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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Aberrant DNA methylation of promoter region CpG islands is associated with gene silencing and serves as an alternative to mutations in the inactivation of tumor suppressor genes in human cancers. Our lab has identified a novel gene, TMS1, that is aberrantly methylated and silenced in human breast tumors. TMS1 is a bipartite signalling molecule that functions in the regulation of apoptosis and inflammation. It is our hypothesis that epigenetic silencing of TMS1 contributes to breast carcinogenesis by allowing cells to bypass normal apoptotic cues, and, as a consequence may cause cells to be more resistant to chemotherapeutic agents. The goals of this proposal are to study the role of TMS1 in breast cell apoptosis by developing breast cells stably knocked down for TMS1 expression using siRNA technology and to determine the impact of TMS1 loss on the response of breast cancer cells to anticancer agents and other apoptotic stimuli. Thus far, we have determined that overexpression of TMS1 induces a time-dependent apoptotic response characterized by cleavage of the initiator caspase-8, the downstream caspase-3, and the death substrate PARP. TMS1-induced apoptosis was blocked by inhibitors of caspase-8, suggesting a role in death receptor mediated apoptosis. The death receptor ligands TNFα and TRAIL upregulate TMS1 expression in breast epithelial cells. The activation of TMS1 by death receptors is mediated at the level of transcription and requires the activity of the NF-κB and JNK signalling pathways. Upregulation of TMS1 by TNFα and TRAIL and subsequent activation of caspase-8 could function as an amplification loop necessary to achieve cell death by the death receptrors in some cell types, including breast cells. These results may also have therapeutic implications in that the methylation status of TMS1 in human tumors may influence sensitivity to TRAIL, which is currently undergoing clinical trial.

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A. Introduction

Breast and other cancers arise from mutations in tumor suppressor genes and oncogenes that accumulate in normal tissue over time. Aberrant methylation of promoter region CpG islands is associated with gene silencing and serves as an alternative to mutations in the inactivation of tumor suppressor genes in human cancers. Our lab has identified a novel gene, TMS1 (for Target of Methylation-mediated Silencing 1), that is aberrantly methylated and silenced in 40% of human breast cancers. Subsequent work showing that TMS1 is a target of methylation-mediated silencing in lung cancers, ovarian cancers, melanomas, and glioblastomas suggest that silencing of TMS1 contributes to the pathogenesis of a number of different tumor types.

At present, the consequences of TMS1 silencing and its role in breast carcinogenesis are not known. The TMS1 protein contains a caspase-recruitment domain and a pyrin domain, two protein-protein interaction motifs found in intracellular signaling molecules involved in the regulation of apoptosis and inflammation. Previous work in the lab has shown that overexpression of TMS1 promotes apoptosis and inhibits the colony-forming ability of breast cancer cells, consistent with a tumor suppressor role. Similarly, work by others indicates that reduced expression of TMS1 renders HL-60 cells resistant to apoptosis induced by etoposide, indicating a role in drug-induced apoptosis. Recent studies showing that TMS1 modulates the activity of caspase-1 and can block the downstream activation of NF-κB suggest that methylation-mediated silencing of TMS1 could promote tumorigenesis by allowing cells to bypass apoptosis, to evade a local immune response and by allowing NF-κB-dependent survival signals to go unchecked. However, the exact role of TMS1 in breast cell apoptosis has not been clearly defined.

It is our hypothesis that epigenetic silencing of TMS1 contributes to carcinogenesis by allowing breast cells to bypass normal apoptotic cues and, as a consequence, may cause some cancers to be resistant to chemotherapy. The goals of this proposal are to develop breast cells lacking TMS1 expression using siRNA technology and to determine the impact of TMS1 loss on the response of breast cancer cells chemotherapeutic agents and other apoptotic stimuli. In this annual summary report, I will discuss the progress made on this project, recent accomplishments, and an additional novel direction that this project has taken.

B. Body

To study the role of TMS1 in breast carcinogenesis and the response to chemotherapy, I proposed to develop a model of TMS1 loss-of-function in human breast cancer cells. To create this system, I proposed to generate breast cancer cell lines stably knocked down for TMS1 using RNA interference technology. The specific goals within Task 1 were to a.) determine the target site within TMS1 mRNA for blocking TMS1 expression with short interfering RNA, b.) construct a TMS1 siRNA plasmid containing a U6 promoter driving the expression of a short hairpin RNA (shRNA) directed at the target site of TMS1, c) stably transfect MCF7 and MB468 breast cancer cell lines with the TMS1 shRNA plasmid or vector alone to create TMS1-null breast cancer cells and isolate neo-resistant clones, and d.) test individual clones for decreased TMS1 by Western analysis. The majority of these goals have been met. A target site in TMS1 mRNA was identified empirically in transient transfection experiments using various synthetic siRNAs targeting different regions of the TMS1 mRNA. An optimal RNAi targeting sequence was identified that decreased TMS1 protein levels by over 90%. An shRNA plasmid directed at the same sequence was then generated in the pSilencer 1.0 vector (Ambion), and MCF7 cells were stably transfected with this plasmid or with vector alone. A large number of neo-resistant clones were isolated and tested for TMS1 expression by Western blot analysis. A large majority of the isolated clones displayed some reduction in TMS1, with several exhibiting no detectable TMS1 protein. Unfortunately, the knockdown of TMS1 appeared to be transient, as all of the knock down clones regained TMS1 expression after 10-20 passages in culture.

As an alternative to the siRNA approach, I proposed to create knockdown cell lines using an antisense approach. A construct containing the TMS1 message in an antisense orientation driven by the CMV promoter was constructed and stably introduced into MCF7 cells. Neomycin resistant clones were isolated and tested for TMS1 expression by Western blot analysis. Although a number of positive clones were identified, the knockdown of TMS1 expression was again transient in nature, with most cells regaining TMS1 expression levels after several passages. Taken together, these results may indicate that loss of TMS1 represents an unfavorable condition for breast cancer cells.

Thus, it would appear that it will be necessary to use a transient approach to study the impact of TMS1 silencing. To this end, I have optimized the transfection of synthetic siRNA to TMS1 in MCF7 cells, and am beginning to study the effects of TMS1 silencing on response of breast cancer cells to apoptotic agents. In addition, the lab has created an shRNA adenoviral construct that will allow infection of 100% of cells and efficient knockdown of TMS1 expression over 7-10 days. This should be sufficient to examine the impact of TMS1 loss on apoptosis induced by chemotherapeutic agents and other apoptotic stimuli (e.g. death receptors). Therefore, the goals proposed in Tasks 2 and 3 of the approved statement of work, which are 1.) to determine whether loss of TMS1 causes resistance of breast cancer cells to anticancer agents and proapoptotic stimuli and characterize the resulting apoptotic response using cell lines in which TMS1 expression is reduced, and 2.) to determine the effects of TMS1 loss on anchorage-independent growth potential, will still be attainable.

Importantly, despite the problems encountered during Task 1, this project has made significant progress in other areas. In an effort to examine the role of TMS1 in apoptosis (Task 2), I have characterized the downstream events in TMS1-induced apoptosis. I found that inhibition of caspase-8 (but not caspase-1, or-9) inhibits TMS1 induced apoptosis, suggesting a role in signaling from death receptors. As a result, I have begun to study the role of TMS1 in the cellular response to TRAIL and TNFα. During the course of these experiments, I made an important and novel observation that TMS1 expression is induced in breast epithelial cells by TNFα and TRAIL, further supporting a role for TMS1 in death receptor-mediated apoptosis. My

most recent work has focused on the regulation of TMS1 by death receptor signaling. I found that the induction of TMS1 by TNFα is mediated at the level of transcription and requires the activity of NF-κB and JNK signaling pathways. I have further identified a putative TNF response element in intron 2 of the TMS1 gene. Our continued efforts should shed light on the role of TMS1 in death receptor mediated apoptosis. In addition, these results may also have therapeutic implications in that the methylation status of TMS1 in human tumors may influence sensitivity to TRAIL, which is currently undergoing clinical trial. This work was recently presented as a poster at the 95th Annual AACR Meeting, in Orlando, FL in March 2004 and at the AACR Pathobiology of Cancer Workshop, in Snowmass, CO in July 2004. Furthermore, a manuscript describing these results (Parsons, M.J. and Vertino, P.M. Regulation of TMS1 by Death-Receptor Signaling. in preparation) is currently in preparation and will be submitted within the next few months.

C. Key Research Accomplishments

- Development of a model of TMS1 loss-of-function in human breast cancer cells
  - Identification of target sites within TMS1 mRNA amenable to blocking TMS1 expression with short interfering RNA
  - Optimization of transient siRNA transfection to knock down TMS1 expression
  - Generation of MCF7 cells stably expressing shRNA to TMS1
  - Generation of MCF7 cells stably expressing antisense TMS1

- Determination that TMS1 is regulated by death receptor signaling in breast epithelial cells
  - Identified TNFα and TRAIL as positive regulators of TMS1 expression
  - Determined that TNFα-mediated upregulation of TMS1 occurs at the message level
  - Determined that TNFα-mediated upregulation of TMS1 requires NF-κB and AP-1 activity
  - Identified putative TNFα regulatory element in intron 2 of TMS1 gene

D. Reportable Outcomes

Abstracts:

- Parsons, M.J., B.B. McConnell and P.M. Vertino. "Role of TMS1/ASC in Death Receptor Mediated Apoptosis and Survival Pathways" 95th Annual AACR Meeting, Orlando, FL (March, 2004).

- Parsons, M.J., B.B. McConnell and P.M. Vertino. "Role of TMS1/ASC in Death Receptor Mediated Apoptosis and Survival Pathways" AACR Pathobiology of Cancer Workshop, Snowmass, CO (July, 2004).

Manuscripts:

- Parsons, M.J. and Vertino, P.M. (2004) Regulation of TMS1/ASC by death receptor signaling in breast cancer cells. In preparation.

Presentations:

- "Role of TMS1 Silencing in Breast Carcinogenesis" Graduate School Seminar, February 2004
- "Role of TMS1/ASC in Death Receptor Mediated Apoptosis and Survival Pathways" Poster Presentation, 95th Annual AACR Meeting, Orlando, FL (March 2004)
- "Role of TMS1/ASC in Death Receptor Mediated Apoptosis and Survival Pathways" Poster Presentation, AACR Pathobiology of Cancer Workshop, Snowmass, CO (July, 2004)

Resources Developed:

- siRNA and shRNA vectors against human TMS1
- TMS1 promoter/ reporter constructs
- MCF7 cells stably expressing shRNA to TMS1
- MCF7 cells stably expressing antisense TMS1

E. Conclusions

My overall goal is to determine how the silencing of TMS1 contributes to breast carcinogenesis. To do this, I proposed in Task 1 to create breast cancer cells that exhibited reduced expression of TMS1 by stable siRNA technology. Although stable knockdown of TMS1 using shRNA vectors has proven not to be prudent, I have developed and optimized two alternative methods of transient knockdown of TMS1. I have also made progress on elucidating the function of TMS1 in apoptosis (Task 2). I have established a potential role for TMS1 in death-receptor mediated apoptosis as an upstream activator of caspase-8 and I have further found that TMS1 levels are positively regulated by death-receptor ligands. Death receptor mediated activation of TMS1 occurs at the level of transcription, and is dependent upon both NF-κB and JNK signalling pathways. My work on this project over the last year is has resulted in the submission of two abstracts, two presentations at national scientific conferences and a manuscript in preparation. In addition, I have given one oral presentation to the Emory University Graduate School of Arts and Sciences on my work. A number of valuable resources have also been generated including siRNA and shRNA vectors targeting the TMS1 gene, TMS1 promoter/reporter constructs, and MCF7 cells stably expressing shRNA against TMS1. Having established effective means to knockdown TMS1 expression in breast epithelial cells using RNAi, I will focus over the next year on the analysis of drug-and death receptor-induced apoptosis in TMS1 knockdown and control breast cancer cells (Tasks 2&3).

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Role of TMS1/ASC in death receptor mediated apoptosis and survival pathways

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Breast and other cancers arise from mutations in tumor suppressor genes and oncogenes that accumulate in normal tissue over time. Aberrant methylation of promoter region CpG islands is associated with gene silencing and serves as an alternative to mutations in the inactivation of tumor suppressor genes in human cancers. Our lab has identified a novel gene, TMS1 (Target of Methylation-induced Silencing 1), that is aberrantly methylated and silenced in 40% of primary breast tumors. TMS1 contains a caspase-recruitment domain and a pyrin domain, and is thought to function as an intracellular signaling molecule mediating apoptosis and/or inflammation. However, its precise role in these processes is not known. Using an inducible expression system in HEK293 cells, we have characterized the impact of TMS1 expression on apoptosis and survival pathways. We found that overexpression of TMS1 induced a time-dependent apoptotic response characterized by cleavage of the initiator caspases –8 and –9, the downstream caspase –3, and the death substrate PARP. TMS1-induced apoptosis was blocked by specific inhibitors of caspase-8, but not inhibitors of caspases-1 or –9. We therefore examined the role of TMS1 in death receptor signaling. Intracellular levels of TMS1 had no impact on the apoptotic response to the death receptor ligands Fas, TNFα, or TRAIL. However, TMS1 suppressed the activation of NF-κB induced by TRAIL, but not TNFα or Fas, in a dose-dependent manner. The data indicate that TMS1 acts as a negative regulator of TRAIL-induced NF-κB activation, and suggest that epigenetic silencing of TMS1 in breast and other cancers contributes to carcinogenesis by allowing NF-κB dependent survival signals to go unchecked. These results may also have therapeutic implications in that the methylation status of TMS1 in human tumors may influence sensitivity to TRAIL, which is currently undergoing clinical trial. Supported by grants from the American Cancer Society (RSG- 02-144-01), the Department of Defense (DAMD-17-03-1-0578), and the Avon Foundation.

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