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Spatial control of gene expression within a scaffold by localized inducer release

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ABSTRACT

Gene expression can be controlled in genetically modified cells by employing an inducer/promoter system where presence of the inducer molecule regulates the timing and level of gene expression. By applying the principles of controlled release, it should be possible to control gene expression on a biomaterial surface by the presence or absence of inducer release from the underlying material matrix, thus avoiding alternative techniques that rely upon uptake of relatively labile DNA from material surfaces. To evaluate this concept, a modified ecdysone-responsive gene expression system was transfected into B16 murine cells and the ability of an inducer ligand, which was released from elastomeric poly(ester urethane) urea (PEUU), to initiate gene expression was studied. The synthetic inducer ligand was first loaded into PEUU to demonstrate extended release of the bioactive molecule at various loading densities over a one year period *in vitro*. Patterning films of PEUU variably-loaded with inducer resulted in spatially controlled cell expression of the gene product (green fluorescent protein, GFP). In porous scaffolds made from PEUU by salt leaching, where the central region was exclusively loaded with inducer, cells expressed GFP predominately in the loaded central regions whereas expression was minimal in outer regions where ligand was omitted. This scaffold system may ultimately provide a means to precisely control progenitor cell commitment in a spatially-defined manner *in vivo* for soft tissue repair and regeneration.

1. Introduction

The inherent complexity associated with the structure of a tissue poses a major design challenge to engineering strategies for tissue repair and regeneration. Embryogenesis demonstrates the commitment of cells to multiple lineages in a well-regulated spatial and temporal manner, and differentiated cells must perform specific functions in synchrony to form a functional tissue or organ structure. Growth factor gradients and spatial patterning of gene expression not only direct cell differentiation, but also regulate processes such as vascularization and wound repair [1–8]. Therefore, tissue engineered constructs capable of spatially and temporally controlling cell behavior may more closely mimic the natural tissue microenvironment, thereby resulting in a more successful *ex vivo* development of functional tissues and *in situ* tissue repair and regeneration.

Controlling the location and timing of biomolecule delivery from tissue engineering scaffolds has been the subject of many reports in the literature. Scaffolds have been used for the sustained delivery of numerous growth factors [1,9,10], and the spatial organization of growth factors on substrates has been achieved through a number of fabrication processes to modulate cell behaviors including proliferation, differentiation, and migration *in vitro* [11–19]. In lieu of growth factor presentation, natural and synthetic polymers have also been used for controlled non-viral gene delivery [20–22]. Inducing cells to express a gene of interest can effectively alter local cell behavior and function leading to, for example, neovascularization following myocardial infarction or cell death in a tumor [23,24]. Adsorption of DNA to specific regions of a tissue engineered construct is also under investigation to encourage cells to express different genes depending on their location in the scaffold. Some of this work has been aimed at mimicking the organization of motor

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and sensory neurons in a spinal cord bridge following injury with promising results [25–27].

Inducer molecules capable of regulating gene expression in specific cell populations have thus far proven effective in basic science and preclinical studies [28–31]. One such inducible gene expression system is based on a modified version of the ecdysone-responsive gene expression system which mimics the action of 20-hydroxyecdysone, a steroidal hormone which regulates genes necessary for metamorphosis in *Drosophila melanogaster* [32–34]. In this gene expression system, which uses a non-steroidal analog of 20-hydroxyecdysone as the inducer ligand, transcription is repressed in the absence of inducer, however, when the ligand is added to the system, it binds to the receptor and induces gene expression over a broad range of concentrations and in a dose-dependent manner. High induction potential coupled with very low basal expression makes this an attractive gene expression system. The use of such regulatable gene expression systems has many advantages relative to traditional DNA delivery approaches. First, because all cells used in these applications are stably transfected with the gene of interest prior to use, the problem of low transfection efficiency from delivering plasmid *in situ* is circumvented. Second, because the inducer molecules provide a direct method to regulate both the level and timing of gene expression, these systems offer the potential for greater control. Third, a well-designed inducer will have no effect on cells that lack the transfected plasmid, thus assuring a response only in the designated cells.

The objective of this study was to combine the benefits of local controlled release with a gene expression system to provide spatial control of gene expression in a tissue engineered construct. To our knowledge, this approach has not been investigated previously, and is a potential step towards generating complex tissue architectures without the need for direct DNA delivery. To this end, the ability to achieve controlled release of an inducer ligand in an active form over extended periods of time was demonstrated from a biodegradable poly(urethane). Two-dimensional polymer films and three-dimensional porous scaffolds were fabricated with spatially-defined regions containing or omitting ligand. Cells stably transfected with a gene under control of the inducer ligand were seeded onto biomaterials variably loaded with inducer, and spatial patterning of gene expression was achieved.

2. Materials and methods

2.1. Gene expression system

The inducible gene expression system (RheoSwitch, Intrexon Corp) was composed of an engineered nuclear receptor and a highly specific synthetic ligand inducer (Fig. 1a). In *Drosophila melanogaster,* 20-hydroxsyecdysone allows gene expression by binding to a nuclear receptor heterodimer consisting of an ecdysone receptor (EcR) and a retinoid X receptor (RXR). The RheoSwitch system uses a hybrid EcR (RheoReceptor-1, RR1), composed of the ligand binding domain of EcR fused to the yeast GAL4 binding domain. RR1 binds to RheoActivator (RA), which fuses the ligand binding domain of RXR with the activation domain of viral transactivator VP16. The RR1-RA bipartite holoreceptor has also been engineered to bind to the 5X RE (contains 5 copies of the GAL4 response element) promoter and induce gene expression only in the presence of the non-hormonal, synthetic inducer ligand RheoSwitch Ligand 1 (RSL1) [N-(2-ethyl-3-methoxybenzoyl)-N′-(3,5-dimethylbenzoyl)-N′-tert-butylhydrazine] (MW 382.5) (Fig. 1b). Briefly, cells were stably transfected with both the pNEBR-R1 and pNEBR-X1 plasmids (New England Biolabs), which constitute the RheoSwitch system. pNEBR-R1 constitutively produces RA and RR1 under control of the ubiquitin B (UbB) and ubiquitin C (UbC) promoters, respectively. Once formed, the holoreceptor regulates transcription of the gene of interest which was cloned into the pNEBR-X1 expression vector. For the selection and generation of stable cell lines, pNEBR-R1 and pNEBR-X1 plasmids also provide neomycin and hygromycin resistance, respectively. In the absence of inducer ligand, the holoreceptor binds with negative regulatory cofactors to the 5X RE promoter, preventing transcription. However, when present, inducer tightly and selectively binds to the holoreceptor, changing the conformation of and activating the receptor protein so that it releases bound negative regulatory cofactors, resulting in a highly induced transcriptional state. The level of gene expression can be controlled by adjusting the concentration of inducer ligand present [34].

A B16 murine melanoma cell line was obtained from Intrexon Corporation. These cells were stably transfected with both the pNEBR-R1 and pNEBR-X1 plasmids. pNEBR-X1 plasmids were cloned to express a reporter gene, green fluorescent protein (GFP), in the presence of inducer ligand. Cells were maintained on tissue culture polystyrene (TCPS) in growth medium (RPMI-1640 supplemented with FBS, gentamycin, G418 sulfate, non-essential amino acids, and beta-mercaptoethanol), and varying amounts of RSL1 ligand (1–5 μM final concentration) were added directly to growth medium in order to induce gene expression. After 48 h, cells were fixed with 2% paraformaldehyde for 10 min, and nuclei were stained with Hoechst dye (1:100 in PBS) or 4′,6-diamidino-2-phenylindole (DAPI, 1:100 in PBS). Cell nuclei and GFP expression were visualized using fluorescent microscopy.

2.2. Polyurethane synthesis

Poly(ester urethane) urea (PEUU) based on polycaprolactone diol (PCL, MW 2000, Aldrich), butane diisocyanate (BDI, Fluka), and putrescine (Aldrich) was synthesized in a two-step solution polymerization as previously reported [35]. Briefly, PCL in dimethyl sulfoxide (DMSO) was stirred with BDI in DMSO. A catalyst, stannous octoate (Aldrich) was added, and the reaction was allowed to continue at 80 °C for 3 h. Putrescine in DMSO was then added to the pre-polymer solution, and the reaction was allowed to continue at room temperature for 12–18 h. The stoichiometry of the reaction was 2:1:1 BDI:PCL:putrescine. PEUU was precipitated in distilled water and immersed in alcohol to remove unreacted monomers. Finally, polymer was dried under vacuum at 50 °C for 4–5 days in order to completely remove solvent.

2.3. Preparation of RSL1-loaded PEUU films

RSL1-loaded PEUU films were fabricated using a solvent casting method. PEUU was dissolved in DMSO at 80 °C to obtain a 2 wt% polymer solution. Predetermined amounts of RSL1 (reconstituted in DMSO) were added to polymer solutions (0–150 μM final ligand concentration), and 0.5 mL polymer solution was cast into each well of 24 well culture plates or glass vials for gene expression and ligand release studies, respectively. PEUU films were dried under vacuum at 50 °C prior to the initiation of experimental protocols.

2.4. RSL1 release kinetics

RSL1-loaded PEUU films were incubated *in vitro* at 37 °C in 1 mL release fluid (90% v/v PBS and 10% v/v acetonitrile) in order to determine ligand release kinetics. A solubility enhancer, in this case acetontrile, was required in the release fluid to encourage sink conditions for the hydrophobic RSL1 [36,37]. Releasate was collected and stored at −20 °C at pre-determined time points and was replaced with fresh release fluid. A ligand standard curve was obtained by adding known concentrations of RSL1 to releasate collected from polymer films that were not loaded with ligand and by assaying absorbance using UV spectrometry (Lambda 2, Perkin Elmer) at 250 nm. Absorbances at 250 nm for releasate collected from RSL1-loaded PEUU films ($n = 4$) were compared to the ligand standard curve to determine the amounts of RSL1 released over time.

2.5. Degradation of PEUU films

Degradation of PEUU films was determined over time by measuring the intrinsic viscosity of polymer films dissolved in hexafluoroisopropanol (HFIP). Films were soaked in either PBS or a solution of 90% v/v PBS with 10% v/v acetonitrile. Degradation fluid was replaced weekly. At designated time points over one year, polymer films ($n = 3$) soaked in each degradation fluid were collected, dried, and dissolved in HFIP to a concentration of 1.5 mg/mL. Polymer solutions were put into a modified Ubbelohde viscometer (Ace Glass Inc.), and the time for solution to travel between markings was measured and compared to the time for pure HFIP solvent. These data were used to determine the intrinsic viscosity of polymer solutions using a single-point calculation as a representation of polymer chain degradation [38].

2.6. Bioactivity of released RSL1

The bioactivity of released RSL1 was determined by the ability of the ligand to induce GFP expression in B16 cells plated on TCPS. For short-term bioactivity studies (3 week endpoint), 24 well tissue culture plates containing PEUU films ($n = 4$) loaded with 0–10 μM RSL1 were incubated in 1 mL B16 growth medium per well at 37 °C. Growth medium was removed at pre-determined time points from polymer films and transferred to B16 cells on 24 well tissue culture plates. For long-term (greater than 3 weeks) bioactivity studies, films ($n = 4$) loaded with high RSL1 content (25, 75, 150 μM) were prepared. Releasate from polymer films was dried in a vacuum oven at 50 °C overnight to remove acetonitrile and subsequently reconstituted in 4 mL B16 growth medium per sample prior to transfer to B16 cells on 24 well plates. In order to visualize cells and quantify gene expression, cell monolayers were fixed with 2% paraformaldehyde and stained with nuclear dye 48 h after