AD_____

Award Number: DAMD17-03-1-0545

TITLE: Engineered Autologous Stromal Cells for the Delivery of Kringle 5, A Potent Endothelial Cell Specific Inhibitor, for Anti-Angiogenic Breast Cancer Therapy

PRINCIPAL INVESTIGATOR: Sabrina R. Perri, Ph.D.

CONTRACTING ORGANIZATION: Lady Davis Institute
Montreal, Quebec H3T 1E2
Canada

REPORT DATE: August 2005

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-08-2005	Annual Summary	10 Jul 2004 – 9 Jul 2005

4. TITLE AND SUBTITLE
Engineered Autologous Stromal Cells for the Delivery of Kringle 5, A Potent Endothelial Cell Specific Inhibitor, for Anti-Angiogenic Breast Cancer Therapy

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0545

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Sabrina R. Perri, Ph.D.

E-mail: sabrinaperri@yahoo.com

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Lady Davis Institute
Montreal, Quebec H3T 1E2
Canada

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT: Neoangiogenesis has been strongly correlated with aggressive breast tumor growth and metastasis. The fifth kringle (K5) domain of human plasminogen is distinct from angiostatin (K1-4), and has been shown - on its own - to act as a potent suppressor of angiogenesis. We propose that the K5 domain may serve as a potent angiostatic agent and that it may act as a useful therapeutic transgene within a breast cancer gene therapy strategy. To test this hypothesis, we have developed a K5-expressing retroviral vector, gene-modified murine DA3 mammary cells to produce soluble human K5 protein and characterized the anti-tumor potency of the *de novo* produced K5 peptide *in vivo*. The 381bp K5 domain cDNA was His-tagged at the C terminus and cloned into a bicistronic retroviral vector comprising the enhanced green fluorescent protein reporter (GFP) gene. Upon transfection of the K5 retrovector plasmid into 293GPG retroviral packaging cells, single clones were drug selected and characterized. Stable K5-expressing 293GPG cells were utilized to transduce murine DA3 mammary cancer cells. Gene-modified polyclonal DA3-K5-GFP cells were GFP positive as assessed by flow cytometry analysis and capable of secreting soluble K5 protein as detected by anti-His immunoblot analysis. Upon subcutaneous implantation of one million DA3-K5-GFP cells in immunocompetent BALB/c mice, tumor growth was strongly suppressed as early as 7 days post-implantation ($P = 0.01$ by t test) as compared to DA3 control implanted mice and the anti-tumor effect remained sustained for over 6 months. As a result, the DA3-K5-GFP implanted mice possessed a clear survival advantage with 100% of mice surviving over 6 months as compared to 20% in the control cohort ($P = 0.0002$ by log-rank). Conversely, upon subcutaneous implantation of one million DA3-K5-GFP cells in immunodeficient NOD-SCID mice, tumor growth was delayed, probably due to the anti-angiogenic effect of K5, however tumor suppression was not sustained and all mice had to be sacrificed by 2 months post-implantation. Experiments are underway to characterize which host-derived immune effector cells are essential to induce an anti-tumor effect. We provide compelling evidence that soluble K5 protein expressed by retrovirally-engineered mammary cancer cells possesses potent anti-tumor properties which induces long-term tumor regression in immunocompetent mice. These findings demonstrate for the first time that soluble K5 protein holds promise in breast cancer gene therapy and further supports its potency as an anti-angiogenic agent.

15. SUBJECT TERMS
Human plasminogen kringle 5, gene transfer, immune modulation

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	13	

Table of Contents

INTRODUCTION:

Breast cancer has become a serious public-health concern with more than 4.4 million women living with the disease worldwide (1). Current therapeutic modalities primarily target rapidly dividing malignant cells and include combinations of surgery, radiotherapy and chemotherapy (2;3). It is now an established concept that breast cancer progression and metastasis is dependent upon the formation of new blood vessel growth, a process known as angiogenesis (4;5). Indeed, the urgent need for the development of potent anti-breast therapeutics as well as the importance of angiogenesis in breast cancer growth (4;6) has fueled the identification and characterization of numerous anti-angiogenic agents, aimed at interrupting new vessel formation and ultimately arresting tumor growth. The use of anti-angiogenic agents as therapeutics is an appealing approach over conventional chemotherapeutic drugs since it targets nonmalignant endothelial cells that form the tumor vasculature, indirectly affecting tumor cells and thus minimizes the risk of toxicity. In addition, the problem of drug resistance associated with chemotherapy agents, is avoided since normal endothelial cells are genetically stable unlike tumor cells (7). In this regard, several reports have demonstrated that breast cancer progression and metastasis is suppressed upon systemic administration of angiogenic inhibitors or blockers of angiogenic inducers (8-11). Although angiogenesis inhibition offers several advantages over traditional therapeutic approaches, it is expected to induce a cytostatic effect resulting in tumor stabilization not eradication. Furthermore, single agent anti-angiogenic therapy may lead to a compensatory increase in the production of other angiogenic factors, which may then sustain angiogenesis. Despite these limitations, it is now well recognized that angiostatic agents either singly or in combination with other treatment modalities could offer superior therapeutic benefits than currently attainable (12-18), converting the tumor into a controlled, quiescent chronic disease. Direct delivery of purified recombinant anti-angiogenic proteins however necessitates large quantities and repeated administration of the therapeutic gene product for a prolonged period of time (19;20). Therefore, development of a gene therapy strategy incorporating tumor targeted antiangiogenic transgenes may address some of the pharmacokinetic shortcomings of longterm and constant delivery of antiangiogenic proteins and peptides *in vivo*, a strategy we wish to develop as part of this proposal.

Several inhibitors of angiogenesis exist endogenously as proteolytic cleavage products of larger precursor molecules. Angiostatin, a well characterized anti-angiogenic agent discovered by O'Reilly and colleagues in Folkman's laboratory, was initially identified as an endothelial cell growth inhibitor present in urine and plasma of animals harboring solid tumors (7;21), is an internal cryptic fragment of human plasminogen (Plg) encompassing the first four kringle (K1-4) domains **(Fig. 1*A*).** The K5 domain of Plg has been expressed as a recombinant protein in bacteria and been found to be more potent than K1-4 or any of the Plg kringles expressed individually in inhibiting growth factor stimulated proliferation of endothelial cells *in vitro* (22;23). It has also been demonstrated that kringle domains 1-5 (K1-5) act as more potent endothelial cell inhibitors *in vitro* and are more effective in suppressing fibrosarcoma tumor growth *in vivo* as compared to K1-4 alone (24). Data recently published in *Cancer Research* by our laboratory validates the use of tumor-targeted K5 cDNA expression as an effective therapeutic intervention as part of a cancer gene therapy strategy in an orthotopic brain cancer model (25). We demonstrate that the K5 domain **(Fig. 1*B*)** can serve as a potent angiostatic agent -on its own- to induce *in vivo* tumor regression.

BODY *(Completion of Tasks 1, 2a-b & 3a-c):*

The 381bp K5 domain cDNA - Pro^{447}–Asp^{546} of human plasminogen (InvivoGen, San Diego, CA) was His-tagged at the C terminus and cloned into a bicistronic retroviral vector comprising the enhanced green fluorescent protein reporter (GFP) gene. Upon transfection of the K5 retrovector plasmid into 293GPG retroviral packaging cells, single clones were drug

selected and characterized. Tetracycline-withdrawal from the culture media led to the production of VSVG-typed hK5His-GFP retroviral particles which were subsequently concentrated 100-fold by ultracentrifugation and a viral titer of ~$2.5x10^6$ infectious particles/mL was obtained. Concentrated VSVG-typed hK5His-GFP retroviral particles were utilized to transduce mammary tumor cells. The balb/c-compatible DA/3 murine mammary adenocarcinoma cell line is estrogen independent and serves as a murine model of locally advanced breast cancer (28-31). Following retroviral transduction, polyclonal gene-modified DA/3 cells were assessed for GFP expression by flow cytometry and sorted to obtain a 100% GFP-positive population. To ensure hK5His transgene expression and proper secretion, anti-His immunoblot analysis was performed on conditioned supernatant collected from hK5His transduced murine DA/3 mammary tumor cells and detects a major 15kDa protein consistent with the predicted molecular weight of soluble hK5His **(Fig. 2).**

Stable K5-expressing 293GPG cells were utilized to transduce murine DA3 mammary cancer cells. Gene-modified polyclonal DA3-K5-GFP cells were GFP positive as assessed by flow cytometry analysis and capable of secreting soluble K5 protein as detected by anti-His immunoblot analysis. Upon subcutaneous implantation of one million DA3-K5-GFP cells in immunocompetent BALB/c mice, tumor growth was strongly suppressed as early as 7 days post-implantation ($P = 0.01$ by t test) as compared to DA3 control implanted mice and the anti-tumor effect remained sustained for over 6 months. As a result, the DA3-K5-GFP implanted mice possessed a clear survival advantage with 100% of mice surviving over 6 months as compared to 20% in the control cohort ($P = 0.0002$ by log-rank) **(Fig. 3*A*).**. Conversely, upon subcutaneous implantation of one million DA3-K5-GFP cells in immunodeficient NOD-SCID mice, tumor growth was delayed, probably due to the anti-angiogenic effect of K5, however tumor suppression was not sustained and all mice had to be sacrificed by 2 months post-implantation **(Fig. 3*B*).**. Experiments are underway to characterize which host-derived immune effector cells are essential to induce an anti-tumor effect.

Our preliminary data suggests that soluble K5 monomer modulates the immune system in order to induce tumor regression. We characterized more specifically which immune effector cells are implicated in eliciting an anti-tumor response. To analyze the immune cellular infiltrate that is recruited by soluble K5 *in vivo*, we matrix-embedded K5 monomer-expressing DA/3 cells in Matrigel™ (a semi-solid matrix derived from a murine sarcoma cell line) and implanted the cells subcutaneously. The implants were retrieved, a total cell count was performed with a hemacytometer and the cellular infiltrate was analyzed by staining the single cell suspension with antibodies enabling the identification of different immune subsets by flow cytometry analysis. We have preliminary evidence indicating that DA/3-K5 monomer-containing implants retrieved 3 days post-implantation contain significantly more total cells and a trend toward an increased recruitment of lymphoid (CD3+, CD3+CD4+, CD4+CD25+, CD3+CD8+, NK/NK-T+) and myeloid (CD45+Mac3+, CD45+Gr-1+) cellular subtypes as compared to DA/3 control implants, suggesting that soluble K5 monomer is capable of inducing a potent acute innate inflammatory response, which leads to strong tumor regression. Our data also indicates that once tumor regression is achieved there is decreased recruitment of lymphoid and myeloid cellular subtypes, as was demonstrated 6 days post-implantation **(Fig. 4).**

The sources of all reagents utilized in the study are included in the *Materials & Methods* of the manuscript. Upon manuscript submission, the PI will provide a copy to your office if necessary.

Task 3d *(In progress)*

- ***Effect on Host-Derived Vascular Response:*** We also plan to assess the *in vivo* antiangiogenic potency of DA/3 K5 monomer cells by analyzing the explants from control

and test cohorts at fixed time points (2, 7,14 days) using the following experimental techniques: ***(i)*** explants will be quickly fixed in formalin, paraffin-embedded, sectioned and stained with hematoxylin and eosin followed by analysis by expert pathologist Dr. Daniel Martineau, a close collaborator from Université de Montréal, to decipher further immune-associated mechanistic insights; ***(ii)*** von Willebrand Factor (vWF, Neomarkers) immunostaining (a specific endothelial cell marker) to quantify the section surface area occupied by blood vessels and ***(iii)*** collagenase digestion of Matrigel explants, antibody staining followed by quantification of absolute cell number of CD31+CD45- cells by flow cytometry analysis.

KEY RESEARCH ACCOMPLISHMENTS:

- We genetically engineered balb/c-compatible DA/3 mouse breast carcinoma cells to express K5 and measured their growth *in vivo*. We found that tumor growth was profoundly suppressed by K5 in balb/c mice. Remarkably, the anticancer effect was severely curtailed when the same cells were implanted in immunedefective NOD-SCID mice. This observation suggested that the immune system is playing a role in the tumoricidal effects of K5.
- We have performed further studies which reveal that K5 initiates a robust recruitment of cytotoxic lymphocytes in the tumor microenvironment and that this effect may be due in part on upregulation of inflammatory cytokines (such as IL-1) from K5 stimulated tumor cells (data not shown). Indeed, a recent publication from Abbott Laboratories (26) demonstrates that hypoxic cancer cells translocate GRP78 to the cell membrane which then serves as a ligand for K5. K5-bound GRP78 thereafter initiates an apoptotic cascade of the tumor cell. Taken together, our observation and the recent GRP78:K5 interaction suggests that K5 may initiate a direct apoptotic and inflammatory response in tumor cells as well as a host-targeted antiangiogenic effects.

REPORTABLE OUTCOMES:

MANUSCRIPTS :

1. S. R. Perri, M. François, L. Lejeune, D. Martineau and J. Galipeau. Kringle 5-Engineered Cells Recruit Innate Effector Cells and Suppress Mammary Adenocarcinoma Growth (working title). *(manuscript in preparation).*

2. S. R. Perri, J. Nalbantoglu, B. Annabi, Z. Koty, L. Lejeune, M. François, M. R. Di Falco, R. Béliveau, and J. Galipeau. Plasminogen Kringle 5-Engineered Glioma Cells Block Migration of Tumor-Associated Macrophages and Suppress Tumor Vascularization and Progression. *Accepted in Cancer Research.*

ABSTRACTS:

1. S. R. Perri, M. François, L. Lejeune, D. Martineau and J. Galipeau. Engineered Murine Mammary Cancer Cells Producing Soluble Human Plasminogen Kringle 5 Peptide Arrest Tumor Growth. *This abstract was accepted for poster presentation at the Era of Hope Department of Defense Breast Cancer Research Program held in Philadelphia.*

2. S. R. Perri, M. François, L. Lejeune, D. Martineau and J. Galipeau. Human Plasminogen Kringle 5-Engineered Murine Mammary Cancer Cells Arrest Tumor Growth and Promote Long-Term Survival. Molecular Therapy. May 2005.
This abstract was accepted for poster presentation at the 8th Annual Meeting of the American Society of Gene Therapy (ASGT) held in St. Louis (June 2005).

3. S. R. Perri, J. Nalbantoglu, B. Annabi, Z. Koty, L. Lejeune, M. François, M. R. Di Falco, R. Béliveau, and J. Galipeau. Soluble Human Plasminogen Kringle 5 Domain Acts as a 2-Pronged Anti-Cancer Agent Inhibiting Both Endothelial Cells and Tumor-Associated Macrophages. 2005 Proceedings of the American Association for Cancer Research. April 2005.
This abstract was accepted for poster presentation at the 2005 American Association for Cancer Research Meeting (AACR) held in Anaheim (April 2005).

4. S. R. Perri, J. Nalbantoglu, B. Annabi, Z. Koty, L. Lejeune, M. François, M. R. Di Falco, R. Béliveau, and J. Galipeau. Kringle 5-Engineered Glioma Cells Block Migration of Tumor-Associated Macrophages and Suppress Tumor Progression and Angiogenesis
*This abstract was presented at the **5th Annual McGill Biomedical Graduate Conference** and was **awarded the 1st Poster Prize** (Feb. 2005).*

CONCLUSIONS:

This study focuses on the development of an innovative strategy for the treatment of advanced refractory breast cancer and exploits the use of a potent endogenous soluble protein to inhibit angiogenesis and stimulate an antibreast cancer immune response. To date, there are no studies describing the immune effects of K5 for the treatment of cancer. Concepts and biopharmaceuticals developed in this proposal will serve as stepping stones for the development of therapeutics for Phase I/II clinical trials in breast cancer and other advanced malignancies.

REFERENCES:

1. Veronesi U, Boyle P, Goldhirsch A, Orecchia R, Viale G. Breast Cancer. *The lancet* 2005;365:1727-41.

2. Holland EC. Glioblastoma multiforme: the terminator. *Proc.Natl.Acad.Sci.U.S.A* 2000;97:6242-4.

3. Bello L, Giussani C, Carrabba G, Pluderi M, Costa F, & Bikfalvi A. Angiogenesis and Invasion in Gliomas. In: Kirsch M, Black PM. Norwell: Kluwer, 2004:263-84.

4. Folkman J. Tumor angiogenesis: therapeutic implications. *N.Engl.J.Med.* 1971;285:1182-6.

5. Folkman J. What is the evidence that tumors are angiogenesis dependent? *Journal of the National Cancer Institute* 1990;82:4-6.

6. Maher EA, Furnari FB, Bachoo RM, Rowitch DH, Louis DN, Cavenee WK et al. Malignant glioma: genetics and biology of a grave matter. *Genes Dev.* 2001;15:1311-33.

7. O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M et al. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* 1994;79:315-28.

8. Gasparini G. Clinical significance of determination of surrogate markers of angiogenesis in breast cancer. *Crit Rev.Oncol.Hematol.* 2001;37:97-114.

9. Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS et al. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. *Nature* 1993;362:841-4.

10. Griscelli F, Li H, Bennaceur-Griscelli A, Soria J, Opolon P, Soria C et al. Angiostatin gene transfer: inhibition of tumor growth in vivo by blockage of endothelial cell proliferation associated with a mitosis arrest. *Proc.Natl.Acad.Sci.U.S.A* 1998;95:6367-72.

11. Rosen L. Antiangiogenic strategies and agents in clinical trials. *Oncologist.* 2000;5 Suppl 1:20-7.

12. Bello L, Carrabba G, Giussani C, Lucini V, Cerutti F, Scaglione F et al. Low-dose chemotherapy combined with an antiangiogenic drug reduces human glioma growth in vivo. *Cancer Res.* 2001;61:7501-6.

13. Bello L, Giussani C, Carrabba G, Pluderi M, Lucini V, Pannacci M et al. Suppression of malignant glioma recurrence in a newly developed animal model by endogenous inhibitors. *Clin.Cancer Res.* 2002;8:3539-48.

14. Gorski DH, Beckett MA, Jaskowiak NT, Calvin DP, Mauceri HJ, Salloum RM et al. Blockage of the vascular endothelial growth factor stress response increases the antitumor effects of ionizing radiation. *Cancer Res.* 1999;59:3374-8.

15. Griscelli F, Li H, Cheong C, Opolon P, Bennaceur-Griscelli A, Vassal G et al. Combined effects of radiotherapy and angiostatin gene therapy in glioma tumor model. *Proc.Natl.Acad.Sci.U.S.A* 2000;97:6698-703.

16. Hwu WJ, Raizer J, Panageas KS, Lis E. Treatment of metastatic melanoma in the brain with temozolomide and thalidomide. *Lancet Oncol.* 2001;2:634-5.

17. Li L, Rojiani A, Siemann DW. Targeting the tumor vasculature with combretastatin A-4 disodium phosphate: effects on radiation therapy. *Int.J.Radiat.Oncol.Biol.Phys.* 1998;42:899-903.

18. Mauceri HJ, Hanna NN, Beckett MA, Gorski DH, Staba MJ, Stellato KA et al. Combined effects of angiostatin and ionizing radiation in antitumour therapy. *Nature* 1998;394:287-91.

19. O'Reilly MS, Holmgren L, Chen C, Folkman J. Angiostatin induces and sustains dormancy of human primary tumors in mice. *Nat.Med.* 1996;2:689-92.

20. O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS et al. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell* 1997;88:277-85.

21. Kirsch M, Strasser J, Allende R, Bello L, Zhang J, Black PM. Angiostatin suppresses malignant glioma growth in vivo. *Cancer Res.* 1998;58:4654-9.

22. Cao Y, Chen A, An SS, Ji RW, Davidson D, Llinas M. Kringle 5 of plasminogen is a novel inhibitor of endothelial cell growth. *J.Biol.Chem.* 1997;272:22924-8.

23. Cao Y, Ji RW, Davidson D, Schaller J, Marti D, Sohndel S et al. Kringle domains of human angiostatin. Characterization of the anti-proliferative activity on endothelial cells. *J.Biol.Chem.* 1996;271:29461-7.

24. Cao R, Wu HL, Veitonmaki N, Linden P, Farnebo J, Shi GY et al. Suppression of angiogenesis and tumor growth by the inhibitor K1-5 generated by plasmin-mediated proteolysis. *Proc.Natl.Acad.Sci.U.S.A* 1999;96:5728-33.

25. Perri, SR, Nalbantoglu, J, Annabi, B, Koty, Z., Lejeune, L, Francois, M, Di Falco, M, Beliveau, R, and Galipeau, J. Plasminogen Kringle 5-Engineered Glioma Cells Block Migration of Tumor-Associated Macrophages and Suppress Tumor Vascularization and Progression. Cancer Research . 2005.

26. Davidson DJ, Haskell C, Majest S, Kherzai A, Egan DA, Walter KA et al. Kringle 5 of human plasminogen induces apoptosis of endothelial and tumor cells through surface-expressed glucose-regulated protein 78. *Cancer Res.* 2005;65:4663-72.

APPENDIX

Figure 1:

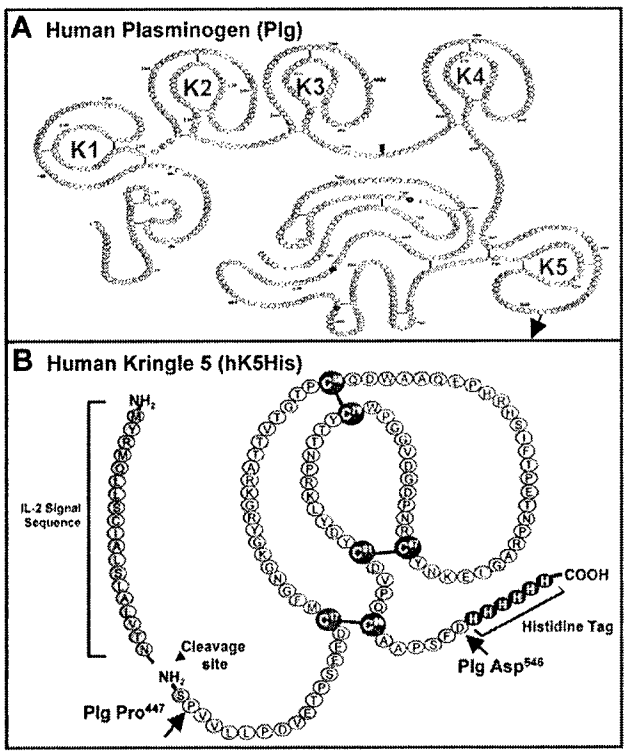

Fig. 1. Schematic illustration of human Plg and its kringle domains. (*A*) Plg is composed of 791 amino acids with a single *N*-linked glycosylation at Asn^{289} and a single *O*-linked glycosylation at Thr^{346}. Angiostatin, a well known inhibitor of angiogenesis, encompasses the first three to four kringle structures of Plg. This is a modified schematic courtesy of Dr. M. R. Llinás and co-workers, Carnegie Mellon University, Pittsburgh, PA *(www.chem.cmu.edu/groups/Llinas/res/structure/hpk.html)*. (*B*) Human kringle 5 (hK5) consists of the last cryptic fragment of Plg (Cys^{462}-Cys^{541}) and is composed of 80 amino acid residues with 3 distinct disulfide bonds.

Figure 2:

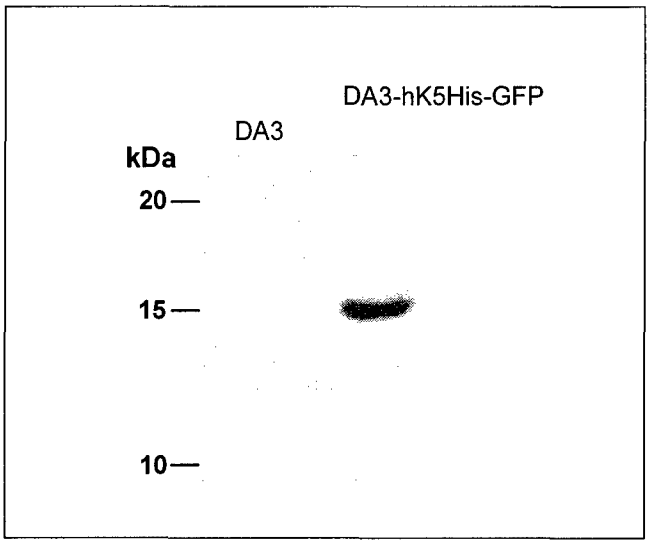

Fig. 2. Anti-His immunoblot analysis reveals functional secretion of human kringle 5 protein migrating at ~15kDa.

Figure 3:

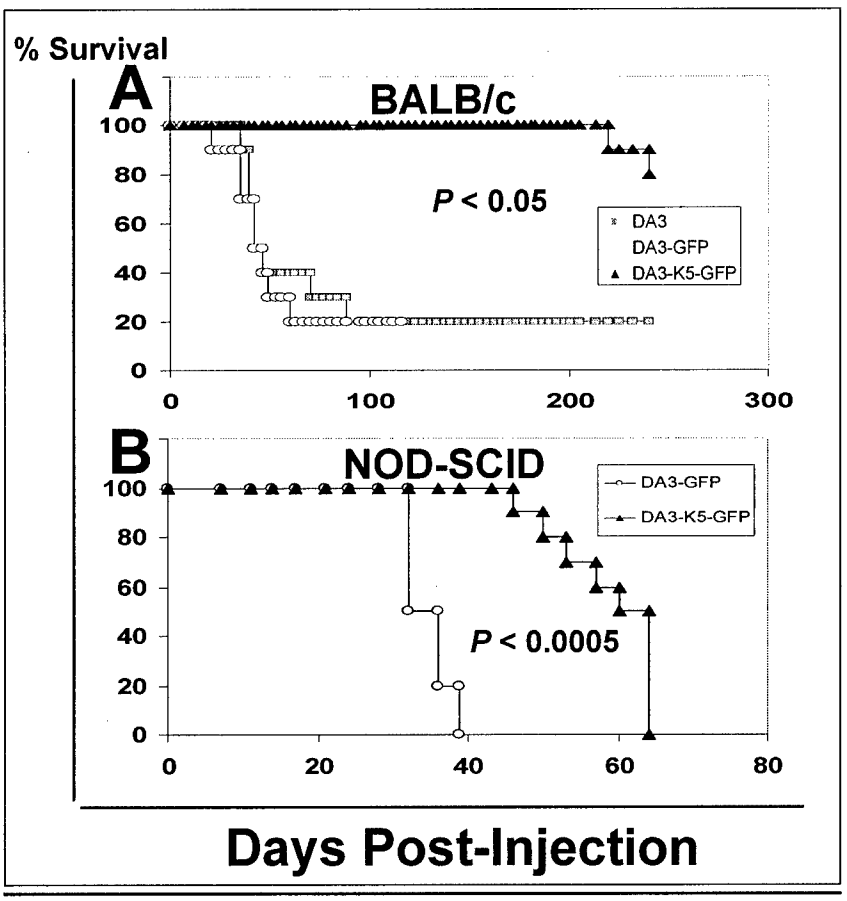

Fig. 3. Kaplan-Meier survival curves for subcutaneous implantation in ***(A)*** immunocompetent Balb/c mice and ***(B)*** immunodeficient NOD-SCID mice.

Figure 4:

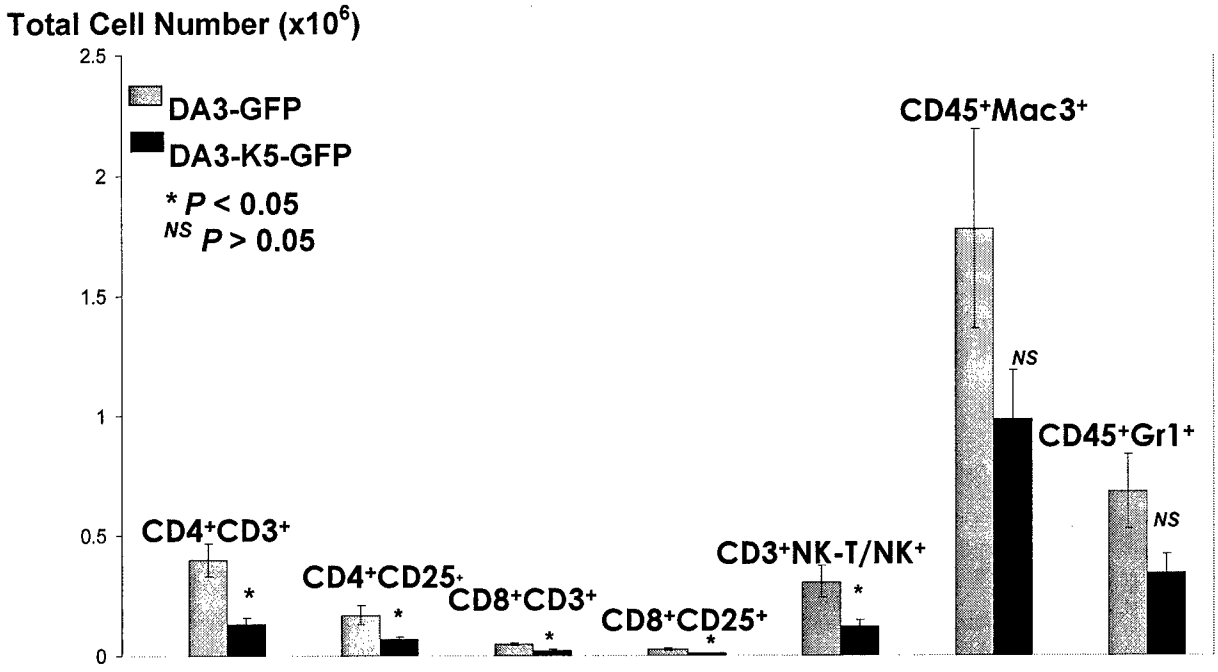

Fig. 4. Matrigel Immune Cellular Infiltrate Analysis. At 6 days post-implantation, once tumor regression is achieved there is decreased recruitment of lymphoid and myeloid cellular subtypes.