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Award Number: DAMD17-00-1-0532

TITLE: Identification of Alternative Splicing Factors Involved in Prostate Cancer Progression

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REPORT DATE: October 2001

TYPE OF REPORT: Annual

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2001	Annual (1 Oct 00 - 30 Sep 01)

4. TITLE AND SUBTITLE
Identification of Alternative Splicing Factors Involved in Prostate Cancer Progression

5. FUNDING NUMBERS
DAMD17-00-1-0532

6. AUTHOR(S)
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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

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

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

The goal of this project is to identify and characterize proteins factors that regulate splicing of fibroblast growth factor 2 (FGFR2) in prostate cancer cells. A change in the splicing of this transcript has been shown to accompany the transition of model prostate cancers from an androgen dependent to androgen independent phenotype. It is hypothesized that the factors that control this splicing "switch" may be involved in the acquisition of androgen independent growth. To accomplish these goals, we are making use of a genetic screening strategy to identify splicing factors that specifically block the splicing of an exon, IIIc, whose inclusion is seen in androgen independent cancers. We have established reporter constructs in which splicing is required in order to restore an open reading frame (ORF)to a green fluorescent protein (GFP) expression plasmid. An adenoviral derived intron has placed in the GFP ORF and been shown to be efficiently spliced to yield GFP expression that can be identified by flow cytometry. We have inserted FGFR2 sequences containing this intron and been able to show cell-type specific splicing of exon IIIc. Currently we are further establishing the screen by optimizing exon IIIc splicing in GFP reporter contructs prior to performing cDNA screening.

14. SUBJECT TERMS
prostate cancer, alternative splicing, androgen independent prostate cancer, protein factors, fibroblast

15. NUMBER OF PAGES
8

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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INTRODUCTION

The progression of prostate cancers from indolent, localized and androgen dependent tumors to aggressive, metastatic and androgen independent tumors involves a series of poorly understood biological and genetic events. Work from A rat prostate cancer model has demonstrated a change in the alternative splicing pathway of fibroblast growth factor receptor 2 (FGFR2) that accompanies this transition. Through a process of mutually exclusive alternative splicing of two exons (IIIb or IIIc) encoding an extracellular domain of FGFR2 gene, two different receptor isoforms (FGFR2 (IIIb) or FGFR2 (IIIc)) are produced which differ in their ligand binding specificities. FGF-R2 (IIIb), expressed in normal prostatic epithelial cells and androgen dependent tumors, is responsive to fibroblast growth factors 7 and 10 (FGF-7 and FGF-10), which are expressed in an androgen dependent manner by prostatic stromal cells and appears to promote epithelial cell differentiation. Androgen independent tumors express FGFR2 (IIIc), and it is hypothesized that this change in isoforms may render these cells unresponsive to this pathway of growth regulation. We recently have shown that this transition appears to occur in human prostate cancers cells as well. We are investigating the mechanism which leads to this change in alternative splicing of FGFR2 and seek to identify specific protein factors which direct the splicing pathways of this gene in prostate cancer cell lines. The androgen dependent DT3 rat prostate cancer cell line expresses exclusively FGFR2 (IIIb) and the AT3 cell line is an androgen independent line expressing only FGFR2 (IIIc). We are in the process of carrying out a strategy using FGFR2 minigenes to set up a genetic screen to identify and characterize proteins which cause a switch in alternative splicing.

BODY

The body of work demonstrating that a change in the alternative splicing of FGF-R2 may be a key step involved in prostate cancer progression led us to consider strategies towards identifying factors which mediate this splicing choice. It is clear that a loss in expression of FGF-R2 (IIIb) by itself is unlikely to completely explain the development of androgen independent and/or metastatic growth. Nonetheless, the involvement of this receptor in pathways of growth regulation in the prostate suggests that loss of the normal alternative splicing choice for this receptor may be one molecular event in a series of steps in the progression of prostate cancer. This hypothesis is supported by studies using the Dunning rat prostate tumor model showing that some reversal of the more aggressive phenotype can be achieved through restoration of the FGFR2 (IIIb) expression in androgen independent, metastatic AT3 cells. Therefore, we suspect that identification of the protein factors which result in maintenance (or loss) of FGF-R2 regulated splicing may further contribute to our understanding of prostate cancer. Our published studies strongly suggest that a key component of regulation is achieved through the expression of at least one factor in DT3 cells which is required in order to repress exon IIIc splicing and/or activate splicing of exon IIIb. While a single factor may not achieve both of these effects, it would appear that the presence of a factor mediating IIIc repression is necessary for regulation to be maintained in DT3 cells to prevent loss of FGF-R2 (IIIb) expression. It is hypothesized that identification of a factor mediating IIIc repression in

DT3 cells will demonstrate a protein whose function or activity is lost in the progression of many prostate cancers, with loss of splicing regulation being one consequence. We have made progress using the approach outlined in the proposal to begin to identify proteins that specifically repress exon IIIc splicing in DT3 cells. Our findings and progress to date will be included in an outline of the statement of work.

Task 1. Set up and perform a genetic screen to identify proteins involved in alternative splicing of FGF-R2.

- **Use PCR to create an intron within the coding sequence of pEGFP-N1 which disrupts the open reading frame and test this construct for splicing in DT3 and AT3 cells using transfection (months 1-4).**

We have used a PCR based method to engineer an adenoviral intron into the open reading frame of EGFP in this GFP expression construct. This construct has been stably tranfected into DT3 and AT3 cells and we have shown that in both cell types this constitutively spliced intron is completely spliced as shown by RT-PCR and furthermore that this splicing can also be demonstrated using flow cytometry using the University of Pennsylvania Flow cytometry facility. More recently we have also generated additional reporter constructs in which the an adenoviral pre-mRNA sequences with the same intron has been placed upstream of the normal GFP ORF such the ORF begins in the 5' adenoviral exon. The reason for this alternative approach will be described below.

- **Introduce FGF-R2 minigene sequences containing exon IIIc into the artificial intron of pEGFP-N1 to obtain plasmid pEGFP-IIIc (months 4-5).**

FGFR2 sequences containing intron sequences upstream of exon IIIc that repress exon IIIc spliced have been introduced into the intron of the EGFP expressing plasmid Different versions have been created including both those that contain all sequences required for exon IIIc repression (pEGFP-IIIc-plus) as well as those that have deletions of some such elements (pEGFP-IIIc-minus) for use as controls.

- **Transfect DT3 and AT3 cells with pEGFP and confirm efficient exon IIIc splicing in AT3 and repression in DT3. Prepare DT3 cDNA library in mammalian expression vector (months 5-7).**

pEGFP-IIIc-plus and pEGFP-IIIc-minus have been transfected into AT3 and DT3 cells and the resulting splicing pattern has been assessed by RT-PCR and by flow cytometry. The expectation was that in DT3 cells exon IIIc would be inefficiently included, whereas in AT3 cells exon IIIc would be included and thus GFP activity abolished. While we continued to observe complete skipping of exon IIIc in DT3 cells as expected, we observed variable levels of exon IIIc inclusion in AT3 cells. While this clearly resulted in differential splicing that could be distinguished by

RT-PCR and differences in GFP that could be detected by flow cytometry, there was nonetheless sufficient skipping of exon IIIc inclusion that GFP activity in AT3 cells transfected with this plasmid still had some residual GFP. Because the success of the screen will be significantly enhanced if we can achieve complete exon IIIc inclusion in AT3 cells we have thus generated additional expression constructs in which exon IIIc inclusion can also be assessed following Transfection. One version involves placement of a pre-mRNA sequences upstream of the EGFP ORF and in which splicing of this pre-mRNAs intron generates an open reading frame that originated in the 5' exon of this pre-mRNA that maintains the same ORF into the GFP sequence. We have thus also cloned exon IIIc and flanking intron sequences into this intron and are currently assessing exon IIIc inclusion in stably transfected AT3 and DT3 cells. Furthermore, current experiments in the lab are testing FGFR2 intron sequences for repression of heterologous exons. In fact we have found that intron sequences located upstream of exon IIIc are sufficient to repress heterologous exons in stably transfected DT3 cells, but not in AT3 cells. Thus, we should be able to substitute a more efficiently spliced exon for exon IIIc in its place that is more efficiently spliced. This same exon should thus be repressed in DT3 cells. We are currently adapting these constructswith heterologous exons for use in the GFP based screen.

- **Set up series of transfections with pEGFP-IIIc in AT3 cells, screen with DT3 cDNA library and sort cells by FACS analysis (months 7-18).**

Because we are still characterizing the best reporter construct to be used with the screen we have not as yet begun screening with DT3 cDNA libraries in AT3 cells. However, we have made use of a recent consulting arrangement with another lab at the University of Pennsylvania to assist in the generation of a cDNA library and to establish the best screening methodology. We have considered also attempting the screen using a retroviral approach and are in the planning stages of generating the cDNA library such that retroviral constructs can also be used to carry out the screen.

- **Isolate cDNAs from fluorescent FACS sorted AT3 cells and sequence the cDNAs. Perform Northern blot analysis in AT3 and DT3 cells to look at differences in expression. (months 18-24).**

<u>Task 2</u>. **Rescreen candidate cDNAs to confirm role in splicing repression and examine effects on splicing of endogenous FGF-R2 *in vivo*.**

- **Transfect AT3 cells expressing pEGFP-IIIc with candidate cDNAs to confirm repression of exon IIIc splicing (months 20-24).**
- **Test cDNAs for specificity of splicing repression using FGF-R2 sequences which lack ISAR and using constructs with heterologous exons (months 22-28).**
- **Test cDNAs for ability to activate exon IIIb splicing and to change splicing of the endogenous FGF-R2 gene in AT3 cells from use of exon IIIc to IIIb**

(months24-30).

- **Analyze the effects of transfection of DT3 cell derived cDNAs on AT3 cell growth, including restoration of FGF-7 dependent growth inhibition (months 30-36).**

Because the remaining tasks are dependent of completion of earlier tasks and steps outlined we have not as yet addressed these steps nor have results to report, but expect further progress in these tasks over the coming year.

KEY RESEARCH ACCOMPLISHMENTS

- Generation of a GFP reporter construct that contains an intron that is efficiently spliced and correspondingly restores the GFP open reading frame and GFP that can be assayed by flow cytometry.
- Demonstration that exon IIIc splicing in a GFP reporter contstruct is spliced in a cell type specific manner and that reduction in GFP can also be assayed by floe cytometry.
- Demonstration that intron sequences from FGFR2 that repress exon IIIc likewise repress heterologous exons when placed in a similar location upstream of such heterologous exons. Such results will permit additional screening methodologies.

REPORTABLE OUTCOMES

Abstracts:

Intronic FGFR2 cis-elements confer cell-type specific splicing regulation to heterologous exons in vivo and in vitro
Ruben Hovhannisyan, Stephanie J. Muh, and Russ P. Carstens.

A novel genetic screen for protein factors that regulate fibroblast growth factor receptor 2 (FGFR2) splicing
Stephanie J. Muh, Ruben Hovhannisyan, and Russ P. Carstens.

Both abstracts accepted for poster presentation at the RNA Society Meeting to be held in Madison, WI May, 28-June 2, 2002.

CONCLUSIONS

Identification of molecular mechanisms and genes whose expression is involved in prostate cancer progression are crucial for progress in generating future therapies and disease markers. Loss of factors that maintain the epithelial specific FGFR2 (IIIb) isoform and that lead to splicing of the mutually exclusive exon IIIc are hypothesized to be one step in the acquisition of androgen independent growth by aggressive prostate

cancers. We are continuing work to identify such factors using a novels genetic screening methodology. Thus far we have been able to show that expression of GFP can be easily assayed by flow cytometry in response to cell type specific splicing pathways and are beginning to screen for cDNAs encoding splicing regulatory factors. Several modifications of the original approach are also being tested in order to make the screen more robust. Specifically we are generating additional reporter constructs that demonstrate more efficient exon inclusion in AT3 cells that abolishes GFP activity. Significant progress has also been made to identify with greater precision the FGFR2 intronic sequence elements that mediate exon IIIc repression and this information will allow us to further tailor our methods to identify the factors that carry out splicing repression.