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Collagen VI: A New Candidate Breast Cancer Marker Linked to Resistance to Platinum-Based Cancer Drugs

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**14. ABSTRACT:** We have observed a dramatic increase of adipocyte-derived matrix protein collagen VI (COL6) level during cancer progression, particularly it relates to a discrete C-terminal domain of the alpha3 subunit of COL6. I have established 2 lines of transgenic mice which overproduce C-terminal domain, called "C5", of the COL6A3 (COL6A3-C5) under the control of MMTV promoter and crossed with MMTV-PyMT mice to see the C5 effects on mammary tumor progression *in vivo*. Our results indicate that COL6A3-C5 augments primary tumor growth and pulmonary metastasis in MMTV-PyMT mammary tumor mice model *in vivo*. Based on the cDNA microarray data with tumor tissues, COL6A3-C5 seems to be a signaling molecule regulating a kinase (or a phosphatase) activity which may be through a specific receptors that remains to be identified. Furthermore, treatment with COL6A3-C5 neutralizing antibodies in MMTV-PyMT mice protects mammary tumor progression. I have determined the effects of COL6 on the TZD mediated enhancement of cisplatin susceptibility with PyMT/COL6$^{-/-}$ mice. Tumor growth in PyMT/COL6$^{-/-}$ mice was significantly attenuated either by cisplatin or by a combination of cisplatin and TZD compared to PyMT/COL6$^{+/+}$. To better understand the COL6 effects on drug resistance, I have tested COL6A3-C5 neutralizing antibodies in combination with cisplatin and TZD using the primary cultured tumor cells implantation system. To visualize the tumor progression *in vivo*, I generated MMTV-F635 transgenic mice which overproduce an infrared fluorescence protein specifically in the mammary epithelial cells. This allows me for the first time to monitor efficacy of treatment modalities longitudinally in mice.

**15. SUBJECT TERMS** – None provided.

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## INTRODUCTION

**Subject:**

The interactions between malignant ductal epithelial cells and the surrounding stromal cells play a crucial role in mammary tumor progression (*1*). The adipocyte is one of the predominant stromal cell types in the tumor microenvironment. The adipocyte is a highly active endocrine cell secreting numerous signaling molecules and profoundly shaping stromal-epithelial interactions (*2, 3*). However, it remains unclear which specific adipocyte-derived factors are involved and how malignant cells are regulated by these factors *in vivo*. We have previously identified a prominent adipocyte-derived extracellular matrix (ECM) protein, type 6 Collagen (COL6), as an important stimulator of mammary tumor growth. It is highly up regulated in human breast cancer patients (*4, 5*). Our previous works of COL6 knock-out (KO) mice in the background of MMTV-PyMT (mammary tumor virus-polyoma middle T antigen) demonstrated significantly attenuated rates of early hyperplasia and primary tumor formation (*5*).

**Purpose:**

In this project, I have focused on the carboxy-terminal domain of COL6 alpha3 subunit named COL6A3-C5, because I previously determined that the carboxy-terminal domain of COL6 is highly enriched in malignant tumor tissues relative to full length COL6. The COL6A3-C5 domain is cleaved off from the COL6 microfilament ECM structure upon secretion from the adipocyte (*6*). We believe that the adipocyte-derived, cleaved form of the C5 protein acts as a signaling molecule, influencing tumor growth and metastasis through various downstream signaling pathways. Therefore, I have explored the roles of **adipocyte-derived COL6A3-C5 as a novel mitogen** in mammary tumor progression *in vivo*. Furthermore, it has been suggested that increased levels of COL6 are involved in chemo-resistance to platinum-based therapeutic approaches in cancers, which is a widely used chemotherapeutic agent (*7, 8*). The molecular mechanisms that explain platinum resistance in tumor cells remain largely unknown. Anti-diabetic agents, thiazolidinediones ("TZD's", PPARγ agonists) have been studied in human cancer therapy based on anti-mitogenic and terminal differentiation therapy as well as combination therapy with platinum-based regimen (*9, 10*). Our observation that COL6 level is decreased by TZD treatment let us to investigate whether **COL6 is involved in the synergistic effects of TZD in combination with platinum-based therapy *in vivo*.**

**Scope of research:**

Here, we highlight the adipocyte-derived extracellular matrix as an important and novel site of modulation of cancer cell behavior within the tumor microenvironment. Contributions of stromal adipocytes on cancer cell behavior are expected to have more relevance to obesity-related cancers such as colon, renal, pancreas, and post-menopausal breast cancer. Our study will highlight novel mechanisms linking obesity and aggressive cancer progression and provide therapeutic strategies for these obesity-related cancers.

## BODY

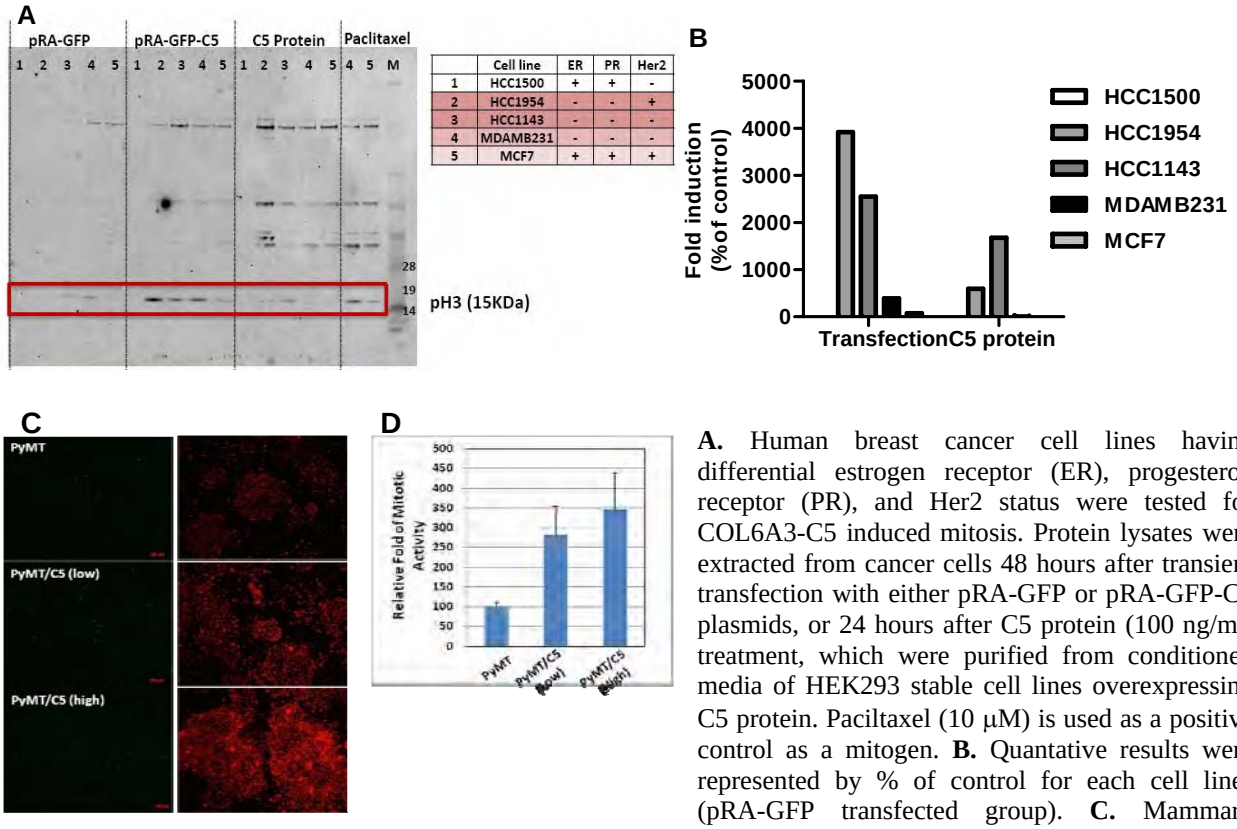
The specific aims outlined in the original SOW are restated below. New SOWs, which include modified methodologies for Aim3.2 and Aim4.2, are attached in the APPENDIX section.

***Specific Aim1*. Analyze the phenotype of MMTV-COL6A3-C5 transgenic mouse in the background of MMTV-PyMT breast cancer model.**

Task 1. Determine the pro-mitotic activities of COL6A3-C5 in mammary cancer cells (1-9 months): *completed.*

We have determined the mitotic activity of COL6A3-C5 with various types of human breast cancer cell lines and primary cultured mammary epithelial tumor (MET) cells from tumor tissues of control versus COL6A3-C5 transgenic mice in the MMTV-PyMT background. Phosphorylation of Histone3 ("pH3") is used as a mitotic marker. Mitotic activities of human breast cancer cells were highly upregulated either by transient transfection or by recombinant C5 protein treatment as judged by immunoblotting with pH3 antibody (**Fig. 1A-B**). In addition, primary cultured mammary epithelial tumor cells overexpressing COL6A3-C5 proliferate faster compared to control cells (MMTV-PyMT derived tumor cells) as determined by immunostaining of pH3 (**Fig. 1C**).

**Figure 1. Proteolytic fragment of COL6 (COL6A3-C5) *augments* mitotic activity of breast cancer cells.**




**A.** Human breast cancer cell lines having differential estrogen receptor (ER), progesteron receptor (PR), and Her2 status were tested for COL6A3-C5 induced mitosis. Protein lysates were extracted from cancer cells 48 hours after transient transfection with either pRA-GFP or pRA-GFP-C5 plasmids, or 24 hours after C5 protein (100 ng/ml) treatment, which were purified from conditioned media of HEK293 stable cell lines overexpressing C5 protein. Paciltaxel (10 μM) is used as a positive control as a mitogen. **B.** Quantative results were represented by % of control for each cell lines (pRA-GFP transfected group). **C.** Mammary epithelial tumor cells are isolated from 13-week-old control and COL6A3-C5 transgenic mice (low and high expressors) in the background of MMTV-PyMT. The same number of cells was seeded into 12-well plates and mitotic activity is measured by immunostaining of phsopho-Histone3 (pH3). Fluorescence values of pH3 positive cells are normalized with propodium (staining nuclei) positive cells. **D**. Quantitative results are represented by

Mean±SEM. (n=5 per each group). *p<0.001 by *t-test*.

Task 2. Characterization of the VIa3-C5 transgenic mouse model in the MMTV-PyMT background (1-24months): *ongoing*

We have established 2 lines of COL6A3-C5 transgenic mice out of the 7 positive founder lines, which we refer to as "high" (M46) and "low" (F45) expressors. The COL6A3-C5 transgene is highly expressed in the mammary gland and also expressed at very low levels in other fat pads and lung tissue under the control of MMTV promoter (**Fig. 2**).

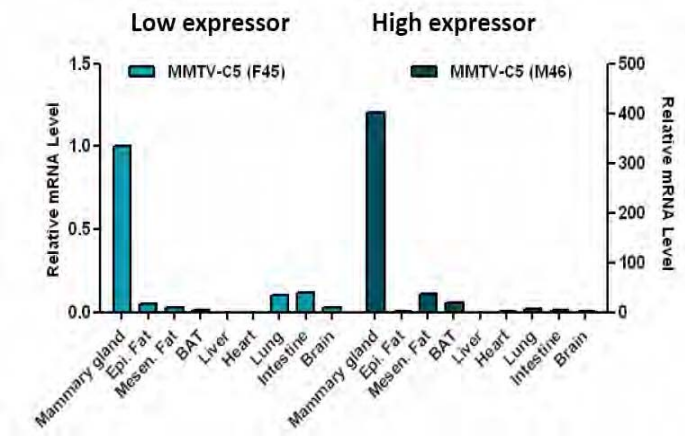



**Figure 2. Tissue distributions of C5 transgene in the low (F45) and high (M46) expressor.** Total RNA was extracted from the various tissues and cDNAs were synthesized to analyze mRNA levels of the C5 transgene. mRNA levels were determined by Q-PCR and normalized with β-actin (left).

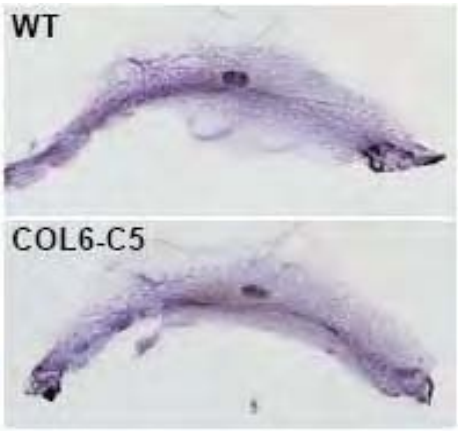



**Figure 3. Ductal epithelial growth and branching in COL6A3-C5 transgenic mice is similarly developed compared to wild-type mice.** Whole mammary gland tissues from the COL6A3-C5 transgenic mice and WT mice were stained with carmine-alum (right) to monitor ductal epithelial development.

The complete development of the mammary ductal epithelium is crucial to properly examine mammary tumor behavior, since mammary tumors originate from the ductal or intraductal epithelium. To see whether C5 affects the ductal epithelium development, we performed whole mount staining with mammary gland tissues from C5 transgenic mice compared to wild-type littermates. Ductal epithelial growth and branching were completely normal compared to wild-typein both high and low expressors of the C5 transgenic mice (**Fig. 3**). However, high expressers showed low fertility and reduced locomotion as well as spontaneous tumor development, a phenomenon not observed in low expressers (**supporting data 1**) or wild-type mice. The spontaneous development of tumors in the high expressors highlights the potent nature of this new mitogen. Nevertheless, we have been using the low expresser line to examine the C5 effects on tumor behavior in crosses with the MMTV-PyMT, mammary tumor mice model. Age- matched PyMT female mice were used for analysis in all experiments. Early neoplastic lesion area was dramatically increased in PyMT/COL6A3-C5 mice compared to PyMT (**Fig. 4A**). For quantification, primary tumor growth was determined by caliper measurements from mice 6- to 13-weeks of age in both groups. PyMT/COL6A3-C5 showed a tendency of increased primary tumor growth compared to PyMT (**Fig. 4B**).

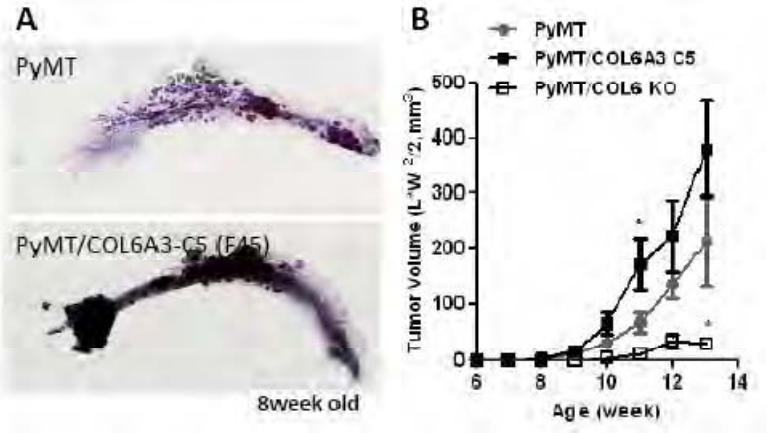



**Figure 4. COL6A3-C5 augments early neoplastic lesion areas and primary tumor growth in PyMT mice. A**. Early neoplastic lesion areas were presented by whole mount staining of mammary gland tissues from PyMT/COL6A3-C5 and PyMT mice at 8-weeks of age. **B**.

Primary tumor growth was determined by caliper measurements once a week from 6- to 13-weeks. PyMT/COL6$^{-/-}$ mice were compared in parallel with both groups. Results are represented by Mean ± SEM (n=35, PyMT; n=38, PyMT/COL6A3-C5). $^{*}p<0.05$ vs. PyMT by 2-way ANOVA (no-matching).

We determined metastatic growth by histological analysis of H&E-stained pulmonary tissues, by counting the incidence of pulmonary metastasis in PyMT/COL6A3-C5compared to PyMTduring tumor progression (**Fig. 5A**). The number of metastatic lesions observed was significantly higher in PyMT/COL6A3-C5mice compared to PyMT mice at 16-weeks of age (**Fig. 5B**). Therefore, we conclude that COL6A3-C5 dramatically increases the primary tumor growth and pulmonary metastasis in MMTV-PyMT mammary tumor mice *in vivo*.

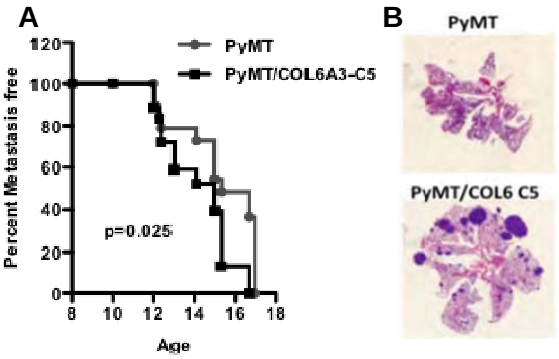



**Figure 5. Pulmonary metastasis is dramatically increased in PyMT/COL6A3-C5 compared to PyMT. A**. The ratio of pulmonary metastasis was determined from 8 to 17-week-old PyMT and PyMT/COL6A3-C5 (n=23-25 per group). The graph shown represents the percentage of mice that are metastasis-free. The survival curve was analyzed with a Log-rank test, P=0.025 vs. PyMT. **B.** Degree of pulmonary metastasis was also assessed by H&E stain of lung tissues from PyMT and PyMT/COL6A3-C5 at 17 weeks of age.

To identify the molecular pathways involved in C5-induced tumor progression and metastasis, we performed cDNA microarray analysis with tumor tissues from PyMT/COL6A3-C5 and PyMT at 12 weeks of age. A significant number of genes on our 47K gene array were modulated and 4.7% and 3.16% of these genes were either up- and down-regulated in PyMT/COL6A3-C5 tumor tissues, respectively. Based on the functional annotation analysis, 45 % of genes altered in their expression were associated with phosphorylation events, suggesting that COL6A3-C5 seems to be involved in regulation of kinase and phosphatase activities (**Fig. 6**). We currently focus on several candidates to verify the signaling pathways.

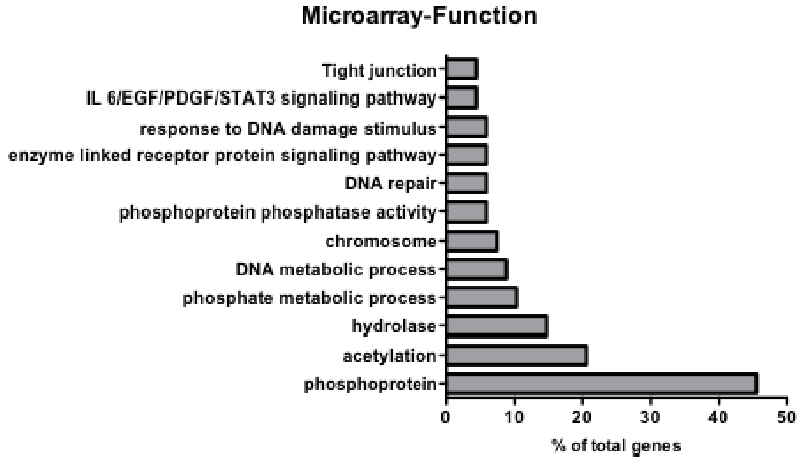



**Figure 6. Microarray analysis:** Total RNA was extracted from tumor tissue from 12-week-old PyMT and PyMT/COL6A3-C5 (n=9 /group). Microarrays were performed in our UTSW Microarray Core. The mouse Illumina Bead Array platform (47K array) (Illumina, Inc., San Diego, CA) was used. Gene lists and cluster analyses of the data sets were performed using Ingenuity pathway software (Ingenuity systems) and David Bioinformatics Resource. Cut off values are 1.2 fold and $p<0.05$ for functional annotation.

***Specific Aim2*. Generation of polyclonal antibodies and monoclonal antibodies that can neutralize the activity of the collagen VIα3-C5 domain, the region of the protein that we believe to confer induction of metallothioneins**

Task 1. Generate VIa3-C5 specific polyclonal antibodies(1-12 months): *completed*

We generated rabbit anti-mouse COL6A3-C5 polyclonal antibodies that are working very well for all applications, including immunoblotting (IB), immunoprecipitation(IP), and immunohistochemistry. Mammary gland tissues from wild-type, COL6A3-C5 transgenic mice (high and low expressors), and $COL6^{-/-}$ mice were immunostained with this anti-C5 antibody (**Fig. 7A**). COL6A3-C5 is abundantly expressed in adipose tissues of wild-type mice and in COL6A3-C5 transgenic mice, it can also be found in both ductal epithelial and adipose tissues, whereas C5 could not be detected in $COL6^{-/-}$ mice. The cleaved form of C5 protein is specifically detected by western blotting (**Fig. 7B**).

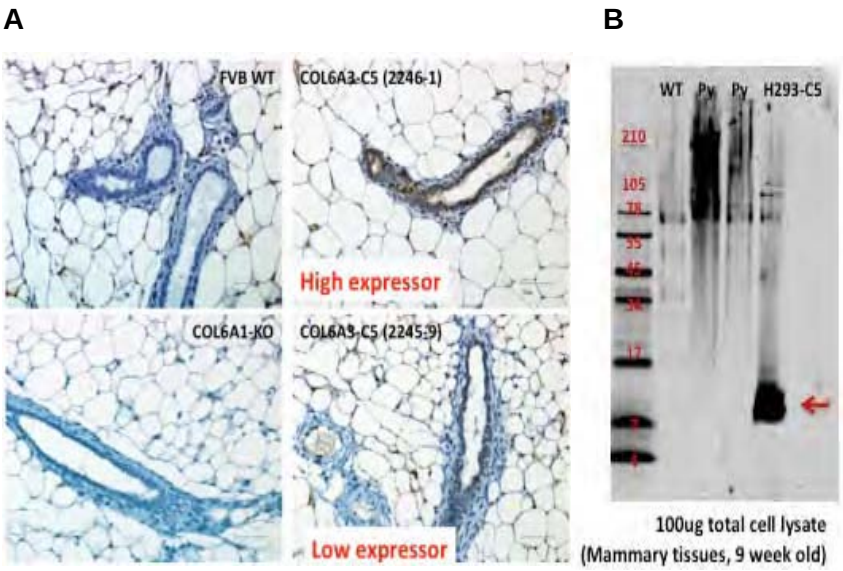



**Figure 7. Applications of COL6A3-C5 specific polyclonal antibodies. A**. Immunohistology: Paraffin embedded mammary gland tissue slides from WT, COL6A3-C5 transgenic lines, and $COL6^{-/-}$ were used for immunostaining with C5 polyclonal antibodies. Dark brown color represents C5 positive regions. **B**. Western blot: Protein lysates extracted from mammary gland tissues of FVB wild-type (lane 1) and MMTV-PyMT (lane 2-3) mice at 9-weeks of age as well as C5 over-expressing HEK293 cells (lane 4) were subjected to western blotting. Arrow indicates cleaved form of C5 protein.

Task 2. Generation of VIa3-C5 specific monoclonal antibodies (1-12 months): *completed*

Initially, we have generated mouse anti-mouse COL6A3-C5 monoclonal antibodies using $COL6^{-/-}$ mice as a host and purified the native form of the C5 protein as an antigen. As we previously stated in the original SOW, 2 hybridoma cell lines were selected for highest activity in ELISA assays and immunoblotting. However, these monoclonal antibodies did not work well after they were established as hybridoma cell lines. Therefore, we retried to generate C5-specific monoclonal antibodies with rat as a host. At present, we have established total 5 hybridoma cell lines out of 19 clones, selected by highest activity of ELISA and dot-blotting (data not shown) and 3 clones show high activity of C5 capturing as determined by IP (**Fig. 8**). Therefore, we currently use the 10B6 clone for further studies.

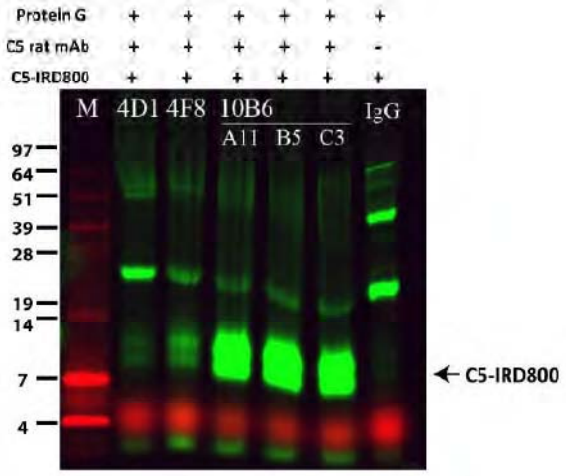



**Figure 8. Rat anti-mouse C5 monoclonal antibodies efficiently capture native C5 protein.** IRD (infrared dye)-800 labeled native form of C5 protein was incubated with either C5 monoclonal antibodies including 4D1, 4F8, 10B6-A11, 10B6-B5, and 10B6-C3 or rat-IgG for 2 hours at room temperature and subsequently incubated with protein G sepharose for 1 hour with rotation. Protein-sepharose complex were separated on 10-20 % Tricin gel after 3 times washing with PBS. Captured C5 protein by C5 monoclonals

or IgG was visualized on the Licor Odessey Infrared Scanner (Licor Bioscience). Green color represents IRD800 channel.

Task 3. Verify the neutralizing activities of VIα3-C5 monoclonal antibodies (1-12 months): *on-going*

This task has been delayed due to regeneration of C5 monoclonal antibodies from the rat. To find the optimal read-out of neutralizing activity of C5-monoclonal antibodies, I focus on the mitotic activity of COL6A3-C5 protein. Thus, I will test whether C5 monoclonal antibodies abolish the C5-stimulated mitotic activity in cancer cells by immunoblotting or immunocytochemistry of pH3 which is a well established mitosis marker.

Although we do not know exactly how COL6A3-C5 stimulates tumor progression and metastasis, we tested the effects of C5 monoclonal antibodies on mammary tumor progression. As shown in **Fig. 9**, tumor growth was significantly decreased by 10B6 treatment compared to IgG treatment, suggesting that 10B6 actively neutralizes the COL6A3-C5 stimulated tumor growth *in vivo*. This represents a **major breakthrough** in our studies and bears great promise for further analysis.

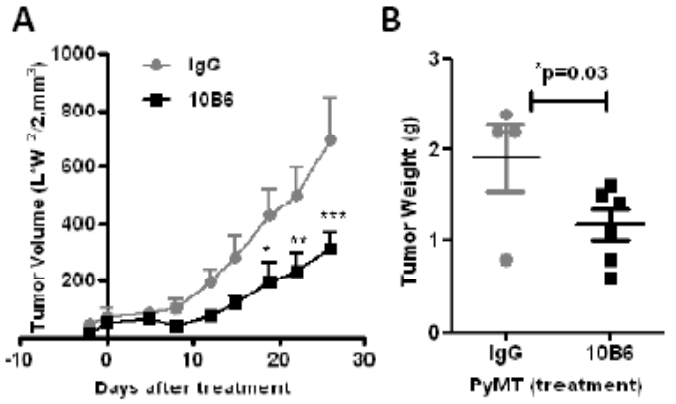



**Figure 9. C5 monoclonal antibody (10B6) attenuates mammary tumor growth in MMTV-PyMT mice.** 9-week-old MMTV-PyMT mice were treated with either 10B6 (200 μg/mice) or rat-IgG (200 μg/mice) by intraperitoneal injection twice a week and tumor growth was monitored by caliper measurements. **A.** Tumor growth. Graph is represented as Mean ± SEM (n=4 for IgG and n=6 for 10B6 group). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. IgG by 2-way Anova (no-matching). **B.** Tumor was excised and weighted 4 weeks after 10B6 or IgG treatment. *p=0.03 vs. IgG by *t-test*.

To analyze the expression levels of metallothioneins, mammary glands from MMTV-PyMT mice at various stages with or without treatment of 10B6 will be dissected and extracted for both RNA and protein. Expression levels of metallothioneins will be investigated using RT-PCR and western blot analysis. Furthermore, we will further analyze the COL6A3-C5 mediated downstream signaling pathways, including the canonical β-catenin pathway which we previously reported as a candidate pathway (*5*). Once we get additional insights into the downstream pathways, we will test the neutralizing activity of C5 monoclonal antibodies targeting the selected pathways.

***Specific Aim3*. Compare the susceptibility to platinum-based cancer regimens in wild-type vs. collagen VI null mice ($COL6^{-/-}$) in the MMTV-PyMT (6-24 months).**

Task 1. Generate collagen VI null mice and breed with MMTV-PyMT mice (6-12 months): *completed*

We currently have $COL6^{-/-}$ mice which are crossed with MMTV-PyMT. Male $COL6^{-/-}$ in the background of MMTV-PyMT (PyMT/$COL6^{-/-}$) have been crossed with female $COL6^{-/-}$ to generate female PyMT/$COL6^{-/-}$. The resulting cross generates 25 % of PyMT/$COL6^{-/-}$ female mice. Age matched PyMT female mice are used for analysis.

Task 2. Characterize the collagen VI effects on cisplatin resistance in MMTV-PyMT mice model (9-24 months): *on-going*

2a. Evaluate cisplatin drug toxicity and set up the optimal protocol of cisplatin treatment in wild-type and collagen VI null mice in the MMTV-PyMT background (9-18 months): *completed*

We have tested various ranges of cisplatin dosages from 1 to 2.5 mg/kg. Therefore, we decided to apply either 2.5 mg/kg or 1 mg/kg by intraperitoneal injection (I.P) two times per week for cisplatin mono-therapy or cisplatin and TZD combination therapy, respectively. Cisplatin is treated from 10-weeks-of-age to 16-weeks. We do not treat with cisplatin for more than 2-months because of drug toxicity.

2b. Cisplatin treat with experimental cohorts and measure tumor volumes, rates of apoptosis, and expression levels of metallothioneins (18-24 months). To get improved quantitative results, I will perform these experiments in triplicate using independent cohorts: *on-going*

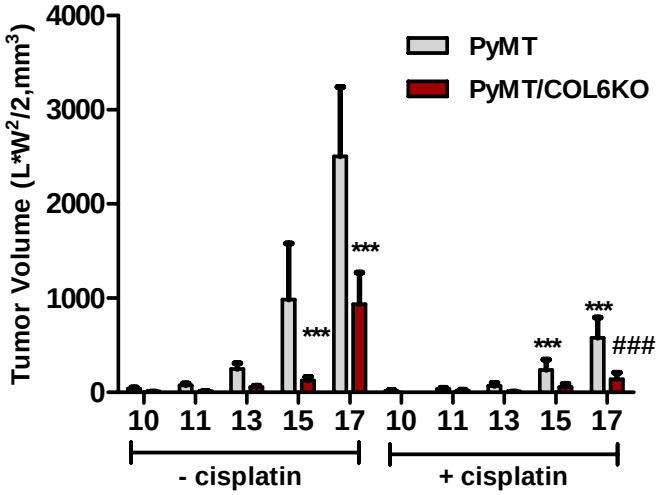



**Figure 10. PyMT/COL6$^{-/-}$ mice are more susceptible to cisplatin treatment.** 10-week-old PyMT/COL6$^{+/+}$ vs. PyMT/COL6$^{-/-}$ mice were treated with 2.5 mg/kg cisplatin by intraperitoneal injection (i.p.) twice a week. Tumor progression was measured by caliper during cisplatin treatments. n=7 for cisplatin$^{-}$ and n=10 for cisplatin$^{+}$ group. $^{***}p<0.001$ vs. PyMT (-cisplatin), $^{###}p<0.001$ vs. PyMT/COL6KO (-cisplatin) by 2-way Anova (no-matching).

We showed that primary tumor growth in PyMT/COL6$^{-/-}$ mice is significantly attenuated after cisplatin treatment compared to PyMT/COL6$^{+/+}$ suggesting that adipocyte derived COL6 affects cisplatin susceptibility in mammary cancer at the morphological level (**Fig. 10**). However, we realized that the current experimental setting has a potential drawback. First, the rate of tumor growth in PyMT/COL6$^{-/-}$ is significantly attenuated even in the cisplatin free groups because COL6 affects tumor growth itself. Thus, we could not compare the cisplatin resistance with the same size of tumors. Second, tumor progression in MMTV-PyMT mice is very aggressive. They develop early carcinoma to late carcinoma at 10-weeks and 13-weeks, respectively, which is a very narrow time frame to determine drug resistance. To resolve these problems, we have tested cisplatin resistance with the FVB wild-type mice after implantation of primary cultured mammary epithelial tumor cells (MET cells) from tumor tissues of PyMT/COL6$^{+/+}$ vs. PyMT/COL6$^{-/-}$ mice. I will start cisplatin treatment when the tumor size reaches 5 mm diameter and stop treatment when the tumor is regressed by cisplatin. This treatment protocol will be repeated until they show cisplatin resistance to determine whether or not COL6 affects cisplatin susceptibility in this setting. Our alternative experimental setting will allow us to investigate the cisplatin resistance with more accuracy and longer time frame of tumor latency.

*Development in vivo imaging tools to quantify tumor volume:*

The detailed quantification of lesion size remains a challenge. If the differences in lesion growth are visually apparent, the appropriate quantification is fairly straightforward. For cases in which the differences are more subtle, I am currently establishing new protocols for volume integration of lesions using an infrared scanner. I have generated the MMTV-infrared fluorescence transgenic mice which will allow us to visualize breast tumor progression in whole animals with the Maestro scanner. I have already tested the feasibility of this approach in a few preliminary experiments (**Fig. 11**). We will breed MMTV-F635 mice with COL6A3-C5 mice and COL6$^{-/-}$ mice to quantify tumor volume at the whole animal level.

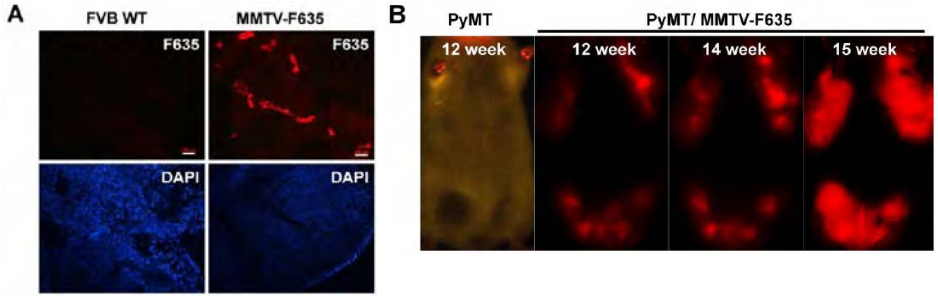



**Figure 11. Quantification of tumor progression with MMTV-F635 infrared-fluorescence mice. A.** Infrared fluorescence protein (F635) is exclusively expressed in the mammary ductal epithelium under the control of MMTV promoter. We have established a MMTV-F635 transgenic mouse line from 7 founders by screening for F635 fluorescence protein expression with frozen sectioned mammary glands. Images were acquired using the Leica confocal microscope. The DAPI stain highlights nuclei. **B.** ***In vivo*** **tumor imaging with MMTV-F635 transgenic mice.** Female MMTV-F635 mice are crossed with male MMTV-PyMT mice to obtain female PyMT/F635 mice. Tumor volume in PyMT/F635 mice was quantified by infrared fluorescence signal intensity which expresses in ductal epithelium during tumor progression. Images are acquired with the Maestro Fluorescence scanner (available at the Simmons Cancer Center at UTSW Medical Center).

***Specific Aim4.*** **Examine the roles of collagen VI on synergistic effects between thiazolidinediones (TZDs) and cisplatin therapy (3-36 months)**

Task 1. Examine the regulation of VIα3-C5 by TZDs treatment *in vivo* (3-8 months): *on-going*

1a. Obtain experimental cohorts (3-6 months): At least 5 mice will be used in the experiments.

1b. Perform immunohistochemistry (IHC) with histology samples of mammary gland tissues of MMTV-PyMT mice with or without TZD treatments for 2 to 4 weeks (4-8 months): slides are ready for these experiments (*ongoing)*

Task 2. Compare cisplatin susceptibility in collagen VI null and wild type mice in the background of MMTV-PyMT mice with or without TZDs treatments (6-24 months): *ongoing*

2a. Get experiment cohorts (6-12 months): 7-10 mice in each group will be used in the experiments.

2b. Optimize the protocols of cisplatin treatment in combination with TZD as mentioned in Specific Aim3- Task 2 (12-18 months)

2c. Perform cohort experiments (18-24 months): Collect samples and analyze it.

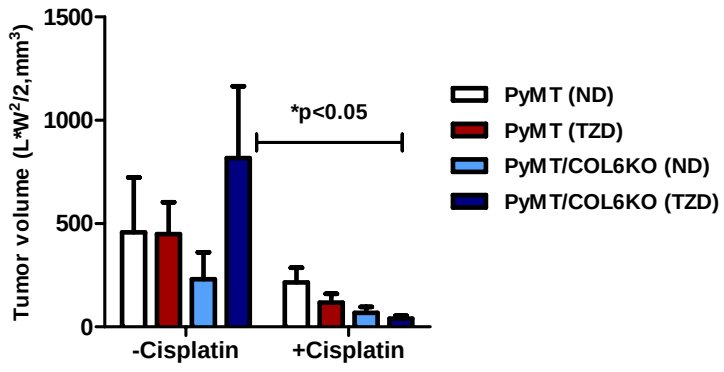



**Figure 12. Comparison of cisplatin resistance between PyMT/COL6$^{-/-}$ and PyMT/COL6$^{+/+}$ in combination with TZD treatment.** Rosiglitazone (20 mg/kg/day) was used for 2 weeks before cisplatin treatment by inclusion in the diet and cisplatin (1 mg/kg) was injected by i.p. twice a week. Tumor volume was determined by caliper measurements during cisplatin treatment from 10 weeks to 17 weeks. The graph represents Mean ± SEM (n= 7-10 mice per each group) at the time of end of study, 17 weeks. $^{*}p<0.05$ vs. PyMT/COL6KO (-cisplatin), by 2-way Anova (no-matching).

We investigated cisplatin susceptibility with or without TZD treatment in the PyMT/COL6$^{-/-}$ compared to PyMT/COL6$^{+/+}$. The results indicated that tumor growth is attenuated in the PyMT/COL6$^{-/-}$ by either cisplatin treatment or cisplatin in combination with TZD (**Fig. 12**). Therefore, we decided to use tumor cell implantation as an alternative method as described in SA3-Task2, since the MMTV-PyMT mouse model is not an optimal setting to see the cisplatin resistance.

***Specific Aim5*. Verify the relevance of VIα3-C5 levels in clinical samples (6-36 months).**

Task 1. Verify the levels of VIα3-C5 in breast cancer mouse subjects (6-12 months).

1a. Measure the VIα3-C5 protein levels in MMTV-PyMT mice: *completed*

Immunohistochemistry was performed with mammary gland tissues of MMTV-PyMT mice compared to wild-type litter-mates with COL6A3-C5 polyclonal antibodies (**Fig. 13, upper panel**). At least 5 mice are used in these experiments. Human breast cancer samples were obtained from UTSW human sample Bank and examined C5 expression level with C5 specific antibodies (**Fig. 13, bottom panel**). COL6A3-C5 levels are highly increased in the tumor tissues in both MMTV-PyMT mice and human breast cancer patients as determined by immunohistochemistry with our COL6A3-C5 specific antibodies described above.

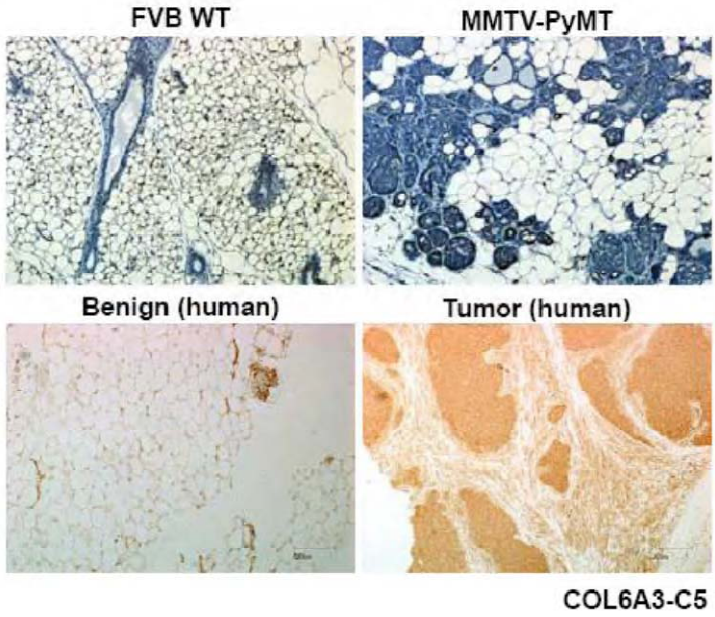



**Figure 13. Expression levels of COL6A3-C5 are highly up-regulated in both mouse mammary tumors (MMTV-PyMT) and human breast tumor tissues compared to those of control or benign tumor tissues, respectively.** Mammary gland tissues from 8-week-aged FVB WT and MMTV-PyMT mice were used. Human breast cancer tissues were acquired from the UTSW human tissue bank. Formalin-fixed paraffin-embedded tissue sections were used for immunostaining with the rabbit polyclonal antibody against COL6A3-C5 (Covance). The reaction was visualized by the DAB Chromogen-A system (DakoCytomation). Mouse tissues were counterstained with hematoxylin. Images were acquired using a Nikon Cool Scope.

1b. Measure the circulating levels of VIα3-C5 (6-12 months): Development of ELISA method to detect circulating VIa3-C5 proteins in the serum samples of mouse and human breast cancer subjects. At least 5 samples in each group will be used in this experiment: *ongoing*

We have been developing the sandwich-ELISA method using COL6A3-C5 specific rat monoclonal antibody and rabbit polyclonal antibody as a capturing and detection antibodies. We expect to have optimal conditions shortly for ELISAs to measure the COL6A3-C5 in circulation.

Task 2. Verify the levels of VIα3-C5 in breast cancer human subjects (12-36 months): *on-going*

2a. Collect human tissue samples from UTSW cancer tissue bank and analyze the levels of VIα3-C5 by immunohistochemistry using VIa3-C5 antibodies (12-18 months): *completed.*

See Figure 13.

2b. Measure the VIα3-C5 levels in both serum and breast tumor samples of breast cancer patients at UTSW medical center (24-36 months): ***ongoing***

We have developed an ELISA system to measure COL6A3-C5 levels in human serum samples.

2c. Measure the VIα3-C5 levels in human samples after platinum-based chemotherapy in combination with TZDs to characterize VIα3-C5 effects on drug resistance (24-36 months). Our collaborator Dr. David Euhus in UTSW Medical Center will play a critical role in spearheading and facilitating these efforts.

## KEY RESEARCH ACCOMPLISHMENTS

- Established COL6A3-C5 transgenic mice under the control of MMTV promoter: low and high expressors identified.
- Generated both polyclonal and monoclonal antibodies against COL6A3-C5 domain.
- Determined the COL6A3-C5 protein levels in both human and mouse mammary tumor tissues by immunohistochemistry with COL6A3-C5 specific polyclonal antibodies.
- Determined anti-apoptotic and pro-mitotic activities of COL6A3-C5 protein *in vivo.*
- Determined the ratio of tumor progression and pulmonary metastasis in COL6A3-C5 transgenic mice in the background of MMTV-PyMT (PyMT/COL6A3-C5) compared to control littermates *in vivo.*
- cDNA microarray analysis successfully performed with tumor tissues from PyMT/COL6A3-C5 and PyMT mice.
- Generation of MMTV-F635 (infrared fluorescence protein) transgenic mice for *in vivo* imaging of mammary tumor progression successfully accomplished.
- Optimized the protocols for immunotherapy with COL6A3-C5 monoclonal antibodies.
- Investigated the cisplatin susceptibility in combination with TZD to examine the effects of COL6 on drug resistance (large cohort experiments).

## REPORTABLE OUTCOMES

**Reportable outcomes that have resulted from this research project:**

- Manuscript : NONE (in preparation).
- Abstracts: NONE
- Presentations: Touchstone Diabetes Center Seminar Series at UTSW.

## CONCLUSION

We have characterized the properties of the adipocyte-derived type 6 collagen-alpha3-C5 domain (COL6A3-C5) on mammary tumor progression and metastasis *in vivo*. We established COL6A3-C5 transgenic mice under the control of MMTV promoter which were bred with MMTV-PyMT mice to investigate COL6A3-C5 effects on mammary tumor progression and metastasis. Our results indicate that **COL6A3-C5 is a potent new mitogen**, stimulating cancer cell proliferation as well as it also enhances the pulmonary metastasis *in vivo*. We will pursue to quantify the properties of cancer cell motility and invasion regarding metastasis with primary cultured mammary epithelial tumor cells (MET cells) from PyMT/COL6A3-C5 and PyMT mice. To identify the molecular mechanisms on COL6A3-C5 mediated cell proliferation and survival, we performed cDNA microarray with tumor tissues from 12-weeks of aged PyMT/COL6A3-C5 and PyMT mice. Based on our microarray results, 45 % of genes significantly modulated by COL6A3-C5 are related to phosphorylation, suggesting that COL6A3-C5 is a signaling molecule stimulating a kinase (or a phosphatase) activity. We currently analyze the microarray data and will focus on several target molecules regulated by C5-mediated signaling pathways.

For the therapeutic purposes, we generated COL6A3-C5 specific monoclonal antibodies and have tested these on a mouse mammary tumor model, MMTV-PyMT. Our preliminary results suggest that COL6A3-C5 monoclonal antibodies **have potent protective effects on mammary tumor progression**. We will pursue to determine the efficacy of COL6A3-C5 monoclonal antibodies more carefully.

We have investigated cisplatin resistance with COL6 knock-out mice in the background of MMTV-PyMT mice and compared that to control MMTV-PyMT mice. PyMT/COL6$^{-/-}$ mice were more susceptible to cisplatin treatment relative to PyMT as judged by tumor volume during cisplatin treatment. However, we noticed that it is challenge to determine drug resistance with MMTV-PyMT mice model because the tumor growth of these mice is so aggressive. To better address these questions, we will use tumor cell implantation system with primary cultured mammary epithelial tumor cells from PyMT/COL6$^{-/-}$ vs. PyMT which will allow us to determine cisplatin resistance with a longer time period of tumor progression.

Finally, I will measure COL6A3-C5 levels in human cancer patient serum samples with the ELISA which we are currently optimizing for the conditions with monoclonal- and polyclonal antibodies against COL6A3-C5. If I can detect the COL6A3-C5 level in human samples, this has the potential to be a useful therapeutic marker for cancer patients.

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## APPENDICES

### Statement of Work (SOW)- 1st Annual Report

Modified methodology is highlighted in red.

***Specific Aim1.*** Analyze the phenotype of MMTV-Collagen VIα3-C5 transgenic mouse in the background of MMTV-PyMT breast cancer model (1-24 months).

Task 1. Determine the pro-mitotic activities of VIα3-C5 in mammary cancer cells (1-9 months).

1a. Measure the mitotic activities of breast cancer cell lines such as MCF7 and Met1 with or without recombinant protein of VIα3-C5 treatment (Completed).

1b. Measure the mitotic activities using transient transfection of VIα3-C5 plasmid into MCF7 cells (alternative method, 1 month).

1c. Measure the mitotic activities of primary cultured mouse cancer cells from wild-type versus VIα3-C5 transgenic mice in the MMTV-PyMT background (6-9 months). 5 mice in each group will be used. Mitotic activities are measured by phospho-Histon3, BrdU or Ki67 immunostaining as well as live cell counting with trypan blue staining.

Task 2. Characterization of the VIa3-C5 transgenic mouse model in the MMTV-PyMT background (1-24 months).

2a. Generate a construct and VIa3-C5 transgenic mice

2b. Establish founder lines: Out of the 94 founders, we obtained 7 positives. I bred each one into a F2 generation and established 3 individual founder lines already.

2c. Breed VIa3-C5 transgenic mice with MMTV-PyMT mice to get the experiment cohorts (completed)

2d. Examinethe phenotypes regarding cancer progression (9-18 months): Measure the tumor weight and tumor volumes as well as marker gene expressions. 5 to 10 mice in each group will be used.

2e. Microarray analysis to identify the molecular pathways regarding cancer progression (12-24 months): UTSW microarray core facility will perform the cDNA microarray analysis with illumine bead chips. At least 9 mice in each group will be used in microarray analysis.

***Specific Aim2***. Generate polyclonal antibodies and monoclonal antibodies that can neutralize the activity of the collagen VIα3-C5 domain, the region of the protein that we believe to confer induction of metallothioneins. (1-12 months)

Task 1. Generate VIa3-C5 specific polyclonal antibodies.

1a. Generate a construct of GST fused VIα3-C5and purify recombinant protein from E.coli system.

1b. Immunize 2 rabbits with GST fused VIα3-C5 recombinant protein.

1c. Collect serum samples containing VIα3-C5 antibodies

Task 2. Generate VIa3-C5 specific monoclonal antibodies.

2a. Generate a construct of prolactin signal sequence fused VIα3-C5

2b. Establish stable cell lines expressing prolactin signal sequence fused VIα3-C5 results in secretion of VIα3-C5 to cultured medium.

2c. Purify native form of VIα3-C5 protein from supernatants of stable cell lines and used as an antigen. 5 female collagenVI null mice were used and highest immunized mouse was used to make hybridomas.

2d. Ascites production of 2 hybridomas having highest activities in ELISA and immunoblotting.

Task 3. Verify the neutralizing activities of VIα3-C5monoclonal antibodies (1-12 months)

3a. Verify VIα3-C5 activities in induction of canonical β-catenin pathway in breast cancer cell lines (1-2 months): Perform immunoblotting or immunocytochemisty of β-catenin accumulation in VIα3-C5 transient transfected breast cancer cell lines compare with wild type.

3b. Verify neutralizing activities of VIα3-C5monoclonal antibodies in breast cancer cell lines (2-4 months): Measure canonical β-catenin pathway with or without VIα3-C5monoclonal antibodies treatment in VIα3-C5 transient transfected MCF7 breast cancer cell lines.

3c. Verify neutralizing activities of VIα3-C5monoclonal antibodies in MMTV-PyMT mice (4-12 months): Measure the activation of β-catenin pathway in the MMTV-PyMT mice with or without VIα3-C5monoclonal antibodies injection. 5 mice in each group will be used.

***Specific Aim3***. Compare the susceptibility to platinum-based cancer regimens in wild-type vs. collagen VI null mice in the MMTV-PyMT (6-24 months).

Task 1. Generate collagen VI null mice and breed with MMTV-PyMT mice (6-12 months).

1a. We already have collagen VI null mice (Completed).

1b. Breed collagen VI null mice with MMTV-PyMT. At least 7 to 10 mice in each group will be used.

Task 2. Characterize the collagen VI effects on cisplatin resistance in MMTV-PyMT mice model (9-24 months).

2a. Evaluate cisplatin drug toxicity and set up the optimal protocol of cisplatin treatment in wild-type and collagen VI null mice in the MMTV-PyMT background (9-18 months).

**2b. Tumor tissues or MET cells (mammary epithelial cells) from PyMT/COL6$^{+/+}$ vs. PyMT/COL6$^{-/-}$ mice will be implanted into WT mice. Cisplatin treatment will be performed on the tumor bearing mice when the tumor size reaches 5 mm diameter and treatment will be ceased when the tumor regression is apparent. (12-24 months)**

2c. Treat experimental cohorts with cisplatin and measure tumor volumes, rates of apoptosis, and expression levels of metallothioneins (18-24 months). To obtain quantitative results with significance, I will perform these experiments in triplicates using independent cohorts.

***Specific Aim4***. Examine the roles of collagen VI on synergistic effects between thiazolidinediones (TZDs) and cisplatin therapy (3-36 months).

Task 1. Examine the regulation of VIα3-C5 by TZDs treatment *in vivo* (3-8 months).

1a. Obtain experimental cohorts (3-6 months): At least 5 mice will be used in the experiments.

1b. Perform immunohistochemistry (IHC) with histology samples of mammary gland tissues of MMTV-PyMT mice with or without TZD treatments for 2 to 4 weeks (4-8 months).

Task 2. Compare cisplatin susceptibility in collagen VI null and wild type in the background of MMTV-PyMT mice with or without TZDs treatments (6-24 months).

2a. Obtain experimental cohorts (6-12 months): 7-10 mice in each group will be used in the experiments.

2b. Optimize the protocols of cisplatin treatment in combination with TZD as mentioned in Specific Aim3- Task 2 (12-18 months)

2c. Perform cohort experiments (18-24 months): Collect samples and analyze it.

**Task 3. Characterize the effects of VIα3-C5 neutralizing monoclonal antibodies in combination with TZDs to demonstrate synergistic enhancement susceptibility to cisplatin treatment for breast cancer (18-36 months).**

**3a. Tumor tissues or MET cells (mammary epithelial cells) from PyMT/COL6$^{+/+}$ mice will be implanted into WT mice.**

**3b. Perform the treatment with TZD and VIα3-C5 monoclonal antibodies in combination with cisplatin**

**3c. Collect samples and analyze the tumor progression and cisplatin susceptibility (24-36 months).**

***Specific Aim5***. Verify the relevance of VIα3-C5 levels in clinical samples (6-36 months).

Task 1. Verify the levels of VIα3-C5in breast cancer mouse subjects (6-12 months).

1a. Measure the VIα3-C5protein levels in MMTV-PyMT mice: Immunohistochemistry or immunocytochemistry will be investigated in mammary gland tissues of MMTV-PyMT mice during cancer progression with VIα3-C5 antibodies. Total 10 mice will be used in the experiments.

1b. Measure the circulating levels of VIα3-C5 (6-12 months): Development of ELISA method to detect circulating VIa3-C5 proteins in the serum samples of mouse and human breast cancer subjects. 10 samples in each group will be used in this experiment.

Task 2. Verify the levels of VIα3-C5in breast cancer human subjects (12-36 months).

2a. Collect human tissue samples from UTSW cancer tissue bank and analyze the levels of VIα3-C5 by immunohistochemistry using VIa3-C5 antibodies (12-18 months).

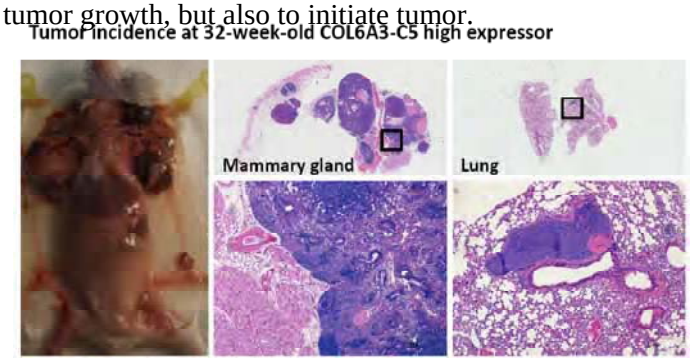
2b. Measure the VIα3-C5 levels in both serum and breast tumor samples of breast cancer patients at UTSW medical center (24-36 months).

2c. Measure the VIα3-C5 levels in human samples after platinum-based chemo-therapy in combination with TZDs to characterize VIα3-C5 effects on drug resistance (24-36 months). Our collaborator Dr. David Euhus in UTSW medical center will play a critical role in mediating these efforts.

## SUPPORTING DATA

### S1. MMTV-COL6A3C5 high-expressor develop tumor spontaneously.

High and low expressors of COL6A3-C5 transgenic mice were maintained until 12 months to see whether they have any phenotypic differences. Surprisingly, about 5 % of the high expressors develop tumors at 6 to 8 months-aged mice. This is specific to the transgene, since this is never seen in the low expressors or their control littermates. This result indicates that high levels of COL6A3-C5 are not only able to enhance tumor growth, but also to initiate tumor.




**S1. Phenotypic and histological analysis of high expresser of COL6A3-C5 transgenic mice.** The high expresser mice are examined at 32-week-old for analysis. Picture of whole body (left panel). Paraffin embedded mammary glands and lung tissues from the 32-week-old mice were stained with H&E. Images were obtained with a Nikon Coolscope. Higher magnification pictures for the regions highlighted by a square in the top are at the bottom of each picture.