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13. ABSTRACT (Maximum 200 Words)

The major goal of this study was to investigate whether the estrogen-inducible transcription factor ZEB-1 contributed to the progression of breast carcinoma. For this study, we pursued and obtained sufficient numbers of staged, matched breast cancer biopsy tissues. I developed a novel protocol for multiplex real-time PCR so we could analyze the expression of four genes in one reaction simultaneously. The second year of work on these projects showed great progress and completion of our goals. Keeping in mind a new family member, ZEB-2, I designed and performed the real-time assays on a number of breast tissue samples. We found that the expression of both ZEB-1 and ZEB-2 are significantly increased in invasive breast cancers, compared to their normal match tissue, and ZEB-1 is no longer under the regulation of estrogen. To investigate the possibility of gene amplification, I created a ZEB-1 specific DNA program for quantitative Southern blot analysis and have successfully tested this probe on ovarian tissue biopsies. I have also constructed the ecdysone-inducible MCF-7 cell line that is needed for Task 4. In this line, ZEB-1 expression can be turned on or off with the presence or absence of ecdysone, and thus the ability of ZEB-1 to promote cell invasion can now be studied by Matrigel invasion assay. In support of our hypotheses, we have shown that ZEB -1 expression correlates with invasive breast cancer and that its expression is no longer regulated by estrogen.

14. SUBJECT TERMS

Gene expression, invasive breast cancer, estrogen signaling, quantitative PCR, DNA transfections

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INTRODUCTION

The steroid hormone estrogen is reported to contribute to the etiology of breast cancer, though relatively little is known about the downstream targets of the estrogen receptor. We have previously shown that estrogen induces the expression of the AREB6/ZEB (ZEB-1) transcription factor at the transcriptional level within two hours of treatment (1). Our unpublished data indicate that expression of ZEB-1 is deregulated, and high, in rapidly proliferating endometrial and ovarian cancers. Furthermore, microarray data suggest that ZEB-1 is more highly expressed in invasive/metastatic breast cancer lines compared to poorly invasive lines (2). In the systems where it has been tested, ZEB-1 is thought to act to promote cell proliferation and prevent differentiation (3-5). These observations led to our hypothesis that *ZEB-1 contributes to the progression and metastasis of breast cancer by promoting cell invasion and proliferation.* I propose that this is a consequence of ZEB-1 gene expression becoming independent of regulation by estrogen so that ZEB-1 is expressed at inappropriately high levels. Also, I anticipate that it is the deregulated expression of ZEB-1 that contributes to the estrogen-independent phenotype observed with some advanced breast cancers.

BODY

The specific research aims of this proposal were to I). *determine whether the level of expression of ZEB-1 correlates with the invasive and metastatic potential of breast cancers*, II). *investigate whether the ZEB-1 gene is amplified during breast cancer progression*, and III). *ascertain whether overexpression of ZEB-1 converts MCF-7 cells from a noninvasive to an invasive phenotype.* To complete these aims, four major Tasks were developed:

Tasks 1 and 2:

The first two tasks were designed to determine whether ZEB-1 gene expression becomes independent of estrogen in breast carcinomas and, if so, at which stage. The plan was to measure the amount of ZEB-1 mRNA in staged human breast cancers and normal breast samples and to correlate that with serum estrogen levels. The accomplishment of these tasks had been somewhat delayed in the first year because of three changes in strategy: 1) switching our control method of testing estrogen responsiveness from measuring serum estrogen levels to measuring expression of a known estrogen-responsive gene; 2) switching our design and analysis to include consideration of the other family member, ZEB-2 (SIP1), which may play a role in oncogenesis (6); 3) switching our assay method from quantitative competitive PCR to quantitative real-time PCR, which is far more sensitive and can be multiplexed. These changes in strategy promoted a massive effort in terms of redesigning the experimental conditions, reagents, and analyses.

After testing PR, pS2, and SDF-1α primers, the estrogen-responsive control was chosen to be SDF-1α, a gene known to be very sensitive to estrogen in breast tissue. I designed and tested optimal real-time PCR primers and a dual-lableled TaqMan™ probe for SDF-1α. Two different breast tissues were harvested for RNA, which were then reverse transcribed into cDNA, and the real-time assay was run on these in duplicate. The resulting chart yielded smooth, reproducible curves and revealed that these primers and probe are effective for detecting SDF-1α expression (**Appendix 1**). The same process was carried out for the ZEB-2 gene, with the added concern of cross-detection with ZEB-1. These two

genes are 80% identical at the nucleotide level, raising the issue of primer specificity. After careful design and exhaustive testing, appropriate primers and probe were selected that were both effective and mutually exclusive with ZEB-1. I repeated the design and testing process for the remaining genes, RPL32 and ZEB-1, with equal success. All primer and TaqMan™ probe sequences are listed in **Appendix 2.** Switching to the quantitative real-time PCR method required a major intellectual and technical undertaking, culminating in my successful optimization of the method for multiple genes. I am one of the first and few investigators in the country to work out this technique to assay four simultaneous genes, so that their cDNAs can be amplified in the same polymerase chain reaction at the same time. Upon the completion of this massive optimization and troubleshooting effort, I was prepared to investigate the relative expression of both ZEB family members along with an estrogen-responsive control gene, as well as the loading control RPL32 gene for normalization.

These efforts were rewarded when I finally ran the project samples. Seventy breast biopsy tissues were harvested for total RNA. Reverse transcription reactions were performed to create cDNA from these RNA. I performed real-time quantitative PCR on these cDNA using primers and TaqMan™ probes specific for ZEB-1, ZEB-2, SDF-1α, and RPL32. Data for the samples that were matched (meaning both a normal tissue and a diseased tissue were available from the same patient) were pulled out and analyzed. All values were normalized to RPL32 expression using the following equation:

$$\text{Gene Expression} = \frac{(\%\ \text{efficiency RPL32})^{CT[RPL32]}}{(\%\ \text{efficiency Gene})^{CT[Gene]}}$$

where Gene is ZEB-1, ZEB-2, or SDF-1α. After normalization, the expression of these three genes in invasive breast cancers was plotted relative to their expression in matched normal tissues. The chart in **Appendix 3** reveals that *ZEB-1 expression increases 17- to 22-fold in invasive breast cancer relative to normal tissue.* ZEB-2 expression also increases, approximately 5-fold, in the invasive samples relative to normal. It is important to note that these increases do not correlate with simultaneous increases in estrogen responsiveness. In one patient, SDF-1α expression increases 3-fold in the invasive tissue, while in the other patient, in decreases by 50%. This lack of parallel trends indicates that while expression of the ZEB genes are induced significantly in the invasive tissues, ZEB-1 is no longer under the control of estrogen. As I stated in the Introduction, I propose that in invasive breast cancers, ZEB-1 gene expression becomes independent of regulation by estrogen so that ZEB-1 is expressed at inappropriately high levels. These results confirm our hypothesis.

Task 3:

The third task is to determine whether the ZEB-1 gene is amplified in our breast cancer samples. This is being assayed by quantitative Southern blot. I have learned the Southern blotting procedure and have designed and synthesized a ZEB-1-specific DNA probe and a β-globin control DNA probe. I have used these probes successfully to detect the ZEB-1 and β-globin genes in a number of ovarian samples. I am in the process of harvesting DNA from the appropriate breast samples, those showing high ZEB-1 expression by real-time PCR, to run the remaining quantitative Southern blots. The blots will be performed on the invasive and normal tissue for each patient.

Task 4:

The goal of this task is to determine whether overexpression of ZEB-1 can make MCF-7 cells invasive. This requires a two-stage procedure to make a cell line in which the level of ZEB-1 is controlled by ecdysone treatment. In the first stage, a stable MCF-7 cell line is constructed containing an ecdysone and retinoic acid receptor. The cells are then screened to identify those that have stably integrated the receptors. The next step is to create an ecdysone-inducible ZEB-1 expression vector and stably transfect this vector into those cells. Both of these stages have been successfully completed. The next task is to identify and isolate individual clones of the double-stable transfectants. Twelve clones were isolated and these 12 clonal lines were then expanded and propagated in separate flasks until they reached confluence. Each line was split into two flasks and one of the flasks from each line was treated with ecdysone. All of the lines were harvested and I made nuclear extracts from each flask. The proteins were run and analyzed by western blot using a ZEB-1 antibody. The blots reveal that Clonal Line 3 expresses ZEB-1 in response to ecdysone treatment (**Appendix 4**). This original Line will now be grown up and used in Matrigel invasion assays to determine whether ZEB-1, in response to ecdysone induction, can promote cell invasion through collagen.

Task 5:

Manuscript is in press (**Appendix 5**).

KEY RESEARCH ACCOMPLISHMENTS

- Development of an alternative strategy (measurement of the amount of the estrogen-regulated mRNAs for SDF-1α) for assessing estrogen levels in breast tissue.
- Learned the real-time PCR technique, which is more sensitive and technically challenging than competitive PCR.
- Collection of staged breast cancer samples, isolation of RNAs, and creation of cDNAs.
- Design and synthesis of gene-specific real-time PCR primers and TaqMan™ dual-labeled probes for ZEB-1, ZEB-2, SDF-1α and RPL32.
- Development and optimization of a novel multiplex real-time PCR protocol for assaying four genes in one tube at one time.
- Performed real-time PCR on 67 breast staged breast biopsy tissues with four probes.
- **Analysis of matched normal and diseased breast tissues by real-time PCR to reveal ZEB-1 expression is dramatically raised in invasive breast cancers and no longer regulated by estrogen. ZEB-2 expression is also significantly increased.**
- Learned the quantitative Southern blotting technique.
- Design and synthesis of ZEB-1 and β-globin DNA probes for Southern blot.
- Tested Southern probes successfully on ovarian biopsy samples.
- Creation of an ecdysone-inducible ZEB-1 expression vector.
- Development of MCF-7 cell lines that express stable EcR-RXR and ZEB-1 vectors.
- Selection by western blot of optimal MCF-7 Clonal Line that expresses ZEB-1 in response to ecdysone treatment for use in Matrigel invasion assay.

KEY TRAINING ACCOMPLISHMENTS

- Selected for and attended the AACR Pathobiology of Cancer Workshop in Keystone, Colorado. Presented a research poster and learned a great deal about cancer biology and pathogenesis. Became an Associate Member of the AACR.
- Attended and presented a research poster at the IVth International Congress on Hormonal Carcinogenesis in Valencia, Spain. Won an abstract award.
- Attended and presented a research poster at the 94th Annual Meeting of the AACR in Washington, D.C.
- Attended weekly seminars in the University of Minnesota Cancer Center, especially those that pertained specifically to breast cancer.

REPORTABLE OUTCOMES

Manuscripts:

Anose, B.M., M.P. Linnes, and M.M. Sanders (2004). Hormonal Regulation of ZEB-1 and Implications for Progression of Human Reproductive Cancers. Hormonal Carcinogenesis J.J. Li and S.A. Li (eds). Vol 4. New York: Springer-Verlag (in press).

Abstracts:

Anose, B.M., Landry, M.M., and Sanders, M.M. (2004). The androgen-regulated ZEB-1 gene is a biomarker for metastatic prostate cancer. 12th International Congress of Endocrinology. Lisbon, Portugal (accepted).

Anose, B. M. and M. M. Sanders (2003). Steroid Hormones Regulate Expression of Human ZEB-1: Implications for Cancer Progression. 94th Annual Meeting of the American Association for Cancer Research. Abstract R5066. Washington, D.C.

Anose B.M., M.P. Linnes and M. M, Sanders (2003). Hormonal regulation of ZEB-1 and implication for progression of human reproductive cancers. 4th International Symposium on Hormonal Carcinogenesis. Abstract 54. Valencia, Spain.

Invited Presentations:

03/28/03 "Steroid hormone regulation of human ZEB-1 and implications for cancer progression." Cancer Biology Research Club, University of Minnesota Cancer Center

05/21/04 "The androgen-regulated ZEB-1 gene in reproductive cancer progression." Cancer Biology Research Club, University of Minnesota Cancer Center

Reagents:

- Ecdysone-inducible ZEB-1 expression vector
- Double stable ZEB-1 inducible MCF-7 cell line
- Real-time PCR primers specific for ZEB-1, ZEB-2, SDF-1α and RPL32
- Real-time PCR TaqMan™ probes specific for ZEB-1, ZEB-2, SDF-1α and RPL32

CONCLUSIONS

The first year of progress on these projects was made primarily in the areas of reagent creation and technical development. Significant changes were made in the protocols for Tasks 1 and 2, requiring the design and synthesis of effective primers and probes for multiplex real-time PCR. To actually run the study, we pursued and eventually obtained sufficient numbers of staged, matched breast cancer biopsy tissues. I developed a novel protocol for multiplex real-time PCR so we could analyze the expression of four genes in one reaction simultaneously. The second year of work on these projects showed great progress and completion of our goals. Keeping in mind a new family member, ZEB-2, I designed and performed the real-time assays on a number of breast tissue samples. I found that *the expression of both ZEB-1 and ZEB-2 are significantly increased in invasive breast cancers, compared to their normal match tissue, and ZEB-1 is no longer under the regulation of estrogen.* For Task 3, I created a ZEB-1-specific DNA probe for quantitative Southern blot analysis, and have successfully tested this probe on ovarian tissue biopsies. I have also constructed the ecdysone-inducible MCF-7 cell line that is needed for Task 4. In this line, ZEB-1 expression can be turned on or off with the presence or absence of ecdysone, and thus the ability of ZEB-1 to promote cell invasion can now be studied by Matrigel invasion assay. In support of our hypotheses, we have shown that ZEB-1 expression correlates with invasive breast cancer and that its expression is no longer regulated by estrogen.

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APPENDICES

Appendix 1. Chart of Successful Test of SDF-1α Primers and Probe in Real-Time PCR

Appendix 2. Table of Entire Set of Real-Time PCR Primers and TaqMan™ Probes

Appendix 3. Real-Time PCR Results Showing Increased ZEB-1 and ZEB-2 Expression in Invasive Breast Carcinomas

Appendix 4. Western Blot of ZEB-1 Protein Extracts from MCF-7 Inducible Cell Line

Appendix 5. Chapter to be Published in the 4th Edition of Hormonal Carcinogenesis

Anose, B.M., M.P. Linnes, and M.M. Sanders (2004). Hormonal Regulation of ZEB-1 and Implications for Progression of Human Reproductive Cancers. Hormonal Carcinogenesis J.J. Li and S.A. Li (eds). Vol 4. New York: Springer-Verlag (in press).

Appendix 6. Abstract to be Presented at the 12th International Congress of Endocrinology

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Appendix 7. Abstract Presented at the Annual Meeting of the American Association for Cancer Research

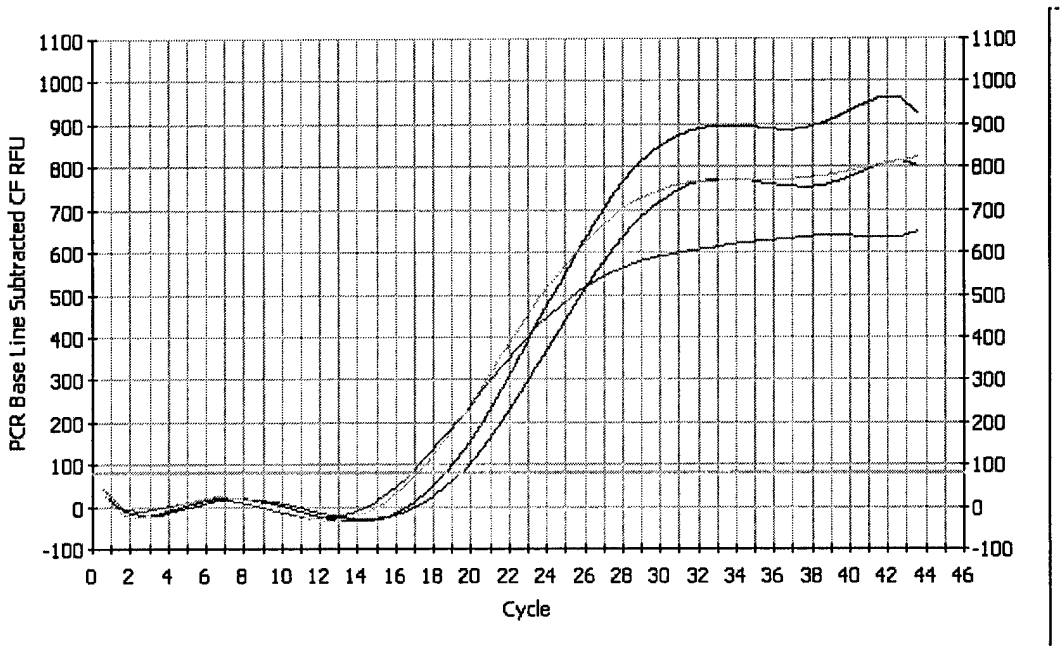
Anose, B. M. and M. M. Sanders (2003). Steroid Hormones Regulate Expression of Human ZEB-1: Implications for Cancer Progression. 94th Annual Meeting of the American Association for Cancer Research. Abstract R5066. Washington, D.C.

Appendix 8. Abstract Presented at the 4th International Symposium on Hormonal Carcinogenesis

Anose B.M., M.P. Linnes and M. M, Sanders (2003). Hormonal regulation of ZEB-1 and implication for progression of human reproductive cancers. 4th International Symposium on Hormonal Carcinogenesis. Abstract 54. Valencia, Spain.

Anose, Bynthia M.
DAMD17-02-1-0493

Appendix 1. Testing SDF-1α Primers and Probe



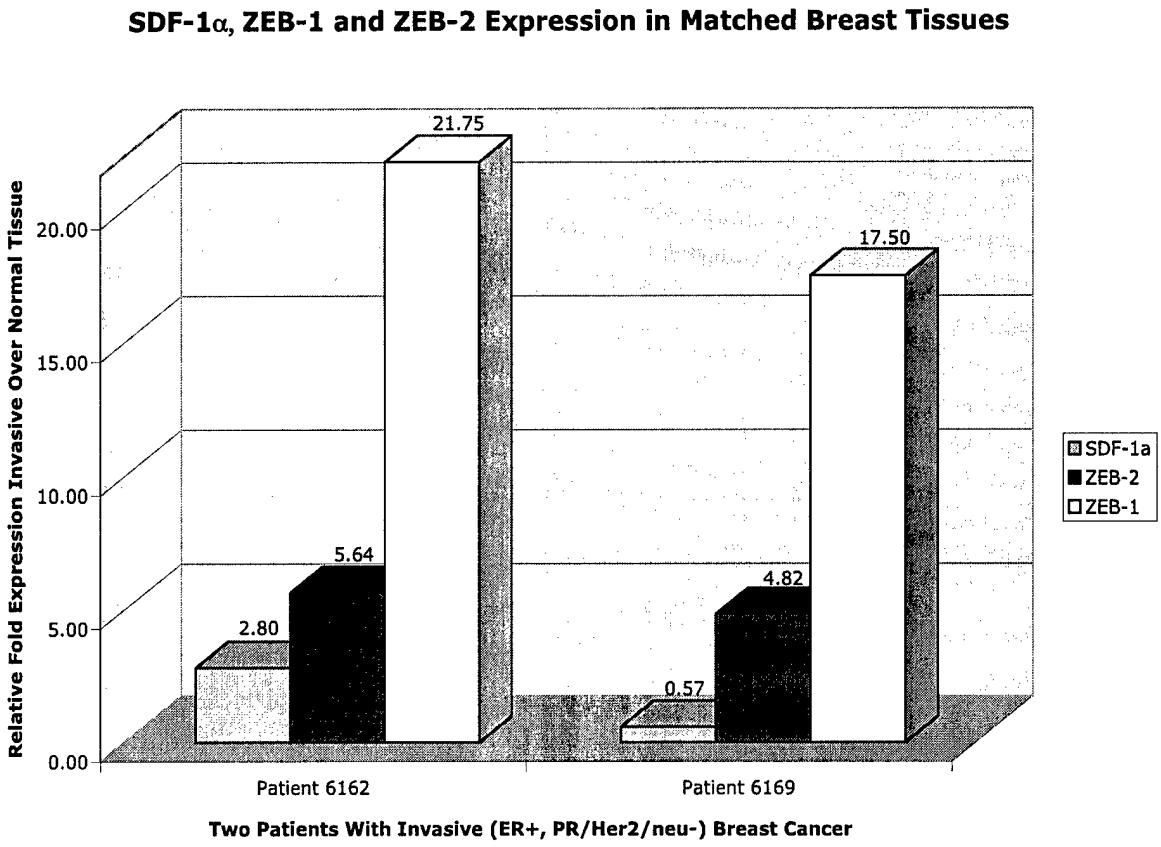
RNA was extracted from two breast cancer biopsy samples and subjected to reverse transcription to create cDNA. Real-time PCR was performed using the SDF-1α primers and TaqMan™ probe. Samples were run in duplicate. The curves are exactly the type of profile that one would expect if the primers/probe are performing as desired, and the replicates are within the optimal 1-C_T error. Thus, both the technique and the reagents are suitable and effective.

Appendix 2. Real-Time PCR Primers and TaqMan™ Probes

Oligomer Name	Primer or TaqMan™ Probe Sequence	T_M
ZEB-1 Forward Primer	5' TCC TGA GGC ACC TGA AGA GG 3'	58.9°C
ZEB-1 Reverse Primer	5' CAG AGA GGT AAA GCG TTT ATA GCC 3'	58.2°C
ZEB-1 Probe	5' CCA GAG GCA GGG CAC ACC AGA AGC 3'	68.5°C
ZEB-2 Forward Primer	5' CAC AGC CAT TAT TTA CCC AGA AGC 3'	59.2°C
ZEB-2 Reverse Primer	5' AGT TCC AGG TGG CAG GTC AT 3'	59.1°C
ZEB-2 Probe	5' AGC TGT CTC GCC TTG GCA CGC C 3'	68.5°C
SDF-1α Forward Primer	5' GTG GCA CTC AGA TAC CGA CTG 3'	58.8°C
SDF-1α Reverse Primer	5' GAG CCC ACA GAG CCA ATC AC 3'	59.0°C
SDF-1α Probe	5' CGC CGC CAC TGC CTT CAC CTC C 3'	68.8°C
RPL32 Forward Primer	5' CCA TCT CCT TCT CGG CAT CAT G 3'	59.4°C
RPL32 Reverse Primer	5' GGT TTC CGC CAG TTA CGC TTA 3'	59.2°C
RPL32 Probe	5' CCG CCC TCA GAC CCC TTG TGA AGC 3'	68.2°C

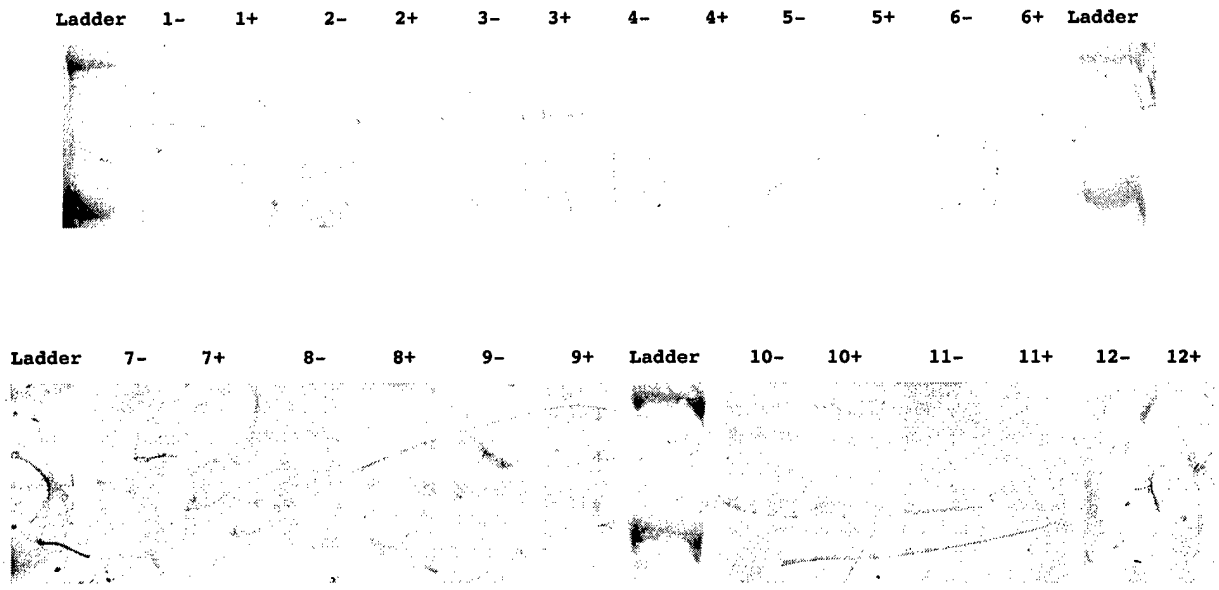
All of the above PCR primers and TaqMan™ probes have been tested in real-time PCR reactions on reproductive carcinomas. Conditions for all gene combinations have been determined such that a single curve is generated for each gene with no contamination or cross-reaction.

Appendix 3. Real-time PCR Results



Real-time PCR was performed on matched normal and diseased breast tissues from the same patients. The results indicate that expression of ZEB-1 in the invasive cancer tissue is increased 22-fold in one patient and 17.5-fold in the other patient relative to their normal tissue matches. SDF-1α expression does not follow a similar trend, suggesting ZEB-1 is no longer under the control of estrogen. ZEB-2 expression is also higher, by approximately 5-fold, in the invasive cancer tissues.

Appendix 4. ZEB-1 Protein Expression in Twelve MCF-7 Ecdysone-Inducible Clonal Cell Lines



MCF-7 cells were stably transfected with an ecdysone receptor construct and an ecdysone-inducible ZEB-1 construct. Twelve clonal lines were selected, expanded, and treated +/- ecdysone. Nuclear protein extracts were western blotted with α-ZEB-1 antibody. Clonal Line 3 shows the strongest expression of ZEB-1 in response to ecdysone induction.

Appendix 5. Chapter to be Published in the 4th Edition of Hormonal Carcinogenesis

Hormonal Regulation of ZEB-1 and Implication for Progression of Human Reproductive Cancers

Bynthia M. Anose, Michael P. Linnes, and Michel M. Sanders

Summary

The human Zinc finger E-box Binding (ZEB)-1 protein belongs to a family of transcription factors involved in critical developmental processes. Yet little is known of the mechanisms by which ZEB-1 is regulated. Our lab has recently demonstrated that the expression of ZEB-1 is induced by estrogen (E) in the ovarian cancer cell line Ov266 and that it is regulated by dihydrotestosterone (DHT) in the human PC-3/AR prostate carcinoma cell line. Interestingly, a dose-response assay indicates the expression of ZEB-1 is induced by 5 nM DHT and repressed at higher dosages. Cloning and analysis of approximately 1000 bp upstream of the translation start site of hZEB-1 revealed a number of putative E and androgen (A) response elements. Transient transfection assays indicate that this region is sufficient to confer responsiveness to both steroids. To determine whether expression of ZEB-1 could serve as a marker of tumor progression, real-time PCR (rtPCR) assays were performed on staged human reproductive carcinomas. Preliminary results indicate that expression of ZEB-1 increases as the normal ovary transforms to a primary carcinoma and continues to increase as the cancer progresses to an invasive and finally a metastatic state. There is an approximate 12-fold elevation in the expression of ZEB-1 in metastatic ovarian carcinoma relative to its expression in *in situ* cancers. rtPCR is currently being utilized to investigate the potential changes in ZEB-1 expression in breast and prostate cancer during the progression of these diseases. These data raise the possibility that overexpression of ZEB-1 contributes to the progression of reproductive carcinomas.

Introduction

Nuclear receptors (NR) comprise a large family of ligand-activated transcription factors known as the steroid hormone receptor superfamily; this is the largest eukaryotic family of transcription factors (1). The ability of these factors to influence gene transcription and other downstream events relies on the binding of their cognate ligands, the steroid hormones (2). Steroids facilitate the changes in conformation and dissociation of chaperone proteins from the NR that allow it to bind its respective hormone response element (HRE). The resultant changes in gene expression lead to varied and important physiological consequences.
Although considerable information is known about how NR regulate transcription, relatively little is known about the transcriptional cascades that they activate in vertebrates. Interestingly, we have found that E (3) and A (B.M. Anose and M.M. Sanders, unpublished) induce the ZEB-1/dEF1 (ZEB-1) transcription factor. This establishes a novel signaling cascade for these steroids. ZEB-1 is ubiquitously expressed and is required for mesodermal development (4, 5). Homologues of this important transcription factor have been cloned in many species: mouse, *Drosophila*, rat, hamster (6-10). Of particular interest is the observation that it can both repress (11-16) and activate transcription (3, 17). Understanding the actions of ZEB-1 has been complicated by the presence of a second family member, SIP1 or ZEB-2 (18 and references therein).

ZEB-1 is 1114 amino acids long, and it contains numerous functional domains that are reminiscent of classical transcriptional regulators (Fig. 1). The 7 zinc fingers are of particular note because of their potential roles in determining whether ZEB-1 activates or represses transcription on a specific target gene.

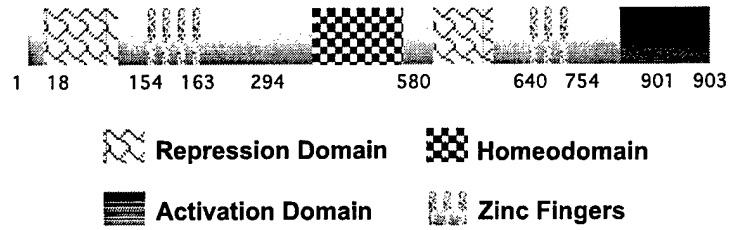


Figure 1. Human Zinc finger E-box Binding (ZEB)-1 protein contains 1114 amino acids. It is a typical zinc finger homeodomain protein containing two sets of Krüppel-type zinc fingers and a central homeodomain.

Materials and Methods

Transient Transfections. The PC-3/AR prostatic carcinoma cell line (kindly provided by K. Burnstein) was cultured as described (19). Transfections were carried out with Lipofectamine 2000 reagent (Invitrogen 2003) per the manufacturer's protocol. 6 hours post-transfection, steroid treatments were administered. The ovarian carcinoma cell line Ov266 (kindly provided by K. Kalli) was maintained as described (20). Transfections were carried out by electroporation in phenol red-free media containing the appropriate steroids. After 24 h, both cell lines were harvested. b-galactosidase assays were performed on total cell lysates with detection by luminometer, measuring 1sec/tube.

Real-Time RT PCR. Snap-frozen biopsy tissue samples were obtained through the University of Minnesota Cancer Center Tissue Procurement Facility (TPF) and the Cooperative Human Tissue Network (CHTN). Total RNA was harvested from each sample using the RNeAsy Extraction kit (Qiagen 2003) per the manufacturer's protocol. 2 mg RNA aliquots were reverse transcribed to create template cDNAs. 5 mL of each reaction was transferred to a SYBR Green rtPCR reaction and run on the *iCycler* machine (BioRad) using primers specific to human ZEB-1.

Results

Regulation of the hZEB-1 Promoter by Sex Steroids. Approximately 1 kb of the human ZEB-1 5'-flanking region was cloned by us from human genomic DNA by PCR and subcloned into the pBlueTOPO vector (Invitrogen). This construct was named pBlueZEB974. To test the responsiveness of this DNA region to A, transient transfections were carried out in the PC-3/AR prostate carcinoma cell line, and the cells were treated with 100 nM DHT for 24 h prior to harvesting for analysis of b-galactosidase expression. The high, constitutive expression of b-galactosidase from the control pCMV*SPORTbGal plasmid confirms that the transfections were performed successfully. The highly inducible expression of the pMMTV-LacZ plasmid confirms the cells are A-responsive. Interestingly, 100 nM DHT represses expression of the ZEB-1 construct by 10-fold.

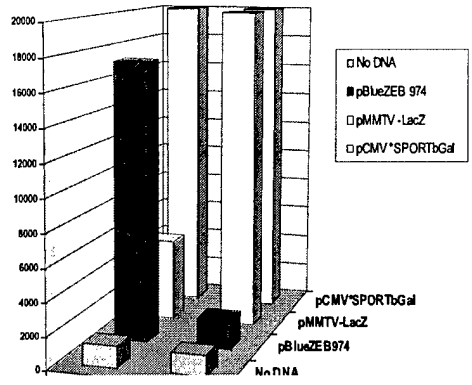


Figure 2. The PC-3/AR A-responsive prostate cell line was transiently transfected with 3 *LacZ* reporter plasmids: pBlueZEB974, which contains 974 bp of the hZEB-1 5'-flanking region, pMMTV-LacZ which is an A-responsive control, or with pCMV*SPORTbGal, which is a transfection control. The cells were then treated with 100 nM DHT for 24 h.

Similarly, the E-responsive Ov266 ovarian carcinoma cell line was transfected with pBlueZEB974, pCMV*SPORTbGal and an estrogen-inducible reporter construct (Fig. 3). The cells were treated with 100 nM 17b-estradiol (E_2) for 24 h and

processed as the PC-3/AR cells. In contrast to the effects of A in the PC-3/AR cells, pBlueZEB974 was induced about 5-fold by E in the Ov266 cells.

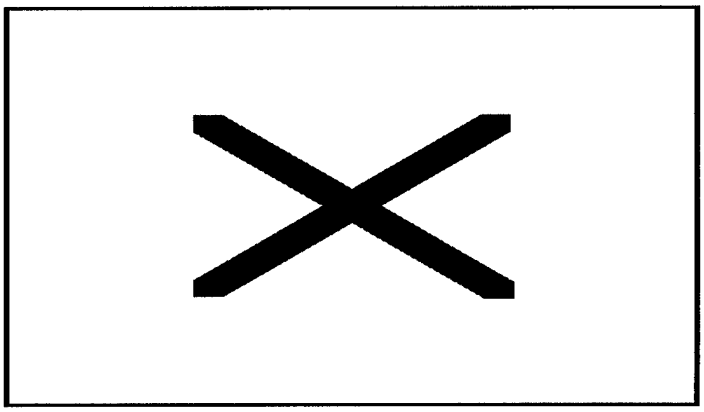


Figure 3. Ov266 cells were transiently transfected with pBlueZEB974, pGL3-900 (an E-responsive positive control), or pCMV*SPORTbGal. The cells were treated with 100 nM E_2 for 24 h.

Expression of hZEB-1 in Human Reproductive Carcinomas. To investigate the possibility that hZEB-1 may be involved in the etiology of cancers in sex steroid-responsive organs, the expression patterns of ZEB-1 in staged human ovarian and prostate carcinomas was studied. RNA was harvested from human biopsy samples and subjected to rtPCR. Based on the threshold crossing values (C_T), it appears that hZEB-1 expression does indeed increase during ovarian cancer progression (Fig. 4). Specifically, its expression in invasive carcinoma is 2-fold higher than its expression in *in situ* carcinoma, and its expression in metastatic carcinoma is 6-fold higher than in invasive carcinoma. Therefore, ZEB-1 expression overall increases approximately 12-fold as a primary ovarian cancer progresses to a metastatic state.
ZEB-1 resides at chromosomal location 10p11.2-p12, which is associated with some prostate cancers. Additionally, human RNA master blots reveal high expression of ZEB-1 in the prostate gland. This suggests, in conjunction with our observation that ZEB-1 is regulated by A, a potential role for ZEB-1 in prostate cancer. To study this possibility, human prostate samples were obtained from the CHTN. Thus far only 2 samples have been obtained and neither contains carcinoma. Analysis of these samples indicates that the expression of hZEB-1 is very similar in the normal prostate and in benign prostatic hyperplasia.

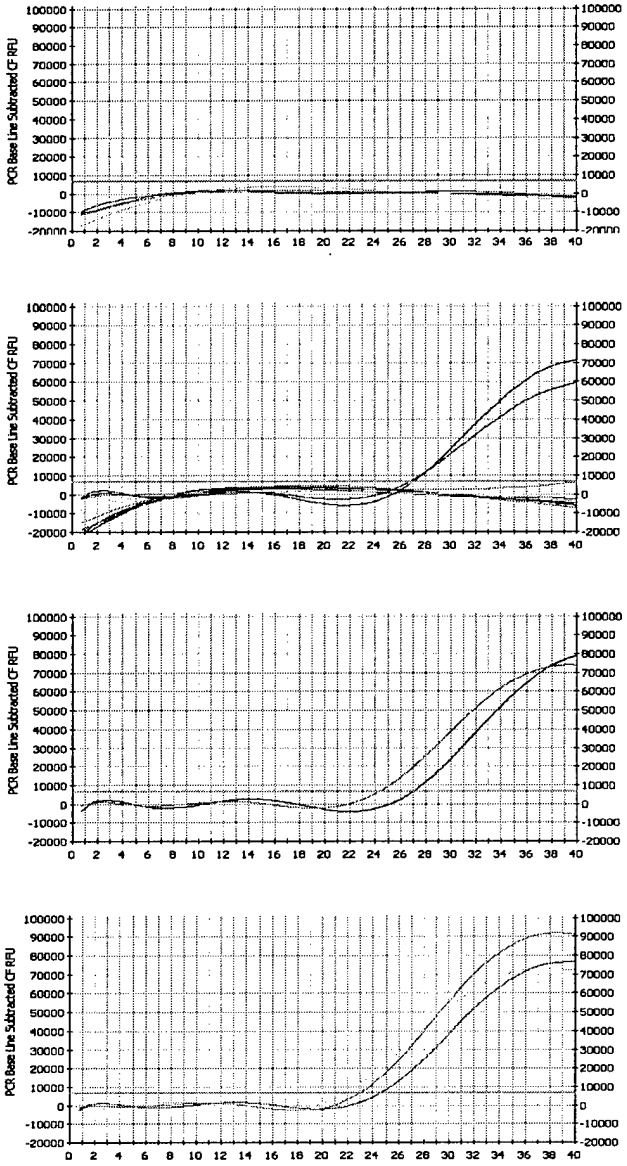


Figure 4. rtPCR was used to sensitively measure expression levels of hZEB-1 mRNA in ovarian biopsy tissues. A. In normal ovary, hZEB-1 expression levels are low, nearly undetectable N = 4 B. In primary *in situ* ovarian carcinoma, hZEB-1 expression is heterogeneous, with some samples having high and some very low levels. N = 12 C. In invasive ovarian carcinoma, hZEB-1 expression levels are approximately 2-fold higher than those in the *in situ* carcinomas. N = 2 D. In metastatic ovarian carcinoma, hZEB-1 expression levels are approximately 6-fold higher than those in invasive carcinomas, and 12-fold higher than those in the primary *in situ* carcinomas. N=3

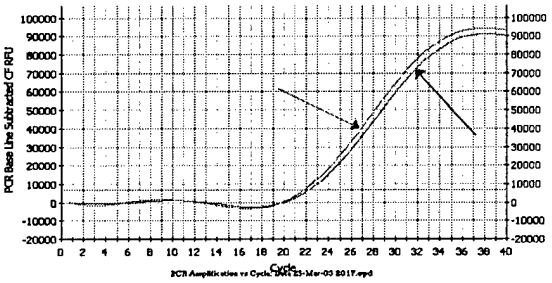


Figure 5. rtPCR was used to sensitively measure expression of hZEB-1 mRNA in prostate biopsy tissues. hZEB-1 expression is nearly identical in normal prostate and benign prostatic hyperplasia. N = 2

Discussion

Steroid hormones influence or control a large number of molecular pathways, and they induce an array of physiological responses in both healthy and disease states. The data presented here demonstrate that human ZEB-1 is a steroid hormone target gene. It is regulated by both A and E, in a dose-dependent manner. Recent dose-response assays (data not shown) demonstrate that the expression from pBlueZEB974 is actually induced at lower concentrations of DHT (1-5 nM) and repressed at doses from 7-100 nM DHT. Thus, it appears the hZEB-1 promoter may be exquisitely regulated by DHT in a highly sensitive manner. Interestingly, expression of ZEB-1 appears to increase as the normal ovary transforms to a primary carcinoma, and continues to increase as the cancer progresses to an invasive and finally a metastatic state. Its expression is unchanged in early stages of prostate cancer but has recently been correlated with progression of these cancers to the metastatic state (B.M. Anose and M.M. Sanders, unpublished). Thus, ZEB-1 is a molecular link between steroid hormones and the progression of various human reproductive cancers.

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Appendix 6. Abstract to be Presented at the 12th International Congress of Endocrinology

THE ANDROGEN-REGULATED ZEB-1 GENE IS A BIOMARKER FOR METASTATIC PROSTATE CANCER

Bynthia M. Anose, Megan M. Landry, and Michel M. Sanders, Ph.D.

Background: Steroid hormones regulate genes critical for both physiological and pathological processes. Our lab has shown that the ZEB (Zinc finger E-box Binding factor)-1 gene is a target of androgen signaling and may be involved in the etiology of male reproductive cancers.

Objectives: Having cloned the ZEB-1 promoter, our objectives are to map the androgen regulatory element(s) and to determine whether a correlation exists between ZEB-1 expression and prostate cancer progression.

Methods: A 974 base pair fragment upstream of the translation start site in the ZEB-1 gene was subcloned into a reporter vector. Two putative androgen response elements (AREs) in this region were then mutated alone and in combination. The wild-type and mutated vectors were transfected into the PC-3/AR prostate carcinoma cell line. Cells were cultured with or without 5 nM dihydrotestosterone (DHT) for 24 hours. To determine whether aberrant ZEB-1 expression is associated with prostate cancer progression, RNA was harvested from prostate biopsy tissues and subjected to real-time PCR using ZEB-1 specific probe and primers.

Results: Transfection experiments determined the wild-type ZEB-1 construct is induced 7- to 9-fold by DHT while none of the three mutated constructs were responsive. Real-time PCR assays revealed expression of ZEB-1 mRNA is unchanged in normal prostate, benign prostatic hyperplasia, and localized prostate cancer. A dramatic decrease in ZEB-1 expression (50- to 500-fold) correlates with progression of prostate cancer to the metastatic state.

Conclusion: The ZEB-1 gene is regulated by androgens acting through two AREs in its 5'-flanking region. ZEB-1 may server as a biomarker for metastatic prostate cancer.

Appendix 7. Abstract Presented at the Annual Meeting of the American Association for Cancer Research

STEROID HORMONES REGULATE EXPRESSION OF HUMAN ZEB-1: IMPLICATIONS FOR CANCER PROGRESSION

Anose, B.M. and M. M. Sanders, Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, Minneapolis, MN 55455

The human Zinc finger E-box Binding (ZEB)-1 transcription factor belongs to an ever-growing family of zinc-finger homeodomain proteins that are involved in critical developmental processes. From flies to humans, these factors have been shown to regulate a number of important pathways in processes as diverse as myogenesis, hematopoeisis, skeletal patterning, and gonadal development. Work in our lab has shown that ZEB-1 is often deleted or deregulated in various female reproductive carcinomas. It has also been reported that expression of ZEB-1 is significantly increased in invasive breast cancers, and even more so in invasive, metastatic tumors. In addition to this, the ZEB-1 gene has been chromosomally mapped to a locus deleted in some prostate cancers. Despite these findings, very little is known of the mechanisms by which ZEB-1 is regulated or the downstream targets of this important transcription factor.

A number of experiments were undertaken by our lab to elucidate the mechanisms of human ZEB-1 gene regulation and to study its role in reproductive carcinomas. We have previously reported that the chick homolog of ZEB-1 is induced by estrogen in primary chick oviduct cells. To investigate whether the human gene is also regulated by estrogen, the human Ov266 ovarian carcinoma cell line was treated with estrogen over a time course spanning 0-24 hours. Reverse Transcription (RT)-PCR analysis revealed the endogenous ZEB-1 gene is induced by estrogen within 3 hours of treatment. To investigate whether the human gene is regulated by any other class of steroid hormones, a dose response assay was performed to study its possible regulation by androgen. RT-PCR analysis revealed the ZEB-1 gene is also induced by 5 nM dihydrotestosterone (DHT) in the human PC-3/AR prostate carcinoma cell line.

As the human ZEB-1 promoter had never been identified, experiments were undertaken to clone this and the upstream regulatory region. Approximately 1000 base pairs of the region upstream of the translation start site of human ZEB-1 were successfully cloned. Analysis of this region reveals a number of putative estrogen, progesterone, and androgen response elements. Experiments are underway to test the functionality of these elements. Concurrently, the transcription start site is being determined. Experiments are also being performed to further investigate the presence and expression of ZEB-1 in various stages of human reproductive carcinomas. Preliminary results indicate that the ZEB-1 gene is present in rapidly proliferating female reproductive carcinomas and deleted in slow-growing cancers. Current experiments involve measuring the levels of ZEB-1 mRNA in reproductive cancers at each stage of progression.

Our results show that the ZEB-1 gene is regulated by at least two classes of steroid hormones. Furthermore, we hypothesize that the loss of regulation by steroid hormones contributes to the progression of reproductive carcinomas due to inappropriate ZEB-1 expression.

Appendix 8. Abstract Presented at the 4th International Symposium on Hormonal Carcinogenesis

HORMONAL REGULATION OF ZEB-1 AND IMPLICATION FOR PROGRESSION OF HUMAN REPRODUCTIVE CANCERS

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The human Zinc finger E-box Binding (ZEB)-1 protein belongs to a family of transcription factors involved in critical developmental processes. Yet little is known of the mechanisms by which ZEB-1 is regulated. Our lab has recently demonstrated the expression of ZEB-1 is induced by estrogen in the ovarian cancer cell line Ov266, and it is regulated by dihydrotestosterone (DHT) in the human PC-3/AR prostate carcinoma cell line. Interestingly, a dose-response assay indicates the expression of ZEB-1 is induced by 5 nM DHT and repressed at higher dosages. Cloning and analysis of approximately 1000 bp upstream of the translation start site of hZEB-1 revealed a number of putative estrogen and androgen response elements. Transient transfection assays indicate that this region is sufficient to confer responsiveness to both steroids.

To determine whether expression of ZEB-1 could serve as a marker of tumor progression, real-time PCR assays were performed on various stages of human reproductive carcinomas. Preliminary results indicate that expression of ZEB-1 increases as the normal ovary transforms to a primary carcinoma and continues to increase as the cancer progresses to an invasive and finally a metastatic state. There is an approximate 12-fold elevation in the expression of ZEB-1 in metastatic ovarian carcinoma relative to its expression in *in situ* cancers. This technique is currently being utilized to investigate the potential changes in ZEB-1 expression in breast and prostate cancer during the progression of these diseases. These data raise the possibility that overexpression of ZEB-1 contributes to the progression of reproductive carcinomas.