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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.

Keywords

gene expression; controlled release; spatial control; elastomer; tissue engineering

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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 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 behavior 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 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 (Figure 1a). In *Drosophila melanogaster* 20-hydroxyecdysone 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) (Figure 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 μ M final concentration) were added directly to growth medium in order to induce gene expression. After 48 hrs, cells were fixed with 2% paraformaldehyde for 10 minutes, 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 hrs. Putrescine in DMSO was then added to the pre-polymer solution, and the reaction was allowed to continue at room temperature for 12–18 hrs. 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 μ M 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 acetonitrile, 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 μ M 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 μ M) 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 hrs after treatment with released RSL1. Cells were visualized (at least 4 fields) for nuclear staining and GFP expression using fluorescent microscopy, and the percentage of GFP-positive cells was quantified.

2.7 Gene expression on PEUU cell-seeded films

Polymer films containing varying concentrations of RSL1 ligand (0–10 μM) were cast into 24 well glass bottom plates (MatTek Corporation), and B16 cells (1000 cells/ cm^2) were plated directly onto PEUU films in 1 mL growth medium per well. Growth medium was exchanged every 3–4 days for a period of three weeks. At pre-determined time points, cells were fixed with 2% paraformaldehyde and stained with a nuclear dye. Cells were visualized for nuclear stain and GFP expression using confocal fluorescent microscopy, and the percentage of GFP-positive cells was quantified.

2.8 Spatial control of gene expression on PEUU films

A 2 wt% PEUU solution in DMSO was obtained as described. RSL1 ligand was added to half of the polymer solution to yield a 10 μM final ligand concentration. Droplets (20 μL s each) of PEUU with RSL1 and PEUU without RSL1 were pipetted onto glass microscope slides in a spatially-defined pattern and allowed to dry under vacuum at 50°C (Figure 7a). Once solvent was evaporated, another droplet was placed on top of the existing one. In total, five droplets of polymer solution (i.e. $5 \times 20 \mu\text{L}$ s) were placed at each spot on each microscope slide. Various configurations of droplets were tested to optimize spatial control. Slides were incubated overnight in 10 mL PBS to remove surface-bound RSL1 and then sterilized under UV light for 20 min. B16 cells were plated onto slides (1000 cells/ cm^2) and cultured in Petri dishes in 10 mL growth medium for up to 72 hrs with culture medium exchanged every 24 hrs. At pre-determined time points, slides were fixed with 2% paraformaldehyde and stained with a nuclear dye. Cells were visualized for nuclear stain and GFP expression using confocal fluorescent microscopy, and the percentage of GFP-positive cells was quantified.

2.8 Fabrication of PEUU scaffolds with localized RSL1 loading

A two-step salt-leaching (SL) processing method was used to obtain porous PEUU scaffolds. Polymer was dissolved in 1 mL HFIP (35% w/v PEUU) and 1000 μM RSL1, mixed with 5 g NaCl (particle size 75–100 μm), forming a paste-like material, and tightly packed into a small diameter (6 mm inner diameter) cylindrical glass mold. The newly formed scaffold was immediately pushed out of the glass mold and exposed to air overnight to allow solvent evaporation. The dried scaffold was then placed in the center of a larger cylindrical glass mold (inner diameter 13 mm), and a polymer and salt mixture identical to that described above, but without RSL1, was packed around the small-diameter scaffold, creating a concentric scaffold with an RSL1-containing core and an outer ring without RSL1. The concentric scaffold was removed from the mold, and solvent was again evaporated. The final scaffold was cut into disks (0.75 mm thickness, 13 mm diameter), and salt was leached from constructs by soaking in deionized water for 30 min immediately before seeding with cells. For comparison, porous scaffolds without any RSL1 or with RSL1 through the entire scaffold were also synthesized and seeded with B16 cells. Scaffold disks were visualized using a JEOL 9335 field emission gun scanning electron microscope (JEOL, Peabody MA) to confirm porous structure and continuity between concentric scaffold regions.

2.9 Spatial control of gene expression within PEUU scaffolds

Stably transfected B16 cells were seeded onto porous PEUU disks with spatially defined RSL1 loading in order to demonstrate the ability of the constructs to elicit spatially defined gene expression. Scaffolds were sterilized under UV light for 20 min, and cells were seeded onto scaffolds at a density of 2×10^6 cells/scaffold using a filtration seeding method [39]. Cell-seeded scaffolds were then statically cultured in 6-well TCPS plates containing 8 mL/well B16 growth medium for up to 7 days with media exchanged every 3–4 days. Scaffolds

were also cultured in a transmural perfusion system to reduce near-surface RSL1 in the fluid phase [40]. At days 3 and 7, scaffolds were fixed with 2% paraformaldehyde, stained with nuclear dye, and mounted onto slides. For each scaffold (n=3), nuclear dye and GFP expression were visualized using fluorescent confocal microscopy, and 10 images were captured from each of the inner and outer regions. The percentage of cells expressing GFP was quantified for each image. One representative scaffold was selected to be imaged in its entirety in a mosaic fashion. Additionally, a Matlab computer program (Mathworks) was written to quantify the location-dependent color intensity of the fluorescent images in order to obtain a quantitative measure of spatial control of gene expression. Specifically, the intensity of the green signal (GFP) was normalized to the intensity of the blue signal (nuclear dye) (G/B ratio) and averaged along a 4 mm thick horizontal band across the center of the scaffold.

2.10 Statistical methods

Statistical analyses were performed by one-way ANOVA with Tukey post-hoc testing of differences at specific times of interest using SPSS software. Linear regression was used to describe the dose-dependent response of GFP expression to RSL1 loading dose in films or culture medium. Differences in GFP expression between inner and outer regions of concentric scaffolds were compared using a student's t-test with $p < 0.05$ considered significant.

3. Results

3.1 Gene expression by media manipulation

Stably transfected B16 cells cultured in growth medium in the absence of RSL1 ligand did not express GFP. GFP expression, however, was evident in cells cultured in growth medium containing varying concentrations of ligand (1–5 μM) 48 hrs post-RSL1 administration. (Figure 2). The percentage of cells expressing GFP was 16.6 ± 4.4 , 32.5 ± 3.0 , 44.0 ± 15.0 , and $63 \pm 7.0\%$ for 1, 2, 3, and 5 μM RSL1, respectively ($R^2 = 0.981$).

3.2 Sustained release of RSL1 from PEUU films

Solvent casting of PEUU into 24 well TCPS plates yielded approximately 50 μm -thick polymer films. PEUU films loaded with 25, 75, or 150 μM RSL1 demonstrated a two-phase *in vitro* release profile with a small burst release of ligand accounting for less than 10% of loaded RSL1 during the first week of incubation followed by near zero-order release kinetics over the next 10 months. A total of 65–80% of loaded RSL1 was released after 10 months *in vitro* (Figure 3), with approximately 1–3% of RSL1 steadily released per week regardless of the initial ligand loading dose. Degradation of PEUU films, as determined by the decrease in polymer viscosity over 1 year, was not different when films were soaked in PBS with 10% v/v acetonitrile compared to pure PBS. After just one week of soaking in each medium polymer intrinsic viscosity was 2.49 ± 0.03 and 2.51 ± 0.08 dL/g for films in PBS and in PBS with acetonitrile ($p > 0.1$), respectively. These values decreased to 2.03 ± 0.14 and 1.91 ± 0.08 dL/g after 1 year ($p > 0.1$). Additionally, RSL1 solubility in PBS/acetonitrile fluid far exceeded the total loading doses used in the films studied.

3.3 Bioactivity of released RSL1

For short-term studies, cells cultured in releasate from all RSL1-loaded PEUU films expressed GFP 48 hrs post-treatment, while cells in releasate from PEUU films without ligand did not. Additionally, dose-dependent gene expression ($R^2 = 0.99$) was evident in cells cultured in releasate collected over a three week period from polymer films containing as little as 0–5 μM RSL1 (Figure 4). For long-term studies, releasate collected from films

loaded with high concentrations of RSL1 ($>25 \mu\text{M}$) was able to induce GFP expression in B16 cells for extended periods of time, with the duration of effective gene expression increasing with increasing RSL1 loading concentration (Figure 5). Gene expression levels were similar between all three concentrations until 300 days, at which point differences in scaffold loading doses had a significant effect on the percentage of GFP expressing cells ($p<.001$ between each of the loading doses). By 300 days *in vitro*, releasate from $25 \mu\text{M}$ -loaded films appeared to lose the ability to effectively induce GFP expression in adherent cells, while releasate from $75 \mu\text{M}$ - and $150 \mu\text{M}$ -loaded films maintained gene induction ability to at least 355 days, the longest timepoint studied. At this time, however, gene expression was reduced compared to earlier time points.

3.4 Gene expression from PEUU films

B16 cells cultured directly on PEUU films containing $1\text{--}10 \mu\text{M}$ RSL1 expressed GFP for up to 21 days *in vitro*, while cells cultured on films without ligand did not express GFP. Furthermore, cells cultured on films containing $0\text{--}5 \mu\text{M}$ RSL1 demonstrated dose-dependent GFP expression ($R^2=0.99$) over this 3 week period, as evidenced by an increasing percentage of GFP-positive cells with increasing ligand loading concentration (Figure 6). While $75.2 \pm 9.0\%$ of cells on PEUU films loaded with $5 \mu\text{M}$ RSL1 expressed GFP, an increase in the GFP-positive cell fraction was not seen in cells cultured on PEUU films loaded with $7 \mu\text{M}$ ($72.2 \pm 7.8\%$) and $10 \mu\text{M}$ ($76.1 \pm 8.3\%$) RSL1.

3.5 Spatial control of gene expression on PEUU films

All cells expressed GFP 24 hrs after being plated onto microscope slides containing distinct regions of polymer with and without RSL1, regardless of the configuration of PEUU droplets. At 48 and 72 hrs after cell seeding, however, cells in regions where polymer contained RSL1 ligand expressed GFP, whereas cells in regions where ligand was not added to the polymer did not express GFP. (Figure 7b)

3.6 Spatial control of gene expression on PEUU scaffolds

Porous scaffolds with or without RSL1 were successfully fabricated using the SL technique. After 72 hrs of cell culture, cells were found on the surface and interior of PEUU scaffolds either with or without RSL1 ligand. Cells cultured in scaffolds without RSL1 did not express GFP, while cells cultured in scaffolds with ligand expressed GFP (images not shown). Concentric porous scaffolds generated with an interior region containing RSL1 were successfully generated (Figure 8a). Electron microscopy revealed that the inner and outer regions were fused well at their interface (white arrows Figure 8b). Material continuity between the regions was further demonstrated by manual stretching of these scaffolds. The scaffold disks did not separate at the boundaries between inner and outer regions, but acted as a continuous material (Figure 8c and supplemental video).

Cells were evident throughout the concentric scaffolds after 72 hrs of static culture. (Figure 9a-c) Cells in the center of the scaffold predominately expressed GFP; however, some GFP positive cells were evident in the non-RSL1 outer ring of the scaffold, radiating outward from the RSL1-containing core. The extent of high GFP expression in non-RSL1 regions varied by location around the scaffold core, in some areas being largely confined to the center and in other areas extending $>1 \text{ mm}$ away from the core. Additionally, the same level of spatial control over gene expression could be maintained up to seven days in static culture. The percentage of cells expressing GFP in the inner and outer regions of the scaffold after 3 days of culture was $87 \pm 2.5\%$ and $14 \pm 2.6\%$, respectively ($p<.001$). The normalized G/B ratio of image intensity data across the center of the scaffold demonstrated the general localization of GFP expression to the middle of the scaffold (Figure 9d), and GFP expression levels rapidly decreased the further cells were from the RSL1-containing

core. Use of the transmural perfusion system led to fewer cells present on the scaffolds and markedly less GFP expression overall (data not shown).

4. Discussion

Gene-regulating systems that utilize an inducer molecule to regulate the timing and level of gene expression have proven effective in various applications [28–31, 34, 41]. Not only do these systems eliminate the need for DNA delivery *in situ*, the inducer molecules are more stable than plasmids, lending themselves to easy incorporation into biomaterials with the potential for maintained bioactivity for extended release. The present study explored the potential for spatiotemporal regulation of gene expression through the use of a biodegradable, elastomeric polyurethane scaffold as a controlled-release system for the delivery of one such inducer molecule, RSL1.

Biodegradable polyurethane scaffolds have been explored for a variety of soft tissue engineering applications including blood vessel, myocardial, cartilage, and abdominal wall reconstruction [42–48]. Linear thermoplastic elastomers are particularly advantageous because they can be easily blended and processed with biomolecules for controlled release applications [49, 50]. In the current report, scaffolds were synthesized using a salt-leaching technique since it provided a simple method to fabricate porous scaffolds containing RSL1 in a step-wise process with good continuity between neighboring regions. Further, this method did not require a liquid phase solvent extraction step which might allow premature release of incorporated ligand.

The release characteristics of RSL1 from PEUU films were studied over an extended period *in vitro*. Release studies were performed under a solution consisting of 10% v/v acetonitrile to simulate sink conditions for the hydrophobic RSL1 inducer. This release medium also proved attractive because it did not influence polymer degradation and subsequently, release rates. The small burst release and subsequent linear release profile of ligand was likely due to the interaction of the hydrophobic RSL1 molecule with hydrophobic regions of the PEUU polymer. It is understood that such positive interactions between drug and carrier can influence drug release kinetics and has been demonstrated in other drug-delivery systems [37, 51]. Furthermore, the lack of porosity in PEUU films may lead to release rates slower than that which would be seen from porous scaffolds.

The diffusion of the RSL1 ligand seems to play an important role in the ability to spatially control gene expression. Varying the spatial patterning of droplets on microscope slides for 2D control had no effect on the spatially-defined gene expression. Rather, spatial control of gene expression in 2D films could be achieved only during the second phase of ligand release in static culture because the initial burst release from areas of film containing RSL1 sufficiently spread throughout the culture medium to cause GFP expression in cells on regions without RSL1. This limitation was less pronounced with three-dimensional scaffolds in static culture. Ligand from the center of the scaffold was not adequate to cause all cells on the scaffold to express GFP. However, the presence of some GFP-positive cells in the non-RSL1-containing region may have been due to the diffusion of the inducer molecule laterally from regions of high RSL1 content to regions of low RSL1 content. The functional consequence of high levels of GFP expression extending about 1 mm out from the scaffold core may be insignificant if larger scaffolds are involved. In contrast, dynamic culture of cell-seeded scaffolds, using a transmural perfusion system [40], did not achieve high levels of GFP expression regardless of cell location on the scaffold, and it is postulated that this was due to RSL1 being washed away from cells too rapidly (i.e. before they could take up ligand to initiate transcription). As such, while the use of static culture was limited in that it may have allowed RSL1 diffusion to trigger GFP expression in bordering non-RSL1

regions, its use led to higher GFP expression overall. Appropriate dynamic culture systems may be explored more fully for the potential to control molecule diffusion while allowing for RSL1 uptake into cells. Other material-based techniques may be employed to control ligand diffusion, for example forming a polymer film around the inner core to discourage lateral RSL1 diffusion or cell migration to the outer regions. Diffusion of ligand at a scaffold implant location *in vivo* may also vary depending on the local tissue environment. Areas with high mass transfer, for example on the luminal side of a blood vessel, may behave differently than other areas.

It has been demonstrated that the activation of just a few genes of interest can cause both precursor and adult cells to differentiate to specific lineages [52–56]. While outside the scope of the current research, it is reasonable to postulate that if precursor cells are loaded with multiple genes which are under control of an inducer ligand, and which cause differentiation to a specific lineage, then those cells would differentiate automatically once in the location of a scaffold loaded with the inducer. For example, cardiac fibroblasts transfected with genes GATA4, Mef2c, and Tbx5 under control of an inducer ligand may automatically reprogram to cardiomyocytes in the location of a scaffold containing the inducer ligand [52]. Additionally, it has been shown that spatiotemporal control of biochemical signals plays a role in influencing appropriate chondrogenesis [57, 58]. Particularly, articular cartilage has a complex three dimensional arrangement wherein the size and shape of chondrocytes, and the content and type of collagen and proteoglycans varies from the subchondral bone to the articulating surface. Using spatial gene expression through an inducer system may more closely generate multifaceted tissue constructs by encouraging cellular differentiation or localized growth factor and extracellular matrix production. Similar complex architectures are found throughout the body including in the gastrointestinal tract, spinal column, vasculature, and bone. Even more advanced scaffold systems may be envisioned wherein different cell types are loaded with different gene expression systems, the inducer ligands for which have been independently loaded into specific regions of a single scaffold. In this way both cell types could be cultured in the scaffold together, yet each will only perform a designated behavior in the location of release for their specific inducer ligand. Cells not in contact with their ligand would remain in their original state or subject to endogenous influences. Overall, such approaches may be promising for the development of a desired tissue type from precursor or adult cells in a three dimensional configuration which is patterned at the time of scaffold fabrication.

While the *in vitro* data of this report demonstrate the potential for extended periods of controlled release of a bioactive inducer ligand, performing *in vivo* studies to evaluate the duration of the gene expression effect for extended periods is a logical next step. Polymer degradation will be accelerated *in vivo* and will be accompanied by local inflammatory phenomena. The inducer ligand release might be accelerated, or potentially the ligand bioactivity might be compromised at extended periods. Data from the *in vivo* administration of RSL1 does not suggest acute lability, however [59]. A more appropriate cell type would also likely be pursued for *in vivo* studies. B16 cells were utilized here for their ease of handling and expansion. Use of a cell whose functionality could be altered in a clinically relevant manner by the expression of a specific gene could be pursued. Furthermore, while B16 cell attachment and growth was qualitatively evident throughout the scaffolds and no reduction in cell numbers was noted over the culture period used in this study, the viability and proliferation of clinically-relevant cell populations will need to be examined with further assessment of the therapeutic potential for this type of scaffold system.

6. Conclusion

A polymer scaffold system capable of spatially controlling gene expression in cells *in vitro* without local DNA delivery has been demonstrated. The biodegradable elastomeric polyurethane employed released an inducer ligand over an extended period while maintaining ligand bioactivity. 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Modified ecdysone-responsive gene expression (RheoSwitch) system (a) and chemical structure of the RSL1 inducer ligand (b). Plasmid pNEBR-R1 uses ubiquitin B (UbB) and ubiquitin C (UbC) promoters to constitutively express proteins RheoActivator (RA) and RheoReceptor-1 (RR1), respectively. RR1 and RA form a bipartite holoreceptor that binds the 5X RE promoter sequence on plasmid pNEBR-X1, which contains the gene of interest (green fluorescent protein, GFP). When bound to inducer ligand RSL1, the holoreceptor exchanges bound negative regulatory cofactors for positive cofactors, allowing high transcription rates of GFP.

Figure 2.

Gene expression control by culture medium manipulation. At 48 hrs post-RSL1 administration, cells in growth medium without ligand did not express GFP (a), while cells in growth medium containing 1, 2, 3, and 5 μM ligand (b-d, respectively) expressed GFP with increasing frequency in a linear fashion (f). Scale bar = 200 μm .

Figure 3. Release of RSL1 from PEUU films *in vitro*. Films had initial loading concentrations of 25, 75, and 150 μ M RSL1.

Figure 4. Gene expression in cells treated with release medium from PEUU films containing (a) 0 μM , (b) 1 μM , (c) 2 μM , (d) 3 μM , (e) 4 μM , and (f) 5 μM RSL1. Scale bar = 500 μm .

Figure 5. Long-term bioactivity of released RSL1. Release fluid from films loaded with 25, 75, or 150 μ M RSL1 at designated time points was able to cause GFP expression in cultured B16 cells. * denotes $p < .001$ between each of the 3 concentrations.

Figure 6. Gene expression on PEUU films containing (a) 0 μM , (b) 1 μM , (c) 2 μM , (d) 3 μM , (e) 4 μM , and (f) 5 μM RSL1. (g) Dose-dependent GFP expression is preserved when cells are maintained on polymer films. Scale bar = 500 μm .

Figure 7.

Spatial control of gene expression on PEUU films. (a) Typical slide layout for 2-dimensional spatial control experiments. (b) Cells on all regions of slides containing PEUU without ligand (grey) did not express GFP (left), whereas cells on all regions of slides containing PEUU loaded with RSL1 (black) clearly expressed GFP (right), demonstrating spatial control of gene expression on 2-dimensional PEUU films. Scale bar = 500 μ m

Figure 8. Macroscopic and scanning electron micrograph of porous scaffold differentially loaded with RSL1. (a) RSL1 was added to a center core of the material whereas no RSL1 was present in the outer ring, (b) Scanning electron micrograph of the boundary between RSL1-containing core and RSL1-free outer ring. White arrows delineate boundary between regions. (c) Mechanical stretching of the scaffold seen in (a) reveals continuity between concentric regions (Also demonstrated in supplementary video). Scale bar = 1 mm.

Figure 9.

Spatial control of gene expression within 3D PEUU scaffolds. GFP expression was concentrated in the inner region of the scaffold where RSL1 inducer was present. (a) Blue - DAPI nuclear staining only, (b) green - GFP expression only, and (c) combined image showing localization of both cells and GFP expression. Dashed white circle represents boundary between inner and outer scaffold regions. Scale bar = 1 mm. (d) Ratio of green to blue (G/B) fluorescence signal with respect to location across center of scaffold, demonstrating spatial control of gene expression.