Volume 1 | Number 1 | Jan 2013 | Pages 1–100

Molecular Biosystems

www.rsc.org/molecularbiosystems

THE BIOLOGY OF PLAGUE

ROYAL SOCIETY OF CHEMISTRY

This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the **Information for Authors**.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard **Terms & Conditions** and the **Ethical guidelines** still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

Molecular BioSystems

COMMUNICATION

Patterning of Cells Through Patterning of Biology

A. Kala[a], P.K. Jain[a], S.H. Friedman[a*]

Figure 1. Synthesis and characterization of modified siRNAs. a) The sequences of both pten targeting siRNA and the control siRNA. b) Reaction scheme, showing DMNPE reacting with the four terminal phosphates in each duplex RNA. c) The HPLC trace of purified, tetra-DMNPE modified pten targeting siRNA. Asterisked peaks were found in the blank injection. d) ESI-MS spectrum of purified tetra-DMNPE modified pten targeting siRNA. Asterisked peaks indicate sodium adducts.
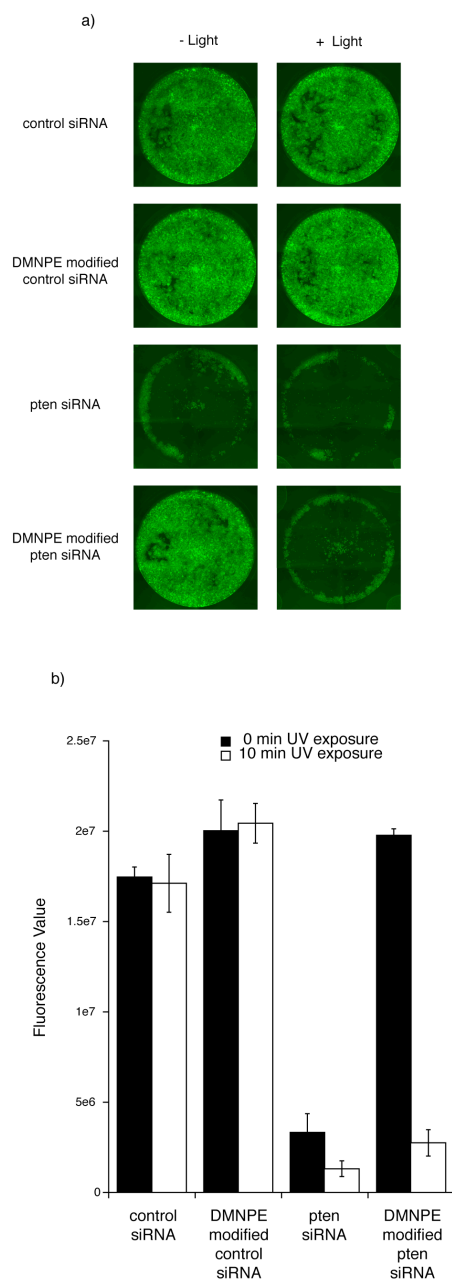
For the first time, cells have been patterned on surfaces through the spatial manipulation of native gene expression. By manipulating the inherent biology of the cell, as opposed to the chemical nature of the surfaces they are attached to, we have created a potentially more flexible way of creating patterns of cells that does not depend on the substrate. This was accomplished by bringing an siRNA that targets the expression of pten under the control of light, by modifying it with photocleavable groups. This pten-targeting siRNA has been previously demonstrated to induce dissociation of cells from surfaces. We modified this siRNA with dimethoxy nitro phenyl ethyl photocleavable groups (DMNPE) to allow the activity of the siRNA, and hence pten knockdown, to be toggled with light. Using this approach we demonstrated light dependent cell dissociation only with a DMNPE modified siRNA that targets pten and not with control siRNAs. In addition we demonstrated the ability to make simple patterns of cells through the application of masks during irradiation.

The patterning of cells is an important technique for the study of a range of phenomenon. It can be used as a tool for tissue engineering, as well as to understand fundamental aspects of cellular behavior and communication. Methods to pattern cells typically rely on some spatial manipulation of the surface upon which cells will be grown [1-10]. Often, this is directed by light, using the tools of photolithography (masks and photochemistry) to prepare surfaces that will have differential binding to cells. While this has proven to be effective, we were interested in developing methods that were more akin to the way nature actually patterns cells in growing tissues, namely by spatially manipulating the expression of native genes. The potential advantage of such an approach is that it eliminates dependency on the specific substrate the cells are being grown on. This in turn increases the likelihood that the cell pattern created will have more relevance to actual tissues, for example by allowing for three dimensional patterning to be effected.

In this work we describe our first efforts to pattern cells by spatially manipulating biology, specifically gene expression. To do this, we have applied a technique developed in our lab, light

Figure 2. Light dependent changes in cell numbers with control and pten targeting siRNA. a) Stitched micrographs of whole wells treated with different siRNA species in the presence (+ right column) and absence (- left column) of light. Cells are stained using calcein AM and visualized by fluorescent microscopy. b) Quantitation of whole well signals. Average of three replicates as well as standard errors indicated.

activated RNA interference (LARI)[11]. We and others have found that photocleavable groups can be used with nucleic acids to control gene expression and other phenomena[11-17]. In LARI, we incorporate four photocleavable groups (typically the dimethoxy-nitro-phenyl ethyl, or DMNPE, group) into the four termini of siRNA or dsRNA. These groups are able to effectively block RNA interference prior to irradiation. Upon irradiation, the groups come off the RNA

exposing native siRNA or dsRNA. These are then able to enter the RNA interference pathway, and effect expression reduction. Using LARI we have previously shown that we can create patterns of gene expression in cell monolayers, including detailed patterns and gradients of expression, using appropriate masks and irradiation[14]. In these studies we have used DMNPE and not CD-DMNPE, a derivative of DMNPE that we have previously shown to have an improved expression window observed in LARI based on dsRNA. In early studies, we did not see a significant difference in light initiated cell dissociation when using CD-DMNPE over DMNPE and so used the more conveniently obtained DMNPE for the work described here.

To apply LARI to the patterning of cells, we used an siRNA that targets pten developed by Doi and coworkers[18]. They found that when HeLa and 293T cells were transfected with pten targeting siRNA (pten-siRNA), the cells became rounded and detached from the cell culture surface. The mechanism by which this happened is not completely clear, but it may be related to the induction of malformation of the cytoskeletal architecture. For our purpose of patterning cells this was a good candidate siRNA to be incorporated into the LARI process.

HeLa cells were plated in 24-well cell culture plates at a confluency of 5%. They were then allowed to culture for 24 hours. At that point different experimental and control transfection solutions were added to different wells. These solutions were a) DMNPE modified pten-siRNA, b) unmodified pten-siRNA, c) DMNPE modified control siRNA d) unmodified control siRNA. The control siRNA used was a GFP targeting siRNA with no homology to the pten-siRNA.

siRNAs incorporating four DMNPE groups were synthesized using the remarkable regiospecificity we have previously described, in which diazo-DMNPE preferentially reacts with terminal phosphates in duplex RNA, over internal phosphates or nucleobase sites[17]. Therefore, siRNA synthesized with four terminal phosphates can be reacted with diazo-DMNPE resulting in the incorporation of four DMNPE groups per duplex. Both experimental and control siRNA were reacted thusly, and the resultant tetra-modified duplexes were purified by HPLC, and characterized by ESI-MS (Fig. 1) (Supplementary Fig. S2 shows the 346 nm chromatogram for DMNPE modified pten siRNA. Supplementary Figs. S3 and S4 show HPLC traces and ESI-MS respectively for the DMNPE modified control siRNA).

DMNPE modified pten-siRNA was the main experimental focus, as we wanted to see if we could toggle pten associated cell detachment with light. Unmodified pten-siRNA was a positive control for pten associated cell detachment, independent of light. The control siRNAs (with and without DMNPE groups attached) were used to confirm that the detachment was specific to pten, and was not associated with irradiation or DMNPE photolysis products. All conditions used oligofectamine and a nucleic acid concentration of 15.69 nM, although we also examined higher concentrations of siRNA and found them to have modestly higher efficacies (see Supplementary Information: Supporting Method and Supporting figure S1).

After 7.5 hours of transfection, the transfection solutions were removed and replaced with fresh media. The cell culture plate was divided into two regions, and half the wells were irradiated for 10' with a 360 nm Blak Ray far UV lamp. The media was then replaced, and the cells allowed to culture for five more days at which point the media was changed. The cells were allowed to culture one more day in this fresh media. At the end of this period, the media was again removed and the cells gently washed twice with PBS. Viable cells were visualized using calcein AM. Stained cells were imaged using fluorescence microscopy, and the amount of

fluorescent signal was quantitated using Photoshop. The microscope image results are shown in Fig. 2a and the quantified data shown in Fig. 2b.

　　　　The fluorescent images are shown in pairs, with irradiated wells to the right, and unirradiated wells to the left. Multiple images of each well were taken and then automerged using Photoshop to show the entire well. These images show that only the DMNPE modified pten targeting siRNA showed a light dependent loss of cells from the cell culture surface. Unmodified pten targeting siRNA showed cell detachment independent of irradiation. We also observed in these samples a "halo" effect, in which cells at the corners of the well remained, even after pten-siRNA treatment. These cells may be more tightly attached to the surface of the well, due to having both well bottom and well side points of attachment. We observe this halo effect with the irradiated DMNPE modified pten-siRNA as well. We varied parameters such as irradiation time and transfection time in an attempt to ameliorate the halo effect, but were unable to eliminate it.

　　　　The control siRNA show little or no detachment, with and without DMNPE group modification, which is consistent with pten targeting being crucial for cell detachment. The quantitated results are shown in Fig. 2b. They strongly parallel the images shown in Fig. 2a. Taken together, the data demonstrate a detachment of cells that is triggered by the light activated knockdown of pten expression.

　　　　We then examined whether we could generate a simple pattern of cells by using the DMNPE modified pten-siRNA and masked irradiation. We previously have generated patterns of gene expression using LARI and masked irradiation of cultured cells.[14] We generate masks using a laser printed pattern on transparencies, which are then treated with "Magic Dark" to increase the density of the mask. For the patterning experiment, we used three kinds of mask: 1) A completely clear control mask, 2) A completely dark ("100%") control mask, and 3) an experimental mask with a dark circle with 20% the diameter of the whole well, positioned over the center of the well (Fig. 3).

　　　　Masks were printed and positioned on the bottom of the
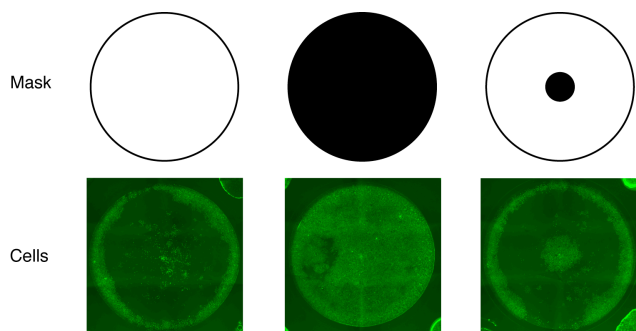
Figure 3. Patterning of cells in monolayers using masks. All wells were treated with DMNPE modified pten targeting siRNA. Viable cells were stained using calcein AM and visualized by fluorescent microscopy. The mask used to irradiate a given well is shown above the stitched micrograph of the well.

culture plates. Cells were plated, and the experiment proceeded identically to the previous description, with the following key difference: irradiation was performed for 20 min, to compensate for light absorption by the transparency. On the sixth day of culture, the mask was removed from the plate bottom, the cells were stained with calcein AM, and the resulting patterns visualized using a fluorescent microscope. The resultant images are shown in Fig. 3.

　　　　In cells treated with DMNPE modified pten-siRNA, and masked with a clear mask, cells were detached (except for the previously described halo effect.) Similarly treated cells with a

100% mask, showed a full well of viable cells, indicating no detachment. This indicates that our masking approach works in the limits. The 20% circular mask showed a circle of cells at the center of the well. Repeat experiments showed a similar pattern (see Supplementary Fig. S5), indicating robust creation of a cell pattern using light activated RNA interference.

　　　　We also examined if we could continuously vary the amount of cells remaining attached by varying the amount of irradiation. Cells were treated with DMNPE modified siRNA targeting pten in a manner similar to the previously described studies. Different wells were irradiated for 0, 2, 4, 6, 8, and 10 min, allowed to culture for six days and the live cells quantitated with calcein AM. Representative wells as well as the signal versus irradiation time are shown in figure 4. They illustrate the variable removal of cells depending on irradiation time.

a)

0 min　　　2 min　　　4 min

6 min　　　8 min　　　10 min

b)
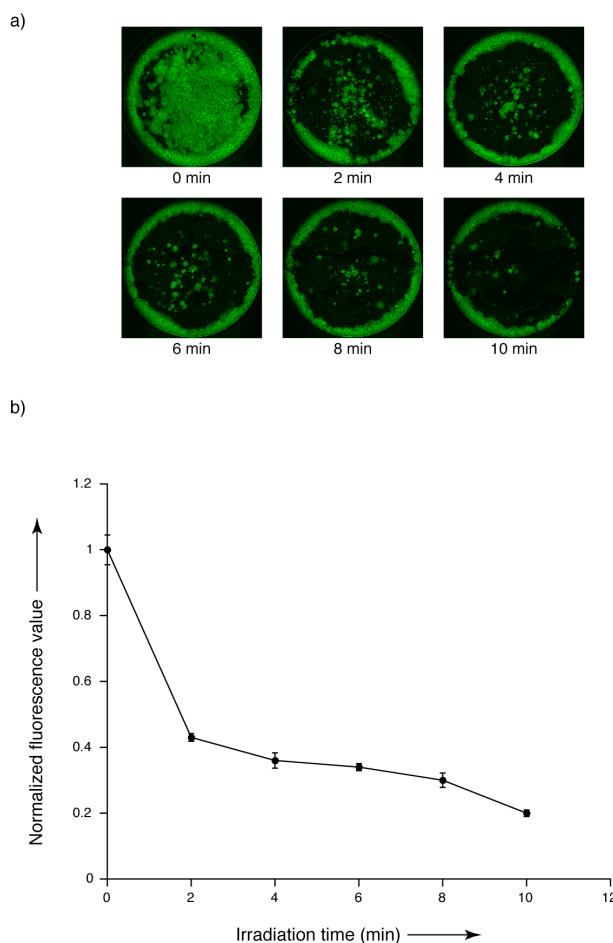
Figure 4. Varying cell density through variation in irradiation time. All wells were treated with DMNPE modified pten targeting siRNA and irradiated for the indicated amount of time after the transfection period. a) Representative whole wells showing live cells labeled with calcein AM. b) Cell density versus well irradiation time.

Conclusions

　　　　In this work, we have shown that cells can be patterned, not by spatially manipulating the surfaces upon which they are grown, but rather by spatially manipulating the biology of the cell

itself, using light activated RNA interference. We believe that this is the first time this approach has been taken on normal cells with unaltered genomes. (Koh and coworkers have also created cell patterns using specifically engineered, light inducible cell lines)[19]. There are unique strengths and weaknesses of our method over the traditional approaches of the past two decades. One of the challenges of the approach as currently manifested is that it requires a low initial confluency of cells to be effective. The detachment of cells due to knockdown of pten by siRNA is most effective when the transfection of cells takes place at low confluency (personal communication from Doi, confirmed by our lab). This may be due to the lower number of interactions with adjacent cells, which makes more densely packed cells more difficult to detach. The result of this is that after irradiation, knockdown and dissociation of cells, the irradiated portions are truly free of cells, but the unirradiated portions are only seeded with cells, that then need to divide to fill the space completely. It is interesting to note that in the case of the 20% circular mask, the cells do not spread to a large extent beyond the limits of the mask edge. This may reveal something about the influence of neighbor cells on the dynamics of cell division.

The unique advantage of spatially manipulating the biology of the cells as opposed to spatially manipulating the culture surface is that the patterning of cells is no longer tied to the specifics of the substrate, which by their nature are unnatural (e.g. gold surfaces, silica surfaces etc.) This increases the relevance of cell patterning for the construction of complex tissues in three dimensions. Why not simply use high powered irradiation to ablate cells in three dimensions? This will result in a mass of necrotic tissue, which may a detrimental effect on a growing three dimensional tissue from a mass of necrotizing cells caused by simple ablation.

We are pursuing the shaping of tissues as Nature herself shapes tissues, both through cell division, and through directed, planned, programmed cell removal. Indeed, the spatial control of *apoptosis* may provide an even more natural way of patterning cells in three dimensions, and may also be amenable to the LARI approach. The spatial manipulation of *biology* as opposed to *surfaces* is a useful lens through which to view the problem of shaping patterns of cells and tissues.

Notes and references

a Division of Pharmaceutical Sciences, School of Pharmacy, University of Missouri, Kansas City.

2464 Charlotte Street, Kansas City, MO 64108-2718 USA

*Correspondence should be directed to: friedmans@umkc.edu

1. F. Brétagnol, A. Valsesia, G. Ceccone, P. Colpo, D. Gilliland, L. Ceriotti, M. Hasiwa and F. Rossi, *Plasma Processes and Polymers*, 2006, 3, 443-455.
2. D. Falconnet, G. Csucs, H. M. Grandin and M. Textor, *Biomaterials*, 2006, 27, 3044-3063.
3. A. Folch and M. Toner, *Annual Reviews in Biomedical Engineering*, 2000, 2, 227-256.
4. A. L. Hook, N. H. Voelcker and H. Thissen, *Acta Biomater*, 2009, 5, 2350-2370.
5. A. Khademhosseini, R. Langer, J. Borenstein and J. P. Vacanti, *Proc Natl Acad Sci U S A*, 2006, 103, 2480-2487.
6. M. Mrksich, L. E. Dike, J. Tien, D. E. Ingber and G. M. Whitesides, *Exp Cell Res*, 1997, 235, 305-313.
7. K. Y. Suh, J. Seong, A. Khademhosseini, P. E. Laibinis and R. Langer, *Biomaterials*, 2004, 25, 557-563.
8. G. M. Whitesides, E. Ostuni, S. Takayama, X. Jiang and D. E. Ingber, *Annu Rev Biomed Eng*, 2001, 3, 335-373.
9. G. Piret, E. Galopin, Y. Coffinier, R. Boukherroub, D. Legrand and C. Slomianny, *Soft Matter*, 2011, 7, 8642-8649.
10. L. Marcon, A. Addad, Y. Coffinier and R. Boukherroub, *Acta Biomater*, 2013, 9, 4585-4591.
11. S. Shah, S. Rangarajan and S. H. Friedman, *Angew. Chem. Int. Edit.*, 2005, 44, 1328-1332.
12. C. Brieke, F. Rohrbach, A. Gottschalk, G. Mayer and A. Heckel, *Angew Chem Int Ed Engl*, 2012, 51, 8446-8476.
13. L. Gardner and A. Deiters, *Curr Opin Chem Biol*, 2012, 16, 292-299.
14. P. K. Jain, S. Shah and S. H. Friedman, *J Am Chem Soc*, 2010.
15. H. M. Lee, D. R. Larson and D. S. Lawrence, *ACS Chem Biol*, 2009, 4, 409-427.
16. D. Puliti, D. Warther, C. Orange, A. Specht and M. Goeldner, *Bioorg Med Chem*, 2011, 19, 1023-1029.
17. S. Shah, P. K. Jain, A. Kala, D. Karunakaran and S. H. Friedman, *Nucleic Acids Res.*, 2009, 37, 4508-4517.
18. S. Mise-Omata, Y. Obata, S. Iwase, N. Mise and T. S. Doi, *Biochem. Bioph. Res. Co.*, 2005, 328, 1034-1402.
19. D. J. Sauers, M. K. Temburni, J. B. Biggins, L. M. Ceo, D. S. Galileo and J. T. Koh, *ACS Chem Biol*, 2010, 5, 313-320.