Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** Thomas Jefferson University

2. **Reporting Period (start and end date of grant award period):** 01/01/2010 – 12/31/2013

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Joy Soleiman, MPA

4. **Grant Contact Person's Telephone Number:** 215-955-5684

5. **Grant SAP Number:** 4100050910

6. **Project Number and Title of Research Project:** 7 - CAV3, Myo-Epithelial Barrier Function and Breast Cancer

7. **Start and End Date of Research Project:** 01/01/2010 – 8/31/2012

8. **Name of Principal Investigator for the Research Project:** Michael P. Lisanti, MD, PhD

9. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $ 871,755.62

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project	Cost
Lisanti	Principal Investigator	2%	5,088
Balliet	Instructor	100%	24,273
Capparelli	Postdoc Fellow	50%	10,192
Guido	Postdoc Fellow	50%	7,269
Eaton	Graduate Student	100%	10,297
Lin	Research Assistant	75%	24,447

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project	Cost
NONE			

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
NONE		

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes_____ No ___X_____

If yes, please indicate the source and amount of other funds:

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes_____ No ____X_____

If yes, please list the applications submitted (column A), the funding agency (National

Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
None	☐NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)		$	$

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes_____ No ___X_____

If yes, please describe your plans:

12. Future of Research Project. What are the future plans for this research project?

A lactation-based therapeutic strategy using recombinant α-casein would provide a more natural and non-toxic approach to the development of novel anticancer therapies.

13. New Investigator Training and Development. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes_____ No ___X_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male				
Female				
Unknown				
Total				

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic				
Unknown				
Total				

	Undergraduate	Masters	Pre-doc	Post-doc
White				
Black				
Asian				
Other				
Unknown				
Total				

14. Recruitment of Out-of–State Researchers. Did you bring researchers into Pennsylvania to carry out this research project?

Yes_____ No ___X_____

If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. Impact on Research Capacity and Quality. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes ___X_____ No_____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

This grant provided funds for the support and development of an important research project.

16. Collaboration, business and community involvement.

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes_____ No ___X_____

If yes, please describe the collaborations:

16(B) Did the research project result in commercial development of any research products?

Yes_____ No ___X_____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No ___X_____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

The two Specific Aims of the project are:

1. Determine the role of Cav-3 and Myo-Epithelial Cells in Mammary Cell Hyperplasia, Dysplasia, and Ductal Carcinoma *In Situ* (DCIS).

2. Determine the role of Cav-3 and Myo-Epithelial Cells in Mammary Tumor Onset, Progression, and Metastasis.

Summary

Materials and Methods

Materials

Antibodies and their sources were as follows:

Cell lines
Met-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 2 mmol/L glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a 5% CO2 incubator. Met-1 cells were the generous gift of Dr. Robert D. Cardiff (University of California-Davis).

FACS-sorted GFP-positive cells

Lentiviral infection
Met-1 stable cell lines were also produced by lentiviral infection. Control empty vector plasmid and human α-casein s1 expression construct, both eGFP-positive, were obtained from Genecopoeia. Lentiviral empty vector and ORF expression vector (with human α casein s1) were transfected into Genecopoeia GCI-L3 chemically competent E. coli cells by Fugene HD transfection reagent (Roche) following the manufacturer's manual. Two days after transfection, cell culture supernatants were collected and centrifuged. Supernatant containing lentiviral particles were used to infect Met-1 target cells. After lentiviral transduction, target Met-1 cells were selected for 10–14 d in 2.5 μg/ml of puromycin. Efficiency of α-casein transfection was determined by immunoblot analysis.

Gene expression profiling
Total RNA (5 μg) was reverse transcribed using the Superscript III first-strand synthesis system (Invitrogen) using a HPLC purified T7-dT24 primer (Sigma Genosys), which contains the T7 polymerase promoter sequence. The single-stranded cDNA was converted to double-stranded cDNA using DNA polymerase I (Promega) and purified by cDNA spin column purification using GeneChip Sample Cleanup Module (Affymetrix). The double-stranded cDNA was used as a template to generate biotinylated cRNA using the Bioarray high-yield RNA transcription labeling kit (Enzo) and the labeled cRNA purified by GeneChip Sample Cleanup Module (Affymetrix). Fifteen μg of cRNA was fractionated to produce fragments of between 35–200 bp using 5× fragmentation buffer provided in the Cleanup Module. The sample was hybridized to mouse 430 2.0 microarray (Affymetrix) representing more than 39,000 transcripts. The

hybridization and washing steps were performed in accordance with Affymetrix protocols for eukaryotic arrays. The arrays were scanned at 570 nm with a confocal scanner from Affymetrix. Analysis of the arrays was performed using the R statistics package and the limma library of the Bioconductor software package. Arrays were normalized using robust multiarray analysis (RMA), and p value of 0.05 was applied as criteria for statistically differentially expressed genes. GO tree analysis of gene function was analyzed by Webgestalt (Vanderbilt University). Outcome analysis (recurrence- and metastasis-free survival) in human breast cancer patients was performed, essentially as previously described (1).

INTRODUCTION

One novel hypothesis is that milk itself contains as-yet-undiscovered components that function as tumor suppressor(s). In direct support of this notion, implantation of mammary tumor cells within the primary duct of the mammary gland of a constitutively lactating mouse model [Cav-3 (−/−) mice] resulted in a > 1,000-fold reduction in tumor growth (2) Furthermore, addition of human milk at low concentrations to cultured mammary tumor cells dramatically inhibited their capacity for migration (2). These preliminary findings provide a simple experimental model to identify which milk components possess tumor suppressor properties.

Further analysis of which gene transcripts are elevated in the mammary glands of constitutively lactating Cav-3 (−/−) mice revealed a significant up regulation of several members of the casein gene family, with α-casein being featured prominently (2). Thus, we speculated that members of the casein gene family might actually function as tumor suppressors.

RESULTS

Our previous studies have suggested that a lactogenic microenvironment might play a role in the protection against breast cancer (Sotgia et al., Am J Pathol. 2009 ;174(2):613-629).
Here, we tested this hypothesis experimentally. First, we show that purified α-, β- and κ-casein proteins all inhibit the migration of mammary tumor cells in vitro, with α-casein being the most effective. Second, we demonstrate that recombinant expression of α-casein in mammary tumor cells significantly decreases both in vivo tumor growth (by > 5-fold) and experimental lung metastasis (by nearly 10-fold), by reducing their proliferative capacity and by increasing their sensitivity to stress-induced apoptosis, under hypoxic conditions. Mechanistically, using genome-wide transcriptional profiling, we highlight that recombinant overexpression of α-casein results in the activation of interferon-based signaling via STAT1 up regulation and hyper activation. Thus, the possibility exists that recombinant casein family members could be used as novel anticancer therapeutics to locally or systemically treat human breast cancers.

DNA microarray analysis: α-Casein activates STAT1 signaling and decreases "stemness" in mammary tumor cells
To understand the mechanism(s) that underlie the protective effect of α-casein expression against the progression of human breast cancer and lung metastasis, we subjected the Met-1 cell lines DNA microarray analysis. Figure1A and 1B show the results from pathways analysis. Interestingly, expression of α-casein induced the up regulation and down regulation of genes

associated with different signaling pathways. This includes, most prominently, the interferon (IFN-α, -β, -gamma) signaling pathways. In particular, given that IFN-mediated signaling involves the activation of the classical Jak/STAT1 pathway, to validate our result obtained from this transcriptional profiling, we performed immunoblot analysis using an antibody directed against STAT1. In this pathway, JAKs associate with IFN-receptors and, following receptor interaction with IFN, Jak phosphorylates STAT1 (3).

Figure 2 shows the results of this immunoblot analysis. Interestingly, Met-1 cells expressing α-casein displayed increased activation of STAT1 (phosphorylated at the Tyr701 position) together with a higher expression level of its non-phosphorylated form. Moreover, DNA microarray analysis also revealed that α-casein expression down regulated genes involved with focal adhesions. Thus, to validate our finding, we evaluated the expression levels of uPAR/PAI-1 that are important in the remodeling of the extracellular matrix during tumor cell invasion. Our results from immunoblot analysis show low or undetectable expression levels of uPAR and PAI-1 proteins after α-casein expression (Figure 3).

Finally, we found that α-casein expression down regulates genes that are highly expressed in stem/progenitor cells. To provide independent validation of this result obtained from our gene expression profiling study, we conducted FACS analysis. In these experiments, the antibody for the stem cell surface marker CD44 was used. Figure 4 shows the result of this analysis. Importantly, α-casein expression resulted in the reduction of the number of CD44(+) cancer stem cells.

Overall, these findings suggest that α-casein expression in mammary epithelial cancer cells may represent a potential therapeutic approach as an antitumor agent in breast cancer.

Our previous studies have also suggested an important role for metabolic function of myofibroblasts in the protection against breast cancer development (Sotgia et al., Am J Pathol. 2009 ;174(2):613-629). The transfer of metabolites between myofibroblasts and cancer cells was further evaluated in the following project.

Recent studies have suggested that cancer cells behave as metabolic parasites, by inducing oxidative stress in adjacent normal fibroblasts. More specifically, oncogenic mutations in cancer cells lead to ROS production and the "secretion" of hydrogen peroxide species. Oxidative stress in stromal fibroblasts then induces their metabolic conversion into cancer-associated fibroblasts. Such oxidative stress drives the onset of autophagy, mitophagy, and aerobic glycolysis in fibroblasts, resulting in the local production of high-energy mitochondrial fuels (such as L-lactate, ketone bodies, and glutamine). These recycled nutrients are then transferred to cancer cells, where they are efficiently burned via oxidative mitochondrial metabolism (OXPHOS). We have termed this new energy-transfer mechanism "Two-Compartment Tumor Metabolism", to reflect that the production and consumption of nutrients (L-lactate and other catabolites) is highly compartmentalized. Thus, high-energy onco-catabolites are produced by the tumor stroma.

Here, we used a genetic approach to stringently test this energy-transfer hypothesis. First, we

generated hTERT-immortalized fibroblasts which were genetically re-programmed towards catabolic metabolism. Metabolic re-programming towards glycolytic metabolism was achieved by the recombinant over-expression of MFF (mitochondrial fission factor). MFF over-expression results in extensive mitochondrial fragmentation, driving mitochondrial dysfunction. Our results directly show that MFF-fibroblasts undergo oxidative stress, with increased ROS production, and the onset of autophagy and mitophagy, both catabolic processes. Mechanistically, oxidative stress induces autophagy via NF-kB activation, also providing a link with inflammation. As a consequence MFF-fibroblasts showed intracellular ATP depletion and the extracellular secretion of L-lactate, a critical onco-catabolite. MFF-fibroblasts also showed signs of myofibroblast differentiation, with the expression of SMA and calponin.

Importantly, MFF-fibroblasts significantly promoted early tumor growth (up to 6.5-fold), despite a 20% overall reduction in angiogenesis. Thus, catabolic metabolism in cancer-associated fibroblasts may be a critical event during tumor initiation, allowing accelerated tumor growth, especially prior to the onset of neo-angiogenesis.

RESULTS

MFF over-expression in fibroblasts induces mitochondrial dysfunction

Mitochondrial Fission Factor (MFF) is involved in the fission process normally required to maintain the physiologic function of mitochondria. However, it remains unknown if MFF over-expression plays a role in the breast cancer tumor stroma. Thus, we stably expressed MFF in hTERT-immortalized human fibroblasts. Figure 5 shows that we successfully over-expressed MFF in fibroblasts, and this is especially evident upon exposure to hypoxic stress.
To evaluate if increased mitochondrial fission alters the bioenergetic state of mitochondria, we first evaluated mitochondrial mass by immuno-staining with antibodies directed against TOMM20, a mitochondrial outer membrane marker. Figure 6A shows that TOMM20 immuno-staining is reduced in fibroblasts over-expressing MFF, relative to controls.
Next, we used MitoTracker to assess the status of mitochondrial activity. Interestingly, relative to control cells, MFF-fibroblasts display a strong reduction in MitoTracker staining, both under normoxic (Figure 6A) and hypoxic conditions (Figure 6B), indicative of decreased mitochondrial membrane potential. Especially under hypoxia, we observed a more pronounced loss of mitochondrial function. To independently validate these data, we evaluated the expression of OXPHOS components upon hypoxic stress. Mitochondrial components of complexes I, II, III, and IV were all dramatically reduced in fibroblasts over-expressing MFF, relative to empty vector controls (Figure 6C).

An imbalance between fusion and fission can cause mitochondrial dysfunction, with decreased ATP production. Thus, we evaluated the steady-state ATP levels in MFF-fibroblasts and empty vector controls under hypoxia. Figure 7 shows that after 12 and 24 hours of hypoxia, MFF-fibroblasts display a 1.7-fold and a 11.5-fold reduction, respectively, in ATP levels, indicating that mitochondrial dysfunction is associated with decrease intracellular ATP, as predicted.

Taken together, these data demonstrate that, as reported in the literature, an increase in mitochondrial fission drives mitochondrial fragmentation associated with mitochondrial

dysfunction, decreased membrane potential, decreased oxidative phosphorylation, and reduced ATP levels.

Fibroblasts over-expressing MFF display metabolic remodeling, with a shift towards glycolytic metabolism

Decreased mitochondrial functional activity is associated with metabolic changes, with a shift towards glycolysis. Thus, we next examined the accumulation of L-lactate in the conditioned media of MFF-fibroblasts versus control fibroblasts. Figure 8A shows that under hypoxic conditions, MFF-fibroblasts display a 2-fold increase in L-lactate secretion, relative to control cells. Interestingly, however, in normoxia, MFF-fibroblasts show a 1.5-fold decrease in lactate generation, likely due to compensatory adaptation. Thus, MFF promotes a glycolytic phenotype under conditions of hypoxia that are usually found within tumors in vivo.

To validate that MFF-fibroblasts are more glycolytic, we performed an immuno-blot analysis with antibodies directed against MCT4. MCT4 is the transporter that extrudes L-lactate from glycolytic cells. MCT4 is also HIF-1a target and is an hypoxia-inducible gene. Consistent with the results of the lactate assay, Figure 8B shows that during conditions of hypoxic stress, MCT4 expression is upregulated in MFF-fibroblasts, as compared to control cells. In normoxia, however, MCT4 levels are decreased in MFF-fibroblasts, as compared to control cells. These results suggest that under the low oxygen tension conditions found in human tumors, MFF-fibroblasts are more glycolytic and display an efflux of high-energy mitochondrial fuels (such as L-lactate) into the extracellular microenvironment.

Fibroblasts over-expressing MFF show increased oxidative stress and autophagy, with NFkB-hyperactivation

It is well known that mitochondrial dysfunction induces oxidative stress and that oxidative stress promotes autophagy, driving the removal of damaged mitochondria, via mitophagy. To evaluate if MFF causes increased oxidative stress in stromal cells, we examined the status of ROS production in fibroblasts carrying the Lv-105 empty vector or MFF. ROS levels were measured by FACS analysis. Interestingly, MFF-fibroblasts show a 50% increase in ROS production, as compared to control cells (Figure 9A).

Next, the activation of autophagy/mitophagy was analyzed on protein extracts of fibroblasts harboring MFF by immuno-blotting. Interestingly, MFF induces an autophagy/mitophagy program in fibroblasts, as judged by the upregulation of markers of autophagy (LC3 I, LC3II and cathepsin B), and mitophagy (BNIP3 and BNIP3L) (Figure 9B). Beclin-1 expression remains unchanged. To gain insights into the mechanism(s) triggering autophagy, we monitored the activation the NF-kB pathway by immuno-blotting. Figure 9C shows that MFF-fibroblasts display the constitutive activation of NF-kB, suggesting that NF-kB activation is likely one of the mechanism(s) driving autophagy.

To further characterize the features of MFF-expressing fibroblasts, we investigated if MFF induces a myofibroblastic phenotype. Figure 10 shows that MFF-fibroblasts display the up-regulation of two myofibroblast markers, namely α-SMA and calponin.

Fibroblasts over-expressing MFF promote tumor growth, especially in the early phases of tumorigenesis, without any increase in neo-angiogenesis

To evaluate the effects of MFF on tumor development in vivo, MFF-fibroblasts or empty vector control fibroblasts were co-injected with MDA-MB-231 human breast cancer cells into the flanks of nude mice. Interestingly, Figure 11A and Table 1 demonstrate that MFF over-expression in fibroblasts increases tumor growth rates (up to 6.5-fold), especially in the early phases of tumor development, suggesting that MFF can act as a stromal tumor promoter.

To evaluate if the MFF-tumor promoting effects are dependent on increased angiogenesis, tumor xenografts were immunostained with antibodies against CD31, an endothelial cell marker. CD31 quantification indicates that the tumors derived from MFF fibroblasts display a 20% decrease in vessel density, as compared to the control tumors, suggesting that increased angiogenesis is not the mechanism promoting tumor growth.

In conclusion, MFF-fibroblasts represent a new model system for studying the role of mitochondrial dysfunction and stromal L-lactate production in tumor initiation, progression, and metastasis, independently of tumor angiogenesis. This phenotype was most evident under hypoxic conditions, which is consistent with the angiogenesis-independent nature of our findings.

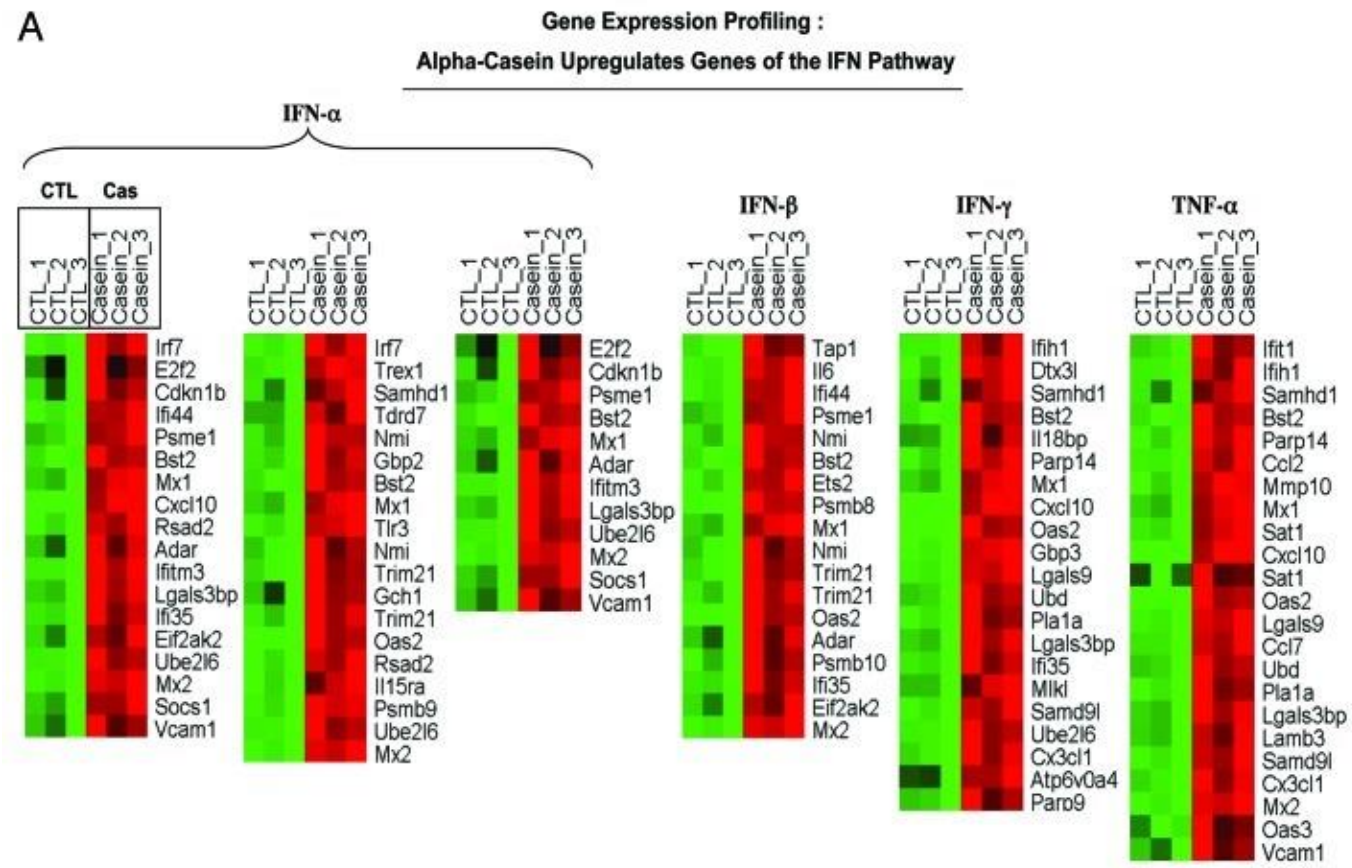

Figure1A**.** α-casein transcriptional profiling. Results from pathway analysis with ASSESS. We performed pathway analysis using ASSESS (analysis of sample set enrichment scores). Note that α-casein upregulates gene transcripts associated with inflammatory interferon/STAT1 signaling (A) and decreases gene transcripts associated with "stemness" (B).

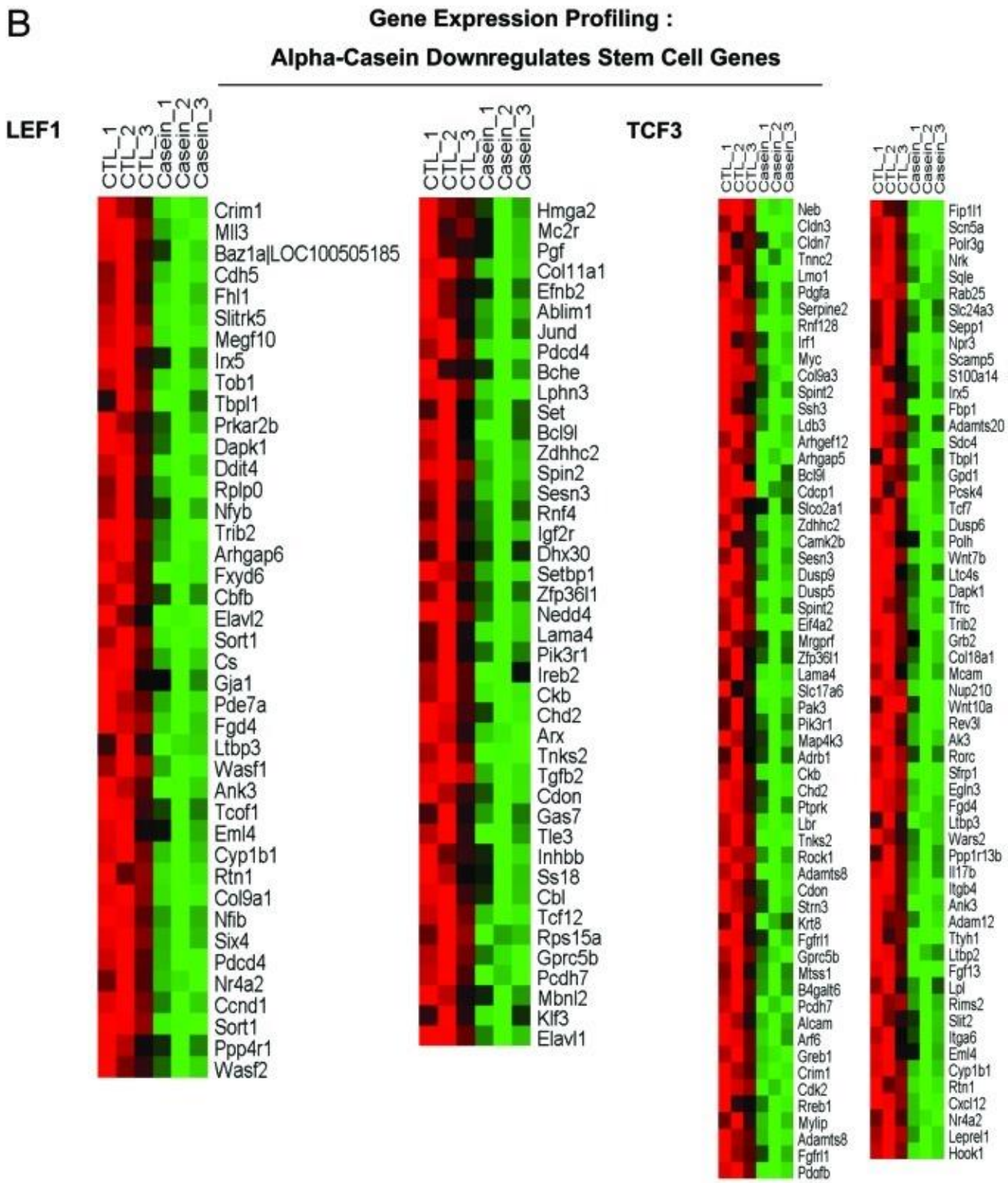

Figure 1B**.** α-casein transcriptional profiling. Results from pathway analysis with ASSESS. We performed pathway analysis using ASSESS (analysis of sample set enrichment scores). Note that α-casein upregulates gene transcripts associated with inflammatory interferon/STAT1 signaling (A) and decreases gene transcripts associated with "stemness" (B).

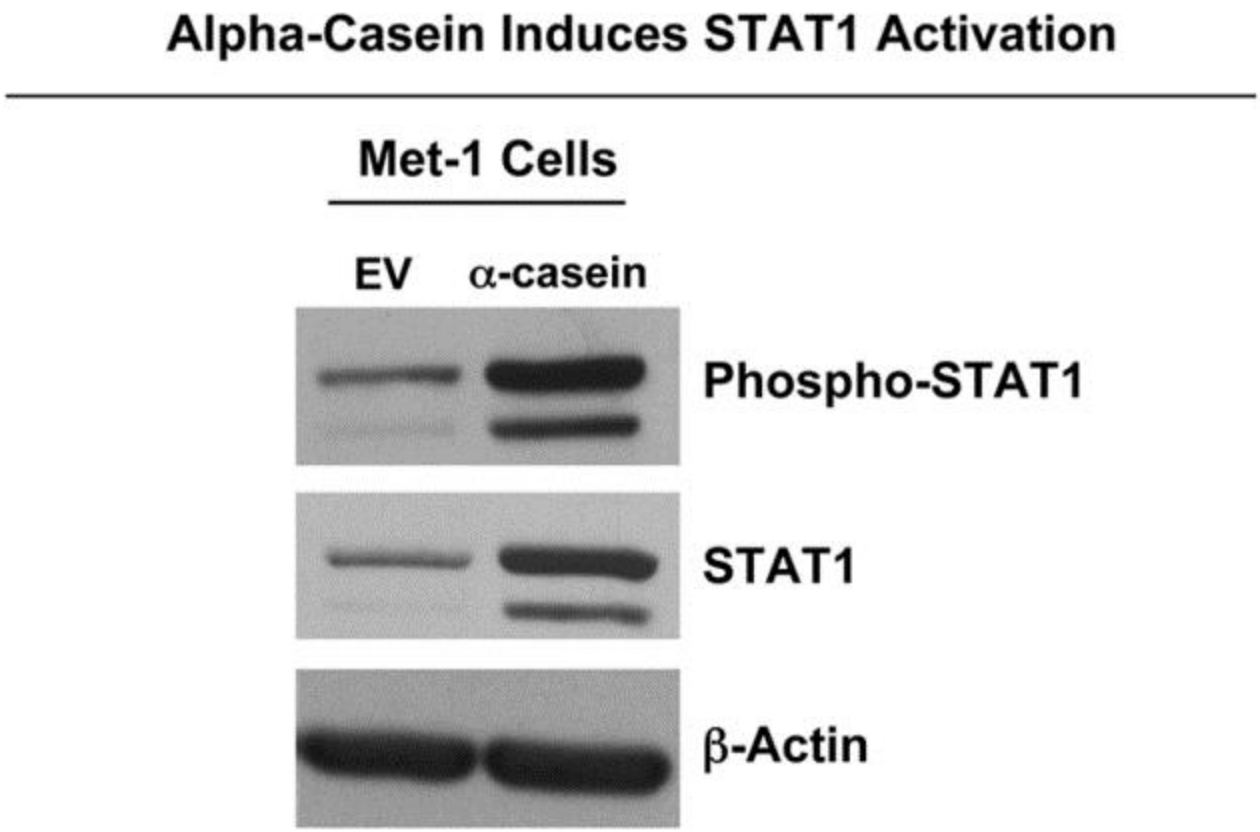

Figure 2. α-casein induces STAT1 activation. Results from DNA microarray analysis suggested that α-casein expression induces the upregulation of the IFN-signaling pathway. Activation of this pathway results in STAT1 phosphorylation. Thus, we performed immunoblot analysis on Met-1 cell lystates. Interestingly, both total STAT1 and phospho-STAT1expression levels were clearly increased by the expression of α-casein.

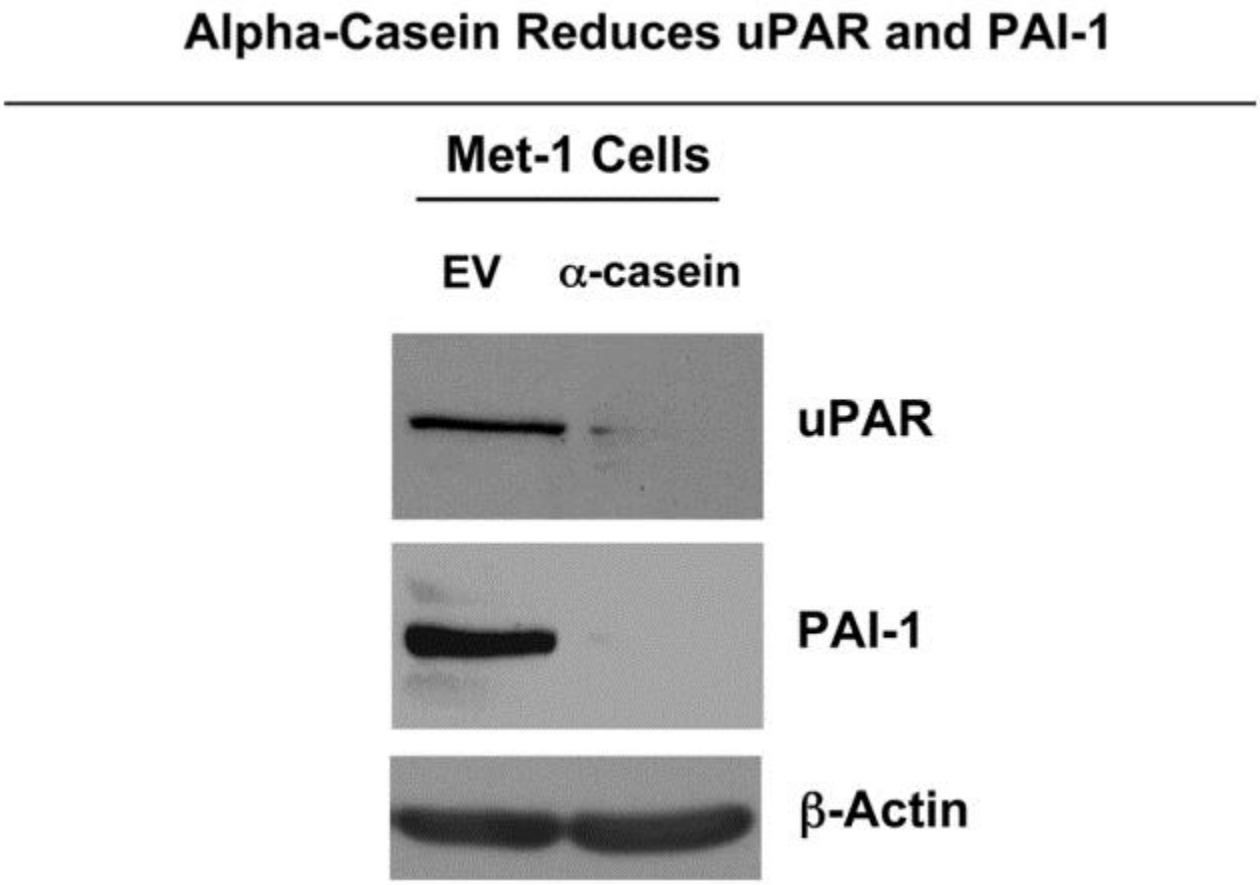

Figure 3**.** α-casein decreases the expression of uPAR and PAI-1. Lysates were prepared from Met-1 cells recombinantly expressing α-casein or the vector alone control. Then, they were subjected to immunoblot analysis with specific antibody probes. Note that expression of α-casein dramatically reduces the expression of uPAR and PAI-1, as predicted. Immunoblotting with β-actin is shown as a control for equal loading.

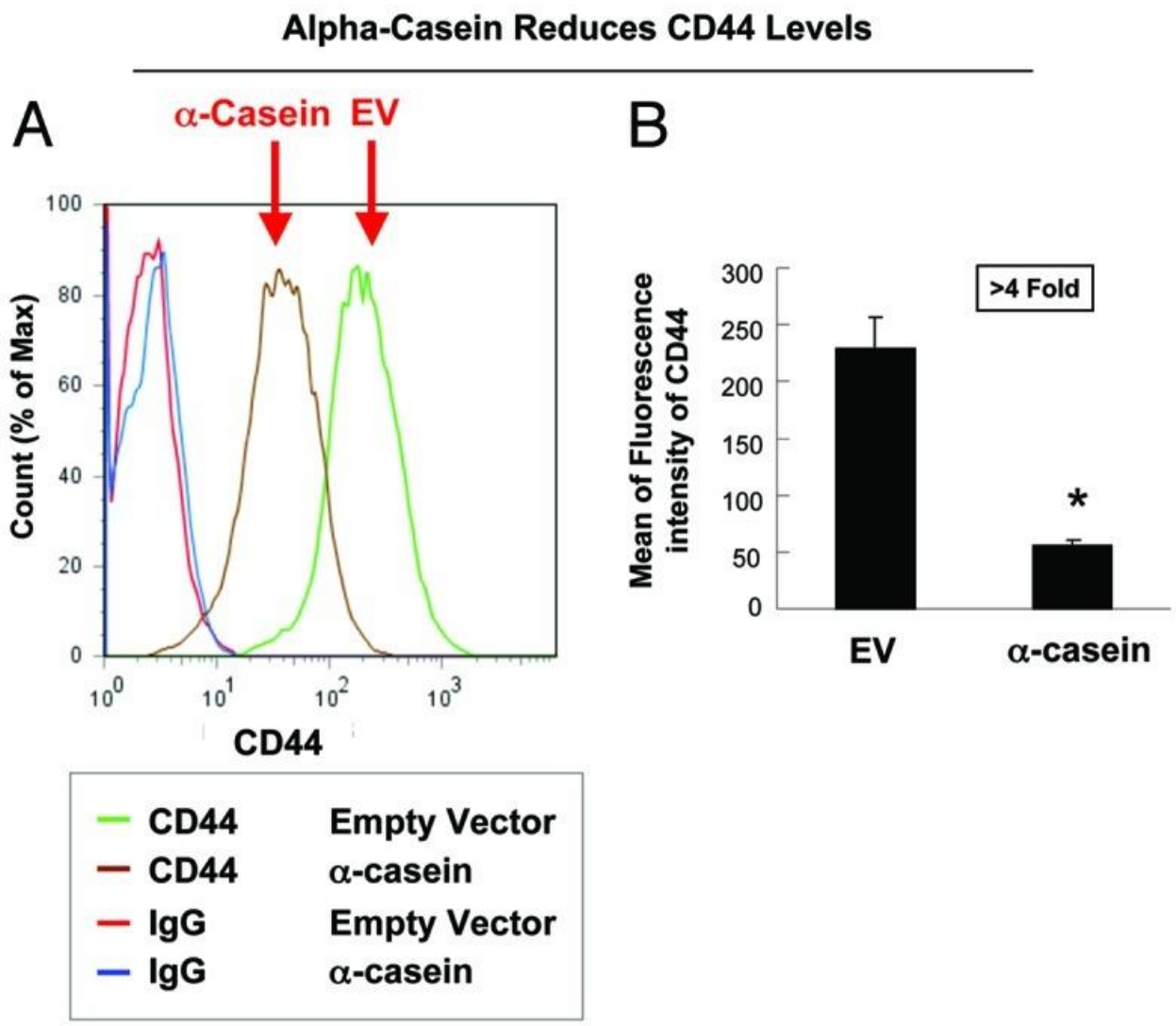

Figure 4**.** α-casein decreases the number of mammary cancer "stem" cells. (A) We performed FACS analysis on Met-1 cell cultures to investigate the expression of the cell surface stem/progenitor marker CD44. Note that the proportion of CD44(+) cells was lower in Met-1 cells expressing α-casein (brown line) as compared with Met-1 empty vector control cells (green line). (B) Graphic representation of the mean values. *$p < 0.01$.

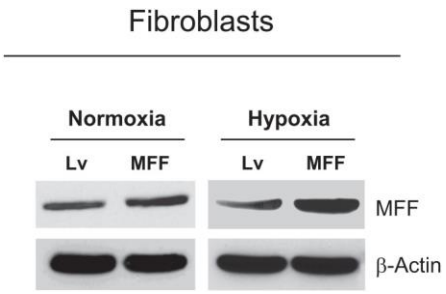

Figure 5: Generation of fibroblasts over-expressing MFF. Fibroblasts over-expressing MFF were generated via lentiviral transduction. Empty vector (Lv) control cells were generated in parallel. Fibroblasts were maintained under conditions of normoxia or hypoxia for 6 hours. MFF protein expression levels were evaluated by immuno-blotting. β-actin was used as an equal protein loading control.

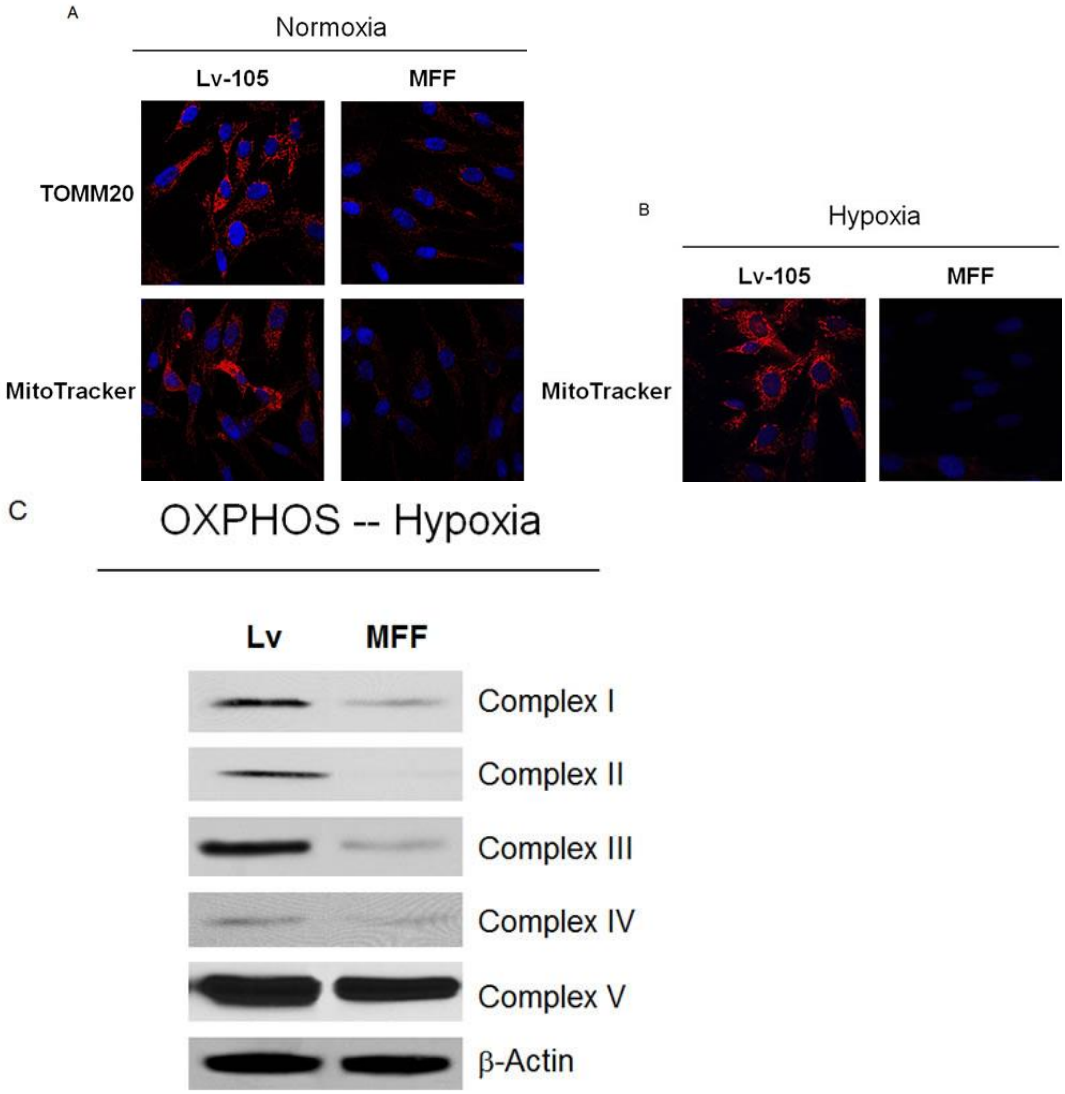

Figure 6: Fibroblasts over-expressing MFF show mitochondrial dysfunction. (A) Normoxia. To determine mitochondrial mass, cells were immuno-stained with TOMM20, a mitochondrial membrane marker. Note that TOMM20 is significantly decreased in MFF-fibroblasts, suggesting that MFF reduces mitochondrial mass. To determine functional mitochondrial activity, cells were independently labeled with MitoTracker. Note the strong reduction in mitochondrial membrane potential. Original magnification, 40X. (B) Hypoxia. MitoTracker staining was performed in control (Lv-105) and MFF-fibroblasts kept under hypoxia for 6 hours (0.5% O2). Note that MFF induces a very strong reduction in mitochondrial membrane potential, particularly upon hypoxic stress. Original magnification, 40X. (C) OXPHOS protein levels were evaluated by immuno-blotting after 6 hours of hypoxia. Note that MFF-fibroblasts display a strong down-regulation of key components of complex I (20 kDa subunit), II (30 kDa subunit), III (Core-2), and IV (COX-II). β-actin was used as an equal protein loading control.

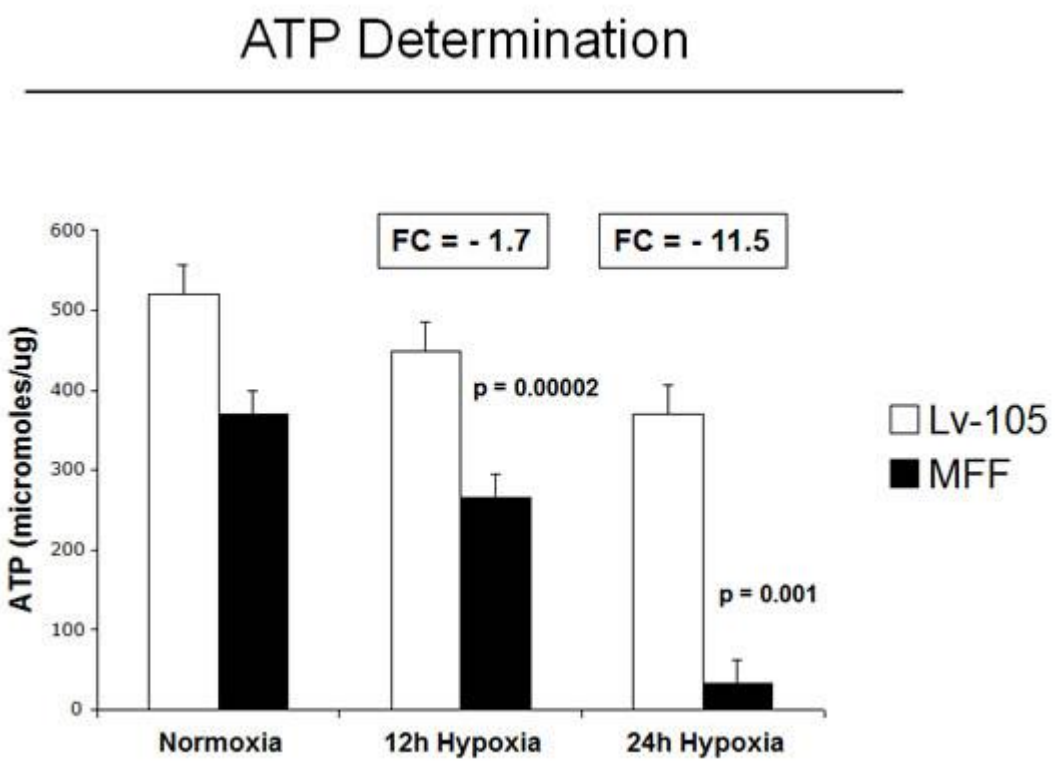

Figure 7: Fibroblasts over-expressing MFF show decreased ATP levels. Intracellular ATP content was measured using empty vector control (Lv-105) and MFF-fibroblasts maintained under normoxia or hypoxic stress (for 12 and 24 hours). Note that as compared to control cells, MFF-fibroblasts display a 1.7-fold and a 11.5-fold reduction in steady-statet ATP leves, respectively, after 12 and 24 hours of hypoxia. These results suggest that the mitochondrial dysfunction in MFF over-expressing fibroblasts is associated dramatic decreases in intracellular ATP.

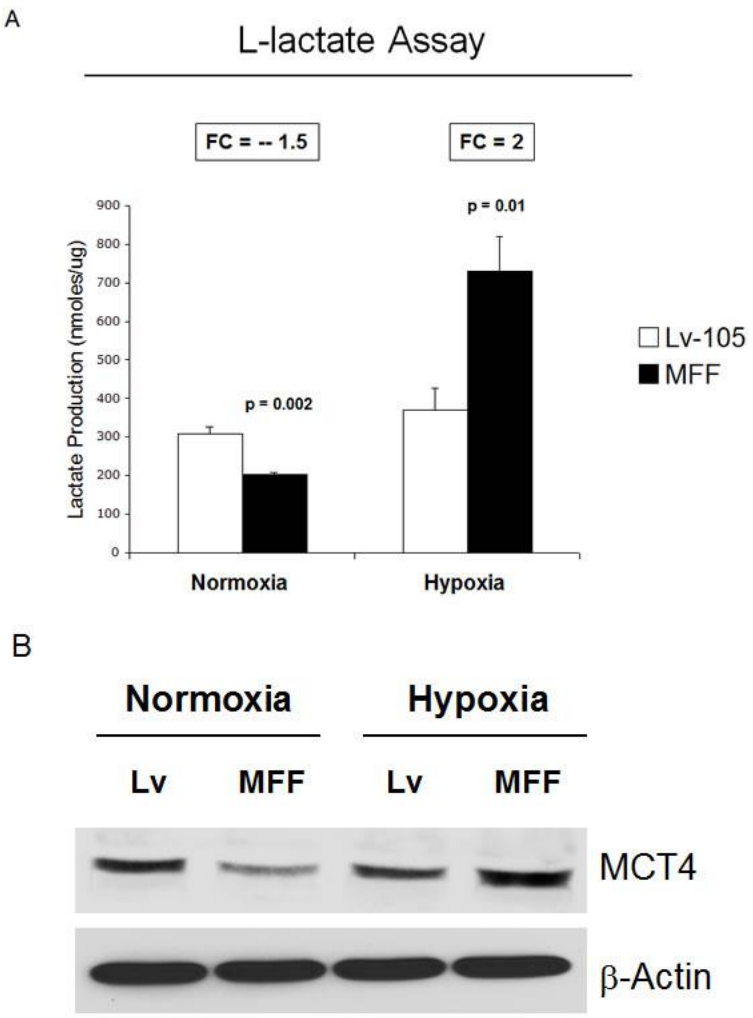

Figure 8: Fibroblasts over-expressing MFF show increased L-lactate generation and MCT4 expression, under hypoxia. (A) Empty vector (Lv-105) and MFF-fibroblasts were grown under normoxic or hypoxic conditions (6 hours), and then subjected to biochemical analysis, to determine L-lactate content in the tissue culture media. The graph shows that MFF-fibroblasts show a 1.5-fold decrease in L-lactate production under normoxia. However, when challenged with hypoxia, MFF-fibroblasts exhibit a 2-fold increase in L-Lactate generation, as compared with empty vector (Lv-105) control cells. (B) MCT4 is a transporter which mediates the efflux of L-lactate and is a marker of oxidative stress and aerobic glycolysis. Empty vector and MFF-fibroblasts were maintained under normoxia or hypoxia (6 hours), and were subjected to immuno-blotting with anti-MCT4 antibodies. Consistent with the L-lactate assay results, MCT4 levels are down-regulated in MFF-fibroblasts under normoxia; in contrast, MCT4 levels are strongly up-regulated in MFF-fibroblasts under hypoxia.

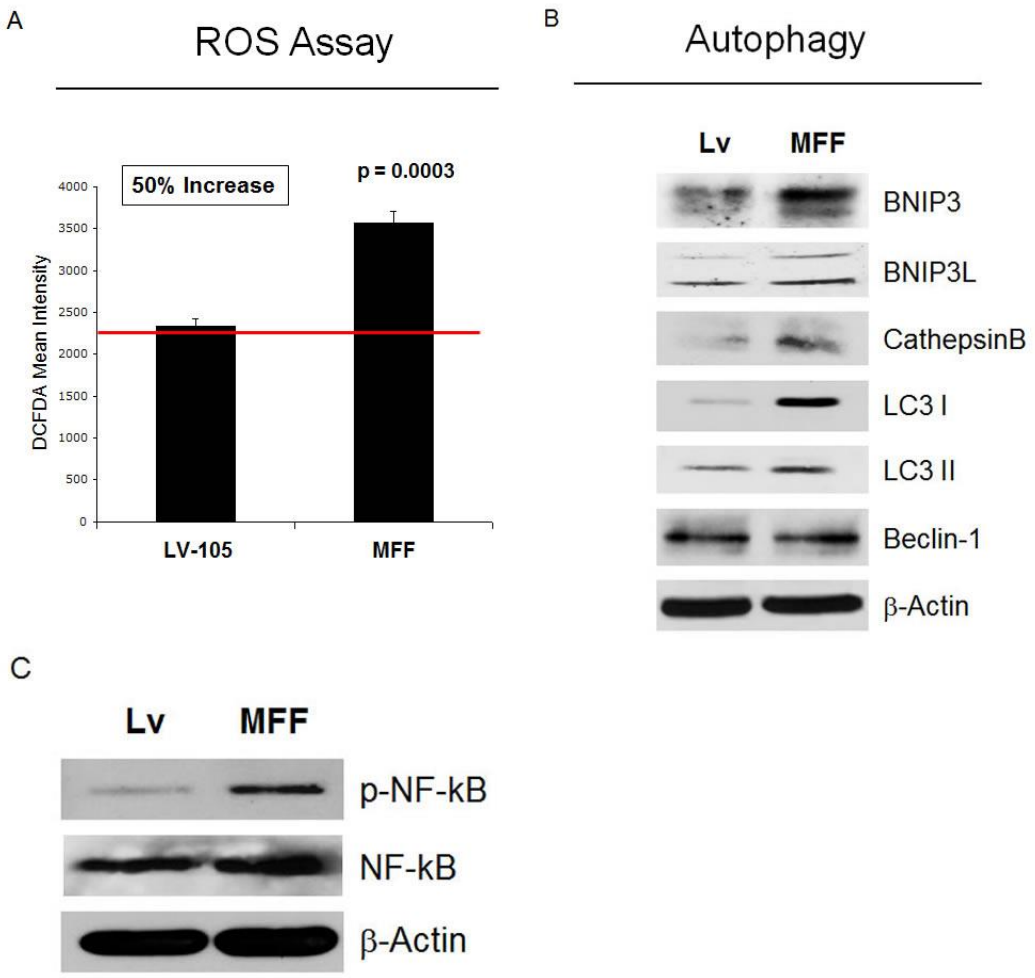

Figure 9: Fibroblasts over-expressing MFF show increased ROS generation, and induction of autophagy, with NF-kB hyper-activation. (A) ROS levels were measured by FACS analysis, as described under Materials and Methods. Note that MFF over-expression induces a 50% increase in ROS production, as compared to control cells. (B) Induction of an autophagic/mitophagic process was analyzed on cellular extracts of fibroblasts expressing MFF and empty vector controls by immunoblotting. Note that MFF-fibroblasts show the up-regulation of several markers of autophagy (LC3I/II and cathepsin B), and mitophagy (BNIP3 and BNIP3L), as compared to control cells. No changes were observed for Beclin-1 expression. β-actin is shown as an equal loading control. (C) NF-kB activation was monitored by immuno-blotting with phospho-NF-kB antibodies. Note that MFF-fibroblasts show constitutive NF-kB activation, relative to control cells, suggesting that NF-kB activation maybe the mechanism driving the induction of autophagy. β-actin was used as an equal protein loading control.

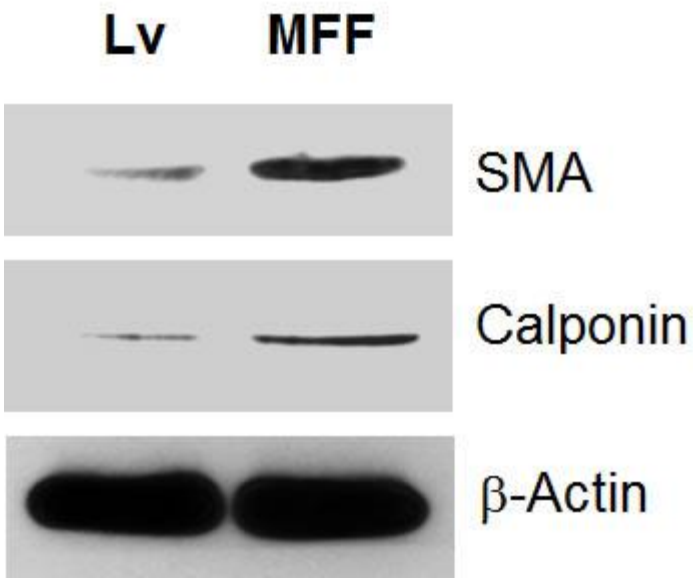

Figure 10: Fibroblasts over-expressing MFF show myofibroblastic features. To evaluate if MFF-fibroblasts acquire myofibroblastic features, cells were analyzed by immuno-blotting with antibodies directed against α-SMA and calponin. Note that MFF-fibroblasts show increased expression of two myo-fibroblast markers, namely α-SMA and calponin, indicating that MFF promotes a myofibroblastic differentiation. β-actin was used as an equal protein loading control.

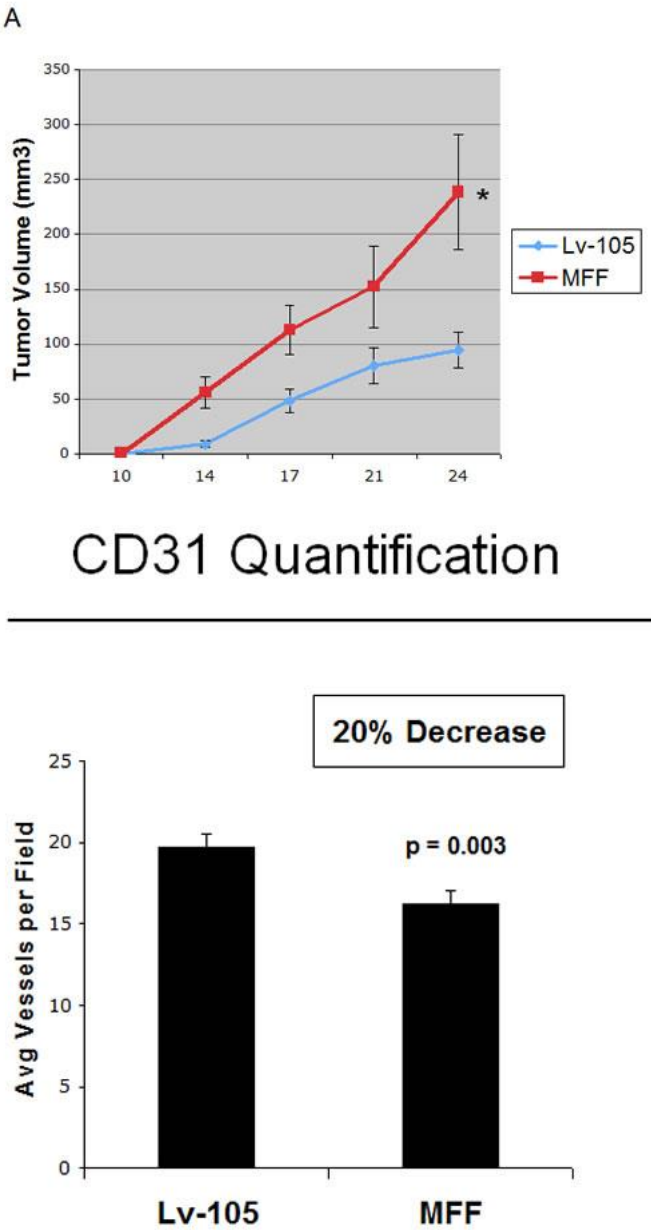

Figure11: Fibroblasts over-expressing MFF promote tumor growth, independently of neo-angiogenesis. (A) To assess the functional in vivo effects of MFF over-expression in fibroblasts, we co-injected MFF-fibroblasts or vector alone control fibroblasts with MDA-MB-231 breast cancer cells into the flanks of nude mice. Then, tumor growth rates were monitored every 3-4 days for 24 days. Note that MFF-fibroblasts promote a signficant increase in tumor growth rates, as compared with control fibroblasts. N = 10 tumors per experimental group. (B) Tumor angiogenesis was monitored by CD31 immuno-staining on tumor frozen sections. Note that MFF-xenografts show a 20% decrease in tumor neo-vascularization, as compared to controls. These data indicate that the MFF-induced tumor growth is independent of angiogenesis.

Table 1: MFF over-expressing fibroblasts increase tumor growth, especially during the early phase of tumorigenesis. Note that fibroblasts over-expressing MFF greatly increase tumor growth. Fold increases in tumor growth rates for MDA-MB-231 xenografts are shown (co-injected with MFF over-expressing fibroblasts versus empty vector controls (Lv-105)). Interestingly, the role of MFF is most relevant in the early phase of tumor development, suggesting that increased mitochondrial fission in the stroma can metabolically promote and support tumor initiation.

Days Post-Injection	Lv-105 Tumor Volume	MFF Tumor Volume	Fold-Increase (MFF/control)	p-values
14	8.51	55.37	6.5	0.003
17	48.14	112.42	2.3	0.01
21	80.40	152.32	1.9	0.047
24	94.38	238.42	2.5	0.009

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1. Ertel A, Tsirigos A, Whitaker-Menezes D, Birbe R, Pavlides S, Martinez-Outschoorn U, et al. Is cancer a metabolic rebellion against host aging? In the quest for immortality, tumor cells try to save themselves by boosting mitochondrial metabolism. Cell Cycle 2012; 11:253 – 63.
2. Sotgia F, Casimiro M, Bonuccelli G, Liu M, Whitaker-Menezes D, Er O, et al. Loss of caveolin-3 induces a lactogenic microenvironment that is protective against mammary tumor formation. Am J Pathol 2009; 174:613 – 29.
3. Kaplan D, Shankaran V, Dighe A, Stockert E, Aguet M, Old L, et al. Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. Proc Natl Acad Sci USA 1998; 95:7556 – 61.

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes

__X__ No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
__X__ No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
__X__ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, and an abbreviated title of the publication. For example, if you submit two publications for Smith (PI for Project 01), one publication for Zhang (PI for Project 03), and one publication for Bates (PI for Project 04), the filenames would be:

Project 01 – Smith – Three cases of isolated
Project 01 – Smith – Investigation of NEB1 deletions
Project 03 – Zhang – Molecular profiling of aromatase
Project 04 – Bates – Neonatal intensive care

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed

acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1. The milk protein α-casein functions as a tumor suppressor via activation of STAT1 signaling, effectively preventing breast cancer tumor growth and metastasis.	Bonuccelli G, Castello-Cros R, Capozza F, Martinez-Outschoorn UE, Lin Z, Tsirigos A, Xuanmao J, Whitaker-Menezes D, Howell A, Lisanti MP, Sotgia F.	Cell Cycle	Sept 2012	☐Submitted ☐Accepted ☒Published
2. Mitochondrial fission induces glycolytic reprogramming in cancer-associated myofibroblasts, driving stromal lactate production, and early tumor growth.	Guido C, Whitaker-Menezes D, Lin Z, Pestell RG, Howell A, Zimmers TA, Casimiro MC, Aquila S, Ando' S, Martinez-Outschoorn UE, Sotgia F, Lisanti MP.	Oncotarget	July 2012	☐Submitted ☐Accepted ☒Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes_____ No ___X_____

If yes, please describe your plans:

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

Ultimately, we believe that these studies will lead to the development of new biomarkers for treatment stratification of breast cancer patients.

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

Overall, these findings suggest that α-casein expression in mammary epithelial cancer cells may represent a potential therapeutic approach as an antitumor agent in breast cancer.

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No___X_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No____

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes___ No____

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No ___X_____

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Michael P. Lisanti, M.D.-Ph.D.	**PI: Professor of Cancer Biology**
eRA COMMONS USER NAME **MPL001**	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
New York University, NY, NY	BA	1985	Chemistry
Cornell University Medical College, NY, NY	MD-PhD	1992/1991	Cell Biology & Genetics
Rockefeller University, NY, NY	Visiting Sci.	1992	Molecular Parasitology
Whitehead Institute/ MIT, Cambridge, MA	Fellow	1992-97	Molecular Cell Biology

A. Professional Experience

2006-2012 Leader/Director, *Program in Cancer Genetics and Molecular Biology*, Kimmel Cancer Center, Thomas Jefferson University (TJU)

2006-2012 Member, *Executive Steering Committee*, Kimmel Cancer Center, TJU

2006-2012 Professor, Dept., of *Cancer Biology*, Kimmel Cancer Center, TJU

2006-2012 Professor, Dept., of *Biochemistry and Molecular Biology,* Kimmel Cancer Center, TJU

2002-2006 Professor, Dept. of *Medicine,* The Albert Einstein College of Medicine (*Secondary Appointment*)

2001-2006 Professor, Dept. of *Molecular Pharmacology*,The Albert Einstein College of Medicine;
Member, Albert Einstein's *Cancer, Diabetes and Liver Centers*

1998-2001 Associate Professor, Dept. of *Molecular Pharmacology*,The Albert Einstein College of Medicine;
Member, Albert Einstein's *Cancer, Diabetes and Liver Centers*

1997-1998 Assistant Professor, Dept. of *Molecular Pharmacology*,The Albert Einstein College of Medicine;
Member, Albert Einstein's *Cancer, Diabetes and Liver Centers*

1992-1997 Whitehead Institute Fellow, Whitehead Institute for Biomedical Research, Massachusetts Institute of Technology (MIT). Affiliated with Dr. Harvey Lodish's laboratory

July 1991-June 1992 Visiting Scientist, with Dr. A. K. Menon, Laboratory of Molecular Parasitology, The Rockefeller University

1988-1991 Ph.D., with Dr. Rodriguez-Boulan, Dept. of Cell Biology, Cornell University Medical College

Honors and Awards. 1981, Top 40 Winner, Westinghouse Science Talent Search, National Navy Science Award; 1984, American Chemical Society Analytical Chemistry Award, Lucy G. Moses Scholarship; 1985, University Honors Scholar, George Granger Brown Prize for Chemistry and Physics, Merk & Co. Book Award for Chemistry, graduated Magna cum laude, Phi Beta Kappa; 1988, Dean's Research Prize; 1990, Julian R. Rachele Prize, Dean's Research Prize and Vincent du Vingneaud Prize; 1991, Dean's Research Thesis Award; 1993, NIH FIRST

Award; 1995, Elsa U. Pardee Foundation Grant; 1997, Charles E. Culpeper Foundation--New Faculty Award, Mathers Charitable Foundation Grant; 1998, Sidney Kimmel Cancer Research Foundation Award, Melini Award; 1999, Albert Einstein Research Achievement Award--External selection committee consisted of Drs. Levine, Sabatini, and Silverstein; given to the most outstanding faculty members at Einstein; 2001, The Bensley Award, American Association of Anatomists

B. Publications (Dr. Lisanti has over 245 publications.

1. Martinez-Outschoorn UE, Lin Z, Flomenberg N, Wang C, Pavlides S, Pestell RG, Howell A, Sotgia F, Lisanti MP. 2011. Understanding the metabolic basis of drug resistance: Therapeutic induction of the Warburg effect kills cancer cells. Cell Cycle, 10: 2521-28.
2. Bryant KG, Camacho J, Jasmin JF, Wang C, Addya S, Casimiro MC, Fortina P, Balasubramaniam S, Knudsen KE, Schwarting R, Lisanti MP, Mercier I. 2011.
3. Caveolin-1 overexpression enhances androgen-dependent growth and proliferation in the mouse prostate. Int J Biochem Cell Biol., 43: 1318-29.
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5. Martinez-Outschoorn UE, Whitaker-Menezes D, Lin Z, Flomenberg N, Howell A, Pestell RG, Lisanti MP, Sotgia F. 2011. Cytokine production and inflammation drive autophagy in the tumor microenvironment: Role of stromal caveolin-1 as a key regulator. Cell Cycle. 10: 1784-93.
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11. Martinez-Outschoorn, U.E., Pavlides, S., Howell, A., Pestell, R.G., Tanowitz, H.B., Sotgia, F., and Lisanti, M.P. 2011. Stromal –Epithelial Metabolic Coupling in Cancer: Integrating Autophagy and Metabolism in the Tumor Microenvironment. Int J Biochem Cell Biol., 43: 1045-51.
12. Sotgia F, Martinez-Outschoorn UE, Lisanti MP. 2011. Mitochondrial Oxidative Stress Drives Tumor Progression and Metastasis: Should We Use Antioxidants As A Key Component of Cancer Treatment and Prevention? BMC Med. 9(1):62.