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14. ABSTRACT
We hypothesized that specific *ERG* splice forms in *TMPRSS2-ERG* fusion transcripts are selectively expressed in CaP cells and are functionally relevant in CaP. The specific aims of this study were to characterize full length sequences of *TMPRSS2-ERG* transcripts; to quantitatively evaluate selected *TMPRSS2-ERG* variants in CaP specimens and their prognostic features; and to defined the functional significance of specific splice variants of the rearranged *ERG* locus in CaP. We have identified two types of *TMPRSS2-ERG*,*Type I*, which encodes full-length ERG protein consisting SAM and ETS domains (ERG1, ERG2, ERG3), and *Type II,* encoding ERG proteins lacking the ETS domain (ERG8 and a new variant, TEPC). Increased ratio of *Type I* over *Type II* variants showed a correlation with poorly differentiated pathology /high Gleason score and outcome. We found that ERG3 a product of *Type I* splice variant, transcriptionally activates gene expression through ETS-regulated enhancers and ERG8 encoded by the *Type II* splice variant abrogated the transcriptional activator function of ERG3. We have generated an anti-ERG antibodies to detect the protein products of both *Type I* and *Type II* splice variants and demonstrated the correlation between detecting *ERG* genomic rearrangement and ERG oncoprotein human prostate tumors. We showed that *Type I/Type II ERG* ratios may play a role in defining the levels of *C-MYC* oncogene in a *TMPRSS2-ERG* fusion harboring prostate cancer cell culture model and revealed that *Type I/Type II* ratio correlated with *C-MYC* levels, higher Gleason sum and poor overall prognosis of prostate cancer patients.

TABLE OF CONTENTS

INTRODUCTION

We and others have shown that *ETS*-related gene (*ERG*) proto-oncogene is overexpressed in the prostate cancer (CaP) transcriptome using micro-dissected prostate tumor specimens (Ernst *et al.,* 2002; Vanaja *et al.,* 2003; Petrovics *et al.,* 2005). Our studies focusing on comparative quantitative expression of *ERG* in epithelial cells of matched benign and malignant prostate cells from a large patient cohort highlighted that CaP cells harbor frequent overexpression of *ERG* (60-70%) (Petrovics *et al*., 2005). Subsequent landmark study showed *ERG* gene as a common partner among gene fusions described in CaP (Tomlins *et al*., 2005). Genomic fusion of the androgen regulated *TMPRSS2* promoter (Lin *et al*., 1999; Nelson *et al*., 1999) to the *ERG* proto-oncogene protein coding sequences (Reddy *et al*., 1987; Rao *et al*., 1987) in CaP is now established as one of the most common mechanisms of the an oncogenic activation (Tomlins *et al.,* 2005; Kumar-Sinha *et al*., 2008; Rubin *et al*., 2011; Chinni *et al*., 2012). Detection and characterization of the ERG oncoprotein in CaP and other neoplasms has recently been reported by our group (Furusato *et al*., 2010; Miettinen *et al.,* 2011, Braun *et al.,* 2012, Rosen *et al.,* 2012) and by others (Park *et al.,* 2010; Yaskiv *et al* 2011; Magi-Galluzzi *et al.*, 2011; Minner *et al*., 2011). Activation of *ERG* has been recognized as a causal oncogenic alteration in CaP (Klezovitch *et al.,* 2008; Tomlins, *et al.,* 2008; Sun *et al.,* 2008; Carver *et al*., 2009; King *et al.*, 2009; Goldstein *et al.,* 2010). *ERG* gene is a member of the *ETS* family of transcription factors showing diverse expression patterns in human tissues (Turner and Watson, 2008). *ERG*, similar to other members of the ETS family, has been described as a mediator of mitogenic signals, such as mitogen activator protein kinases (Hart *et al.*, 1995). The multi-exon (17 exons) structure of *ERG* spans about 300 kb, and is transcribed to nine alternative splice variants and isoforms by a combination of alternative transcription initiation, mRNA splicing and transcription termination. The translated products of various *ERG* transcripts can function as oncoproteins with transforming activity (Rao *et al*., 1987; Sementchenko *et al*., 1998; Oikawa and Yamada, 2003; Rainis *et al*., 2005). Thus, due to its significance in CaP, *ERG* continues to be the subject of intense research investigations. In recent years, various *ERG* splice variants have been described, and their relative abundance in CaP cells have been examined (Wang *et al*., 2006 and 2008, Hu *et al*., 2008). However, much remains to be defined with respect to expression and functions of the *ERG* splice variants in CaP. This knowledge will further our understanding of *ERG* towards its clinical utility including patient stratification, treatment monitoring and therapeutic targeting of CaP.

To address these goals, the DoD-PCRP Idea grant award focused on the following specific aims:

- ***Characterization of full length sequences of TMPRSS2-ERG transcripts.***
- ***Quantitative evaluations of selected TMPRSS2-ERG variants in CaP specimens and prognostic features.***
- ***Defining the functional significance of specific splice variants of the rearranged ERG locus in CaP***.

The hypothesis of this proposal was that specific *ERG* splice forms in *TMPRSS2-ERG* fusion configuration are selectively expressed in CaP cells and are functionally relevant in CaP. Since the discovery of *TMPRSS2-ERG* fusions, several fusion transcripts were identified by our laboratory as well as others. The expression of some of these transcripts was shown to be associated with poor prognosis in previous studies (Wang *et al*., 2006 and 2008; Hu *et al*., 2008). However, careful evaluation of individual full length *TMPRSS2-ERG* transcripts is necessary to understand their biologic functions. Towards defining their functional significance, we generated a cDNA library from the *TMPRSS2-ERG* positive tumor specimens of CaP patients to identify and isolate full length *TMPRSS2-ERG* transcripts. Our characterization of the structure, expression and functions of full length *TMPRSS2-ERG* transcripts in CaP has led to a better understanding the relative expression and function of individual *TMPRSS2-ERG* splice variants in prostate cancer.

BODY

The findings reported here reflect major advances towards our understanding of the key *TMPRSS2-ERG* splice variants expressed in prostate cancer cells.

Aim #1: Characterization of full length sequences of *TMPRSS2-ERG* transcripts in prostate cancer. *The overall aim was to perform:*

- *Innovative analyses of CPDR-CaP cDNA library (at least 1,000,000 pfus) that would facilitate the identification of relatively common TMPRSS2-ERG splice and fusion variants in human CaP.*
- *Define ERG protein products encoded by TMPRSS2-ERG splice variants*
- *Develop ERG specific antibodies for the detection of ERG oncoprotein in prostate cancer*

Accomplished

- A new CPDR CaP cDNA library was developed from a pool of six *TMPRSS2-ERG* positive prostate cancer specimens.
- We for the first time established the expression of two major types of *TMPRSS2-ERG* transcripts in CaP: Type I included near full length transcripts that coded ERG protein with amino-terminal deletion of 32 amino acids; Type II included shorter transcripts encoding carboxy-terminal truncated proteins that lacked the ETS binding domain.
- We have defined the transcription initiation site of *TMPRSS2-ERG* fusion transcripts in prostate cancer cells towards the functional assessment of the *TMPRSS2* promoter and upstream sequences.
- Protein encoding products from the *TMPRSS2-ERG Type I* and *Type II* cDNAs were defined. We also established the distinct sub-cellular localization of the ERG Type I (nuclear) and Type II (cytoplasmic) proteins.
- First highly specific ERG monoclonal antibody (CPDR ERG-MAb) was developed that exhibited >99% specificity for detecting ERG expressing tumor cells in the prostate. There was almost complete concordance between the presence of the ERG fusion and ERG oncoprotein in early stage tumors. CPDR ERG-MAb showed superior specificity for ERG in comparison to commercially available ERG antibodies. This antibody has led to streamlined evaluation of ERG oncoprotein in prostate cancers world-wide.
- Using the CPDR ERG-MAb, we also developed the first mouse body map of the ERG protein showing normal expression of ERG in endothelial cells and specialized hematopoietic cells and lack of ERG in epithelial glands including prostate.

Novel Findings

Identification of *TMPRSS2-ERG* fusion variants in prostate cancer specimens. The CPDR CaP cDNA library was screened with both the *ERG* and *TMPRSS2* cDNA probe to identify *TMPRSS2-ERG* fusion cDNA clones. Clones were sequenced and analyzed for ERG protein coding reading frames. The identified clones contained 3' polyadenylation signals that clearly defined the 3' UTRs (Figure 1). The 5' transcription start sites of fusion transcripts within the *TMPRSS2* promoter downstream sequences were precisely defined by the 5'-RACE method that can enhance further evaluation of the gene regulatory sequences of *TMPRSS2-ERG* fusions (Figure 2). From the phage DNA sequences, plasmid (cDNA) clones were generated by using phage excision strategy. The most common *TMPRSS2-ERG* splice variants characterized from the CPDR

CaP cDNA library are summarized in Figure 1. As noted above, we identified near full length, *Type I* and shorter, *Type II TMPRSS2-ERG* transcripts. We obtained a representative number of full-length *TMPRSS2-ERG* clones of all major types. **Identification of these clones provides first of its kind information and reagents in defining complete coding sequences of *TMPRSS2-ERG* fusion transcripts in prostate cancer specimens.**

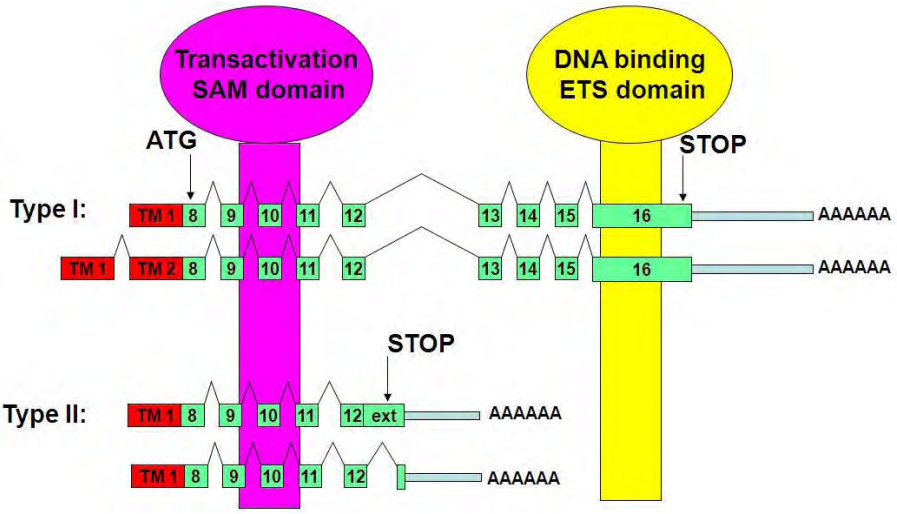

Figure 1. Common TMPRSS2-ERG splice variants expressed in prostate cancer.

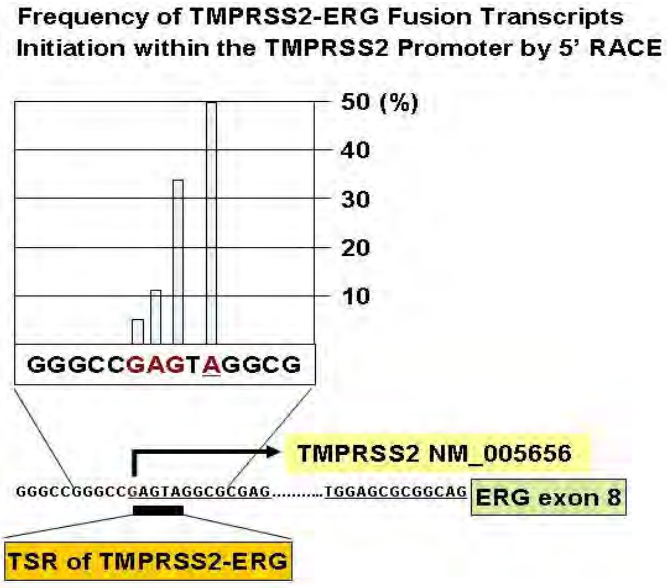

Figure 2. Frequency of transcription initiation of TMPRSS2-ERG fusion transcripts was defined by the 5' RACE method. Transcription initiation site of the wild type (NM_005656) TMPRSS2 transcript relative to the transcription start region (TSR) of TMPRSS2-ERG fusion transcripts and the TMPRSS2-to-ERG exon 8 junction are also shown.

In addition to expected near full length *TMPRSS2-ERG* transcripts, careful analysis of the cDNA clones also revealed tumor specific novel sequences with *TMPRSS2* gene fusions such as *TMPRSS2-ERG8, TMPRSS2-EPC1* and *TMPRSS2-EPC2*. Translational capabilities of these identified clones were analyzed by subcloning the cDNA in pIRES-EGFP expression vectors and transfecting into HEK293 and LNCaP cells (Figure 3.) Of note, characterization of these clones for the first time provides information on the full length protein coding sequences of *TMPRSS2-ERG* fusion transcripts which will aid in developing more precise bio-marker and therapeutic strategies.

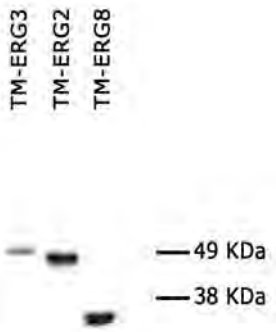

Figure 3. Detection of full-length ERG proteins products of TMPRSS2-ERG3, TMPRSS2-ERG2 and TMPRSS2-ERG8 coding sequences by expression of these cDNAs in HEK293 cells. Proteins of different molecular weights encoded by TMPRSS2-ERG3 (predicted MW: 54 kDa), TMPRSS2-ERG2 (predicted MW: 52 kDa) and TMPRSSS2-ERG (predicted MW: 37 kDa) were detected in immunoblot assay using the CPDR ERG monoclonal antibody.

Development of ERG specific monoclonal antibodies. On the basis of ERG translational products defined in this aim, our laboratory undertook the challenging task of developing ERG specific antibodies. Although the ERG oncogene was discovered in 1987, there has been no reliable ERG specific antibody available for evaluations of the endogenous ERG protein in cancer cell lines or clinical specimens. None of the commercially available ERG antibodies up until 2009 reliably detected the ERG protein even in the *TMPRSS2-ERG* positive VCaP cells. Since *ERG* is a member of the *ETS* gene family, that comprises of 30 closely related genes, cross reactivity of existing ERG antibodies to other ETS related proteins remained a challenge. **Our laboratory led the development of the first highly ERG specific monoclonal antibody (ERG-MAb) to detect ERG oncoprotein in clinical specimens.** The first ERG oncoprotein expression portrait was unveiled in the prostate, towards the goal of assessing its utility in prostate cancer diagnosis and prognosis (Furusato *et al.*, 2010). Specificity of the ERG-MAb was established by using cell culture models harboring endogenous or ectopic expression of *TMPRSS2-ERG* fusion (Figure 4).

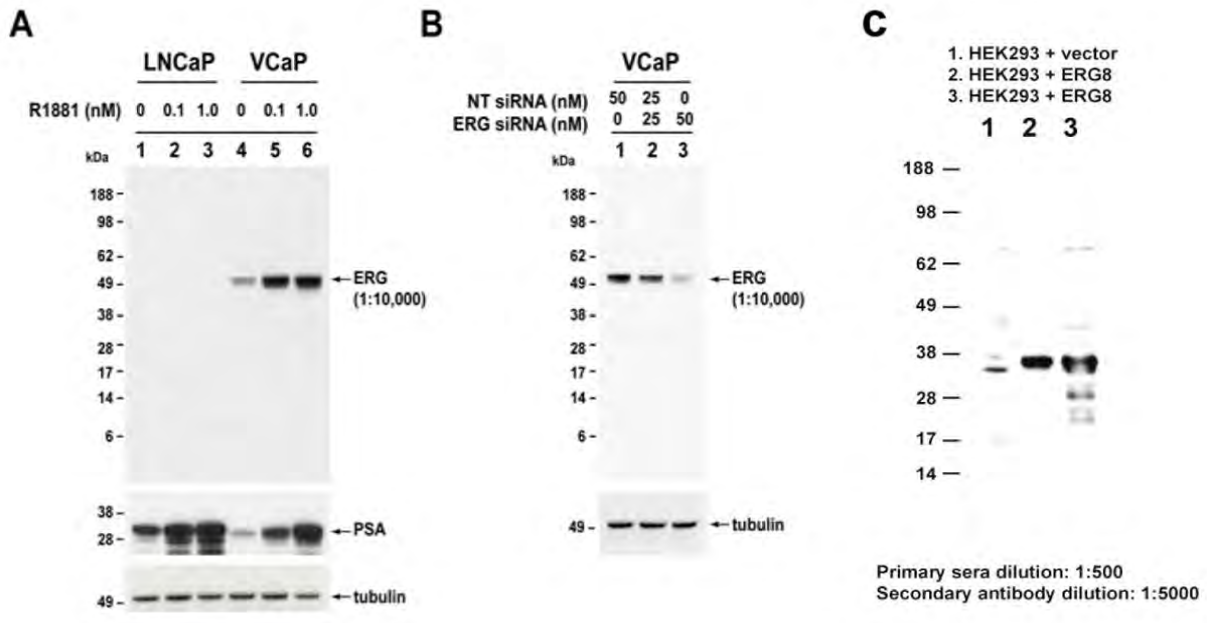

Figure 4. Evaluation of ERG specific monoclonal antibody and the ERG 8 specific polyclonal antibody. A, Detection of endogenous ERG protein expression in TMPRSS2-ERG harboring VCaP cells in response to androgen treatment (R1881) by the CPDR ERG monoclonal antibody (clone 9FY). LNCaP cells are TMPRSS2-ERG negative and do not express ERG protein. B, Evaluation of

knock-down of ERG protein expression by specific ERG siRNA using CPDR ERG monoclonal antibody (clone 9FY). C, Immunodetection of Type II ERG8 protein by a CPDR polyclonal anti-ERG8 antibody raised against an ERG8-specific C-terminal peptide.

Using representative whole-mount prostate sections from 132 patients, ERG protein expression was analyzed in PIN, tumor foci, benign glands and other cell types in the prostate. In randomly selected cases, ERG protein expression was correlated with *ERG* fusion status. ERG-MAb showed a striking specificity for detecting prostate tumor cells (>99.9%) (Figure 5). Specimens from 65% of patients had one or more ERG positive tumor focus. Examination of the ability to detect ERG positive PINs and ERG positive tumors within the same whole-mount sections revealed a 97% concordance.

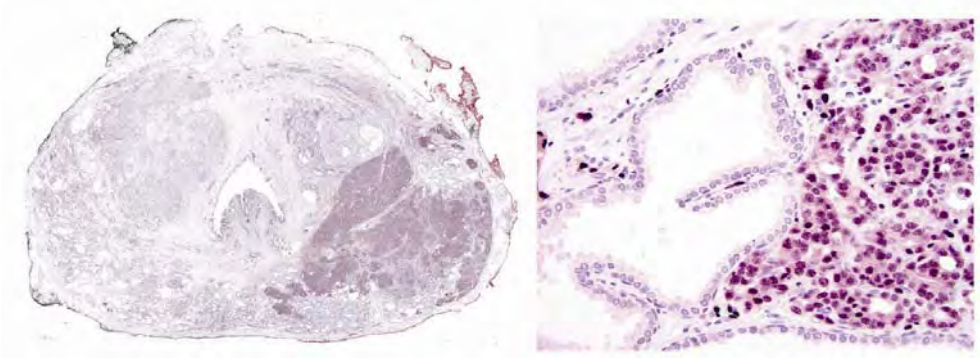

Figure 5. Whole-mount section (left) of representative prostate section from radical prostatectomy specimen was stained with ERG-MAb. Of the two main tumor foci in this section, one tumor focus was* ERG *positive. In ERG positive tumor focus, malignant epithelium or normal endotheials were ERG positive (right).

P	Benign glands	LGPIN	T1 PIN	T1	T2 PIN	T2	T3 PIN	T3	T4 PIN	T4
1			0	6						
2			1	6						
3		LGPIN	1	6						
4			1	6						
5			1	6						
6			1	6						
7			1	6						
8			0	7a						
9			0	7a						
10			0	7a						
11			0	7a						
12			1	7a						
13			1	7a						
14			1	7a						
15			1	7a						
16			1	7a						
17			1	7a						
18			1	7a						
19			1	7a						
20			1	7a						
21			1	7a						
22			1	7a						
23			1	7a						
24			1	7a						
25			1	7a						
26			1	7a						
27			1	7a						
28			1	7b						
29			0	7b						
30			0	7b						
31			0	7b						
32			0	8						
33			0	8						
34			0	8						
35			0	8						
36			1	8						
37			1	8						
38			1	8						
39			1	8						
40			1	8						
41			1	9						
42			0	9						
43			0	9						
44			0	9						
45			0	9						
46			0	9						
47			1	9						
48			1	9						
49			1	9						
50			1	10						
51			1	10						
52			0	6	0	6				
53			0	6	NP	6				
54			1	6	1	6				
55			1	6	1	6				
56	Benign		1	6	1	6				
57			1	6	1	6				
58			1	6	0	6				
59			1	6	0	6				
60			1	6	0	6				
61			1	6	0	6				
62			1	6	0	6				
63			NP	6	0	6				
64			0	7a	0	7a				
65			0	7a	0	6				
66			0	7a	0	6				

P	Benign glands	LGPIN	T1 PIN	T1	T2 PIN	T2	T3 PIN	T3	T4 PIN	T4
67			0	7a	0	6				
68			0	7a	0	6				
69			0	7a	0	6				
70			0	7a	0	6				
71			0	7a	NP	6				
72			0	7a	1	6				
73			0	7a	0	6				
74			1	7a	NP	6				
75			1	7a	1	6				
76			1	7a	1	6				
77			1	7a	1	7a				
78			1	7a	0	6				
79			1	7a	0	6				
80			1	7a	0	6				
81			1	7a	0	6				
82			1	7a	0	6				
83			NP	NP	0	6				
84			0	8	0	6				
85			0	8	0	6				
86			0	8	1	NP				
87			0	8	1	6				
88			1	8	1	7a				
89			1	8	1	6				
90			1	8	1	6				
91			0	9	0	6				
92			1	9	0	6				
93	Benign		1	9	0	6				
94			1	9	1	6				
95			0	6	0	9	1	6		
96		LGPIN	0	6	1	6	1	6		
97			1	6	1	6	1	6		
98			1	6	1	6	1	6		
99			1	6	0	6	0	6		
100			1	6	0	6	0	6		
101			0	7a	0	6	0	6		
102			0	7a	0	6	0	6		
103			0	7a	0	6	0	6		
104			1	7a	0	6	0	6		
105			0	7a	1	6	0	6		
106			0	7a	1	6	NP	6		
107			1	7a	0	6	NP	6		
108			1	7a	NP	NP	0	6		
109			1	7a	0	6	0	6		
110			1	7a	1	6	1	6		
111			1	7a	1	6	0	6		
112			NP	NP	1	6	0	6		
113			1	7a	0	7b	NP	6		
114			0	9	0	6	0	6		
115			0	9	0	6	0	6		
116			1	9	1	6	0	6		
117			0	6	0	6	0	6	0	6
118			0	6	0	6	0	6	0	6
119			0	6	0	6	0	6	0	6
120			0	6	1	6	0	6	1	6
121			1	6	1	6	1	6	0	6
122			1	6	1	6	0	6	NP	6
123			1	6	1	6	0	6	0	6
124			1	6	1	6	0	6	0	6
125	Benign		0	7a	0	6	0	6	0	6
126			0	7a	0	6	0	6	1	NP
127			1	7a	1	6	0	6	0	6
128			NP	7b	1	6	1	6	0	6
129		LGPIN	0	8	0	6	0	6	0	6
130			0	8	0	6	0	6	0	6
131			1	8	0	6	1	6	NP	6
132			NP	NP	0	6	0	6	0	6

ERG Type I splice variants encode the prototypical ERG protein (ERG1, ERG2, and ERG3) which includes the ETS DNA-binding domain and the nuclear localization signal (NLS), and *Type II* encodes the shorter version lacking both the ETS domain and NLS (ERG8 and a new variant, TEPC). To determine the subcellular localization of ERG proteins encoded by *Type I* and *Type II* splice variants, we expressed the protein products encoded by the *TMPRSS2-ERG3* and *TMPRSS2-ERG8* cDNAs in HEK293 cells and evaluated the subcellular localization by immunofluorescence assay. Transient expression in HEK293 cells revealed the presence of *TMPRSS2-ERG3* coded protein in the nucleus and *TMPRSS2-ERG8* product in the cytoplasm (Figure 7).

Figure 6. Summary of the ERG oncoprotein status in individual tumors of whole-mount prostate sections. Abbreviations: Red: ERG positive IHC; Green: ERG negative IHC; NP: not present in the section; LGPIN: "low grade PIN"; 1: ERG expression in PIN; Gleason score (7a: 3+4; 7b: 4+3) annotated in tumor columns (T1 to T4).

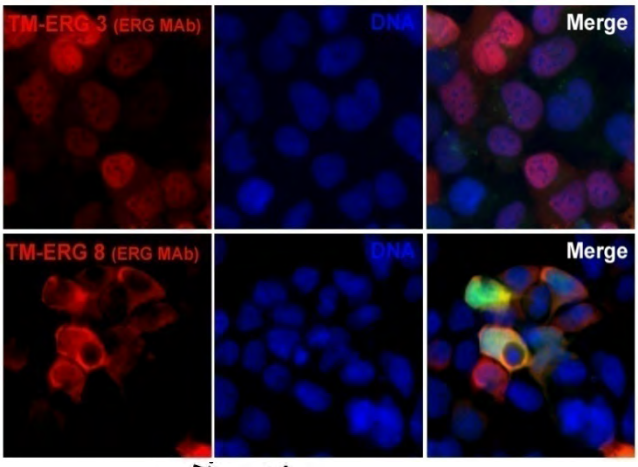

Figure 7. Immunofluorescence analysis of prototypic Type I and Type II ERG proteins revealed distinct sub-cellular localization of ERG proteins, in which Type I (TMPRSS2-ERG3) containing the NLS, was primarily localized to the nucleus, and Type II (TMPRSS2-ERG8), lacking the NLS, was found in the cytoplasm.

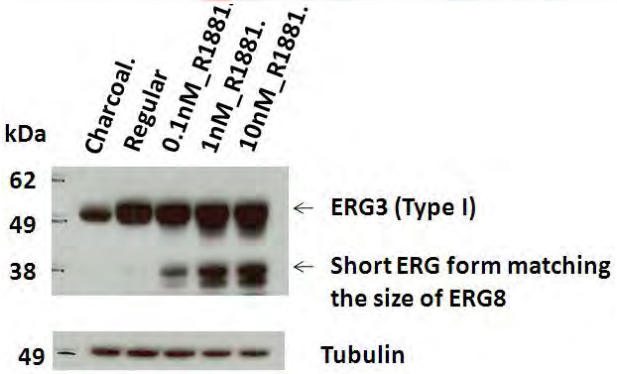

Figure 8. Androgen dose dependent expression of ERG3 protein, a product of Type I splice variant and the expression of a 38 kDa ERG protein matching the expected size of ERG8 (Type II).

Expression of ERG proteins in cancer cell lines. Since the CPDR ERG-MAb epitope is common to the ERG proteins encoded by both *Type I* and *Type II* splice variants, the availability of this ERG MAb provides unique opportunity for the evaluation of all ERG protein forms in CaP (Furusato *et al*., 2010; Miettinen *et al*., 2011). Theerfore, we evaluated ERG protein translated from *Type I* and *Type II* splice variants in various cancer cell lines. We have examined the androgen inducible expression of ERG proteins in the TMPRSS2-ERG harboring VCaP cell line, in response to increasing doses of the synthetic androgen hormone R1881. As expected, we observed the dose dependent increase in ERG3 protein expression. Under these experimental conditions, we also observed the increased expression of a protein product with molecular weight similar to the protein product of *Type II* transcript, ERG8 (Figure 8). This observation further highlights the need for evaluations of ERG 8 protein that has not yet been studied.

Similarly, we have observed ERG3 and shorter protein products in cell lines derived from diverse cancers: acute myeloid leukemia, KG-1; colon cancer, COLO 320; acute T lymphoblast leukemia, MOLT4; and prostate cancer, VCaP. In addition to the expression of *Type I* splice variant product,we consistently detected shorter protein products (Figure 9).

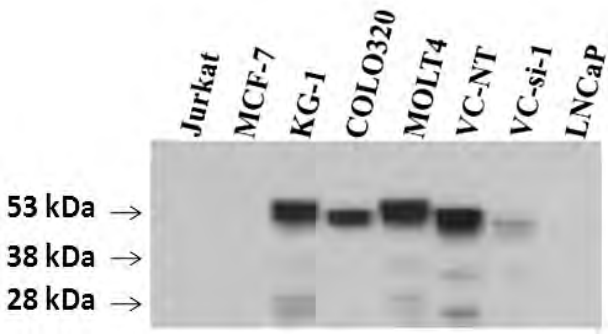

Figure 9. Detection of ERG protein in prostate and non-prostate cancer cell lines, by CPDR ERG-MAb. ERG protein expression was analyzed by using cell lysates from cell lines derived from diverse cancers: Jurkat (acute T cell leukemia); MCF7(breast cancer), KG1 (acute myelogenous leukemia), COLO320 (colon carcinoma); MOLT-4(acute lymphoblastic leukemia); VC-NT (VCaP prostate cancer cells treated with non target siRNA); VC-si-1(VCaP prostate cancer cells treated with ERG specific siRNA); LNCaP (prostate cancer cell line). Jurkat, MCF7, and LNCaP cells were used as negative controls.

Temporal/spatial expression of Erg proteins in developing mouse. Using the CPDR ERG-MAb, we also analyzed the normal expression pattern of the mouse Erg protein in developing and adult mouse tissues. The most striking of these observations was the highly selective and abundant expression of ERG protein in endothelial cells of mouse tissues (Figure 10). For the first time, we also illustrated that endogenous ERG was not expressed in normal mouse prostate epithelium (Mohamed *et al*., 2010). Similar findings were noted for human prostate (Furusato *et al.,* 2010). These observations highlight the cancer-specific aberrant expression and function of ERG in the prostate epithelium.

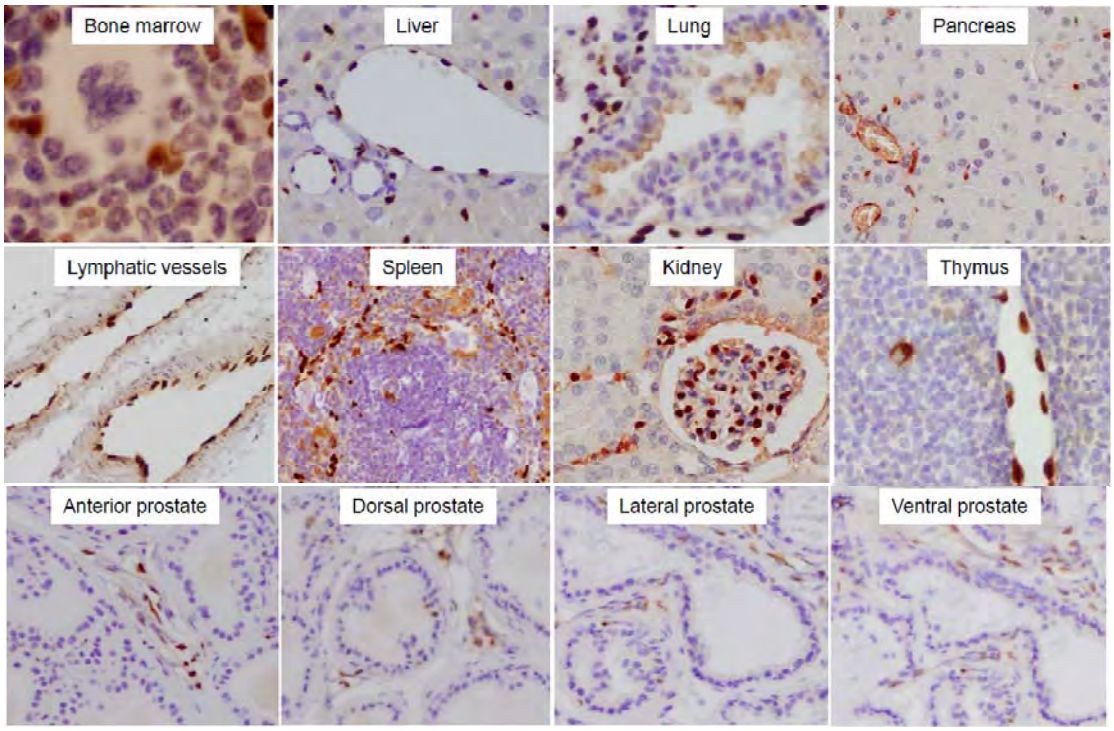

Figure 10. Widespread immunolocalization of ERG proteins was observed in endothelial cells and restricted expression in precartilage and hematopoietic tissues. ERG is not expressed in any epithelial tissue including prostate epithelium (lower panels), or in infiltrating lymphocytes that are occasionally seen in the prostate, a common site of tumors with ERG rearrangements and unscheduled ERG expression.

Aim #2: Quantitative evaluations of selected *TMPRSS2-ERG* variants in prostate cancer specimens and prognostic features.

Towards this aim, we had proposed to perform:

- *Parallel quantitative analyses of up to 6 selected most abundant TMPRSS2-ERG variants in LCM tumor and matching benign epithelial cells from 150 patients representing primary prostate cancer specimens.*
- *Correlations of quantitative expression of the most abundant TMPRSS2-ERG variants with clinico-pathologic parameters including patient age, race, pre-treatment PSA level, Gleason score, CaP family history, tumor stage, surgical margin status, seminal vesicle invasion, PSA recurrence, bone metastasis, nuclear grade, differentiation, and a follow-up for cancer recurrence by serum PSA after surgery PSA doubling time and prostate cancer associated death.*

Accomplished

- Quantitative RT-PCR analyses of fusion transcripts from 122 evaluable patients have shown the relative abundance of three *Type I ERG* transcripts (*TMPRSS2-ERG 1, 2, 3*) and two *Type II ERG* transcripts (*TMPRSS2-ERG8 and TMPRSS2-EPC1*) in prostate tumor cells.
- Significant correlation was found between increased ratios of *Type I* (*ERG*1-3) over *Type II* (*ERG*8 and *TEPC1*) splice variants and higher Gleason sum and poorly differentiated phenotype.

Novel Findings

Evaluation of quantitative gene expression of fusion transcripts in matched patient specimens. Transcripts of the ERG locus were defined by quantitative PCR, using the CPDR prostate cancer mRNA bank. Towards this task, we performed parallel quantitative analyses of *ERG 1/2, ERG 3, TEPC and ERG 8* splice variants in laser capture micro-dissected, tumor and matching benign, epithelial cells from 122 patients representing primary prostate cancer specimens (Figure 11). In general, detectable expression of an ERG mRNA splice variant in CaP cells correlated with the ERG fusion status. Surprising findings was the detection of high levels of *ERG Type II* transcripts in prostate tumor specimens in relation to *Type I* transcripts. The distribution and level of various *ERG* splice variants in tumor specimens of each patient is shown in Figure 11.

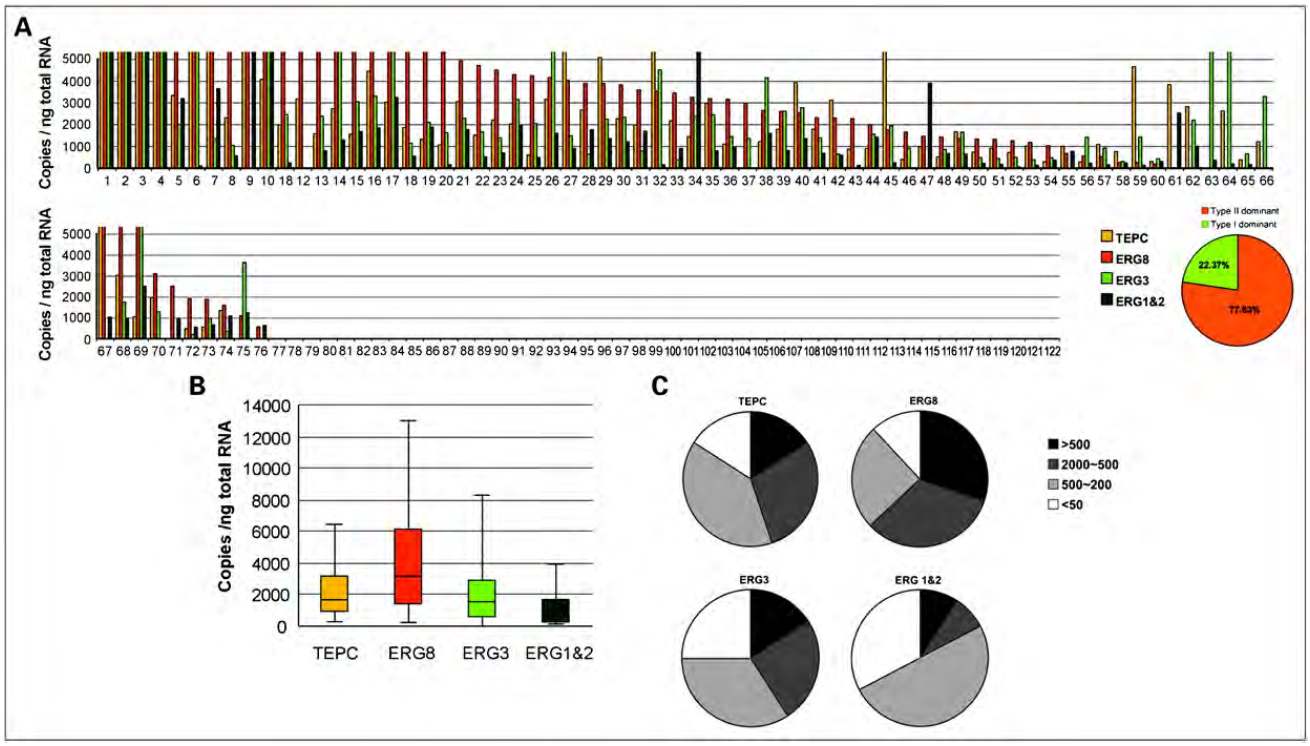

Figure 11. Quantitative expression of ERG splice variants in prostate cancer patients. A, Quantitative expression of ERG splice forms ERG8, TEPC, ERG3 and ERG 1&2 (represented by columns with different colors) were determined in micro-dissected tumor cells of CaP patients (N=122). The graph depicts relative expression levels (normalized to GAPDH) in patients with (upper panel, N=66), and

without (lower panel, N=56, detectable TMPRSS2-ERG fusion A transcript. In the pie chart, the percentage represents CaP patients with either higher (~77%) Type II (ERG8 and TEPC) expression, or higher (~23%) Type I (ERG1-3) expression level. B, Relative abundance of various ERG splice forms (ERG8, TEPC, ERG3 and ERG 1-2) depicted by boxplots, represents the copy numbers determined in the 76 CaP patients overexpressing ERG. C, Pie charts illustrate the distribution of CaP patients with various levels of expression (copy number/ng total RNA) of ERG8, TEPC, ERG3 and ERG 1&2 splice forms in CaP cells.

Prognostic potential of ratios of *TMPRSS2-ERG* variants in prostate cancer patients. Towards defining the relative abundance and the ratios of the full-length fusion transcripts, we evaluated the association of various *ERG* variants with clinico-pathologic parameters. Quantitative expression was assessed for patient age, race, pre-treatment PSA level, Gleason score, CaP family history, tumor stage, surgical margin status, seminal vesicle invasion, PSA recurrence, bone metastasis, nuclear grade, differentiation, PSA doubling time and prostate cancer associated death. This study addressed the potential of ERG splice variants for important biological and clinical relevance. A remarkable finding of this study is that an increased ratio of *Type I ERG* (*ERG*1-3) over *Type II* (*ERG*8 and *EPC*1) splice variants tightly correlates with higher pathological Gleason sum and poor LCM differentiation (Table 1), (Hu *et al.*, 2008).

Clinico-pathological characteristics	**ERG splice variant expression**			**Type I/Type II ratio**		
	No (46)	**Yes (76)**	**P value****	**N (76)**	**median**	**P value***
Race			**0.0345**			0.1142
Caucasian	29 (33%)	59 (67%)		59	0.51	
African American	15 (56%)	12 (44%)		12	0.33	
Family history			0.6312			0.3259
No	28 (38%)	45 (62%)		45	0.52	
Yes	10 (33%)	20 (67%)		20	0.51	
Pathological T stage			0.5318			0.2541
pT2	14 (36%)	25 (64%)		25	0.44	
pT3	29 (42%)	40 (58%)		40	0.51	
Pathological Gleason sum			**0.0023**			**0.0323**
2 to 6	9 (24%)	29 (76%)		29	0.35	
7	20 (36%)	36 (64%)		36	0.46	
8 to 10	14 (70%)	6 (30%)		6	0.70	
LCM differentiation			**0.0058**			**0.0067**
Well	31 (32%)	66 (68%)		65	0.45	
Poorly	15 (62%)	9 (38%)		9	0.76	
Margin status			0.9436			**0.0032**
Negative	28 (38%)	45 (62%)		45	0.39	
Positive	16 (39%)	25 (61%)		25	0.57	
PSA recurrence			0.7312			**0.0456**
No	33 (37%)	56 (63%)		56	0.42	
Yes	11 (41%)	16 (59%)		16	0.61	

*One-sided test, $P<0.05$ was considered statistically significant.

** Two-sided test, $P<0.05$ was considered statistically significant.

Table 1. Type I/Type II ratio (N=76): The ratio of ERG Type I/Type II splice variants in CaP cells is increased in patients with poor tumor cell differentiation and with PSA recurrence.

Detection of genomic rearrangements in prostate specimens with FISH. Prostate cancer specimens were assessed for their genomic *ERG* rearrangement status by applying dual-color fluorescence in situ hybridization (FISH) ERG break-apart assay to consecutive sections (Figure 12). There was high concordance (>95%) of ERG rearrangement detected by FISH assay and ERG protein detection by CPDR-ERG MAb (Furusato *et al.*, 2010; Braun *et al.*, 2012).

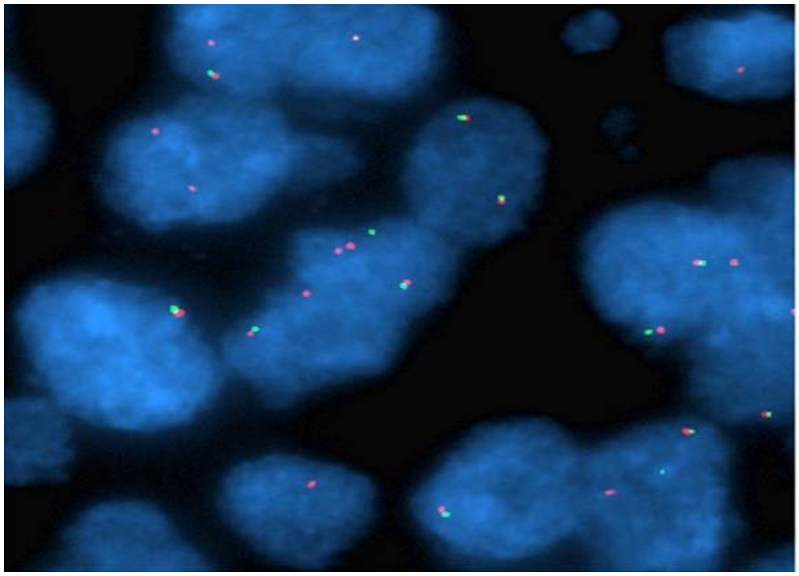

Figure 12. Separate and distinct red and green signals indicate a fusion of the TMPRSS2 and ERG loci. Loss of the green signal complementary to the telomeric 5'ERG represents a deletion in this chromosomal region. For the non-rearranged ERG locus, the set of probes appear either as juxtaposed red and green signals or yellow spots due to the overlap between the red centromeric or green telomeric probes.

Aim #3: Defining the functional significance of specific splice variants of the cancerous ERG locus.

Towards this specific aim we had proposed:

- *Evaluation of multiple inhibitory siRNA molecules for targeting specific ERG splice variants expressed in CaP.*
- *Evaluation of transcription factors activity of proteins coded by ERG Type I and Type II splice variants.*
- *Assessment of the potential dominant negative function of ERG Type II for ERG Type I.*
- *Evaluation of cancer biology related features (cell growth, soft agar colony formation, cell invasion and changes in cell cycle of cell) of the cells in response to knock- down or heterologous expression of specific TMPRSS2-ERG transcripts.*

<u>Accomplished</u>

- Inhibitory siRNA molecules were successfully designed and tested to selectively target the *ERG1-3 (Type I)* and *ERG8 (Type II)* variants.
- The transcription factor activity of *Type I* and *Type II* products were tested by using different ERG targeted promoters in luciferase assay systems. In contrast to nuclear ERG Type I variants, ERG Type II variants with cytoplasmic localization were inactive for transcription factor activity.
- Ectopic expression of the *ERG Type II variant (TMPRSS2-ERG8)* in *TMPRSS2-ERG* positive VCaP cells led to the inhibition of levels of ERG Type I protein products, suggesting for negative regulation of functional *ERG* Type I variant by the *ERG Type II variant*.

- Higher ratios of *ERG Type I* vs. *ERG T*ype II variants in patient specimens correlated with increased expression of the ERG target , *C-MYC* an important observation suggesting that levels of ERG *Type I* and *Type II* variants may determine overall biological function of *ERG* in prostate tumorigenesis.

Novel Findings

Knock-down of *ERG* using siRNA for the inhibition of *Type I* and *Type II* splice variants. To define the functional significance of *ERG* and its splice variants, *ERG* shRNA and *ERG* siRNA molecules were used in the *TMPRSS2-ERG* positive VCaP cell model. **Simultaneous inhibition of both Type I and Type II variants by ERG siRNA and shRNA molecules resulted in significant morphological alterations and growth inhibition of VCaP cells, supporting the overall ERG inhibition as a rational strategy for ERG targeted therapy for CaP (Figure 13).**

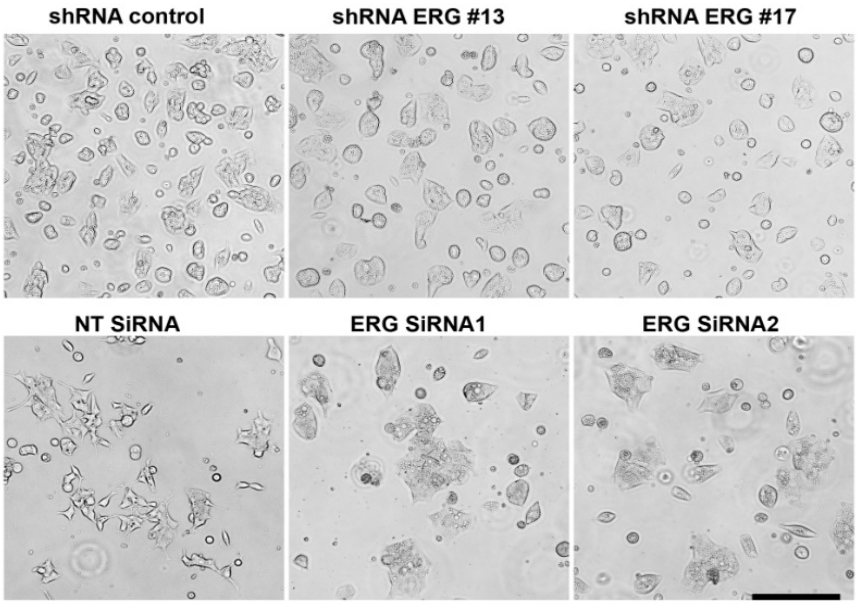

Figure 13. ERG knockdown by various siRNA and shRNA molecules in VCaP cells show striking alteration of cellular morphology. Cells depleted in all ERG splice forms show a more rounded morphology instead of the less differentiated phenotype observed in the control.

In addition to changes in cellular morphology, ERG knock-down in VCaP cells exhibited concomitant depletion of C-MYC and over-expression of intercellular tight junctions marker, such as ZO-1 (Figure 14).

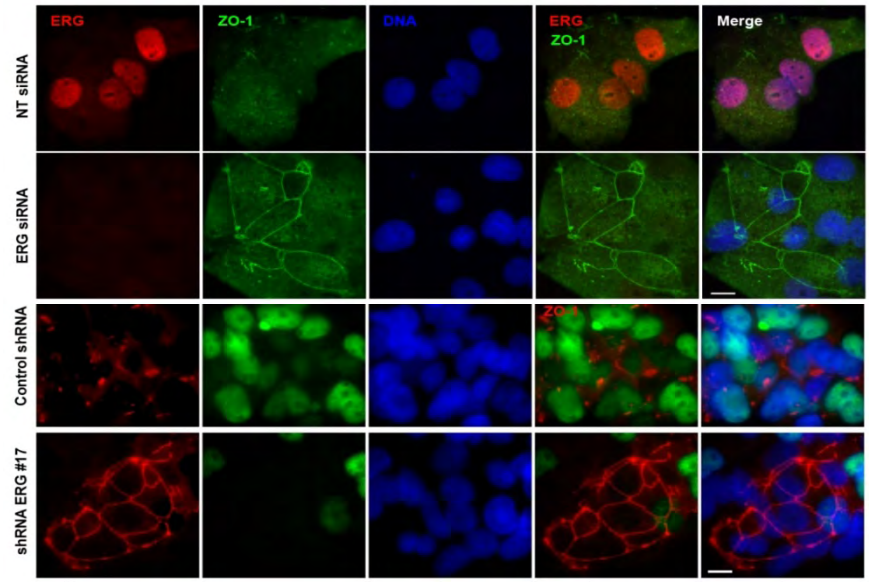

Figure 14. Deposition of ZO-1 indicates the formation of tight junctions and establishment of new permeability barrier, suggesting that inhibition of morphological differentiation by ERG was rescued by ERG knockdown. Downregulation of C-MYC in response to the inhibition of ERG shown at the lower panels.

Towards the goals to generate siRNAs that specifically inhibit *Type II* variant, we have generated a siRNA for the knock-down of ERG8 without interfering with the expression of protein products of *Type*

I transcripts. We designed and evaluated specific siRNA molecules for knocking down the *Type II* splice variant, *ERG8*, in HEK293 cells transiently co-transfected with *TMPRSS2-ERG8* and *TMPRSS2-ERG3* plasmid expression vectors. Inhibition of *ERG8* siRNA markedly reduced the expression of ERG8 protein levels in comparison to ERG 3 protein levels (Figure 15).

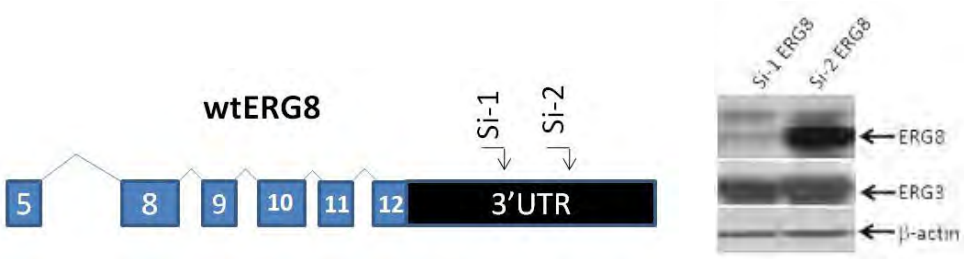

Figure 15. Inhibition of ERG8 protein in response to ERG8 selective siRNA. We have designed siRNA molecules for targeting specific ERG splice variants which were assayed for inhibiting ERG8. Selective inhibition of the ERG8 protein in immunoblot assay (right panel) demonstrates the identification of a siRNA specific for ERG8. The ERG3 protein, encoded by Type I, was minimally affected by ERG8 siRNA.

In addition, we have developed a rabbit polyclonal anti-ERG8 antibody in order of thoroughly evaluate the *Type II* splice variant, ERG8. Initial characterization of the anti-ERG8 antibody confirmed the specificity of the antibody in detecting ERG8 protein by Western blot assays.

Evaluation of the transcription regulatory function of *ERG Type I* and *Type II* products by using luciferase assay systems. The Myocyte Enhancer Factor 2 (MEF2) family of transcription factors are expressed in multiple cell types and are important regulators of development and differentiation (DeVal *et.al*, 2004; Wei *et al*., 2010). The enhancer from the mouse *mef2c* gene is a well characterized enhancer that has been shown to be active in the vascular endothelium during embryogenesis and in adulthood where endogenous ERG protein is highly expressed (Mohamed *et al.*, 2010; Furusato *et al*., 2010; Miettinen *et al.*, 2011). Therefore, we have utilized the *mef2c* vascular endothelial enhancer sequence, which harbors a cluster of four conserved elements for ETS factors, including ERG, to evaluate the

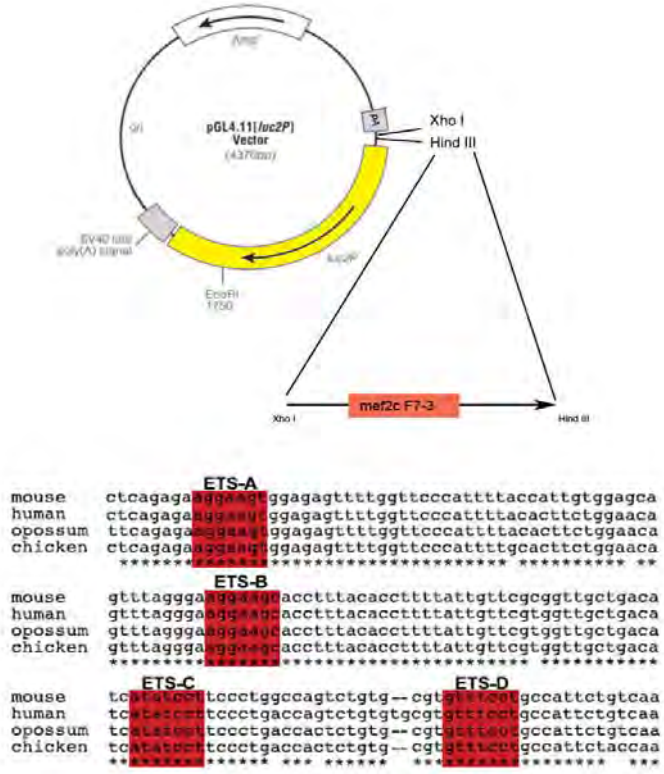

Figure 16. Design of a Luciferase reporter vector using the mouse mef2c vascular endothelial enhancer, for evaluation of transcriptional regulatory function of ERG splice variants.

transcriptional regulatory function of ERG splice variants (Figure 16). Although we had originally intended to use other regulatory sequences (*TGF-beta*, *MMP3* and *collagen*), based on studies of *mef2c* gene expression regulation, we anticipated a stronger read out using a *mef2c* enhancer based luciferase reporter construct, for assessing the regulatory activities of *ERG* splice variants. The *mef2c* enhancer was cloned into the pGL4.24[*luc2P*/minP] vector that carries a minimal promoter upstream of the luciferase reporter gene *luc2P*, which is designed for high expression, reduced anomalous transcription, and sensitivity to activation and repression.

In addition to the *mef2c* enhancer, we also examined the transcriptional regulation of *MASPIN-297* by *ERG* splice variants (Figure 17). The maspin gene encodes a 42-kDa protein and belongs to the serine protease inhibitor (serpin) superfamily. MASPIN expression was originally detected in normal breast and prostate epithelial cells, whereas tumor cells exhibited reduced or no expression. Maspin has been shown to inhibit tumor cell invasion and metastasis in breast tumor cells. Previous studies have shown that maspin is a target of prostate derived ETS factor, PDEF, and transcriptionally regulated though ETS binding domains (Zhang *et al*., 1997; Zou *et al.*, 2000) of which there are two within 297 bp of the start site. The promoter region of Maspin was amplified by PCR according to reported DNA sequences. The pM-Luc(−759;+87) was generated and cloned into the *Xho*I and *Hin*dIII sites of the pGL3 basic vector (Promega). pM-Luc(−297;+87) was then made by deleting the *Pst*I fragment from pM-Luc(−759;+87).

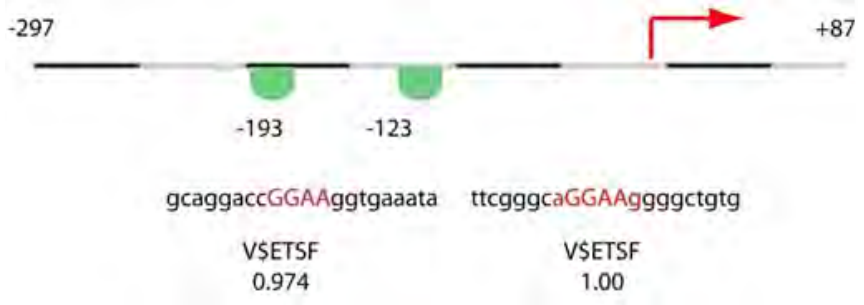

Figure 17. MASPIN(-297) promoter containing two ETS binding sites within 297 bp of the transcription initiation site, used to design a luciferase reporter vector to evaluate the regulatory function of ERG splice variants.

The pGL4.24-*mef2c*-[*luc2P*/minP] and pM-*Maspin* -Luc(-297) reporter constructs were individually transfected with 0-80 ng of expression vectors encoding Type I or Type II ERG proteins in HEK293 cells. Both full length (wt ERG3) and the N-terminus truncated Type I ERG protein (product of *TMPRSS2-ERG* fusions) activated the transcription from the *mef2c* and *MASPIN* driven luciferase reporter constructs. Consistent with the absence of nuclear localization signal in the *Type II* product, encoded by *TMPRSS2-ERG8*, the ERG8 protein did not alter the basal promoter activity of the reporter constructs which was similar to the frame shift *TMPRSS2-ERG3* mutant that served as a negative control (Figure 18).

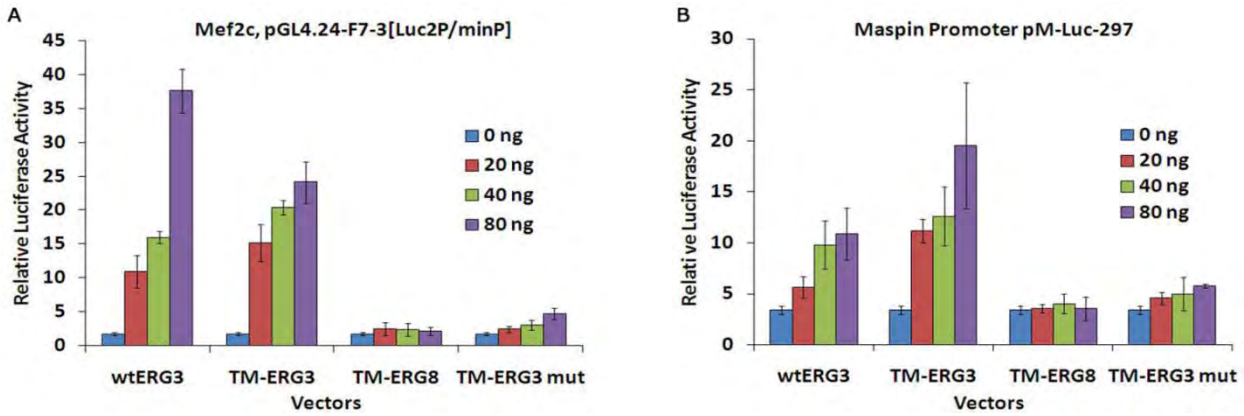

Figure 18. Type I transcript encoded ERG protein (wt ERG3 and the N-terminus truncated ERG3 protein, a product of TMPRSS2-ERG3 fusion) activates the transcription of luciferase reporter through A, the mef2c promoter, and B, the MASPIN promoter, in a dose dependent manner. Consistent with the absence of nuclear localization signal in Type II encoded TMPRSS2-ERG8, ERG8 protein does not alter the basal promoter activity of the reporter constructs.

Interaction between *Type I* splice and *Type II* splice variants in transcriptional regulation. In an experiment addressing the interaction of *Type I* and *Type II* splice variants, we co-transfected *TMPRSS2-ERG3* and increasing concentrations of *TMPRSS2-ERG8* expression vectors in HEK293 cells (Figure 19). We found a dose dependent inhibition of ERG3-mediated activation of the *mef2c* reporter construct in response to increasing doses of ERG8. This observation supports the hypothesis of our proposal that the *Type II* splice form may interfere with *Type I* function. However, this interference was not observed in the activation of the *MASPIN* reporter construct. These experiments were then repeated in BPH prostate cells, and we again observed consistent dose dependent inhibition of ERG3-mediated activation. In this experiment, the activity of the *MASPIN* reporter construct was decreased, while *mef2c* expression was largely unaffected by *TMPRSS2-ERG8*. These experiments suggest that the interference of *Type I* splice variants by *Type II* variants is both cell type, and promoter context dependent.

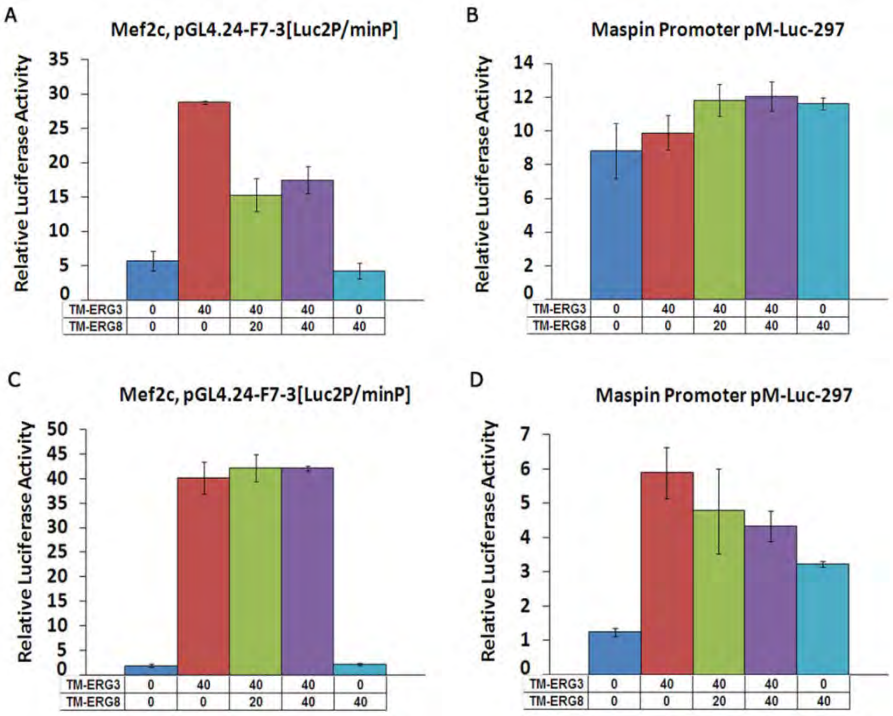

Figure 19. Interaction of Type I and Type II ERG splice variants. TM-ERG8 dose dependent abrogation of Type I ERG3 mediated transcriptional activation of mef2c in HEK293 cells (A), while transcriptional activation of MASPIN is relatively unaffected (B). Dose dependent inhibitory effects of TM-ERG8 on TMPRSS2-ERG3 mediated mef2c transcription activation was not seen in BPH cells, (C), while a decrease of TMPRSS2-ERG3 mediated transcriptional activation of MASPIN was observed (D). Cells were co-transfected with TMPRSS2-ERG3 and TMPRSS2--ERG8 expression vectors and luciferase activity was measured.

C-MYC levels are upregulated by the increased ratio of *ERG Type I* and *Type II* splice variants. To further elucidate the cross talk between *ERG* splice variants, we explored the interactions of proteins of *Type I* and *Type II* variants in VCaP cells (Figure 20). As these cells endogenously produce *Type I* and *Type II* splice variants, only exogenous *TMPRSS2-ERG8* plasmid, supplemented with empty vector, was transfected in VCaP cells in increasing concentrations to examine the effect on endogenous *TMPRSS2-ERG3*. An increase in ectopic expression of *TMPRSS2-ERG8* resulted in a decrease in endogenous *TMPRSS2-ERG3* protein in VCaP cells. Remarkably, *C-MYC* expression mirrored the decrease in *ERG3* protein levels. These results corroborated the results observed with the luciferase reporter constructs, in which an inhibition in the transcriptional activity of *Type I* variants was observed in response to increasing dosage of *Type II* variants. Further these data support the functional role of the relative levels of ERG Type I and Type II proteins in regulating a known ERG target, C-MYC in *TMPRSS2-ERG* positive prostate cancer cell culture model.

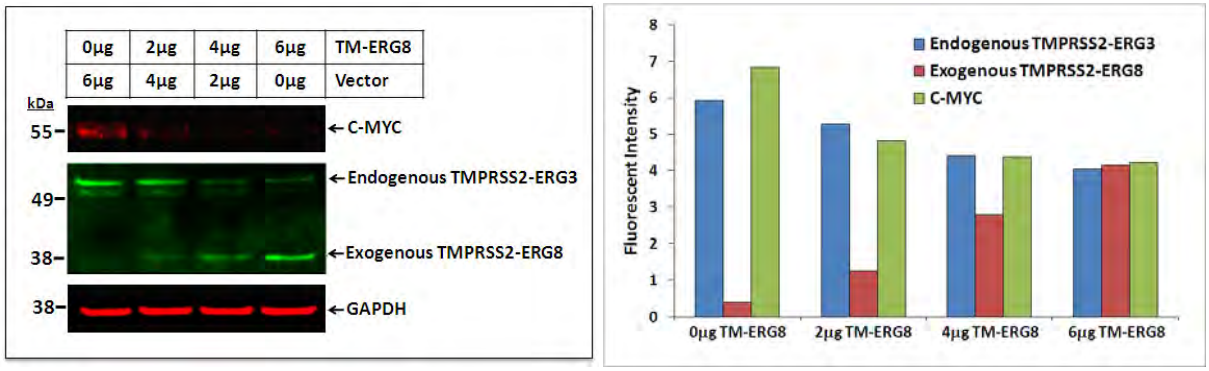

Figure 20. Decreases in ERG3 protein levels in response to exogenous ERG8 expression in VCaP cells is shown by immunoblot assay (left panel). C-MYC follows the decreases in ERG3 levels as indicated by the relative fluorescence intensities (right panel).

Model of an ERG transcriptional target regulation by changes in the relative levels of *TMPRSS2-ERG Type I* and *Type II* splice variants prostate cancer cells. In an attempt to understand the mechanism behind the association of high *ERG Type I* to *Type II* with poor prognostic features of prostate cancer, the following model is proposed:

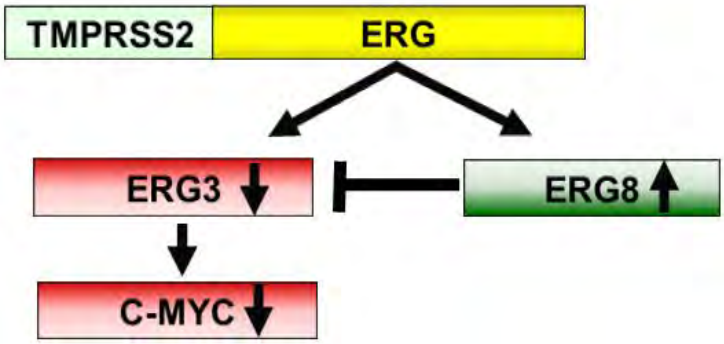

Figure 21. Mechanistic model of the ERG mediated regulation of C-MYC. Levels of ERG3 and C-MYC decrease in response to ERG8.

Correlation of *Type I/Type II* ratio and *C-MYC* mRNA levels in human prostate cancer. The intriguing observation that *Type I/Type II* ratios can significantly alter *C-MYC* levels prompted us to examine quantitative RT-PCR data of splice variants and *C-MYC* expression in LCM selected prostate cancer cells (Table 2). As previously reported, an increased ratio of *Type I ERG* variants over *Type II ERG* variants associated with higher Gleason sum and poorly differentiated phenotype (Hu *et al*., 2008). In contrast, decreases in *Type I /Type II* was associated with favorable clinical-pathologic data. Consistent with these observations we also noted a positive correlation between *Type I/Type II* ratio and *C-MYC* levels by Spearman's correlation analysis (ρ = 0.37 and P = 0.0134). Moreover, T-test analysis showed similar relationship between the *Type I / Type I*I ratios and *C-MYC* mRNA levels in patient specimens (Table 2). Thus, the increased *C-MYC* does corroborate the association of a high *Type I/Type II* ratio with a higher Gleason sum and poor overall patient prognosis.

Clinicopathologic characteristics	*ERG* splice variant expression			*Type I/Type II* ratio		
	No (46)	Yes (76)	P*	*n* (76)	Median	P†
Pathologic Gleason sum			0.0023			0.0323
2-6	9 (24%)	29 (76%)		29	0.35	
7	20 (36%)	36 (64%)		36	0.46	
8-10	14 (70%)	6 (30%)		6	0.7	
LCM differentiation			0.0058			0.0067
Well	31 (32%)	66 (68%)		65	0.45	
Poorly	15 (62%)	9 (38%)		9	0.76	
PSA recurrence			0.7312			0.0456
No	33 (37%)	56 (63%)		56	0.42	
Yes	11 (41%)	16 (59%)		16	0.61	

Gene Expression, qPCR	*Type I/Type II* ratio			
	n (45)	Median		P†
C-MYC fold difference				0.063
				**0.056
<0.5 (down-regulation)	8	0.28	**	
0.5-2.0 (relatively unchanged)	23	0.46		
>2.0 (up-regulation)	14	0.72		

NOTE: *ERG* splice variant expression (*N* = 122): In comparison with patients with no detectable expression of *ERG* in their prostate cancer cells (*n* = 46), the *ERG*-positive patient cohort (*n* = 76) has a decreased proportion of patients with high Gleason grade and poor prostate cancer cell differentiation. *Type I/Type II* ratio (*n* = 76): The ratio of *Type I/Type II ERG* splice variants in prostate cancer cells is increased in patients with poor tumor cell differentiation and with prostate-specific antigen recurrence. Gene Expression: Of the patients that have qPCR data for *C-MYC* (*n* = 45), the patient cohort with down-regulated *C-MYC* has a decreased ratio of *Type I/Type II ERG* splice variants in prostate cancer cells, whereas patients with up-regulated *C-MYC* also have an elevated *Type I/Type II* ratio.

Abbreviation: PSA, prostate-specific antigen.

* Two-sided test, P < 0.05 was considered statistically significant.
†One-sided test, P < 0.05 was considered statistically significant.

Table 2. Correlation analysis of ERG splice variant expression and Type I/Type II ratios with clinicopathologic characteristics and gene expression in prostate cancer specimens.

KEY RESEARCH ACCOMPLISHMENTS

Aim #1 In Depth Characterization of full length sequences of *TMPRSS2-ERG* transcripts (Type I and Type II) in prostate cancer

- A new CaP cDNA library was generated from the pooled RNA, isolated from *TMPRSS2-ERG* positive tumors of six patients. *TMPRSS2-ERG* cDNA clones were isolated by screening the library with *ERG* and *TMPRSS2* probes to enrich for *TMPRSS2-ERG* fusion cDNA clones. The *TMPRSS2-ERG* cDNA clones were characterized by DNA sequencing to provide first insights into the relative levels of *TMPRSS2-ERG* mRNA splice variants commonly expressed in prostate cancer.

- Novel findings revealed the expression of two major types of *TMPRSS2-ERG* transcripts in CaP: *Type I* included near full length transcripts that coded ERG protein with amino-terminal deletion of 32 amino acids; *Type II* included shorter transcripts encoding carboxy-terminal truncated proteins that lacked the ETS binding domain.

- Protein products from the *TMPRSS2-ERG Type I* and *Type II* cDNAs were defined. Distinct subcellular localization of protein encoded by *ERG Type I* (nuclear) and *Type II* (cytoplasmic) splice variants was observed.

- The first highly specific and clinically useful ERG monoclonal antibody (CPDR ERG-MAb) has been developed. CPDR ERG-MAb exhibits >99% specificity for detecting ERG expressing tumor cells in the prostate gland. There was almost complete concordance between the presence of the *ERG* fusion and ERG oncoprotein in prostate tumors. CPDR ERG-MAb showed superior specificity for ERG in comparison to commercially available ERG antibodies. This antibody has led to the world-wide collaborations of ERG oncoprotein evaluations in prostate cancers.

- Using the CPDR ERG-MAb, the first mouse body map the Erg protein expression showed normal expression of ERG in endothelial cells and in specialized hematopoietic cells. Of note, ERG is not expressed in any normal epithelial tissue including prostate epithelium, a common site of human prostate tumors with ERG rearrangements. These findings support the concept of out of context expression of ERG in malignant prostate epithelium.

Aim #2 Quantitative assessment of *TMPRSS2-ERG* splice variants reveals association of high ratios of Type 1/Type II ratios with poor prognostic features of prostate cancer.

- Parallel quantitative analyses was performed for the most abundant *TMPRSS2-ERG Type I* (ERG 1/2; ERG 3) and *Type II* (ERG8 and TEPC) splice variants in laser capture micro dissected paired normal and tumors cells from 150 patients and data was analyzed from 122 evaluable patients representing primary prostate cancer specimens.

- The association of various *TMPRSS2-ERG* variants with clinico-pathologic parameters was evaluated, such as, patient age, race, pre-treatment PSA level, Gleason score, CaP family history, tumor stage, surgical margin status, seminal vesicle invasion, PSA recurrence, bone metastasis, nuclear grade, differentiation, PSA doubling time and prostate cancer associated death.

- Novel findings revealed a significant correlation between increased ratio of *Type I* (*ERG*1-3) over *Type II* (*ERG*8 and *TEPC) ERG* splice variants and poor prognostic features (higher pathological Gleason sum, poorly differentiated tumor cells and PSA recurrence).

- A high concordance (>95%) between the presence of *ERG* gene rearrangement and ERG oncoprotein expression has been established in prostate cancer, as determined by dual-color interphase fluorescence *in situ* hybridization (FISH) and ERG immuno-histochenistry (IHC) based evaluations of whole-mount prostates and tissue micro-arrays.

Aim #3: Defining the functional significance of specific splice variants encoded by *ERG* gene Fusions .

- Simultaneous inhibition of both *Type I* and *Type II* variants by ERG siRNA and shRNA molecules resulted in significant morphological alterations and growth inhibition of VCaP cells, supporting the overall *ERG* inhibition as a rational strategy for *ERG* targeted therapy for over half of all CaP. Mechanisms of *ERG* knockdown by siRNA revealed downregulation of *C-MYC*, overexpression of cell differentiation markers and a marker of intercellular tight junctions, *ZO-1*.

- Transcriptional regulatory activities of *ERG* splice variants were assayed by using the murine *mef2c* promoter-luciferase reporter and *maspin* promoter-luciferase constructs. *Type I* variant encoded *ERG3* protein activated the transcription of luciferase reporter through the *mef2c* enhancer and *maspin* promoter. In contrast, *Type II* encoded *ERG8* did not alter the basal promoter activity. *Type II* splice variants of *ERG* inhibited with *Type I* driven transcriptional regulation of *mef2c* enhancer and *MASPIN* promoters.

- Important biological observations of the influence of *Type II* over *Type I ERG* variants also include: (a) decreased levels of *ERG Type I* variant encoded protein in response to exogenous *ERG8* expression in *TMPRSS2-ERG* positive VCaP cell model and (b) association of increased *Type I/Type II* ratio with higher *C-MYC* levels (ERG downstream target) and overall poor prognosis of prostate cancer patients.

- Taken together, these compelling biological studies support the central hypothesis of this proposal that changes in ratios of *Type 1 and Type II ERG* splice variants in prostate tumor cells influence the overall oncogenic function of ERG in prostate tumorigenesis. Importantly, increased *ERG Type I over Type II* ratios associate with poor prognostic features of CaP. Finally, strategies inhibiting all of *ERG* variants may contribute to developing ERG targeted CaP therapy.

REPORTABLE OUTCOMES

Publications

1) Braun M, Goltz D, Shaikhibrahim Z, Vogel W, Scheble V, Böhm D, Sotlar K, Fend F, Tan S-H, Dobi A, Wernert N, Perner S.: ERG protein expression and genomic rearrangement status in primary and metastatic prostate cancer - a comparative study of two monoclonal antibodies. *Prostate Cancer and Prostatic Diseases*, 15,165-9 (2012).

2) Rosen P, Sesterhenn IA, Brassell S, McLeod DG, Srivastava S, Dobi A.: Clinical potential of the ERG oncoprotein in prostate cancer. *Nature Reviews Urology*, 9, 131-7 (2012) [Review].

3) Sreenath T, Dobi A, Petrovics G, Srivastava S.: Oncogenic activation of ERG, a predominant mechanism in prostate cancer. *Journal of Carcinogenesis*, 10, 10-21(2011) [Review].

4) Mohamed AA, Tan SH, Mikhalkevich N, Ponniah S, Vasioukhin V, Bieberich CJ, Sesterhenn IA, Dobi A, Srivastava S, Sreenath TL. Ets family protein, erg expression in developing and adult mouse tissues by a highly specific monoclonal antibody. *J Cancer*. 1, 197-208 (2010).

5) Furusato B, Tan SH, Young D, Dobi A, Sun C, Mohamed AA, Thangapazham R, Chen Y, McMaster G, Sreenath T, Petrovics G, McLeod DG, Srivastava S, Sesterhenn IA.: ERG oncoprotein expression in prostate cancer: clonal progression of ERG positive tumor cells and potential for ERG based stratification. *Prostate Cancer and Prostatic Diseases* 13, 228-237 (2010).

6) Hu Y, Dobi A, Sreenath T, Cook C, Tadese AY, Ravindranath L, Cullen J, Furusato B, Chen Y, Thangapazham R, Mohamed A, Sun C, Sesterhenn IA, McLeod DG, Petrovics G, Srivastava S.: Delineation of *TMPRSS2-ERG* splice variants in prostate cancer. *Clinical Cancer Research* 14, 4719-4725 (2008)

Podium presentations

1) *United States & Canadian Academy of Pathology Meeting Feb. 26 – March 4, San Antonio, TX, 2011*
Albert Dobi, Shyh-Han Tan, Bungo Furusato, Denise Young, Ahmed Mohamed, Yongmei Chen, Gary McMaster, Taduru Sreenath , Gyorgy Petrovics, David G. McLeod, Shiv Srivastava and Isabell A. Sesterhenn. **Defining ERG Oncoprotein in Prostate Cancer by the Anti-ERG Monoclonal Antibody, CPDR ERG-MAb, and Evaluation for Clinical Utilities.**

2) *American Association for Cancer Research, Washington, DC, April 18-22, 2010*
Shyh-Han Tan, Bungo Furusato, Denise Young, Albert Dobi, Chen Sun, Ahmed Mohamed, Rajesh Thangapazham, Yongmei Chen, Gary McMaster, Taduru Sreenath, Gyorgy Petrovics, David G. McLeod, Shiv Srivastava and Isabell A. Sesterhenn. **ERG oncoprotein expression in prostate cancer: Potential for ERG-based stratification**

3) *XVIII. International Semmelweis Symposium: New Trends, Innovation & Technology in Urology, Budapest, Hungary, November 18-21, 2009*
Albert Dobi: **Prevalent Alterations of the ETS Related Gene (ERG) in Prostate Cancer: A Promising Biomarker and Therapeutic Target**

4) *American Association for Cancer Research, Denver, CO, April 18-22, 2009*
Ying Hu, Lakshmi Ravindranath, Bungo Furusato, Chen Sun, Albert Dobi, Isabell A Sesterhenn, David G. McLeod, Gyorgy Petrovics, Shiv Srivastava. **Expression and sub-cellular localization of predominant TMPRSS2-ERG transcripts with and without ETS domain in prostate cancer**

5) *9th Asian Congress of Urology New Delhi, India, Oct 4, 2008*
Shiv Srivastava: **Bio-marker and Therapeutic Target Potential of Frequent Gene Alterations in Prostate Cancer**

Poster presentations

1) *American Urological Association Annual Meeting, May 19 - 23, Atlanta, GA, 2012*
Shyh-Han Tan, Christian Ruiz, Martin Braun, Veit Scheble, T Zellweger, CA Rentsch, A Bachmann, Albert Dobi, Sven Perner, Isabell Sesterhenn, Shiv Srivastava, L Bubendorf. **Comprehensive analysis of the TMPRSS2-ERG translocation during prostate cancer progression**

2) *American Association for Cancer Research, "Advances in Prostate Cancer Research" February 6-9, Orlando, FL, 2012*
Shyh-Han Tan, Ruiz C, Braun M, Scheble VJ, Zellweger T, Rentsch CA, Bachmann A, Albert Dobi, Perner S, Sesterhenn I, Srivastava S, Bubendorf L. **Comprehensive analysis of the *TMPRSS2-ERG* translocation during prostate cancer progression**

3) *American Association for Cancer Research, "Advances in Prostate Cancer Research" February 6-9, Orlando, FL, 2012*
Taduru Sreenath, Shyh-Han Tan, Martin Braun, Diane Goltz, Zaki Shaikibrahim, Wenzel Vogel, Diana Boehm, Veit Scheble, Albert Dobi, Falko Fend, Nicolas Wernert, Glen Kristiansen, Sven Perner. **ERG protein expression and genomic rearrangement status in primary and metastatic prostate cancer - a comparative study of two monoclonal antibodies.**

4) *Society for Basic Urologic Research (SBUR) Fall Symposium November 10-13, Las Vegas, NV, 2011*
Ahmed A. Mohamed, Shyh-Han Tan, Natallia Mikhalkevich, Sathibalan Ponniah, Valeri Vasioukhin, Charles J. Bieberich, Isabell A. Sesterhenn, Albert Dobi, Shiv Srivastava and Taduru L. Sreenath. **Ets-Related Gene (ERG) Expression in Mouse Organogenesis.**

5) *American Association for Cancer Research, April 2-6, Orlando, FL, 2011*
Ahmed A. Mohamed, Shyh-Han Tan, Natallia Mikhalkevich, Sathibalan Ponniah, Valeri Vasioukhin, Charles J. Bieberich, Isabell A. Sesterhenn, Albert Dobi, Shiv Srivastava and Taduru L. Sreenath. **ETS-Related Gene (ERG) Expression in Developing and Adult Mouse Tissues**

6) *United States & Canadian Academy of Pathology Meeting Feb. 26 – March 4, San Antonio, TX, 2011*
Albert Dobi, Shyh-Han Tan, Bungo Furusato, Denise Young, Ahmed Mohamed, Yongmei Chen, Gary McMaster, Taduru Sreenath , Gyorgy Petrovics, David G. McLeod, Shiv Srivastava and Isabell A. Sesterhenn.
Defining ERG Oncoprotein in Prostate Cancer by the Anti-ERG Monoclonal Antibody, CPDR ERG-MAb, and Evaluation for Clinical Utilities.

7) *Innovative Minds in Prostate Cancer/DoD-PCRP, March 9–12, Orlando, FL, 2011*
Zainab Afzal, Tseday Zewdu Tegegn, Ahmed Mohamed, Taduru Sreenath, Deepak Kumar, Shiv Srivastava and Shyh-Han Tan
Sub-cellular Localization and Transcriptional Activity of ERG Protein Encoded by the Common *TMPRSS2-ERG* Splice Variants Expressed in Prostate Cancer.

8) *Innovative Minds in Prostate Cancer/DoD-PCRP, March 9–12, Orlando, FL, 2011*
Taduru Sreenath, Albert Dobi, Ying Hu, Shyh-Han Tan, Yongmei Chen, Chen Sun, Isabell A. Sesterhenn, David G. McLeod, Gyorgy Petrovics and Shiv Srivastava
Structure and Function of the Splice Variants of TMPRSS2-ERG, Prevalent Genomic Alteration in Prostate Cancer.

9) *58th James C. Kimbrough Urological Seminar Jan. 16-21, Seattle, WA, 2011*
Albert Dobi, Shyh-Han Tan, Bungo Furusato, Denise Young, Ahmed Mohamed, Yongmei Chen, Gary McMaster, Taduru Sreenath, Gyorgy Petrovics, David G. McLeod, Isabell A. Sesterhenn, and Shiv Srivastava. **ERG oncoprotein in pre-invasive and invasive prostate cancer.**

10) *American Urologic Association Annual Meeting, San Francisco, CA, May 29-June 3, 2010*
Bungo Furusato, Shyh-Han Tan, Denise Young, Albert Dobi , Chen Sun, Ahmed Mohamed , Rajesh Thangapazham , Yongmei Chen , Gary McMaster , Taduru Sreenath , Gyorgy Petrovics, David G. McLeod, Shiv Srivastava and Isabell A. Sesterhenn. **Robust ERG oncoprotein expression in prostate cancer: potential for ERG based stratification**

11) *American Association for Cancer Research, Washington, April 18-22, 2010*
Shyh-Han Tan, Ying Hu, Albert Dobi, Gyorgy Petrovics, Shiv Srivastava. **Functional Assessment of ERG Proteins Encoded by *ERG* Splice Variant Transcripts in Prostate Cancer Cells.**

12) *American Association for Cancer Research, Denver CO, April 18-22, 2009*
Ying Hu, Albert Dobi, Taduru Sreenath, Atekelt Tadese, Shyh-Han Tan, Lakshmi Ravindranath, Bungo Furusato, Yongmei Chen, Rajesh Thangapazham, Amina Ali, Ahmed Mohamed, Chen Sun, Isabell Sesterhenn, David McLeod, Gyorgy Petrovics, Shiv Srivastava: **Predominant *TMPRSS2-ERG* Transcripts in Prostate Cancer**

13) *Advances in Prostate cancer (AACR) San Diego, CA Jan 21-24, 2009*
Taduru L. Sreenath, Ying Hu, Albert Dobi, Atekelt Tadese, Shyh-Han Tan, Lakshmi Ravindranath, Bungo Furusato, Youngmei Chen, Rajesh Thangapazham, Amina Ali, Ahmed Mohamed, Chen Sun, Isabell Sesterhenn, David G. McLeod, Gyorgy Petrovics, Shiv Srivastava: **Expression and Sub-**

cellular Localization of Predominant *TMPRSS2-ERG* Transcripts with and without ETS domain in Prostate Cancer

14) *Kimbrough Urological Seminar-Washington DC, Jan 11-16, 2009*
Won Best Poster Award
Albert Dobi, Chen Sun, Ying Hu, Rajesh Thangapazham, Ahmed Mohamed, CPT Eric J. Whitman, CPT Dorotha Hawksworth, Bungo Furusato, Shyh-Han Tan, Atekelt Y. Tadase, Lakshmi Ravindranath, Jennifer Cullen, Yongmei Chen, Gyorgy Petrovics, Taduru Sreenath, Isabell A. Sestrehenn, COL David G. McLeod, Shiv Srivastava:
Splice Variants and Functions of *TMPRSS2-ERG* Fusion, a Common Genomic Alteration in Prostate Cancer

15) *Society for Basic Urology Research, Phoenix, AZ, Nov 20-23, 2008*
Shyh-Han Tan, Ying Hu, Albert Dobi, Taduru Sreenath, Christopher Cook, Atekelt Y. Tadase, Lakshmi Ravindranath, Jennifer Cullen, Bungo Furusato, Yongmei Chen, Rajesh Thangapazham, Ahmed Mohamed, Chen Sun, Isabell Sesterhenn, David G. McCleod and Shiv Srivastava:
***TMPRSS2-ERG* Splice Variants In Prostate Cancer**

Press Releases

1) Scientists develop highly specific ERG monoclonal antibody for detecting common oncogenic alterations in prostate cancer.

   Released by the Uniformed Services University, Bethesda, Maryland, www.usuhs.mil, June 29, 2010.

2) Biocare Medical and the Henry M. Jackson Foundation for the Advancement of Military Medicine announce exclusive distribution agreement for a highly specific ERG monoclonal antibody to detect prevalent oncogenic alterations in prostate cancer.

   Released by the Uniformed Services University, www.usuhs.mil, Bethesda, Maryland, January 20, 2011.

Funding Applied for Based on Work Supported by this Award

Consistent with the central hypothesis of this proposal, our data strongly support the functional and prognostic significance of *ERG Type I and Type II* splice variants in prostate cancer. To define the role of *TMPRSS2-ERG* splice variants in prostate cancer, we have proposed to generate a transgenic mouse model that will approximate the expression of *ERG Type I* and *Type II* as noted in tumors specimens of CaP patients (NIH RO1/NCI: 9RO1CA162383; Cell Specific Expression Signatures in Prostate Cancer; PI: Dr. Shiv Srivastava). The pertinent aim of the study is to develop an *Erg* transgenic mouse that recapitulates the expression of Erg splice variants *in vivo*. Another grant application focusing on prognostic marker utility of ERG splice variants will be developed.

Employment or research opportunities applied for and/or received based on experience/training supported by this award

1) This award supported the employment and post-doctoral training of Anshu Rastogi, PhD. Dr. Rastogi joined the CPDR in August 2011 after obtaining her Ph.D. from the University of Maryland. She has completed the analysis of reporter assays of *Type I* and *Type II* splice variants. Her research results revealed that modulating the ratios of *Type I* and *Type II* splice variants in cell culture models affect the expression levels *C-MYC* oncogene. Dr. Rastogi's research prompted us to propose that the expression levels of *C-MYC* oncogene may mirror *Type I* and *Type II* ratios. Consistent with the proposed model we found that *Type I/Type II* ratio correlated with higher *C-MYC* levels, and with a higher Gleason sum and poor overall prognosis of prostate cancer patients. Based on her successful training under this award, Dr. Rastogi has been offered new job opportunity at CPDR.

2) This award supported the research project and post-doctoral training of Dr. Ying Hu, MD, MPH, PhD (2008-2010). Dr. Hu published the ground breaking study showing for first time qualitative and quantitative features of the ERG splice variants in prostate cancer (Hu *et al*., Clin Cancer Res. 2008). She also developed the original concept, as well as, very promising data related to biological relevance of increased *ERG Type I and II* ratios with poor prognosis of prostate cancer. Based on her training Dr. Ying Hu received an independent investigator position in the Department of Epidemiology and Biostatistics, School of Public Health, Medical Center of Wuhan University, Wu Chang Wuhan, China.

List of Personnel (not salaries) receiving pay from the research effort

Co-PI: Taduru Sreenath, PhD.
Co-I: Albert Dobi, PhD.
Post-doctoral Fellow: Ying Hu, MD, PhD.
Post-doctoral Fellow: Anshu Rastogi, PhD.

CONCLUSIONS and SO WHAT

Unscheduled expression of the *ETS* related genes as a result of fusion of androgen responsive promoters (predominantly *TMPRSS2*) and the *ETS* related genes (predominantly *ERG*) are the most prevalent oncogenic activations described in CaP till date. Due to the tumor specific and causal nature of these alterations, *ERG* is one of the most studied and validated cancer genes in CaP. **There is tremendous interest in biological and clinical evaluations of the ERG as a biomarker or therapeutic target, as *ERG* alterations are present in two thirds of prostate cancer patients in the USA.**

Our laboratory has been engaged in the comprehensive evaluations of *ERG* alterations in CaP, beginning with our in-depth study highlighting overexpression of the *ERG* proto-oncogene in over two thirds of CaP patients.

Our research has continued to focus on biological characteristics and translational utility of *ERG* in CaP. The specific focus of this proposal was to address the qualitative and quantitative nature of full length *ERG* fusion transcripts in the context of RNA splice variants in CaP. Since majority of studies focusing on *ERG* fusions defined the fusion junctions, the nature of full length *ERG* fusion transcripts in the context of RNA splice variants remained to be elucidated. Although *ERG* splice variants were described in other tissues and cell types, there was no information about the major *ERG* RNA splice variants in prostate cancer cells. We envisioned that characterization of *ERG* splice variants in CaP would lead to better understanding of its role in CaP.

The proposal was successful from achieving technical objectives and from the perspective of novel findings. A new CaP cDNA library was generated and characterized from the pooled RNA, isolated from *TMPRSS2-ERG* positive tumors of six patients. **Novel findings revealed the expression of two major types of *TMPRSS2-ERG* transcripts in CaP: *Type I* included near full length transcripts that coded ERG protein with amino-terminal deletion of 32 amino acids; *Type II* included shorter transcripts encoding carboxy-terminal truncated proteins that lacked the ETS binding domain.** Protein encoding products from the *TMPRSS2-ERG Type I* and *Type II* cDNAs were defined. We also established the distinct sub-cellular localization of the ERG Type I (nuclear) and Type II (cytoplasmic) proteins.

Parallel quantitative analyses was performed for the most abundant *TMPRSS2-ERG Type I* (*ERG 1/2*; *ERG 3*) and *Type II* (*ERG8* and *TEPC*) splice variants in laser capture micro dissected paired normal and tumors cells from 150 patients and data was analyzed from 122 evaluable patients representing primary prostate cancer specimens. **Novel findings revealed a significant correlation between increased ratio of *Type I* (*ERG*1-3) over *Type II* (*ERG*8 and *TEPC*) *ERG* splice variants and poor prognostic features (higher pathological Gleason sum, poorly differentiated tumor cells and PSA recurrence).** Despite the very promising observation, highly complex nature of this assay has precluded its validation by other investigators. The assay needs to be streamlined for FFPE specimens to further enhance its practical utility.

The first highly specific and clinically useful ERG monoclonal antibody (CPDR ERG-MAb) has been developed. CPDR ERG-MAb exhibits >99% specificity for detecting ERG expressing tumor cells in the prostate gland. There was almost complete concordance between the presence of the *ERG* fusion and ERG oncoprotein in prostate tumors. CPDR ERG-MAb showed superior specificity for ERG in comparison to commercially available ERG antibodies. This antibody has led to the world-wide collaborations of ERG oncoprotein evaluations in prostate cancers. Using the CPDR ERG-MAb, **the first mouse body map the Erg protein expression showed normal expression of ERG in endothelial cells and in specialized hematopoietic cells.** Of note, ERG is not expressed in any normal epithelial tissue including prostate epithelium, a common site of human prostate tumors with *ERG* rearrangements. These findings support the concept of out of context expression of *ERG* in malignant prostate epithelium.

Functional studies suggest that shorter *Type II ERG* splice variants may negatively influence the near full length *Type 1 ERG* splice variants. Transcriptional regulatory activities of *ERG* splice variants were assayed by using murine *mef2c* promoter-luciferase reporter construct and *maspin* promoter-luciferase constructs. *Type I* variant encoded *ERG3* protein activated the transcription of luciferase reporter through the *mef2c* enhancer. In contrast, *Type II* encoded *ERG8* did not alter the basal promoter activity. *Type II* splice variants of *ERG* inhibited with *Type I* driven transcriptional regulation of *mef2c* enhancer. Important biological observations of the influence of *Type II* over *Type I ERG* variants also include: (a) decreased levels of *ERG Type I* variant encoded protein in response to exogenous *ERG8* expression in *TMPRSS2-ERG* positive VCaP cell model and (b) association of increased *Type I/Type II* ratio with higher *C-MYC* levels (*ERG* downstream target) and overall

poor prognosis of prostate cancer patients. While these functional studies are promising more in-depth study of biological functions of *ERG* splice variants is warranted.

Taken together, these compelling studies support the central hypothesis of this proposal that changes in ratios of *Type I and Type II ERG* splice variants in prostate tumor cells influence the overall oncogenic function of *ERG* in prostate tumorigenesis. Importantly, increased *ERG Type I over Type II* ratios associate with poor prognostic features of CaP. Finally, these data may aid in refining ERG targeted prognostic marker and therapeutic target strategies.

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Delineation of *TMPRSS2-ERG* Splice Variants in Prostate Cancer

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Abstract

Purpose: The expression of the *ETS*-related gene (*ERG*) is low or undetectable in benign prostate epithelial cells. High prevalence of *ERG* overexpression in prostate cancer cells due to *TMPRSS2-ERG* fusions suggest for causal roles of ERG protein in the neoplastic process. *TMPRSS2-ERG* fusion junctions have been extensively studied in prostate cancer. However, virtually nothing is known about the nature of full-length transcripts and encoded proteins. This study focuses on qualitative and quantitative features of full-length *TMPRSS2-ERG* transcripts in prostate cancer.

Experimental Design: Full-length *TMPRSS2-ERG* transcripts were cloned and sequenced from a cDNA library generated from pooled RNA of six *TMPRSS2-ERG* fusion–positive prostate tumors. The encoded ERG proteins were analyzed in HEK293 cells. Copy numbers of *TMPRSS2-ERG* splice variants were determined by quantitative reverse transcription-PCR in laser capture microdissected prostate cancer cells.

Results: Two types of *TMPRSS2-ERG* cDNAs were identified: type I, which encodes full-length prototypical ERG protein (*ERG1, ERG2, ERG3*), and type II, encoding truncated ERG proteins lacking the ETS domain (*ERG8* and a new variant, *TEPC*). In microdissected prostate tumor cells from 122 patients, relative abundance of these variants was in the following order: *ERG8* > *TEPC* > *ERG 3* > *ERG1/2* with combined overexpression rate of 62.3% in prostate cancer. Increased ratio of type I over type II splice forms showed a trend of correlation with less favorable pathology and outcome.

Conclusions: Qualitative and quantitative features of specific *ERG* splice variants defined here promise to enhance the utility of *ERG* as a biomarker and therapeutic target in prostate cancer.

Molecular genetic evaluations of prostate cancer are defining mutational and expression alterations of critical oncogenes involved in disease onset and/or progression (reviewed in refs. 1–3). Discovery of prevalent chromosomal rearrangements/translocations leading to the activation of ETS transcription factors (predominantly *ERG*) through the androgen receptor–regulated *TMPRSS2* gene promoter underscore the critical roles of *ERG*-encoded protein in prostate cancer (4–7). Because *ERG* represents the majority of *TMPRSS2-ETS* factor alterations described thus far (6, 7), we have focused on the expression and regulation of *TMPRSS2-ERG* in prostate cancer. Oncogenic functions of ETS factors, including ERG, have also been implicated in diverse cancers (8).

Structure and function of *ERG*-encoded proteins remain to be defined in prostate cancer. *ERG* consists of 17 exons spanning about 300 kb and generates at least nine alternate splice forms, seven of them coding for protein products of varying sizes (9). These *ERG* splice variants have been primarily described in nonprostate tissues. Despite the large body of data on the *TMPRSS2-ERG* fusion junctions in prostate cancer (reviewed in refs. 6, 7), virtually nothing is known about the full-length *TMPRSS2-ERG* transcripts in prostate cancer, including the existence and relative abundance of specific splice variants. In this context, it is important to note that the cancer-associated splice variants of numerous genes, e.g., *androgen receptor*, *fibroblast growth factor receptor*, *survivin*, and *MDM2*, have functional implications (10, 11). Thus, characterization of full-length *TMPRSS2-ERG* transcripts is essential to better understand *ERG* function(s) in prostate cancer and to further enhance its utility as biomarker and therapeutic target.

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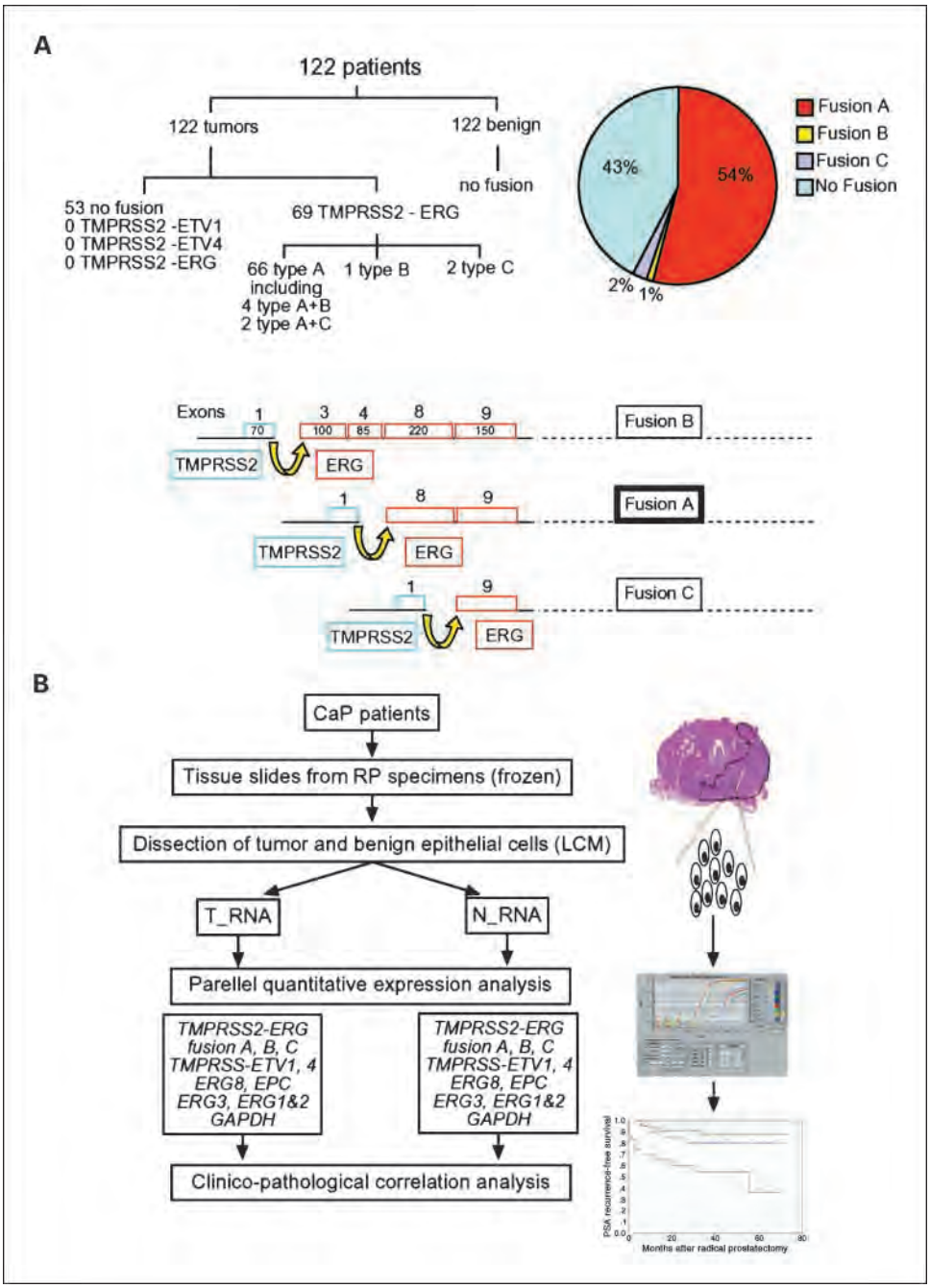

Fig. 1. Expression of *TMPRSS2-ERG* fusions in prostate cancer specimens. *A,* evaluation of the prevalence of *TMPRSS2-ERG* fusion A transcript junctions in prostate cancer patients (N = 122): Tumor and matching benign cells were assayed for *TMPRSS2-ERG, TMPRSS2-ETV1*, and *TMPRSS2-ETV4* fusion transcript junctions by quantitative reverse transcription-PCR. The schematic representation depicts the distribution of the various fusions in the patient specimens. The pie chart summarizes the *TMPRSS2-ERG* fusion junction types in the 122 tumor specimens. inset, *TMPRSS2-ERG* fusion A (*T1-E8*), B (*T1-E3*), and C (*T1-E9*) detected in this study (exon numbering is according to ref. 9). *B,* schematic representation of the experimental strategy and workflow of quantitative gene expression analysis in prostate tumor specimens.

In this study, we have cloned and sequenced full-length cDNAs from *TMPRSS2-ERG* fusion–positive prostate tumors, and from the VCaP cell line. We have identified two types of *TMPRSS2-ERG* cDNAs, one (type I) encoding full-length prototypical ERG protein (*ERG1*, *ERG2*, *ERG3*) and the other (type II) encoding a shorter version lacking the ETS domain (*ERG8* and a new variant, *TEPC*). We have further quantified and validated the expression of these *ERG* splice forms in a large cohort of prostate cancer specimens. The *ERG* exons at the *TMPRSS2-ERG* fusion junction have been the subject of a number of studies (fusion junction variants; refs. 5–7, 12–19). However, these exons are present in all *ERG* splice forms and do not identify specific splice variants. In recent *in vivo* models assessing the role of ERG in prostate cancer, only a type I splice variant, specifically NH_2-terminally truncated *ERG3*, was tested (20, 21). Intriguingly, the data presented here shows a more abundant expression of type II splice variants in prostate cancer cells. Our new findings on *ERG* splice variants in prostate cancer have promise in improving the understanding of *ERG* functions and its therapeutic targeting in prostate cancer, as well as in enhancing the detection of *ERG* alterations in clinical specimens.

Materials and Methods

Tissue specimens, laser capture microdissection, and quantitative gene expression analysis. The prostate tissue specimens used in this study

were obtained from radical prostatectomy procedures under an Institutional Review Board–approved protocol at Walter Reed Army Medical Center. Laser capture microdissection (LCM) of tumor and benign epithelial cells from optimum cutting temperature–embedded frozen tissues obtained from the radical prostatectomy specimens, RNA isolation from the LCM samples, and real-time quantitative reverse transcription-PCR (TaqMan) were essentially done as described previously (4, 22). The differentiation status of microdissected cells was recorded independently from the overall pathologic Gleason grade of the prostate, which was determined from whole-mounted, formalin-fixed, paraffin-embedded prostate specimens of each patient. The small amounts of tissue specimens (~ 5 mm^3) were obtained for optimum cutting temperature embedding from radical prostatectomy specimen before whole-mount prostate processing. Selection of specimens for LCM was primarily driven by the presence of sufficient amount of tumor cells for the LCM. The predominant tumor cell type (by differentiation) present in a frozen section was microdissected. Most of the time, but not always, the predominant differentiation grade in frozen tissue section represented the prevalent differentiation grade of the tumor cells in the prostate. Overall, 88.4% of LCM samples were collected from the primary Gleason pattern of the index tumor. Because of this, we have compared the quantitative gene expression of *TMPRSS2-ERG* splice variants to the differentiation grade of the microdissected cells, as well as to the overall differentiation grade of the of tumor cells in the prostate. Overall conclusions were similar by two-way comparisons but an increased statistically significant relationship was noted when the gene expression ratios of ERG I/II in LCM-RNAs was correlated with the differentiation grade of the LCM-dissected cells. TaqMan primers and probes are listed in the Supplementary data.

Detection of the *TMPRSS2-ERG* and *TMPRSS2-ETV* fusion transcripts was done essentially as described (5). The different *TMPRSS2-ERG* fusion junction types (A, B, and C) are described in Fig. 1A in a schematic diagram. All three fusion types have been previously described (5, 19). The expression of *GAPDH* was simultaneously analyzed as endogenous control, and the target gene expression in each sample (in duplicates) was normalized to *GAPDH*. RNA samples without reverse transcription were included as the negative control in each assay.

Generation and screening of cDNA library from prostate tumors. For the generation of the cDNA library, frozen tumor tissues from index tumors of six patients were selected based on available tissue size (over 30 mg), highest tumor cell content (over 70%), and the presence of *TMPRSS2-ERG* fusion transcripts by reverse transcription-PCR. Polyadenylated RNA was isolated from the optimum cutting temperature–embedded frozen tumor tissues. A cDNA library was generated from the pooled RNA (Lofstrand Laboratories) and cloned into the *Xho*I-*Eco*RI sites of lambdaZAP Express vector (Stratagene). Screening of the expression library was carried out according to the protocol described by the manufacturer (Stratagene). The primary library of about 400,000 plaques were screened by *ERG2* probe (NM_004449; cDNA obtained from Dr. Dennis Watson, Medical University of South Carolina, Charleston, SC) and found 84 hybridized with different intensities. The positive plaques were further screened for the presence of *TMPRSS2* fusions by fusion-specific PCR (5). A total of 12 plaques showed

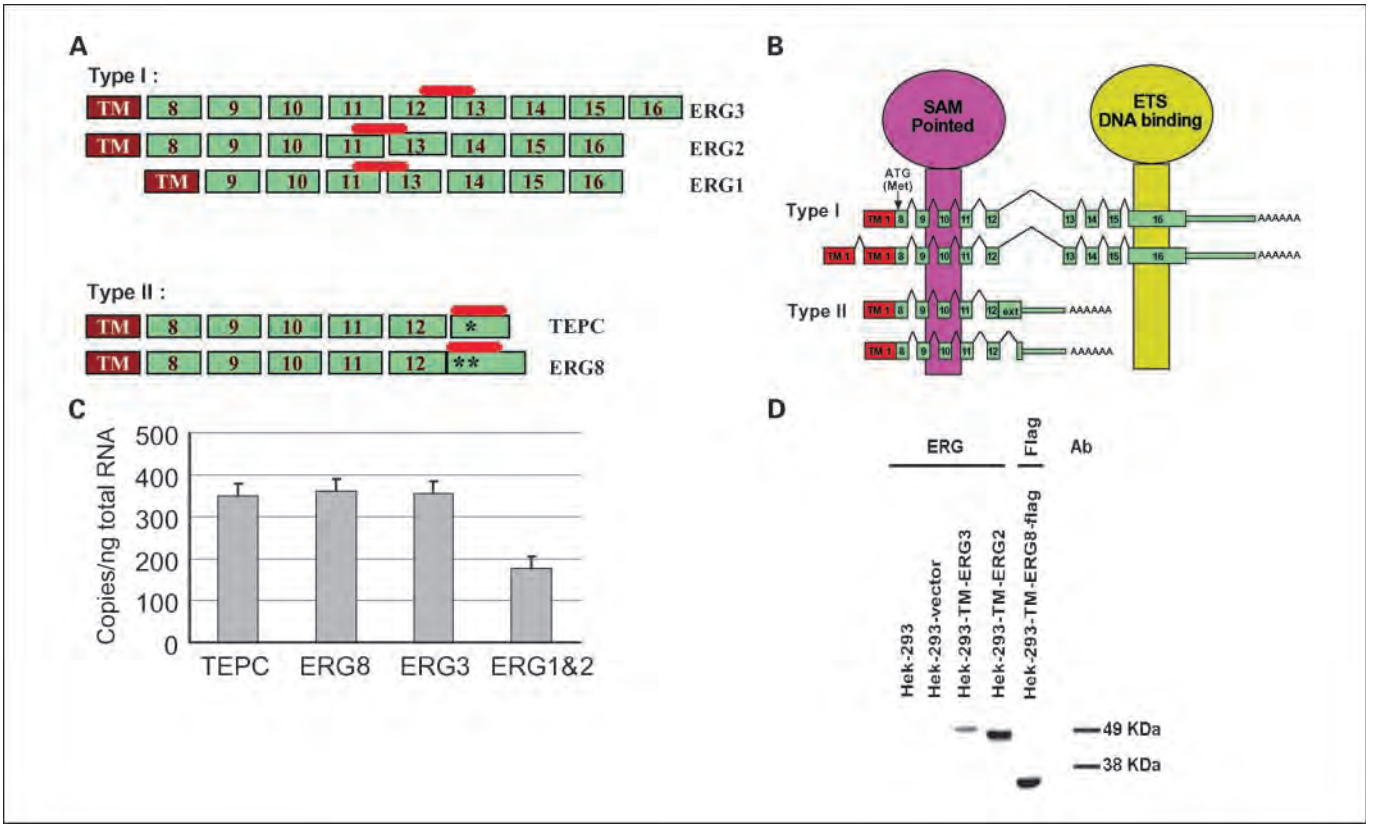

Fig. 2. *ERG* splice forms in prostate tumors and their expression in the VCaP prostate cancer cell line. *A,* schematic representation of full-length type I and type II *ERG* transcripts expressed in prostate cancer cells with *TMPRSS2-ERG* fusion. Numbered boxes, *ERG* exons (9); boxes with * and **, unique regions of *TEPC* and *ERG8*, respectively. Solid lines above the exons, TaqMan primers and probes used for the detection of the *ERG* splice variants. *B,* type I transcripts code for both transactivation (*SAM Pointed*) and DNA-binding (*ETS*) domains. In contrast, type II variants lack the coding sequence for the DNA-binding domain. The relative positions coding for the two major functional domains of ERG protein are shown in type I and type II splice variants. *C,* columns, copy numbers of the *ERG* splice forms in VCaP cells determined by TaqMan quantitative reverse transcription-PCR. The median of three experiments using triplicates are shown. *D,* protein products expressed from *TMPRSS2-ERG2, TMPRSS2-ERG3*, and *TMPRSS2-ERG8* clones transiently transfected into HEK293 cells are shown by Western blot analysis. Anti-Flag antibody was used for the detection of Flag-tagged ERG8 protein.

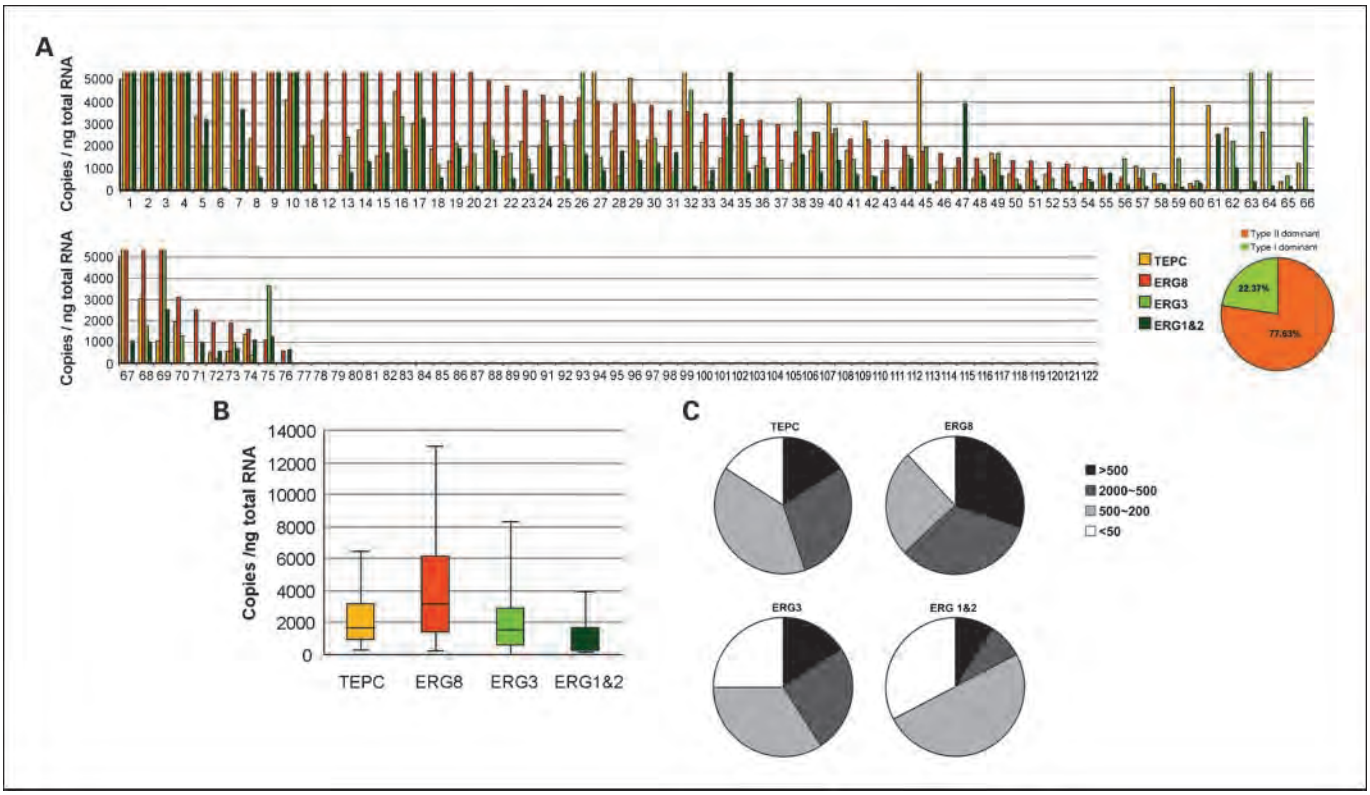

Fig. 3. Quantitative expression of *ERG* splice variants in prostate cancer patients. *A,* quantitative expression of *ERG* splice forms *ERG8, TEPC, ERG3*, and *ERG1&2* (*columns with different colors*) were determined in microdissected tumor cells of prostate cancer patients ($N = 122$). The graph depicts relative expression levels (copy number/ng total RNA, normalized to *GAPDH*) in patients with (*top*, $n = 66$) and with no detectable (*bottom*, $n = 56$) *TMPRSS2-ERG* fusion A transcript junction. Due to wide dynamic range of copy numbers, the values above 5,000 are not shown here (the range of expression for each splice variant is depicted in *B*). In the pie chart, the percentage of prostate cancer patients are represented either with higher (~77%) or with lower (~23%) expression level of type II than type I *ERG* transcripts. *B,* relative abundance of various *ERG* splice forms (*ERG8, TEPC, ERG3*, and *ERG1&2*) is depicted by box plots representing the copy numbers determined in 76 prostate cancer patients overexpressing *ERG*. *C,* pie charts illustrate the distribution of prostate cancer patients with various expression levels (copy number/ng total RNA) of *ERG8, TEPC, ERG3*, and *ERG1&2* splice forms in prostate cancer cells.

amplification. Detailed cDNA sequence analysis revealed the presence of two types of *TMPRSS2-ERG* fusion transcripts. Within the positively identified plaques, three represented type I (with both SAM domain and DNA-binding ETS domain) and five type II (without ETS domain). Fusion-positive type I– and type II–containing phages were amplified with T3 and T7 primers, subcloned into TOPO vector (Invitrogen), and verified by DNA sequencing.

Cell culture and Western blot. The prostate cancer cell line VCaP, which has type A *TMPRSS2-ERG* fusion (5), and human embryonic kidney HEK 293 cells were obtained from the American Type Culture Collection. Cells were cultured according to the provider's instructions[5] and harvested upon confluence of 70%. RNA was isolated by RNAzol B method (Tel-Test, Inc.). *TMPRSS2-ERG2*, *TMPRSS2-ERG3*, and *TMPRSS2-ERG8* (Flag-tagged) constructs were cloned from prostate cancer cDNA library into pIRES-EGFP plasmid vector (Clontech) and were verified by DNA sequencing. HEK293 cells transfected with the constructs were lysed in M-PER mammalian protein extraction reagent (Pierce) supplemented with protease and phosphatase inhibitor cocktails (Sigma). ERG2 and ERG3 proteins were detected by Western blot (NuPAGE Bis-Tris gel, Invitrogen) using immunoaffinity-purified anti-ERG peptide polyclonal antibody prepared in our laboratory (DFHGI AQALQ PHPPE SSLYK YPSDL PYMGS YHAHP QKMNF VAPHP PAL). The tagged ERG8 protein was detected by Flag-tag antibody (Sigma).

Statistical analyses of clinical and gene expression data. Measures of central tendency (median) and dispersion (range) are used to describe continuously measured patient characteristics, whereas frequencies and percentages are used to describe categorical patient characteristics. χ^2 and Fisher's exact tests were conducted to compare *TMPRSS2-ERG* splice variant transcript expression across patient clinical and demographic characteristics. P values <0.05 are considered statistically significant.

Results

Quantitative analysis of TMPRSS2-ERG expression in prostate tumors. Quantitative analyses of the transcript levels of various *TMPRSS2-ETS* fusion genes were done in LCM matched benign and tumor epithelium of prostate cancer specimens (122 patients; 244 specimens; Fig. 1A). The demographic, clinical, and pathologic variables of the patient cohort are summarized in Supplementary Table S1. The workflow of LCM and quantitative reverse transcription-PCR analysis is summarized in Fig. 1B. The most frequently observed *TMPRSS2-ERG* fusion transcript junctions (6, 7) were detected in 57% of the patients, and among these 95% expressed *TMPRSS2-ERG* fusion type A (Fig. 1A). Fusions with other ETS family members, such as *TMPRSS2-ETV1* or *TMPRSS2-ETV4*, were not detected in this cohort. No fusions were detected in matched benign prostate epithelial cells dissected from the same prostate.

Identification of full-length TMPRSS2-ERG transcripts in prostate tumors. To investigate the nature of *TMPRSS2-ERG*–encoded proteins in prostate cancer, a cDNA library was generated from RNA pooled from six prostate tumors with

[5] http://www.atcc.org

TMPRSS2-ERG fusion. Screening of the library (see flow chart in Supplementary Fig. S1) by both *ERG* and *TMPRSS2* probes resulted in the identification of the following *ERG* splice variants: *ERG1* (M21535), *ERG2* (NM004449), *ERG3* (NM182918), *ERG8* (AY204742), and *TEPC*, a novel splice variant (EU432099; Fig. 2A). *ERG1*, *ERG2*, and *ERG3* contain both SAM (pointed) and ETS (DNA-binding) domain (type I); however, *ERG8* and *TEPC* lack the ETS domain (type II; Fig. 2B). Among the positively identified cDNA library clones, 30% were type I and 70% were type II. Both types of *ERG* transcripts are expressed in VCaP cells, a human prostate cancer cell line derived from vertebral metastasis that harbors *TMPRSS2-ERG* fusion, with the type II transcripts being more abundant (Fig. 2C).

HEK293 cells were transiently transfected with the *TMPRSS2-ERG2*, *TMPRSS2-ERG3*, and *TMPRSS2-ERG8* constructs and the expressed ERG proteins were detected by Western blot showing the expected molecular weight of type I and type II proteins (Fig. 2D). For the detection of type I splice forms (ERG2 and ERG3), an anti-peptide polyclonal ERG antibody was used, which was developed in our laboratory. ERG8, a type II splice form, was Flag-tagged and detected by anti-Flag antibody, because ERG8 lacks part of our ERG peptide epitope.

Relative abundance of type II ERG splice forms in tumor cells of prostate cancer patients. Quantitative expression of the *ERG* splice variants were determined in microdissected tumor cells of 122 prostate cancer patients: 66 with *TMPRSS2-ERG* fusion A transcript junction and 56 with no detectable fusion A transcript (Fig. 3A). At least two or more *ERG* splice variants were detectable in all *TMPRSS2-ERG* fusion A–positive prostate cancer patients. *ERG8* and *TEPC* represented the most abundant *ERG* splice forms analyzed (Fig. 3B) and were detected in 65 of 66 *TMPRSS2-ERG* fusion A expression–positive patients (Fig. 3A). Expression of at least two of the *ERG* splice forms was detected in 10 of 56 fusion A expression–negative cases. Three of these tumors were positive for *TMPRSS2-ERG* fusion types B or C. It is likely that other such tumors may harbor other *TMPRSS2-ERG* fusion junctions. Thus, quantitative analysis of *ERG* splice variants, especially *ERG8* and *TEPC*, provide a reliable surrogate for *TMPRSS2-ERG* fusion in prostate cancer, and in addition it detects *ERG* overexpression even if the fusion junction type is unknown. The order of median abundance (copies/ng total RNA) of *ERG* splice forms in prostate cancer cells of 76 patients with detectable *ERG* expression was *ERG8* (~3,200) > *TEPC* (~1,800) > *ERG3* (~1,500)> *ERG1&2* (~800; Fig. 3B). Overall, the type II splice variants (with no ETS domain) were present in higher copy numbers in prostate cancer cells than the type I splice forms (Fig. 3B and C), and 77% of *ERG*-positive prostate cancer patients tested have more copies of type II than type I splice forms (Fig. 3A). We conclude that quantitative detection of *ERG* splice variants, especially *ERG8* and *TEPC*, may provide increased sensitivity in assessing overall frequency of *TMPRSS2-ERG* fusions in prostate cancer cells.

Expression of ERG splice forms in relation to clinicopathologic variables of prostate cancer patients. In comparison with

Table 1. Correlation of *ERG* splice variant expression and type I/type II ratio with clinicopathologic characteristics

Clinicopathologic characteristics	*ERG* splice variant expression			Type I/ type II ratio		
	No (46)	Yes (76)	*P**	*n* (76)	Median	*P* †
Race			0.0345			0.1142
Caucasian	29 (33%)	59 (67%)		59	0.51	
African American	15 (56%)	12 (44%)		12	0.33	
Family history			0.6312			0.3259
No	28 (38%)	45 (62%)		45	0.52	
Yes	10 (33%)	20 (67%)		20	0.51	
Pathologic T stage			0.5318			0.2541
pT_2	14 (36%)	25 (64%)		25	0.44	
pT_3	29 (42%)	40 (58%)		40	0.51	
Pathologic Gleason sum			0.0023			0.0323
2-6	9 (24%)	29 (76%)		29	0.35	
7	20 (36%)	36 (64%)		36	0.46	
8-10	14 (70%)	6 (30%)		6	0.70	
LCM differentiation			0.0058			0.0067
Well	31 (32%)	66 (68%)		65	0.45	
Poorly	15 (62%)	9 (38%)		9	0.76	
Margin status			0.9436			0.0032
Negative	28 (38%)	45 (62%)		45	0.39	
Positive	16 (39%)	25 (61%)		25	0.57	
PSA recurrence			0.7312			0.0456
No	33 (37%)	56 (63%)		56	0.42	
Yes	11 (41%)	16 (59%)		16	0.61	

NOTE: *ERG* splice variant expression (N = 122): In comparison with patients with no detectable expression of *ERG* in their prostate cancer cells (n = 46), the *ERG*-positive patient cohort (n = 76) has a decreased proportion of patients with high Gleason grade, poor prostate cancer cell differentiation, and African American ethnicity. Type I/type II ratio (n = 76): The ratio of *ERG* type I/type II splice variants in prostate cancer cells is increased in patients with poor tumor cell differentiation and with prostate-specific antigen recurrence.
Abbreviation: PSA, prostate-specific antigen.
*Two-sided test, $P < 0.05$ was considered statistically significant.
† One-sided test, $P < 0.05$ was considered statistically significant.

prostate cancer patients with no detectable *ERG* expression ($n = 46$), the *ERG* expression–positive patient cohort ($n = 76$) has a smaller proportion of patients with high pathologic Gleason grade (8–10), poor prostate cancer cell differentiation, or African American ethnicity (Table 1). The levels of type I or type II *ERG* splice forms in the cohort of prostate cancer patients with *ERG* expression ($n = 76$) did not show significant correlations with clinicopathologic variables. However, there was a trend of correlation of higher copy number ratio of type I over type II splice forms with poor differentiation of prostate cancer cells, higher pathologic Gleason sum, positive margin, and biochemical recurrence (Table 1).

Discussion

ERG overexpression as a result of *TMPRSS2-ERG* fusion represents a highly prevalent oncogenic alteration in prostate cancer. Remarkable progress has been made in just over 2 years in establishing the diagnostic and prognostic features of *TMPRSS2-ERG* fusion in prostate cancer (6, 7). Despite the large body of data on the *TMPRSS2-ERG* fusion junctions, virtually nothing is known about the full-length *TMPRSS2-ERG* transcripts, including the existence and relative abundance of specific splice variants in human prostate tumors. However, splice variants of numerous genes, e.g., *androgen receptor*, *fibroblast growth factor receptor*, *survivin*, and *MDM2*, are known to play critical roles in various human cancers (10, 11).

This study establishes the nature of full-length *TMPRSS2-ERG* transcripts and encoded proteins in prostate cancer cells. In addition to expected full-length *TMPRSS2-ERG* transcripts, we have identified relatively abundant *ERG* splice forms with unique 3′ sequences that lack a conserved region coding for the DNA binding ETS domain. Parallel quantitative analyses of *ERG* splice variants in precisely microdissected cells from well-defined histologic features of the tumor provided accurate data with respect to the presence, abundance, and distribution of various *ERG* splice forms in prostate cancer in relation with clinicopathologic status.

Monitoring the expression of *ERG* splice variants, we detected more prostate cancer cases than by monitoring the fusion transcript junctions, likely because unknown or undetected fusions are present in a subset of cases. Furthermore, the number of various fusion junctions in prostate cancer is far more than the number of *ERG* splice variants.

Recent reports revealed that specific junction types of *TMPRSS2-ERG* fusion transcripts, genomic deletions, or the presence of *TMPRSS2-ERG* fusion are associated with poor prognosis (reviewed in refs. 6, 7). However, others reported that fusion-positive tumors were associated with lower Gleason grade and/or better disease outcome (12). In this study, we found that compared with patients with no detectable expression of *ERG* in their prostate cancer cells, the *ERG* expression–positive patient cohort has a decreased proportion of patients with high Gleason grade, poor prostate cancer cell differentiation, and African American ethnicity ($N = 122$). This is in agreement with our previous study on *ERG* expression in prostate cancer (4). Lower or no *ERG* expression in a subset of aggressive tumors with *TMPRSS2-ERG* fusion may reflect attenuation of androgen signaling pathway during prostate cancer progression (23). The levels of type I or type II *ERG* splice forms did not show significant correlations with clinicopathologic variables. It will be useful to combine multiple approaches, including quantitative assessment of *TMPRSS2-ERG* expression levels, evaluation of genomic rearrangements, and different types of transcripts in multicenter cohort to confirm prognostic values of qualitative and quantitative aspects of *ERG* alterations in prostate cancer.

The diversity of *TMPRSS2-ERG* fusion transcripts has recently been emphasized focusing on the fusion junction region of the transcripts (13, 14) and by using exon arrays (15) that did not allow for the discovery of the type II splice variants described here. Our results highlight the importance of understanding the expression and distribution of full-length splice forms of *ERG*, including variants with no DNA binding domain, in the tumor cells. Our data show a trend of correlation of relatively more type I over type II splice forms, with less favorable pathology and outcome that need to be confirmed in a larger patient cohort. The heterogeneity of *TMPRSS2-ERG* rearrangements in multifocal prostate cancer reported by our group and others (18, 19) further adds to the complexity of understanding the roles of *ERG* in prostate cancer.

In conclusion, this study establishes two major types of full-length transcripts from the *TMPRSS2-ERG* locus in prostate cancer. Further, we establish the protein products translated from type I and type II transcripts. The presence of these specific *ERG* splice forms, especially the more abundant type II splice forms, may provide new opportunities in as prostate cancer biomarker. Finally, overall status of the type I and II forms in prostate cancer cells, such as the ratio of their expression levels, has potential to enhance our understanding of the biology of prostate tumors with *TMPRSS2-ERG* fusion.

Disclosure of Potential Conflicts of Interest

None of the authors have competing financial interests.

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ORIGINAL ARTICLE

ERG oncoprotein expression in prostate cancer: clonal progression of ERG-positive tumor cells and potential for ERG-based stratification

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Gene fusions prevalent in prostate cancer (CaP) lead to the elevated expression of the *ERG* proto-oncogene. *ERG* activation present in 50–70% of prostate tumors underscores one of the most common oncogenic alterations in CaP. Despite numerous reports of gene fusions and mRNA expression, ERG oncoprotein status in CaP still remains to be defined. Furthermore, development of ERG protein-based assays may provide a new dimension to evaluation of gene fusions involving diverse androgen-regulated promoters and the ERG protein-coding sequence. Through exhaustive evaluations of 132 whole-mount prostates (261 tumor foci and over 200 000 benign glands) for the ERG oncoprotein nuclear expression, we demonstrated 99.9% specificity for detecting prostate tumor cells using a highly specific anti-ERG monoclonal antibody. The ERG oncoprotein expression correlated well with fusion transcript or gene fusion in randomly selected specimens. Strong concordance of ERG-positive foci of prostatic intraepithelial neoplasia (PIN) with ERG-positive carcinoma (82 out of 85 sections with PIN, 96.5%) affirms the biological role of ERG in clonal selection of prostate tumors in 65% (86 out of 132) of patients. Conversely, ERG negative PINs were associated with ERG-negative carcinoma. Taken together, the homogeneous and strong ERG expression detected in individual tumors establishes the potential for ERG oncoprotein-based stratification of CaP.
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Keywords: ERG; oncoprotein; prostatic intraepithelial neoplasia; clonal selection; patient stratification

Introduction

Prevalent gene fusions involving regulatory sequences of the androgen receptor (AR) regulated prostate-associated genes (predominantly *TMPRSS2*) and protein-coding sequences of nuclear transcription factors in the *ETS* gene family (primarily *ERG*), result in frequent overexpression of *ERG* in prostate tumors.[1–5] Emerging studies suggest oncogenic functions of *ERG* and *ETV1* in prostate cancer (CaP).[1,6–11] Previous studies including our report have analyzed *ERG* gene fusions at genomic or mRNA levels in the context of multi-focal CaP and these data showed inter-tumoral heterogeneity within the same prostate.[12–15] Despite numerous reports of gene fusions and mRNA expression, ERG oncoprotein in CaP still remains to be defined. Using an anti-ERG monoclonal antibody (ERG–MAb) developed by our group, a global view of ERG oncoprotein expression has been established in the context of multi-focal CaP.

Materials and methods

Cell culture and androgen treatment

LNCaP (ATCC, no. CRL-1740) cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum and 2 mM glutamine. Cells (2×10^6) were seeded onto 10 cm dishes and maintained for 5 days in media containing 10% charcoal-stripped fetal bovine serum (c-FBS; no. 100119 Gemini Bio-Products, Calabasas, CA, USA). For androgen induction, fresh media was supplemented with 0.1 nM R1881 or 1 nM R1881 synthetic androgen for 48 h. VCaP cells (ATCC, no. CRL-2876)

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were grown in DMEM medium supplemented with 10% fetal bovine serum and 2 mM glutamine. Cells (2×10^6) were seeded onto 10 cm dishes and maintained for 3 days in media containing 10% charcoal-stripped fetal bovine serum. For androgen induction, fresh media were supplemented with 0.1 nM R1881 or 1 nM R1881 for another 48 h. At the end of the incubation period, cells were harvested and analyzed by western blots and by microscopy.

ERG *siRNA treatment of prostate cancer cells*
VCaP cells were seeded onto 10 cm tissue culture dishes in DMEM medium containing 10% c-FBS for 3 days. Cells were transfected with *ERG* siRNA or non-targeting control RNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as described before.[7] Twelve hours after transfection with siRNAs, the cell culture medium was replaced with DMEM containing 10% charcoal-stripped serum and 0.1 nM R1881 and maintained for 4 days before harvest and analysis by western blots and microscopy.

Immunoblot analysis
Cells were lysed in M-PER mammalian protein extraction reagent (Thermo, Rockford, IL,USA) containing protease and phosphatase inhibitor cocktails (Sigma, St Louis, MO, USA). Proteins were measured with Bradford Assay reagent (BioRad, Hercules, CA, USA) and lysates equivalent to 25 μg proteins were separated on NuPAGE Bis-Tris (4–12%) gels (Invitrogen, Carlsbad, CA, USA) and blotted onto PVDF membranes (Invitrogen). Immunoblot assays were performed with ERG–MAb (CPDR) mouse monoclonal antibody generated against immunizing polypeptide GQTSKMSPRVPQQDWLSQPP ARVTI, anti-PSA (Cat # A056201–2, DAKO, Carpinteria, CA, USA) and anti–tubulin (Cat no. sc-5286, Santa Cruz, CA, USA) antibodies. Clustal W[16] alignment did not reveal a significant homology of the ERG–MAb peptide antigen with 29 other protein sequences belonging to the human ETS family. Of note, FLI1 protein sequence, which showed 48% identity with the ERG-immunizing peptide was not recognized by the ERG–MAb (Supplementary Figure S1).

Immunofluorescence assay
Cells were fixed in fresh 4% formaldehyde in phosphate-buffered saline (PBS) and permeabilized in PBS-T (PBS + 0.1% Triton X-100) and then centrifuged onto glass slides with a Cytospin 4 centrifuge. Cells were blocked in PBS-NT20 (PBS supplemented with 0.1% Tween-20 and 1% normal horse serum (Vector Laboratories, Burlingame, CA, USA). After incubation with a primary antibody, cells were rinsed and then treated with goat anti-mouse Alexa-594 (Cat no. A11302, Invitrogen) followed by DAPI staining. Images were captured using a $\times 40/0.65$ N-Plan objective on a Leica DMIRE2 inverted microscope equipped with a QImaging Retiga-EX CCD camera (Burnaby, BC, Canada), operated by OpenLab software (Improvision, Lexington, MA, USA). Images were converted into color and merged by using Photoshop (Adobe, San Jose, CA, USA). For ERG peptide competition experiments, the ERG–MAb antibody was pre-incubated with 2000-fold molar excess of competing or non-competing peptide on ice for 30 min.

Prostate specimens
Under an Institutional Review Board-approved protocol, radical prostatectomy specimens from patients enrolled in the Center for Prostate Disease Research program were obtained by pathologists within 30 min after the surgical removal of the specimens. Prostates were processed as whole-mounts according to the Armed Forces Institute of Pathology (AFIP) protocol.[15] From each of 132 patients, one whole-mount cross section containing one to four tumors (mostly two foci) was selected and tumors represented different grades and stages. Each tumor was separately diagnosed in the prostatectomy specimens and slices with more than one tumor focus represented separate tumors. The cohort includes 50 stage and grade matched patients of whom 25 developed metastasis and 25 had no recurrence with a mean follow up of 46.5 months with the intent to address possible prognostic features of ERG. To assess the relationship between mRNA and ERG oncoprotein data specimens from patients were included from previous studies investigating *ERG* transcripts by quantitative RT-PCR or by GeneChip (2, 5).

Immunohistochemistry (IHC) for ERG
Following deparaffinization, 4 μm sections were dehydrated and blocked in 0.6% hydrogen peroxide in methanol for 20 min. Sections were processed for antigen retrieval in EDTA (pH 9.0) for 30 min in a microwave followed by 30 min of cooling in EDTA buffer. Sections were then blocked in 1% horse serum for 40 min followed by incubation with the ERG–MAb mouse monoclonal antibody at a dilution of 1:1280 for 60 min at room temperature. Sections were incubated with the biotinylated horse anti-mouse antibody at a dilution of 1:200 (Vector Laboratories, Burlingame, CA, USA) for 30 min followed by treatment with the ABC Kit (Vector Laboratories) for 30 min. The color detection was achieved by treatment with VIP (Vector Laboratories) for 5 min. Sections were then counterstained in hematoxylin for 1 min, dehydrated, cleared and mounted. The ERG–MAb staining was determined according to percent of cells positive: up to 25% (1+), >25–50% (2+), >50–75% (3+) and >75% (4+). The intensity was scored as mild (1+), moderate (2+) and marked (3+). A combination of both measurements was calculated by multiplying the percent of positive cells with the degree of intensity, which resulted in a score. As, most of the tumors showed positivity in over 75% of cells and the intensity was uniform, we expressed the staining results as ERG positive or negative.

Analysis of ERG mRNA by branched-chain DNA (bDNA) signal amplification
One 4-μm thick section was selected from each of the 35 FFPE whole-mount prostate samples. Areas identified as tumors were marked, removed by scraping and were homogenized and processed as described previously[17] and in the Supplementary Materials and Methods. The geometric mean of the expression of three housekeeping

genes (*ACTB, B2M, RPL19*) was determined and only samples with this mean value of minimum three-fold over background signal were included in the analysis. The *TMPRSS2–ERG* expression data was normalized to the geometric mean of the three housekeeping genes in each sample. Samples with normalized *TMPRSS2–ERG* expression over an arbitrary cutoff of 0.5 were considered positive for the fusion. The *TMPRSS2–ERG* expression data tightly correlated with similarly normalized *ERG* mRNA expression in the same samples. The blinded transcript expression data were then compared with the protein expression data.

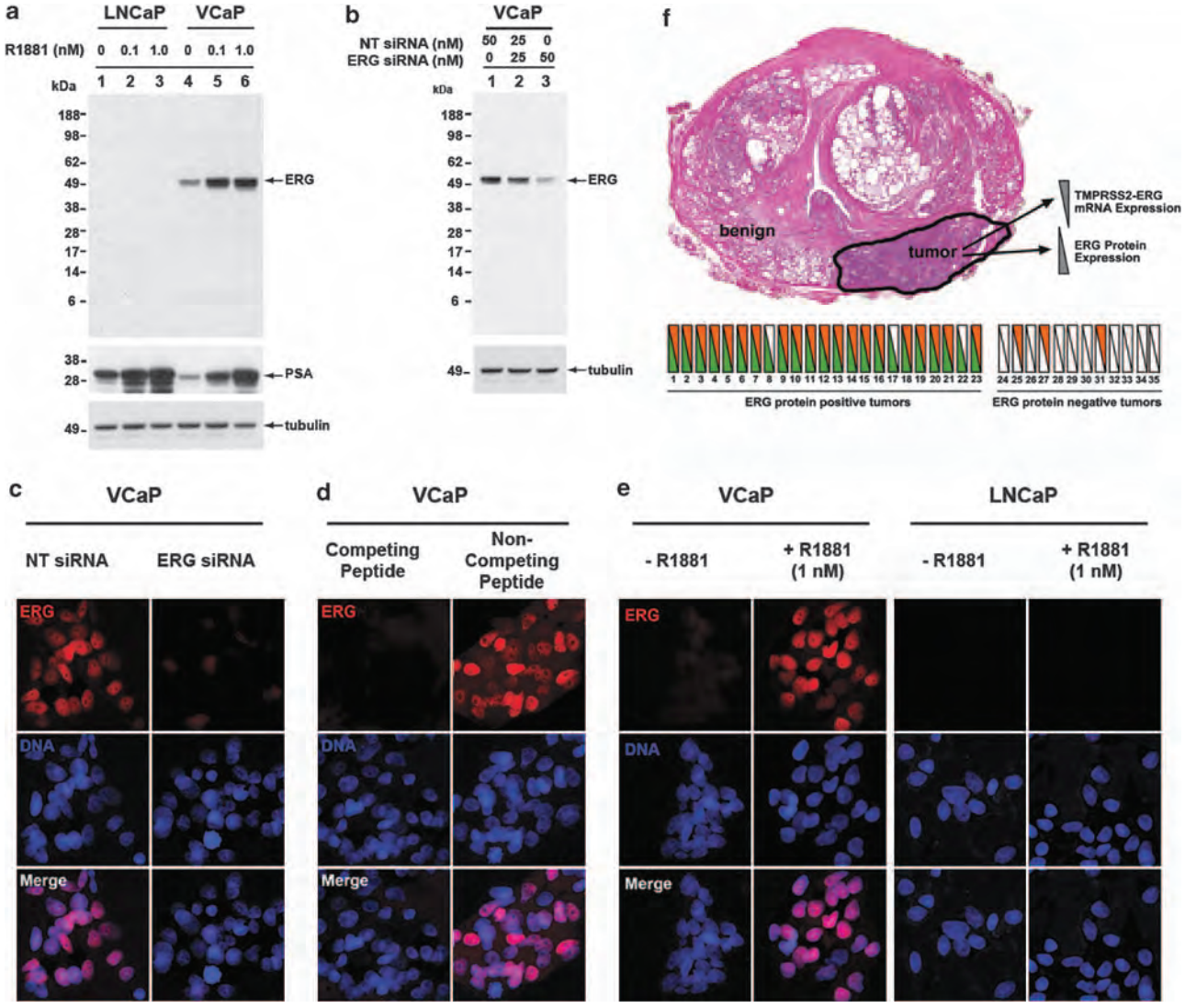

Figure 1 Detection of ERG oncoprotein by ERG–MAb in prostate cancer cells. (**a**) LNCaP cells treated with 0.1 nM R1881 (lane 2) or 1 nM R1881 (lane 3) and VCaP cells treated with 0.1 nM R1881 (lane 5) or 1 nM R1881 (lane 6) were analyzed for ERG oncoprotein by using ERG–MAb as described in Materials and Methods. LNCaP (lane 1) and VCaP cells (lane 4) were processed in parallel without R1881 served as controls. (**b**) ERG oncoprotein was analyzed in VCaP cells transfected with non-targeting (NT) or *ERG* siRNA oligonucleotides. Cell lysates were prepared 4 days after transfection with 50 nM NT siRNA (lane 1), 25 nM NT siRNA and 25 nM *ERG* siRNA (lane 2) or 50 nM *ERG* siRNA oligonucleotides (lane 3). Twenty-five micrograms of cell lysates were separated on NuPAGE Bis-Tris (4–12%) gels, transferred onto PVDF membrane and immunoblotted ERG–MAb. Identical samples were transferred onto PVDF membranes and probed with ERG–MAb, anti-PSA and anti–tubulin antibodies. The apparent size of the ERG protein products in the western blots correspond to predicted molecular weights of *TMPRSS2* (exon 1)-*ERG3* (exons from 8 to 16, GenBank accession number NM_001136154) *or TMPRSS2* (exon 1)-*ERG2* (exons from 8 to 16 lacking exon 12, GenBank accession number NM_004449). (**c**) VCaP cells transfected with either NT siRNA (left panel) or ERG siRNA (right panel) were immunostained with mouse ERG–MAb followed by goat anti-mouse Alexa-594 (red). (**d**) VCaP cells were grown in DMEM supplemented with 10% fetal bovine serum. Cells were incubated with ERG–MAb, pre-treated with competing or non-competing peptide. (**e**) VCaP or LNCaP cells treated with or without 1 nM of R1881 were analyzed for ERG oncoprotein by ERG–MAb. (**f**) Schematic representation of the expression of ERG oncoprotein (IHC) and *TMPRSS2–ERG* fusion mRNA was determined in prostate tumors of 35 CaP patients treated with radical prostatectomy by using bDNA assay as described in Materials and Methods. Consecutive tissue slides from whole-mounted FFPE prostate specimens were used for the two assays in a blinded fashion. Green triangles represent positive ERG oncoprotein staining, orange triangles represent the detection of *TMPRSS2–ERG* fusion mRNA. Hollow triangles indicate specimens with undetectable ERG oncoprotein or *TMPRSS2–ERG* fusion transcript.

Statistical analysis

Sensitivity and specificity of ERG oncoprotein expression were analyzed for distinguishing all tumor foci from benign glands in whole-mount prostates (261 tumor foci and over 200 000 benign glands). Chi square test was used to test the association of ERG oncoprotein status with tumor differentiation and Gleason score for individual tumors. *P*-value of 0.05 was adopted as statistically significant. The SAS version 9.2 was used for all data analyses.

Results

Characterization of ERG oncoprotein by ERG monoclonal antibody in cancer cell lines

In *TMPRRS2–ERG*-positive VCaP cells, a mouse monoclonal anti-ERG antibody, ERG–MAb recognized predicted sizes of full length protein products (50–52 kDa) encoded by *TMPRSS2–ERG2* and *TMPRSS2–ERG3* fusion transcripts (Figure 1 and Supplementary Figure S2). As expected, ERG–MAb did not detect ERG oncoprotein in LNCaP cells, which do not harbor *TMPRSS2–ERG* fusion (Figure 1a and Supplementary Figure S2b). To further show the specificity of ERG–MAb, a significant inhibition of the endogenous ERG oncoprotein was noted in *ERG* siRNA[7] transfected VCaP cells (Figure 1b). ERG protein was also detected in tumor cell lines (KG1, COLO 320, MOLT4) previously described to express *ERG* (Supplementary Figure 2b). Specificity of the ERG–MAb for ERG oncoprotein detection in VCaP cells was further validated by immunofluorescence (IF) assays (Figures 1c–e). These data together established the specificity of the ERG–MAb in detecting ERG oncoprotein in CaP cells.

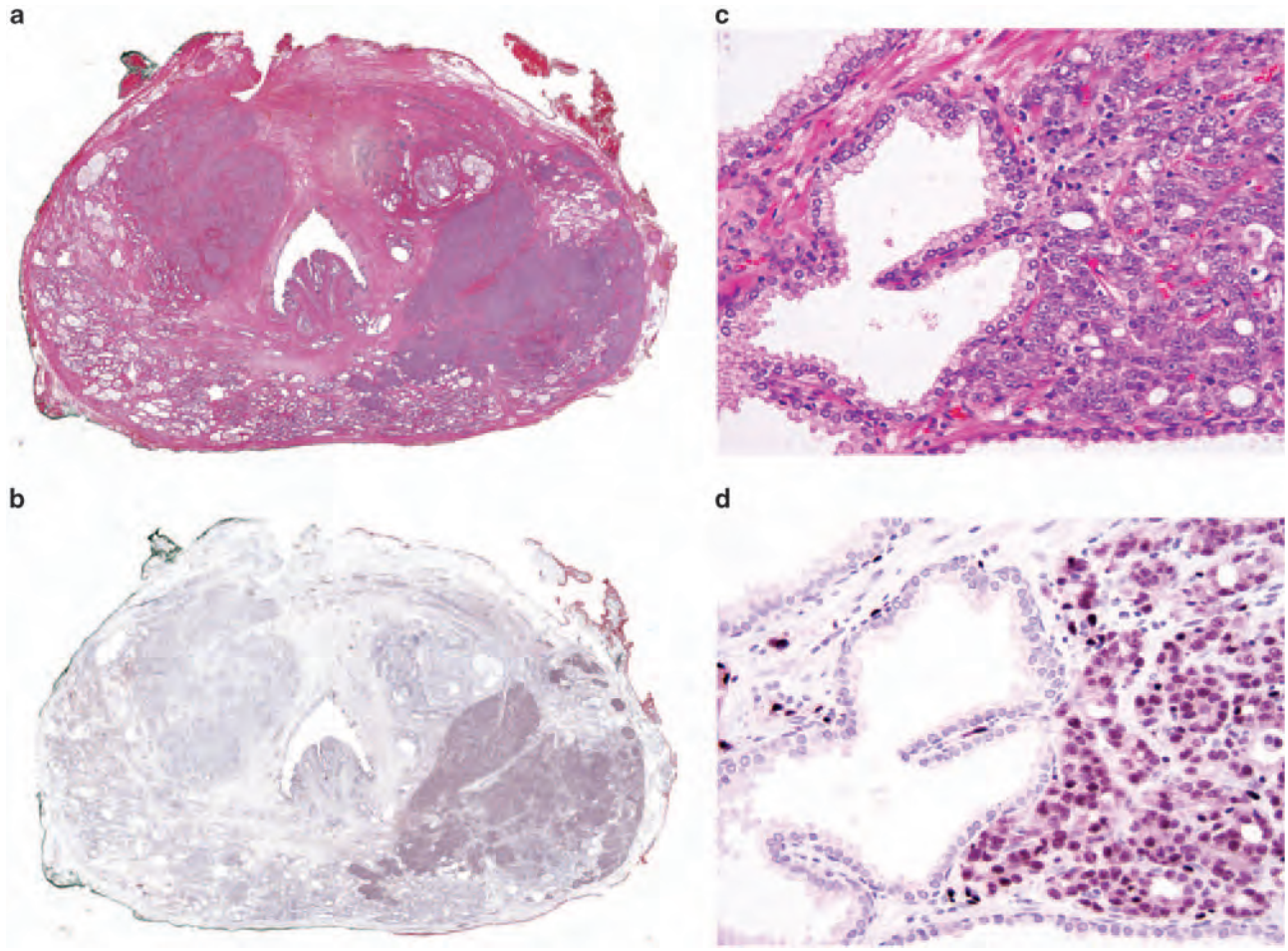

Figure 2 Distribution of ERG oncoprotein in a patient's whole-mount cross section of the prostate. (**a**) Whole-mount cross section of one prostate with two tumors: left upper quadrant and right lower quadrant, H&E × 1. (**b**) Same section as (**a**). The tumor in the left upper quadrant is ERG negative, whereas the tumor in the right lower quadrant is ERG positive. Note that the entire tumor is positively outlined including the irregularly infiltrating borders, ERG–MAb × 1. (**c**) The tumor infiltrates as densely packed simple glands between benign glands, H&E × 20. (**d**) Same field as (**c**). Only tumor cells are positive for ERG. Note the strong reactivity in endothelial cells of the capillaries, some of which are in intimate proximity to benign glands, ERG–MAb × 20. (**e**) Native glands lined by secretory cells with nuclear anaplasia and recognizable basal cells are diagnostic of high-grade prostatic intraepithelial neoplasia. They are associated with infiltrating carcinoma, H&E × 20. (**f**) Same field as Figure (**e**). The nuclei of both the prostatic intraepithelial neoplasia and infiltrating carcinoma, are positive for ERG, but with variable intensity. Basal cells are negative. Note the uniformly strong nuclear staining in capillaries (arrows). (**g**) A cluster of benign glands appears to be prominent based on the dark staining cytoplasm, H&E × 20. (**h**) Same field as (**g**). Rare benign secretory cells show nuclear reactivity, ERG–MAb × 20. (**i**) Left upper quadrant tumor. Note benign glands in the lower left. H&E × 1. The inset shows infiltrating carcinoma at the left adjacent to a benign gland, H&E × 20. (**j**) Same field as (**i**). The tumor is negative for ERG. In the inset, both the benign gland and tumor are negative for ERG. ERG–MAb × 1 inset ERG–MAb × 20.

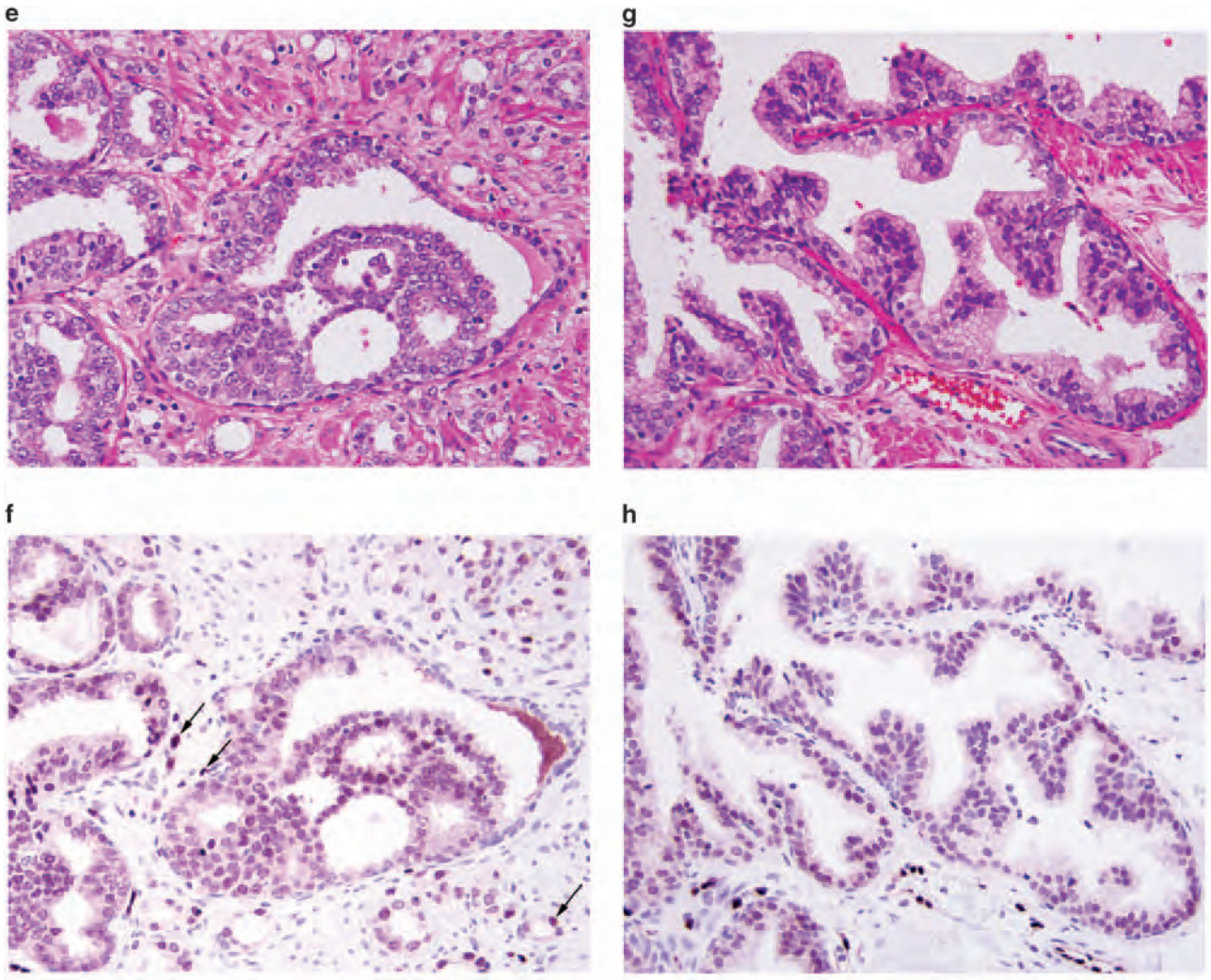

Figure 2 Continued.

Relationship of ERG oncoprotein and TMPRSS2–ERG fusion status in prostate specimens

To determine the clinical utility of ERG–MAb, it was critical to establish the specificity of the ERG oncoprotein staining in tumor specimens in relation to *TMPRSS2–ERG* fusion status. A comparative analysis was performed on consecutive tissue sections of the ERG oncoprotein positive or negative FFPE specimens for the detection of *TMPRSS2–ERG* mRNA. Analysis of 35 evaluable specimens revealed a strong correlation between mRNA levels of *TMPRSS2–ERG* fusion type A transcript and ERG oncoprotein immunohistochemistry (Figure 1f). A concordance rate of 82.8% was noted between mRNA and protein data despite the expected differences in the sensitivity as well as read-outs of the two techniques. A comparative evaluation of *TMPRSS2–ERG* gene fusion analysis by fluorescence *in situ* hybridization (FISH) and ERG oncoprotein expression by ERG–MAb IHC in 10 specimens revealed no discrepancies (Supplementary Figure S3).

Expression map of the ERG oncoprotein in multi-focal prostate cancer

To delineate the expression map of ERG in benign glands, carcinoma and prostatic intraepithelial neoplasia (PIN), we utilized one entire cross section of each whole-mount radical prostatectomy from 132 patients with prostatic carcinoma. Each tumor was individually measured and graded. On average, one whole-mount section (3.5×2.5 cm or 4.0×3.5 cm) is equivalent to approximately 800–1400 tissue microarray cores of 1 mm diameter. In addition to index tumors, most of these cross sections contained benign prostatic tissue of the peripheral and the transition/periurethral zone as well as the urethra, utricle, ejaculatory ducts (Figures 2a and b), and seminal vesicles. A single tumor was present in 51 sections, and multiple individual tumors were present in 81 sections. Tumor grade, pathological stage, margin status and clinical data are summarized in Supplementary Table S1. In prostatic adenocarcinomas (Figures 2c and d) and in PIN (Figures 2e and f) the epithelial cells showed nuclear staining. ERG was positive in 117 of 261 (44.8%) individual tumors (Table 1a). ERG oncoprotein expression was highly specific (99.9%) in detecting carcinoma (Table 1a). Of 132 specimens only six specimens showed rare ERG-positive non-malignant cells. In three specimens, a single group of benign glands (average seven glands, raging from five to eight glands) each was positive for ERG in addition to carcinoma (Figures 2g and h). In

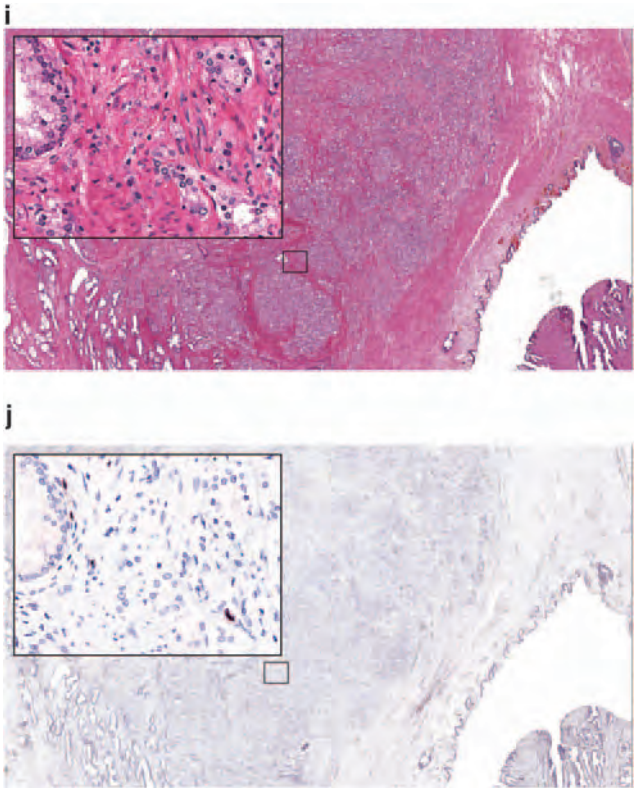

Figure 2 Continued.

Table 1a Frequency of ERG oncoprotein expression in whole-mount prostatectomy specimens

ERG	*Individual tumors*	*Benign glands*
Positive	117	22
Negative	144	200 000

Specificity = 99.99%; Sensitivity = 44.83%; PPV 84.17% and NPV = 99.93%

Sensitivity and specificity of the ERG oncoprotein nuclear staining distinguishing tumor foci from benign glands in 132 whole-mounted prostate sections (261 tumor foci and about 200,000 benign glands). Number of benign glands represents an estimate based on counting of the number of benign glands in three average size sections of this cohort (average 1550 benign glands/section) multiplied by 132 sections.

Table 1b Association of ERG oncoprotein status with tumor differentiation and Gleason pattern of individual tumors ($N = 261$)

Tumor grade	*ERG status*		*P-value*
	Negative (N = *144*)	*Positive* (N = *117*)	
Tumor differentiation			
Gleason pattern 3 (Well differentiated)	100 (62.5%)	60 (37.5%)	0.0027
Gleason pattern 4/5 (moderate/poorly differentiated)	44 (43.6%)	57 (56.4%)	
Tumor Gleason sum			0.0094
6	100 (62.5%)	60 (37.5%)	
7	26 (41.3%)	37 (58.7%)	
8–10	18 (47.4%)	20 (52.6%)	

three additional specimens, ERG was present in small aggregates of native glands (3–5 glands) with increased cellularity and nuclear enlargement and mild atypia, changes previously referred to as 'low grade PIN'. Eight of the nine anterior/transition zone tumors were negative (Figures 2i and j). In all but five cases, over 85% of tumor cells showed moderate to strong nuclear staining with cytoplasmic blush (Figures 2c and d).

Association of the ERG oncoprotein status was evaluated with various clinico-pathological features (Supplementary Table S1a and b). Although, ERG expression did not show correlation with most clinico-pathological features, when all of the tumor foci in a given whole-mount section were taken into account, higher Gleason sum and less-differentiated tumors showed significant correlation with ERG-positive immunostaining (Table 1b).

Eighty-two of eighty-five (96.5%) evaluable specimens with ERG-positive tumor foci contained ERG-positive PIN lesions, and all of the ERG-positive PIN foci were co-located with ERG-positive tumors (Table 2). Eighty-one sections contained multiple tumors; in 15 of these all tumors were positive; in 31 all tumor foci were negative and in 35 some tumors were diffusely positive and others completely negative. Thus, in a multi-focal tumor context, 50 of 81 sections (61.7%) had one or more ERG-positive tumors. In the 51 sections containing only one tumor, 36 (70.6%) were ERG positive, and two of these contained clones of completely ERG-negative tumor cells embedded in the positive areas (Figures 3a and b). A weak non-discriminatory cytoplasmic staining was observed in all epithelial cell types (prostatic and non-prostatic), which was consistent with the cell line data (Figures 3c and d).

The ERG–MAb consistently detected ERG in the nuclei of all endothelial cells (lympho/vascular), which served as intrinsic positive control for the ERG IHC assay. ERG expression in endothelial cells has also been noted previously in other contexts; however, its significance remains to be defined.[18–20] Endothelial cells can be easily identified by ERG-positive nuclei in cells with very little discernible cytoplasm in contrast to carcinoma, in which most of the tumor cells have ERG-positive nuclei and easily identifiable cytoplasm (Figures 2c and d). In ERG negative poorly differentiated/Gleason pattern 4 or 5 carcinomas, positive nuclei of endothelial cells often have a linear narrow distribution (Supplementary Figure S4a and b).

Tumor cells with amphophilic cytoplasm were more strongly positive than those with pale or foamy cytoplasm (Supplementary Figure S5a and b). Three of the four mucinous carcinomas were positive for ERG (Supplementary Figure S5c and d). Only two of the five tumors with a ductal component were positive for ERG (Supplementary Figure S5e–h). One tumor with vacuolated/signet ring-like appearance was positive for ERG. The focus with lymphoepithelioma-like features was negative. In seven patients with lymph node metastases at the time of prostatectomy, the ERG expression mirrored the expression status of the index tumor. Four ERG-positive primary tumors had ERG-positive metastases, and conversely, three ERG-negative primary tumors had ERG-negative metastases (Supplementary Figure S3). By FISH assay, ERG-positive primary tumors (Supplementary Figure S3a) and the corresponding metastases (Supplementary Figure S3b) showed identical fusion patterns.

Basal cells, urothelial cells of the prostatic urethra and periurethral prostatic ducts were non-reactive. Ejaculatory

Table 2 Summary of the ERG oncoprotein status in individual tumors of whole-mount prostate sections

P	Benign glands	LGPIN	T1 PIN	T1	T2 PIN	T2	T3 PIN	T3	T4 PIN	T4
1			0	6						
2			1	6						
3		LGPIN	1	6						
4			1	6						
5			1	6						
6			1	6						
7			1	6						
8			0	7a						
9			0	7a						
10			0	7a						
11			0	7a						
12			1	7a						
13			1	7a						
14			1	7a						
15			1	7a						
16			1	7a						
17			1	7a						
18			1	7a						
19			1	7a						
20			1	7a						
21			1	7a						
22			1	7a						
23			1	7a						
14			1	7a						
25			1	7a						
26			1	7a						
27			1	7a						
28			1	7b						
29			0	7b						
30			0	7b						
31			0	7b						
32			0	8						
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36			1	8						
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41			1	8						
42			0	9						
43			0	9						
44			0	9						
45			0	9						
46			0	9						
47			1	9						
48			1	9						
49			1	9						
50			1	10						
51			1	10						
52			0	6	0	6				
53			0	6	NP	6				
54			1	6	1	6				
55			1	6	1	6				
56	Benign		1	6	1	6				
57			1	6	1	6				
58			1	6	0	6				
59			1	6	0	6				
60			1	6	0	6				
61			1	6	0	6				
62			1	6	0	6				
63			NP	6	0	6				
64			0	7a	0	7a				
65			0	7a	0	6				
66			0	7a	0	6				

P	Benign glands	LGPIN	T1 PIN	T1	T2 PIN	T2	T3 PIN	T3	T4 PIN	T4
67			0	7a	0	6				
68			0	7a	0	6				
69			0	7a	0	6				
70			0	7a	0	6				
71			0	7a	NP	6				
72			0	7a	1	6				
73			0	7a	0	6				
74			1	7a	NP	6				
75			1	7a	1	6				
76			1	7a	1	6				
77			1	7a	1	7a				
78			1	7a	0	6				
79			1	7a	0	6				
80			1	7a	0	6				
81			1	7a	0	6				
82			1	7a	0	6				
83			NP	NP	0	6				
84			0	8	0	6				
85			0	8	0	6				
86			0	8	1	NP				
87			0	8	1	6				
88			1	8	1	7a				
89			1	8	1	6				
90			1	8	1	6				
91			0	9	0	6				
92			1	9	0	6				
93	Benign		1	9	0	6				
94			1	9	1	6				
95			0	6	0	8	1	6		
96		LGPIN	0	6	1	6	1	6		
97			1	6	1	6	1	6		
98			1	6	1	6	1	6		
99			1	6	0	6	0	6		
100			1	6	0	6	0	6		
101			0	7a	0	6	0	6		
102			0	7a	0	6	0	6		
103			0	7a	0	6	0	6		
104			1	7a	0	6	0	6		
105			0	7a	1	6	0	6		
106			0	7a	1	6	NP	6		
107			1	7a	0	6	NP	6		
108			1	7a	NP	NP	0	6		
109			1	7a	0	6	0	6		
110			1	7a	1	6	1	6		
111			1	7a	1	6	0	6		
112			NP	NP	1	6	0	6		
113			1	7a	0	7b	NP	6		
114			0	9	0	6	0	6		
115			0	9	0	6	0	6		
116			1	9	1	6	0	6		
117			0	6	0	6	0	6	0	6
118			0	6	0	6	0	6	0	6
119			0	6	0	6	0	6	0	6
120			0	6	1	6	0	6	1	6
121			1	6	1	6	1	6	0	6
122			1	6	1	6	0	6	NP	6
123			1	6	1	6	0	6	0	6
124			1	6	1	6	0	6	0	6
125	Benign		0	7a	0	6	0	6	0	6
126			0	7a	0	6	0	6	1	NP
127			1	7a	1	6	0	6	0	6
128			NP	7b	1	6	1	6	0	6
129		LGPIN	0	8	0	6	0	6	0	6
130			0	8	0	6	0	6	0	6
131			1	8	0	6	1	6	NP	6
132			NP	NP	0	6	0	6	0	6

Abbreviations: Red: ERG-positive IHC; Green: ERG-negative IHC; NP: not present in the section; LGPIN: 'low grade PIN'; 1: ERG expression in PIN; Gleason score (7a: 3+4; 7b: 4+3) annotated in tumor columns (T1 to T4).

A comprehensive analysis of benign glands, PIN and tumor foci for ERG oncoprotein status is summarized in the heat map. Eighty-two of eighty-five (96.5%) evaluable specimens with ERG-positive tumors contained ERG-positive PIN lesions and most of the time focally ERG-positive PIN foci co-located with ERG-positive tumors. In contrast, ERG-positive PIN foci were present in only 3 of 45 (6.6%) sections with ERG-negative tumors. In the entire study cohort of 132 cases, six cases (4.5%) were ERG positive in rare benign glands or in atypical (LGPIN) foci and four of the six cases had ERG-positive tumors.

ducts, seminal vesicles, nerve bundles, fibromuscular stroma, variants of glandular hyperplasia including microacinar hyperplasia (synonyms: adenosis, atypical adenomatous hyperplasia), sclerosing adenosis and basal cell hyperplasia were all negative for ERG. Different patterns of atrophy including proliferative inflammatory atrophy and evolving or partial atrophy were also negative for ERG.

Discussion

As the gene fusion events in CaP commonly involve regulatory sequences of AR-regulated prostate-associated genes, for example, *TMPRSS2*, *SLC45A3* or *NDRG1* along with protein coding sequences of the nuclear transcription factors in the *ETS* gene family (*ERG, ETV1, ETV4–6* and *ELK4*), the resultant protein products are ETS-related oncogenic transcription factors with ERG being the most common.[1] The ERG–MAb described herein exhibits a high degree of specificity and sensitivity in recognizing ERG oncoprotein. Positive nuclear staining for the ERG oncoprotein is highly specific (99.9%) in identifying tumor cells in 65% of patients. Nuclear ERG staining is virtually absent in benign epithelial cells. Overall 44.8% of all 261 individual tumors were ERG positive in this cohort, whereas 70.6% of 51 specimens with single tumor were ERG positive and 62% of 81 specimens with more than one tumor were ERG positive. Overall frequencies of ERG expression in CaP specimens noted here are similar to the reported rate of gene fusions involving *ERG* locus reviewed in Kumar Sinha *et al*., and Clark and Cooper.[1,21] Furthermore, this study points to the potential contribution of sample bias in assessing frequency of ERG alterations in CaP. In previous studies specificity and sensitivity of ERG protein detection was not addressed due to limited number of specimens examined.[6,22] In general, tumors are either homogeneously positive or negative for ERG expression. This study highlights the association (96.5%) of ERG-positive PINs with ERG-positive tumors (Table 2).

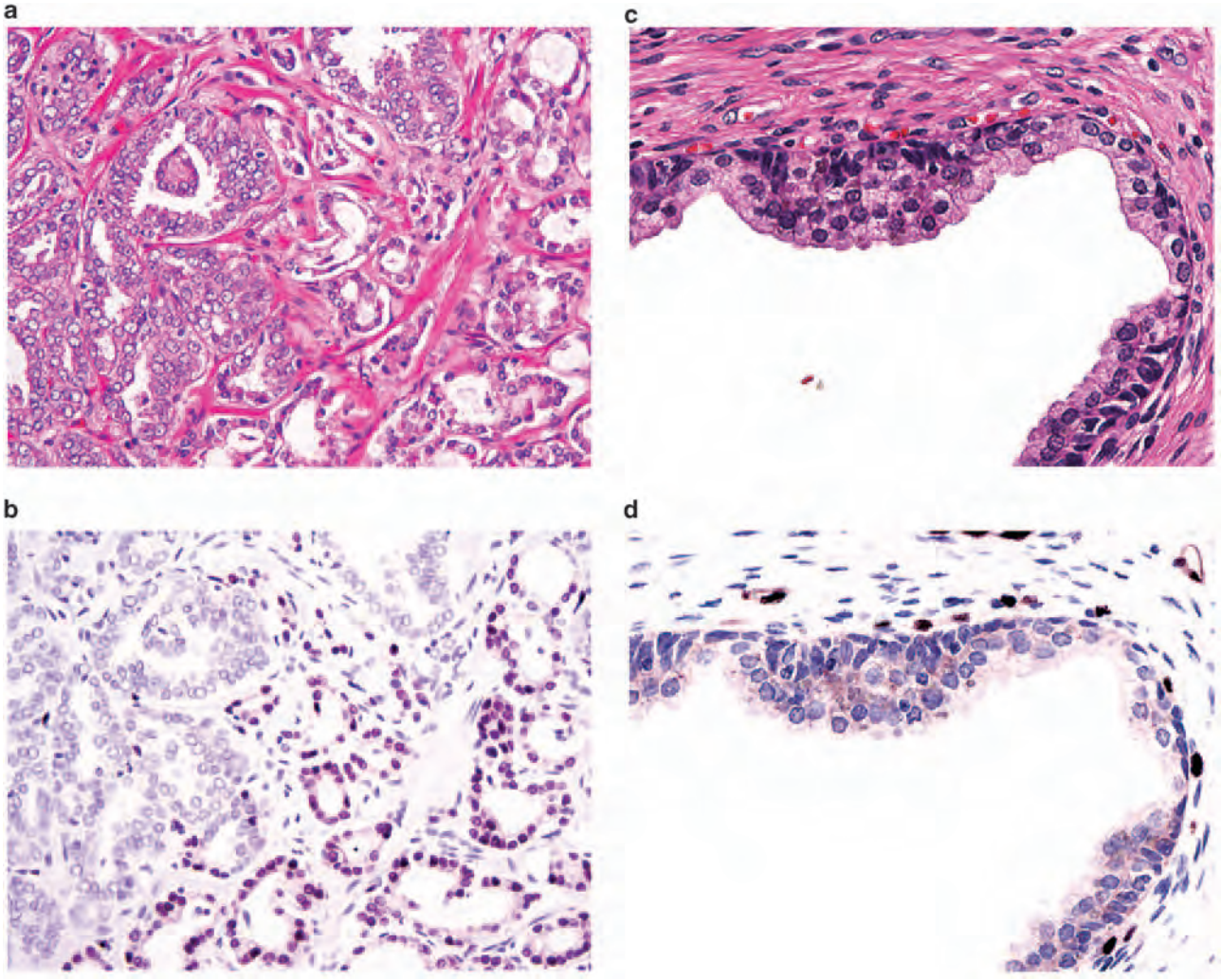

Figure 3 ERG oncoprotein in carcinoma with heterogeneous expression and non-discriminatory staining of benign glands. (**a**) Tumor shows a 'diverse' ERG expression pattern with ERG positive alternating with ERG negative clones, H&E × 20. (**b**) Same field as (**a**). Although, the tumor cells appear similar in the H&E-stained section, they differ in their ERG oncoprotein distribution, ERG–MAb × 20. (**c**) Benign gland with basal and secretory cells, H&E × 20. (**d**) Same field as (**c**). The nuclei of the secretory and basal cells are negative for ERG. Note weak cytoplasmic reactivity in the secretory cells. The endothelial cells show strong nuclear positivity for ERG, ERG–MAb × 20.

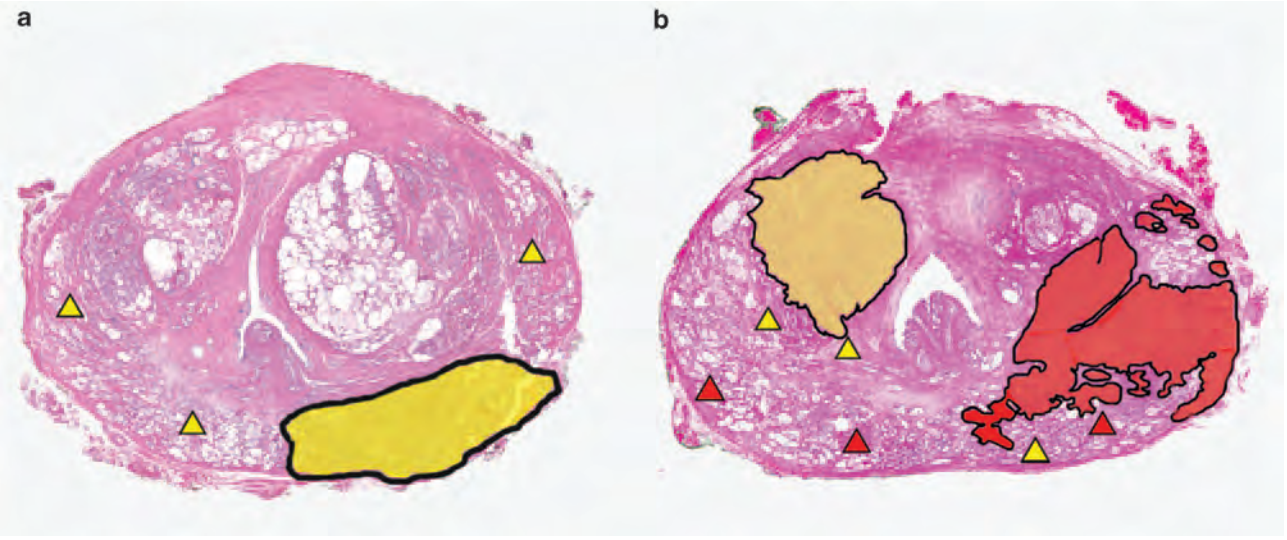

Figure 4 Schematic representation of the concordance of ERG status between PIN and carcinoma in whole-mount prostates. Two scenarios of whole-mount radical prostatectomy specimens are represented with carcinoma and PIN areas are marked. (**a**) Specimens with ERG (−) carcinoma (yellow areas) and ERG (−) PIN foci (yellow triangles) in the same prostates. (**b**) Specimens with at least one ERG (+) carcinoma (red areas) and PIN foci (red triangles) in the same prostates.

Although other studies[1,23,24] have shown lower frequency of *ERG* fusion-positive PIN (15–20%), this study of whole-mount prostate sections allows more comprehensive evaluation of PIN and tumors in the context of ERG oncoprotein expression (Figure 4).

The rare ERG-positive benign glands and the rare atypical native glands, referred to as low-grade PIN, may harbor sub-morphological molecular alterations, particularly in view of their topographical relationship to PIN and/or carcinoma. This finding is in agreement with previous studies reporting the presence of *TMPRSS2–ERG* fusion transcripts in rare instances of benign prostatic glands.[12,15] The confirmation of *TMPRSS2–ERG* fusions in these foci is challenging due to their small size. When considering the high concordance rate between ERG oncoprotein expression and *TMPRSS2–ERG* gene fusion transcript status, one could employ the ERG IHC as an excellent surrogate marker for gene fusions leading to ERG overexpression. Thus, in addition to complementing genomic and mRNA-based assays ERG oncoprotein detection provides a significant advance in assessing ERG alterations in CaP. For example, translational products resulting from genomic fusion events of *ERG* protein-coding sequence and regulatory sequence of any 5′ fusion partners (*TMPRSS2, SLC45A3 and NDRG1*)[3,25,26] can be detected by ERG–MAb. On the practical side, evaluation of ERG protein by IHC will be more rapid and informative for morphological assessment of ERG oncogenic activation in 'front-end' pathology setting.

Among the currently used diagnostic markers, α-methylacyl-CoA racemase detects approximately 80% of prostatic carcinomas and a variety of other carcinomas.[27] However, the specificity of α-methylacyl-CoA racemase is lower than that of the ERG, because 25–30% of benign prostatic glands may stain for α-methylacyl-CoA racemase. Thus, inclusion of ERG–MAb in a diagnostic IHC panel may increase the specificity for tumor detection. The strong positive reaction of ERG–MAb in endothelial cells observed highlights many more capillaries in the prostate than were previously appreciated using conventional endothelial cell markers (CD 31, CD 34 and Factor VIII-related antigens). However, this feature of ERG expression may cause some difficulties in the interpretation of the ERG IHC staining. For example, capillaries in intimate contact with glands may suggest basal cell staining, or dilated capillaries with reactive endothelium may mimic small tumor glands or atrophy. This initial limitation can be overcome by gaining experience recognizing ERG-positive vascular patterns (Supplementary Figure S4a and b).

Although prognostic features of ERG alterations in CaP remain to be better understood, both positive and negative associations have been reported and reviewed in Kumar Sinha *et al*. and Clark and Cooper.[1,21] In this evaluation of ERG oncoprotein, when all of the tumor foci in a given whole-mount section were taken into account, higher Gleason sum and less-differentiated tumors showed correlation with ERG immunostaining (Table 1b). However, there was no significant correlation with progression (Supplementary Figure S6). Considering the ERG expression in the multi-focal tumor context, further independent evaluations in larger and better-defined cohorts are warranted.

In summary, among the currently known CaP protein biomarkers the detection of the homogeneous, strong and highly specific ERG oncoprotein offers unprecedented opportunities in CaP diagnostic setting. These findings substantiate the role of *ERG* activation in clonal selection and expansion of ERG-positive tumor cells during the transition from pre-invasive to invasive CaP in two-thirds of patients. Finally, with a better understanding of ERG functions in prostate tumor biology, ERG–MAb-based stratification of prostate tumors in the future may be used in the context of imaging, targeted therapy or monitoring efficacy of androgen ablation therapy.

Conflict of interest

The Henry M Jackson Foundation for the Advancement of Military Medicine filed a patent on ERG–MAb, in which AD, ST and SS are co-inventors. GM is an employee of the Affymetrix.

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Disclaimer

The views expressed in this article are those of the authors and do not reflect the official policy of the Department of the Army, Department of Defense or the US Government.

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Research Paper

Ets Family Protein, Erg Expression in Developing and Adult Mouse Tissues by a Highly Specific Monoclonal Antibody

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Abstract

Oncogenic activation of the ETS Related Gene (*ERG*) in humans was originally identified in subsets of Ewing sarcomas, myeloid leukemias and, recently, in the majority of prostate cancers. Expression of human ERG protein and consequently its functions in normal and disease states needs to be better understood in light of its suggested role in cell differentiation and proliferation. Here, we analyzed temporal and spatial expression of the Erg (mouse protein) by immunohistochemical analysis during mouse embryonic and adult organogenesis using a highly specific ERG monoclonal antibody (ERG MAb). This study establishes widespread immunolocalization of Erg protein in endothelial cells and restricted expression in precartilage and hematopoietic tissues. Intriguingly, Erg is not expressed in any epithelial tissue including prostate epithelium, or in infiltrating lymphocytes that are occasionally seen in the prostate environment, a common site of tumors with *ERG* rearrangements and unscheduled *ERG* expression. These findings will further aid in investigations of Erg functions in normal and disease conditions.

Key words: Ets Related Gene, ERG, Expression, ERG MAb, Mouse, Development.

Introduction

Chromosomal translocations leading to gene fusions have been well characterized in variety of malignancies [1]. Gene fusions often result in chimeric proteins with aberrant functions and/or ectopic expression. Frequent elevated expression of the *ETS* related genes (*ERG*) due to chromosomal rearrangements resulting into the fusion between androgen regulated promoters (predominatly*TMPRSS2*) and protein coding sequence of *ETS* transcription factors has been established in prostate cancer [2, 3]. *ETS* (Erythroblast Transformation Specific family of transcription factors) genes are a large family with at least thirty members that function as transcription factors [4]. All *ETS* transcription factors share a highly conserved DNA binding domain, the ETS domain [5] and at least *Ets1, Erg, Fli1* and *Etv2* are expressed in embryonic endothelial cells of mouse [6, 7]. *ERG* is well conserved in evolution and its expression and poten-

tial functions have been studied in xenopus, zebrafish, mouse and humans [8-12]. The results from these studies suggest an emerging role for *ERG* in the transcriptional regulation of endothelial specific genes [13-16] and in definitive hematopoiesis [17, 18]. Both hematopoietic and endothelial cells are of mesodermal origin and are derived from the hemangioblast, a common precursor, suggesting a shared developmental pathway [19]. Knock-down of *Erg* is associated with a significant reduction in the formation of vascular structures and the number of endothelial cells [20] and with apoptosis [21]. These studies indicate that Erg may have important implications in vascular development during mouse embryogenesis. Although *Erg* does not appear to be required for hematopoiesis during embryonic stem cell differentiation, it may play a role in endothelial cell differentiation [20]. Hematopoietic stem cells give rise to both T- and B-lymphocytes in embryogenesis and throughout adult life. Although mature T-lymphocytes do not express *Erg*, expression is detected transiently during T-lineage specification and is silenced after their commitment [22]. During B-cell development in the mouse, *Erg* expression was detected in early pre-B cells, pre-B and in mature B cells [23]. In developing mouse, *Erg* mRNA is expressed in mesodermal tissues such as endothelial cells, mesenchymal condensations during precartilaginous depositions, and in urogenital regions [11]. All of the expression studies were carried out by using RT-PCR or in situ hybridization. However, the protein expression and its cellular distributions could not be performed due to a lack of an Erg-specific antibody.

The goal of this study was to establish the expression pattern of Erg protein in developing and adult mouse tissues by using an ERG-specific antibody. These data would serve as a basis to understand the function of Erg during normal development in many organs and pathological conditions, such as its cancer-specific expression in prostatic adenocarcinoma. Although several antibodies for detecting human ERG protein and mouse Erg protein have been described, due to high degree of homology among *ETS* family members, in particular its closest homologue *Fli-1*, antibody cross reactivity has become a major concern in detection of the ERG protein. Recently, we have generated and characterized an ERG-specific mouse monoclonal antibody that showed high specificity towards ERG protein that does not cross react with FLI-1 protein [24]. In the present study, we examined the detailed expression of the Erg protein during prenatal and adult mouse organogenesis.

Results and discussion

Evaluation of ERG Monoclonal Antibodies for the Specificity of ERG Protein Detection: We evaluated the specificity of three recently available ERG monoclonal antibodies including the ERG MAb that we recently reported [24]. As noted previously, the ERG MAb detected ERG protein products in MOLT4, KG1, COLO 320 and VCaP tumor cell lines, whereas LNCaP, MCF7 and Jurkat cell lines were negative for ERG. The ERG MAb did not show cross reactivity to FLI-1 in LNCaP cells infected with a FLI-1 adenovirus expression vector (Fig 1A). Under similar assay conditions, rabbit monoclonal antibodies to ERG (EPR 3864 and EPR 3863) obtained from Epitomics (Burlingame, CA) detected FLI-1 in LNCaP cells infected with a FLI-1 adenovirus expression vector (Fig 1B, 1C). In addition, a rabbit monoclonal ERG antibody EPR 3864 detected a protein in Jurkat cell line (acute T cell leukemia) that was not recognized by either the rabbit monoclonal antibody EPR 3863 or our ERG MAb suggesting potential cross reactivity to other ETS related proteins by EPR 3864. Interestingly, other monoclonal ERG antibody EPR 3863 recognized FLI-1 in LNCaP cells infected with a FLI-1 adenovirus expression vector. Taken together, the results obtained from immunoblot analyses suggest that the ERG MAb we developed is highly specific for ERG protein detection and was further assessed in other immunoassays.

To determine the efficiency of ERG MAb antibody in a prostate tumor model, we analyzed ERG expression in ERG-positive VCaP and ERG-negative LNCaP prostate cancer cell tumor xenografts in SCID mice. The ERG MAb antibody detected ERG protein in VCaP xenografts, staining primarily the nuclei with some cytoplasmic reactivity (Fig 2A). As expected, ERG negative LNCaP xenografts did not show ERG expression. The endothelial cells lining the blood vessels and capillaries showed positive reactivity to ERG MAb in tumors as well as normal adjacent tissue (Fig 2B). Further, we evaluated the ERG MAb for the detection of ERG protein the prostates of ERG-transgenic mice [25]. Transgenic ERG expression was detected in the prostate luminal epithelial cells of ERG-transgenic mice (Fig 2C). In addition, endogenous Erg protein was detected only in the endothelial cells of blood vessels and capillaries (Fig 2D). Infiltrating lymphocytes did not show immune-reactivity to ERG MAb (Fig 2 E, G). Consistent with a recent report [26], rabbit monoclonal antibodies EPR 3864 showed a strong staining of lymphocytic infiltration in prostate gland (Fig 2F, H). Interestingly, both the rabbit monoclonal ERG antibodies (EPR 3864, EPR

3863) and FLI-1 antibodies have shown reactivity to infiltrating lymphocytes (Fig 2I, J). It is interesting to note that ERG is turned on at transition stage (between precursor and pro-T stage 1) of T-cell differentiation and does not persist stably to define T-cell identity, and is shut off after T cell lineage commitment [22, 27, 28]. In this study, we have not characterized the transient expression of Erg during T-cell development and differentiation.

To study the utility of the ERG MAb to detect the expression of ERG in cells using flow cytometry, seven human cancer cell lines, VCaP and LNCaP (prostate cancer), T2 and Jurkat (T lymphoblastoid cells), KG-1 and KG-1a (myeloblastic) were analyzed. All cell lines were permeabilized by standard cell intracellular staining methods by using detergent followed by incubation with the ERG MAb and secondary fluorochrome-conjugated antibodies. As shown in Fig 3C and D, expression of ERG was clearly detectable in the TMPRSS2-ERG fusion-harboring VCaP cells and was not observed in the TMPRSS2-ERG negative LNCaP cells (Fig 3A, 3B). In the case of the hematopoietic/lymphoblastoid cancer cells of the T lymphocyte lineage, ERG expression was detected neither in T2 (Fig 3I, 3J) nor in Jurkat cells (Fig 3K, 3L). With the hematopoietic cell lines of myeloid lineage, KG-1 and KG-1a, the expression of ERG was clearly detectable in both cell lines (Fig 3E, 3F and Fig 3G, 3H). Interestingly, the KG-1a cells, which are considered to be less mature or differentiated than the KG-1 cells, expressed much higher amounts of the ERG protein. Taken together, the consistent results of Western blot, IHC and FACS assays established the specificity of the ERG MAb in detecting ERG protein in different assay platforms and biological specimen contexts.

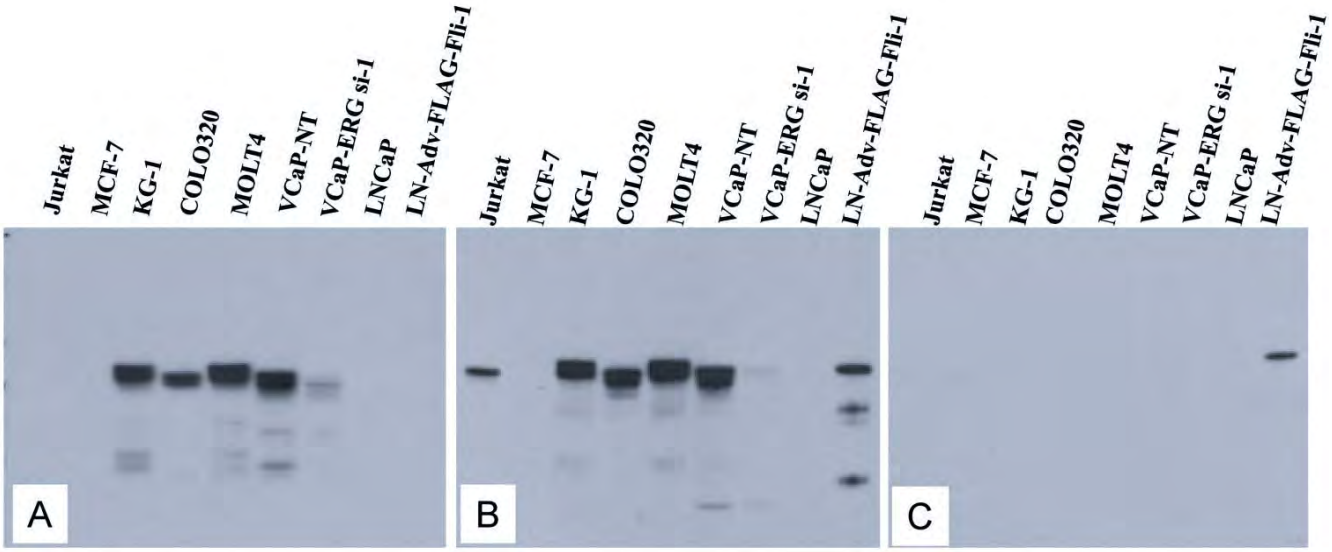

Figure 1: Expression of human ERG protein in cancer–derived cell lines: ERG protein expression was analyzed by using total cell lysates from acute T cell leukemia (Jurkat), breast cancer cell line (MCF7) acute myelogenous leukemia cell line (KG1), colon carcinoma cells (COLO320), acute lymphoblastic leukemia (MOLT-4), ERG expressing prostate cancer cell line (VCaP) treated with non target siRNA (VCaP-NT), prostate cancer cell line (VCaP) treated with ERG specific siRNA (VCaPERG-si-1), prostate cancer cell line LNCaP, prostate cancer cell line LNCaP transduced with adenoviral FLI-1 expression vector. Extracted proteins were processed for immunoblot assay by using (A) mouse monoclonal ERG antibodies ERG MAb, (B) rabbit monoclonal anti-ERG antibodies Epitomics EPR 3864 Cat.No 2805-1, (C). Rabbit monoclonal anti-ERG antibodies Epitomics EPR 3863 Cat.No 2849-1. Note the lack of immunoreactivity to the protein extracts from Jurkat, LNCaP and LNCaP transduced with adenoviral Fli-1 expression vector with ERG MAb in panel A, and reactivity with other antibodies in panel B and C.

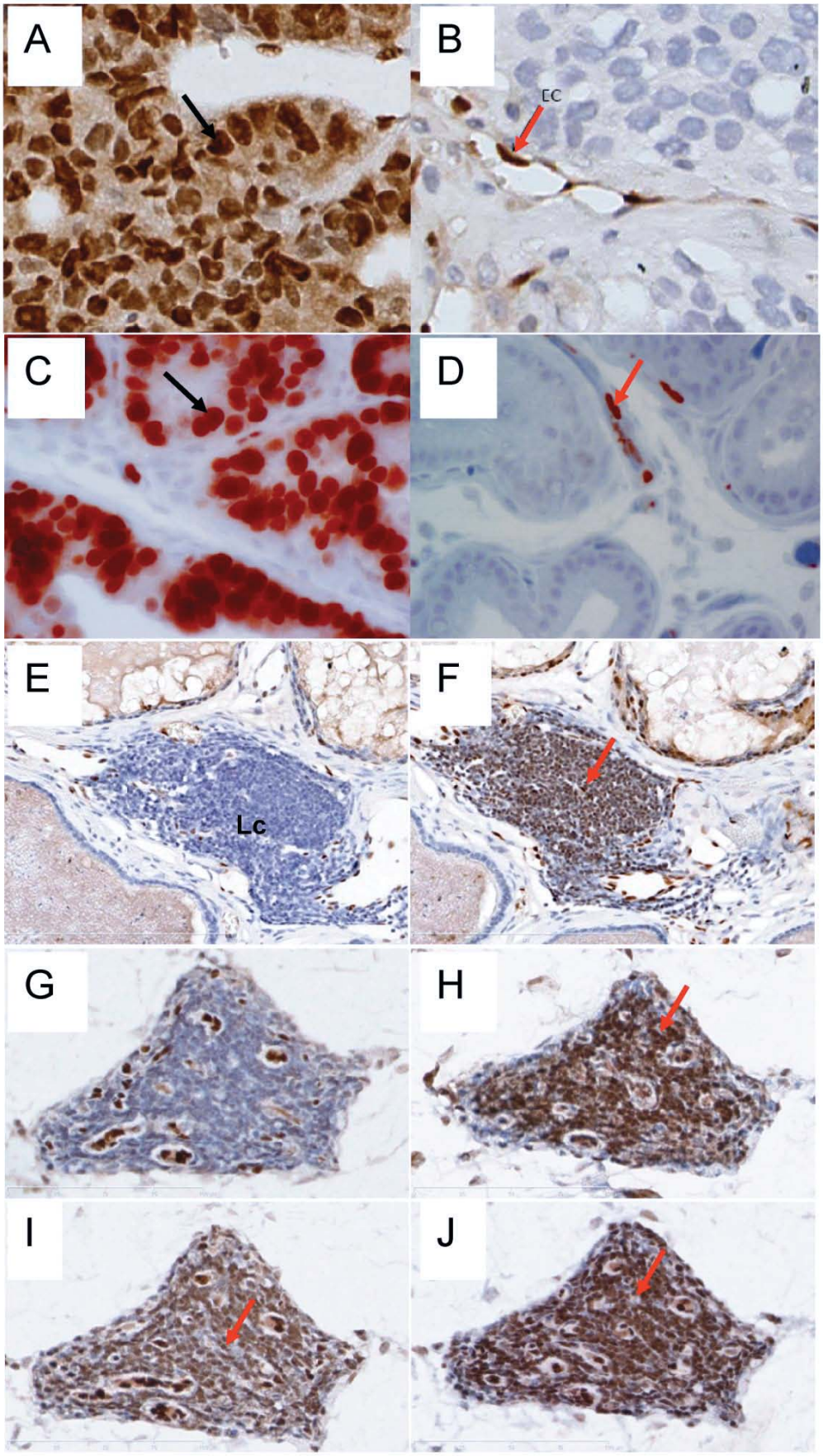

Figure 2: Expression of ERG protein in FFPE tissue: ERG protein expression in (A) VCaP and (B) LNCaP xenograft tumors obtained from SCID mice. Strong expression of ERG in VCaP cells (black arrow). ERG staining is seen only in the endothelial cells (arrow) of LNCaP tumors but not in the epithelial cells. Expression of ERG protein is detectable in the ARR2PBΔERG transgenic mouse prostates (C) compared to wild-type littermate control (D). Infiltrating lymphocytes are occasionally seen in the prostate glands and show no reactivity to ERG MAb (E, G). However, strong staining is seen in the infiltrating lymphocytes with Epitomics EPR 3864 (F, H), EPR 3863 (I) and FLI1 antibody (J).

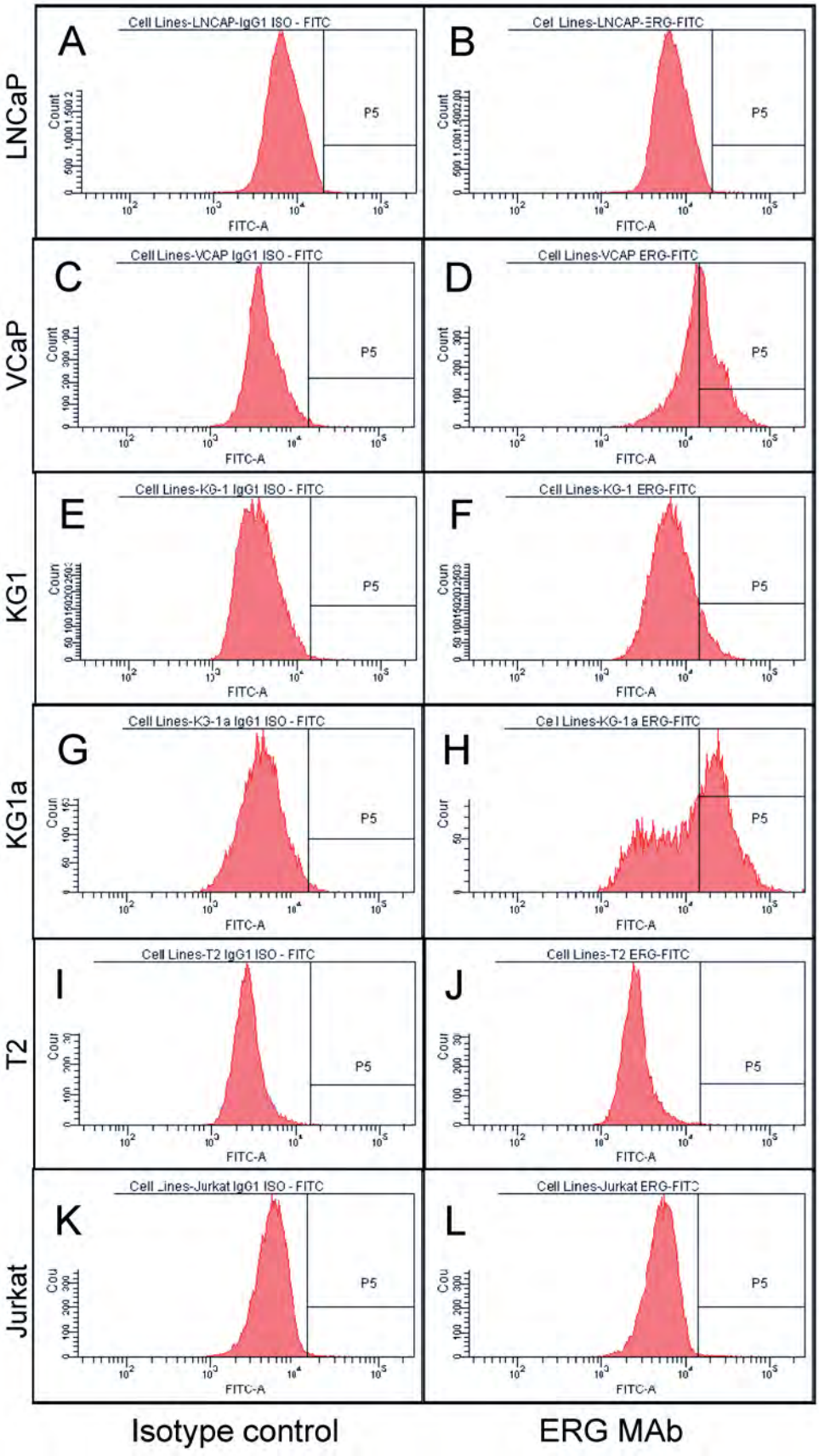

Figure 3. ERG expression in cancer cell lines. Intracellular staining was carried out in permeabilized cells with an IgG1 isotype control antibody (A, C, E, G, I and K) or for ERG with ERG MAb (B, D, F, H, J and L) and detected by using FITC-conjugated secondary antibody and flow cytometry analysis.

Erg protein expression in prenatal mouse development: During E9.5d, strong expression of Erg protein was detected by the ERG MAb mainly in the endothelial cells of blood vessels around the neural tube (Fig 4A, 4B). Additionally, expression was also prominent in the cells that line the amnion (Fig 4A). At this stage, during heart development, the ventricle begins trabeculation to demarcate this region from the primitive heart. Expression of Erg was observed in endothelial cells that line the trabeculated regions of the ventricle (Fig 4C). Endothelial cells present in the inter-somitic capillaries show strong expression of Erg (Fig 4D). Similar endothelial-specific expression was found in the dorsal aorta and around the neural tube (Fig 4E). The distribution observed with ERG MAb antibody is consistent with earlier reported studies of *Erg* mRNA expression [11, 20]. Similar to earlier stages of development, at E12.5d, Erg expression was endothelial cell-specific in the majority of the tissues (Fig 5). In addition to endothelial expression, Erg expression was detected in the precartilage/ cartilage primordium of the nasal septum, neural arch and rib (Fig 5A, 5B, 5C). Mesenchymal condensations are required at this stage to initiate the paving cartilage path for both transient and permanent cartilage. The transient cartilage will undergo ossification to form bone. Interestingly, Erg expression was observed only in the precartilage primordium suggesting that Erg may have critical role in the differentiation of cartilage. Heart development at this stage exhibited extensive trabeculation of the ventricle and showed clear lining of endothelial cells with positive Erg staining along the trabeculated endocardium (Fig 5B). Lungs at this stage were not yet divided into lobes and the stroma with enriched capillaries exhibited strong expression of Erg in developing lung (Fig 5D). Epithelial cells of segmental bronchus did not show Erg expression (Fig 5D). Kidney at this stage starts subdividing into cortical and medullary regions. Expression was detected only in the blood vessels and capillaries uniformly throughout the kidney and not in the kidney cortex or medulla (Fig 5E).

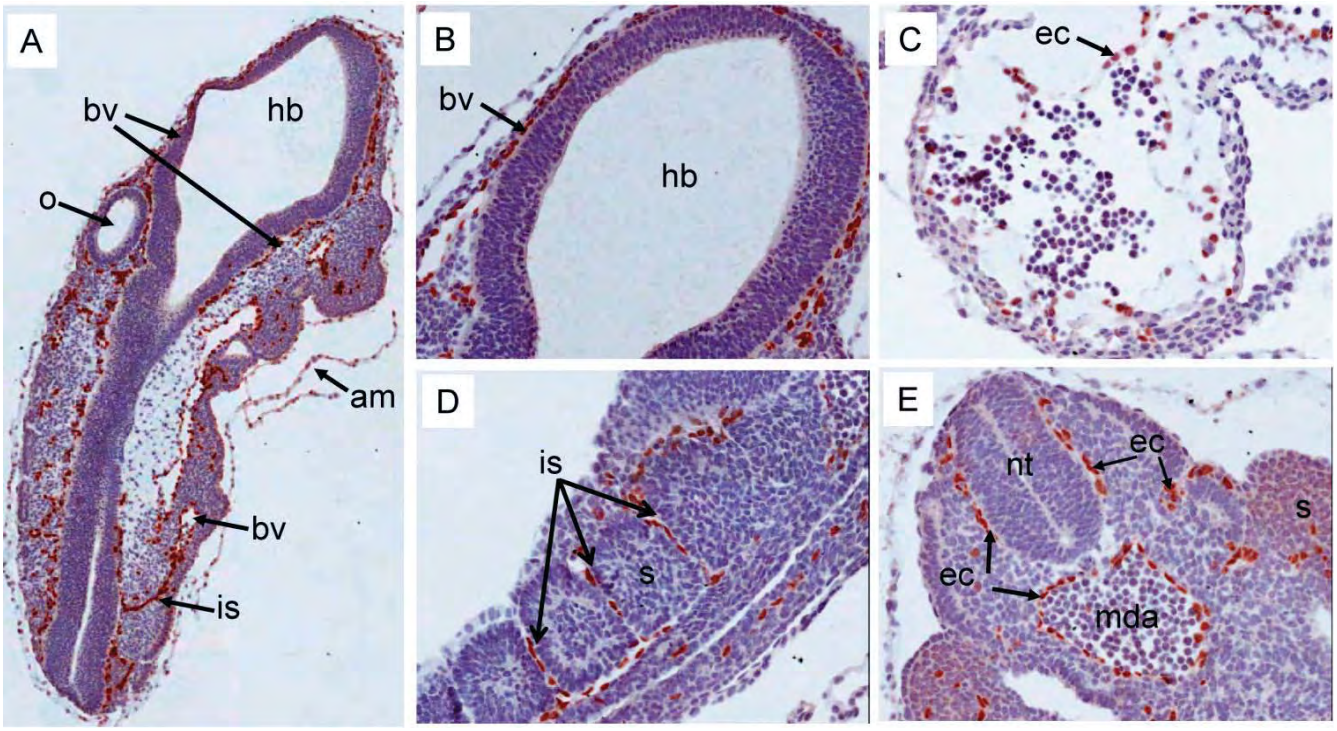

Figure 4. Expression pattern of Erg protein during mouse embryogenesis (E9.5d): Embryonic 9.5d mouse showing the expression of Erg protein by immunohistochemistry with ERG MAb. (A) Coronal section of an E9.5 embryo showing a specific staining in blood vessels (bv) , inter-somitic vessels (is) and in the amnion (am). (B) Higher magnification of hind brain. Expression is not seen in the hind brain (hb), neural tube (nt) and optic vesicle (o). (C). Higher magnification of ventricle (vt) region of the heart showing strong signal in the endothelial cells (ec)along the trabeculated endochordium (D) Hihger magnification of somites in the caudal region showing Erg expression in the inter somatic blood vessels (sv) . (E). Tail region of the embryo showing neural tube (nt) midline dorsal aorta (mda). Erg expression was detectable only in the endothelial cells of dorsal aorta. Somites (s).

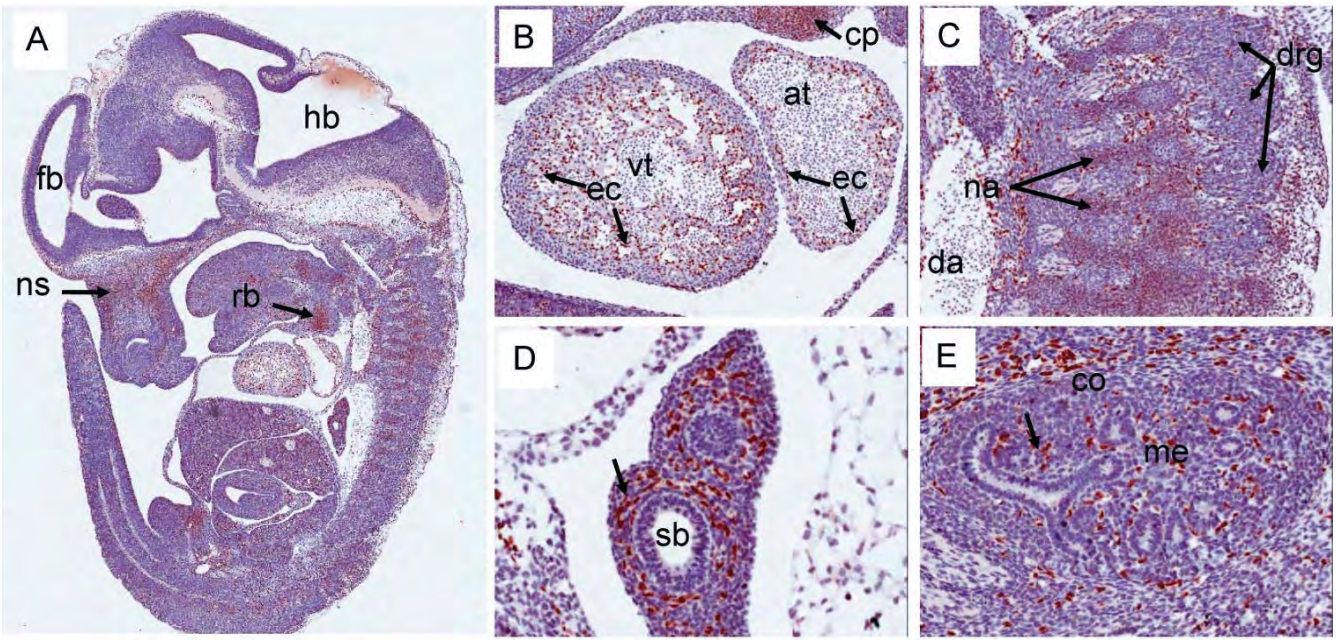

Figure 5. Expression pattern of Erg protein during mouse embryogenesis: (E12.5). (A) Sagittal section of an E12.5d embryo showing a specific staining in cartillage primordium (cp) of the nasal septum (ns), and the mid shaft region of the rib (rb). (B) Higher magnification of ventricle (vt) region of the heart showing strong expression in the endothelial cells (ec) along the trabeculated endocordium. (C) Erg protein was detectable in the precartillage condensations in the neural arch (na). (D) Higher magnification of developing lungs (not yet divided into lobes) show lack of expression in the epithelial cells of segmental bronchus (sb). Surrounding stroma with enrihed capillaires exhibit strong staining. (E). Expression is seen only in endothelial cells of the blood vessels and capillaries uniform throughout the kidney.

Erg expression in E14.5d was found mostly in the endothelial cells of variety of tissues (Fig 6). In developing liver, about 1-2% of liver cells exhibited reactivity with ERG MAb antibody and megakaryocytes did not show Erg expression. Expression in the cartilage appeared to be reduced significantly compared to E12.5d in the rib, nasal septum and vertebrae. We found significant differences between ERG MAb and rabbit monoclonal ERG antibodies EPR 3864 megakaryocytic immunostaining. No significant changes in the Erg expression patterns were observed in the later stages of mouse development. As the overexpression of ERG in the prostate is implicated in the oncogenic process, we examined the normal expression of Erg protein in developing prostate glands. In the mouse, the prostatic buds first emerge at the rostral end of the urogenital sinus at approximately 17.5 days of gestation and subsequently, the prostatic epithelial buds undergo extensive ductal outgrowth and branching morphogenesis into the surrounding mesenchyme during the first three weeks of postnatal development. Interestingly, Erg staining was observed only in the mesenchymal compartment and restricted to capillary endothelial cells (Fig 7) suggesting that Erg may not be critical in normal prostate development or differentiation.

In liver of E17.5d mice, the expression of Erg is restricted to very few cells such as endothelial and other non-parenchymal cells which later differentiate into Kupffer cells and hepatic stellate cells. It is not clear at this stage which type of cells in addition to endothelial cells express Erg protein. Similar to E14.5d liver megakaryocytes, ERG MAb did not show reactivity with E17.5d hepatic megakaryocytes (Fig 8A). However, rabbit monoclonal ERG antibodies EPR 3864 showed a strong staining in megakaryocytes, in addition to endothelial and other cell types (Fig 8B).

Expression of Erg protein in adult mice: We extended our study to adult mouse tissues to analyze the expression of Erg protein (Fig 9). Similar to embryonic tissues, the expression of Erg was observed in the endothelial cells of the adrenal gland, cartilaginous component of bone, heart muscle, kidney, liver, lung, spleen urinary bladder. Erg expression was also evident in the lymphatic endothelial cells in adult mouse. As expected, expression was undetectable in the prostate epithelium. Detailed examination of these tissues has revealed that the expression was mostly restricted to hematopoietic and endothelial compartments. In liver, the expression appeared to be in the Kupffer cells. In bone marrow, ERG MAb did not show reactivity with megakaryocytes. Interestingly,

eosinophils, with a characteristic ring shaped or segmented/multilobed nuclei stained the nuclei with ERG MAb. Eosinophils are derived from hematopoietic stem cells initially committed to the myeloid line and then to the basophil-eosinophil granulocyte lineage. The presence of Erg in these cells suggest its potential function in either differentiation or maintenance of differentiation state. During development, Fli1 is preferentially expressed in hematopoietic cells, endothelial cells and in the mesenchyme which is mainly derived from neural crest cells [29]. Similar to earlier observations [11] we also observe the co-expression of the *Erg* and *Fli1* genes in both endothelial and mesodermal tissues, including urogenital tract and precartilaginous areas. Similar to Erg, Fli1 expression also appeared transient during mouse embryogenesis. This result is consistent with subtle temporal regulation. Whether this spatiotemporal overlap determines redundant functions or not remains to be determined. Due to specificity and clarity of Erg detection in developing and adult mouse tissues, these findings will be valuable in further assessing in vivo functions of Erg in normal and malignant tissues.

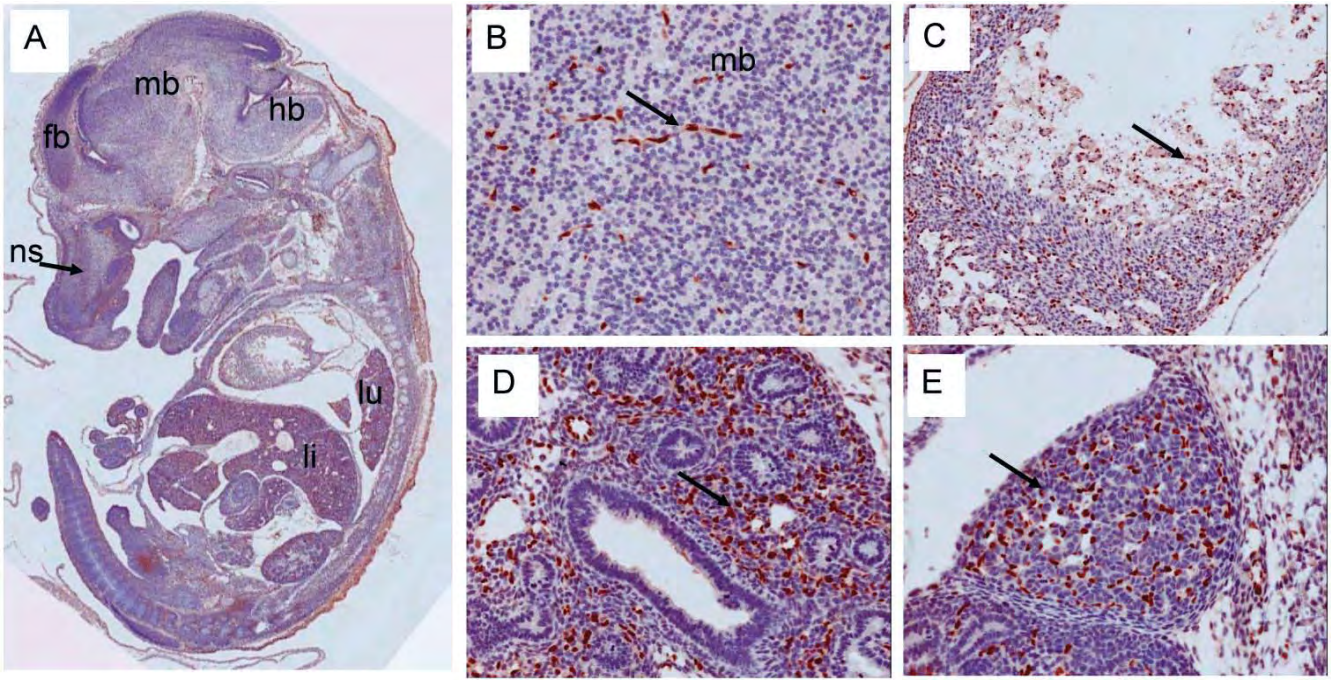

Figure 6. Expression pattern of Erg protein during mouse embryogenesis (E14.5): (A) Sagittal section of an E14.5 embryo showing a specific staining in cartillage primordium (cp) of the nasal septum (ns) similar to E12.5d. Higher magnification of midbrain showing capillaries staining of Erg. (C) Higher magnification of ventricle (vt) region of the heart showing strong ractivity in the endothelial cells (ec) along the trabeculated endocordium. (D) Higher magnification of developing lungs shows lack of expression in the epithelial cells of segmental bronchus (sb). Stroma with enrihed capillaires exhibit strong staining. (E). Higher magnification of adrenal gland with endothelial cell specific Erg staining.

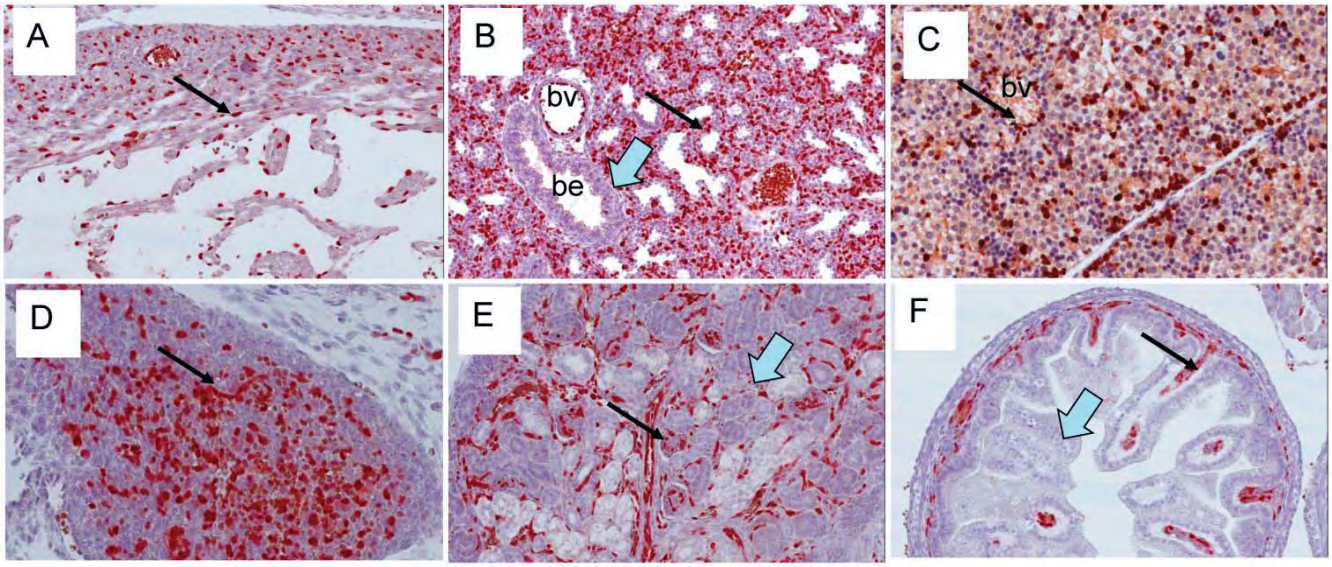

Figure 7. Expression pattern of Erg protein during mouse embryogenesis(E17.5): (A) Ventricle showing trebeculated endocordium with endothelial specific staining of Erg. (B). High magnification of lung showing capillaries and blood vessel specific Erg reactivity. Note the lack of staining in the lung epithelial. (C) High magnification of liver showing a specific staining is non-hepatocytes in addition to endothelial cells. (D) High magnification of adrenal gland showing random Erg in both cortex and medullary region consistent with fenestrated vessels. Similarly, kidney (E) and intestine (F) show endothelial specific staining, Open arrows show the lack of expression in the bronchial epithelial cells (B), kidney tubules (E) and intestinal epithelial cells in the cripte (F). Blood vessel (bv), bronchial epithelium (be).

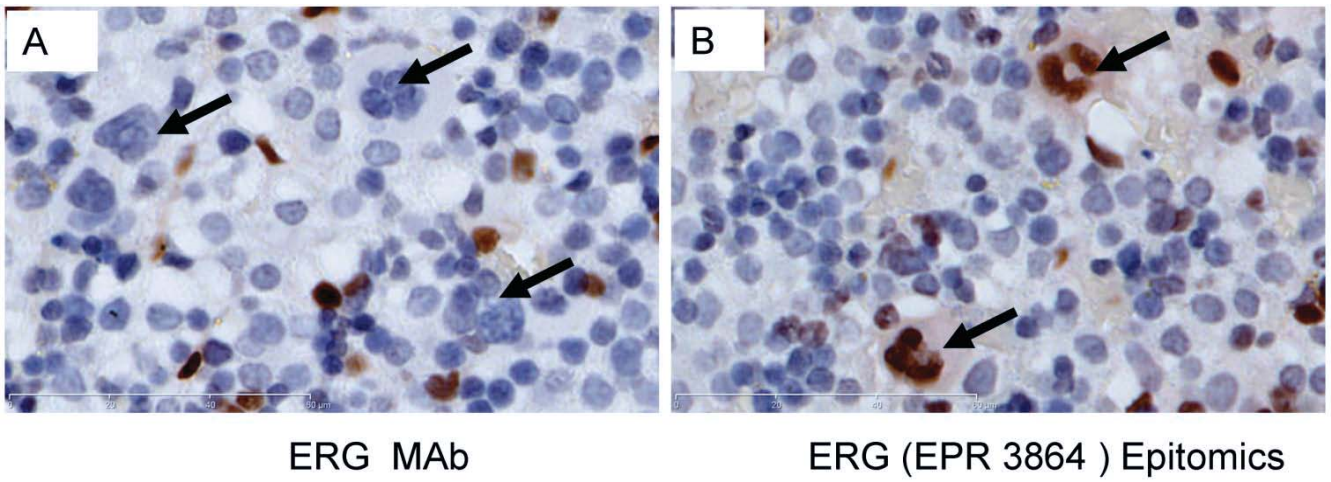

Figure 8. Expression of Erg protein in developing liver (E17.5). (A) Detection of Erg protein in endothelial and non-paranchymal cells by ERG MAb. The arrows point to megakaryocytes that lack the expression of Erg protein. (B). Erg staining with rabbit monoclonal ERG antibodies Epitomics EPR 3864 show endothelial and non-paranchymal cells staining. Megakaryocytes know to have expression of Fli1 also are detected with EPR 3864. Similar megakaryocyte specific expression is also observed with EPR 3863 and FLI1 antibodies (data not shown).

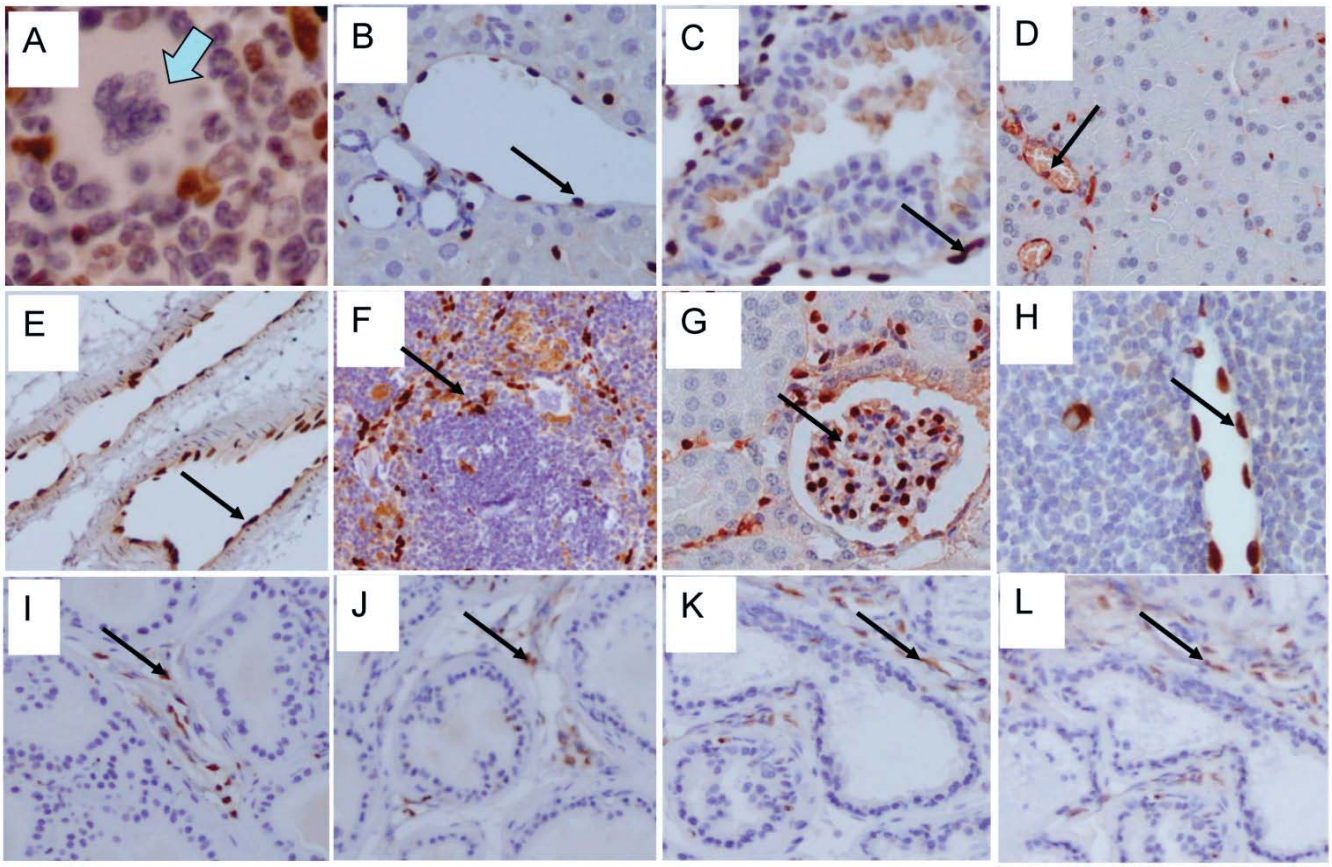

Figure 9. Expression of Erg protein in adult tissues: (A) Bone marrow (B) Liver (C) Lung (D) Pancreas (E) Lymphatic vessels (F) Spleen (G) Kidney (Glomeruli) (H) Thymus (I) Anterior prostate (J) Dorsal prostate (K) Lateral prostate (L) Ventral prostate. Expression is seen mainly in the endothelial cells in blood vessels and capillaries (arrows). In bone marrow, megakaryocyte show lack of Erg expression (open arrow).

Materials and Methods

Antibodies: Recently, we have reported the generation and characterization of mouse monoclonal antibodies to ERG, showing higher specificity [24]. In this study, we have used mouse monoclonal ERG antibodies along with the other commercially available ERG antibodies, a rabbit anti-ERG monoclonal antibody clone EPR 3864 (Cat No. 2805-1) [26] and clone EPR 3863 (Cat No. 2849-1) obtained from Epitomics, Burlingame, CA and FLI-1 antibodies from Dr. Denis Watson, University of South Carolina, Charleston, SC. Anti-GAPDH (sc-25778, Santa Cruz Biotechnology, Santa Cruz, CA) sheep anti-mouse IgG-HRP (NXA931, GE Health Care, Buckinghamshire, UK) donkey anti-rabbit IgG-HRP (NA934, GE Health Care, Buckinghamshire, UK)

Western blot assays. Cells (Jurkat, MCF7, KG1, Molt4, VCaP, LNCaP) were lysed in Mammalian Protein Extraction Reagent (M-PER) (Pierce, Rockford, IL) containing protease inhibitor cocktail and phosphatase inhibitor cocktails I & II (Sigma, St Louis, MO). Cell lysates equivalent to 50 µg of protein were separated on 4-12% Bis-Tris Gel (Invitrogen, Carlsbad, CA) and transferred to PVDF membrane (Invitrogen, Carlsbad, CA). Membranes were incubated with primary antibodies: Anti-ERG mouse monoclonal antibody (ERG MAb)[24] (1:500 dilution), rabbit monoclonal ERG (EPR 3864 and EPR 3863) antibodies (1:500 dilution) anti-GAPDH (sc-25778, Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for overnight. Membranes were washed three times for 5 minutes each at room temperature followed by treatment with secondary antibodies: sheep anti-mouse IgG-HRP or donkey anti-rabbit IgG-HRP at 24°C for 1 hour. Finally membranes were washed three times and bands were visualized with ECL Western blot detection reagent (GE Health Care, Buckinghamshire, UK).

Immunofluorescence staining for ERG in cell lines and flow cytometry analysis. The cancer cell lines used in this study were obtained from ATCC. VCaP cells were grown in DMEM/10%FCS+Penicillin/Streptomycin/L-Glutamine. LNCaP, T2, Jurkat, KG-1 and KG-1a cells were

cultured in RPMI/10%FCS/Penicillin/ Streptomycin/L-Glutamine. On the day of the assay adherent cell lines (VCaP, LNCaP) were trypsinized to yield single cell suspensions while the non-adherent cells (T2, Jurkat, KG-1 and KG-1a) were utilized after a washing step. For each cell line, two aliquots of $5x10^5$ cells/tube were permeabilized with freshly prepared permeabilization buffer as directed by the manufacturer (eBioscience). One aliquot was stained with an IgG1 isotype control antibody (Invitrogen) and the second aliquot was stained with the anti-ERG antibody at a 1:20 dilution for 1 hour at 4°C. Then cells were washed and stained with FITC-conjugated rat anti-mouse IgG1 antibody (Clone A85-1, BD Pharmingen) for an additional 30 minutes at 4°C. Cells were then washed and analyzed by using a BD FACS Canto II flow cytometer. Data was collected on the total cell population and the analysis was performed by using FACS Diva software (version 5 .03) (Becton Dickinson).

Tissues and processing for Immunohistochemistry: Fixation and impregnation FVB/N and C57BL6 mice embryos from various stages of development were dissected from pregnant females, washed in ice-cold phosphate-buffered saline (PBS) and fixed in ice-cold 4% paraformaldehyde overnight, sequentially dehydration, embedding, and sectioning steps were performed according to established protocols.

Preparation for staining: Slides (with 5-mm sections) were warmed at 60°C for 15 minutes and immediately immersed in xylene for effective removal of paraffin and processed additionally twice in xylene for 5 min each, washed twice in 100% ethanol for 5 min each, and subsequently rehydrated in 95, 75% ethanol, and PBS for 5 min at room temperature.

Immunohistochemistry: Antigen retrieval was performed on these slides in Antigen unmasking solution (Vector Biolabs, Burlingame, CA) by using vegetable steamer for 45 minutes and the slides were allowed to cool to room temperature for about 30 min. Slides were washed twice in 1xPBS and treated with 3% H_2O_2 in ultrapure water for 15 minutes to quench the endogenous peroxidase activity. Immunodetection was performed using Mouse–to-mouse detection system kit according manufacturer's instructions (Millipore Inc. Billerica, MA). Briefly, nonspecific binding of the antibody was blocked by incubating the slides with pre-blocking solution for 10 minutes at RT, primary antibodies (200 pg/ml in 10% normal goat serum) at 4°C overnight or room temperature for 2 hours. Slides were washed twice in 1xPBS sites 5 min each, incubated with post-antibody blocking solution for 10 min. Slides were washed twice in 1xPBS sites 5 min each and incubated with ready-to-use poly-HRP-Anti-Mouse/Rabbit IgG for 30 minutes, washed twice with 1xPBS for 5 minutes each. Color development was performed by using DAB as a substrate for peroxidase enzyme activity. The color reaction was stopped by washing/rinsing slides in tap water several times. Slides were counterstained with Hematoxylene for 2 minutes, rinsed in tap water for 5 mins, dehydrated sequentially in ascending concentration of alcohol, cleared in xylene and permanently mounted with Permont solution. The slides were scanned in Biomedical Instrumentation Center at Uniformed Services University of the Health Sciences using Olympus NanoZoomer Digital Pathology microscope at 40X magnification and digital images were taken from the scans.

Acknowledgements

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Conflict of Interest

The authors do not have any conflict of Interest.

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ORIGINAL ARTICLE

ERG protein expression and genomic rearrangement status in primary and metastatic prostate cancer—a comparative study of two monoclonal antibodies

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BACKGROUND: Overexpression of the ERG protein is highly prevalent in prostate cancer (PCa) and commonly results from gene fusions involving the ERG gene. Recently, N-terminal epitope-targeted mouse and a C-terminal epitope-targeted rabbit monoclonal anti-ERG antibody (ERG-MAbs) have been introduced for the detection of the ERG protein. Independent studies reported that immunohistochemistry (IHC) with both ERG-MAbs highly correlates with the underlying ERG gene rearrangement status. However, comparative studies of both antibodies are lacking. Here, we are among the first to compare the mouse ERG-MAb with the rabbit ERG-MAb for their concordance on the same PCa cohort. Furthermore, we assessed whether the ERG protein expression is conserved in lymph node and distant PCa metastases.

METHODS: We evaluated tissue microarrays of 278 specimens containing 265 localized PCa, 29 lymph node, 30 distant metastases and 13 normal prostatic tissues. We correlated ERG protein expression with ERG rearrangement status using an ERG break-apart fluorescence *in-situ* hybridization assay and IHC of both ERG-MAbs.

RESULTS: ERG expression and ERG rearrangement status were highly concordant regardless of whether the mouse or rabbit ERG-MAb was used (97.8% versus 98.6%, respectively). Of interest, both ERG antibodies reliably detected the ERG expression in lymph node and distant PCa metastases, of which a subset underwent decalcification. Lymphocytes only revealed immunoreactivity using the rabbit ERG-MAb. If ERG protein expression was present in localized PCa, we observed the same pattern in the corresponding lymph node metastases.

CONCLUSIONS: By demonstrating a broad applicability of IHC to study ERG protein expression using either antibody, this study adds an important step toward a facilitated routine clinical application. Further, we demonstrate that the clonal nature of the ERG rearrangement is not restricted to the genomic level, but proceeds in the proteome. Together, our results simplify future efforts to further eliucidate the biological role of ERG in PCa.

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Keywords: ERG rearrangement; ERG protein expression; gene fusions; ERG immunohistochemistry

INTRODUCTION

The discovery of highly prevalent and recurrent gene rearrangements in prostate cancers (PCa) challenged previously assumed paradigms, and prompted detailed examination of their biological role, diagnostic and therapeutic potentials.[1-3] The most prominent gene rearrangement in PCa arises between the androgen-regulated serine protease *TMPRSS2* and the transcription factor *ERG*, resulting in an overexpression of a truncated oncoprotein.[2,4-6] Depending on factors, such as, cohort design (PSA-screened PCa versus incidentally diagnosed PCa), zonal tumor origin (PCa originating from the transitional zone versus PCa originating from the peripheral zone)[7,8] and various histologic variants of PCa,[9-12] *ERG* rearrangements occur in 15–80% of PCa. Furthermore, despite its high prevalence and clonal nature,[13-17] the *ERG* rearrangement is highly specific to PCa.[18] Thus, a diagnostic as well as companion diagnostic utilization of the *ERG* rearrangement in PCa is imminent, whereas a prognostic and predictive relevance is still controversial.

Of interest, within the last 1 year, ERG immunohistochemistry (IHC) has been introduced as a promising tool to detect an ERG expression on the protein level. So far, two monoclonal ERG-specific antibodies (that is, mouse monoclonal anti-ERG antibody (ERG-MAb) and rabbit ERG-MAb) have been established on prostatic tissues. Furusato *et al*.[19] reported a highly specific mouse ERG-MAb performing a comprehensive evaluation of ERG protein expression using whole mount prostate sections from 132 PCa cases. The ERG protein expression pattern showed a strong concordance with *ERG* fusion transcripts by branched DNA assay or *ERG* rearrangement by fluorescence *in-situ* hybridization (FISH) in selected specimens. By IHC using a rabbit ERG-MAb, Park *et al*. and

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van Leenders *et al*. have provided a comprehensive data showing that virtually all *ERG* rearranged PCa exhibit an ERG overexpression on the transcriptional and translational level.[20,21] He *et al*.[22] confirmed the feasibility of the ERG IHC on an independent cohort containing a broader spectrum of prostatic lesions.

In this study, we are among the first to compare the mouse ERG-MAb with the rabbit ERG-MAb and for their concordance on the same cohort of 265 PCa specimens, and matched these results with the respective *ERG* rearrangement status as defined by FISH. Furthermore, we assessed whether the ERG protein expression is conserved in lymph node and distant PCa metastases, of which a subset underwent decalcification.

MATERIALS AND METHODS

Cohort

We assessed 265 PCa specimens for their *ERG* rearrangement status and ERG protein expression levels under an approved protocol from the University Hospital of Tuebingen. In detail, the cohort comprises 206 localized PCa samples, 29 lymph node metastases and 30 distant metastases. For 16 localized PCa specimens matching lymph node metastases were available. Among the distant metastases, 26 were of osseous origin and 4 were derived from the brain. Furthermore, 13 benign prostatic specimens were included. Patients were diagnosed and treated at the University Hospital of Tuebingen.

Decalcification

All prostatic specimens from osseous origin were decalcified by using the chelating agent EDTA. We used a 10% EDTA solution in distilled water (pH 7.4), for a period of 1–3 weeks at 4 °C, depending on degree of mineralization. EDTA was replaced every week. After decalcification, specimens were routinely processed and embedded in paraffin.

Tissue microarray construction

FISH and IHC experiments were performed on tissue microarrays (TMA). TMA construction was performed as described earlier.[12,18] Briefly, formalin-fixed paraffin-embedded PCa tissue blocks were sectioned at 3 μm thick sections, mounted on slides and stained with haematoxylin and eosin. Prior to TMA construction, representative cancer areas were marked by a pathologist (S P). Three cores, each 0.6 mm in diameter, were taken from the corresponding donor block and placed into a TMA recipient block using a semiautomatic tissue arrayer (Beecher Instruments, Sun Prairie, WI, USA). Of the resulting TMA, tissue sections (3 μm thick) were cut and placed onto superfrost slides.

ERG break-apart fluorescence *in-situ* hybridization assay

A previously described *ERG* break-apart FISH assay was used to detect the *ERG* rearrangement at the chromosomal level on formalin-fixed paraffin-embedded specimens.[1,13] In short, this assay employs a split-signal approach, with two probes spanning the *ERG* locus. BAC clones RP11-24A11 were used for centromeric labelling with biotin and RP11-372O17 for telomeric labelling with digoxigenin. This FISH assay allows for *ERG* rearrangement status assessment (that is, rearrangement versus no rearrangement of *ERG*). The assay is also capable of differentiating between two different mechanisms of *ERG* rearrangement (that is, *ERG* rearrangement through deletion versus *ERG* rearrangement through insertion) (Figures 1e–f). The samples were analysed under a 63× oil immersion objective using a fluorescence microscope (Zeiss, Jena, Germany) equipped with appropriate filters, a charge-coupled device camera and the FISH imaging and capturing software Metafer 4 (Metasystems, Altlussheim, Germany). The evaluation of the tests was carried out independently by two experienced observers (V J S and M B). At least 100 nuclei per case were evaluated.

IHC

Sections mounted on superfrost slides were deparaffinized. IHC was conducted with the Ventana Discovery XT automated staining system (Ventana Medical Systems, Tucson, AZ, USA) using Ventana reagents. The following clones and primary antibodies were used: anti-ERG rabbit monoclonal antibody (1:100 dilution, clone EPR3864, Abcam, Cambridge, MA, USA)[21] and anti-ERG mouse monoclonal antibody (1:1000 dilution, CPDR ERG-MAb, clone 9FY, CPDR, Rochville, MD, USA).[19] Dilution was

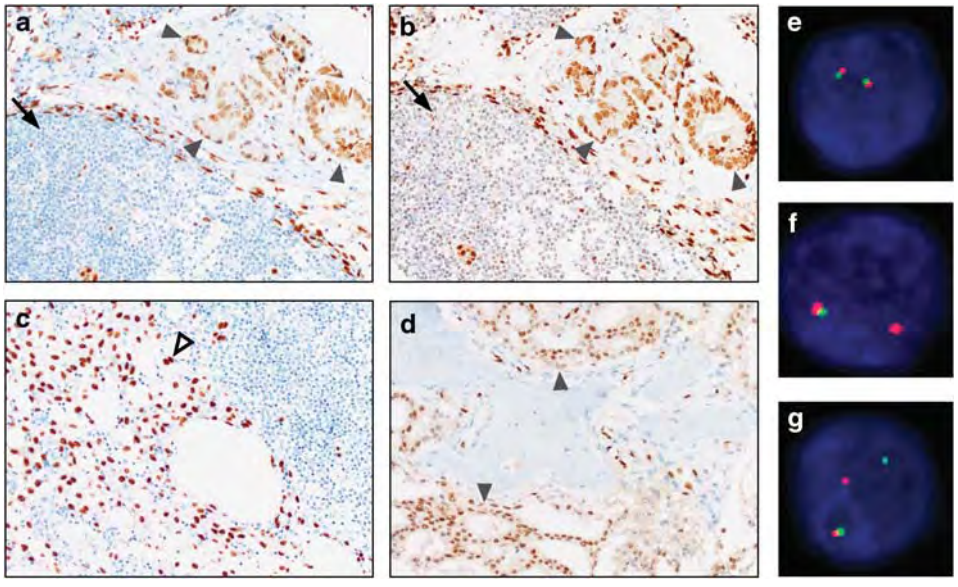

Figure 1. A lymph node metastasis of an *ERG*-rearranged prostate cancer (PCa) (arrowheads) stained using the mouse monoclonal anti-ERG antibody (ERG-MAb) (**a**) and the rabbit ERG-MAb (**b**), respectively. Follicular lymphocytes (arrows) stained positively using the rabbit ERG-MAb. Sinosoidal histiocytes (white arrowheads) within a lymph node strongly express endogenous ERG protein using the rabbit ERG-MAb (**c**), but not with the mouse ERG-MAb (not shown). A decalcified vertebral metastasis (arrowheads) of an *ERG*-rearranged PCa-expressing ERG protein, using the rabbit ERG-MAb (**d**). Notably, the mouse ERG-MAb works equally good in a decalcified setting and results in the same expression pattern (not shown). Representative images of the *ERG* break-apart fluorescence *in-situ* hybridization (FISH) assay as used for the indirect detection of *ERG* rearrangements (**e**–**g**): interphase nuclei displaying a wild-type *ERG* status (**e**), a heterozygous *ERG* rearrangement through deletion (**f**) and a heterozygous *ERG* rearrangement through insertion (**g**).

performed by using a Ventana diluent. Heat-induced epitope retrieval was performed for both anti-ERG antibodies. Primary antibodies were incubated for 60 min at room temperature. For IHC using anti-ERG rabbit monoclonal antibody (rabbit ERG-MAb), a secondary antibody (UltraMap anti-Rabbit HRP; Ventana Medical Systems, Tucson, AZ, USA) was applied for 16 min at room temperature. For IHC of the anti-ERG mouse monoclonal antibody (mouse ERG-MAb), a secondary antibody (UltraMap anti-Mouse HRP) was applied for 16 min at room temperature. Secondary antibody detection was performed by using the ChromoMap DAB detection kit (Ventana Medical Systems). Slides were counterstained with Hematoxylin II for 8 min followed by Bluing Reagent (Ventana Medical Systems) for 4 min at 37 °C.

The samples were analyzed under a 10 × dry objective using a standard bright field microscope. The evaluation of the tests was carried out independently by two experienced observers and blinded to the *ERG* rearrangement status (M B & D G). Nuclear immunoreactivity was scored as negative, weak, moderate or strong. As the vast majority of immunoreactive PCa specimens showed a homogeneous intensity in most tumor cells, any nuclear immunoreactivity of weak, moderate or strong intensity was considered to be positive for ERG protein expression. Strongly ERG protein expressing vascular endothelia was used as an intrinsic positive control for the staining procedure.

Bioinformatics analysis

The amino-acid sequence of ERG was retrieved from the NCBI database. The sequence was analyzed to map the exon locations within the protein by using the Ensembl Genome Browser. The peptide regions of the antibodies were matched with the amino-acid sequence of ERG (NM_004449).

Mouse ERG-MAb: immunizing peptide (amino acids 49–73): gqtskmsprvpqqdwlsqpparvti[19]

Rabbit ERG-MAb: immunizing peptide (amino acids 243–465): vypeatqr ittrpdlpyepprrsawtghghptpqskaaqpspstvpktedqrpqldpyqilgptssrlanpgsgqi qlwqfllellsdssnsscitwegtngefkmtdpdevarrwgerkskpnmnydklsralryyydknimt kvhgkryaykfdfhgiaqalqphppesslykypsdlpymgsyhahpqkmnfvaphppalpvtsss ffaapnpywnsptg [21]

Statistical analysis

For statistical analysis, SPSS Statistics 18 was used (SPSS, Chicago, IL, USA). FISH results were compared for their concordance with the IHC results performing Pearson's correlation test. A *P*-value <0.05 was considered as statistically significant.

RESULTS

A total of 278 samples were assessed for the *ERG* rearrangement and ERG protein expression, consisting of 206 localized PCa samples, 29 lymph node metastases, 30 distant metastases and 13 benign prostatic tissues. Among the distant metastases, 26 were of osseous origin and 4 were derived from brain.

By FISH analysis, *ERG* rearrangements could be detected in 45.3% (120/265 cases) of PCa specimens. Within these cases, 69.2% (83/120) harbored an *ERG* rearrangement through deletion and 30.8% (37/120) harbored an *ERG* rearrangement through insertion. As expected, none of the 13 benign samples showed evidence of an *ERG* rearrangement. Among the lymph node and distant metastases, *ERG* rearrangements could be detected in 24.1% (7/29) and 26.7% (8/30), respectively. *ERG* rearrangement status was conserved in all matching lymph node metastases (43.8%, 7/16).

Using the rabbit ERG-MAb, ERG protein expression was detected in 44.5% (118/265) of PCa specimens and 0% (0/13) of benign prostatic tissues. Using the mouse ERG-MAb ERG protein expression was found in 45.3% (120/265) of PCa specimens and 0% (0/20) of benign prostatic tissues.

Assessing the concordance between ERG protein expression levels and *ERG* rearrangement status (that is, positive ERG protein expression and positive *ERG* rearrangement status versus negative ERG protein expression and no *ERG* rearrangement versus discrepant results), we found a highly significant correlation between *ERG* rearrangement status and ERG protein expression status for both the rabbit ERG-MAb and mouse ERG-MAb (Pearson's correlation coefficient 0.971 and 0.956, respectively; $P<0.001$). Using the rabbit ERG-MAb, ERG protein expression correctly predicted an *ERG* rearrangement status in 98.6% (274/278) of cases, whereas discordant results were seen in four cases. Within these, three cases exhibited no ERG protein expression despite a positive *ERG* rearrangement status. In one case, a weak ERG protein expression was observed in the PCa tissue without any evidence of an *ERG* rearrangement, as determined by FISH. Using the mouse ERG-MAb, ERG protein expression correctly predicted the *ERG* rearrangement status in 97.8% (272/278) of cases. Discordant results were observed in only six cases. Three of these cases did not reveal any ERG protein expression despite harboring the *ERG* rearrangement. In the remaining three cases, an ERG protein expression was present in the PCa tissue without evidence of an *ERG* rearrangement. In 100% (7/7) of ERG-overexpressing PCa, the same ERG expression pattern was present in the corresponding lymph node metastases.

Assessing ERG protein expression by using either the rabbit ERG-MAb or the mouse ERG-MAb, a highly significant correlation was observed (Pearson's correlation coefficient 0.985, $P<0.001$). In 99.2% (276/278) of cases, IHC of either antibody revealed the same ERG protein expression pattern (that is, ERG protein expression versus no ERG protein expression), whereas only in three specimens differing results were observed. In all two cases, the PCa tissue displayed an ERG protein expression using the mouse ERG-MAb, but no ERG protein expression was found by using the rabbit ERG-MAb. Both cases did not exhibit an *ERG* rearrangement in the FISH analysis.

Despite an ERG protein expression in a considerable number of PCa tissues, a small subset of high-grade prostatic intraepithelial neoplasia revealed a moderate-to-strong ERG protein expression. Interestingly, in all these cases, an *ERG*-rearranged PCa tumour focus with an at least moderate ERG protein expression was present. In concordance with previous reports, most vascular endothelia displayed a strong expression of the ERG protein, regardless whether in proximity of malignant or benign tissue. Of interest, lymphocytes revealed immunoreactivity using the rabbit ERG-MAb, but not by using the mouse ERG-MAb (Figures 1a and b). As expected, FISH analysis could not reveal evidence of an *ERG* rearrangement in any of the above-mentioned non-neoplastic tissues. Of note, in two lymph node metastases, sinushistiocytes were present exhibiting a strong ERG protein expression (Figure 1c). Notably, decalcification procedures of the bone metastases had no influence on the staining results (Figure 1d). Cross tables summarize the correlation between the mouse and rabbit ERG-MAb and underlying *ERG* rearrangement status. (Table 1–3).

Table 1. Correlation between *ERG* rearrangement (by FISH) and ERG protein expression (by IHC using the rabbit MAb)

ERG rearrangement status	*ERG protein expression*		
	Positive	*Negative*	*Total*
Positive	117	3	120
Negative	1	157	158
Total	118	160	278

Abbreviations: FISH, fluorescence *in-situ* hybridization; IHC, immunohistochemistry; MAb, monoclonal antibody.

Table 2. Correlation between *ERG* rearrangement (by FISH) and ERG protein expression (by IHC using the mouse MAb)

ERG rearrangement status	*ERG protein expression*		
	Positive	*Negative*	*Total*
Positive	117	3	120
Negative	3	155	158
Total	120	158	278

Abbreviations: FISH, fluorescence *in-situ* hybridization; IHC, immunohistochemistry; MAb, monoclonal antibody.

Table 3. Correlation between ERG protein expression by IHC using the rabbit ERG-MAb and mouse ERG-MAb

Rabbit ERG-MAb	*Mouse ERG-MAb*		
	Positive ERG protein expression	*No ERG protein expression*	*Total*
Positive ERG protein expression	118	0	118
No ERG protein expression	2	158	160
Total	120	158	278

Abbreviations: IHC, immunohistochemistry; MAb, monoclonal antibody.

DISCUSSION

Recurrent gene rearrangements involving members of the *ETS* gene family are a hallmark of PCa.[13] Among these, the most commonly observed and highly PCa-specific event is the promoting linkage of the androgen-regulated gene *TMPRSS2* to the oncogenic transcription factor *ERG*.[18] Other, but rarely observed 5′ fusions partners of *ERG,* are *SLC45A3* and *NDRG1*.[2,3] In the vast majority of PCa, *ERG* rearrangements result in a clonal overexpression of chimeric fusion transcripts encoding truncated variants of the ERG protein.[2,4-6,23] Consequently, with the recent advent of ERG-specific IHC, a potential clinical utilization of ERG-IHC in PCa is straight forward.[19-22,24]

Two ground-breaking studies by Furusato *et al.* and by Park *et al.* on two different ERG-MAbs gave first insights into the landscape of ERG oncoprotein expression in PCa.[19,21] Furusato *et al.*[19] introduced and tested a mouse ERG-MAb by providing a comprehensive evaluation of 132 whole mount PCa sections. Park *et al.*[21] demonstrated high concordance between ERG protein expression and underlying *ERG* gene rearrangement status in 210 cases by using a rabbit ERG-MAb. Van Leenders *et al.* and He *et al.* confirmed the feasibility of the rabbit ERG-MAb IHC in two independent cohorts containing a wide spectrum of prostatic lesions.[20,22] Although the recent reports uniformly indicated a high potential for a clinical application of the ERG-IHC in PCa, a comprehensive and comparative side-by-side validation of both antibodies was necessary to perform.

Consequently, the objective of this IHC study was to compare ERG protein expression by using both the mouse ERG-MAb and rabbit ERG-MAb on the same large PCa progression cohort with known *ERG* rearrangement status. So far, this report is the first to include brain and skeletal metastases. Specifically, we addressed three questions: first, the correlation of ERG protein expression with the *ERG* rearrangement status; second, whether ERG protein expression was conserved in metastatic *ERG* rearranged PCa; third, whether ERG IHC expresion is possible on decalcified specimens.

Our results demonstrate that both ERG-MAbs exhibited a high degree of specifity and sensitivity in detecting *ERG*-rearranged PCa. A positive nuclear immunostaining using either the rabbit ERG-MAb or the mouse ERG-MAb predicted an *ERG*-rearranged PCa with a sensitivity of 97.5% (specificity 99.4%) and 97.8% (specificity 98.1%), respectively. These findings also account for the assessed lymph node and distant metastatic PCa, where we observed a concordant immunostaining in all cases. Of note, decalcification procedures of the bone metastasis had no influence on staining results (Figure 1d). Of interest, both ERG-MAbs showed a strong ERG expression in vascular endothelia and sinusoidal histiocytes that can be used as an intrinsic positive control for the staining procedure described in this study. As far as the rabbit ERG-MAb is concerned, also lymphocytes qualify as an intrinsic positive control for the ERG immunoreactivity.

Taken together, our results confirm that ERG-IHC allows reliable and robust detection of ERG protein expression in localized and metastatic PCa.

ERG-rearranged PCa express various fusion transcripts. The most frequently observed *TMPRSS2-ERG* fusion transcript results in a combined expression of exon 1 of *TMPRSS2* and exon 4 of *ERG*.[3,25-27] According to our bioinformatics analysis, the mouse and the rabbit ERG-MAb detect sequences within ERG-exon 4 and exons 8–11, respectively. C-terminal amino-acid sequences are well conserved within the truncated oncoprotein variants. Apparently, both ERG-MAbs are similarly applicable to detect the most common truncated ERG proteins. As expected, we showed an almost perfect agreement between ERG protein expression patterns of the two antibodies (98.9%). Comparing ERG protein expression utilising the rabbit and mouse ERG-MAb with the underlying *ERG* rearrangement status, discordant findings were observed in only six cases. Among these, three cases uniformly (that is, with both the rabbit and mouse ERG-MAb) revealed no immunoreaction despite harboring the *ERG* rearrangement, suggesting the expression of a highly truncated ERG protein lacking all target binding sites of both ERG-MAbs (for example, transcripts exclusively involving ERG-exons 1–3 or ERG-exons 5–7). More likely, our findings provide evidence that a very small subset of *ERG*-rearranged PCa are not overexpressing fusion transcripts, for example, because of dysfunctional androgen signaling.[28] Alternatively, the negative immunoreaction could be because of a very low protein expression, which would not exceed the detection threshold. One case uniformly displayed a strong immunostaining but no evidence of an *ERG* rearrangement, suggesting that mechanisms other than the *ERG* rearrangement may lead to an overexpression of the ERG protein. Lastly, two cases displayed an ERG protein expression by using the mouse ERG-MAb, but no ERG protein expression was found by using the rabbit ERG-MAb. Both cases (weak ERG protein immunostaining) showed no *ERG* rearrangement. These cases may be because of a weak ERG protein expression, which is independent of an *ERG* rearrangement, and does not exceed the detection threshold of the rabbit ERG-MAb.

Of note, if the *ERG* rearrangement and ERG protein expression was present in localized PCa, we observed the same pattern in the corresponding lymph node metastases. These findings provide further insight into recent observations that the *ERG* rearrangement status within PCa tumor foci shows clonality during disease progression–in contrast to a commonly occurring change of features like androgen receptor, phosphatase and tensin homolog copy number changes or Gleason pattern.[13-16] In this respect, our results demonstrate that the clonal nature of the *ERG* rearrangement is not restricted to the genomic and transcriptional levels, but proceeds in the proteome. This observation supports that the *ERG* rearrangements is rather a driver than a passager event in PCa.

In concordance with previous findings, nuclear expression of the ERG protein was present in endothelial cells. Furthermore, by

observing that lymphocytes exclusively revealed immunostaining using the rabbit ERG-MAb, but not using the mouse ERG-MAb, we confirm a recent report by Mohamed *et al.*[29] (Figures 1a and b). Additionally, within lymph nodes, we were the first, to our knowledge, to observe a strong nuclear ERG protein expression in sinushistiocytes (Figure 1c). The characteristically morphological pattern allowed precise differentiation between mononuclear phagocytes and PCa infiltrates. However, this needs to be kept in mind as a pitfall for the unexperienced observer. A recent multitumor ($n = 1864$) evaluation of ERG protein expression by mouse ERG-MAb showed that in addition to PCa, ERG protein expression is a highly specific marker for vascular endothelium and vascular tumors. Of note, angiomatoid and cutaneous fibrous histiocytoma both did not exhibit ERG immunoreactivity.[24]

In summary, by demonstrating a broad applicability of both, the rabbit and mouse ERG-MAb, we confirm the feasibilty of ERG IHC in PCa. Given the comfort of performing IHC versus FISH, ERG protein-based stratification of PCa in formalin-fixed paraffin-embedded tissue and in decalcified material may evolve into a valuable tool for routine diagnostics. Furthermore, results of this study facilitate recent efforts to understand the causative role, diagnostic utility and potential applications for monitoring therapeutic efficacy of ERG and the recurrent gene rearrangements in PCa.

CONFLICT OF INTEREST

The Brigham and Women's Hospital and the University of Michigan have filed a patent on ETS gene rearrangements in PCa, on which S P is a co-inventor and the diagnostic field of use has been licensed to GenProbe (San Diego, CA, USA). GenProbe did not have a role in the design and conduct of the study, in the collection, analysis or interpretation of the data and had no involvement in the preparation, review or approval of the manuscript. The Henry M Jackson Foundation for the Advancement of Military Medicine has filed a patent application on the mouse monoclonal antibody, ERG-MAb, on which S T and A D are co-inventors and has been licensed to the Biocare Medical (Concord, CA, USA). This study was conducted independent of any involvement from Biocare Medical.

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Review Article

Oncogenic activation of *ERG*: A predominant mechanism in prostate cancer

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Abstract

Prevalent gene fusions involving regulatory sequences of the androgen receptor (AR) regulated genes (primarily *TMPRSS2*) and protein coding sequences of nuclear transcription factors of the *ETS* gene family (predominantly *ERG*) result in unscheduled androgen dependent *ERG* expression in prostate cancer (CaP).Cumulative data from a large number of studies in the past six years accentuate *ERG* alterations in more than half of all CaP patients in Western countries. Studies underscore that *ERG* functions are involved in the biology of CaP. *ERG* expression in normal context is selective to endothelial cells, specific hematopoetic cells and pre-cartilage cells. Normal functions of *ERG* are highlighted in hematopoetic stem cells. Emerging data continues to unravel molecular and cellular mechanisms by which *ERG* may contribute to CaP. Herein, we focus on biological and clinical aspects of *ERG* oncogenic alterations, potential of *ERG*-based stratification of CaP and the possibilities of targeting the *ERG* network in developing new therapeutic strategies for the disease.

Keywords: ERG, prostate cancer, TMPRSS2-ERG, oncoprotein, androgen receptor, patient stratification

BACKGROUND

Key molecular genetic alterations in prostate cancer

Prostate cancer (CaP) is the most common malignancy that affects men worldwide, with high frequency in the United States, Western Europe[1] and low reported frequency in Asia.[2,3] Risk factors associated with CaP include age, family history and ethnicity.[1,4] Although precise molecular events that contribute to such variation in the CaP incidence are not well established, the differences may be attributed to factors such as genetics, diet, lifestyle, and male hormone levels.[4-6] Despite the recent advances in early detection and continued refinements in treatment strategies, CaP is still the second leading cause of cancer mortality in American men.[1] Discovery of CaP-specific gene expression and/or mutational alterations have contributed to a significant impact on designing molecular markers to distinguish indolent from more aggressive forms of cancers as well as molecular pathways to develop effective novel therapeutic approaches to combat the disease.[7-12]

CaP susceptibility loci with germ-line mutations of *RNAseL, ELAC2, MSR1, BRCA* 1 and *2, HPCX, KLF6,* and *HPC20*have been reported in primary CaP.[13,14] However, low penetrance and disease heterogeneity have precluded the validation of CaP susceptibility genes. Recent genome wide association studies (GWAS) have identified multiple CaP risk alleles towards defining genetic determinants of CaP risk.[15,16] A "gene less 1.18 Mb region" between FAM84B at centromeric end and *C-MYC* at telomeric end on chromosome 8q24

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has been consistently found to be associated with CaP risk.[17-21] The8q risk allele specific for African ancestry showed an association with higher pathologic stage of CaP in African American men.[22] Functional evaluations of a risk allele on chromosome 10 suggested its impact on regulation of expression of *NCOA4* (*AR* co-activator) and *MSMB*.[23] Overall, a combinatorial assessment of the risk alleles has shown a significantly increased predictive power of CaP risk.[19,24]

Chromosome loci harboring putative proto-oncogenes or tumor suppressor genes (TSGs) have been extensively evaluated toward identifying specific gene mutations and expression signatures in CaP. Mutations, amplifications or over-expression of the androgen receptor (*AR*), and mutations in tumor suppressors such as *p53* and *PTEN,* are frequently identified subsets of advanced CaP.[8,9,25-28] Among the recurrent allelic losses of 8p21-22, 6q16, 7q31, 10q23-25 and 16q24 loci detected in primary CaP,[8,29] deleted 8p21-22 locus harbors a widely studied tumor suppressor gene *NKX3.1*.[30] While early studies showed *PTEN* mutations in subset of advanced cancers, more recent reports underscore higher frequency of *PTEN* hemizygous deletions in primary CaP.[31] In addition, frequent gains of chromosome 8q24, as well as over-expression of *C-MYC* and prostate stem cell antigen (*PSCA*) within this locus have been reported.[13]

Identification of common CaP specific gene signatures have enriched mechanistic as well as translational research investigations. Expression of genes such as *NKX3.1*[32] and *GSTP1*[33] have been studied extensively for their biological roles in onset of CaP. The virtual absence of *GSTP1* expression due to promoter methylation has led to blood- and urine-based assays for diagnosis.[34] Overexpression of AMACR and absence of p63 in most prostate tumors have already led to the use of these two proteins in diagnostic pathology.[35] Striking overexpression of a prostate tissue specific gene, *DD3/PCA3* in CaP have led to extensive evaluations for its diagnostic utility as a marker in urine based assays.[36] Although CaP specific gene alterations are increasingly studied, the most validated oncogenic alteration to date is *ERG*. This observation led to multi-faceted investigations towards defining the cancer specific characteristics of *ERG,* and is discussed in the following sections.

Prevalence of *TMPRSS2-ERG* fusion in prostate cancer

Identification of *ERG* proto-oncogene overexpression in CaP transcriptome led to focused evaluations of *ERG* alterations in CaP.[37-39] Quantitative expression assessment of *ERG* mRNA in matched benign and malignant prostate cells from a large patient cohort confirmed the tumor cell specific *ERG* overexpression in 60-70% patients.[39] Over expression of *ERG* due to fusions between androgen regulated *TMPRSS2* gene promoter and the coding regions of *ERG* has been identified as the most common genomic alteration.[40] These observations also led to the development of a combined CaP gene panel (*PCA3, ERG* and *AMACR*) with diagnostic potential in which overexpression of at least one of three genes associated with virtually all of prostate tumor specimens.[39] Discovery of prevalent gene fusions involving promoters of the androgen receptor (*AR*) regulated genes (*TMPRSS2, SLC45A3, NDRG1, Herv-K22q11.23, CANT1 and KLK2*) and coding sequences of *ETS* gene family (*ERG, ETV1, ETV4, ETV5*)marked a major milestone towards defining molecular mechanisms of prostate carcinogenesis.[11,41] Of the fusions involving *TMPRSS2* and *ETS* factors in CaP, majority (>90%) involve *ERG,* and *ETV1, ETV4* and *E*TV5 represent very low frequency (1-5%).[11] *TMPRSS2* gene is mapped to 21q22.3 between markers *ERG* and D21S56, and transcribed as 3.8 kb mRNA.*TMPRSS2* promoter analysis revealed the presence of a non-canonical ARE as a *CIS*-regulatory target of AR action.[42] *TMPRSS2* is predominantly expressed in prostate tissues with low levels of expression in pancreas, kidney, lung, colon and liver.[43,44] Gene fusions between *TMPRSS2* and *ERG* or *ETV1* appears to be CaP specific and are potentially mediated by AR-induced proximity of fusion gene partners in the presence of genotoxic factors[45,46] followed by topoisomerase-2b-mediated recombination event.[47] Comprehensive evaluations of gene fusions involving *ETS* factors have been covered in excellent reviews.[11,48]

ERG gene structure and transcription

ERG is a member of the *ETS* gene family[49,50] which is one of the largest families of transcriptional regulators consisting of at least 27 members, subdivided into 5 subfamilies.[51] Conserved PNT/SAM domain and an ETS domain are the common features of members of ETS related proteins. These domains play key roles in regulating downstream target genes that are crucial for several biological processes such as cellular proliferation, differentiation, development, transformation, and apoptosis.[52] *ERG* consists of 17 exons and is transcribed to generate several alternately spliced forms[53] [Figure 1]. At least five splice variants are translated into proteins: ERG-1 (p41), ERG-2 (p52), ERG-3 (p55), ERG-4 (p49) and ERG-5 (p38)[54] by a combination of alternative mRNA splicing and/or use of alternative polyadenlyation sites.[50,55] Most characteristic of the family is the evolutionarily conserved 85–amino acid ETS domain, which facilitates binding to purine-rich DNA with a GGAA/T core consensus sequence.[51,56]

ERG is among a small number of transcription factors

that exhibit an endothelial cell and hematopoietic cell restricted expression pattern in various species. In developing mouse, *Erg* mRNA is expressed in mesodermal tissues such as endothelial cells, mesenchymal condensations during precartilaginous depositions, and in urogenital regions.[57] Similarly, *ERG* protein is predominantly detected in endothelial cells, hematopoietic tissues and transiently in pre-cartilage.[58] *Erg* is expressed transiently during early T-cell development, early pre-B and continue to express in mature B cells.[59,60] Later in development, *Erg* functions in cell survival maintaining the differentiation of endothelial cells of vascular and lymphatic origins.[61,62] Thus, highly restricted expression of *Erg* mRNA or *ERG* protein during early phases of lymphocytic, hematopoietic, chondrocytic and endothelial lineage differentiations appears to be crucial in lineage specification function.[58,63-65] Intriguingly, *ERG* protein is not detected in any epithelial tissues including prostate epithelium, or in infiltrating lymphocytes that are occasionally seen in the prostate environment.

Normal biological functions of *ERG*

Biological functions of *ERG* have been studied in xenopus, zebra fish, mouse and humans.[57,66-71] Angiogenesis is an essential process by which new vessels are developed from preexisting ones, during normal development, as well as in pathologic conditions, including tumor development. Widespread expression of *ERG* in endothelial cells suggests for its biological roles in these specialized cells. In addition to VE-cadherin, other endothelial specific factors such as, von Willebrand factor, endoglin, and intercellular adhesion molecule-2 are also regulated by *ERG* supporting its role in endothelial cell differentiation and angiogenesis.[62,72] Endoglin is an accessory receptor for TGF-β and both endoglin and TGF beta receptor type II are positively regulated by Erg.[73,74] Recently, using a functional mutation in mouse models, *Erg* has been shown to regulate the normal platelet development, stem-cell function, definitive hematopoiesis and the normal megakaryopoiesis.[70] Although, *ERG* is considered as critical regulator of hematopoiesis, *Erg* is dispensable during early embryonic hematopoietic development, hematopoietic specification from the mesoderm and is required to sustain definitive hematopoiesis. During this process, *ERG* acts as a direct regulator of critical transcription factors such as Runx1 and Gata2.[75] During hematopoiesis, adult hematopoetic stem cells require *ERG* for self-renewal and differentiation.[76] *ERG* is also documented as a transcription regulator of embryonic stem cell (ES) towards differentiation of early endothelial lineage[77] and exhibits anti-inflammatory responsein endothelial cells by suppressing IL 8.[72]

Prostate cancer associated *TMPRSS2- ERG* transcripts

Several types of *TMPRSS2-ERG* fusion transcripts involving various exons of the *TMPRSS2* and *ERG* have been identified in CaP specimens.[66,78-83] These transcripts were identified on the basis of *TMPRSS2* fusions with the 5' end of the *ERG* and are broadly classified into 8 different groups. In the context of full length transcripts, 2 major forms were identified on the basis of mRNA splicing, cDNA and deduced amino acid sequences.[81] Although, several fusion transcripts are generated from *TMPRSS2-ERG* fusions, it is not clear whether these transcripts are expressed from a single or multiple foci of CaP. Evaluation of *TMPRSS2-ERG* transcripts in multi-focal CaP have shown inter-focal heterogeneity with respect to the presence of fusion positive or negative foci in malignant prostate glands.[82,84-86]

Despite the heterogeneity of *TMPRSS2-ERG* fusions, most common fusion is in between *TMPRSS2* exon 1 and *ERG* exon 4, which results in the deletion of first 32 amino acids from the N-terminus of *ERG* protein.[87] The expression of *TMPRSS2* exon 2 with *ERG* exon fusion 4 mRNA associated with PSA recurrence and seminal vesicle invasion.[78] The most common full length *TMPRSS2-ERG* transcripts (Type I) translate into full length proteins (ERG1, ERG2, ERG3) containing protein-protein interacting (pointed/SAM) and DNA-binding (ETS) domains.[81,87] The most predominant of the proteins generated from the fusions is the N-terminal truncated ERG3 protein. Whereas the type II *TMPRSS2-ERG* transcripts code for ERG8 and a new variant, TEPC, with deletion of 32 amino acids at N-terminus and contain only pointed/SAM domain[81] [Figure 1]. Importantly, higher ratio of type I over type II *TMPRSS2-ERG* splice forms are shown to correlate well with unfavorable prognostic features of CaP, such as poorly differentiated tumors, higher Gleason sum, positive margin, and biochemical recurrence.[81] Additional studies are needed to assess prognostic association specific *TMPRSS2-ERG* fusion transcripts with CaP progression. Since *ERG* is the most common cancer gene activation in CaP, *ERG* expression and function in normal and other cancer contexts may be illustrative in further understanding the biological roles of *ERG* in CaP.

Prostate cancer associated functions of *ERG*

Since the discovery of *ERG*, several reports have shown that *ERG* transforms epithelial cells[49,88-91] and functions through mitogenic signals including the *MAP* kinases.[88] Acute myeloproliferation and megakaryocytic differentiation are the main features of hematologic diseases associated with Down syndrome (trisomy of chromosome 21), in which *ERG* expression is found to be elevated.[92] Myeloproliferation and acute megakaryocytic leukemia were experimentally demonstrated in a genetically engineered Down syndrome mouse model Ts(17(16))65Dn.[92] Similarly, in cell culture system, over expression of *ERG* in erythroleukemia cell line,

K562 induced erythroid to megakaryoblastic phenotype[91] suggesting a critical role for *ERG* in malignant hematologic disorders in Down syndrome. In addition, *ERG* promotes expansion of megakaryocytes from hematopoietic progenitor cells[93] and function as a megakaryocyte oncogene.[94]

In diverse neoplasms, *ERG* is either over expressed abnormally or fused to other genes due to chromosomal translocations and expressed as a chimeric protein. *ERG* gene fusions were initially described in Ewing's sarcoma (EWS) and acute myeloid leukemia (AML).[90,95] In a small subset (about 5-10%) of Ewing's sarcoma, *EWS-ERG* fusions resulted into a chimeric protein containing amino-terminal end of EWS and the carboxy-terminal *ERG* including the DNA binding ETS domain.[96] Majority (95%) of *EWS* fusion involve *EWS* and *FLI*, the closest homolog of the *ERG*.[97] Similarly, *ERG* fuses with *TLS/FUS* in certain acute myeloid leukemias.[98] These fusions generate chimeric proteins abnormally regulate downstream genes due to altered transactivation and DNA binding activities.

As noted above *TMPRSS2-ERG* fusions in CaP leading to androgen dependent expression of *ERG* are exclusive to prostate tumor cells. *ERG* regulates the expression of *C-MYC*, a widely studied oncogene, by physically interacting with the ETS binding element within the P2 promoter region.[71] Consistent with the above observations a positive correlation between *ERG* and C-*MYC* expression suggests that *ERG* mediates oncogenic process through *C-MYC* and may be one of the potential mechanisms in CaP. In addition to the positive regulation of *C-MYC*, *ERG* negatively regulates the expression of a number of prostate differentiation genes such as *KLK3/ PSA, SLC45A3/ Prostein* and abrogates the prostate epithelial differentiation program.[71,99] Of note, knock-down of either *ERG* or *C-MYC* in *TMPRSS2-ERG* positive CaP cells showed similar effects on cellular morphology and expression of prostate differentiation related genes.[71]

In the majority of cancers, cell invasion and migration are the key features of aggressive nature of tumors towards metastasis. *ERG* regulates invasion and migration related genes in CaP

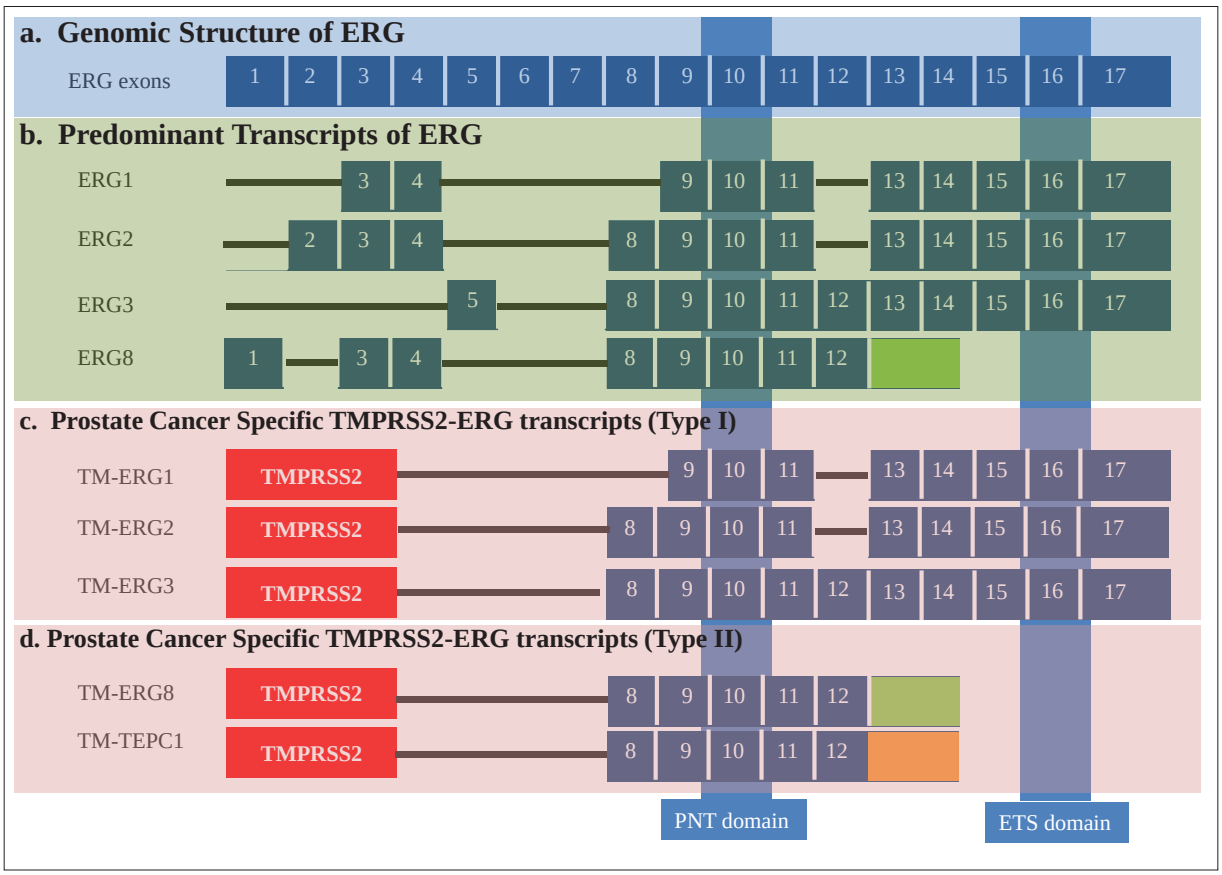

Figure 1:Genomic structure and transcripts of human ERG gene. (a) Genomic structure depicting ERG Exons (blue boxes) numbered from 1-17.[53] (b) Structure of expressed ERG transcripts.[53] (c) Prostate cancer specific TMPRSS2-ERG fusion transcripts containing protein-protein interaction domain (pointed/SAM) and DNA binding (ETS) domain (Type I).[81] (d). TMPRSS2-ERG fusion Type II transcripts containing only pointed/SAM without ETS domain.[81] Note: In prostate cancer, the original ERG exon 8[53] is numbered as 4.[40,78,87]

such as *MMP1, MMP3, MMP9,* and *ADAM19*, the urokinase plasminogen activator (*PLAU*), and the plasminogen activator inhibitor type1 in CaP.[99-101] *ERG* enhances cell invasion and metastasis through regulating *CXCR4*, a chemokine receptor.[27,102] *ERG* also induces the expression of osteopontin (*OPN*) through ETS binding sequences within the promoter.[103] *OPN*, a member of a Small Integrin-Binding Ligand, N-linked Glycoprotein (SIBLING),and a key regulator of metastasis of a wide variety of cancers is up-regulated in several cancers including prostate. Phenotype of human prostate cancer such as metastasis has been correlated with increasing levels of *OPN* expression.[104]

Accumulating data suggests that *ERG* mediates epigenetic regulatory function[105] through *EZH2*, a polycomb group (PcG) protein in CaP.[106] *EZH2* promotes cancer formation and progression through activation of oncogenic signaling cascades and inhibition of pro-differentiation pathways.[10] In CaP, *NKX3.1* expression is negatively regulated by *ERG* induced *EZH2* interactions.[106] Interestingly, *NKX3.1* negatively regulates *TMPRSS2* promoter that is frequently fused to *ERG*.[107] Therefore inhibition of *NKX3.1* either by *ERG/EZH2* or loss of *NKX3.1* due to recurrent 8p21 deletions may fuel *TMPRSS2* dependent *ERG* expression in CaP. Other epigenetic factors include histone acetyl transferases (*HATs*) and histone deacetylases *(HDACs*) which are frequently altered in majority of cancers including CaP.[108] *ERG* binds to and inhibits HAT activity to deregulate protein acetylation and also activates *HDAC* to deacetylate histone proteins.[109,110] Interestingly, *ERG* has been shown to play critical role in epithelial-to-mesenchymal transition (EMT) by repressing epithelial specific genes and inducing mesenchymal specific genes through *WNT* signaling components.[109,111] EMT has received considerable attention as a conceptual paradigm to explain invasive and metastatic behavior during cancer progression. During this transition, the epithelial cells lose their polarity and cohesiveness, acquiring migration and invasive properties.[112] Recent genome wide screening of *ERG* candidate genes and subsequent validation revealed ERG-enriched targets that include both canonical and non-canonical *WNT* signaling genes: *WNT11, WNT2, WNT9A, CCND1* and *FZD7*.[113] Both *ERG* and *WNT11* expression were elevated in high-grade prostate tumors.[114,115] *FZD4*, one of the members of *WNT* signaling pathway, is often co-expressed with *ERG* in clinical specimens. Down regulation of *ERG* or *FZD4* releases the transcriptional block on both *β1-Integrin* and *E-cadherin* to maintain epithelial phenotype.[109] Interestingly, *ERG* also up regulates EMT facilitators such as *ZEB1* and *ZEB2* that negatively control the *E-cadherin*[111] potentially through *SNAIL1* and *2* pathway in CaP.[116] Although EMT is not a prerequisite for invasive cancer development, this process can play an important role in cancer cell dissemination from the tumor due to altered expression of *E-cadherins.*

ERG has also been shown to interface with genes linked to inflammation and DNA damage repair pathways. *ERG* activates *NF-kB* pathway through *toll-like receptor 4* suggesting for its role in inflammation related pathways.[117] *15-hydroxy-prostaglandin dehydrogenase (HPGD),*a tumor suppressor and prostaglandin catabolizing enzyme, is down regulated in variety of cancers such as lung, colon, breast and bladder cancers. Recent studies have shown a potential link between *ERG* and prostaglandin signaling and inflammation pathways in which *ERG* down-regulates the *HPGD* expression to induce carcinogenesis.[118] Proteomics evaluations of *ERG* binding proteins show that *ERG* interacts with Poly (ADP-ribose) polymerase (PARP) and catalytic subunit of DNA protein kinase (DNAPKcs) in a DNA independent manner.[119] This complex formation is required for *ETS* gene mediated transcription and cell invasion. *ERG* induced DNA damage in CaP cells can further be potentiated by PARP1 inhibition, an observation similar to effects of these inhibitory compounds in breast cancer with *BRCA1/2* mutations. As noted, most of studies addressing biochemical and cell biological functions of *ERG* in CaP have used VCaP cell line as this is the only well characterised TMPRSS2-*ERG* positive CaP cell line.[120] Since *ERG* downstream targets may be cellular context dependent, these data need to be interpreted with caution especially in cases when, findings have not been validated in human CaP specimens or complementary experimental models. Development of additional *ERG* positive CaP cell lines will also facilitate cell biologic evaluations of ERG.

Although, the presence of elevated expression of *ERG* in large number of CaP patients have been well characterized by several groups, it is not clear whether *ERG* is an initiating factor or expressed as a consequence of other aberrant genetic events. Towards this, several groups have developed *ERG* transgenic mice by prostate targeted expression of *ERG* driven by rat probasin promoter.[27,87,99,101] Prostatic intraepithelial neoplasia (PIN), a pre-invasive lesions of CaP was reported in the prostates of transgenic mice, which surprisingly did not progress to adenocarcinoma.[99,101] On the contrary, other studies did not observe PIN phenotype, however, developed of adenocarcinoma in combination with either phospho *AKT* overexpression or with loss of *PTEN*.[27,87] Similarly, in prostate tissue dissociation/ regeneration system, high levels of *ERG* expression could induce the initiation of neoplastic transformation of adult prostate epithelial cells and further developed adenocarcinoma in combination with *pAKT* or *AR*.[121] Recent evaluations of the association

*TMPRSS2-ERG*fusion with other genomic alterations in human CaP revealed significant associations with deletions of chromosomal regions, 10q23.31 and 17p13.1 harboring PTEN and p53 respectively.[122] Further, *ERG* fusions showed an intriguing association with CaP specific focal deletion of 3p14.1-p13 harboring several candidate TSGs.[122] While cooperation of *ERG* with PTEN/p-AKT has been shown in enhancing prostate tumorigenesis, interaction of *ERG* with other cancer genes needs to be further defined in engineered mouse models. Taken together, the studies focusing on *ERG* functions provide an emerging picture of the *ERG* network involved in the regulation of differentiation, cell invasion, epigenetic control, EMT inflammation and DNA damage, all of these support the biological role of *ERG* in CaP [Figure 2]. Further, interactions/cooperation of *ERG* with genes (*AR, C-MYC, NKX3.1* and *PI3K/PTEN* axis) functionally significant in CaP, defines potential role of *ERG* in common CaP pathways. These findings have potential to provide new therapeutic approaches for CaP.

ERG as diagnostic/prognostic marker for prostate cancer

Detection of gene fusions has led to a paradigm shift in the diagnosis, classification, and treatment options for hematologic cancers.[123-125] These gene fusions provide CaP specific markers which have promise in improving diagnosis, as well as molecular classification of prostate tumors.[126,127] The feasibility of detecting *TMPRSS2-ERG* fusion by FISH in prostate biopsies and prostatectomy specimens enhances the detection of CaP in diagnostic and prognostic settings.[128-131] The clinical value of *ERG* fusion in prostate biopsies needs to be further explored and validated in larger prospective studies.

Interrogation of the presence of *TMPRSS2-ERG* fusion or *ERG mRNA* in CaP was initially believed to provide prognostic information. However, in retrospective prostatectomy cohorts conflicting results have been reported regarding associations between *ETS* fusions and cancer aggressiveness.[11,48] For example, presence of *TMPRSS2-ERG* fusion predicted cancer recurrence after surgery or lethal outcome in a watchful waiting cohort.[79,132] However, association of the fusion or *ERG* expression with favorable outcome was also reported.[39,133,134] Since *ERG* expression in CaP is androgen dependent due to *TMPRSS2-ERG* fusion, alterations of AR transcription factor activity may result in altered *ERG mRNA* expression as noted in poorly differentiated tumors.[135] These data also suggest that *ERG* in combination

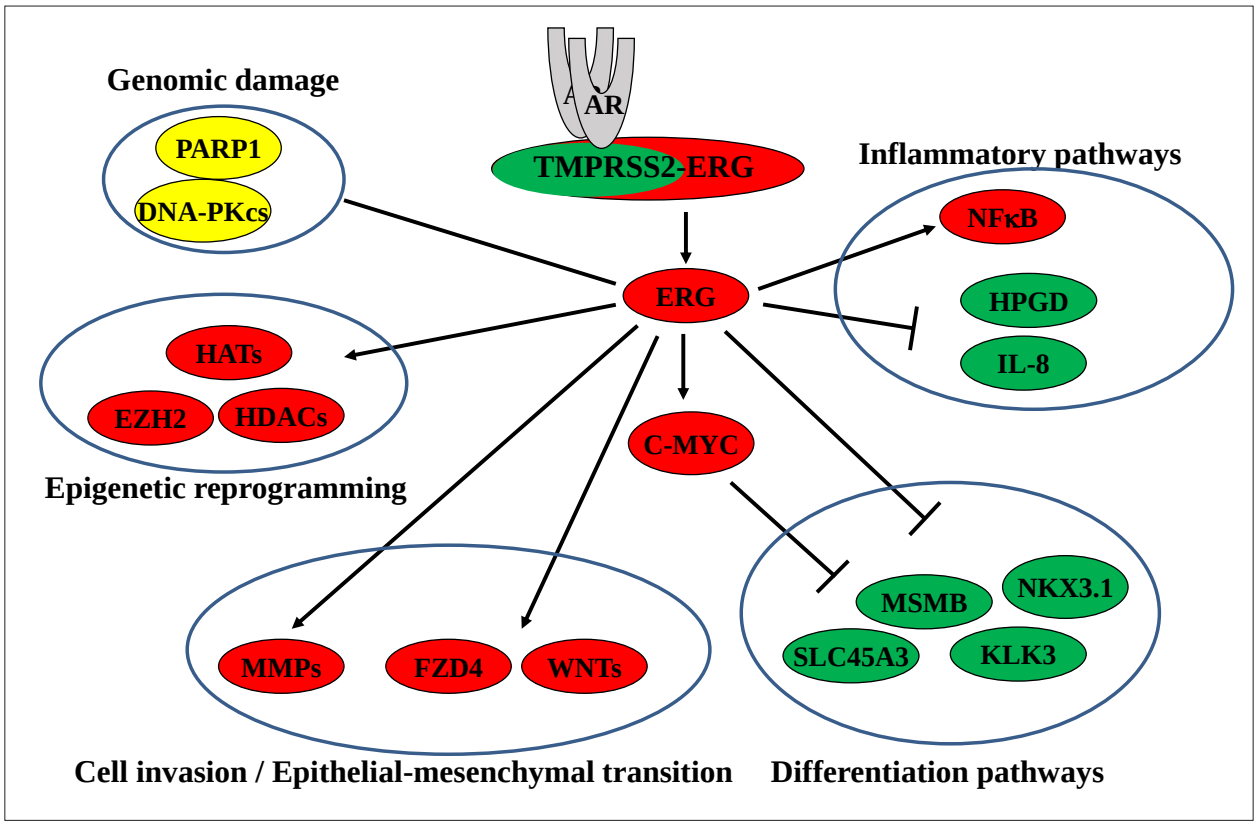

Figure 2: ERG regulated prostate cancer pathways.ERG regulates the expression of target genes associated with cancer initiation and progression pathways such as DNA damage, inflammation,epigenetic control,regulation of differentiation, EMT, cell proliferation and cell invasion. (Red: upregulated;Green:down regulated;Yellow: protein-protein interactions)

with a panel of androgen receptor regulated genes (*PSA, PMEPA1, NKX3.1, ODC, AMD*) may serve as a biomarker panel for Androgen Receptor Function Index (ARFI) in CaP. Thus, ARFI may provide new opportunities in AR function based stratification of CaP, where *ERG* expression evaluation could play important role in over half of CaP.[135] These findings may provide potential biologic basis for initial observations on association of decreased or no *ERG mRNA* expression with poor prognosis of CaP.[39] *TMPRSS2-ERG* fusion isoforms have variable tumor promoting biological activities and certain isoforms are correlated well with more aggressive disease[55] and others with favorable prognosis.[136] Similarly, the ratios of full length splice forms type I and type II also shown to have prognostic association.[81] However, some studies have reported no significant association of *TMPRSS2-ERG* fusion or *ERG* expression with disease progression after prostatectomy.[83,137,138] Therefore, larger and better designed studies are needed for further clarification. The observations of combination of *TMPRSS2-ERG* fusion and *PTEN* deletions associating with poorer prognosis have been supported with functional studies showing cooperation of these genes in mouse models of CaP.[27,87,121,139] Further assessment of the utility of combinatorial prognostic markers is warranted.

Utility of detection of *TMPRSS2-ERG* fusion or *ERG* transcripts in post-digital rectal examination (post-DRE) urine are also being evaluated for improving CaP diagnosis using minimally invasive assays.[140-142] Promising results from evaluations of highly CaP specific non-coding RNA, *PCA3*, in post-DRE urine specimens, have led the way for evaluation of additional CaP specific expression markers.[143-145] A CaP gene panel (*PCA3, ERG* and *AMACR*) with diagnostic potential in which overexpression of at least one of three genes associated with virtually all of the LCM derived prostate tumor specimens suggested for careful evaluation of such panels in post-DRE urine.[39] Evaluation of ERG[141] or *TMPRSS2-ERG*[140] transcripts in post-DRE urine have provided promising data on diagnostic potential of *ERG* in this minimally invasive bio-specimen. A recent multi-center study of 1312 men showed promising data with respect to association of *TMPRSS2-ERG* in post-DRE urine with clinically significant CaP.[142] This study further showed utility of the combination of *TMPRSS2-ERG* and *PCA3* in post-DRE urine in comparison to serum PSA for detecting clinically significant CaP in specimens.[142]

New insights into detection of *ERG* oncoprotein in prostate cancer

Accurate molecular analysis of ERG oncoprotein in CaP has been a challenge as ETS family of proteins share high homology among the family members. Recent development and evaluation anti-ERG monoclonal antibodies have paved the way for evaluation of ERG protein in routine pathologic specimens. Through exhaustive analysis of 132 whole-mount prostates sections (261 tumor foci and over 200,000 benign glands) for the ERG oncoprotein nuclear expression by an anti-ERG mouse monoclonal antibody (clone 9FY), this study demonstrated 99.9 % specificity for detecting tumor cells in prostate.[138] The ERG oncoprotein expression correlated well with fusion transcript or gene fusion in selected specimens. Strong concordance of ERG positive prostatic intraepithelial neoplasia (PIN) lesions with ERG positive carcinoma (82 out of 85 sections with PIN, 96.5%) affirmed the biological role of ERG in clonal selection of prostate tumors in 65% (86 out of 132) of patients[138] [Figure 3]. These observations lend a support to the functional role of ERG in initiation of preneoplastic lesions.[99,101] Evaluations of anti-ERG rabbit monoclonal antibody (EPR 3864) in CaP tissue microarrays from 207 established correlation between detection of ERG protein expression by IHC and ERG rearrangement by using fluorescence *in situ* hybridization (FISH). Detection of the ERG protein expression in CaP exhibited 95.7% sensitivity and 96.5% for the presence ERG rearrangement. Further, presence of ERG protein in CaP also correlated with less common ERG rearrangements. Since ERG expression is almost exclusive to prostate tumor cells and IHC is easier to perform in comparison to FISH. It is expected that ERG protein detection in pathologic specimens will greatly facilitate the evaluations of biological and clinical utility of ERG antibodies in CaP. Among the currently known CaP biomarkers, detection ERG oncoprotein offers unprecedented opportunities in the diagnostic setting [Figure 4]. With the availability of highly specific ERG monoclonal antibodies, better and more effective monitoring, treatment, and therapies

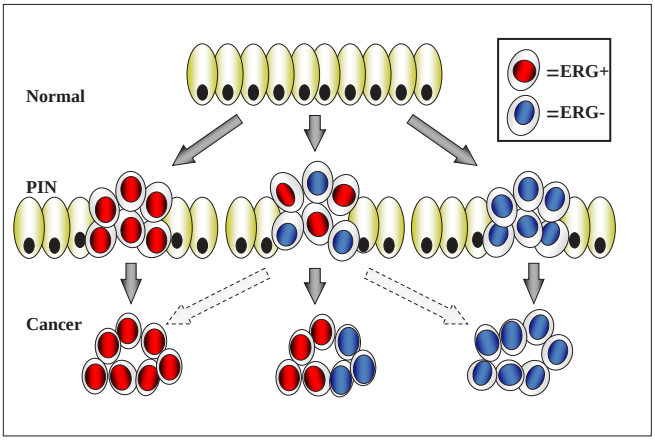

Figure 3: ERG-dependent Clonal Selection of Prostate Tumors. Model describing the ERG-dependent clonal selection of prostate tumors from prostatic intraepithelial neoplasia (PIN) to prostate cancer. Other precursor lesions which may not progress through the PIN morphological stage are not represented by this model. Normal prostate epithelial cells are marked by green color

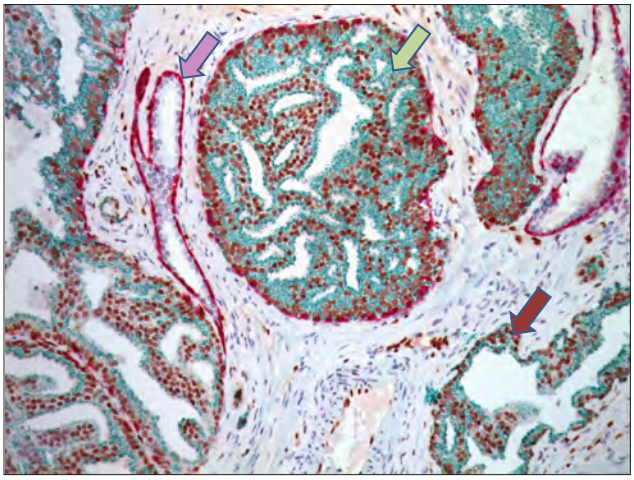

Figure 4: Detection of PIN and prostatic adenocarcinoma by the combination of ERG, AMACR, p63 and CK5 markers in immunohistochemistry.Tumor cells are positive for nuclear ERG (brown) and cytoplasmic AMACR (green), whereas, absence of p63 (purple) and CK5 (purple) indicate the lack of basal cell layer. By contrast, in normal prostatic glands prominent staining with p63 and CK5 distinctively demarcate intact basal cell layer.In PIN disrupted basal cell layer and prominent ERG and AMACR staining is apparent (×400). (Image: Courtesy of Dr. David Tacha, Biocare Medical Inc, Concord, CA, USA)

may also be available in future to patients with CaP.[146,147]

Since ERG MAb 9FY is highly ERG specific as illustrated by lack of recognition of its closest homolog, FLI,[58,138] the presence of ERG protein in hemangiomas, lymphangiomas, angiosarcomas, epithelioid hemangio-endotheliomas and Kaposi sarcomas[148] serve as an excellent new marker for vascular tumors. Similar studies are also warranted in Acute Myeloid leukemia where *ERG* has been suggested as prognostic marker based on mRNA based studies.[58,148]

New therapeutic opportunities targeting *ERG* in prostate cancer

Studies have shown growth inhibitory effects of the *ERG si RNA* in *TMPRSS2-ERG* positive VCaP cells and VCaP derived tumors in SCID mice suggesting for therapeutic potential of *ERG* inhibition in CaP.[66,71] Further, these mechanistic data delineated the effects of *ERG siRNA* through inhibition of C-MYC and induction of prostate epithelial cell differentiation markers.[71] Recent reports in transgenic mice have shown cooperative effects of *ERG* overexpression with *PTEN/PI3K* axis alteartions, leading to progressive features of CaP.[27,87] Thus targeting the inhibition of *ERG* pathway may provide a promising therapeutic strategy. In addition to siRNA as a potential molecule to interfere with the *ERG* expression, YK-4-279, a derivative of the lead compound from the small molecule screen, has proven to effectively bind to ERG and subsequently down regulate its transcriptional activity as well as tumor cell invasion in cell culture model.[149,150] Inhibitors of HDACs are currently being considered as one of the potent anti-cancer agents. HDAC inhibitors, such as SAHA, MS-275, TSA and VPA have been evaluated both *in vitro* and *in vivo* prostate cancer models[108] and in a number of clinical trials.[151] HDAC inhibitors (VPA, TSA) induce apoptosis of prostate cancer cells (VCaP) through up-regulation of *p21/Waf1/CIP1* pathway. These inhibitors alsodown-regulate*TMPRSS2-ERG* and alter the acetylation status of p53.[110] Targeting nuclear transcription factors is often difficult in designing therapeutic strategies; hence, targeting components of the "*ERG* Network" may serve as an effective alternative strategy to combat the CaP. Recent findings showed physical interaction of ERG protein with PARP in inducing DNA damage and inhibition of PARP impaired *ERG* mediated cell invasion and tumorigenesis.[119] These findings suggest a promising therapeutic potential for PARP inhibitors for a large subset of CaP harboring oncogenic activation of the *ERG* or *ETV1*. In recent years, PARP inhibitors have been increasingly considered as a viable option in exploiting the DNA-repair defects of *BRCA1/2*-deficient tumors to induce cell death.[152-154] As CaP is heterogeneous and potentially involves multiple molecular pathways leading to complex phenotypes, development of small molecule inhibitors targeting multiple targets (*AR, ERG, PARP, PTEN, PI3K, AKT* and *mTOR*) may incorporate new therapeutic strategies for CaP.[155,156] Importantly, *ERG* network targeted therapy may be an effective strategy for more than half of CaP in early stages when cancer cells may be more responsive to treatment.

Concluding remarks

Androgen dependent expression of *ERG* transcription factor as a result of *TMPRSS2-ERG* fusion is detected in 50-70% of CaP patients in Western countries. Evaluations of *ERG* fusions represent one of the most studied and validated genomic alterations in CaP. Other gene fusions are low frequency events in CaP and need to be better understood. Since *ERG* fusions described in CaP are highly specific to this cancer type, numerous studies have evaluated clinical utility of *ERG* as a diagnostic or prognostic biomarker in CaP. Detection of *ERG* rearrangement by FISH or immunostaining of *ERG* protein has been streamlined in pathologic specimens and results from these studies suggest the role of *ERG* in clonal expansion of *ERG* positive PIN (pre-invasive lesion) to carcinoma. While *ERG* alteration is homogenous with in a tumor focus, heterogeneity of *ERG* alteration is apparent in mutli-focal tumor context by simultaneous presence of *ERG* positive and negative tumor foci in the malignant prostate of a patient. Detection of *ERG* alterations in tissue or urine based assays have promise in improving prostate cancer diagnosis and continued investigations are anticipated along these lines. Prognostic value of *TMPRSS2-ERG* fusion or

ERG protein expression is uncertain, however, combination of *ERG* alteration with other CaP gene alterations such as PTEN may define prognostic marker panels for progressive disease. Additional studies are also warranted to further assess the prognostic properties of specific *ERG* fusion type or relative abundance of type I and II splice *ERG* splice variants in CaP. *ERG* mRNA or *ERG* protein expression may serve as a surrogate of AR functional status in prostate tumors and therefore evaluation of *ERG* mRNA or protein expression in prostate tumors has potential in companion diagnostic setting for therapeutics targeting androgen/AR axis.

Functional evaluations of *ERG* in experimental models suggest causal role of *ERG* oncogenic activation in prostate tumorigenesis. *ERG* induces pre-invasive lesions and *ERG* in combination with *PTEN* loss, *AKT* or *AR* cooperate in neoplastic transformation. *ERG* knock-down inhibits prostate cancer cell growth. Studies focusing on *ERG* transcriptional targets in prostate cancer cells suggest role of *ERG* in regulating genes involved in oncogegnesis, differentiation, cell invasion, DNA damage, epigenetic control, inflammation and epithelial-mesenchyme transition. The emerging "*ERG* network" defines new facets of *ERG* functions in CaP and underscores the functional interface of *ERG* with genes (*AR, C-MYC, NKX3.1*, and *PI3K/PTEN* axis) known to have critical functions in CaP. Studies focusing on therapeutic targeting of *ERG* or its network are promising as shown by therapeutic potential of *PARP* inhibitors for *ERG* and *ETV1* positive tumors in preclinical models. Taken together, strategies developing *ERG* based biological classification of prostate tumors and therapeutic targeting of the *ERG* network in prostate cancer represent new paradigm in prostate cancer stratification and treatment.

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Clinical potential of the ERG oncoprotein in prostate cancer

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Abstract | Oncogenic activation of *ERG* resulting from gene fusion is present in over half of all patients with prostate cancer in Western countries. Although the underlying genetic mechanisms have been extensively studied, evaluation of the ERG oncoprotein—the translational product of *ERG* gene fusions—has just begun. The robust correlation between ERG oncoprotein detection and gene fusion status enables rapid characterization of this protein in large patient cohorts. Recent studies have focused on characterizing the ERG oncoprotein and determining its potential role in the diagnosis and biological stratification of prostate cancer.

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Introduction

Prostate cancer continues to be the most prevalent cancer among men, with an estimated 240,890 newly diagnosed cases in the USA in 2011.[1] However, compared to other major cancers, it is responsible for a relatively low number of deaths—estimated at 33,720 in the USA in 2011. These data might reflect substantial variation in the natural progression of prostate cancer, which ranges from indolent to aggressive forms of the disease. Given the wide variety of treatments available for prostate cancer—each with unique adverse health-related quality of life effects—researchers have long been searching for clues to help individualize treatment strategies for patients.

Analyses of prostate cancer transcriptomes have revealed that *ERG* mRNA is overexpressed in approximately two-thirds of patients with prostate cancer.[2–4] Landmark studies have identified prevalent gene fusions in prostate cancer between genes encoding ETS transcription factors (predominantly *ERG* but also *ETV1*, *ETV4*, and *ETV5*) and promoter or other upstream sequences of androgen-inducible genes (predominantly *TMPRSS2* but also *SLC45A3*, *NDRG1*, and *C15orf21*).[5–8] Cumulative evidence from the past 6 years has established the predominant involvement of *ERG* gene fusions that lead to *ERG* overexpression in two-thirds of patients with prostate cancer.[7,9–12] Oncogenic activation of other members of the ETS family (*ETV1*, *ETV4*, and *ETV5*) account for less than 10% of prostate cancers and demonstrate mutual exclusivity with *ERG* overexpression.[5,7]

Given the propensity at which oncogenic activation of *ERG* occurs in prostate cancer, it is not surprising that the majority of studies within this field have focused on the role of this oncogene in prostate tumor development. Functional evaluation of *in vivo* models suggests a causal role of *ERG* oncogenic activation in prostate tumorigenesis. *ERG* overexpression has been shown to induce preinvasive lesions in *ERG* transgenic mice.[13,14] ERG in combination with *PTEN* loss or *AKT* overexpression has been implicated in neoplastic transformation.[15–17] Consistent with these findings, *ERG* knockdown inhibits tumor cell growth in xenografts[18] and in cell culture models of prostate cancer cell growth.[13,18] Studies of ERG transcriptional targets in cultured prostate cancer cells suggest that this protein regulates genes involved in oncogenesis, differentiation, cell invasion, DNA damage, epigenetic control, inflammation, modulation of immune response, and epithelial–mesenchyme transition.[13,18–24] The emerging ERG network includes proteins and protein complexes known to have critical functions in prostate cancer, such as the androgen receptor (AR), C-MYC, NKX3.1, and the PI3K–PTEN axis. Therapeutic targeting of the *ERG* network via the inhibition of poly(ADP-ribose) polymerase (PARP) has shown promise in preclinical models and could represent a new paradigm in prostate cancer stratification and treatment.[19]

Although *ERG* activation is thought to be central to the development of a large proportion of prostate cancers, particularly in Western countries, the prognostic value of *ERG* gene fusions remains uncertain.[9–11] Comparing *ERG* mRNA expression levels in prostate cancer cells to matched benign cells revealed an intriguing association between higher *ERG* expression (with favorable pathologic features) and increased recurrence-free survival following prostatectomy.[4] These observations have been confirmed in subsequent studies assessing the prognostic utility of gene fusions and fusion transcripts.[25–27] Given that AR-regulated genes are attenuated in a subset of poorly differentiated tumors, AR status could in part explain the reported association between decreased *ERG* expression and more-advanced prostate cancers.[28–30]

Competing interests
S. Srivastava and A. Dobi declare associations with the following company: Biocare Medical. See the article online for full details of the relationships. The other authors declare no competing interests.

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Key points

- The highly specific detection of ERG oncoprotein by anti-ERG mAbs offers unprecedented opportunities for the diagnosis of prostate cancer in a large proportion of patients
- Strong concordance between focally ERG-positive prostatic intraepithelial neoplasia and homogeneously ERG-positive carcinoma suggests a role for ERG in clonal selection of prostate cancer cells during progression from preinvasive to invasive disease
- Detection of ERG oncoprotein provides an opportunity for stratifying prostate cancers on the basis of a causative oncogenic activation
- Decreased *ERG* expression in a subset of advanced tumors may reflect attenuated AR status indicating the dysfunction of androgen signaling
- Although the prognostic value of *ERG* gene fusions remains uncertain, careful evaluation of ERG oncoprotein expression levels in combination with other markers may have a potential in prognosing and monitoring disease progression

In contrast to these findings, several other reports have shown that the *TMPRSS2–ERG* rearrangement associates with a poorer prognosis.[31–35] Indeed, the seemingly paradoxical data need to be further clarified in well-defined study cohorts by evaluating *ERG* alterations at the transcriptional and translational, as well as genomic, levels.

Until recently, most commonly used assays for studying *ERG* alterations in prostate cancer specimens have utilized fluorescence *in situ* hybridization (FISH) or reverse transcription PCR (RT-PCR).[10,11,36] Studies of the ERG protein in prostate cancer tissue have been limited, largely owing to a lack of well-characterized antibodies and optimized assays,[14,37] because *ERG* belongs to a gene family consisting of 27 closely-related members.[38] Since the discovery of ERG almost a quarter of a century ago,[39] two highly specific anti-ERG monoclonal antibodies (mAbs) have been characterized that have shown promise in evaluating ERG protein expression in prostate cancer specimens (Table 1).[40,41] These findings have fuelled a rapidly growing interest in the ERG oncoprotein. In this Review, we discuss some of the recently published and ongoing studies that have provided noteworthy insights into the expression of the ERG oncoprotein in prostate cancer. We also consider the clinical potential of ERG protein detection, examining the advantages and limitations of its application to the diagnosis and prognosis of prostate cancer.

Anti-ERG antibodies: 9FY and EPR 3864

Recent reports describe two distinct mAbs that detect the ERG oncoprotein with a high degree of concordance; clones 9FY and EPR 3864. A comprehensive evaluation of the ERG oncoprotein in prostate cancer has been performed using clone 9FY—a highly specific mouse mAb that binds near to the N-terminus of the most common forms of ERG oncoprotein encoded by *TMPRSS2–ERG* and evades crossreaction with FLI-1 (the closest ERG homolog).[40,42] 9FY recognizes the protein products of *ERG* gene fusions in prostate cancer, including translational products of predominant type I (near full length) and type II (truncated ERG that lacks the ETS domain) splice variants.[43] While the function of proteins encoded by type II ERG splice variants is unclear, an increased ratio of type I to type II mRNA has been associated with poor prognostic features of prostate cancer.[43]

Upon staining with the 9FY mAb, predominant nuclear expression of the ERG oncoprotein has been observed in prostatic adenocarcinomas (Figure 1) and in a subset of prostatic intraepithelial neoplasia (PIN) lesions.[40] Cytoplasmic blush was also noted in association with strong overall nuclear ERG expression in tumor cells and, consistent with other reports, strong ERG expression was reported in the nuclei of the lymphovascular endothelium.[42,44–47] In general, prostate cancer cells were found to be uniformly ERG-positive or uniformly ERG-negative in a single tumor focus. Examination of over 200,000 benign glands (across 132 whole mount prostate specimens) revealed ERG positivity in just 22 glands (from three patients), demonstrating a specificity of 99.9% for detecting tumor cells in the prostate.[40] In a subset of whole mount sections, ERG oncoprotein detection correlated with *ERG* fusion or rearrangement—most commonly with *TMPRSS2–ERG* fusion transcript status.

The rabbit anti-ERG mAb EPR 3864 maps to a C-terminal epitope of ERG and detects the ERG

Table 1 | Comparison of the 9FY and EPR 3864 anti-ERG monoclonal antibodies

Study	Standard	*n*	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
9FY (mouse)						
Furusato *et al.*[40]	mRNA	35	87.0	75.0	87.0	75.0
Furusato *et al.*[40]	FISH	10	100	100	100	100
Braun *et al.*[52]	FISH	278	97.8	98.1	97.5	98.1
EPR 3864 (rabbit)						
Braun *et al.*[52]	FISH	278	97.5	99.4	99.1	98.1
Park *et al.*[41]	FISH	207	95.7	96.5	95.7	96.5
van Leenders *et al.*[48]	mRNA	41	100	84.6	81.8	100
Chaux *et al.*[49]	FISH	427	85.6	89.2	87.0	88.0
Falzarano *et al.*[50]	FISH	305	96.1	99.5	99.0	98.0
Minner *et al.*[55]	FISH	453	99.1	92.3	93.1	99.0

Abbreviations: FISH, fluorescence *in situ* hybridization; NPV, negative predictive value; PPV, positive predictive value.

oncoprotein with a high specificity in prostate cancer cells.[41] Extensive characterization of EPR 3864 has shown that this antibody recognizes ERG protein products encoded by *ERG* fusions involving different 5' partners (*SLC45A3*, *NDRG1*, or *TMPRSS2*). By comparing ERG protein expression to *ERG* gene rearrangements in tissue microarray (TMA) analysis of 207 specimens, Park *et al.*[41] demonstrated that EPR 3864 can detect ERG oncoprotein expression with a sensitivity of 95.7%, a specificity of 96.5%, and an overall concordance of 96.1%.[41] In another recent report, EPR 3864 staining was shown to predict ERG oncoprotein expression status with a sensitivity of 100% and a specificity of 85% when compared to *ERG* rearrangement status (determined by quantitative RT-PCR).[48] A strong correlation between ERG protein expression and *TMPRSS2–ERG* status (defined by FISH) has been established in various studies. In one such study, involving 427 radical prostatectomy tissue specimens, a sensitivity of 86% and a specificity of 89% were reported.[49] Another report, involving 305 tumor foci, demonstrated a sensitivity of 96% and specificity of 99% for ERG oncoprotein detection (when compared to FISH analysis of fusion status).[50] Furthermore, in a study of 453 prostate cancer specimens, ERG oncoprotein detection reflected *TMPRSS2–ERG* fusion status with a sensitivity of 99.1% and a specificity of 92.3%.[51]

One key difference between 9FY and EPR 3864 is that the former antibody does not stain lymphocytes,[40,47,52] whereas the latter does.[41,42,52–54] A plausible explanation for this observed difference is that lymphocyte staining by EPR 3864 results from FLI-1 crossreactivity—an artifact that does not occur with 9FY.[42] This specificity makes 9FY particularly useful for recognizing metastatic prostate cancer cells in lymph nodes and in other lymphocyte-rich tissue contexts.[52] The remarkable specificity of 9FY for ERG was also critical in demonstrating that ERG is normally expressed in vascular endothelia during development[42] and to define ERG as a marker for vascular endothelial malignancies.[47] ERG protein expression has been analyzed in a wide variety of human neoplasms in a total of 1,880 specimens.[47] Among epithelial cancers, ERG protein was detected only in prostate tumors, supporting previous reports demonstrating specificity of the *ERG* rearrangement for prostate cancer.[55] 9FY staining revealed expression of the ERG protein in 70% of patients with recurrent acute myeloid leukemia (AML).[47] ERG mRNA is known to be expressed in patients with AML, correlating with poor prognosis in the upper 25% of ERG overexpression.[56–58] ERG protein detection could also enhance the diagnosis of endothelial tumors, including Kaposi's sarcoma, and improve prognosis for patients with AML.[47]

A recent comparative study of 9FY and EPR 3864 reported similar levels of sensitivity and specificity for detecting the ERG oncoprotein in prostate cancer.[52] 278 specimens taken from 265 patients (including 29 lymph nodes, 30 sites of metastasis, and 13 cases of benign prostatic tissue) were evaluated for *ERG* rearrangement status (by FISH) and ERG protein expression (by immunohistochemical analysis). ERG detection by 9FY correctly predicted 272 of 278 (97.8%) cases, with three false positives (98.1% specificity) and three false negatives (97.8% sensitivity). EPR 3864 correctly predicted 274 of 278 (98.6%) cases with one false positive (99.4% specificity) and three false negatives (97.5% sensitivity). However, it is worth noting that rare discordant findings between immunohistochemical and FISH results were consistent between the two antibodies in four of the six cases in this study indicating that 9FY and EPR 3864 have comparable sensitivity and specificity for detecting ERG oncoprotein in prostate cancer.

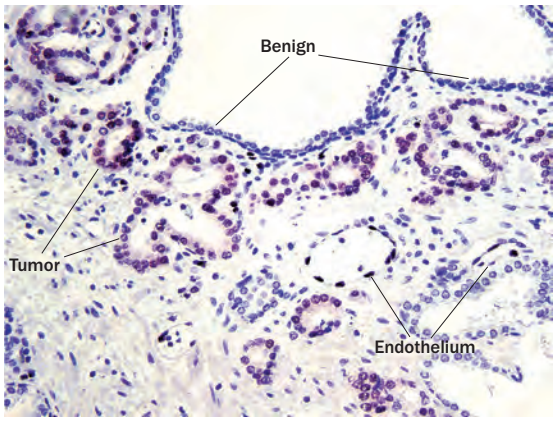

Figure 1 | Detection of ERG protein expression using the mouse anti-ERG mAb 9FY. Prostate cancer cells exhibit nuclear staining with occasional cytoplasmic blush. Endothelial cells exhibit strong nuclear staining. Epithelial cells with normal appearance are uniformly negative for ERG.

Overall, these data suggest that ERG oncoprotein detection in prostate cancer is highly concordant with *ERG* gene fusion status. Some observed differences between studies may be explained by technical or biological issues.[52,59] Although FISH remains the gold standard for detecting *ERG* rearrangements,[60] ERG oncoprotein detection by highly specific anti-ERG mAbs now provides an important tool for evaluating the functional products of *ERG* fusions in prostate cancer. Combinatorial evaluations of *ERG* rearrangements and ERG oncoprotein expression are beginning to provide information about the tumor biology of prostate cancer that has not previously been accessible.[61] For example, preliminary studies of ERG oncoprotein function have contributed to our understanding of processes associated with prostate cancer development, such as clonal expansion and prostatic inflammation.

ERG oncoprotein in prostate cancer

Role of ERG in prostatic clonal expansion

Previous FISH-based evaluations of the genomic rearrangements associated with prostate cancer development have revealed that approximately 20% of PIN lesions in proximity to prostate tumors with *ERG* gene fusions are also positive for *ERG* rearrangement.[16,60,62–66] These findings indicate a clonal relationship between fusion-positive prostate tumors and PIN lesions. Evaluation of ERG oncoprotein expression in whole mount sections using clone 9FY has revealed a strong concordance between focally ERG-positive PIN and homogeneously

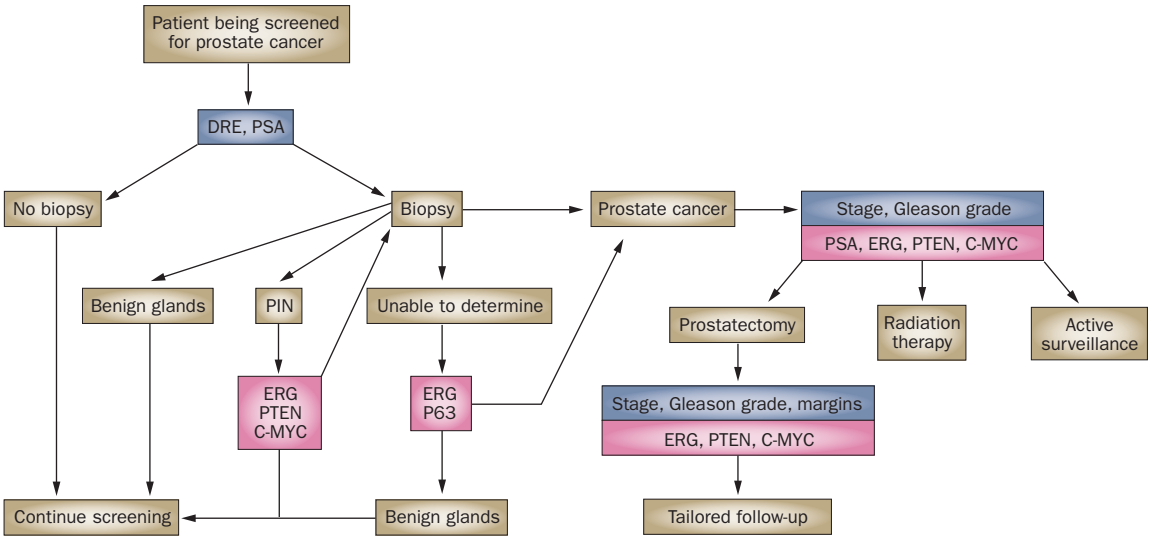

Figure 2 | Potential clinical utility of ERG. Hypothetical algorithm to represent the potential roles of ERG oncoprotein detection in assisting clinical decision making for the diagnosis and treatment of prostate cancer. Blue boxes show biomarkers and investigations with established roles in clinical management. Red boxes show biomarkers with novel or potential roles in clinical management. Abbreviations: DRE, digital rectal examination; PIN, prostatic intraepithelial neoplasia.

ERG-positive carcinoma (present in 96.5% of cases), further supporting a biological role for ERG in the clonal selection of prostate cancer cells.[40] Moreover, this study of whole mount prostate sections containing multiple tumor foci has shown that index tumors are frequently positive for ERG oncoprotein expression. The presence or absence of ERG oncoprotein in different prostate tumor foci suggests a multiclonal origin for prostate cancer.[40,41,52] Taken together, these findings suggest a role for *ERG* in the progression of preinvasive to invasive prostate cancer. Thus, understanding the biochemical functions of ERG in prostate cancer progression is a major focus of recent efforts.

An association between ERG expression and epigenetic reprogramming has also been identified.[67] Overexpression or inhibition of *ERG* has been shown, in various cell culture models, to result in alterations of invasion-associated and differentiation-associated gene expression programs, respectively.[13,18,68] Analyses of the underlying mechanisms suggest that ERG may disrupt the normal prostate differentiation program by activating the *C-MYC* oncogene and interfering with the DNA-binding function of AR.[18] Genome-wide assessment of ERG and AR recruitment to cognate elements supports the notion that ERG interferes with the DNA-binding function of AR,[21] and the significance of *C-MYC* activation by ERG continues to be a focus of interest in current studies.[15,17,69,70]

Role of ERG in prostatic inflammation

Investigations into the role of ERG in the inflammatory mechanisms associated with prostate cancer have shown that the 15-hydroxy prostaglandin dehydrogenase (*HPGD*) gene is negatively regulated by ERG.[22] Furthermore, prostaglandin E2 (PGE_2) synthesis and PGE_2-dependent cell growth is increased in the presence of ERG. Given that *HPGD* downregulates PGE_2 synthesis by antagonizing COX-2 function,[71,72] rates of PGE_2 synthesis and PGE_2-dependent cell growth increase in ERG-expressing prostate cancer cells. A role for the ERG oncoprotein in the activation of NFκB by TLR4, which has a role in the immune response to Gram-negative bacterial infection, has also been recently revealed.[23]

The potential implication of these collective findings is that ERG may contribute to the activation of inflammatory pathways in prostate cancer.[73] Likewise, inflammatory signals may contribute to the generation of the *TMPRSS2–ERG* fusion by interfering with DNA-damage-sensing pathways in the androgen-responsive prostate epithelium.[74] A recent report showed an interaction between ERG and PARP—a DNA damage repair protein—and demonstrated the therapeutic potential of PARP inhibitors on ERG-positive and ETV1-positive tumors in a preclinical model.[19]

Clinical application of ERG oncoprotein

Utility of ERG in prostate biopsy

The diagnosis of prostate cancer from a biopsy specimen is relatively straightforward in more than half of all patients with prostate cancer. However, biomarkers—such as loss of basal cell cytokeratins and overexpression of AMACR—are frequently needed to assist the pathologist in diagnosing prostate cancer. The high specificity of ERG oncoprotein detection in prostate cancer cells has led to evaluation of the diagnostic clinical utility of anti-ERG mAbs (Figure 2). Using the EPR 3864 mAb, assessment of ERG oncoprotein expression in 83 prostate biopsy samples from patients with previously confirmed prostate cancer has shown a positive predictive value of 92% and a negative predictive value of 72% for the presence of prostate cancer.[48] Another study evaluated expression levels of ERG and p63 in 77 prostate biopsies

from patients with previously diagnosed prostate cancer and less than 1 mm of cancerous gland in at least one core.[54] Although only 42% of specimens tested positive for ERG expression, the assay was shown to be highly specific, and all of the cancerous glands were negative for p63. Thus, p63 staining—which is highly sensitive—and ERG oncoprotein detection by anti-ERG mAbs—which is highly specific—could be used in combination to improve the accuracy of prostate cancer diagnosis.

One of the challenges encountered during biopsy evaluation is the diagnosis of atypical glands suspicious for cancer (ATYP). A repeat prostate biopsy after 3–6 months is recommended for patients with ATYP.[75] In a study of follow-up biopsies from 103 patients with a preliminary diagnosis of ATYP, ERG oncoprotein expression was detected in only nine patients.[53] Five of these nine patients (55.6%) had cancer on repeat biopsies, compared with 48.3% of the patients with ERG-negative preliminary biopsies. The report concluded that ERG detection is unlikely to help identify patients suitable for subsequent biopsies. However, in this study the repeat biopsies were not directed to the ERG-positive ATYP area. Further biopsy studies assessing ERG-positive ATYP sites are thus needed to evaluate the utility of measuring ERG expression in patients with ATYP more thoroughly.

Studies showing an association between ERG-positive PIN lesions and ERG-positive prostate tumors indicate the importance of *ERG* activation during the early stages of tumorigenesis. Thus, ERG oncoprotein detection might be useful for stratifying patients according to their need for close follow-up or repeat biopsy after the initial detection of ERG oncoprotein in PIN.[40,41,54] Although the absence of ERG oncoprotein in biopsy specimens might not be conclusive, its presence in PIN or tumor cells could help to characterize these lesions based on tumor biology. However, the focal positivity of ERG in PIN lesions may pose sampling challenges for biopsies. Indeed, future studies are needed to assess the biologic features of biopsy-sampled ERG-positive PIN lesions and prostate tumors. Moreover, further evaluation of the ERG oncoprotein and other proteins relevant to prostate cancer, such as PTEN, C-MYC, and AR, might enable us to predict the aggressiveness of tumor cells.

Prognostic role of ERG after surgery

The prognostic application of ERG oncoprotein detection, in conjunction with other prostate cancer biomarkers, could lead to improved prediction of disease progression after radical prostatectomy (RP; Figure 2). Cooperativity between *ERG* activation and loss of the *PTEN* tumor suppressor gene has been demonstrated in genetically engineered mouse models.[15–17] Studies have also shown an increased rate of *PTEN* loss in patients with *TMPRSS2–ERG*-positive prostate cancer.[66,76]

In a recent report, a combination of wild-type *PTEN* and the absence of *ERG* genomic rearrangement was shown to associate with longer time to biochemical recurrence.[77] Subsequent studies have supported this finding, as well as revealing that allelic loss of *PTEN* in the absence of *ERG* rearrangement is associated with the worst outcome.[78] Examining both ERG oncoprotein expression and genomic rearrangements, Minner *et al.*[51] analyzed TMAs of radical prostatectomy specimens from 2,805 patients with prostate cancer over a mean follow-up period of 72 months.[51] Importantly, none of the patients received neoadjuvant treatment. In this study, ERG oncoprotein expression status did not correlate with biochemical recurrence; a finding that is consistent with many other reports.[40,41] Although a striking association was noted between ERG and AR expression levels, the combined presence of both proteins did not convey an increased risk of biochemical recurrence in this study cohort.

Indeed, the prognostic value of *ERG* alterations remains uncertain in prostate cancer. Carefully designed studies that evaluate ERG oncoprotein expression levels, as well as the multifocal nature and clinical heterogeneity of prostate cancer, are needed to enhance our understanding of whether ERG oncoprotein detection has a viable role in prognosing patients with prostate cancer and monitoring disease progression following RP.

Conclusions

The highly specific detection of ERG oncoprotein (using anti-ERG mAbs) in a large proportion of patients with prostate cancer offers unprecedented opportunities for the diagnosis and stratification of this disease. A high degree of concordance (approximately 96%) between ERG oncoprotein detection and *ERG* gene fusion status has been established and evaluation of these parameters could support an ERG-based approach to prostate cancer stratification. Despite the sampling issues associated with biopsy specimen analysis, addition of the ERG oncoprotein to a prostate cancer biomarker panel has the potential to enhance diagnosis.

Emerging studies are helping to delineate the functions of ERG and its cooperation with other cancer-associated proteins, such as PTEN and C-MYC, in prostate tumorigenesis. Potential roles of ERG and ERG-interacting proteins, such as PARP, have been suggested for the therapeutic targeting of prostate cancer. Evaluation of ERG oncoprotein expression, in diverse tumors and in normal tissue, is likely to benefit greatly from the development of highly specific anti-ERG mAbs such as 9FY and EPR 3864. Accurately defining the expression status of ERG oncoprotein, as well as other oncoproteins and tumor suppressor proteins associated with prostate cancer, could be of substantial use in diagnosing and prognosing prostate cancer, as well as in developing new treatment strategies to complement current approaches to care (Figure 2).

Review criteria

We searched for original articles focusing on ERG and prostate cancer in MEDLINE and PubMed published between 1987 and 2012. The search terms we used were "ERG", "prostate cancer", and "cancer". All papers identified were English-language full text papers. We also searched the reference lists of identified articles for further papers.

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