxidase detection kit and DAB substrate kit (both from Vector Labs), following the manufacturer's instructions. The slides were counterstained with hematoxylin QS (Vector Labs), dehydrated, and mounted with Histomount (Invitrogen). Sections stained for CD31 were treated similarly, except that antigen retrieval was performed after the peroxidase quench and consisted of a digestion with 40 μg/ml proteinase K for 15 min at room temperature. The anti-CD31 rat polyclonal antibody (ab56299; Abcam, Cambridge, MA) or a nonimmune rat IgG (Vector Labs) was diluted to 1.25 μg/ml. Images of the sections were captured on an Eclipse 50i microscope (Nikon Instruments, Melville, NY) with 4×, 10×, and 20× objectives (Nikon) and a DP72 digital camera (Olympus America, Inc., Center Valley, PA). Areas of vWF^+ or $CD31^+$ structures in imaged muscle were calculated using Fiji software after digitally establishing a threshold for DAB staining intensity normalized to H&E counterstain.

Statistical analysis

Statistical analysis was performed using Student's *t*-test to measure statistical differences between groups. Unless indicated otherwise, experimental data are reported as means and SDs of triplicate independent samples.

Results

In vitro *characterization of a heat-activated and rapamycin-dependent gene switch*

Figure 1a outlines the operation of a Hsp-based, rapamycin-dependent gene circuit. In this system, heat treatment activates the human HSP70B promoter to drive expression of a bimodular synthetic transactivator. This factor has separate DNA-binding and activation domains that are expressed as fusion proteins with the rapamycin-binding domains of the human proteins FKBP12 and FRAP (mTOR), respectively. Rapamycin reversibly cross links both modular proteins, resulting in the reconstitution of a functional transcription factor capable of inducing expression of a target gene of choice. Target gene responsiveness is controlled by a minimal IL-12 promoter supplemented with ZFHD1 elements recognized by an engineered DNA-binding domain (Pomerantz *et al*., 1995). These synthetic DNA elements were also inserted into the HSP70B RNA leader region to confer both heat and autoactivated expression on the linked transactivator gene. The highly potent activation domains incorporated into this system, derived from the activation domain of the p65 subunit of human NF-κB and human HSF1 (Schmitz and Baeuerle, 1991; Pollock *et al*., 2000), typically lead to high levels of induced gene expression, whereas in the absence of rapamycin the system displays extremely low basal activity since the transactivator modules have no affinity for one another in the absence of dimerizer (Rivera *et al*., 1999, 2005; Auricchio *et al*., 2002). A mutation of the rapamycin-binding region of the mTOR-activation domain allows the use of certain rapamycin analogs (rapalogs) to specifically heterodimerize the engineered transcription factor without interfering with the activity of endogenous mTOR (Pollock *et al*., 2002). Rapalogs are unable to bind to endogenous mTOR protein and therefore have little immunosuppressive or antiproliferative activity (Indraccolo *et al*., 2006), yet are able to function as chemical inducers of this system (Liberles *et al*., 1997; Stankunas *et al*., 2003). Rapamycin and rapalogs were previously shown to function *in vivo* after oral administration or ip injection (Pollock *et al*., 2000).

To test the functionality of this expression system, we generated a cell line (C3HT101/2-fLuc) that stably harbors a firefly luciferase reporter gene (fLuc), under control of the gene switch. In the absence of HS, increasing doses of heterodimerizer were unable to induce fLuc activity (Fig. 1b). However, heating at 45°C for 30 min in the presence of rapamycin or the rapalog AP21967 resulted in a clear concentration-dependent induction of luciferase activity (Fig. 1b). Consistent with previous reports, rapamycin was slightly more active than AP21967, with half maximal induction seen with 1 n*M* rapamycin versus 5 n*M* for rapalog (Koh *et al*., 2006). As shown in Fig. 1c, a minimum of 20 min at 45°C was required for induction of fLuc activity. Heat treatment for longer than 35–40 min led to degradation of the response, likely due to cell damage. To characterize the kinetics of the target gene induction, a time–course experiment was performed. Peak levels of luciferase activity were detected 24 hr after HS with values gradually decreasing at later times (Fig. 1d). Withdrawal of rapamycin from induced cultures accelerated the rate of decay. Control cultures consisting of vehicle-treated cells with or without exposure to HS or rapamycin-treated cells not exposed to hyperthermia showed negligible levels of background luciferase activity for the entire observation period, confirming the tight control of target gene expression exerted by this gene circuit.

Heat-activated, rapamycin-dependent gene expression in vivo

C3HT101/2-fLuc cells were suspended in a fibrin scaffold and subcutaneously implanted on the backs of adult C3H/HeNCrl mice. After ip administration of heterodimerizer or vehicle, the lower back of each mouse was exposed to

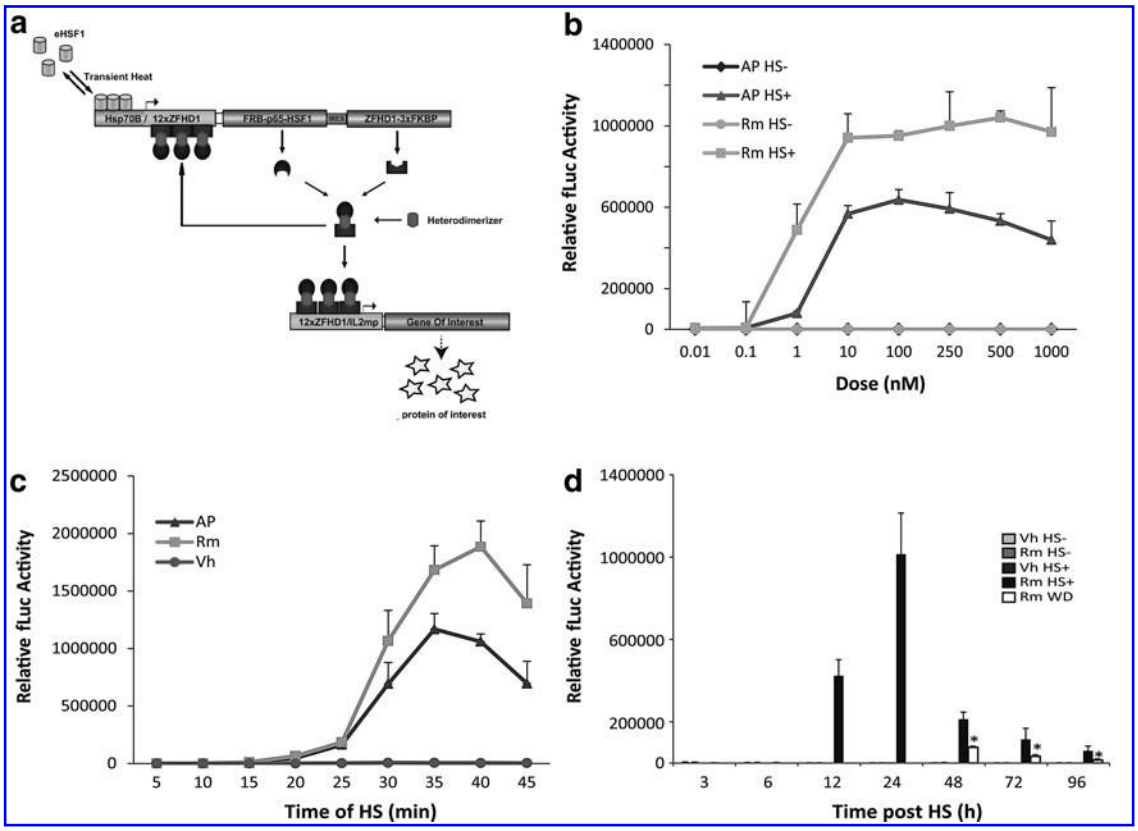

FIG. 1. Development and characterization of a heat-activated, rapamycin-dependent gene switch. **(a)** Outline of the two-component heat shock protein (Hsp)-based circuit that employs a heterodimerizer-regulatable transactivator. **(b)** Ligand concentration dependence. C3HT101/2-fLuc cells were treated with the indicated doses of rapamycin (Rm) or AP21967 (AP) with (+) or without (−) heat shock (HS) at 45°C for 30 min. Relative fLuc activities were assayed 24 hr after HS. **(c)** HS time dependence. C3HT10-1/2-fLuc cells were exposed to vehicle (Vh), Rm, or AP (10 n*M* each), and then heat treated at 45°C for the indicated times. Relative fLuc activities were assayed 24 after HS. **(d)** HS and ligand dependence of gene switch. C3HT10-1/2-fLuc cells were treated with 10 n*M* Rm or vehicle Vh with (+) or without (−) heat treatment at 45°C for 30 min (HS). In one set of Rm HS+ cultures, the medium was changed to an Rm-free medium 24 hr after HS (Rm WD). Relative fLuc activities were assayed at the indicated times after HS. *Significantly different from the Rm HS+ group, $p < 0.05$. Relative fLuc activity was expressed as the number of light units per microgram of cell lysate protein. Error bars depict mean + SD.

localized HS by partial immersion in a 44.5°C water bath; 24 hr later, luciferase activity was assessed by bioluminescence imaging (Fig. 2). Heat activation and administration of 1 mg/kg rapamycin or 10 mg/kg AP21967 resulted in >250-fold induction of fLuc activity. In contrast, fLuc activity in upper-back implants that did not receive any thermal treatment was below the limits of detection and indistinguishable from implants in the vehicle-treated group (Fig. 2a). The experiment presented in Fig. 2b was performed to determine whether gene switch activity induced by a single HS could be sustained in animals receiving a daily rapamycin dose (1 mg/kg ip) and whether activation could be reversed upon discontinuation of heterodimerizer treatment. Bioluminescence image analysis revealed a progressive decrease of luciferase activity during the days following the peak of induction. Similar to the *in vitro* result shown in Fig. 1d, discontinuation of rapamycin administration 24 hr after activation of the gene switch accelerated the decay of fLuc activity, with the luminescent signal approaching basal uninduced levels within 2 days of dimerizer withdrawal. Long-term monitoring of heat-activated, rapamycin-dependent gene switch functionality *in vivo* demonstrated that after activation, fLuc levels remained elevated over uninduced controls for at least 8 days in animals treated with rapamycin every other day (Fig. 2c). To determine if transgene activity can be repeatedly activated, mice in the HS/rapamycin group were given a second HS exposure after 13 and 27 days. At both times, luciferase activity was reinduced within 24 hr and reached levels comparable those observed after the initial rapamycin/HS exposure. Thus, the HS-activated, rapamycin-dependent gene switch was shown to stringently regulate target gene expression *in vivo* and was capable of repeated activation over a 4-week period.

Gene switch regulation of VEGF secretion

To evaluate whether the Hsp-based, rapamycin-dependent gene expression system is an appropriate tool for delivery of a regenerative factor, we developed a cell line, C3H/10T1/2-VEGF, stably harboring hVEGF165 under the control of the gene switch. *In vitro,* engineered cells responded to thermal stress by increasing levels of VEGF secretion in a heterodimerizer-dependent fashion (Fig. 3a). Dose–response curves for rapamycin and AP21967 were

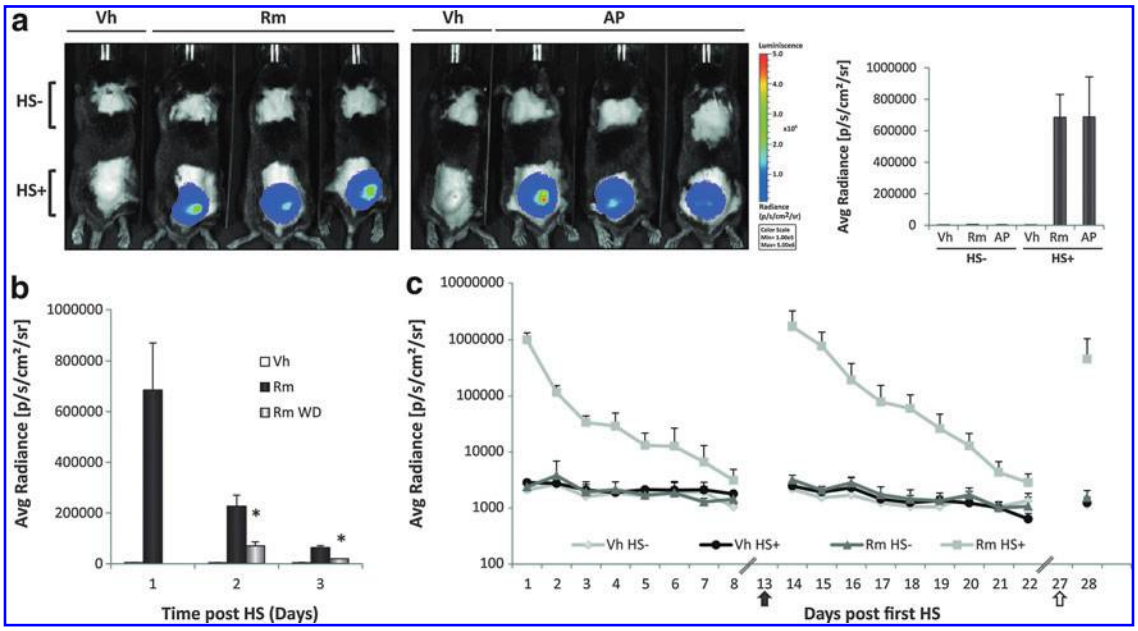

FIG. 2. *In vivo* performance of heat-activated and rapamycin-dependent gene switch. **(a)** *In vivo* induction. C3HT10-1/2-fLuc cells were subcutaneously implanted on the backs of C3H/HeNCrl mice using an *in situ*-polymerizable fibrin scaffold. Animals were injected with rapamycin (Rm), AP21967 (AP), or vehicle (Vh), and lower-back implants were immersed in a water bath at 44.5°C for 20 min (HS+). Upper-back implants did not receive heat shock (HS−). Bioluminescence images (BLI) were obtained 24 hr after HS (left), and average radiance from the region of implantation was analyzed using specific software for BLI signal quantification (right). **(b)** Periodic administration of heterodimerizer maintains the induced state of C3HT10-1/2-fLuc cells *in vivo*. Animals implanted with C3HT10-1/2-fLuc cells were injected with Rm or Vh, subjected to HS at the implantation site, and then administered daily with Rm or Vh as in the initial treatment. In another set of animals, Rm administration was discontinued 1 day after HS (Rm WD). Average radiances from implants subjected to hyperthermia were measured by BLI at the indicated times after HS. *Significantly different from the Rm group, $p < 0.05$, $n = 4$/group. **(c)** Long-term performance and reactivation of the gene switch. Mice were implanted as in **(a)** and treated with Rm or Vh with (+) or without (−) HS. fLuc activity of implanted cells was monitored by BLI measurements at the times indicated after first HS. Rm or Vh were administered every other day after HS treatment. Black arrow and open arrow indicate the day that a second or a third HS was performed, respectively, after Rm or Vh administration. Error bars depict mean+SD. Color images available online at www.liebertpub.com/hgtb

similar to those observed for induction of the luciferase reporter in Fig. 1. Similarly, a HS time of approximately 35 min was required for maximal VEGF induction (Fig. 3b). In contrast to the longer exposure required for activation of fLuc activity, elevated VEGF secretion was observed after as little as 10 min of hyperthermic stimulus. The VEGF expression system could also be reactivated after an initial stimulation (Fig. 3c). In this experiment, cell culture media were collected and replaced daily by fresh media, starting 24 hr after HS. Measurement of VEGF levels in the harvested media revealed a time-dependent decrease in the rate of secretion that decayed to basal levels after 6 days. Withdrawal of rapamycin from induced cultures accelerated the rate of decay such that VEGF levels were 30% of those seen in samples with continued rapamycin treatment 2 days after induction. A second HS treatment 1 week after the initial HS was able to reinduce VEGF secretion to levels greater than those achieved by the initial induction. To test whether induced VEGF is biologically active, we rely on the premise that this growth factor is an important mediator in EC proliferation and survival (Gupta *et al.*, 2002). HUVECs exposed to the conditioned medium from induced C3H/10T1/2-VEGF cells for 7 days exhibited metabolic activities similar to those in cultures treated with commercial EGM and significantly higher than HUVECs exposed to the medium obtained from C3H/10T1/2-VEGF exposed to heat or heterodimerizer alone (Fig. 3d).

Regulation of angiogenic activity and vascular perfusion by the VEGF gene switch in vivo

A final series of experiments examined whether the VEGF gene switch is active *in vivo*. C3H/10T1/2-VEGF cells encapsulated in an *in situ* polymerizable fibrin gel were subcutaneously injected in the posterior dorsal region of C3H/HeNCrl mice. Animals were treated with vehicle or rapamycin with or without localized heat treatment (44.5°C for 25 min). After 24 hr, rapamycin-treated mice exposed to HS exhibited a strong angiogenic response that increased with time (Fig. 4a). Most obvious was an erythema that reached a maximum average size of $1.12 \pm 0.51\ cm^2$ and led to erosion of the skin after 1 week. None of the animals exposed to either HS or rapamycin alone showed any evidence of skin lesions in the implant area. Quantification of VEGF contained in explanted fibrin constructs revealed a 150-fold increase of protein levels in the rapamycin-treated group 1 day after localized hyperthermia, as compared with implants not subjected to heat treatment (Fig. 4b). Although VEGF levels decayed at later times, they consistently remained elevated in rapamycin/HS mice relative to controls. Histological

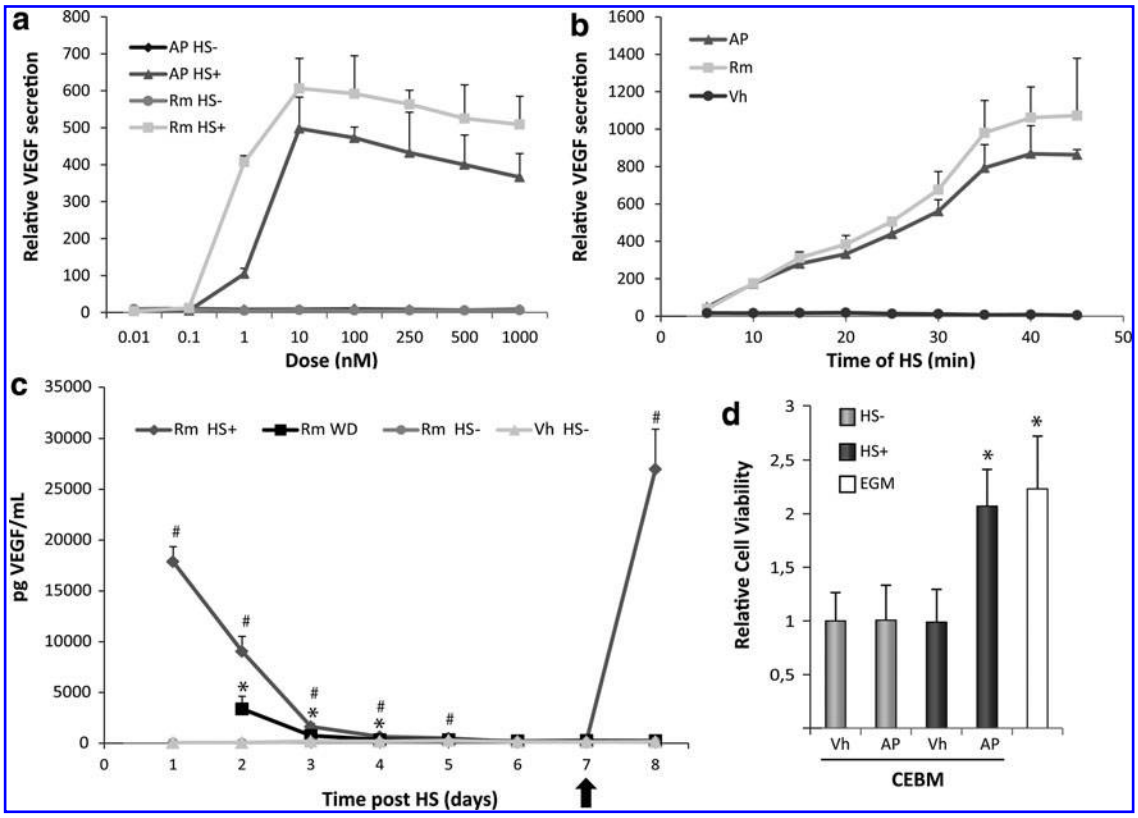

FIG. 3. Gene-switch-mediated *in vitro* induction of bioactive vascular endothelial growth factor (VEGF). **(a)** VEGF secretion as a function of ligand dose. C3HT10-1/2-VEGF cells were treated with the indicated concentrations of rapamycin (Rm) or AP21967 (AP) with (+) or without (−) heat treatment at 45°C for 30 min (HS). Media were collected 24 hr after HS and VEGF secretion was measured. **(b)** VEGF secretion as a function of HS intensity. C3HT10-1/2-VEGF cells were exposed to vehicle (Vh), 10 n*M* Rm, or AP and then heated at 45°C for the indicated times. VEGF secretion was assayed in media collected 24 hr after HS. Relative VEGF secretion was expressed as picogram of VEGF secreted per microgram of cell lysate protein. **(c)** Time–course of VEGF secretion. C3HT10-1/2-VEGF cells were treated with Vh or 10 n*M* Rm, with (+) or without (−) heat treatment at 45°C for 30 min (HS) and further cultured for 24 hr. Media were collected daily after HS and replaced with a fresh medium containing Rm or Vh as in the initial treatment. A set of cultures (Rm WD) was fed with a fresh medium and treated with Vh 1 day after HS treatment in the presence of Rm. Black arrow indicates the day that a second HS was performed. *Significantly different from the Rm HS+ group, $p<0.05$. #Significantly different from Vh HS− or Rm HS− groups, $p<0.05$. **(d)** Bioactivity of induced VEGF. C3HT10-1/2-VEGF cells cultured in EBM in the presence of Vh or 10 n*M* AP with (+) or without (−) HS. Conditioned media (CEBM) were collected 24 hr after HS. HUVECs were cultured with CEBM or EGM for 1 week and cell viability was measured. *Significantly different from Vh HS−, Vh HS+, and AP HS− groups, $p<0.05$. Error bars depict mean+SD.

sections of skin overlying the implant or muscle from beneath the fibrin–cell construct were obtained 3 days after induction and immunohistochemically stained for vWF, a glycoprotein involved in hemostasis that is exclusively expressed by ECs (Yamamoto *et al.*, 1998). Strong vWF staining was observed in skin and muscle sections from the HS-activated, rapamycin-treated group (Fig. 4c). Staining for vWF dissipated with distance from the activated implant. In addition, muscle subjacent to activated implants showed up to a 300-fold increase of the vWF^+ area compared with control implants. Only weak staining for vWF was observed for fibrin-C3HT10-1/2-VEGF constructs exposed to either HS or rapamycin alone. It is worth noting the presence of mononuclear cell infiltrates exclusively in the muscular layers associated with activated implants (Fig. 4c), likely indicating a local increase in vascular permeability, a well characterized response to VEGF (Connolly *et al.*, 1989). The increased hyperemic surface of the gluteal muscles from HS/rapamycin-treated group shown in Fig. 4c is also consistent with a VEGF-induced increase in vascular permeability. Expression of CD31, also known as platelet EC adhesion molecule 1, is indicative of microvasculature (Albelda *et al.*, 1991; Horak *et al.*, 1992). To account for tissue differences in EC distribution after activation of the gene switch, we performed CD31 immunostaining of muscle sections underlying the fibrin constructs. The ratio of $CD31^+$ structures to muscular fibers quantified in the samples showed a threefold increase in activated implants (Fig. 4c), while no substantial differences were found between the samples biopsied from uninduced control groups. Taken together, these results show that the engineered gene switch is capable of selectively inducing biologically active VEGF *in vivo*, leading to dramatic changes in microvasculature dynamics and blood vessel permeability.

Discussion

A particularly attractive aspect of ligand-dependent, HS-inducible gene expression systems is that they can

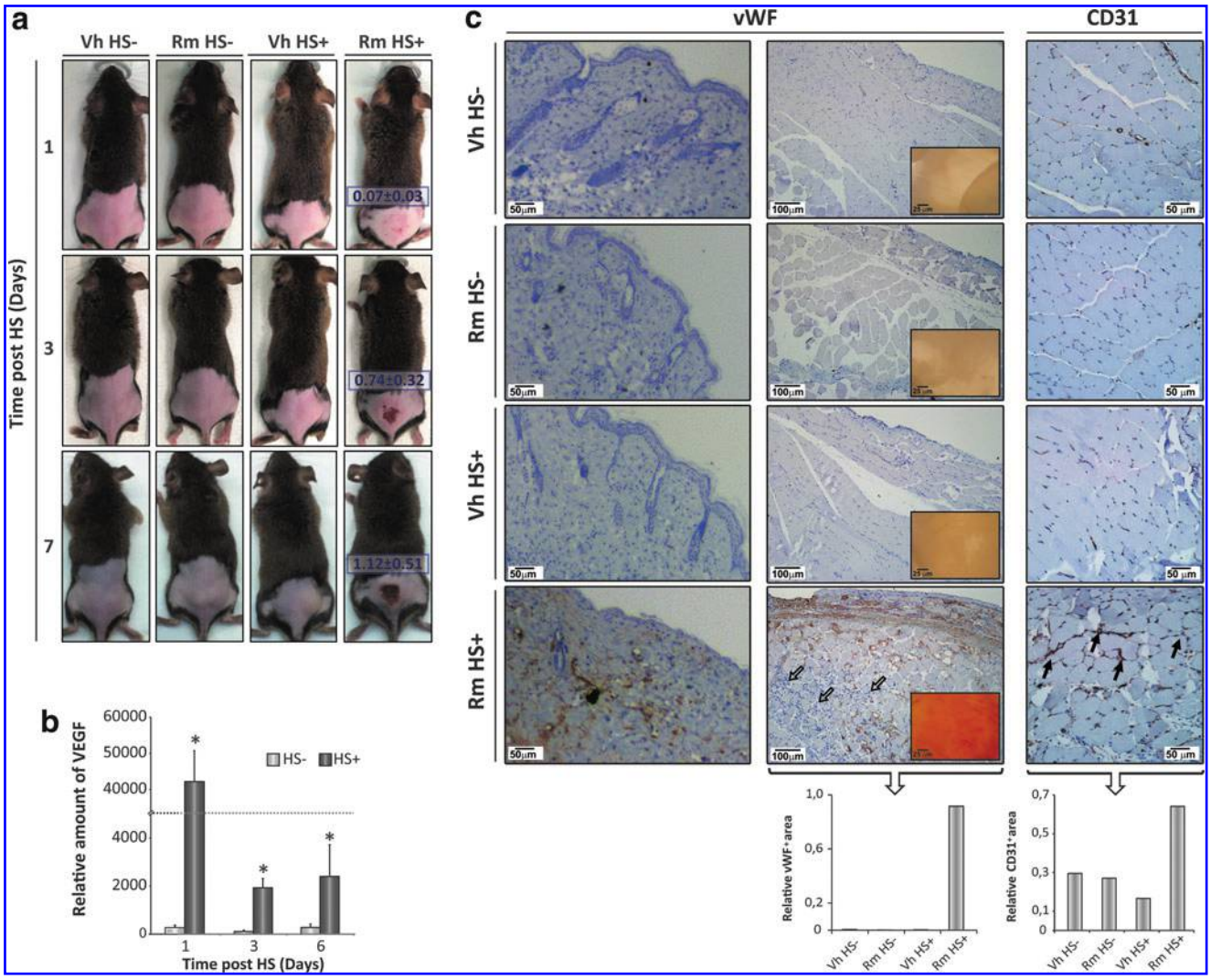

FIG. 4. Gene switch induction of vascular endothelial growth factor (VEGF) *in vivo*. **(a)** *In vivo* induction. C3HT10-1/2-VEGF cells were subcutaneously implanted on the backs of C3H/HeNCrl mice as in Fig. 2. Animals were treated with rapamycin (Rm) or vehicle (Vh), and the lower-back implants were either kept at ambient temperature (HS−) or exposed to heat treatment at 44.5°C for 25 min (HS+). Animals were photographed 1–7 days after HS, and the extent of skin lesions (indicated as erythematous area in cm^2) was calculated ($n=4$/group). **(b)** Measurement of induced levels of VEGF *in vivo*. Subcutaneous fibrin implants were explanted 1–6 days after HS, and processed to quantify the levels of secreted VEGF. Growth factor levels were expressed as picogram of protein per wet weight of fibrin (g). *Significantly different from the HS− group, $p<0.05$, $n=3$/group. **(c)** Histology. Cross sections of skin overlying the fibrin/C3HT10-1/2-VEGF cell implants (left column; scale bar = 50 μm) and muscle underlying the implant (central and right columns; scale bar = 100 and 50 μm, respectively) were obtained from Rm- or Vh-treated mice 3 days after HS. Samples were processed for immunohistochemical detection of von Willebrand factor (vWF) or CD31. Open arrows indicate infiltration of mononuclear cells. Black arrows indicate $CD31^+$ capillary-like structures. Quantification of the area of vWF^+ or $CD31^+$ structures in muscle sections was calculated by image analysis software and indicated as arbitrary units. Microscopy images of muscle surface in contact with fibrin construct are presented as boxed images in the central column (scale bar = 25 μm). Error bars depict mean + SD.

theoretically use hyperthermia to trigger the expression of any number of ligand-activated transcription factors that, in the presence of the appropriate inducer, can each activate a unique promoter and associated transgene. Our initial demonstration of this concept used HS to induce a mifepristone-activated transcription factor (Vilaboa *et al.*, 2005). Here we describe a second HS-inducible system that combines the stringent control of rapamycin/rapalog-dependent transactivators with the spatial control provided by hyperthermic induction. This system requires both HS and ligand for transcriptional activity and has extremely low background in the uninduced state. Luciferase or VEGF expression was shown to decay to basal levels within 1 week, but could be repeatedly reactivated by subsequent heat treatments. Furthermore, use of this approach to induce VEGF *in vivo* resulted in clear temporal and spatial control of a biological response.

Polymeric scaffolds have previously been used to directly deliver bioactive protein cargos such as recombinant VEGF, BMPs, insulin-like growth factors, fibroblast growth factors,

and/or platelet-derived growth factors. The proteins are typically encapsulated within scaffolds or bound to the surface via electrostatic interactions or covalent conjugation. Methods of regulating growth factor release from such scaffolds generally include tuning the spontaneous, cell-mediated, or physically inducible (e.g., light, enzyme, or drug) degradation of the scaffold (Leach *et al*., 2006; Kempen *et al*., 2009; Mercado *et al*., 2009; Jay *et al*., 2010; Singh *et al*., 2012). Although some success has been achieved with these approaches, they have certain inherent limitations, including restriction in the amount and stability of growth factor incorporated into the scaffold, inability to vary release kinetics in real time to compensate for a changing regeneration environment, and difficulties with repeated on/off control of factor release. The regulated gene expression system we developed has many advantages over these protein delivery schemes. Specifically, scaffolds containing a uniform distribution of genetically modified cells can be implanted within a regeneration site and desired subregions within the scaffold can be activated by local thermal treatment. In addition, concentration and duration of growth factor delivery can be controlled by both the extent of HS and the concentration of rapamycin/rapalog ligand.

Prior studies showing that rapamycin-dependent gene circuits are able to precisely control the temporal availability of therapeutic transgenes (i.e., growth hormone, insulin, erythropoietin, IFNα, BMP-2, or cell-line derived neurotrophic factor) (Rivera *et al*., 1999, 2005; Auricchio *et al*., 2002; Johnston *et al*., 2003; Indraccolo *et al*., 2006; Koh *et al*., 2006; Hadaczek *et al*., 2011) indicated that this is an excellent ligand-activated system to combine with Hsp-based systems. Detailed *in vitro* characterization of C3HT101/2-fLuc and VEGF cells showed strong thermal inducibility of the gene switch in the presence of nanomolar concentrations of heterodimerizer, but negligible transgene expression when one or both stimuli are absent. Consistent with previous reports (Jullien *et al*., 2007), we found the activity of AP21967 to be approximately one-fifth the activity of rapamycin. However, this compound does not inhibit endogenous mTOR and has less than one thousandth the immunosuppressive activity of rapamycin (Indraccolo *et al*., 2006). Therefore, it is a preferred ligand to activate this system *in vivo*.

Cell lines expressing either fLuc or hVEGF165 under the control of the HS- and rapamycin-activatable gene circuit demonstrate that this switch is capable of regulating the expression of various transgenes. Interestingly, we found that these cell lines exhibited different sensitivities to hyperthermic stimulus. In the presence of heterodimerizer, VEGF-engineered cells secreted detectable quantities of hVEGF with HS exposure times as short as 10 min, whereas fLuc-engineered cells required twice the time to yield measurable increases in luciferase activity. This difference is particularly impressive given the substantially higher sensitivity of luciferase assays compared with ELISA. Both lines were derived from a common parental line harboring stably integrated copies of the transactivator that is expressed from an activated HSP70B promoter, which suggests that the observed differences in inducibility are not related to the HS-sensitive component of the switch. Rather, we attribute this divergent behavior to variations in the stability of the transgene mRNAs and/or protein products. These results underscore the importance of endogenous mechanisms of post-transcriptional and/or post-translational (trans)gene regulation in the overall behavior of a given transgene.

To assess the functionality of this gene switch *in vivo*, cells containing the inducible system were encapsulated in a biodegradable fibrin hydrogel scaffold. This scaffold material is approved for clinical use by the U.S. Food and Drug Association, polymerizes by an enzymatic mechanism under mild conditions, and permits facile delivery by subcutaneous injection. Fibrin and its degradation products also support angiogenesis and tissue repair (Amrani *et al*., 2001), and can be used as an autologous scaffold to support the growth of seeded cells with low risk of a foreign body reaction (Shaikh *et al*., 2008). We chose to use fibrin in these studies for the reasons described above, but our approach to controlling the delivery of regeneration growth factors is, in principle, compatible with many currently used natural and synthetic tissue engineering scaffolds such as collagen and alginate hydrogels and biodegradable polymers including poly lactic-co-glycolic acid. The only possible restrictions we anticipate would be related to the thermal absorption properties of certain scaffolds such as ceramics that might draw thermal energy away from targeted cells and disrupt activation.

Translation of this platform technology for therapeutic applications will require delivery of the switch components to a clinically relevant cell source, such as primary mesenchymal progenitor cells. We generated clonally derived cell lines harboring the gene switch from a C3H10T1/2 parent line because these cells resemble, in terms of multipotency, primary progenitor cells, are readily available, and elicit modest immune responses when implanted in syngeneic (C3H) immunocompetent mice (unpublished observations). Thus, the C3H10T1/2 lines served as convenient and controlled experimental systems for evaluating the performance of the gene switch *in vitro* and *in vivo*. Ongoing studies are focused on the construction of viral vectors capable of efficiently transducing primary cells with the gene switch and characterizing its performance in a population of transduced cells. Evaluation of switch function in primary cells and *in vivo* models of tissue injury (e.g., hind limb ischemia) will be important next steps in assessing the clinical potential of this platform.

In vivo activation of C3HT101/2-derived cells seeded within an injectable fibrin scaffold demonstrated the ability of the heat-activated rapamycin-dependent gene switch to induce transgene activity at specific sites exposed to hyperthermia. The stringent requirement for both heat and dimerizer stimuli for activation prevents the inadvertent production of therapeutic proteins at distal sites or as a consequence of physiological hyperthermia associated with inflammation, strenuous exercise, or fever. Administration of a heterodimerizer modulates the temporal window during which the gene circuit remains active through the autoregulatory loop defined by the Hsp70B/12xZFHD1 promoter cassette, thereby ensuring the replacement synthesis of the transactivator. Heterodimerizer withdrawal *in vitro* or *in vivo* increases the rate of decay of induced protein. This mechanism does not interfere with the ability of the gene circuit to be reactivated, since maximal transgene induction was seen after successive thermal treatments in the presence of heterodimerizer. This approach was also able to control the release of bioactive VEGF from engineered scaffolds, as evidenced by the local response of ECs to conditioned

medium *in vitro* and the vascular response observed *in vivo*. In the latter case, the host tissue response to induced VEGF was characterized by increased vWF and CD31 staining indicative of vascular reorganization and mononuclear cell infiltration into areas adjacent to activated implants. These signs of increased vascular permeability and hyperemia were macroscopically visible as an erythematous wound bed on muscle and skin, a clear indication that the induced VEGF was biologically active *in vivo*.

Our data indicate that levels of transgene production can be modulated by duration of hyperthermia and heterodimerizer dose. Future studies will focus on fine-tuning regenerative factor release kinetics and localization by precisely controlling thermal dose and spatial localization. This can be achieved using focused ultrasound heating, a technology that can be combined with magnetic resonance temperature imaging to guide the deposition of thermal energy and gene activation (Deckers *et al*., 2009). The gene expression system we developed holds much promise for applications where the temporal and spatial control of VEGF delivery is desired such as in ischemia or applications where a vascular bed is needed for tissue regeneration. This approach is to be contrasted with the systemic delivery of VEGF where hypotension, tachycardia, and reduced cardiac output are common side effects (Yang *et al*., 1996, 1998). Moreover, the heat- and heterodimerizer-dependent system may be used in combination with the previously described Hsp-based and mifepristone-dependent system to sequentially control the expression of two transgenes in a regenerative medicine scenario where precise timing and localization of therapeutic factors expression is critical for therapeutic impact.

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Author Disclosure Statement

R.V. declares the following conflict of interest: he is the founder and CEO of HSF Pharmaceuticals S.A. All other coauthors have no conflicts of interest.

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