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PRINCIPAL INVESTIGATOR: Sebastian Bredow, Ph,D.

CONTRACTING ORGANIZATION: Lovelace Biomedical and Environmental Research Institute
Albuquerque, NM 87185

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Sebastian Bredow, Ph,D.

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Lovelace Biomedical and Environmental Research Institute
Albuquerque, NM 87185

E-Mail: sbredow@lrri.org

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13. ABSTRACT (Maximum 200 Words)
We had hypothesized that transcriptional regulation of Vascular Endothelial Growth Factor (VEGF) in breast cancer could be different from other types of cancer and tissues: an understanding of how the gene is regulated in this disease could be used as a baseline for developing improved treatment regimens. We have identified promoter elements and transcription factors that contribute to enhanced expression VEGF in breast cancer and other non-mammary cell lines. Under normoxic conditions a drop of ≥ 40% in transcriptional activity could be observed in most cell lines when sequences upstream of the hypoxia-regulatory element (HRE) between positions -1175 and -1010 were deleted. Sequences downstream of the HRE between positions -900 and -790 modulate promoter activity in a more cell type-specific manner; a further reduction in promoter activity by 30% was observed, while deletion of sequences between positions -790 and -700 restores promoter activity in some, but not all cell lines, indicating differences in transcriptional regulation among tissues and within the same cell type. Electrophoretic mobility shift assays (EMSA) identified hence to unknown potentially functional binding sites upstream of the HRE for transcription factors AP-1 and Sp1 between positions -1125 and -1115, and -1098 and -1086, respectively.

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INTRODUCTION

Expression of vascular endothelial growth factor (VEGF) is elevated in malignant cells, and clinical studies correlate increased levels of the protein with poor prognosis in breast cancer (1). Currently little is known about the transcriptional activation of the gene in this particular disease. We hypothesized that regulation of VEGF at the transcriptional level could be different from other types of cancer. Experiments were designed to identify elements in the wild-type (wt) VEGF promoter between positions –1200 and +50 that are responsible for increased transcription in breast cancer cells, and to characterize the transcription factors interacting with these elements. An understanding of how the gene is regulated in this disease could be used as a baseline for developing improved treatment regimens. We planned to synthesize novel expression vectors containing the VEGF promoter elements required for high-level expression in breast cancer cells. Therapeutic genes would thus be expressed specifically in malignant but less so in normal cells.

BODY

In the original proposal's Statement of Work we outlined that year 3 was to be spent with the identification and characterization of transcription factors that interact at functionally relevant VEGF promoter regions (Task 2). Composition and post-translational modifications of transcription factor complexes assembled at these sites were to be characterized by Matrix-Assisted Laser Desorption Ionization/Time Of Flight – Mass Spectroscopy (MALDI/TOF – MS). The Statement of Work further outlines the testing of core VEGF promoter expression vectors, which could be useful in cancer-specific gene therapy. We hypothesized that combining the functionally relevant promoter elements, which are exclusively active in breast cancer cells, in various numbers and orientation would result in high-level expression during transient transfection experiments in these but not control cells (Task 3).

Task 2: Identify and characterize transcription factors that interact at functionally relevant VEGF promoter regions

During year 2 we had identified a 165 base pair (bp) region between positions –1175 and –1010 upstream of the transcriptional VEGF start site whose deletion leads to a previously unknown drop in promoter activity of ≥ 40% in most cell lines (Figure 1). Although we had further

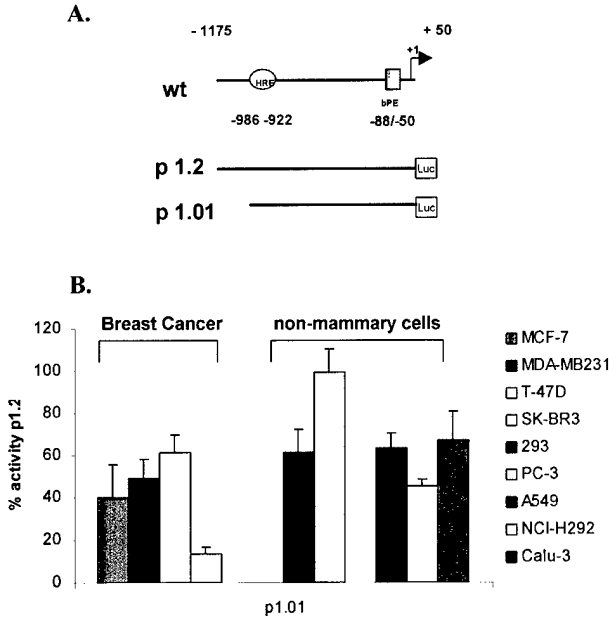
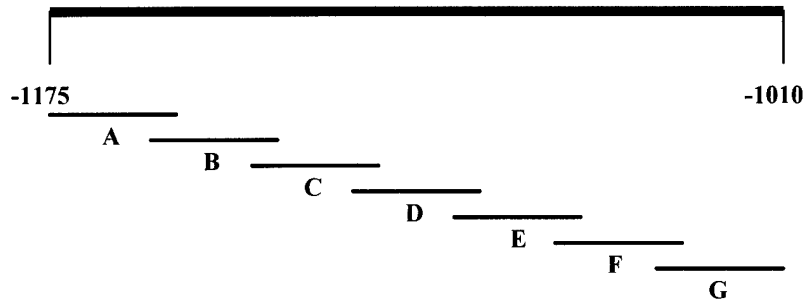


Figure 1: Loss of transcriptional activity in the VEGF wt promoter when sequences upstream of the hypoxia-response element (HRE) are deleted. A. Representative drawing of the VEGF wt promoter (-1175/+50) and promoter constructs (p1.2, p1.01) used to determine activity of the reporter gene Firefly luciferase (Luc) by transient transfection in different cell lines. Within the wt promoter, the positions of the HRE and the basal promoter element (bPE) are indicated; the transcriptional initiation site +1 is marked by the arrow. B. Activity of construct p1.01 normalized to that of construct p1.2 in breast cancer and non-mammary cell lines shows a ≥ 40% loss of promoter activity in most cells when sequences upstream of the HRE are deleted.

identified sequences downstream of the hypoxia-regulatory element (HRE) between positions –900 and –700, whose deletion affect promoter activity in a more cell type-specific manner, we proposed in last year's report to focus mainly on the area upstream of the HRE for the remainder of the research project. Deletion of this region has a greater impact on promoter activity in more cell lines than the downstream sequence, and our intent was to identify elements responsible for increased transcriptional activity for future use in breast cancer-specific gene therapy vectors.

Seven oligonucleotides, approximately 30 bp's in length and with partial overlaps, were designed to span the entire 165 bp upstream sequence (Figure 2A). In addition, the sense-strand was 5'-labeled with Biotin during synthesis. After annealing with their respective antisense-strands, the double-stranded oligonucleotides were used in non-radioactive electrophoretic mobility shift assays (Lightshift™ Chemiluminescent EMSA Kit; Pierce; Rockford, IL) with nuclear extracts from breast cancer cell lines MCF-7 and MDA-MB231. Following incubation, complexes were resolved on 4-20% polyacrylamide gradient gels (BioRad; Hercules, CA) and visualized after electrophoretic transfer onto positively charged Nylon membranes (Roche; Indianapolis, IN) according to the manufacturer's protocol. Unlike the EMSA optimization for the basal promoter element (bPE), optimization of the shift assays for the upstream VEGF promoter sequence was more complex: we observed the formation of several complexes on each oligonucleotide, and assigning specificity to the complexes was time-consuming. In hindsight, it might have been more feasible to identify transcription factor binding sites in this promoter region by DNase I footprinting.

A.



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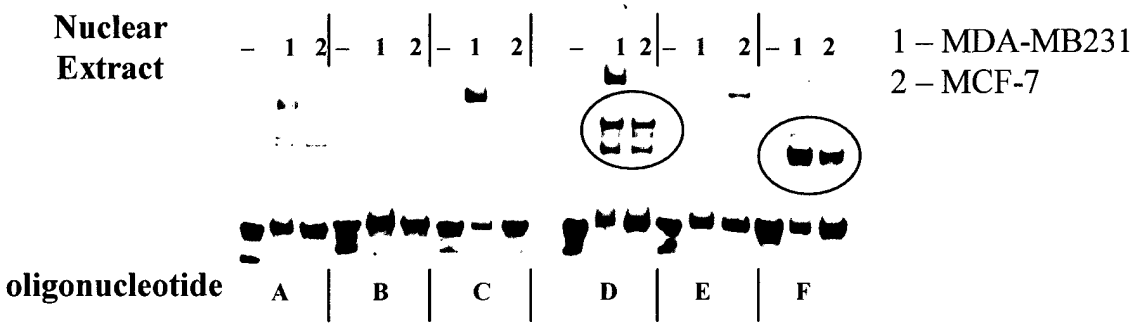

Figure 2: Binding of DNA-binding proteins in nuclear extracts from two breast cancer cell lines (1: MDA-MB231, 2: MCF-7) to the upstream VEGF promoter. A. Seven partially overlapping oligonucleotides were designed to encompass that region between positions –1175 and –1010 upstream of the transcriptional start site. B. Complex formation of 3 µl nuclear extract respectively, with 20 fmol of each of the 5'-biotinylated double-stranded oligonucleotides. No nuclear extract was used in the lanes marked —. Potentially specific complexes are circled.

Results are summarized in Figure 2B (complex formation with oligonucleotide G was never observed, and thus G is not included in the figure). Weak or no binding was observed with oligonucleotidess B and E. Strong complex formation was observed with A and C, albeit with nuclear extract from MDA-MB231 cells only. As we had hypothesized that complexes with similar intensity would be formed on the same oligonucleotide, no matter which extract was used, we excluded complexes with different signal intensities and assumed them to be non-specific. Thus, only the two circled complexes formed on D, and the single circled complex formed on F were considered to be caused by specific transcription factor binding. We also considered the doublet formed on oligonucleotide A as non-specific due to its weak intensity in comparison to the other bands.

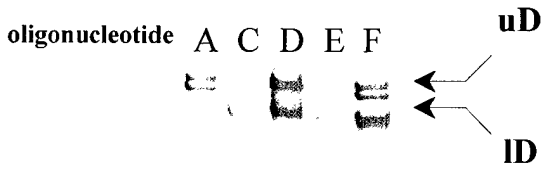

Figure 3: Complex formation seems similar with oligonucleotides A, C, D, E, and F. Results are shown only for the nuclear extract from MDA-MB231 cells. The arrows on the right indicate the positions for the upper (uD) and lower (lD) complex formed on oligonucleotide D.

Further analyses however, made it unlikely that these complexes are formed by specific protein-DNA interactions. First, it appears that similar complexes can also be formed on other oligonucleotides. Figure 3 shows the same, albeit weak, complex formation on oligonucleotide E as on D. The upper complex formed on D (uD) seemed identical with the upper band that is part of a doublet formed on oligonucleotide F (which itself seems identical to the doublet formed on oligonucleotide A). The lower D complex (lD) also appears with oligonucleotide C and could be identical with the complex on F.

Second, a search for potential transcription factor binding sites using two different software programs, MatInspector (2) and AliBaba 2.1 (www.alibaba2.com), indicated that non-specific DNA binding of nuclear proteins likely is responsible for these complexes. Although we found no common binding sites in oligonucleotides D and F, competition experiments with an excess of non-labeled oligonucleotide showed that binding of complex lD can be inhibited by either D or F. Interestingly, formation of complex uD on any of the oligonucleotides could not be inhibited (data not shown).

Oligo	number of binding sites per oligo		common binding sites identified	
	MatInspector	**AliBaba**	**MatInspector**	**AliBaba**
A	**2**	**7**	—	—
B	—	**2**	—	—
C	**3**	**8**	**AP-1**	**AP-1**
D	**6**	**9**	**Sp1**	**Sp1**
E	**4**	**3**	—	—
F	**1**	**3**	—	—
G	—	**1**	—	—

Table 1: Identification of potential transcription factor binding sites in the VEGF promoter upstream of the HRE using two software programs, MatInspector and AliBaba. The number of potential binding sites per oligonucleotide that each program identified is listed. Both programs recognized two identical transcription factor sites, AP-1 in C and Sp1 in D.

The search for potential transcription factor binding site in the upstream VEGF promoter also revealed the existence of two potentially specific sites; results are summarized in Table 1. Not unexpected, each

program identified different numbers of binding sites in each oligonucleotide. Both programs however, identified C and D as the oligonucleotides with the highest number, while B and G contain the least number of binding sites. The two common binding sites that each program recognized are a binding site for Activator Protein 1 (AP-1) in oligo C between positions –1125 and –1115 (3), and a binding site for Stimulating Protein 1 (Sp1) in oligo D between positions –1098 and –1086 [(4); Figure 4A]. Re-analysis of previous blots showed that complexes were also formed with nuclear extract from MCF-7 cells (Figure 4B). However as explained above, these signals were originally considered to be too weak to be caused by specific protein-DNA interactions. Within this context it is interesting to note that we never observed differences in signal intensity for both nuclear extracts when binding to the bPE was assayed.

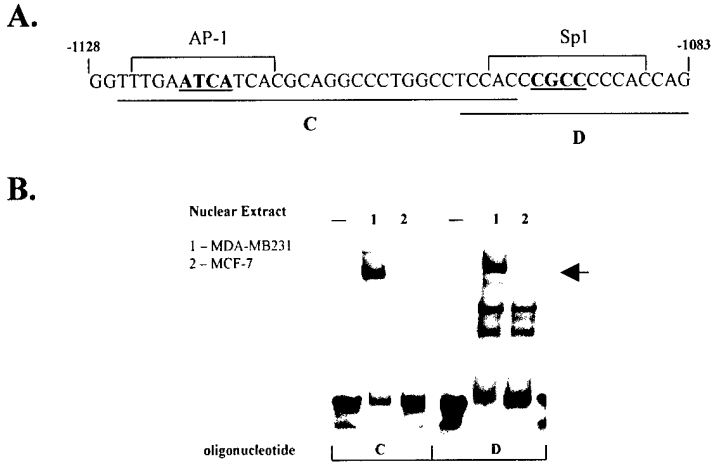

Figure 4: Binding of transcription factors to the VEGF promoter upstream of the HRE. A. Schematic representation of the sequence between positions –1128 and –1083. The binding sites for AP-1 and Sp1 are indicated with important sequence motifs highlighted. The positions of oligonucleotides C and D within this area are also shown. B. Complex formation, as indicated by the arrow on the right, with nuclear extract from MCF-7 cells on C and D (lane 2) is weak in comparison to extract from MDA-MB231 cells (lane 1).

Further shift assays showed that the complex formed on D is identical to a complex formed on an oligonucleotide that contains a functional Sp1 binding site found within the VEGF bPE between positions –67 and –47 upstream of the transcriptional start site (Figure 5A). The complex formed by AP-1 on C is slightly smaller and runs below the Sp1 complex. Preliminary data from competition experiments show that an excess of oligonucleotide, which contains the wt-binding site for AP-1, inhibits formation of this complex (data not shown). On the other hand, binding of Sp1 to the bPE can be competed with excess non-labeled oligonucleotides that contain the wt-sequence for Sp1, but not when this site is mutated, indicating potential functionality of the Sp1 site in the upstream VEGF promoter (Figure 5B). Mutating and deleting the Sp1 binding site in the upstream wt VEGF promoter, followed by transient transfection into breast cancer cells will, in the end, determine whether this transcription factor-binding site is truly functional.

The analyses of the composition of transcription factor complexes that assemble at either oligonucleotide C or D by MALDI/TOF – MS have so far not been undertaken. For one, the assays that would prove functionality of either the AP-1 or Sp1 binding site are not complete, and it does not seem feasible to determine the composition of complexes at either site (or potential post-translational modifications) without prior proof of functionality. Second, as outlined in the original proposal we expected using MALDI/TOF for the analysis of unknown and uncharacterized factors that interact with known DNA-binding proteins. However, our results so far indicate that VEGF regulation in breast cancer could be similar to other cells and tissues, as regulation seems to involve already known and well-characterized transcription factors. Third, the methods, employed to prepare complexes suitable for MALDI/TOF, have not yet resulted in specific signals: neither the originally proposed oligonucleotide pull-down assay (3) nor an EMSA, which would allow visualization of complexes directly in the polyacrylamide gel, have yielded MS data that discriminate between specific and non-specific binding.

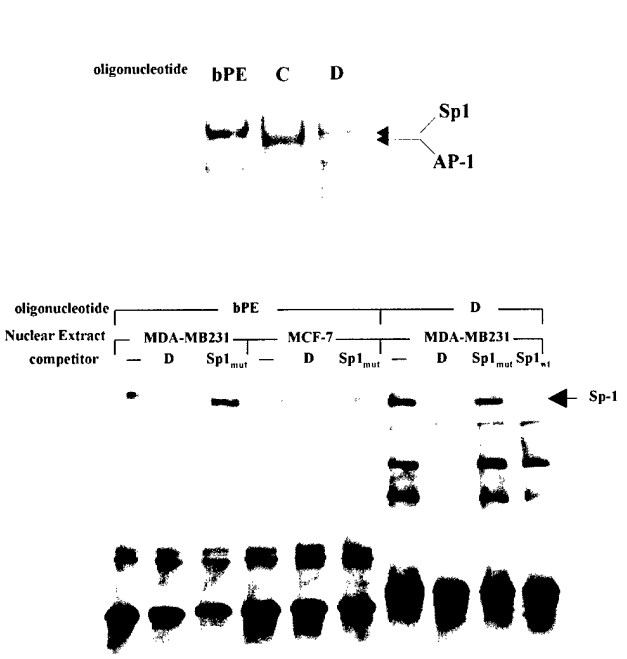

Figure 5: A. High resolution of a shift assay showing that the complex on D is identical to a complex formed on an oligonucleotide that contains a functional Sp1 biding site of the VEGF bPE between positions –67 and –47. The complex formed by AP-1 is smaller than the Sp1 complex (C). B. Inhibition of Sp1 binding reveals potential functionality of the site between positions –1098 and –1086. Binding to the bPE between position –67 and –47 as indicated by the arrow on the right can be competed in both extracts with an excess of D but not with an oligonucleotide, in which the Sp1 binding site is mutated ($Sp1_{mut}$). Binding to D can further be competed with an oligonucleotide that contains the wt binding site for Sp1 ($Sp1_{wt}$) but not $Sp1_{mut}$.

Task 3: Construct core VEGF promoters with high-level activity in breast cancer cells

As outlined in the original proposal, year 3 was supposed to be spent with testing the promoter activity of expression vectors that contain core VEGF promoter sequences in various numbers and orientations by transient transfection in breast cancer and control cells.

This task could not be completed within the proposal's given timeframe. We already stated in last year's report that the promoter regions, which had been identified and are important for transcriptional activation, *i.e.* the areas immediately upstream and downstream of the HRE, are not sufficiently defined yet. This part of the promoter spans approximately 400 bp, and we still consider it to be too large to use it for example in multimerization experiments. Second, most of the identified promoter elements within this area involve binding of already well-known transcription factors, which are also active in many other cell types. It thus appears unlikely that a combination of such elements will result in vectors that are only active in breast cancer tissue.

In last year's report we also suggested the construction and testing of a minimized VEGF promoter prior to changing the numbers and orientation of promoter elements. As shown in Figure 6, which depicts the mean activity of the various promoter deletion constructs in all breast cancer cells analyzed, promoter activity does not change drastically once sequences upstream of

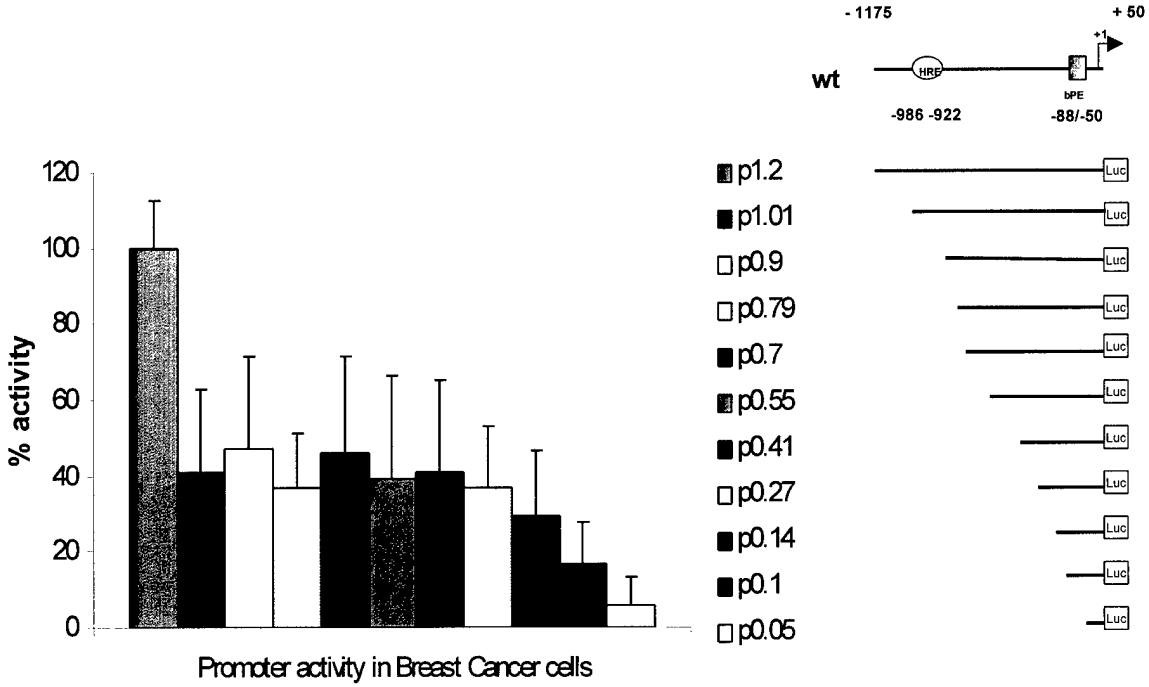

Figure 6: Mean promoter activity of VEGF promoter deletion constructs in all breast cancer cell lines analyzed

the HRE are removed; activity seems identical among the constructs that contain either 1000 (p1.01) or 100 (p0.1) bp upstream of the transcriptional initiation site. This data suggest that a minimized VEGF promoter needs only to contain the bPE, neglecting however, the activating effects mediated through the HRE and other influences such as growth factors, whose sites of interaction within the promoter remain to be characterized (6). Thus the wt promoter, which encompasses the positions between –1175 and +50, is currently the minimal promoter that is needed for high-level expression of VEGF in all cells and tissues.

KEY RESEARCH ACCOMPLISHMENTS

- Identified, through sequential promoter deletion, sequences upstream of the HRE between positions –1175 and –1010 in the VEGF wt gene promoter, which contribute to increased transcriptional activity not only in breast cancer but also non-mammary cell lines. Promoter sequences downstream of the HRE in combination with a potentially negative regulatory element also contribute to transcriptional activation, albeit in a more cell-type specific manner.
- Identified transcription factors that are also ubiquitously present in other cell types and involved in transcriptional activation of many other genes in the promoter sequence upstream of the HRE by EMSA with nuclear extracts from human breast cancer cells. The Zn-finger containing transcription factor Sp1, which also interacts at the bPE, binds to a potentially functional, hence to unknown site between positions –1098 and -1086. The same upstream promoter region also contains a potential binding site for the transcription factor AP-1 between positions –1125 and –1115.

REPORTABLE OUTCOMES

A. Abstracts:

1. Bredow, S., and Malkoski, S.P. Transcriptional Response of VEGF in Breast Cancer Cells. Proceedings, Vol. I, P4-5, Sept. 26, 2002 (Abstract and poster presentation).
2. Bredow, S., Falgout, M.M., and Malkoski, S.P. Transcriptional Regulation of VEGF in Breast Cancer Cells. Proceedings of the American Association for Cancer Research *44*:995, 2003 (Abstract and poster presentation).

B. Presentations:

Seminar – April 27, 2004: Presentation to the Cancer Group at LRRI.

C. Funding applied for:

New Mexico Center for Environmental Health Sciences – April 2004: Pilot Project funded for the period of May 1 – December 31, 2004.

CONCLUSIONS

Since little was known about the regulation of the VEGF gene in breast cancer, we based our hypothesis four years ago on previously available data that suggested that transcriptional regulation of the VEGF gene in this disease could differ from other types of cancer and tissues. We proposed to elucidate these differences and exploit them for developing improved treatment regimens: we hoped to design breast cancer-specific gene therapy vectors through combining functionally important promoter elements to create minimal VEGF core promoters that are capable of driving high-level expression of therapeutic genes exclusively in breast cancer cells. Now, four years later, we can say that this assumption was too optimistic. We had already mentioned in last year's Report that findings began to emerge which indicated that breast cancer cells do not utilize unique cell type-specific promoter sequences for transcriptional activation of VEGF; the overall expression patterns for the different promoter constructs were similar to those found in other non-mammary cell types. The results from this year's experiments further corroborate these findings. Analyses of promoter elements that we had identified to contribute to transcriptional activation of the VEGF gene make it very likely that the same elements are also involved in transcriptional activation of other genes and cell types, as we only identified DNA-binding transcription factors at these elements, which are also ubiquitously found in other cells and involved in the regulation of many other genes.

These results had an obvious impact on the course of the remaining experiments. For one, we decided not to focus on the characterization of the transcription factors and their complexes by MALDI/TOF – MS. As outlined in the original proposal, we felt that this technique was more appropriate to characterize unknown protein-protein interactions than DNA-binding proteins. Most of the DNA-binding proteins that we identified as being potentially important for VEGF expression in breast cancer cells however are already well characterized, making it unlikely that novel protein-protein interactions responsible for increased transcriptional VEGF gene regulation in breast cancer would be identified for these factors. Second, for similar reasons we decided not

to pursue the construction and testing of core VEGF promoters. All of the transcription factors that we identified are also ubiquitously present in other cell types, where they are involved in the regulation of many different genes. It became clear that the construction of therapeutic expression vectors with specific high-level activity in breast cancer cells is not yet possible.

However, within these limitations we have been able to complete most of the work that was outlined in the original proposal. Task 1, the identification of VEGF promoter elements, which are responsible for the elevated transcriptional activation of the gene in breast cancer, is complete. Task 2, the identification and characterization of transcription factors interacting at such functionally relevant promoter sites, will be completed soon. Transient transfections with constructs that contain mutations or deletions of the upstream AP-1 and Sp1 binding sites will prove functionality of either site (and potential synergy). To our knowledge functional transcription factor-binding sites upstream of the HRE in the VEGF wt promoter have yet to be described and characterized. We therefore believe that such data will be of interest to other researchers in the field and be publishable in peer-reviewed journals, bringing this research project to an appropriate closure.

Last, we like to point out that it could be of interest to analyze the promoter sequences downstream of the HRE, which modulate transcriptional activity in a more cell type-specific manner. Unlike the investigation of promoter elements that are involved in gene regulation in most cell types, characterizing the interactions at such *cis*-regulatory sequences may identify novel regulatory pathways whose modulation could be used to decrease the progression of cancer growth. Unfortunately, without any immediate translational potential, it is currently difficult to obtain funding for purely mechanistic research projects.

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APPENDICES

A. BIBLIOGRAPHY

Copies of the abstracts had been attached to the two previous Annual Reports and are therefore not included in this report.

B. LIST OF PERSONNEL

Sebastian Bredow, Ph.D. – Principal Investigator

Melanie M. Falgout, B.S. – Research Technologist