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14. ABSTRACT Breast cancer develops from epithelial lesions in breast ducts and lobules that become invasive and can be metastatic. Breast cancer is a disease of uncontrolled cell division. Cell division normally creates two genetically identical daughter cells through severing of a cytoplasmic bridge that interconnects them. The midbody is an organelle involved in severing. Previously midbodies were thought to be lost from cells after division, but we show they can be retained. Here we show that MBs exhibit stem cell properties and are in breast cancer stem cells (Task 1). They are scaffolds for anchoring breast cancer oncogenes, tumor suppressors and breast cancer stem cell proteins. Increasing MB+ cells increases in vitro tumor potential (Task 2). We activated a MB-degradation pathway that decreased MBs and tumorigenic properties of breast cancer cells (Task 3). Because MBs are in breast cancer stem cells, we believe this MB targeting strategy may be an innovative strategy for therapies focused on the most difficult of all tumors, those thought to be caused by cancer stem cells, namely recurrent, resistant and metastatic cancers.					
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TABLE OF CONTENTS

	Page
Introduction.....	3
Body.....	3-6
Key Research Accomplishments.....	7
Reportable Outcomes.....	7-8
Conclusion.....	9
References.	9
Appendices.....	none

INTRODUCTION. Breast cancer develops from epithelial lesions in breast ducts and lobules that become invasive and can be metastatic. Breast cancer is a disease of uncontrolled cell division. Cell division normally creates two genetically identical daughter cells through severing of a cytoplasmic bridge (Fig. 1, p. 6) that interconnects them. The midbody (Figs. 2, 3) is an organelle involved in severing. Previously midbodies were thought to be lost from cells after division, but we show they can be retained, accumulated and increased with tumor grade (Fig. 2-6). The long-term goal of this project is to identify putative MB-containing breast cells and target them for chemotherapeutic elimination of breast cancer. This Idea Expansion Award is designed to test this idea. We will determine if MBs are present in putative breast cancer stem cells (CSCs) from multiple breast cancer cell lines and tumor types (Task 1), isolate MB-containing breast cancer cells and directly test them for tumorigenic potential in mice (Task 2) and determine if degradation of MBs in breast cancer cells diminishes or eliminates their tumorigenic potential (Task 3). These aims are a logical extension of our previously funded breast cancer grant.

BODY (Figures 1-12, see below, page 6)

Task 1. Test whether MBs are present in breast CSCs in vitro and in vivo.

a. Isolate breast CSCs from cell lines based on the side population and test for enrichment of MBs. We confirmed that MBs were present in putative breast cancer stem cells using the side population approach and MCF cells (SP vs MP, n=4 experiments compared with normal breast epithelial cells, example, Fig. 7). We have confirmed this in other breast cancer cell types (MDA-MB-231, others).

b. Breast CSCs were also isolated from breast cancer cell lines based on surface markers and tested for enrichment of MBs (example, CD44+ CD24- /low, ALDH, Fig. 11). As shown by Morrison and co-workers (Al-Hajj et al., 2003), CD44+ CD24, lineage- appears to be an acceptable combination to use for breast CSCs. In fact, we showed that this subset of cells also has increased MBs; we are testing for tumor-like features in these cells.

c. Determine whether breast tumors are enriched in MBs and increase with increasing tumor grade. We have shown by repeated immunohistochemical staining of mouse and human tumors and but not adjacent nontumor have MBs (Fig. 5) increase with tumor grade. We are testing a larger sample group to get statistically significant data sets.

During the course of these studies, we made a startling discovery that may provide new and highly innovative opportunity for breast cancer therapies. We identified a large number (n=29) canonical oncogenes and tumor suppressors localized to the MB. These include breast cancer specific proteins as well as proteins that are associated with many cancers (examples: BRCA1, BRCA2, p53, ErbB2, Akt, Cdh1, Fig. 12). This new unexpected is important for several reasons. First, it provides a molecular mechanism for the tumorigenic phenotype of MBs; Second, it will lead to a high profile manuscript; Third, it generates a new line of breast cancer research in my laboratory for the future; Fourth, it may serve as a novel therapeutic method for treating breast cancer; Finally, it will be the focus of additional proposal submissions to garner funding based on this discovery.

Task 2. Test the tumorigenic potential of MBs.

a. Test MB+ cells isolated based on MB fluorescence and flow cytometry for growth in soft agar and tumor induction. Using MB+ cells isolated by flow cytometry, we observed an increase in soft agar growth, multicellular breast cancer spheroids, and clonal growth of spheroids (Fig. 8). 100-200 cells were capable of making breast tumor spheroids in vitro compared to MB-low controls. We are retesting and confirming in vitro tumorigenicity using the spheroid assay and by serial passaging. Once we complete the in vitro assays, we will begin testing in mice.

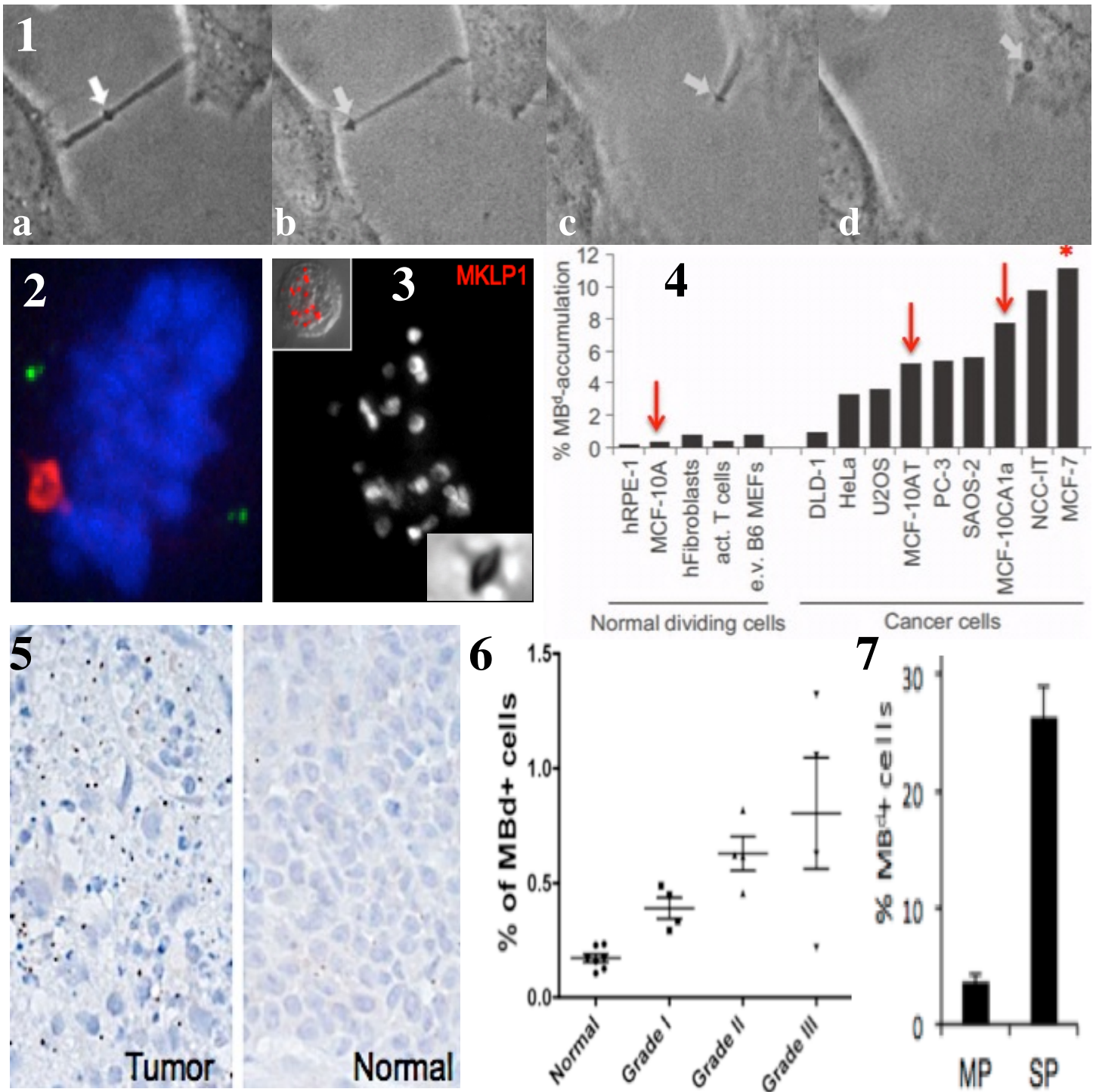
b. Test if NBR1 shRNA increases MB+ cells and growth in soft agar. We show that NBR1 depletion blocks autophagic degradation of MBs (see Fig. 9) and increases the tumor-like properties of cells (Fig. 8). Under separate funding, we performed proteomics to identify NBR1 interacting proteins. We found a protein that will be very useful in this study and for future therapeutic purposes, the A major discovery in this subaim is the finding of another member of the NBR1 MB-autophagy pathway, *NipSnip2*. NipSnip2 depletion has an enhanced effect on MB degradation.

Task 3. Test if specific targeting of MBs for autophagic degradation is a therapeutic strategy for breast cancer.

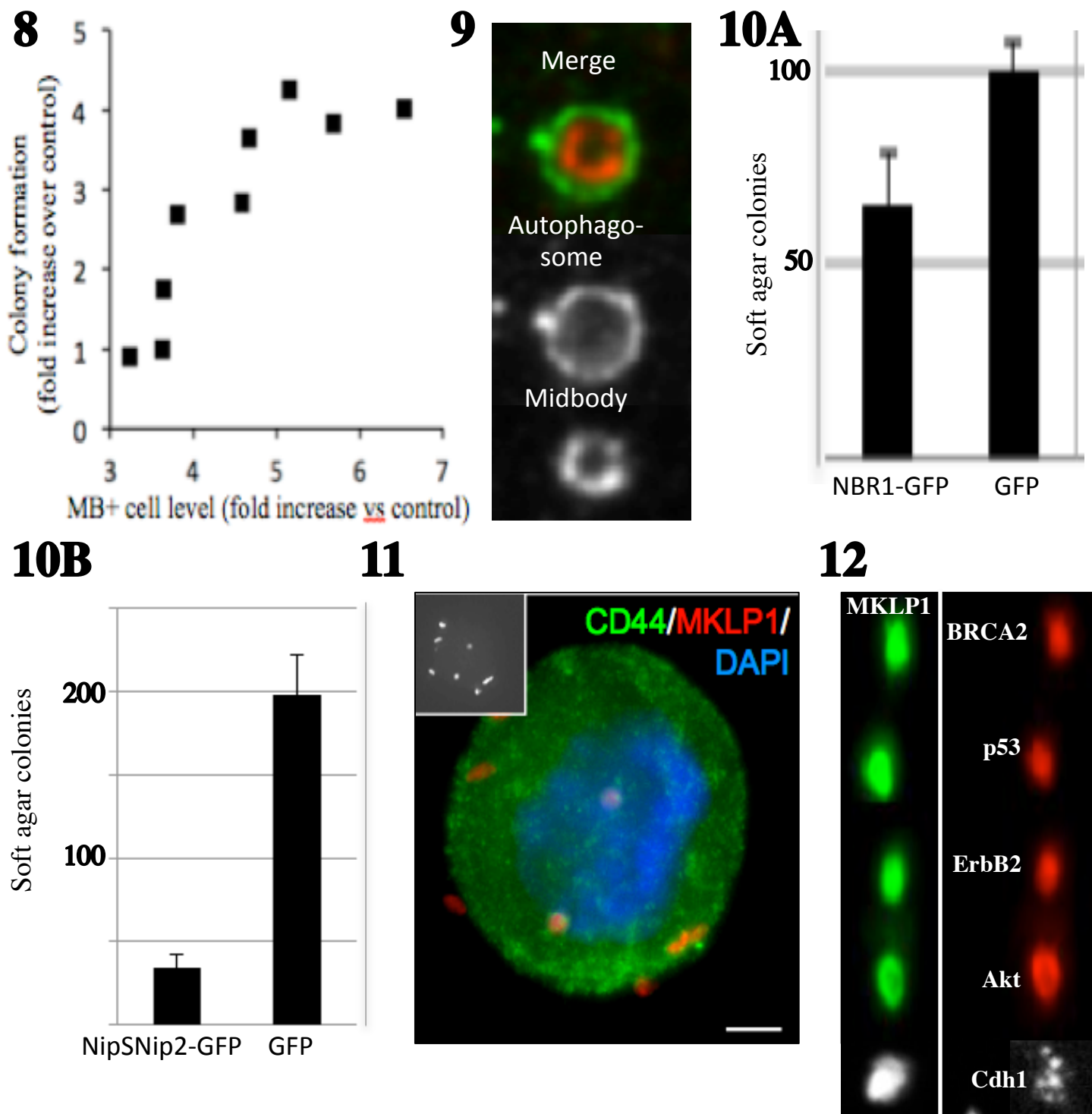
a. Test if NBR1-mediated autophagic degradation of MBs by NBR1 expression inhibits soft agar growth. We showed that NBR1 depletion and the resulting decrease in MB+ cells decreases in vitro tumor potential (Fig. 10A). These studies will be performed using the spheroid assay and mice during the next funding period.

b. We show here that NipSnip2 expression dramatically increases MB degradation leading to decreased tumorigenicity. It is more efficacious than NBR1 in this regard (Fig. 10B).

c. Future studies will utilize a breast cancer model crossed to NBR1 transgenics to test for tumor elimination.



Figures 1-7. **1)** Midbody in intercellular bridge connecting 2 MCF7 cells (a, arrow) moves left (b) then bridge is severed (c) sending the MB on a cytoplasmic tether to the cell on the right (c) where it resides in the cytoplasm (d). **2)** MB (red) in mitotic MCF10CA1a cell. **3)** Multiple MBs; inset bottom, phase contrast image of ring-like MB; inset top MBs overlaid with phase contrast cell image. **4)** % normal or cancer cells with MB accumulation. Arrows and asterisk: MB accumulation in breast cancer cells. **5)** MBs accumulate in HeLa xenograft (dots) compared with adjacent normal tissue. **6)** MBs increase with increasing tumor grade (colon). **7)** The MCF7 cell SP has 8-fold more MB+ cells than the main population (MP). 5



Figures 8-13. **8)** Soft agar colony number increases with increasing percentage of MB+ cells. **9)** Midbody (lower panel) in autophagosome (middle panel) with merge (upper panel). **10A)** NBR1-GFP expression in MCF7 cells decreases soft agar colony # compared to GFP alone (~30% less). **10B)** NipSnip2 GFP expression in MCF7 cells shows a much greater decrease in colony # (~7-fold). **11)** The breast “cancer stem cell” protein, CD44 (green) is present on MBs labeled with the MB marker, MKLP1 (red); inset, CD44 alone. **12)** Breast cancer linked proteins on isolated MCF7 cell MBs (red); MKLP1, MB marker (green). Note dotted staining pattern of Cdh1 (bottom) suggesting discrete binding regions on MBs. 6

KEY RESEARCH ACCOMPLISHMENTS:

Midbodies are inherited by one daughter cell (Fig. 1) where they accumulate in breast cancer cells (Figs. 2-4) and tumors (Fig. 5) and increase with tumor increasing tumor grade (Fig. 6). Midbodies are in breast cancer stem cells (Fig. 7) and their presence is associated with increased in vitro tumor potential over MB-negative breast cancer cells (Fig. 8).

3. Midbodies serve as scaffolds for anchoring breast cancer oncogenes, tumor suppressors and cancer stem cell proteins (Fig. 9).
2. MB-bound breast cancer oncogenes and tumor suppressors could serve as novel targets for breast cancer therapies.
3. Hormone induced recruitment of breast cancer oncogenes and tumor suppressors may serve as a target of anti-breast cancer strategies.
4. NBR1 expression effectively eliminates breast cancer cells (Fig 10A).
5. Expression of the NBR1 interacting protein, NIPSNIP2, outperforms NBR1 alone in decreasing the tumor potential of breast cancer cells in vitro (Fig. 10B).
6. MB+ breast cancer cells contain stem cell antigens CD44.

REPORTABLE OUTCOMES (The following are outcomes of this study):

Manuscripts.

We published papers on many of the findings discussed in this report.

1. Kuo et al., Midbody accumulation through evasion of autophagy contributes to cellular reprogramming and tumorigenicity. *Nature Cell Biology* 13: 1467, 2011. (*See highlights in Current Biology 21, R958-59; Nature Reviews Molecular Cell Biology 12, 690-1, 2010; "The Scientist" September 11, 2001; The Keck Foundation Annual Report 2011, p. 8-10*).
2. Chen C-T, Ettinger A, Huttner, W, and Doxsey S. Resurrecting Remnants: The lives of Post-Mitotic Midbodies. *Trends Cell Biol.* 23, 118-28, 2013.
3. Kuo TC, Doxsey S. Fates and roles of post-mitotic midbodies beyond cytokinesis. *Cell Cycle.* 11, 7-8, 2012.
4. 83. Chen C-T, Hehnly H, Doxsey S, Orchestrating vesicle transport, ESCRTs and kinase surveillance during abscission. *Nature Reviews Molecular Cell Biology*, 13:483-8, 2012.

Cell lines.

We established cell lines expressing GFP-MKLP1, which targets to midbodies.

We established cell lines expressing GFP-Cep55, which targets to midbodies.

We established cell lines expressing GFP-NBR1, which targets to autophagosomes (MCF7).

We established cell lines expressing GFP-NipSnip2, which targets to autophagosomes (MCF7).

Invited seminars based on work in this project.

2011:

03/2011	Frontiers in Science, UMass Medical School, Worcester MA
05/2011	Polycystic Kidney Disease Conf., Harvard Medical School, Boston
08/2011	Biology of Aging, Ellison Foundation Meeting, Woods Hole, MA
06/2011	Plennary Lecture , Molecular Medicine, Istanbul, Turkey
08/2011	Plennary Lecture , Molecular/Cell Biology, Beijing, China
08/2011	Era of Hope Breast Cancer Conference, Orlando, Florida
09/2011	EMBO Workshop, Chromosome Function, Cape Sonio, Greece
10/2011	Centrosomes and Spindle Pole Bodies, Barcelona, Spain
11/2011	American Society of Nephrology, Philadelphia PA
12/2011	American Society of Cell Biology, Denver CO

2012:

04/2012	University of Colorado at Denver, Denver CO
05/2012	Plennary Lecture , Congress, Cell Biology, Rio de Janeiro, Brasil
05/2012	Plennary Lecture , Cilia Structure/Function, Hanko Isle, Norway
05/2012	Cilia in Development and Disease Conference, London England
06/2012	Plennary Lecture , Stockholm, Sweden
06/2012	University of Pennsylvania, Philadelphia, PA
06/2012	National University of Ireland, Chromosome Biology, Galway
10/2012	Plennary Lecture and Teaching Lectures Kisumu Medical Training College, Kisumu, Kenya, East Africa
11/2012	International Drug Discovery Science/Technology, Nanjing China
12/2012	American Society of Cell Biology, San Francisco, CA

2013 (invited and expected to attend)

01/2013	Plenary Lecture , Asian Clinical Congress, Bangkok, Thailand (attended)
03/2013	“Building a Centrosome” Workshop, West Sussex, U.K. (attended)
05/2013	University of Algarve, Portugal (attended)
05/2013	University of Toronto, Toronto Canada (attended)
08/2013	“Anti-Cancer Drugs”, Stockholm, Sweden (scheduled)
09/2013	“Anti-cancer Drugs “Moscow Russia” (scheduled)
09/2013	“Biological and Biomedical Sciences” Dar-Es-Salaam, Tanzania

CONCLUSION:

The results reported in this funding period are very exciting to me, as I am primarily a basic cancer researcher who now has the potential to move into anti-breast cancer strategies. My laboratory has *established a new paradigm for breast cancer treatment* through the exploitation of a midbody-selective autophagy pathway that eliminates post-mitotic midbodies and kills breast cancer cells. To our knowledge, no other lab is working in this area of cancer biology.

Based on the role of MBs in breast cancer and other progress we have made in this regard, we feel we can take our work to a new level that involves breast cancer therapeutics. Several lines of investigation suggest this can be done: MBs enhance breast cancer. However, decreasing MBs kills breast cancer cells. Overexpression of autophagy proteins (NBR1, NIPSNIP2) decreases MBs and kills breast cancer cells. MB-positive breast cancer cells are breast cancer stem cells. This suggests that MB targeting for breast cancer therapy will target the cells that are the most insidious of all breast cancer cells, the breast cancer stem cell. In turn, MB+ breast cancer stem cells are the cells that become resistant to treatment, are recurrent and form metastatic lesions, all of which are the most difficult to treat. We were surprised to find that MBs harbor breast cancer oncogenes, tumor suppressors and cancer stem cell proteins suggesting a mechanism for the tumor potential of MBs. We also believe that this research will have a sustained and significant impact on breast cancer for a number of additional reasons: The work provides a new understanding of breast cancer etiology, namely a novel role for MBs in maintaining the properties of breast cancer cells. We also believe that we have identified a new and effective breast cancer stem cell identification strategy based on novel and atypical biomarkers, MBs.

REFERENCES

Kuo et al., Midbody accumulation through evasion of autophagy contributes to cellular reprogramming and tumorigenicity. *Nature Cell Biology* 13: 1467, 2011.

Muhammad Al-Hajj, Max S. Wicha, Adalberto Benito-Hernandez, Sean J. Morrison, and Michael F. Clarke. Prospective identification of tumorigenic breast cancer cells. *PNAS* 100, 3983-8, 2003.