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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 breast cancer oncogenes, tumor suppressors and 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.

15. SUBJECT TERMS
breast cancer, stem cells, midbody, mitosis, microtubules

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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 (MB, Figs. 2, 3) is an organelle involved in severing the cytoplasmic bridge. 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 cancer cells and target them for chemotherapeutic elimination of breast cancer. 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).

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

Task 1. Test whether MBs are present in breast CSCs in vitro and in vivo.
a. We isolated breast cancer stem cells from breast cancer cell lines based on the side population (SP) and tested for enrichment of MBs. We found that MBs were enriched in these putative breast cancer stem cells in MCF7 cells, compared with the main population (MP); n=4 experiments compared with normal breast epithelial cells, example, Fig. 7).

b. We independently isolated putative breast cancer stem cells using another strategy, namely isolation by sorting for breast cancer stem cell markers (MCF7, CD44+, CD24/- low cells, Fig. 11) as originally shown by Morrison and co-workers (Al-Hajj et al., 2003). Using this method, we showed that there are more MB-positive cells in the breast cancer stem cell population (CD44+, CD24/- low) than in the cell population that was also flow sorted but were MB-negative control (83.1+/-2.1% versus 8.1+/-0.1% for control, ~8-fold greater).

c. We showed that breast cancer stem cells from another breast cancer cell line (MDA-MB-231) isolated by cell surface markers (CD44/CD22-) were enriched in MB-positive cells (61.1+/-0.7% versus 15.0+/-0.2%, ~4-fold). Taken together, these data provide strong evidence that breast cancer stem cells are MB-positive using two methods of isolation, 1) collecting the "side population", 2) collecting cells using stem cell surface markers and 3) by showing they are in multiple different cell breast cancer cell lines.

d. We tested whether breast tumors were enriched in MBs (as in HeLa xenographs, Fig. 5) using immunohistochemical and immunofluorescence staining of mouse and human tumors and adjacent nontumor (control) tissue. We found that 67.3+/- 3.1% of cells in in breast tumors were MB-positive. In contrast, only 4.5+/-0.01% of cells in adjacent breast tumor tissue, representing more than a 10-fold increase in MB+ cells in tumors.

e. In the course of this study, we made an important and unanticipated discovery that may provide a new and highly innovative opportunity for breast cancer therapies. We identified a large number (n=29) of canonical breast cancer oncogenes and tumor suppressors localized to the MB using both immunofluorescence and biochemical approaches (Fig. 12). These include breast cancer specific proteins as well as proteins that are associated with many cancers (examples: BRCA1, BRCA2, p53, ErB2, Akt, Cdh1, Fig. 12). This new unexpected result may provide a molecular mechanism for the tumorigenic phenotype of MBs and could serve as a

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novel therapeutic method for treating breast cancer, a) namely targeting post-mitotic MBs for autophagic degradation and b) displacing MB-bound breast cancer associated proteins.

Task 2. Test the tumorigenic potential of MBs.
A Using fluorescence-activated cell sorting, we isolated for MB-positive cells after labeling MBs with the MB marker, GFP-MKLP1 (also with other MB proteins such as CEP55, MgcRacGAP). These MB+ cells showed an increase in soft agar growth and multicellular breast cancer spheroid formation (Fig. 8). 100-200 cells were capable of making breast tumor spheroids in vitro compared to MB-low controls.

b. The isolated MB+ population of cells also had more tumor-like potential than the MB-negative population (MB+: 41.9+/-2.2, MB-: 7.1+/-0.5% soft agar colonies, Fig. 8). This provides additional evidence that MB-positive cells identify the breast cancer stem cell subpopulation of cancer cells. This suggests that MB-positivity could be exploited to isolate breast cancer stem cells from breast cancer cell lines and human breast tumors (i.e. MBs may have potential as markers for breast cancer stem cells). We have had difficulty with tumor growth in mice, but we now believe increasing cell numbers will allow us to complete this work.

c. 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 breast cancer cells (Fig. 8).

Task 3. *Test if specific targeting of MBs for autophagic degradation is a therapeutic strategy for breast cancer.*
a. Test if autophagic degradation of MBs by expression of GFP-NBR1 inhibits soft agar growth. We showed that NBR1 expression and the resulting decrease in MB+ cells decreases in vitro tumor potential (soft agar growth, Fig. 10A).
b. We identified another member of the NBR1 MB-autophagy pathway by isolating NBR1 binding protein, NipSnip2. This new member of the MB autophagy pathway dramatically increases MB degradation when expressed, leading to decreased tumor-like properties of breast cancer cells. It is more efficacious than NBR1 in this regard (Fig. 10B).
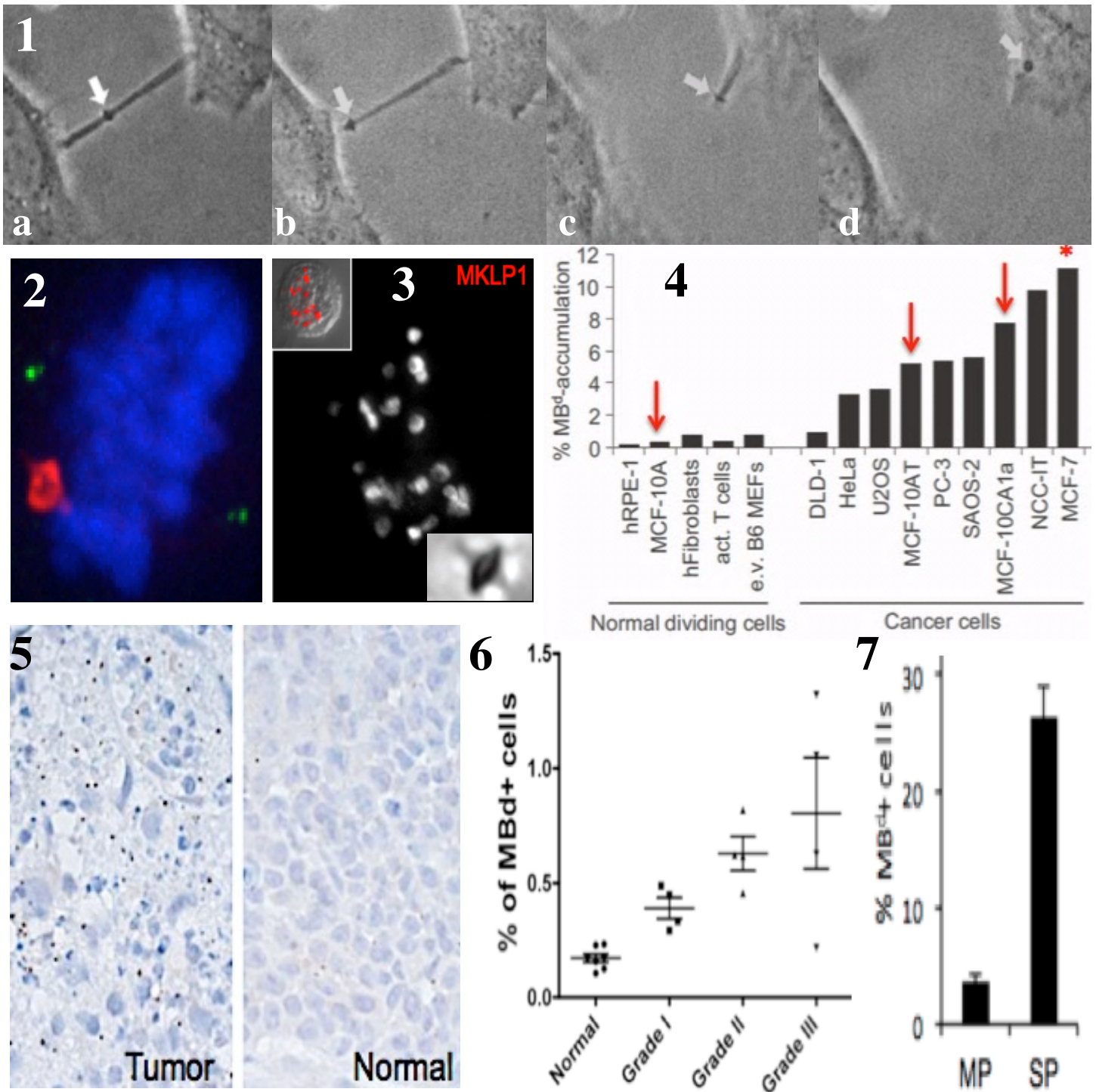
Figures 1-7. 1) Midbody in intercellular bridge connecting 2 MCF7 cells (a, arrow) moves left (b) then bridge is severed (c) sending the MB on a cytoplasmic tether to the cell on the right (c) where it resides in the cytoplasm (d). **2)** MB (red) in mitotic MCF10CA1a cell. **3)** Multiple MBs; inset 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 xenograph (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). 6
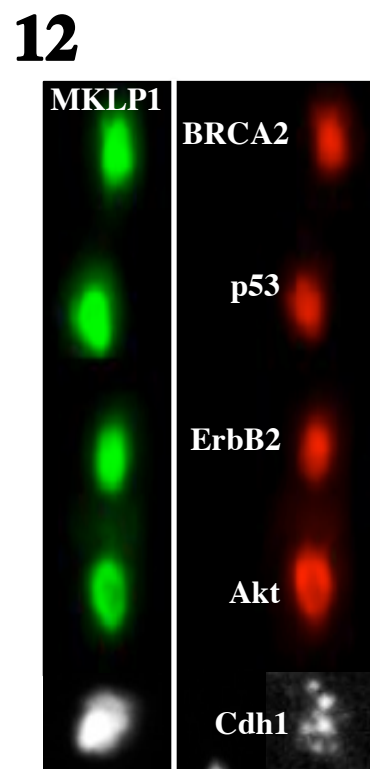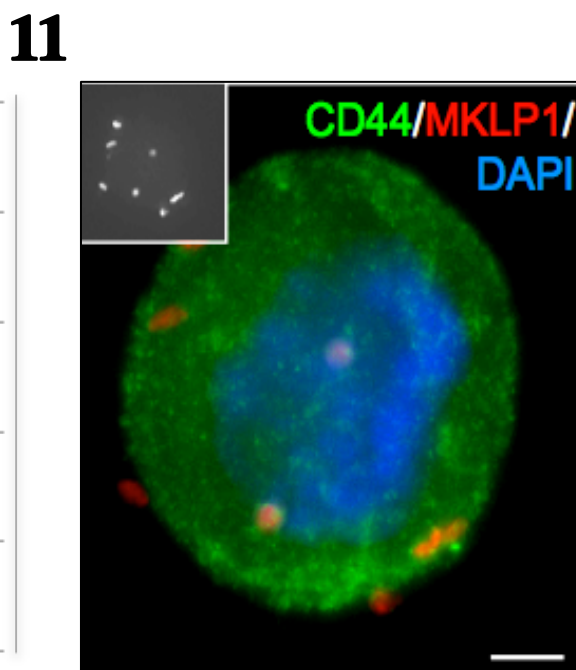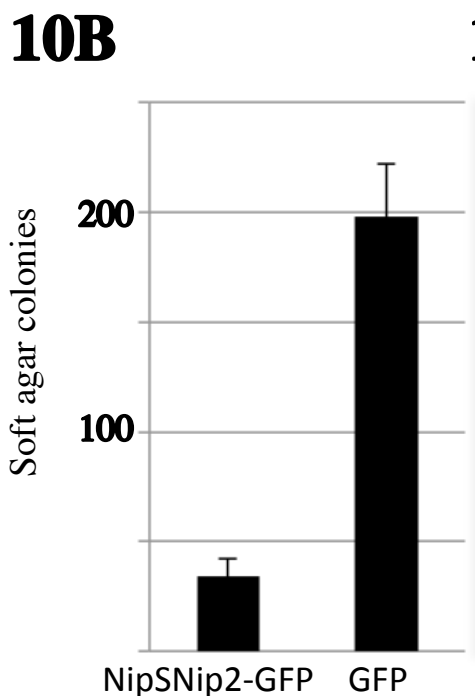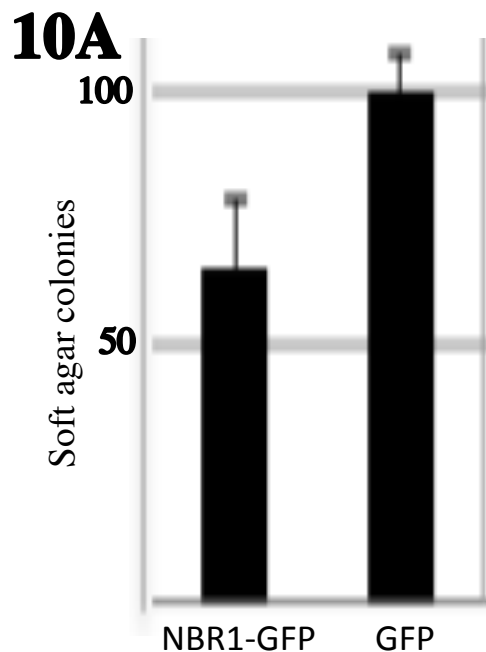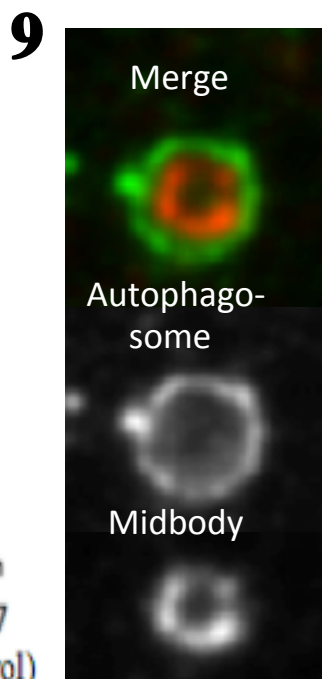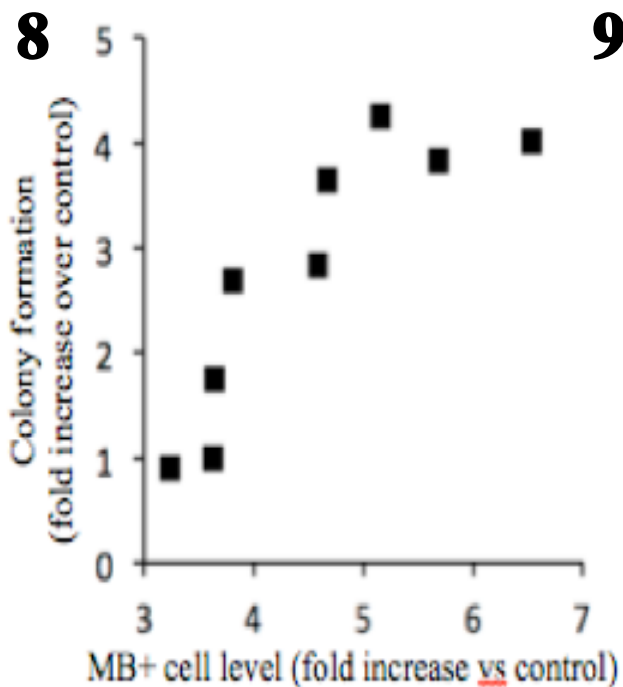
Figures 8-13. **8)** Soft agar colony number increases with increasing percentage of MB+ cells. **9)** Midbody (lower panel) in autophagosome (middle panel) with merge (upper panel). **10A)** NBR1-GFP expression in MCF7 cells decreases soft agar colony # compared to GFP alone (~30% less). **10B)** NipSnip2 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. 7

KEY RESEARCH ACCOMPLISHMENTS:

1. 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).
2. 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. 12).
4. MB-bound breast cancer oncogenes and tumor suppressors could serve as novel targets for breast cancer therapies based on the role of MBs in tumor-like properties.
5. NBR1 expression effectively eliminates breast cancer cells (Fig 10A).
6. Expression of the NBR1 interacting protein, NIPSNIP2, outperforms NBR1 in decreasing the tumor potential of breast cancer cells in vitro (Fig. 10B).
7. MB+ breast cancer cells contain stem cell antigens (e.g. CD44, Fig. 11).

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.
5. Chen C-T, Kuo T-C, Doxsey S. A new potential breast therapeutic strategy: targeting post-mitotic MBs for autophagic degradation and tumor suppression. In preparation.
6. Kuo T-C, Baron, D, Doxsey S. Post-mitotic MBs as markers for breast cancer stem cells. In preparation.
7. Schiel J, Redick S, Asymmetric inheritance of the midbody in breast cancer stem cells requires centrosomal appendages and associated proteins. In preparation.

Cell lines.

1. We established cell lines expressing GFP-MKLP1, which targets to midbodies.
2. We established cell lines expressing GFP-Cep55, which targets to midbodies.
3. We established cell lines expressing GFP-NBR1, which targets to autophagosomes (MCF7).
4. We established cell lines expressing GFP-NipSnip2, which targets to autophagosomes (MCF7).

Invited seminars based on work in this project.

2012:
04/2012   University of Colorado at Denver, Denver CO
05/2012   **Plennary Lecture**, Congress, Cell Biology, Rio de Janeiro, Brazil
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:
01/2013   **Plenary Lecture,** Asian Clinical Congress, Bangkok, Thailand
03/2013   "Building a Centrosome" Workshop, West Sussex, U.K.
05/2013   University of Algarve, Portugal
05/2013   University of Toronto, Toronto Canada
08/2013   "Anti-Cancer Drugs", Stockholm, Sweden
09/2013   "Anti-cancer Drugs "Moscow Russia"
09/2013   "Biological and Biomedical Sciences" Dar-Es-Salaam, Tanzania

2014:
03/2014   Plennary Lecture, Molecular Medicine, Istanbul, Turkey
05/2014   Plennary Lecture, Molecular/Cell Biology, Beijing, China
05/2014   Cancer Therapies, Dailan, China

Personnel paid by this grant.
Chun-Ting Chen
Tse-Chun Kuo
Desiree Baron
Sambra Redick
John Schiel
Alison Bright.

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: 1) MBs enhance breast cancer. 2) Importantly, decreasing MBs kills breast cancer cells. 3) Overexpression of autophagy proteins (NBR1, NIPSNIP2) decreases MBs and kills breast cancer cells. 4) 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. 5) 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. **The "so what section":** We 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. This study could lead to important "clinical products/outcomes" such as novel breast cancer therapies (targeting MBs), and increased understanding of the etiology of breast cancer (modulation by MBs) and new scaffolds/modulators of breast cancer oncogenes and tumor suppressors (MBs).

REFERENCES

Kuo Tc, Chen Ct, Baron D, Onder TT, Loewer S, Almeida S, Weismann CM, Xu P, Houghton JM, Gao FB, Daley GQ, Doxsey S, 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.