

AD_____

Award Number: DAMD17-99-1-9433

TITLE: Anti-Angiogenesis by a Novel VEGF-Intrakine Strategy for Breast Cancer Therapy

PRINCIPAL INVESTIGATOR: Yurong Yang Wheeler
Doctor David Sane

CONTRACTING ORGANIZATION: Wake Forest University
School of Medicine
Winston-Salem, North Carolina 27157

REPORT DATE: July 2001

TYPE OF REPORT: Annual Summary

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE July 2001	3. REPORT TYPE AND DATES COVERED Annual Summary (1 Jul 00 - 30 Jun 01)
----------------------------------	-----------------------------	---

4. TITLE AND SUBTITLE Anti-Angiogenesis by a Novel VEGF-Intrakine Strategy for Breast Cancer Therapy	5. FUNDING NUMBERS DAMD17-99-1-9433
---	--

6. AUTHOR(S)
Yurong Yang Wheeler
Doctor David Sane

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Wake Forest University
School of Medicine
Winston-Salem, North Carolina 27157
E-Mail: yyang@wfubmc.edu

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. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color photos

20011127 028

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

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Angiogenesis plays a pivotal role in tumor growth and metastasis. VEGF, an endothelial specific mitogen and an angiogenesis inducer in vivo, is one of the most important tumor angiogenesis growth factor. KDR, a VEGF receptor, appears to be the major transducer of VEGF signals in endothelial cells. A tethered intracellular antibody ("intrabody") strategy has been used successfully for both phenotypic and functional knockouts of target molecules. In this study, we have targeted a KDR single chain antibody (scFv) p3S5 to the endoplasmic reticulum (ER) using a c-terminal endoplasmic retention signal (KDEL). We hypothesized that the tethered KDR intrabody would bind newly synthesized KDR and block its transport to the surface of endothelial cells, thereby inhibiting VEGF-induced proliferation. The tethered intrabody significantly reduced KDR expression (from $82.5 \pm 12.5\%$ to $27.9 \pm 13.6\%$, $P < 0.005$) in successfully transfected cells. These results demonstrate the potential for using an intrabody strategy to block angiogenesis.

14. SUBJECT TERMS
breast cancer, intrabody, anti-angiogenesis, vascular endothelial growth factor (VEGF)

15. NUMBER OF PAGES
14

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT
Unclassified

18. SECURITY CLASSIFICATION OF THIS PAGE
Unclassified

19. SECURITY CLASSIFICATION OF ABSTRACT
Unclassified

20. LIMITATION OF ABSTRACT
Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

___ Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

___ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

X In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

X For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

<i>Lyg Wheeler</i>	<i>7/5/01</i>
<i>David Sane</i>	<i>7/3/01</i>
PI - Signature	Date

Table of Contents

Cover.....	1
SF 298.....	2
Foreword.....	3
Table of Contents.....	4
Introduction.....	5
Body.....	5-6
Key Research Accomplishments.....	7
Reportable Outcomes.....	7
Conclusions.....	7
References.....	7-8
Appendices.....	9-13

(5). Introduction:

Tumor angiogenesis plays an important role in breast cancer growth and metastasis. Vascular endothelial growth factor (VEGF) stimulates the proliferation of endothelial cells after binding to its receptor (VEGF-R) on cell surfaces, and is a key factor in tumor angiogenesis. **The subject and scope of this research** involves generating various expression vectors for the VEGF-intrakine/intrabody and determining the effects of VEGF-intrakine/intrabody on the VEGF-R expression on endothelial cell surfaces, and evaluating the anti-angiogenesis and anti-tumor activity of this intrakine/intrabody. **The purpose of this study** is to inactivate VEGF-R in vascular endothelial cells by using this intrakine/intrabody strategy and thereby, preventing endothelial cell proliferation, which may lay the groundwork for the development of a novel approach for breast cancer therapy.

(6). Body:

Various VEGF-intrabody expression vectors have been generated by using series PCRs (Fig.1). The original plasmid that contains single chain antibody scFv p3S5 was obtained from Imclone Systems Incorporated (New York, NY). The scFv p3S5 cDNA was amplified by two PCRs with the sense primers (P1:5'-GCTCCCAGATGGG TCCTGTCCCAGGTGAAACTGCAGGAGT CA-3' and P2:5'-TTTGAATTCATGGAA CATCTGTGGTTCTTCTTCT CCTGGTGG CAGCTCCCAGATG GGTCC TGT CC-3'), and antisense primer (5'-TTTCTAGAGGATCC TTAC GCCCGTTTTATTCCAA-3'). An HA tag sequence (YPYDVPDYA) was linked to the scFv gene by a PCR reaction with the sense primer (P1 and P2), and antisense primer (5'-TTTTCTAGAGGATCCTTAAGCATAATCTGGAACATCATATGGATACGCCCGTT TTATTTCCAA-3'). The scFv p3S5 gene tagged with HA was then linked with an ER retention signal (SEKDEL) by a PCR reaction with sense primer (P2) and antisense primer (5'-TTTTCTAGAGGATCCTTACAGCTCGTCCTTCTCGCTAG CATAATCTGGAACATCATA-3'). These DNA fragments were digested with BamHI and EcoRI, and cloned into the expression vector pIRES2-EGFP (Clontech). All of the constructs were identified by restriction enzyme digestion and confirmed by DNA sequencing (Sequencing Core Facility of Wake Forest University School of Medicine).

In order to determine the expression and intracellular localization of these intrabody vectors, these expression vectors were transfected into human umbilical vein endothelial cells using Lipofectamine reagent (Gibco BRL). The procedure followed was "Gibco transient or stable transfection of adherent cells protocol". In brief, in a 35 mm tissue culture plate, 3×10^5 HUVECs were seeded and incubated at 37°C, 5% CO₂ incubator overnight. 2 µg of plasmid DNA and 7 µl of lipofectamine reagent were diluted into 100ul OPTI-MEM I Reduced Serum Medium (Gibco BRL), respectively. The two solutions were mixed and incubated at room temperature for 30 minutes. Following incubation, 0.8ml OPTI-MEM I Reduced Serum Medium was added to the mixture. Then, the final solution was added to the 35 mm HUVECs plate and incubated at 37°C, 5% CO₂ in an incubator for 5 hours. EGM-2 complete medium (0.8 ml) was added at the

last hour of incubation. Following incubation, fresh EGM-2 medium was added to the HUVECs and the cells were incubated in a 37°C, 5% CO₂ incubator. In order to determine the localization of the modified p3S5, immunofluorescent staining was performed using an antibody to the HA tag. Figure 2 demonstrates that cytoplasmic ER staining pattern was observed in the p3S5-HAK transfected HUVECs. The expression of EGFP could be detected in transfected cells using fluorescence microscopy (Figure 2)... Cells that did not express EGFP had no evidence of immunofluorescence with anti-HA (Fig 2), demonstrating specificity of the antibody and the lack of secretion of the intrabody with rebinding to neighboring cells. Cells transfected with the vector expressed EGFP, but did not exhibit immunofluorescence with anti-HA (Fig 2).

To examine the expression of modified p3S5-intrabody further, HUVECs were transfected with either pIRES2-EGFP control or p3S5-HAK and 48 hours later, the cell lysates and concentrated culture medium were immunoblotted with an anti-HA antibody. A 30 kDa protein band corresponding to p3S5 was found exclusively in the cell lysate (Fig 3, lane 3) and not in the culture medium of p3S5-HAK transfected cells (Fig. 3, lane 4). As expected, no immunoreactivity was found in control vector transfected cell lysates (Fig. 3, lane 1) or culture medium (Fig 3, lane 2).

To determine the effects of the modified p3S5-intrabody on KDR expression, HUVECs were transfected with pIRES2-EGFP (control vector), p3S5-HA, or p3S5-HAK, and 48 hour later, the cell surface expression of KDR was examined using flow cytometry (Figure 4). Two-color analysis was performed, with the results summarized in Table 1. Whereas $82.5 \pm 12.5\%$ of cells transfected with pIRES2-EGFP expressed KDR, only $27.9 \pm 13.6\%$ of cells transfected with p3S5-HAK expressed KDR ($p < 0.01$). Transfection with the p3S5-HA vector without the KDEL tag was not effective in suppressing KDR expression with $78.6 \pm 10.7\%$ of the EGFP-expressing cells having detectable KDR ($p > 0.1$). Even when all cells (GFP+ and GFP-) were included in the analysis, p3S5-HAK significantly reduced KDR expression ($p < 0.01$).

To examine the effects of the modified p3S5-intrabody on HUVECs proliferation, HUVECs were transfected with p3S5-HAK or pIRES2-EGFP control. After 48 hours in culture, they were sorted based on EGFP expression into transfected and non-transfected groups, then treated with VEGF₁₆₅ at 15 ng/ml for 30 hours. A [³H] thymidine incorporation assay was performed on these cells. The proliferation rate of the cells that had been transfected with p3S5-HAK was significantly lower than those from the same experiment that were not-transfected ($p < 0.005$) (Fig. 5). Furthermore, there was no significant difference between non-transfected cells in the experimental group and the control vector groups. Thus, only the cells that expressed the p3S5-HAK construct had a significantly reduced response to VEGF₁₆₅.

(7). Key Research Accomplishments:

- Various intrabody expression vectors have been generated and expressed by HUVECs.
- Intrabody significantly decreased HUVECs surface expression of KDR, a VEGF receptor.
- Intrabody significantly inhibited HUVECs proliferation rate.

(8). Reportable Outcomes:

Presentation:

Journal Club, Cancer Biology Department of Wake Forest University School of Medicine: 03/2001.

Poster:

4th Annual American Society of Gene Therapy Meeting, Seattle, WA: 05/2001.

Paper submitted:

Blood: 04/2001.

(9). Conclusions:

Various VEGF-intrabody expression vectors have been generated and successfully transfected into, and expressed by HUVECs. The intrabody has significantly decreased HUVECs surface expression of VEGF receptor, KDR. And they also significantly inhibited HUVECs proliferation rate in vitro. Further studies will be directed to investigate the mechanisms of the intrabody as well as some antitumor effects of intrabody in vivo.

(10). References:

- (1). Plate KH, Breier G, Weich HA, Risau W. Vascular endothelial growth factor is a potential tumor angiogenesis factor in human glioma in vivo. *Nature*. 1992; 359:845-848.
- (2). Bu GJ, Rennke S, Geuze HJ. ERD2 proteins mediate ER retention of the HNEL signal of LRP's receptor associated protein (RAP). *J. Cell Sci*. 1997;110:65-73.
- (3). Townsley FM, Wilson DW, Pelham HRB. Mutational analysis of the human KDEL receptor: distinct structural requirements for Golgi retention, ligand binding and retrograde transport. *EMBO J*. 1993;12:2821-2829.

(4). Lewis MJ, Pelham H R B. Sequence of a second human KDEL receptor. J. Mol. Biol. 1992;226:913-916.

(5). Lewis MJ, Pelham HRB. A human homolog of the yeast HDEL receptor. Nature. 1990;348:162-163.

(6). Marasco WA, Haseltine WA, Chen S. Design, intracellular expression, and activity of a human anti-human immunodeficiency virus type 1 gp120 single-chain antibody. Proc. Natl. Acad. Sci, USA 1993;90:7889-7893.

(11). Appendices:

None.

(12). Final Reports:

One abstract has been accepted by the 4th Annual American Society of Gene Therapy Meeting (05/2001) and published on *Molecular Therapy* (05/2001).

One paper has been submitted to *Blood* (04/2001).

Principle investigator (Yurong Y. Wheeler) has received student payment from this award of \$1, 292.01/month for the last one year.

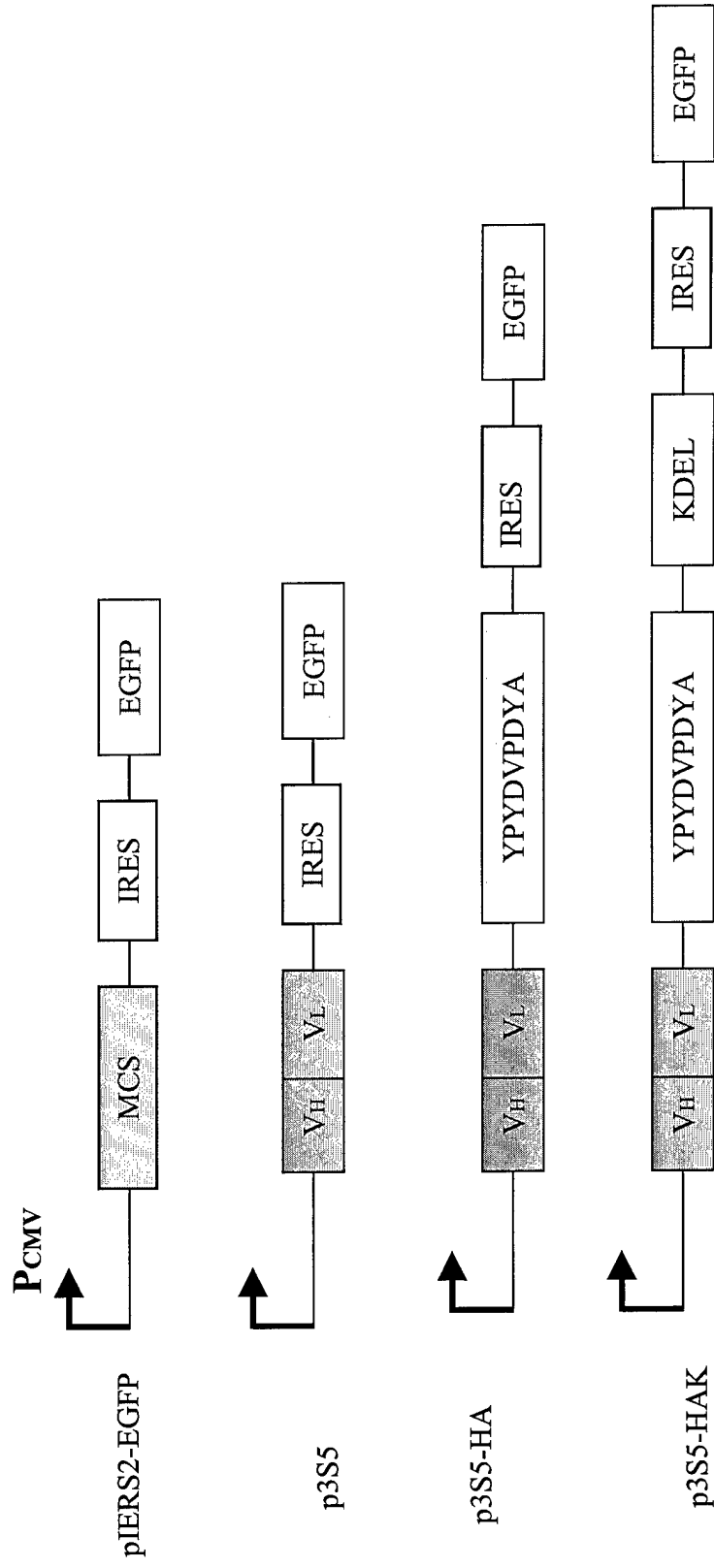


Fig. 1

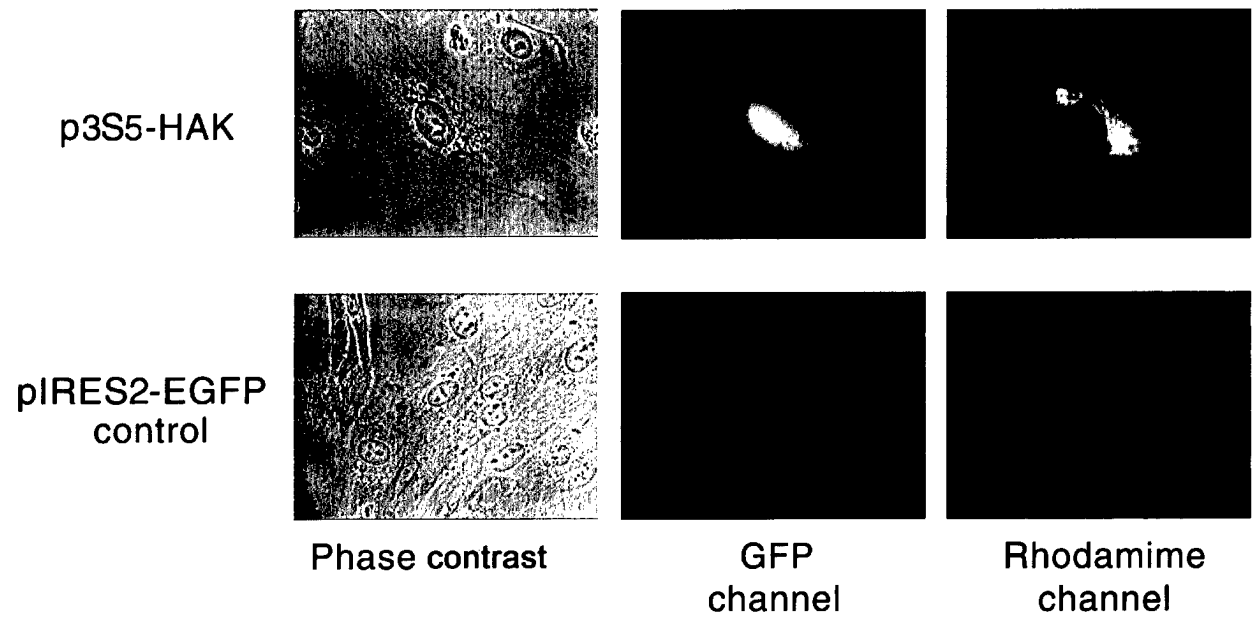


Fig. 2

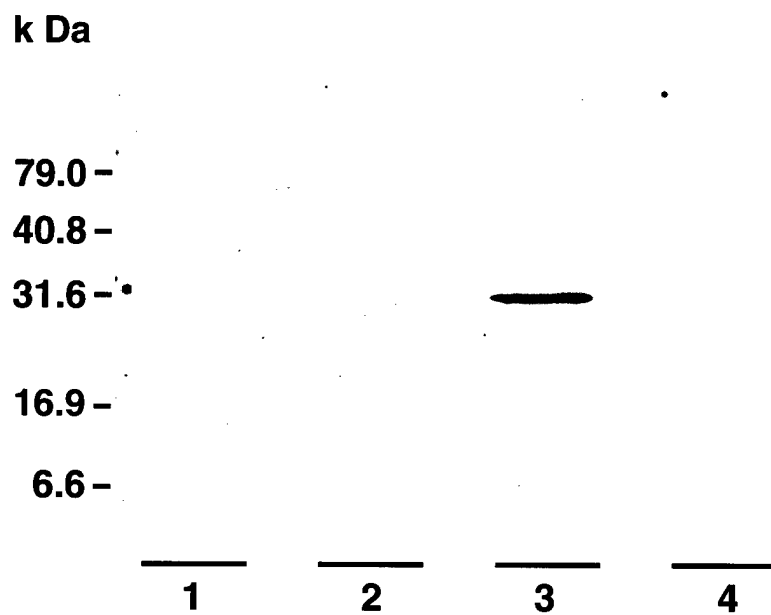
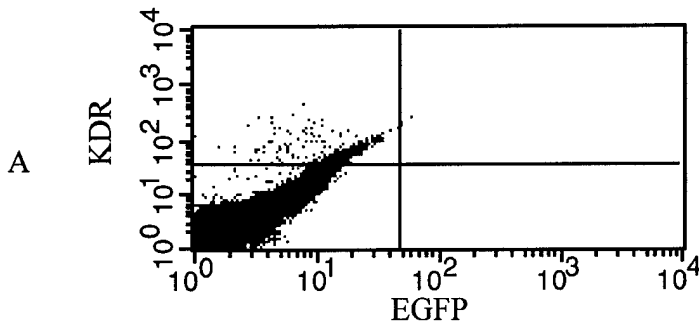
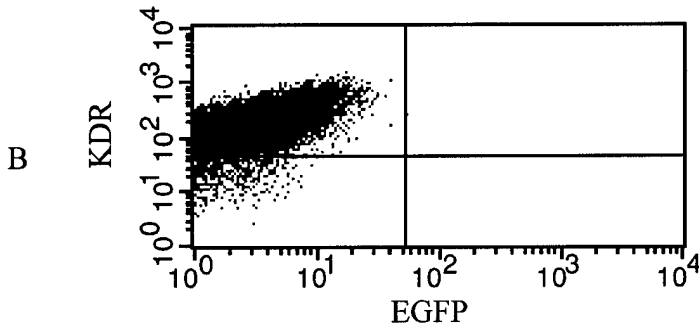


Fig.3

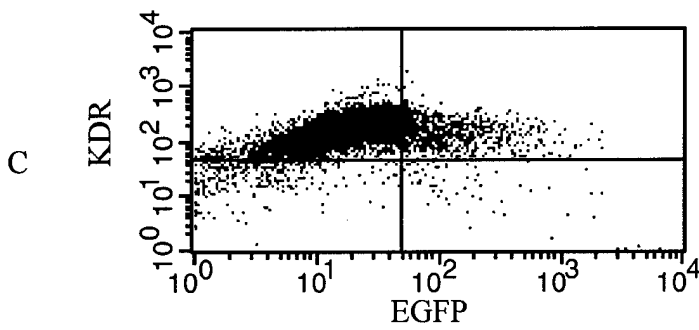
Fig. 4



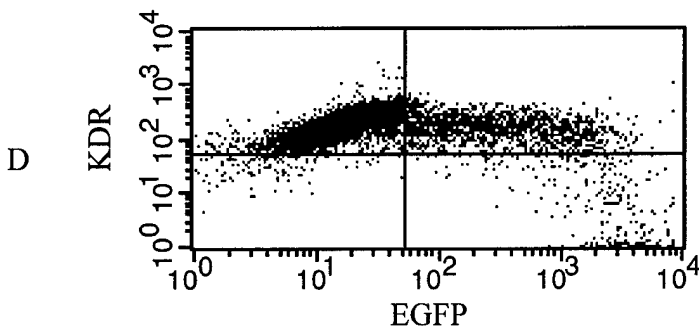
<u>Quad % Gated</u>	
UL	2.85
UR	0.01
LL	97.14
LR	0.00



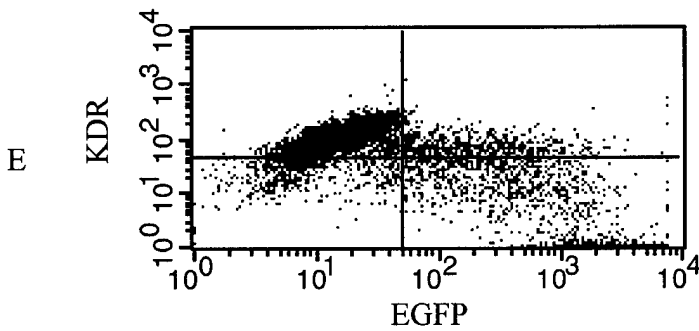
<u>Quad % Gated</u>	
UL	96.56
UR	0.01
LL	3.43
LR	0.00



<u>Quad % Gated</u>	
UL	89.34
UR	7.21
LL	2.92
LR	0.53



<u>Quad % Gated</u>	
UL	82.04
UR	14.11
LL	1.10
LR	2.75



<u>Quad % Gated</u>	
UL	77.23
UR	6.02
LL	5.69
LR	11.05

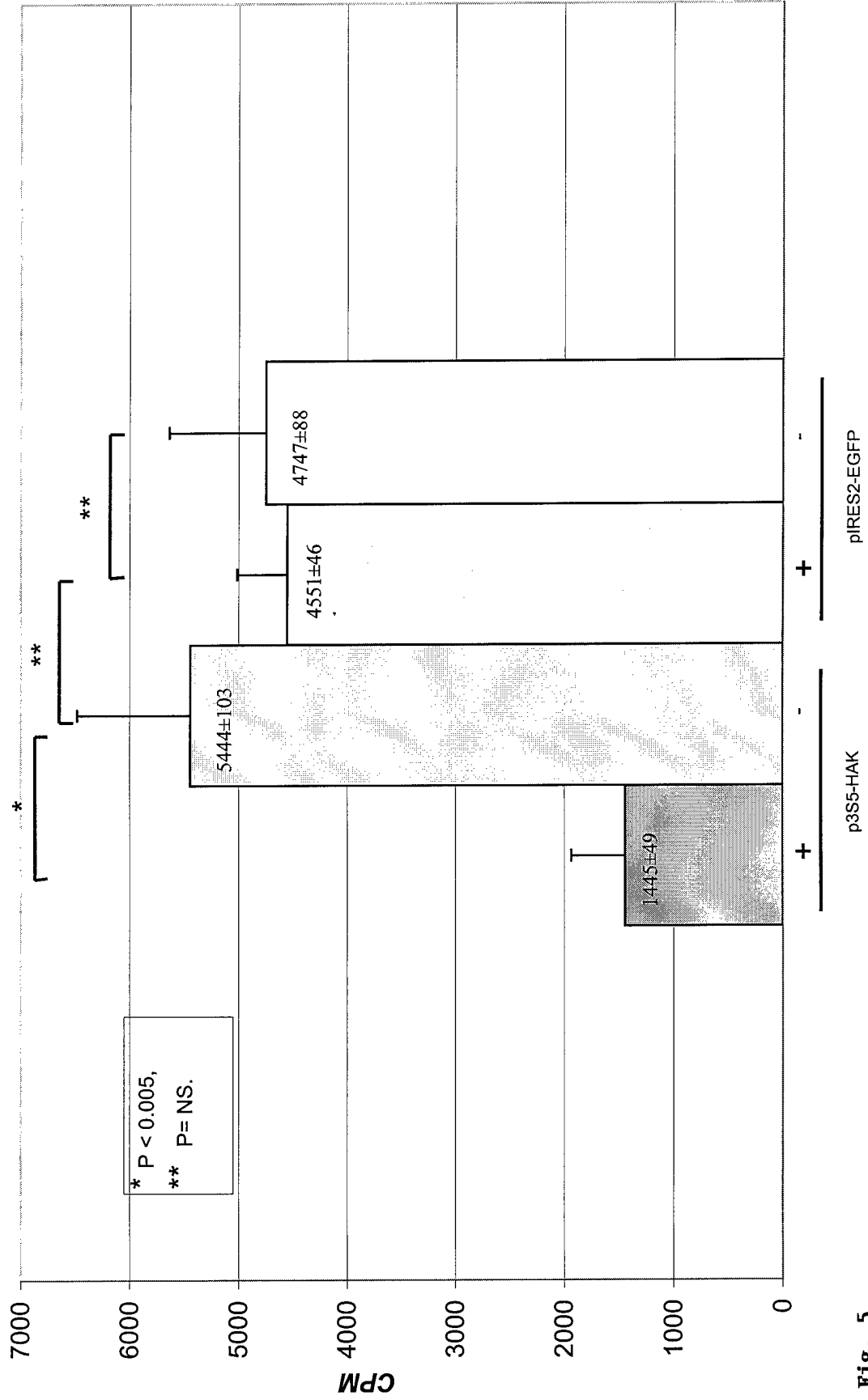


Fig. 5

Table 1.

Analysis of flow cytometry assay of surface KDR expression on p3S5-HA, p3S5-HAK and control transfected HUVECs

Transfected plasmid	Transfection percentage	% KDR positive	
		all cells	transfected cells
pIRES2-EGFP	5.1 ± 2.2	92.5 ± 3.7	(1) 82.5 ± 12.5
p3S5-HA	9.8 ± 6.9	94.1 ± 2.3	(2) 78.6 ± 10.7
p3S5-HAK	13.3 ± 3.1	85.3 ± 1.1	(3) 27.9 ± 13.6

p-values (student t test):

(1) vs (3) $p < 0.01$

(1) vs (2) $p > 0.1$

(2) vs (3) $p < 0.01$