AWARD NUMBER: W81XWH-14-1-0108

TITLE: Integrin Alpha-v and HER2 in Breast Cancer Brain Metastasis

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REPORT DATE: October 2015

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2015	Annual	30 Sep 2014 - 29 Sep 2015

4. TITLE AND SUBTITLE
Integrin alpha v and HER2 in breast cancer brain metastasis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-14-1-0108

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

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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
Brain metastasis is common in patients with breast cancer, and leading to cognitive problems, poor quality of life, and rapid mortality. Despite the fact that brain metastases occur in 5% of breast cancer patients overall and 30% in patients with high expression of HER2, there are few treatment or prevention options. Integrins are a large family of cell surface proteins that are important for the internal structure of cells and also cell interactions with other cells and the biological microenvironment. We have evidences showing that the specific integrin protein αv may be particularly important in cancer metastasis. First, breast cancer cell clones with high levels of αv integrin migrate rapidly and infiltrate in rat brain, while clones with low αv integrin do not, and αv integrin interacts with HER2 to have the highest level of mobility and invasion. Second, decreasing αv integrin also decreases the localization of HER2 in the cell membrane where it is active, and increases the amount of HER2 in lysosomes where it is broken down. Finally, we have new preliminary results showing that metastatic-prone: αv-integrin positive of MDA-MB231BR clones are more resistant to chemo drugs than αv-integrin negative cells with same genetic and molecular origin. Our results suggest that αv integrin has a dual role in breast cancer brain metastasis, first to increase cancer cell binding and invasion into the brain, and second to provide the proper cellular location for HER2 activity.

15. SUBJECT TERMS

Nothing listed

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	U	10	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION:

Brain metastasis is common in patients with breast cancer, occurring sometimes years after diagnosis, and leading to cognitive problems, poor quality of life, and rapid mortality. Despite the fact that brain metastases occur in 5% of breast cancer patients overall and 30% in patients with high expression of HER2, there are few treatment or prevention options. Our objective is to investigate the two aspects of αv integrin in breast cancer brain metastases using cultured cells and rat models of metastasis, with the overall goals of preventing breast cancer brain metastasis and improving therapy and survival. Specific Aim 1 is to investigate the role of αv integrin in the development of breast cancer brain metastases and assess the potential of blocking αv integrin as a mechanism to prevent breast cancer brain metastasis. Specific Aim 2 is to examine the interactions of αv integrin and HER2 in breast cancer brain metastasis and determine their combined role in the metastatic phenotype and response to HER2-targeted therapies.

KEYWORDS: Brain metastasis, breast cancer, αv integrin, HER2, intetumumab, targeted therapy

ACCOMPLISHMENTS:

1. What were the major goals of the project?

Aim 1. The role of αv integrin in the development and prevention of breast cancer brain metastases.
Aim 2. Interactions of αv integrin and HER2 in breast cancer brain metastases.

2. What was accomplished under these goals?

a. Role of αv integrin in the development of breast cancer brain metastases. Knockdown of αv-integrin in MDA-MB231BR clones altered the actin cytoskeleton and cell morphology. After intracerebral inoculation, cells expressing high levels of both αv-integrin and HER2 showed a diffusely infiltrative tumor phenotype, while cells deficient in αv-integrin and/or HER2 showed a compact tumor growth phenotype. In the αv-integrin positive/HER2 positive tumors, infiltrative growth was 57.2 ± 19% of tumor volume, compared to only 5.8 ± 6.1% infiltration in the double (αv-integrin and HER2) deficient tumor cells (**Figure 1**). This work addresses aims 1 and 2 of the grant, and has been published: Lal et al., 2015. In order to investigate the role of αv integrin in the development of breast cancer brain metastases in a hematogenous metastases model, we intra-carotidly infused 10^6 cells of MDA-MB231BR clones with different levels of αv integrins. Unfortunately, female nude rats did not wake up and survive after cell infusion. We are investigating the mechanisms involved in clotting and unexpected death. We extended characterization of breast cancer brain metastases in both hematogenous and xenograft models using 6 additional breast cancer cell lines (MCF7, MDA-361, BT474, SKBR3, HCC1569 and HCC1954). We found that HCC1954 cells with high endogenous αv integrin and HER2 form multiple brain metastases with mean survival time of 39 vs 52 days in MDA-MB231BR-HER2 (MM2BH; Wu et al., 2012). This work addresses aim 1 of the grant. Manuscript is in preparation.

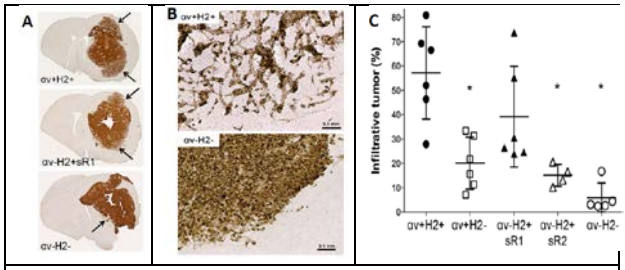

Figure 1. **Characterization of in vivo growth of brain-trophic breast cancer cells**. Brain tumors of 2BH clones were assessed by immunohistochemistry for human mitochondrial antigen 5 wks after intracerebral implantation. **A**) Representative sections from different tumors. Arrows indicate regions of tumor infiltration. **B**) Brain tumor margin showing the infiltrative phenotype in αv+H2+ and αv-H2- tumors. **C**) Quantification of the percent of tumor showing the infiltrative phenotype. Each point indicates the mean value from 2-6 central sections from each rat brain. *p<0.05 compared to control.

b. Effect of αv integrin on cell invasion and migration in vitro. The αv integrin knockdown clones showed significant inhibition of motility compared to control cells; over 80% lower migration and invasion was observed for αv-H2+sR1 cells and 73% and 46% decrease in migration and invasion

respectively, for αv-H2+sR2 cells. The αv+H2- cells, with 21% lower αv-integrin content than control cells, also showed 60% lower migration and 40% lower invasion. The most pronounced decrease in migration and invasion, 90% lower than control cells, was recorded for αv-H2- cells ($p<0.01$). We found that intetemumab decreased cell adhesion and changed cell morphology of HCC1954 breast cancer cells. In addition, cilengitide inhibited MDA-MB231BR cell wound healing assay in dose- and time-dependent manner using IncuCyte ZOOM live cell imaging machine (ESSEN Bioscience; **Figure 2**).

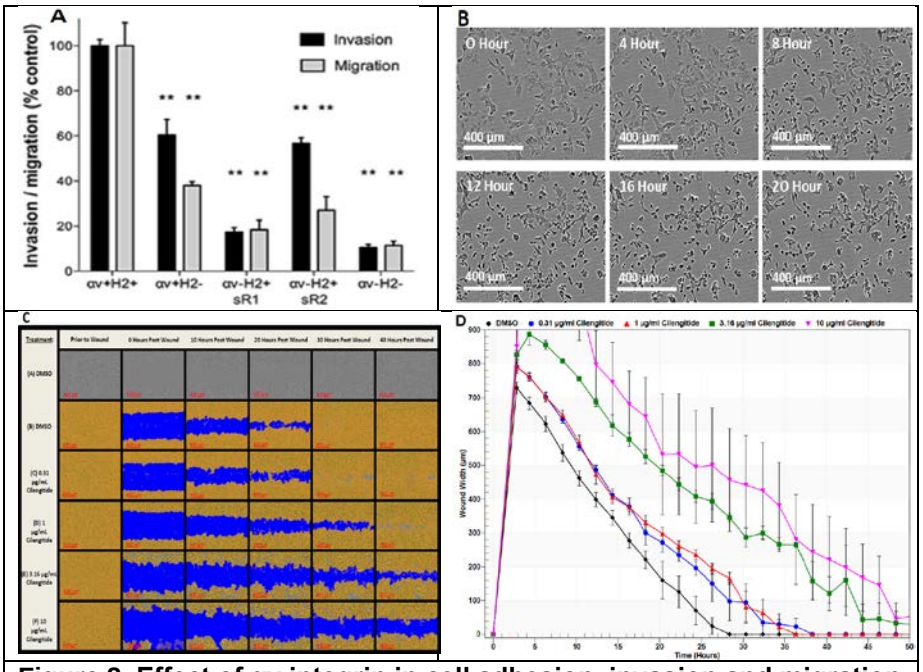

Figure 2. **Effect of αv integrin in cell adhesion, invasion and migration in vitro**. **A**) The motility of MM2BH cell lines was examined in transwell assays in the absence (migration, 8h) and presence of Matrigel extracellular matrix (invasion, 64h) using FBS as chemoattractant. The graph represents average motility from six replicates from independent experiments. Bars show mean ± SEM. **p<0.01 compared to control. **B**) Intetemumab (3.16 µg/mL) decreased cell adhesion and changed cell morphology of HCC1954 breast cancer cells. **C**) Real time live cell imaging of MM2BH cells treated with cilengitide (0, .3, 1, 3, and 10 µg/mL) using IncuCyte ZOOM machine. **D**) Quantification of the gap closure data in (**C**).

c. Interactions of αv integrin and HER2 in breast cancer brain metastases. We found that HER2 co-immunoprecipitated with αv integrin in 4 breast cancer cell lines (MMB2H, HCC1954, SKBR3, and MDA361) in vitro, suggesting they physically complex in cells. Knockdown of αv-integrin altered HER2 localization from its normal membrane position to a predominantly lysosomal localization (**Figure 3**). This work addresses aim 2 of the grant, and has been published: Lal et al., 2015. In addition to HER2, αv-integrin also co-precipitated with EGFR of breast cancer cells in vitro. We are investigating if this observation is true in vivo as well by using intracerebral inoculation of HCC1954 human breast cancer cells in athymic rats.

d. Role of αv integrin of breast cancer cells in chemo-resistance. To explore the potential mechanisms between αv-integrin of breast cancer and brain metastases, we found that metastatic-prone: αv-integrin positive of MDA-MB231BR clones are more chemo-resistant to cisplatin, 5-fluorourical, paclitaxel, and temozolomide than αv-integrin negative cells with same genetic and molecular origin. Pre-treatment of αv-integrin positive MDA-MB231BR cells with intetumumab (10 µg/mL) for 1 hr in vitro enhanced chemo-induced cytotoxicity of cisplatin, 5-fluorourical and paclitaxel but not temozolomide. In addition, cisplatin increased cellular αv-integrin level of MDA-MB231BR cells in dose-dependent manner in vitro (**Figure 4**). This work addresses both aims of the grant. Manuscript is in preparation. However, we did not find any dose-dependent response of HER2 targeting therapies (traszumumab, T-DM1) in either αv-integrin positive or negative breast cancer cells in vitro. Neither T-DM1 (3.6 mg/kg IV) alone nor combined with intetumumab (30 mg/kg IV; 3 d prior to T-DM1) did not reduce tumor volume in both MDA-231BR-HER2 and HCC 1954 breast cancer xenograft models. We will try again in T-DM1 from different providers/vendors.

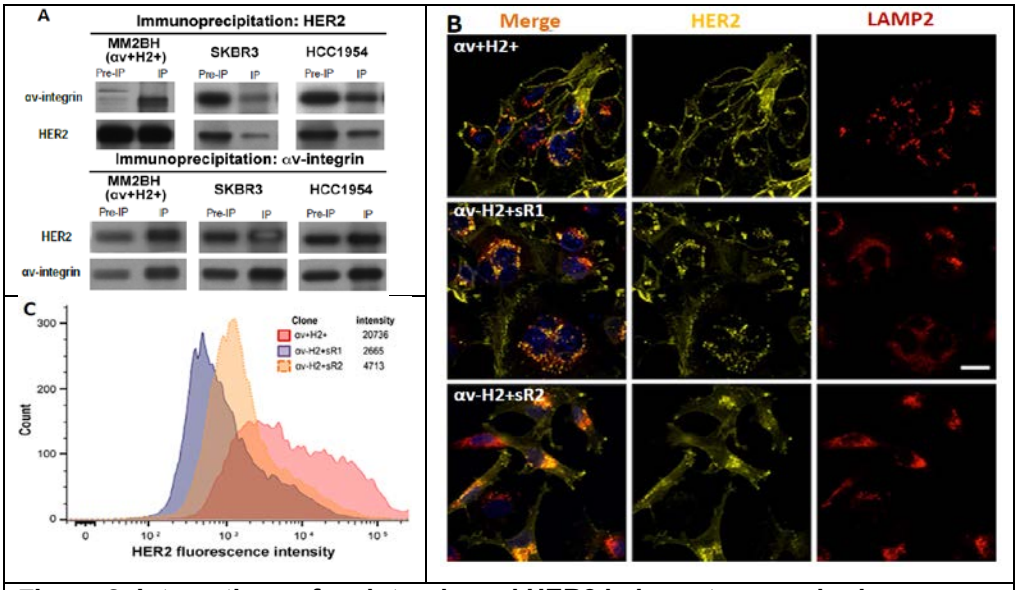

Figure 3. **Interactions of αv integrin and HER2 in breast cancer brain metastases**. **A**) Co-immunoprecipitation of αv-integrin and HER2 proteins in breast cancer. Breast cancer cell lysates were incubated separately with antibodies against HER2 and αv-integrin. Components of the immunocomplex precipitate were analyzed by Western blotting for αv-integrin and HER2 proteins. Protein levels are shown in whole lysate (pre-IP), and the immunoprecipated protein complex (IP). **B**) Localization of HER2 in αv-integrin knockdown breast cancer cells. MM2BH cell clones grown on glass coverslips were fixed, permeabilized and immunostained. HER2 co-localizes with lysosomes in αv-integrin knockdown cells. Yellow: HER2, red: lysosome-associated membrane protein2 (LAMP2), blue: nucleus. The images are representative of three independent experiments. Scale bar = 20μm. (C) Flow cytometry analysis of surface HER2 protein expression in live MM2BH clones.

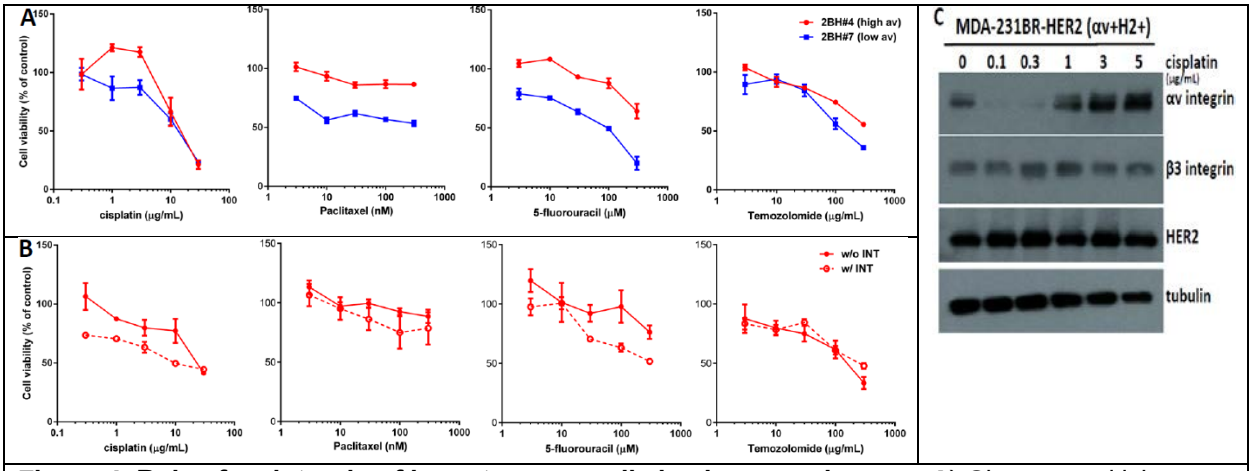

Figure 4. **Role of αv integrin of breast cancer cells in chemo-resistance**. **A**) Chemosensitivity between αv+H2+ and αv-H2+sR1 MM2BH cells. MM2BH cells (10 cells/well) were seeded and treated with different doses of chemo drugs (cisplatin, paclitaxel, 5-flouroucial and temozolomide. Cell viability was measured at 1 d with WST assay. **B**) Intetumumab pretreatment enhanced αv+H2+ MM2BH cytotoxicity induced by chemo drugs. Cells were pretreated with intetemumab (10 μg/mL) for 1 h prior to adding chemo drugs. **C**) Cisplatin treatment increased cellular αv not β3 integrin or HER2 protein expression. Whole cell lysate was collected and subjected to Western blotting at 1d after treatment. Tubulin protein is used as loading control.

3. What opportunities for training and professional development has the project provided?

This project has provided a learning environment for one of our laboratory's graduate student on numerous experimental methods and metastatic brain tumor biology.

4. How were the results disseminated to communities of interest?

This translational preclinical research proposal addresses the overarching challenge of identifying what drives breast cancer growth and metastasis, and identifying why some breast cancers become life-threatening metastases. The proposed research moves beyond an incremental advancement and will have an impact on breast cancer patients by generating data that will allow us to move into clinical trials. These studies are not expected to directly end breast cancer, but will accelerate progress toward ending the devastating complication of brain metastases, which will improve the quality of life and extend survival in breast cancer patients.

5. What do you plan to do during the next reporting period to accomplish the goals?

For Aim1, we will continue to determine the best treatment regime (timing, dose and combination) of αv integrin and HER2 targeting drugs in preventing/treating breast cancer brain metastases by using HCC1954, MDA-231BR-HER2 and other transduced clones. Number of brain metastases and total tumor burden will be used to analyze the treatment efficacy. Studies will be completed for Aim2, to assess the interaction of αv integrin with HER2 and related families both in vitro and in vivo. Additionally, using the clonal cell lines we established with αv-integrin knockdown, we plan to assess the role of αv-integrin in breast cancer cell association with cerebral vasculature in the rat brain to better understand the mechanism by which αv-integrin contributes to brain tumor formation. We will perform co-immunoprecipitation and immunofluorescent staining followed by confocal microscopy on brain tumor xenografts of the breast cancer cell lines to confirm our protein interaction findings in vivo. We will also investigate if the interaction of αv integrin and HER2 will be altered by adding αv integrin targeting drugs (intetumumab or cilengitide) or HER2/EGFR inhibitors (trastuzumab or lapatinib) in vitro and in vivo. Additionally, the role of αv integrin in development of breast cancer brain metastases via chemo-resistant pathway will be further investigated.

IMPACT:

1. What was the impact on the development of the principal discipline(s) of the project?

The proposed studies will delineate mechanisms of αv integrin in metastatic phenotype of breast cancer cell and its interactions with the HER2 growth factor receptor. Our investigation of αv integrins and HER2 in breast cancer brain metastases will also have significant clinical impact in three major areas. First, our findings in the rat model will establish the potential role of αv integrin as a biomarker for breast cancer patients who will develop brain metastases. Second, we will demonstrate the potential of targeting αv integrin with the intetumumab antibody as a mechanism to decrease brain metastasis, which could provide a clinically relevant prevention strategy in breast cancer patients. Third, understanding the interactions of αv integrin and HER2 may aid in the identification of those patients who could benefit from therapy and provide possible mechanisms to improve HER2-targeted therapy.

2. What was the impact on other disciplines?

The interactions of αv integrin and HER2 or other growth factor receptors in breast cancer brain metastasis maybe a novel discovery for protein chemistry and biological functions. To explore the potential mechanisms between αv-integrin of breast cancer and brain metastases, we also found that metastatic-prone: αv-integrin positive of MDA-MB231BR clones are more resistant to chemo drugs than αv-integrin negative cells with same genetic and molecular origin. Cisplatin treatment increased cellular

αv-integrin protein expression. These findings potentially link between chemotherapy treatment, cancer stem cells and development of distant metastases.

3. What was the impact on technology transfer?

Nothing to report.

4. What was the impact on society beyond science and technology?

The proposed research will lead to a solution to the overarching challenge by demonstrating that αv integrin is a biomarker of likely brain metastasis, a potential therapeutic target in HER2-positive breast cancer, and a possible prophylactic agent for the prevention of breast cancer brain metastases. The findings from this research will lead to clinical trials to prevent and treat brain metastasis in breast cancer patients.

CHANGES/PROBLEMS:

1.Changes in approach and reasons for change

No, the major goals have not changed since the initial competing award.

2. Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to report.

3. Changes that had a significant impact on expenditures

Nothing to report.

4. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

No, there are no significant changes in use or care of vertebrate animals, biohazards and select agents. There will be no human subjects involved in this study.

PRODUCTS:

Manuscript publication: Lal S, Kersch C, Beeson KA, Wu YJ, Muldoon LL, Neuwelt EA. Interactions between αv-integrin and HER2 and their role in the invasive phenotype of breast cancer cells in vitro and in rat brain. PLoS One 10(7):e0131842, 2015

Poster presentation at the Cerebral Vascular Biology meeting in Paris, France July 2015

Presentation and poster at OHSU Blood-Brain Barrier Program annual meeting, March 2015

Poster at Tumor Immunology and Immunotherapy: A New Chapter, December 2014

Presentation and poster at OHSU Blood-Brain Barrier Program annual meeting, March 2014

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

1.What individuals have worked on the project?

Name:	Edward Neuwelt
Project Role:	Principal Investigator
Researcher Identifier:	
Nearest person month worked:	12
Contribution to Project:	Dr. Neuwelt, a principal investigator, oversees the overall project.
Funding Support:	

Name:	Leslie L Muldoon
Project Role:	Project coordinator
Researcher Identifier:	
Nearest person month worked:	12
Contribution to Project:	Dr. Muldoon supervises the project and analyzes research data.

Name:	Yingjen Jeffrey Wu
Project Role:	Sr. Research Associate
Researcher Identifier:	
Nearest person month worked:	12
Contribution to Project:	Dr. Wu has performed work in the areas of both in vitro and in vivo animal studies.

Name:	Cymon Kersch
Project Role:	Graduate Student
Researcher Identifier:	
Nearest person month worked:	12
Contribution to Project:	Ms. Kerch has performed work in transducing cell lines with siRNA and immunoprecipitation.

Name:	DreeAna Morris
Project Role:	Research Assistant
Researcher Identifier:	
Nearest person month worked:	6
Contribution to Project:	Ms. Morris has performed work in preparing the tissue samples and immunohistochemistry.

2. Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

3. What other organizations were involved as partners?

Organization Name: Janssen R&D
Location of Organization: 145 King of Prussia Road, Radnor, PA 19087
Partner's contribution to the project:
In-kind support: provide Intetumumab (CNTO 95; anti-human α_v antibody)

SPECIAL REPORTING REQUIREMENTS

Not Applicable

APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc. Reminder: Pages shall be consecutively numbered throughout the report.* ***DO NOT RENUMBER PAGES IN THE APPENDICES.***