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14. ABSTRACT The inhibition of tumor angiogenesis has significant potential as a therapeutic modality in the treatment of breast cancer. Delivering small, interfering RNA (siRNA) to activated breast microvascular endothelial cells (MVEC) can decrease the expression of proteins required during tumor angiogenesis and lead to less toxic and more effective breast cancer treatments but is limited by the absence of efficient targeted drug delivery vehicles. Nanogels (NG) composed of cross-linked polyethylene glycol and polyethylenimine (PEG-cl-PEI) were investigated for targeted siRNA delivery to activated breast MVEC. Targeted NG inhibited activated murine breast MVEC growth and vessel-like formation in vitro with little cytotoxicity irrespective of loaded siRNA. This indicates that unmodified and targeted NG inhibit MVEC by some mechanism unrelated to siRNA or detectable cytotoxicity and that first generation NG are insufficient for siRNA delivery. Similar observations delivering nucleoside analogs in other cells were overcome with biodegradable NG. Therefore, we are now assessing the potential of using biodegradable NG as a platform for further development as a targeted siRNA delivery vehicle.						
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INTRODUCTION

Blood vessel recruitment from existing vasculature (angiogenesis) is a critical factor in breast tumor growth and metastasis. Thus, the inhibition of tumor angiogenesis has significant potential as a therapeutic modality in the treatment of breast cancer. Targeting the delivery of small, interfering RNA (siRNA) to activated breast microvascular endothelial cells (MVEC) in order to decrease the expression of proteins critical to breast MVEC during tumor angiogenesis could lead to less toxic and more effective breast cancer treatments but is limited by the absence of efficient targeted drug delivery vehicles (tDVs)¹. **The long term goal of this proposal** is to develop more efficient tDVs for siRNA delivery to breast tumor vasculature. We changed the order of the original SOW in order to initially screen for a drug delivery vehicle that would be an appropriate platform for further development into a tDV (see below).

BODY

Task 2. Determine the extent that each targeted drug delivery vehicle increases the efficacy of therapeutic siRNA against activated breast microvascular endothelial cell proliferation and migration

Isolation of Murine Breast MVEC We first isolated MVEC from the mammary fat pad (MFP/breast) of the Immortomouse™ strain to serve as a model target cell. The Immortomouse strain² carries a temperature-sensitive SV40 Large T antigen under the control of the H-2K^b promoter (activated by interferon- γ [INF- γ]), which can be used to conditionally immortalize isolated primary cells and decrease the number of primary cell isolations. MVEC³ and other cell types^{4,5} isolated from various Immortomouse tissues have also been shown to retain the functional phenotype of the original primary cells after at least 30 passages. Furthermore, these cells can be grown at 33°C in the absence of INF- γ ³) to avoid the effects of INF- γ on the cell surface and cell function⁶

Heterogeneous populations of cells were isolated from the breast tissue of female Immortomice and grown at permissive temperature (33°C) for ~14-20 d before isolation of breast MVEC. Unlike previous studies^{2,4,5,7}, INF- γ was not added during any stage of isolation to avoid possible long term effects on breast MVEC surface and function. Breast MVEC were isolated by FACS double selecting for the TNF- α -induced expression of V-CAM and E-Selectin³. A cobblestone morphology characteristic of vascular endothelial cells (**Fig.1A**) and the formation of capillary-like structures in Matrigel (**Fig.1B**) were also observed. Thus, a homogeneous population of ImmortoMouse breast MVEC could be isolated without INF- γ .

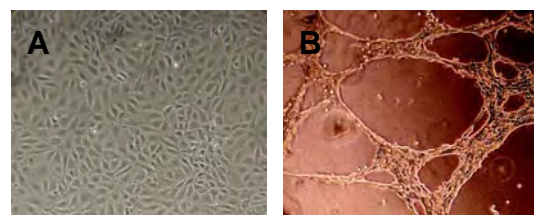


Fig.1 –Light microscopy images of breast MVEC in normal (A) and Matrigel (B) culture conditions

Synthesis and Characterization of Targeted PEG-cl-PEI Nanogels Cross-linked polyethylene glycol/polyethylenimine nanoparticles (“Nanogels”/NG) (**Fig.2**) are promising and novel drug delivery vehicles that have not been used for siRNA delivery or fully optimized for targeted systemic delivery. As such, we wanted to investigate whether NG would be an appropriate platform to develop into a drug delivery vehicle for targeted siRNA delivery to activated breast MVEC and performed Task 2 before Task 1.

Vascular endothelial growth factor receptor 2 (VEGFR2) is expressed on the surface of vascular endothelial cells as well hematopoietic precursors⁸, but downregulated in quiescent adult vasculature⁹. VEGFR2 expression, however, is significantly increased in activated MVEC participating in tumor angiogenesis¹⁰ and anti-VEGFR2 monoclonal antibodies primarily localize to tumor vasculature in a mouse model¹¹. As such, modifying the NG surface with targeting ligands that have a high affinity and specificity for VEGFR2 is expected to increase Nanogel affinity and subsequent transfection efficacy in activated MVEC.

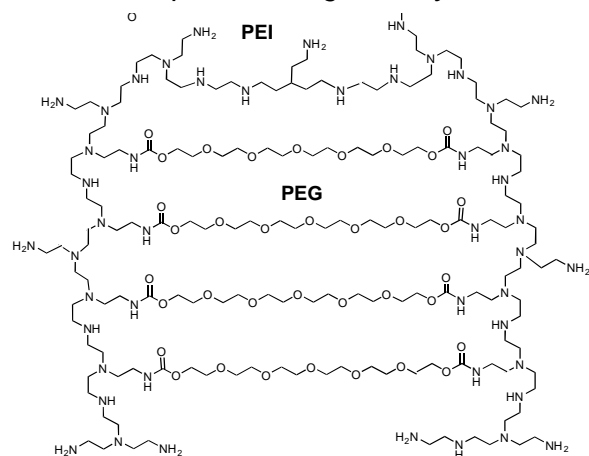


Fig.2 –Basic Nanogel Structure

Conjugating a high affinity, VEGFR2-specific peptide (“V1” = ATWLPPR¹²) to the NG surface is expected to greatly increase Nanogel affinity for the surface of activated MVEC. Nanogels were cross-linked at ~9:1 (PEG/PEI) as this increases the stability of Nanogel formulations in aqueous solution through an increase of hydrophilic PEG chain content¹³. V1 was additionally modified with C-terminal amide groups to ensure stability during systemic delivery¹⁴ and conjugated to the NG surface over a range of surface densities. V1 was attached to free primary amine groups of PEI within the PEI-*c*-PEG NG by N-hydroxysuccinimide (NHS-ester) using a 3.4 kDa linear MAL-polyethylene glycol-NHS (MAL-PEG-NHS) linker (**Fig.3**) to ensure that the V1 is not hindered by the Nanogel surface and able to interact with cell surface VEGFR2. Nanogels were modified with V1 over an initial range of 10, 15, or 20% of Nanogel NH₂ groups / mg NG (~2.3 μmoles NH₂ groups / mg Nanogel).

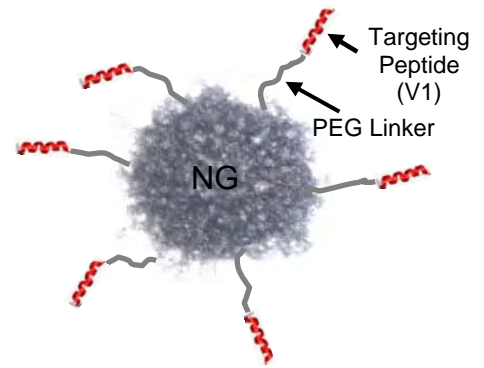


Fig.3 – Targeted Nanogel Structure

Characterization of Targeted Nanogels The degree of peptide substitution was determined for the MAL-PEG-V1 intermediate and each targeted Nanogel construct by the Sakaguchi assay¹⁵ through the presence of arginine in V1. No detectable absorbance was observed with unmodified NG alone and no difference in absorbance was observed between L-Arg and V1 peptide standard curves (data not shown). Predicted and measured arginine concentrations (2 mg NG construct / mL) were in close agreement (**Table 1**). Thus, NG were substituted with V1 peptide at the desired levels.

Table 1 – Comparison of predicted and measured peptide substitution of V1-modified Nanogels

Construct	Pred. [Arg]	Sakaguchi
MAL-PEG(3.4)-V1	0.47 mM	0.38 +/- 0.06 mM
NG (10%)	0.23 mM	0.25 +/- 0.02 mM
NG (15%)	0.28 mM	0.32 +/- 0.05 mM
NG (20%)	0.31 mM	0.36 +/- 0.02 mM

Nanogel Protection of Model siRNA The presence of systemic RNase activity is a major factor in greatly decreasing the plasma half life of siRNA¹⁶. Thus, from a drug delivery standpoint, it is important to form targeted NG-siRNA complexes at N/P ratios that give both maximal siRNA loading and maximal protection from systemic RNase activity. Unmodified NG or NG modified with PEG (3.4 kDa)-V1 at 10, 15, or 20% of total primary amine groups / mg Nanogel were complexed with model siRNA (DNA duplex of 21 nucleotides with two nucleotide 3' overhangs) for 30 min at R/T, then incubated for 1 hr at 37°C in the presence or absence of 8U Turbo DNase. The same concentration of naked dsDNA is completely digested under these conditions. SYBR green 1 was then added to each sample and fluorescence from DNase-treated Nanogel-dsDNA complexes was normalized to fluorescence of the same molar N/P ratio of untreated Nanogel-dsDNA complexes and reported as the mean percentage of treated Nanogel-dsDNA / untreated Nanogel-dsDNA ± propagated SEM (n=3). Unmodified Nanogel protects 90% or greater dsDNA at an N/P ratio of 6:1 or greater (**Fig.4**). A similar trend was observed for Nanogel protection of antisense oligonucleotides¹³. With the exception of NG 20%, targeted Nanogels protect dsDNA to a similar extent as unmodified Nanogels at N/P ratios greater than 8/1 (**Fig.3**). Poor protection by NG 20% suggests a practical limit to Nanogel modification.

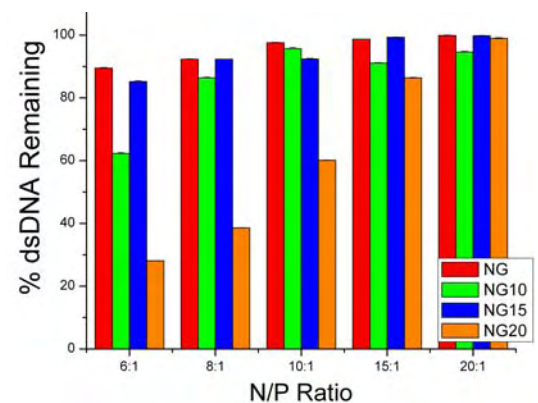


Fig. 4 – Model siRNA protection by unmodified and targeted Nanogels

Dose response of MVEC vessel formation to p73RhoGAP siRNA delivered by unmodified and targeted NGs

siRNA suppression of p73RhoGAP inhibits activated MVEC migration *in vitro* as well as angiogenesis *in vivo*¹⁷. Considering that siRNA suppression is dose-dependent¹⁶, it is expected that targeted NG will deliver a greater amount of p73RhoGAP siRNA to activated MVEC than unmodified NG and consequently inhibit MVEC migration at lower siRNA concentrations. NG were used at the lowest N/P ratio that protects ≥ 95% model siRNA (15/1) (Fig.4). NG20 was not assessed due to its relatively poor ability to protect siRNA (Fig.4). SMARTpool™ p73RhoGAP siRNA (Dharmacon) was used to target p73RhoGAP mRNA and non-targeting SMARTpool™ siRNA (Dharmacon) was used as a control. Subconfluent murine dMVEC were incubated with NG-siRNA complexes in 2D cultures for 2 h, grown for 24h, then transferred to Matrigel to monitor their ability to form vessel-like structures *in vitro*. Dose response curves did exceed 100 nm siRNA to avoid off-targeting reported at siRNA concentrations greater than 100 nm¹⁶.

In contrast to mock-transfected breast MVEC (Fig.5A), 10 nM p73RhoGAP delivered by NG alone (Fig.5C) by NG10 (Fig.5E) or NG15 (Fig.5H) inhibited vessel formation over the range of concentrations (Fig.5). NG alone had the least effect. Similar levels of inhibition, however, were observed between p73RhoGAP and control siRNA for each siRNA concentration (Fig.5A,D,G). Thus, NG alone (to a limited extent) or modified with increasing levels of V1 peptide inhibited the ability of breast MVEC to form vessel-like structures irrespective of the siRNA sequence. This suggested that the NG structures directly affect breast MVEC function.

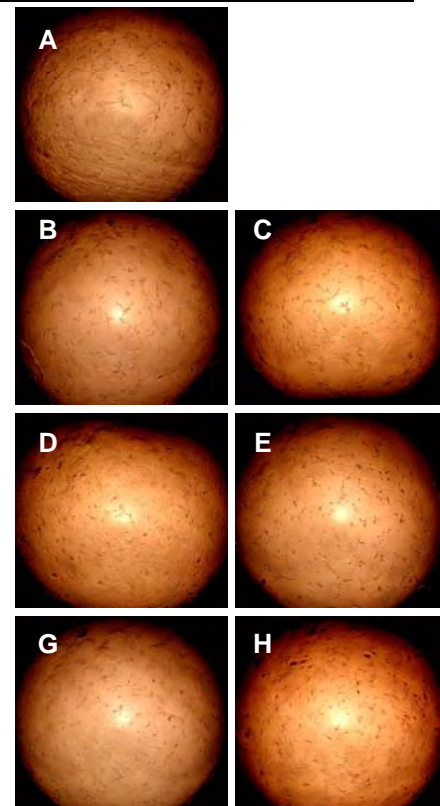


Fig. 5 – Representative response of breast MVEC vessel formation to targeted NG-siRNA delivery

Dose response of breast MVEC proliferation to Kif11 siRNA delivered by unmodified and targeted NG

To address possible direct effects of unmodified and targeted NG on breast MVEC function, breast MVEC were transfected under conditions where both cell growth and cytotoxicity could be monitored. NG were again used at the lowest N/P ratio that protects ≥ 95% model siRNA (15/1) (Fig.4). Kif11 (Eg5) is a motor protein involved in chromosome positioning and bipolar spindle formation during cell mitosis whose reduction causes mitotic arrest¹⁸. SMARTpool™ Kif11 siRNA (Dharmacon) was used to target Kif11 mRNA and non-targeting SMARTpool™ siRNA (Dharmacon) was used as a control.

A slight increase in growth inhibition by Kif11 siRNA over control siRNA was observed for NG alone or NG10 (Fig. 6) that was not observed at the mRNA level (data not shown). A general trend was also observed where an increase in V1 modification resulted in a decrease in breast MVEC growth. The levels of CAM inhibition were similar to levels of growth inhibition, suggesting that growth inhibition was not associated with cytotoxicity. Thus, these results indicate that unmodified and targeted NG inhibit MVEC growth and subsequent vessel-like formation *in vitro* irrespective of loaded siRNA by some mechanism unrelated to detectable cytotoxicity.

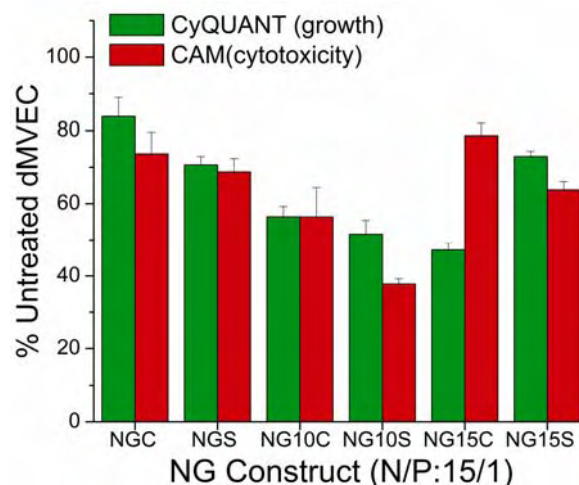


Fig.6 – Representative response of breast MVEC growth and cytotoxicity to targeted NG-siRNA delivery

KEY RESEARCH ACCOMPLISHMENTS

- An experimentally useful and unique murine cell model of breast MVEC was successfully isolated from the Immortomouse™ strain for future targeted nanocarrier delivery studies
- Protocols for the successful isolation of additional cell types from the Immortomouse™ model were established.
- A novel method to assess siRNA degradation within nanocarriers was established
- First generation Nanogels were synthesized and characterized, but found to be insufficient for targeted siRNA delivery to breast MVEC

REPORTABLE OUTCOMES

- Conditionally immortalized murine breast MVEC that are a valuable cell model for correlation with subsequent *in vivo* studies in mouse models of breast cancer were successfully established
- Preliminary data from this grant was used to obtain an NIH R21 (5 R21 EB005683-02) by Dr. Vetro that began in July 2007.
- Preliminary data from this grant will be used in support of a project by Dr. Vetro within an NIH COBRE application in Oct 2007.

CONCLUSION

The inhibition of tumor angiogenesis has significant potential as a therapeutic modality in the treatment of breast cancer. Targeting the delivery of small, interfering RNA (siRNA) to activated breast microvascular endothelial cells (MVEC) in order to decrease the expression of proteins critical to breast MVEC during tumor angiogenesis could lead to less toxic and more effective breast cancer treatments but is limited by the absence of efficient targeted drug delivery vehicles (tDVs)¹. Nanogels hold much promise as targeted siRNA nanocarriers, but first generation Nanogels with stable cross-links were found to be insufficient for delivery to breast MVEC. Given that the first generation NG structure inhibits breast MVEC growth and that similar observations when delivering nucleoside analogs in other cells were overcome with biodegradable NG¹⁹, we are now assessing the potential of using biodegradable NG for targeted siRNA delivery. The targeting ligand was also changed as V1¹² was recently shown to have affinity for purified NRP1 and not purified VEGFR2^{20,21}. Unlike VEGFR2, there is less *in vivo* data to support targeting NRP-1. Therefore, a high affinity peptide (K237) identified by phage display against the interaction of VEGF with VEGFR2²² will be used in place of V1. These studies are currently in progress.

REFERENCES

1. Torchilin, V. P. Drug targeting. *Eur J Pharm Sci* 11 Suppl 2, S81-91 (2000).
2. Jat, P. S., Noble, M. D., Ataliotis, P., Tanaka, Y., Yannoutsos, N., Larsen, L. & Kioussis, D. Direct derivation of conditionally immortal cell lines from an H-2Kb-tsA58 transgenic mouse. *Proc Natl Acad Sci U S A* 88, 5096-100 (1991).
3. Langley, R. R., Ramirez, K. M., Tsan, R. Z., Van Arsdall, M., Nilsson, M. B. & Fidler, I. J. Tissue-specific microvascular endothelial cell lines from H-2K(b)-tsA58 mice for studies of angiogenesis and metastasis. *Cancer Res* 63, 2971-6 (2003).
4. Sweeney, W. E., Jr., Kusner, L., Carlin, C. R., Chang, S., Futey, L., Cotton, C. U., Dell, K. M. & Avner, E. D. Phenotypic analysis of conditionally immortalized cells isolated from the BPK model of ARPKD. *Am J Physiol Cell Physiol* 281, C1695-705 (2001).
5. Takacs-Jarrett, M., Sweeney, W. E., Avner, E. D. & Cotton, C. U. Generation and phenotype of cell lines derived from CF and non-CF mice that carry the H-2K(b)-tsA58 transgene. *Am J Physiol Cell Physiol* 280, C228-36 (2001).
6. Ikeda, H., Old, L. J. & Schreiber, R. D. The roles of IFN gamma in protection against tumor development and cancer immunoediting. *Cytokine Growth Factor Rev* 13, 95-109 (2002).

7. Dory, D., Echchannaoui, H., Letiembre, M., Ferracin, F., Pieters, J., Adachi, Y., Akashi, S., Zimmerli, W. & Landmann, R. Generation and functional characterization of a clonal murine periportal Kupffer cell line from H-2Kb -tsA58 mice. *J Leukoc Biol* 74, 49-59 (2003).
8. Robinson, C. J. & Stringer, S. E. The splice variants of vascular endothelial growth factor (VEGF) and their receptors. *J Cell Sci* 114, 853-65 (2001).
9. Eichmann, A., Corbel, C., Nataf, V., Vaigot, P., Breant, C. & Le Douarin, N. M. Ligand-dependent development of the endothelial and hemopoietic lineages from embryonic mesodermal cells expressing vascular endothelial growth factor receptor 2. *Proc Natl Acad Sci U S A* 94, 5141-6 (1997).
10. St Croix, B., Rago, C., Velculescu, V., Traverso, G., Romans, K. E., Montgomery, E., Lal, A., Riggins, G. J., Lengauer, C., Vogelstein, B. & Kinzler, K. W. Genes expressed in human tumor endothelium. *Science* 289, 1197-202 (2000).
11. Ran, S., Huang, X., Downes, A. & Thorpe, P. E. Evaluation of novel antimouse VEGFR2 antibodies as potential antiangiogenic or vascular targeting agents for tumor therapy. *Neoplasia* 5, 297-307 (2003).
12. Binetruy-Tournaire, R., Demangel, C., Malavaud, B., Vassy, R., Rouyre, S., Kraemer, M., Plouet, J., Derbin, C., Perret, G. & Mazie, J. C. Identification of a peptide blocking vascular endothelial growth factor (VEGF)-mediated angiogenesis. *Embo J* 19, 1525-33 (2000).
13. Vinogradov, S. V., Batrakova, E. V. & Kabanov, A. V. Nanogels for oligonucleotide delivery to the brain. *Bioconjug Chem* 15, 50-60 (2004).
14. Laut, V. M. Pharmacological elements in clinical application of synthetic peptides. *Fundam Clin Pharmacol* 14, 425-42 (2000).
15. Deitch, A. D. An improved Sakaguchi reaction for microspectrophotometric use. *J Histochem Cytochem* 9, 477-83 (1961).
16. Hannon, G. J. & Rossi, J. J. Unlocking the potential of the human genome with RNA interference. *Nature* 431, 371-8 (2004).
17. Su, Z. J., Hahn, C. N., Goodall, G. J., Reck, N. M., Leske, A. F., Davy, A., Kremmidiotis, G., Vadas, M. A. & Gamble, J. R. A vascular cell-restricted RhoGAP, p73RhoGAP, is a key regulator of angiogenesis. *Proc Natl Acad Sci U S A* 101, 12212-7 (2004).
18. Weil, D., Garcon, L., Harper, M., Dumenil, D., Dautry, F. & Kress, M. Targeting the kinesin Eg5 to monitor siRNA transfection in mammalian cells. *Biotechniques* 33, 1244-8 (2002).
19. Kohli, E., Han, H. Y., Zeman, A. D. & Vinogradov, S. V. Formulations of biodegradable Nanogel carriers with 5'-triphosphates of nucleoside analogs that display a reduced cytotoxicity and enhanced drug activity. *J Control Release* 121, 19-27 (2007).
20. Tirand, L., Frochot, C., Vanderesse, R., Thomas, N., Trinquet, E., Pinel, S., Viriot, M. L., Guillemin, F. & Barberi-Heyob, M. A peptide competing with VEGF165 binding on neuropilin-1 mediates targeting of a chlorin-type photosensitizer and potentiates its photodynamic activity in human endothelial cells. *J Control Release* 111, 153-64 (2006).
21. Starzec, A., Vassy, R., Martin, A., Lecouvey, M., Di Benedetto, M., Crepin, M. & Perret, G. Y. Antiangiogenic and antitumor activities of peptide inhibiting the vascular endothelial growth factor binding to neuropilin-1. *Life Sci* (2006).
22. Hetian, L., Ping, A., Shumei, S., Xiaoying, L., Luowen, H., Jian, W., Lin, M., Meisheng, L., Junshan, Y. & Chengchao, S. A novel peptide isolated from a phage display library inhibits tumor growth and metastasis by blocking the binding of vascular endothelial growth factor to its kinase domain receptor. *J Biol Chem* 277, 43137-42 (2002).