AD_____

Award Number: W81WXH-06-1-0308

TITLE: Laminin-5 gamma2 chain in breast cancer metastasis

PRINCIPAL INVESTIGATOR: Shanshan Liu

CONTRACTING ORGANIZATION: Vanderbilt University
Nashville, TN, 37232

REPORT DATE: February 2009

TYPE OF REPORT: Annual Summary

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
01-02-2009	Annual Summary	1 FEB 2006 - 31 JAN 2009

4. TITLE AND SUBTITLE
Laminin-5 gamma2 chain in breast cancer metastasis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81WXH-06-1-0308

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Shanshan Liu

Email: shanshan.liu@vanderbilt.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Vanderbilt University
Nashville, TN, 37232

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

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

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Abstract on next page.

15. SUBJECT TERMS
Laminin-5(Ln-5), breast cancer, Ln-5 gamma2 monomer, Ln-5 heterotrimer, cancer progression

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	10	**19b. TELEPHONE NUMBER** *(include area code)*

Abstract:

To metastasize, cancer cells have to break through the basement membrane. Ln-5 is one o f the basement membrane proteins, consisting of three chains a3,B3 and y2. Ln-5 y2 chain continas DIII domain, a functional EGFR ligand, which can be released by MMP processing. It has been suggested by our lab that DIII domain may facilitate cancer progression by preventing anoikis. However, both the increased and decreased expression levels of Ln-5 subchains are reported in the literature. The fact that y2 chain exists in two different forms (as a secreted monomer, or as a part of the Ln-5 heterotrimer) leads us to hypothesize that those two forms may play different roles in cancer progression. What we report here is that removing Ln-5 heterotrimer (knocking down y2 chain by shRNA) promotes tumor progression by inducing Warburg effect in cancer cells.
The Warburg effect describes that cancer cells consume more glucose than normal cells by converting it to lactate. It has been shown in almost all type of cancer. Reduced secretion of Ln-5, by knock-down of its y2 subunit (LAMC2-kd), caused increased glucose uptake, lactate production, and cytoplasmic NAD(P)H levels. This metabolic shift was dependent upon increased plasma membrane GLUTI. A blocking antibody to the Ln-332 receptor, integrin a3B1, caused GLUTI translocation in control cells (LAMC2-ctrl_, pointing to a signaling pathway that regulates this anti-Warburg effect. LAMC2-kd cells produced tumors ~50 times larger than LAMC2-ctrl. Thus, we conclude that loss of contact with BM-associated ECM can unleash the Warburg effect, promoting tumor progression. The anti-Warburg effect by Ln-332 links ECM to metabolism and adds an unforeseen dimension to physiological functions of ECM and ECM receptors

Table of Contents

Introduction:

To metastasize, cancer cells have to break through the basement membrane. Ln-5 is one of the basement membrane proteins, consisting of three chains α3, β3 and γ2. Ln-5 γ2 chain contains DIII domain, a functional EGFR ligand, which can be released by MMP processing. It has been suggested by our lab that DIII domain may facilitate cancer progression by preventing anoikis.

What we noticed is that there are paradoxical data in regard of the role of Ln-5 in cancer progression. For example, both the increased and decreased expression levels of Ln-5 subchains are reported in the literature. The fact that γ2 chain exists in two different forms (as a secreted monomer, or as a part of the Ln-5 heterotrimer) leads us to hypothesize that those two forms may play different roles in cancer progression.

Therefore, the original aims are expanded and modified as the following two: A). To determine if the expression of Ln-5 γ2 monomer is positively correlated with breast cancer cell line tumorigenecity. B). To determine the role of Ln-5 γ2 chain in cancer progression when it is in the context of Ln-5 heterotrimer. Various phenotypes induced by removing Ln-5 γ2 chain were reported in last report, including the phenotype in glucose metabolism.

Cancer cells are different from normal cells dramatically in cell metabolism. The well known Warburg effect has been shown in almost all type of cancers. And it has been utilized clinically to detect cancer lesion. Therefore we focused on the metabolism phenotype found in 804G-kd cells in the past one year. The unexpected finding is that Ln-5 promotes tumor progression by inducing Warburg effect in cancer cells.

Body:

Accomplishments

To determine the role of Ln-5 γ2 chain in cancer progression in the context of Ln-5 heterotrimer.

Last year, we reported that removing Ln-5 γ2 chain induce tumor progression significantly in mice. To understand the underlying mechanism, various *in vitro* assays were utilized to compare 804G-ctrl (LAMC2-ctrl) and 804G-kd (LAMC2-kd) cells. Phenotypes were summarized and reported in last report.

Among these phenotypes, what interested us most is the phenotype in cell metabolism. It is known that cancer cells and normal cells are different in metabolism. The most famous cancer cell phenotype in metabolism is the Warburg effect, which was described 50 years ago and applied in cancer lesion detection clinically. Therefore, we focused on how Ln-5 facilitates cancer progression through regulating cell metabolism using 804G-ctrl, 804G-kd, MCF10A and its derivative cell line CA1a and CA1d.

In 804G cells:

1. Suppression of Ln-332 induces a metabolic shift

It has been described in last year report that LAMC2-kd cells have higher Glucose uptake rate (GU) and lactate production rate (LP) compared to LAMC2-ctrl cells by novel assays based on a microphysiometer. To confirm those phenotypes, the classical colorimetric assays were performed. Increased GU and LP were observed in LAMC2-kd cells compared to LAMC2-ctrl in the colorimetric assays as well. The increased GU and LP are consistent with what has been described in the classical Warburg effect.(1)

Warburg effect is featured by hyperactive glycolysis in cytoplasm, which is reflected by the level of NADPH. The higher the NADPH level, the more active the glycolysis process in cytoplasm is. NADPH is autofluorescence.(2) To confirm if removing Ln-5 γ2 chain will induce Warburg effect, NADPH autofluorescence was imaged by using two-photon microscope and quantified accordingly in LAMC2-ctrl and LAMC2-kd cells. An increase in cytoplasmic NADPH was observed in LAMC2-kd cells compared to LAMC2-ctrl cells.

Our data indicate that, consequent to loss of Ln-332 secretion, carcinoma cell glucose metabolism switches to aerobic glycolysis, the classic Warburg effect.

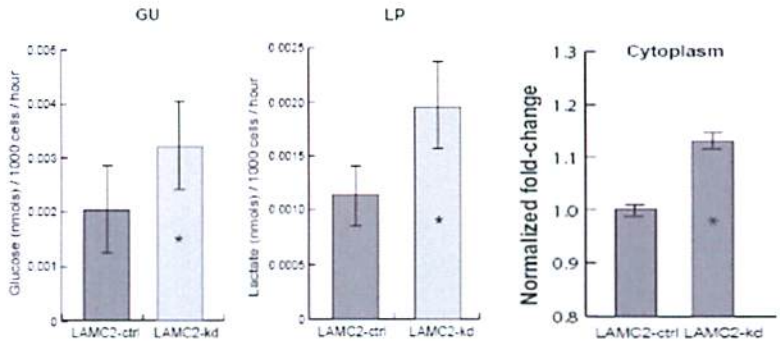


Fig. 1 A Quantification of glucose uptake (GU) and lactate production (LP) rates after 24 h by Biovision colorimetric kits. Data are presented are mean +/- standard deviation. LAMC2-kd cells had significantly higher GU and LP measures than ctrl cells (N=6, p=0.016 and N=6, p=0.022, respectively) B. Data are presented are mean +/- standard deviation. LAMC2-kd cell measurements were significantly greater than LAMC2-ctrl in cytoplasm (N=6, p=0.006), but not in mitochondria (N=6; p=0.695).

2. GLUT1 is increased in 804G-kd (LAMC2-kd) cells.

Glucose uptake can be regulated by different molecules, including glucose transporters (GLUT) and hexokinase etc.(3) Total GLUT1 expression was increased in LAMC2-kd cells by western blotting compared to LAMC2-ctrl. Immunostaining data further indicated that LAMC2-kd cells have more GLUT1 on cell surface. Cell surface labeling confirmed that more surface GLUT1 in LAMC2-kd compared to LAMC2-ctrl cells.

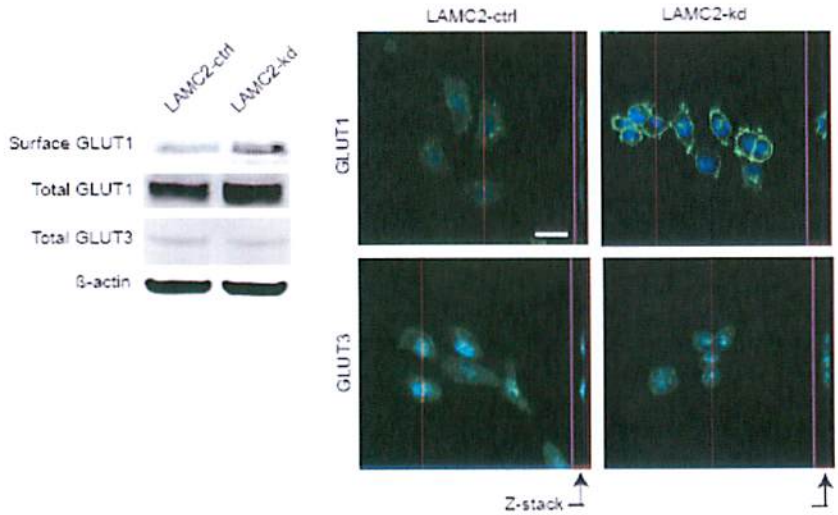


Fig. 2 A Representative western blot of surface GLUT1 and total GLUT1 and GLUT3 protein levels in LAMC2-ctrl and LAMC2-kd cells. Blots were probed for β-actin as loading control. B Representative images of cells stained for GLUT1 (top row; green), GLUT3 (bottom row; green), and

3. Integrin beta1 is involved in GLUT1 localization

Integrin α6β4 and α3β1 are receptors for Ln-5. To investigate whether they are involved in the GLUT1 localization phenotype in 804G-kd cells, those two receptors were either knocked down by shRNA (integrin β4, knockdown cells were named as Vector-ctrl, ITGB-kd) or blocked by blocking

antibody (Ha2/5, integrin β1 blocking antibody). Knocking-down integrin β4 has no impact on GLUT1 localization. However, Integrin β1 blocking antibody treated 804G-ctrl cells acquired more GLUT1 on surface, which is similar to 804G-kd cells. Our data suggest that Integrin β1 is involved in GLUT1 localization.

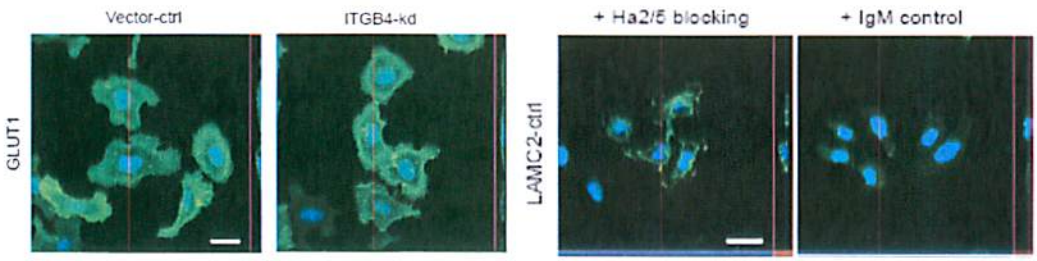
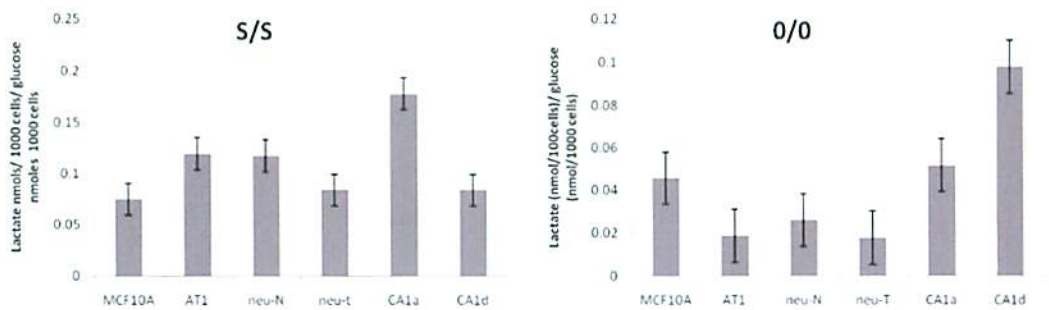


Fig3. (A) Representative images of GLUT1 (green) and GLUT3 (green) immunostaining in Vector-ctrl and ITGB4-kd cells, with nuclear marker (blue) (scale bar=10 μm) (B) Representative images of LAMC2-ctrl cells stained for GLUT1 (green) and nuclei (blue) on Ha2/5 or IgM.. Ha2/5 integrin β1 blocking antibody or IgM control was added to cells 3 h after they attached to dishes. IgM was used as treatment control (scale bar=10 μm).

<u>In MCF10A, CA1a, CA1d, NeuN, NeuT cells (in collaboration with Mohamed Hassanein)</u>

Glucose uptake and lactate production were measured under two conditions (with or without serum supplement S/S and 0/0) in MCF10A, CA1a, CA1d, NeuN and NeuT cells. Glycolytic index (produced lactate/ consumed glucose) was calculated as described before.(4) Our data suggest that more tumorigenic breast cancer cell lines are more glycolytic, in another word, under the Warburg

Fig4. Glycolytic rates were calculated by (A) the glycolytic rates of MCF10A and its derivative cell lines in the presence of serum and supplements. (B) the glycolytic rates of MCF10A and its derivative cell lines in the absence of serum and supplements.



effect.

Key research accomplishment

A. Downregulation of Ln-5 γ2 by shRNA in 804G cells which synthesize Ln-5 heterotrimer induced Warburg effect (increased glucose uptake, lactate production and cytoplasmic NAD(P)H level)

B. Downregulation of Ln-5 γ2 by shRNA in 804G cells induced increased total GLUT1 expression and surface GLUT1, which might contribute to the Warburg effect.

C. Integrin β1 blocking antibody induced increased surface GLUT1 in 804G cells, which suggests that integrin β1 is involved in GLUT1 localization.

D. tumorigenic breast cancer cell lines have higher glycolytic rates (indicated by the

glycolytic index) than the non-tumorigenic cell line MCF10A.

Reportable outcomes

The work so far done in this project has been presented in the department seminar at Vanderblit University.

Manuscript submitted

Conclusions

Overexpression of Ln-5 γ2 chain had been associated with increased tumorigenesis, in breast as well as other cancers. Surprisingly, we have found that deletion of γ2 chain expression, instead, can dramatically upregulate tumorigenesis in *in vivo* models. Ln-5 heterotrimers have a tumor suppressor role in our model. Our current data suggest that Ln-5 can repress the Warburg effect through Integrin α3β1. By removing g2 chain of Ln-5 (elimination of Ln-5 heterotrimer), cells were undergoing Warburg effect which was indicated by increased GU, LP and cytoplasmic NAD(P)H level.

REFERENE

Warburg, O. (1956a). On respiratory impairment in cancer cells. Science *124*, 269-270.

Bennett, B. D., Jetton, T. L., Ying, G., Magnuson, M. A., and Piston, D. W. (1996). Quantitative subcellular imaging of glucose metabolism within intact pancreatic islets. J Biol Chem *271*, 3647-3651.

Berditchevski, F., Chang, S., Bodorova, J., and Hemler, M. E. (1997). Generation of monoclonal antibodies to integrin-associated proteins. Evidence that alpha3beta1 complexes with EMMPRIN/basigin/OX47/M6. J Biol Chem *272*, 29174-29180.

Bissell, M. J. (1976). Transport as a rate limiting step in glucose metabolism in virus-transformed cells: studies with cytochalasin B. J Cell Physiol *89*, 701-709.

Bissell, M. J., Hatie, C., and Rubin, H. (1972). Patterns of glucose metabolism in normal and virus-transformed chick cells in tissue culture. J Natl Cancer Inst *49*, 555-565.

Abstract:

To metastasize, cancer cells have to break through the basement membrane. Ln-5 is one of the basement membrane proteins, consisting of three chains α3, β3 and γ2. Ln-5 γ2 chain contains DIII domain, a functional EGFR ligand, which can be released by MMP processing. It has been suggested by our lab that DIII domain may facilitate cancer progression by preventing anoikis. However, both the increased and decreased expression levels of Ln-5 subchains are reported in the literature. The fact that γ2 chain exists in two different forms (as a secreted monomer, or as a part of the Ln-5 heterotrimer) leads us to hypothesize that those two forms may play different roles in cancer progression. What we report here is that removing Ln-5 heterotrimer (knocking down γ2 chain by shRNA) promotes tumor progression by inducing Warburg effect in cancer cells.

The Warburg effect describes that cancer cells consume more glucose than normal cells by converting it to lactate. It has been shown in almost all type of cancers. Reduced secretion of Ln-5, by knock-down of its γ2 subunit (LAMC2-kd), caused increased glucose uptake, lactate production, and cytoplasmic NAD(P)H levels. This metabolic shift was dependent upon increased plasma membrane GLUT1. A blocking antibody to the Ln-332 receptor, integrin α3β1, caused GLUT1 translocation in control cells (LAMC2-ctrl), pointing to a signaling pathway that regulates this anti-Warburg effect. LAMC2-kd cells produced tumors ~50 times larger than LAMC2-ctrl. Thus, we conclude that loss of contact with BM-associated ECM can unleash the Warburg effect, promoting tumor progression. The anti-Warburg effect by Ln-332 links ECM to metabolism and adds an unforeseen dimension to physiological functions of ECM and ECM receptors

Subjective terms:
Laminin 5 (Ln-5)
Breast cancer
Warburg effect
Glucose transporter 1 (GLUT1)
Integrin alpha3beta1
Cancer progression

Personnel:
Shanshan Liu

Publications:
None