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14. ABSTRACT A significant achievement in the current reporting period is our ability to immunostain both the 12-LOX protein and β 4 integrin in paraffin-embedded prostate tumor tissues circumventing the problems described in the previous report. With the new protocol we have stained about 20 cases so far and the remaining cases are in progress. We have generated several stable transfectants of PC-3 cells expressing mutant forms of the β 4 integrin and studied their interaction with 12-LOX. During this study, we have identified that the peptide spanning between amino acids 1126 and 1315 of the cytoplasmic tail of the β 4 integrin shows strong interaction with 12-LOX. An important observation is that the full-length cytoplasmic tail of β 4 integrin, when expressed ectopically, disrupts the interaction with 12-LOX with β 4 integrin in a dominant negative manner. This interaction also resulted in a decrease in the biosynthesis of the enzymatic product, 12-HETE, as well as reduction in the tumor growth rate from subcutaneously injected PC-3 cells in athymic nu/nu mice.					
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INTRODUCTION

During the progression of human PCa hemidesmosomes, adhesion structures that anchor epithelial cells to basement membrane and function as a tumor suppressor, are lost (1, 2). We found that 12-lipoxygenase directly interacts with $\beta 4$ integrin, an integral part of hemidesmosomes (3, 4). We hypothesized that an increase in 12-LOX activity can cause the disassembly of hemidesmosomes, mobilization of $\alpha 6\beta 4$ integrin from hemidesmosomes to other parts of the cell membrane, and stimulate tumor invasive growth. We proposed to conduct a correlation study to evaluate the relationship between 12-LOX expression and dispersion of $\beta 4$ integrin in clinical tumor specimens. Our proposal further aims to study whether 12(S)-HETE, the enzymatic product of 12-LOX, can disrupt hemidesmosomes and whether 12-LOX inhibitors promote the formation of hemidesmosomes. Then we will study the underlying signaling pathway, especially PKC α , initiated by 12(S)-HETE, in the disassembly of hemidesmosomes. Next we will overexpress $\beta 4$ integrin and study the role of the interaction between 12-LOX and $\beta 4$ integrin in the adhesion, proliferation, migration, and survival, in response to HGF/SF. Finally we will xenograft these transfected cells into mice, to evaluate whether any phenotypic changes of tumor cells in vitro can be recapitulated in vivo. The work will significantly advance our understanding about the complex process of prostate cancer progression as well as the possible role played by dietary fat in the progression of prostate cancer.

BODY OF PROGRESS REPORT

Task 1.

We have attempted several procedures for immunostaining for 12-LOX. In the previous report, the procedure only worked in frozen human prostate tumor tissue. We now worked out the conditions for immunohistochemical analysis of 12-LOX at the protein level in paraffin-embedded human prostate tumor tissues. As shown in the figure 1, 12-LOX immunoreactivity correlated with tumor grade. Neoplastic glands are weakly, moderately or strongly positive for 12-LOX in hyperplastic glands (**Figure 1A**), atrophic glands (**Figure 1 B**), and in tumor Gleason score 4-6 (**Figure 1 C,D**).

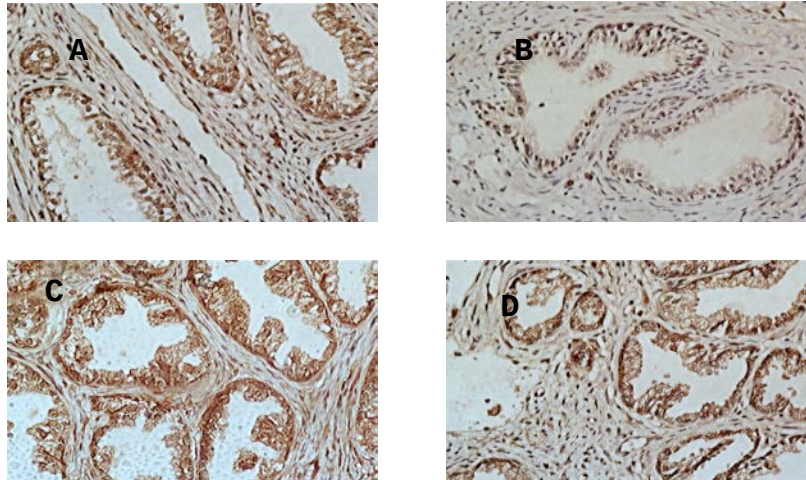


Figure 1. Immunohistochemical staining for 12-LOX in paraffin-embedded prostate tumor tissues. Sections of specimens were probed with α 12-LOX polyclonal antibody (Oxford Biomedical Research Inc, Oxford, MI). Positive immunoreactivity is indicated by staining with brownish color. A, hyperplasia gland; B, atrophic; C/D, tumor with Gleason score 4-6.

We have also attempted several protocols of immunostaining for β 4 integrin in paraffin-embedded material. As shown in figure 2, positive staining was found in tumors and correlated with tumor grade.

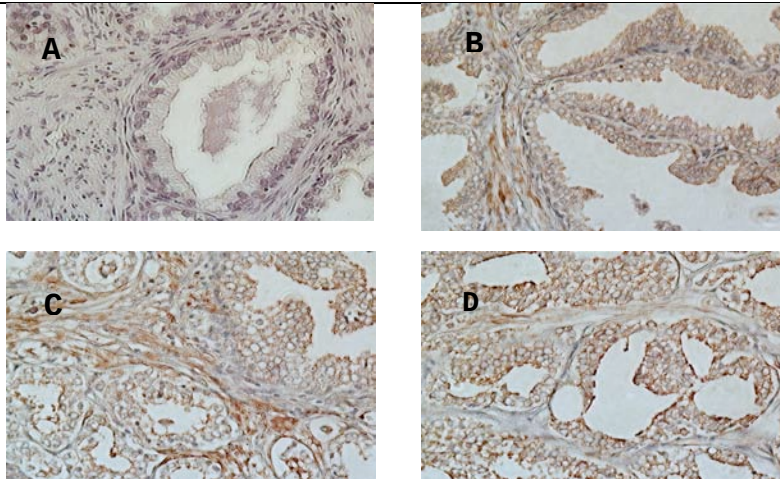


Figure 2. Immunostaining for beta 4 integrin. Brownish color indicates positive staining. Note the pattern of beta 4 integrin positivity. A, normal epithelium; B, tumor with Gleason score 4; C, tumor with Gleason score 6; D, tumor with Gleason score 8.

We have procured 100 cases of prostate tumor specimens. We have analyzed 83 patient samples following the double staining technique. The results are presented in **Table 1**.

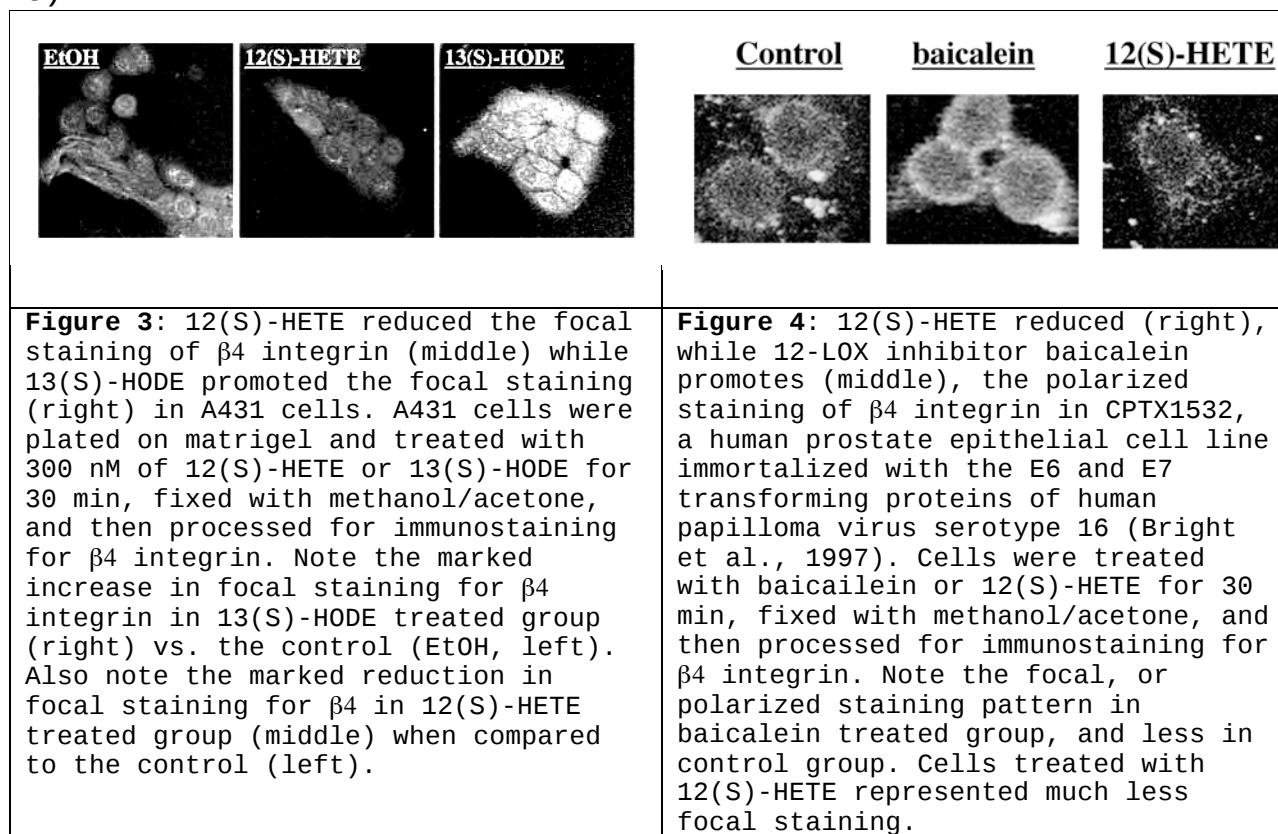
Table 1: 12-LOX and $\beta 4$ Expression in Human Prostate Cancer Compared to Gleason Score

Gleason Score	n	$\beta 4$ Median (range)	12-LOX Median (range)
6	42	30 (5-80)	15 (5-70)
≥ 7	41	70 (5-100)	30 (5-80)
p value		0.0082	0.0109

Mann-Whitney test used for statistical analysis. $p \leq 0.05$ was considered significant.

Task 2. Study the effects of 12-LOX inhibitors and 12(S)-HETE on hemidesmosome in prostate epithelium. Months 1-18:

We studied the effect of inhibitors of 12-LOX as well as 12(S)-HETE on the hemidesmosome-like structures in cultured cells. As shown in Figures 3 and 4, 12(S)-HETE (300 nM) reduced hemidesmosome like structures both in A431 cells and in an immortalized human prostate cancer cell line, CPTX1532. In contrast, baicalein, a 12-LOX inhibitor, promoted the formation of hemidesmosome-like structure (Figure 4). Interestingly, a linoleic acid metabolite from 15-LOX, 13(S)-HODE, which is known antagonist of 12(S)-HETE, promoted the formation of hemidesmosomes (Figure 3).



Task 3. Study the signal transduction pathways that underlie the disassembly of hemidesmosomes by 12(S)-HETE or an increase in 12-LOX activity, Months 12 -24:

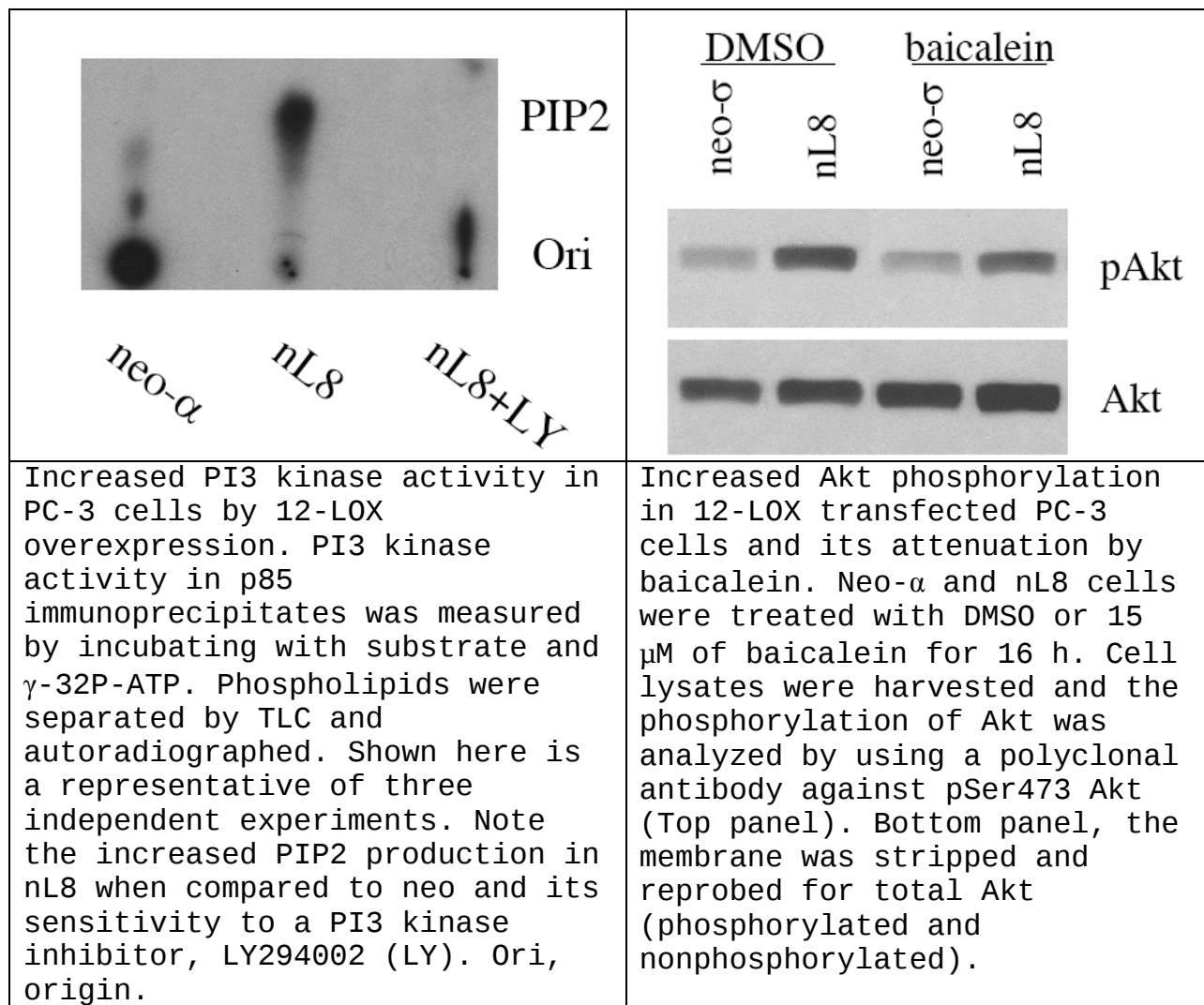


Fig 5. Activation of the PI3K Akt signaling pathway in 12-LOX overexpressing PC-3 cells. **A. B.**

In Vitro PI3 - Kinase Assay - *In vitro* phosphorylation of phosphatidylinositols was carried out with PY20 antibody (Transduction laboratory, KY) for the detection of immunoprecipitated PI 3-kinase essentially as previously described (23). Prostate cancer cells were harvested with PBS containing 1 % Nonidet P-40, 1 mM dithiothreitol, 200 μ M sodium vanadate, 100 μ M 4-(2- aminoethyl) benzenesulfonyl fluoride, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin (lysis buffer). Soluble cell lysates were collected after microcentrifugation at 18,000 g for

5 min. The protein concentration of cell lysates was adjusted to 0.8-1 µg/µl with lysis buffer and PY20 mAb (5 µg) was added to 500 µl of the cell lysate. The mixture was incubated with gentle rocking at 4 °C overnight and then 10 mg of protein A - Sepharose (Amersham Pharmacia Biotech) were added and the incubation was continued for another 2 h. The immunoprecipitates were washed (3 x) with the aforementioned lysis buffer, twice with 0.1 M Tris/HCl, pH 7.5, containing 0.5 M LiCl, and 10 µM sodium vanadate, and twice with 10 mM Tris/HCl, pH 7.5, containing 100 mM NaCl, 10 µM sodium vanadate, and 1 mM EDTA. Then 50 µl kinase buffer [10 mM HEPES (pH7.0), 0.1 mM EGTA, 25 mM MgCl₂, 100 µM ATP, 10 µCi of [³²P]ATP, 10 µg/sample PtdIns] was added to each sample, and incubated at room temperature for 10 min. The assay was terminated by adding 60 µl of 2 µM HCl and 160 µl of chloroform/methanol (1:1) / sample, and followed by vortexing and centrifugation. The chloroform phase which contained the inositides was removed and applied to Silica gel 60 thin layer chromatography (TLC) sheets (Merck). TLC was developed with chloroform : methanol : 24% ammonium hydroxide : H₂O (90 : 90 : 9 : 19). After drying, spots were located by autoradiography and compared with standards.

Task 4. Overexpress b4 integrin in PC-3 cells, in the presence or absence of 12-LOX expression, and evaluate the capacity of transfected cells to form hemidesmosomes and whether an increase in surface expression of α6β4 alters cell proliferation, adhesion, migration, and survival, in response to HGF/SF, Months 18 - 30:

Generation of PC-3 cells expressing various β4 mutants: We constructed a panel of bacterial expression plasmids and mammalian expression constructs that express various b4 mutants of the cytoplasmic tail as shown in Figure 6. Using pGR30-47, we have generated a panel of stable transfectants that express various b4 mutants as GFP fusion proteins through fluorescence activated cell sorting (FACS) and G418 selection for the transfectants.

The expression of β4 mutants as GFP-fusion proteins is shown in figure 7. Co-immunoprecipitation was performed to determine the interaction of 12-LOX with various β4 mutants. As shown in the figure 8, 12-LOX is able to bind to the fusion proteins expressed by pGR40, pGR45, pGR46, and pGR47, but not to the fusion protein with only the linker region (pGR38). 12-LOX weakly binds to the fusion protein encoded by pGR39. Collectively, the data suggest a strong binding site(s) for 12-LOX located between 1126-1315 of β4 cytoplasmic tail.

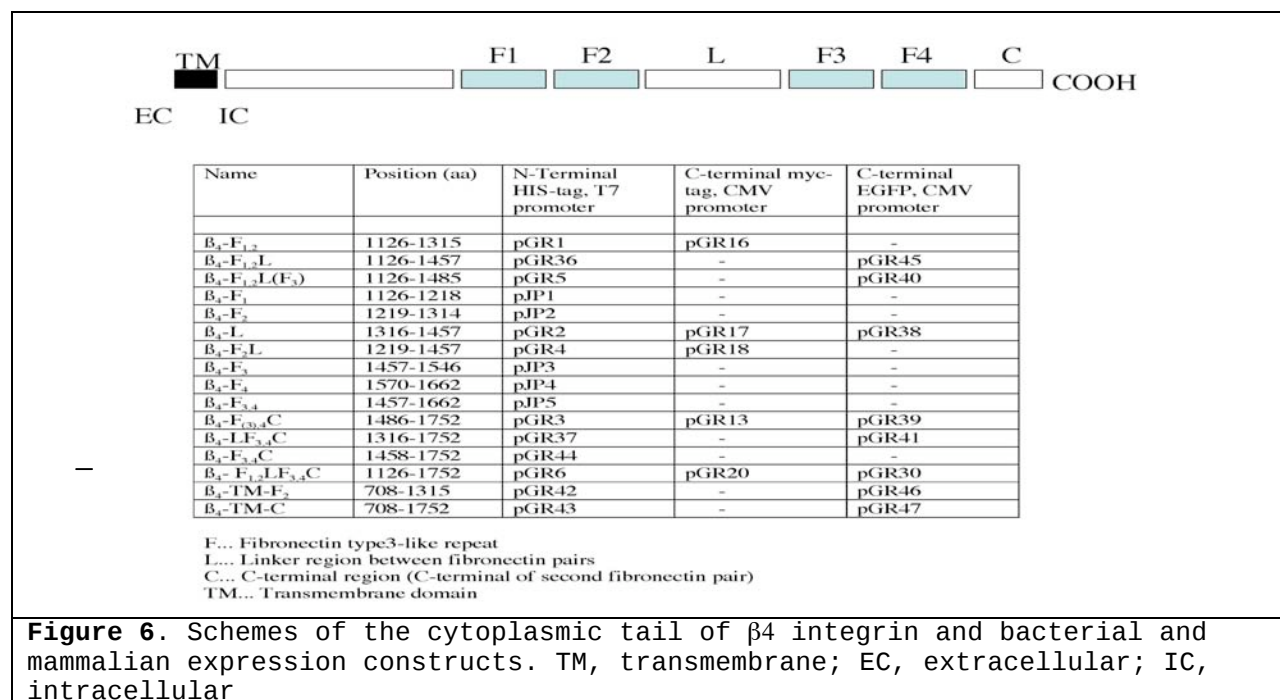
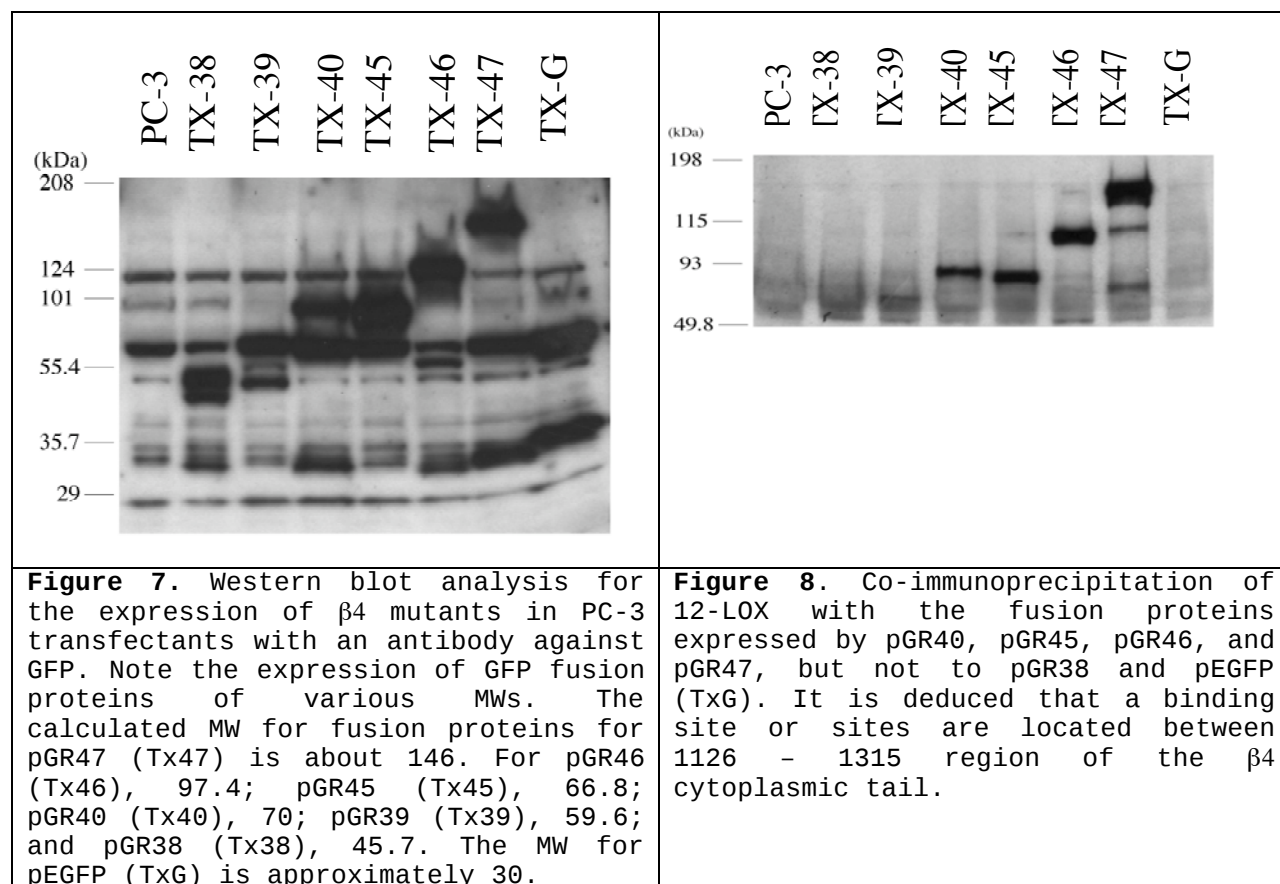
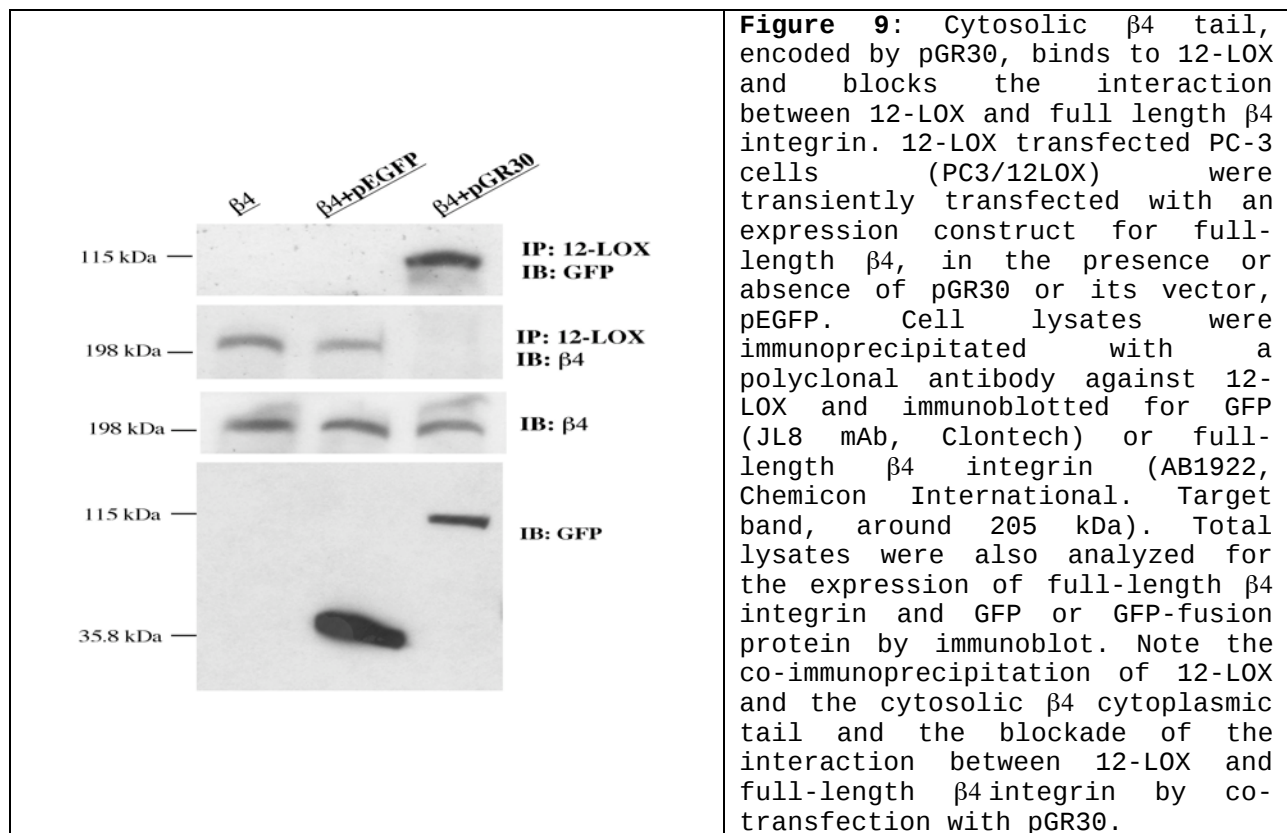
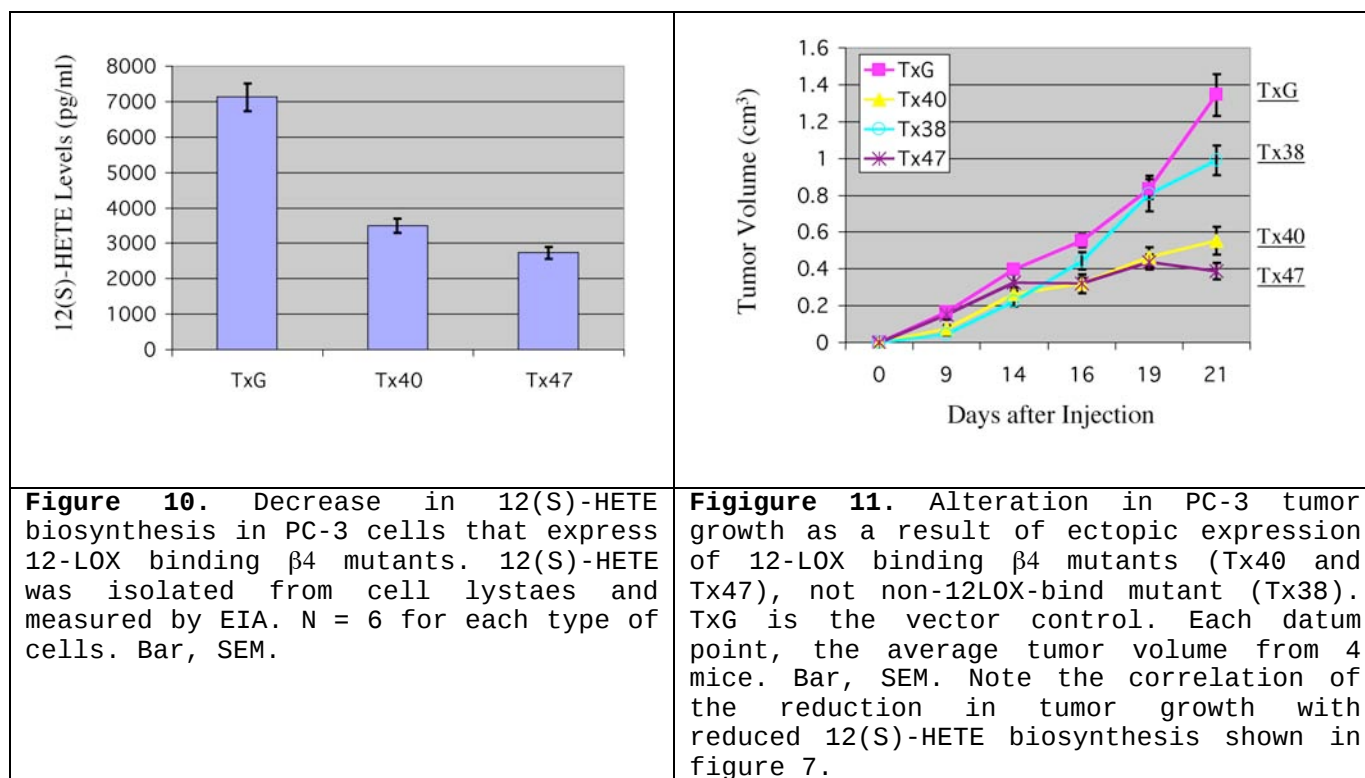


Figure 6. Schemes of the cytoplasmic tail of $\beta 4$ integrin and bacterial and mammalian expression constructs. TM, transmembrane; EC, extracellular; IC, intracellular



Cytosolic $\beta 4$ cytoplasmic tail binds to 12-LOX and blocks the interaction between 12-LOX and full length $\beta 4$ integrin: As shown above, we have constructed a panel of expression constructs encoding various mutants for the $\beta 4$ cytoplasmic tail. The construct pGR30 encodes the cytosolic $\beta 4$ cytoplasmic tail, with TM domain deleted, as a GFP-fusion protein (Figure 6). When ectopically expressed in 12-LOX transfected PC-3 cells, the cytosolic $\beta 4$ cytoplasmic tail interacts with 12-LOX (Figure 9, the IP: 12-LOX / IB: GFP panel). Interestingly the ectopically expressed cytosolic $\beta 4$ tail blocks the interaction of 12-LOX with full-length $\beta 4$ integrin (Figure 9, the IP: 12-LOX / IB: $\beta 4$ panel). The blockade of the interaction between 12-LOX and full-length $\beta 4$ integrin is not due to the lack of $\beta 4$ expression (Figure 9, the IB: $\beta 4$ panel), but due to the presence of the cytosolic $\beta 4$ cytoplasmic tail (Figure 9, the IB: GFP panel). The results suggest that the cytosolic $\beta 4$ mutant, encoded by pGR30, is able to block the interaction between 12-LOX and full-length $\beta 4$ integrin in a dominant negative manner.





Task 5. Evaluate the growth rates of s.c. tumors derived from $\alpha 6\beta 4$ expressing PC-3 cells, in the presence or absence of stable 12-LOX expression, and compare with that of control PC-3 cells, Months 24-36:

We have conducted a preliminary study to determine whether ectopic expression of $\beta 4$ mutants, especially those that can bind 12-LOX, can effect 12-LOX activity. As shown in Figure 10, there was a reduction in 12(S)-HETE biosynthesis in PC-3 cells expressing two 12-LOX binding $\beta 4$ mutants (pGR40 and pGR47). We are still in the process of determining whether 12-LOX cellular localization and interaction with the full-length $\beta 4$ integrin are altered as result of the presence of $\beta 4$ mutants encoded by pGR40 and pGR47. When injected into athymic nu/nu mice, it was found, as shown in Figure 11, that there were a reduction in tumor growth rate in PC-3 cells expressing 12-LOX binding $\beta 4$ mutants (Tx40 and Tx47, see figure 11), when compared to those from vector control (TxG) or a $\beta 4$ fragment that does not interact with 12-LOX (Tx38, see Figure 11).

Key Research Accomplishments:

- Optimized the conditions for immunohistochemical analysis of 12-LOX and $\beta 4$ at the protein level in paraffin-embedded human prostate tumor tissues. This standardized method had enabled

us to continue with the correlation between 12-LOX expression and distribution of $\beta 4$ integrin with Gleason score.

- Constructed several $\beta 4$ cytoplasmic tail mutants and identified the sequence of amino acids on $\beta 4$ integrin that interacts with 12-LOX.
- Preliminary experiments conducted to show the feasibility of disruption of the interaction of 12-LOX with $\beta 4$ integrin in PC-3 cells with ectopic expression of the cytoplasmic tail sequence of $\beta 4$ integrin. This resulted in the reduction of the biosynthesis of 12-HETE as well as tumor growth from PC-3 cells in experimental animals.

Reportable Outcomes:

NONE

Conclusions:

Interaction of 12-LOX with the cytoplasmic tail of $\beta 4$ integrin results in the disruption of hemidesmosomes. Our data presented here demonstrates the potential to exploit the interaction between 12-LOX and $\beta 4$ integrin using ectopically expressed cytoplasmic tail sequence of the integrin to modulate tumor growth.

References:

1. Nievers MG, Schaapveld RQ, Sonnenberg A. Biology and function of hemidesmosomes. *Matrix Biol* 1999;18(1):5-17.
2. Jones JC, Hopkinson SB, Goldfinger LE. Structure and assembly of hemidesmosomes. *Bioessays* 1998;20(6):488-94.
3. Tang K, Finley RL, Nie D, Honn KV. Identification of 12-lipoxygenase interaction with cellular proteins by yeast two-hybrid screening. *Biochemistry* 2000;39(12):3185-91.
4. Tang K, Nie D, Cai Y, Honn KV. The $\beta 4$ integrin subunit rescues A431 cells from apoptosis through a PI3K/Akt kinase signaling pathway. *BiochemBiophysResCommun* 1999;264(1):127-32.

Appendices:

NONE