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14. ABSTRACT In order for prostate cancer to metastasize, it must invade through a laminin-511 rich barrier. We have previously shown that the matrix metalloprotease, MT1-MMP, which is expressed in prostate cancer but not in normal prostate tissue, cleaves the laminin alpha-5 chain into four distinct fragments. This cleavage allows for increased prostate cancer cell migration in vitro. Laminin-511 cleavage also occurs in vivo in human prostate tissue. Cleavage of laminin-511 and release of laminin-511 fragments leads to altered cell function leading to increased cell migration and invasion in in vitro assays. We have demonstrated that prostate cancer cells treated with laminin-511 that has been cleaved by MT1-MMP have increased EGFR phosphorylation compared with cells grown on tissue culture plastic or intact laminin-511 in a Western blot. We have purified a recombinant 45kDa laminin-511 N-terminal cleavage fragment, which contains laminin EGFlke domains. Treatment of prostate cancer cells with soluble recombinant fragment demonstrates that the cleaved laminin fragment acts as a trikin, activating the EGFR on prostate cancer cells in a Western blot. This work demonstrates that increased MT1-MMP expression in prostate cancer not only cleaves the major laminin surrounding prostate cancer to clear a path for migration, but also releases active fragments from the laminin-511 that signal for increased migration.					
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INTRODUCTION

Prostate cancer is currently the most commonly diagnosed neoplasm and the second leading cause of cancer deaths in men in the United States. Due to the aging population, prostate cancer incidence and mortality is expected to increase. Thus, improvements in prevention, early diagnoses, and treatment are needed (1). Hopefully, understanding molecular events of prostate cancer progression will lead to the development of novel therapies for the disease. A prostate cancer's ability to develop an invasive and metastatic phenotype is arguably the most important determinant in the clinical relevance of prostate cancer; and therefore, the study of factors involved in the development of this phenotype is of primary importance. There are at least two recognized phenotypes of prostate cancer. One is a clinically aggressive cancer that can become life-threatening relatively fast, while the other is a slow- growing, "latent" form, which may ever present itself clinically (2).

To date, there is no method to differentiate between these two forms of prostate cancer, leading to potential over- and under- treatment of this disease (3). For this reason, identification and understanding of molecular alterations during prostate cancer progression is essential to distinguish between cases that will progress rapidly to advanced metastatic cancer and those with little likelihood of progressing. This will hopefully lead to more accurate prognoses and appropriate treatments. In addition, identification of biomarkers will be useful for recognizing individuals with a higher risk for prostate cancer and will allow for earlier detection and better treatment of advanced disease. In particular, there is a growing need for biomarkers that access the tumor microenvironment as predictors of metastases (4).

In order for a cancer to metastasize, it must first invade through the basement membrane that surrounds it, migrate through the stroma, invade blood vessels and travel through the bloodstream to a new location where it extravasates the vessel and begins growing at the new site. The mechanisms by which a cancer is enabled to invade and metastasize are currently under intense study. Interactions of the cell with its environment are thought to play a major role in signaling for these invasive processes to occur. Upregulation of proteolytic enzymes, such as the matrix metalloproteases, is suspected of being involved in the metastatic process.

Remodeling of the extracellular matrix (ECM) through proteolysis of ECM proteins is an important step in the metastatic progression of cancer, allowing for invasion of neoplastic cells through the basal lamina (BL) and into the stroma (5). Proteolysis creates paths for migration, releases signaling molecules such as growth factors bound in the ECM, and generates biologically active ECM fragments (6-10).

Prostate cancer is surrounded by a BL composed mainly of laminin-10, laminin-2, type IV collagen, and entactin (11). In order for prostate cancer to invade the stroma and intravasate into the vasculature, it must move through this Ln-10 rich BL either by proteolysis or ameboid movement (12). Ln-10 ($\alpha 5\beta 1\gamma 1$) has been previously identified as a substrate for cell migration and cell adhesion (13). It is now known that MT1-MMP can cleave the laminin-10 $\alpha 5$ chain and this cleavage allows for increased prostate cancer cell migration. We have preliminary evidence that the laminin-10 $\alpha 5$ chain protein cannot be detected in perineural invasive prostate cancer by immunohistochemistry. This apparent loss of laminin-10 protein could be due to complete cleavage by MT1-MMP so that antibodies to the $\alpha 5$ chain cannot bind or it could be due to loss of laminin-10 expression in invasive cancer. We propose that modifications in 3 extracellular matrix proteins surrounding prostate cancer play an important role in the progression of the disease and an understanding in the global changes in cancer that take place as a result of these modifications will be important for future prostate cancer research, treatment, and diagnosis.

BODY

Hypothesis: LM-511 cleavage by MT1-MMP results in decreased adhesion and increased migration of prostate cancer cells due to release of biologically active LM- α 5 fragments containing EGF-like domains that function as matrikines, can bind and activate the EGF receptor.

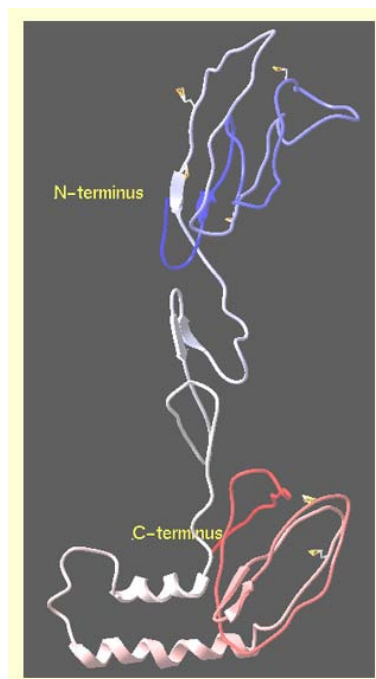
1. **Specific aim 1:** Determine the effects of Laminin-10 fragments on gene expression in prostate Cancer

Tasks Completed:

1. Recombinant 45kD alpha 5 chain of Laminin 10 made and purified.

To test the biologic effects of the laminin-10 cleavage fragments, recombinant proteins corresponding to laminin alpha 5 chain cleavage products are being produced. To date, the 45kDa N-terminal end of the alpha 5 chain has been expressed in E.Coli and purified on a Ni²⁺ column.

Structural representation of LM- α 5 chain 45 KD fragment.



The LM- α 5 chain 45 kDa fragment we have produced in E.Coli has been modeled based on its amino acid sequence. The fragment contains a complete EGF-like domain at its C-terminus.

- Using this fragment (TPOP-1) as an immunogen 2 polyclonal and 6 monoclonal antibodies were made with the help of Tripath.

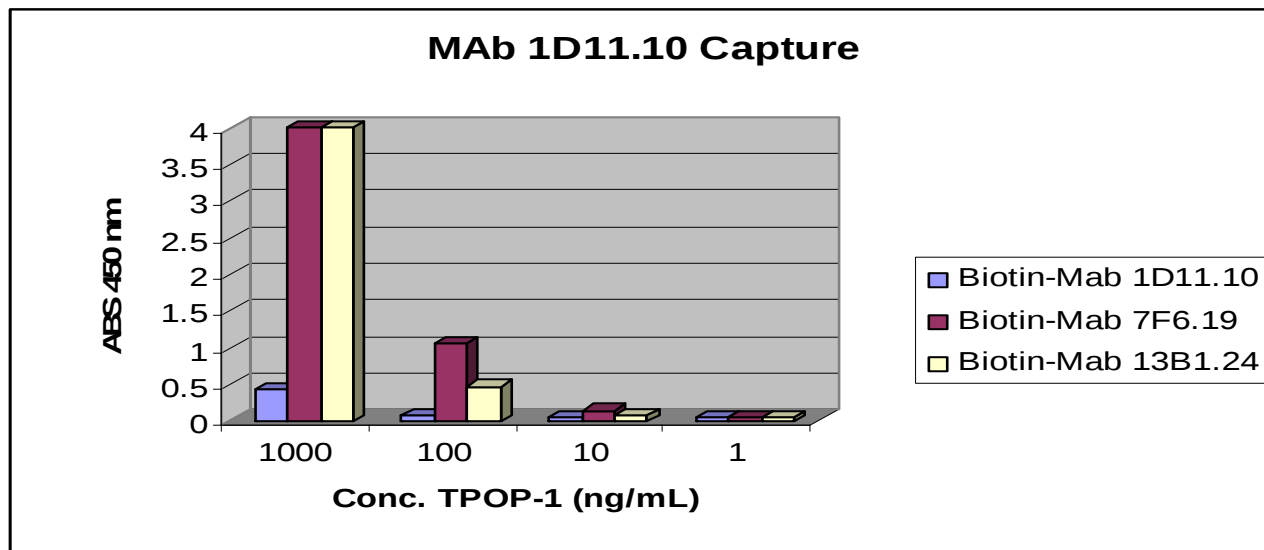
1. Antibody Reactivities in Indirect ELISA and Western Blot

	Indirect ELISA	Western blot
Antibody	TPOP-1	TPOP-1
TPO 508 MAb Clone 7F6.13	+	+
TPO 508 MAb Clone 13B1.24	+	+
TPO 510 MAb Clone 1D11.10	+	+
Polyclonal NC 653-2/20/06	n.t.	n.t.
Polyclonal NC 653-2/20/06	n.t.	n.t.
TPO 508 MAb Clone 7C6.19	+	+
TPO 508 MAb Clone 8E11.12	+	+
Anti-LN α 5-GST MAb 15H5	n.t.	n.t.

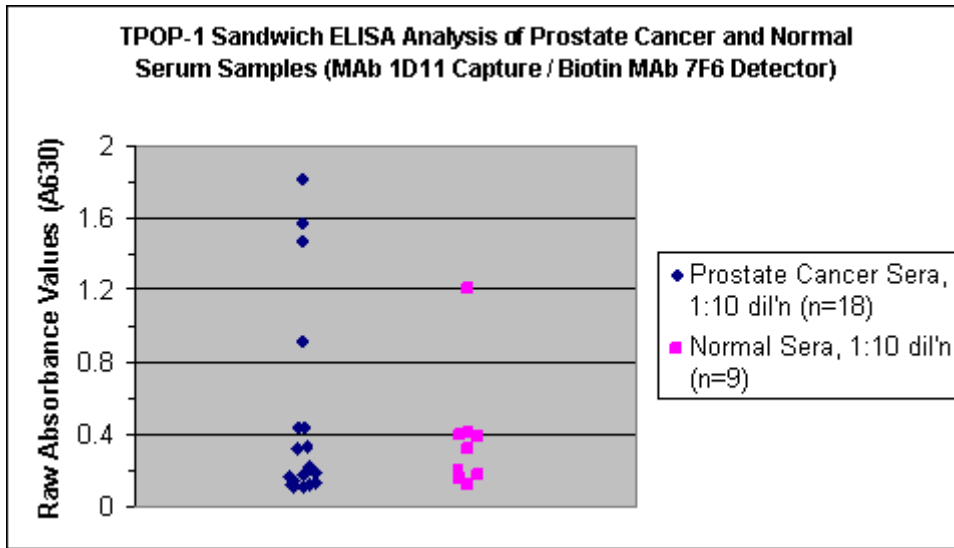
n.t. = not tested

- An ELISA assay using 2 of the monoclonals was developed and revealed the increased 45 kDa Laminin 10 fragment in 4 of 18 prostate cancer patients.

Antibody Reactivities in Sandwich ELISA



Serum samples from 18 prostate cancer patients was analyzed by this ELISA

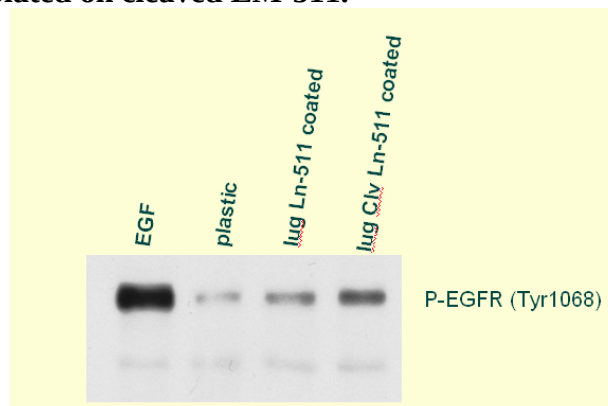


Specific aim 2: Determine the mechanism by which the effects of laminin-10 fragments on prostate cancer are mediated

1. Activation of EGFR in cells grown on cleaved Laminin 10.

The cleaved laminin 10 fragment has been tested to determine whether it is the cause of the biologic effects observed when prostate cancer cells are treated with cleaved laminin-10 (EGFR phosphorylation, proliferation, migration). We have treated DU-145 cells with purified laminin-10 and purified laminin-10 that was treated (cleaved) with MT1-MMP. These experiments demonstrated an increase in EGFR phosphorylation at tyrosine 1068. Purified laminin-10 induced some EGFR activation, but cleaved laminin-10 demonstrated a more robust activation.

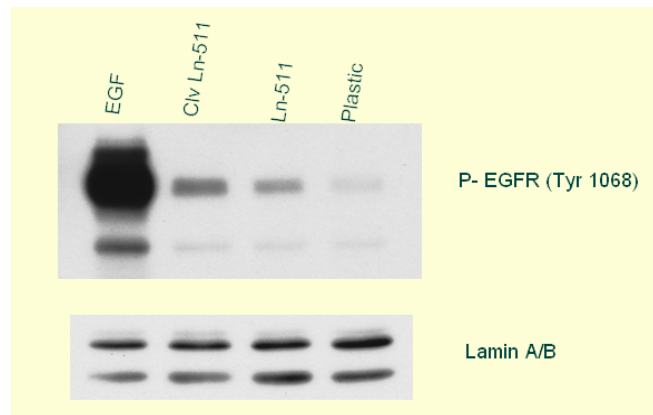
Activation of EGFR on cells plated on cleaved LM-511.



LM-511 (μ g) was coated on a 6-well tissue culture plate and treated with catalytic domain of MT1-MMP (2.1 nmol) or left intact. DU-145 cells were then plated on uncoated and LM-511 coated wells. EGF was added to an uncoated well as a control for activation. 16 hours after plating, cells were lysed and analyzed for EGFR activation at Tyr 1068. Results demonstrate EGFR activation when cells were plated on intact LM-511, with an increase in EGFR activation when cells were plated on cleaved LM-511.

2. Activation of EGFR in cells treated with cleaved, soluble Laminin 10.

Activation of EGFR with soluble LM-511 fragments.

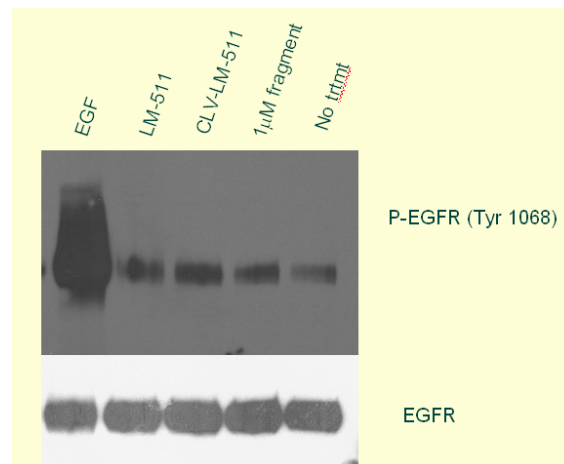


DU-145 cells were seeded in a 6-well tissue culture plate and stimulated with: EGF, LM-511 fragments produced by incubating intact LM-511 with catalytic domain of MT1-MMP 16 hr, intact LM-511, or were left untreated. Results demonstrate that cleaved LM-511 fragments activate EGFR to a greater extent than intact LM-511, and that the receptor is not activated with no treatment. Lamin A/B protein was used as a loading control.

3. Activation of EGFR in cells treated with recombinant alpha 5 chain fragment of Laminin 10.

We have found that treating DU-145 prostate cancer cells with 1 μ M concentration of the 45kDa fragment induces phosphorylation of the EGF receptor at tyrosine 1068, similarly to treating DU-145 cells with 1 μ g purified, cleaved laminin-10.

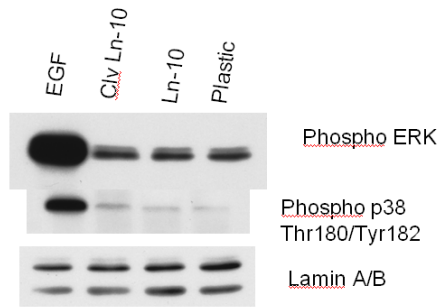
LM- α 5 45 KD fragment activates EGFR.



DU-145 cells were treated with EGF, intact soluble LM-511, cleaved soluble LM-511, 45 kDa soluble fragment, or left untreated. Results indicate that the 45 KD fragment activates EGFR above endogenous levels (untreated). Total EGFR protein is shown as a loading control.

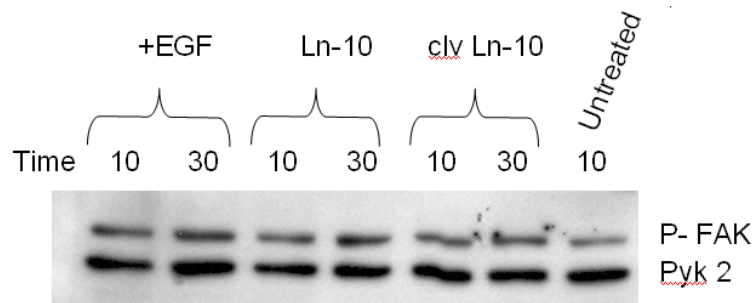
4. No downstream MAP Kinase activation observed.

DU145 on soluble Ln-10, 10 min



5. No FAK activation observed.

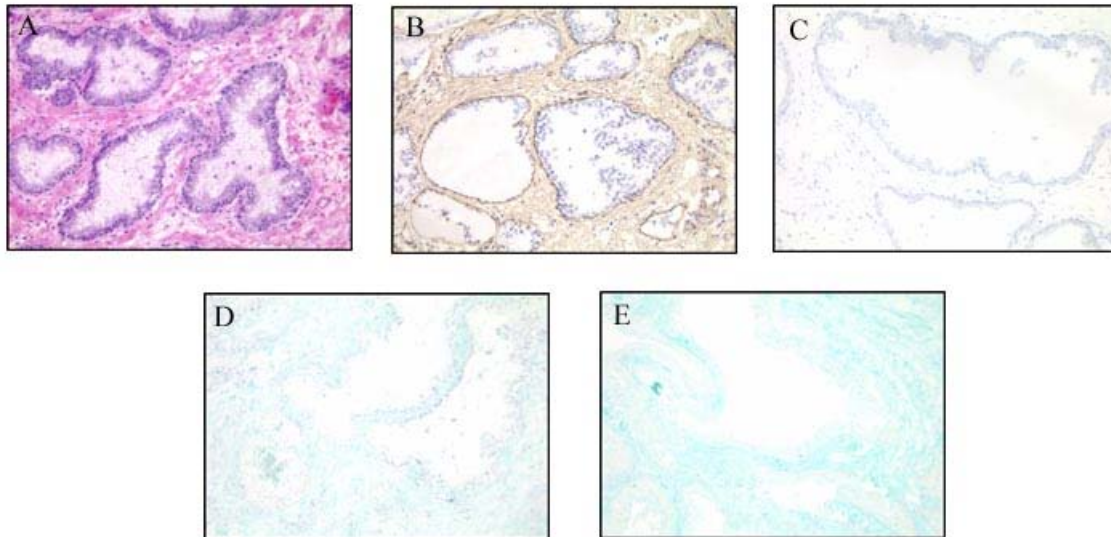
Du-145 plated on Ln-10



Specific aim 3: Examine the apparent loss of laminin-10 expression in perineural invasion of prostate cancer.

1. A digoxigenin labeled probe to alpha 5 chain of Laminin10 generated.
2. Conditions for *in situ* hybridization on frozen prostate tissue using the alpha 5 chain probe were optimized.

Results on frozen tissues with $\alpha 5$ probe



Laminin alpha 5 chain expression in Normal prostate tissue.

A) H&E stain of normal prostate tissue.

B) Immunohistochemistry of the antibody 4C7, which detects laminin alpha 5 chain, demonstrates alpha 5 chain expression surrounding the normal prostate gland and in the stroma of the prostate tissue.

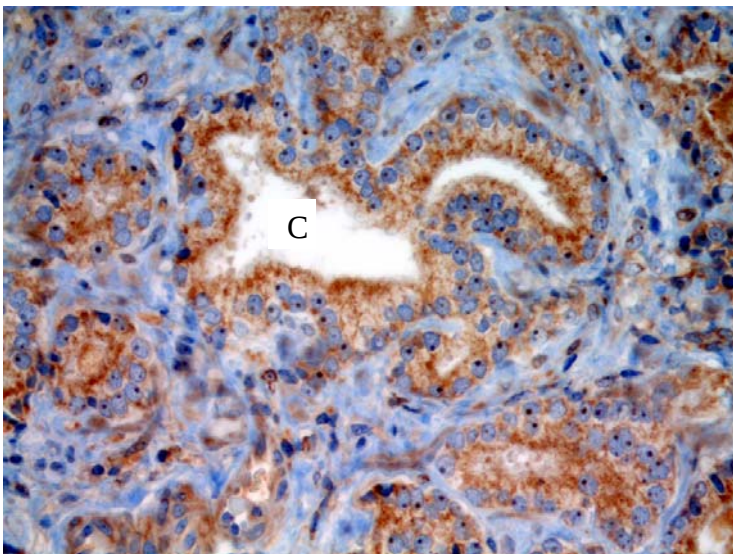
C) Normal prostate tissue hybridized with a DIG-labeled laminin alpha 5 chain antisense probe. Results demonstrate alpha 5 chain message is present in the normal prostate gland with some reactivity in the stroma.

D) Periodic Acid-Schiff (PAS) counterstain on normal prostate tissue hybridized with the laminin alpha-5 DIG-labeled antisense probe shows alpha 5 chain message is present in the normal prostate gland.

E) PAS reaction to counterstain the sense laminin alpha-5 chain DIG-labeled sense probe shows no hybridization to normal prostate tissue.

3. Conditions for detecting MT1-MMP protein on prostate tissue have been developed.

Detection of MT1-MMP in prostate cancer:



Immunohistochemistry was performed using a rabbit polyclonal antibody to the MT1-MMP. Prostate adenocarcinoma Gleason Sum Score 3+3. Cancer is identified as (C).

Methods developed and used:

1. The human prostate carcinoma cell line, DU-145 was maintained in DMEM with 10%FBS, penicillin, streptomycin (all Gibco-BRL). Cells were grown at 37°C in an atmosphere containing 5% CO₂.
2. Recombinant laminin $\alpha 5$ chain fragments were made using Novagen's pET-28c vector system. mRNA was isolated from A549 cells (a human lung cell line that secretes laminin-10) and cDNA was generated via RT-PCR. Sequence specific PCR primers were designed to the N-terminus of the $\alpha 5$ chain and to the MT1-MMP cleavage sites. The PCR product was directly ligated into the pET-28c protein expression vector in-frame with sequences encoding a 6X His tag. The sequence was verified by automated sequencing (Biotechnology Facility, University of Arizona) prior to transformation into M15 [pREP4] E. Coli cells. Transformed cells were screened for the correct insertion of the coding fragment by blotting with an anti-His antibody. The expressed recombinant proteins were isolated using nickel-NTA affinity chromatography.
3. Biochemical assays of ERK phosphorylations were performed by Western blotting with specific antibodies (14).
4. For immunohistochemistry analysis of MT1-MMP expression in prostate tissue, frozen prostate tissue sections were placed on positively charged glass slides and fixed in acetone. Sections will be incubated with primary antibody followed by incubating slides with fluorescent labeled secondary antibodies (Alexa 485 and 565, Molecular Probes, Eugene, OR). Slides were analyzed on a Zeiss LSM 410 UV (Carl Zeiss, Oberkochen, Germany) dual laser confocal microscope using the argon/krypton ion laser operating at 488 and 568 nm.
5. In situ hybridization on sections
Solutions: Ethanol: 100%, 95%, 90%, 80%, 70%, 40% (make in DEPC H₂O)
PBS: 10X stock in DEPC H₂O. Make 1X in DEPC H₂O by adding 50 ml 10X to 450 ml DEPC H₂O.
Proteinase K:
4% paraformaldehyde: make 10% paraformaldehyde in 16 ml DEPC H₂O, add 4 ml 10X PBS DEPC, up to 40 ml DEPC H₂O.
20X SSC:
Hybridization Mix:

	final concentration	for 50 ml
10X salt	1X	5 ml
formamide	50%	25ml
50% dextran sulfate	10%	5g DS
yeast RNA (10mg/ml)	1 mg/ml	5 ml
50X Denhardt	1X	1 ml
DEPC water		

10X salt: for 200 ml (can be kept on shelf long term): NaCl 22.8g, TrisBase 0.268g, TrisHCl 2.8g, NaH₂PO₄ • H₂O / NaH₂PO₄ • H₂O 1.56g / 1.14 g, Na₂HPO₄ 1.42g, 0.5M EDTA 5 ml, up to 200 ml with DEPC
Washing Buffer: 50% formamide /1X SSC / 0.1% tween20
5X MAB stock: (1 liter) 58g maleic acid, 41 g NaCl, equilibrate to pH 7.5 with solid NaOH (approx 5g).
MABT: Dilute 5X MAB to 1X prior to use and all 1 ml Tween20 for 1 liter (final 0.1% tween20).
NTMT: For 200 ml: 5ml 4M NaCl, 20ml 1M Tris, pH. 9.5, 10ml 1M MgCl₂, 200ul Tween20, 168.4ml DEPC H₂O, 96.2mg levamisole
NBT 75mg/ml in 70% dimethylformamide
BCIP 50mg/ml in dimethylformamide

Day 1: Slides were pretreated as follows: frozen tissues was cut 8um thick, rinsed in PBS, fixed in 4% paraformaldehyde/PBS DEPC 50 minutes. The slides were then washed with PBS for 5 min and treated with Proteinase K 20ug/ml in PBS 10 min at 37°C followed by a PBS wash. The slides were then refixed for 20 min. in 4% paraformaldehyde in PBS followed by 1 PBS wash and 1 2X SSC wash.

Hybridization: Prehybe 1 hr in hybridization mix. Slides were placed in 15ml hybe mix and placed upright in water bath 57°C. The probe was denatured for about 5 min at 80°C, put on ice until cooled, 40ul was added to 15ml cold hybridization buffer then heated buffer to hybridization temp - 57°C (between 50-60°C) overnight. (Up to 40 hours).

Day 3: Washing: Blocking reagent was dissolved by warming up to 65°C before adding goat serum. Slides were placed in 50% formamide/ 1x SSC/ 0.1% tween20. Wash 15 min. at 57°C. Slides were treated with 1mg/ml RNase in PBS (DEPC) for 30 min. at 37°C. For Ambion RNase cocktail cat 2286, this is 1ul RNase for every 5 ml PBS. Slides were washed once more in formamide wash buffer for 10 min at 57°C followed by 1X SSC 5 minutes room temp and a 0.5X SSC wash 5 min. room temp. This was followed by two washes in MABT (MAB plus tween20) 15 min. RT.

Anti-digoxigenin immunohistochemistry: Slides are blocked for 1hr30 with 700-800ul of MABT / 2% blocking reagent (from Boehringer Mannheim) / 20% goat serum in a humid chamber. The anti-digoxigenin antibody (Roche) 1:2000 was added to the same blocking mix and put 120ul on each slide. Slides were incubated O/N at 4°C in humid chamber with paraffin coverslips cut to size.

Day 4: Slides were washed 4 to 5 times 30 min. each in MABT at 4°C.

Developing Color: Slides were put for 5 min. in NTMT. The color was developed in the dark in NTMT containing NBT and BCIP (168.75ul and 175ul BCIP in 50ml) then placed in 37°C water bath. Color was observed after 1h, 2hr, 5hr. The slides were then rinsed in 1X PBS, refixed for 20 min in 4% paraformaldehyde. Then washed in PBS and passed through ETOH series (20 sec. each wash), into 100% ETOH 2 x 1 min each, Histo-clear 2 x 5 min each. Permount slides for permanent coverslip.

Key Research Accomplishments

1. Trained in recombinant protein design, synthesis, and purification.
2. Trained in in situ hybridization technique.
3. 2005, NCI-sponsored Tumor Microenvironment Training Program: Organotypic Models Training Program in Dr. Isaiah Fidler's laboratory in Houston, TX participant. .
4. Recombinant 45kD alpha 5 chain of Laminin 10 made and purified.
5. Using this fragment as an immunogen 2 polyclonal and 6 monoclonal antibodies were made.
6. An ELISA assay using 2 of the monoclonals was developed and revealed the increased 45 kDa Laminin 10 fragment in 4 of 18 prostate cancer patients.
7. Testing of 45 kDa fragment effect on gene expression in progress.
8. Activation of EGFR in cells grown on cleaved Laminin 10.
9. Activation of EGFR in cells treated with cleaved, soluble Laminin 10.
6. Activation of EGFR in cells treated with recombinant alpha 5 chain fragment of Laminin 10.
7. A digoxigenin labeled probe to alpha 5 chain of Laminin10 generated.
8. Conditions for *in situ* hybridization on frozen prostate tissue using the alpha 5 chain probe were developed.
9. Conditions for detecting MT1-MMP protein on prostate tissue have been developed.

Reportable Outcomes

Bair EL, Bowden, GT, Nagle, RB “Cleaved Laminin- α 5 β 1 γ 1 Fragment Activates EGFR in Prostate Cancer Cells” An AACR Special Conference in Cancer Research “Cancer, Proteases, and the Tumor Microenvironment” November 30-December 4, 2005, Bonita Springs, Florida.

Pawar S.C., Bair EL, Bowden, GT, Nagle, RB “Cleaved Laminin- α 5 β 1 γ 1 Fragment Activates EGFR in Prostate Cancer Cells” Innovative Minds in Prostate Cancer Today - IMPaCT Congressionally Directed Medical Research Programs U.S. Army Medical Research and Material Command, September 05, 2007 - Saturday, September 08, 2007, Atlanta, Georgia.

Pawar S.C., Bair EL, Bowden, GT, Nagle, RB “Cleaved Laminin- α 5 β 1 γ 1 Fragment Activates EGFR in Prostate Cancer Cells” AHSC Frontiers in Biomedical Research Poster Forum. Wednesday, October 22, 2:00-4:30pm. University of Arizona, Tucson, AZ.

Conclusions

- LM-511 cleaved with MT1-MMP contains potentially bio-active fragments.
- Cleaved LM-511 fragments activate the EGFR at Tyr 1068.
- Recombinant 45 kDa LM- α 5 chain demonstrates activity in activating the EGFR.
- No downstream MAP Kinase signaling observed.
- ELISA to cleaved laminin fragment developed.

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