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14. ABSTRACT

Epidemiologic evidence demonstrates that caloric restriction and physical activity independently reduce breast cancer. Conversely, obesity and insulin resistance are associated with increased breast cancer incidence, metastasis and mortality. To date, no studies have addressed the role of skeletal muscle in breast cancer. To determine the effect of skeletal muscle mass on breast cancer, we are measuring rates of chemically induced mammary tumorigenesis and progression in genetically hypermuscular mice. Mice lacking the skeletal muscle-specific muscle growth inhibitor myostatin and mice expressing a dominant negative form of the myostatin receptor, Activin Receptor Type IIB, display heightened muscle mass. In order to induce mammary cancer in these mice, we administered a combination of a tumor promoter, medroxyprogesterone acetate, and a carcinogen, dimethylbenz-a-anthracene, using a defined protocol. Unfortunately, we have experienced both high non-tumor associated mortality and low fertility, slowing progress of this study and requiring us to seek a no-cost extension of the project. We have resolved the environmental issues leading to high pup mortality and refined the MPA/DMBA model to produce 100% tumor incidence with minimal lethality and are continuing the study. By the completion of this study in 12-18 months, tumor latency, size, stage and burden along with serum hormone/adipokine/myokine levels will be measured. Statistical analyses will be performed to identify relationships among genotypes, hormone/adipokine/myokine levels and rates of breast cancer initiation and progression.

15. SUBJECT TERMS
Breast cancer, skeletal muscle, myostatin, MPA, DMBA, Activin receptor

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A. INTRODUCTION

Epidemiologic evidence demonstrates that caloric restriction and physical activity independently reduce breast cancer. Conversely, obesity and insulin resistance are associated with increased breast cancer incidence, metastasis and mortality. Current data suggest that the heightened glucose and insulin levels of obesity and Type II diabetes synergize with adipose-derived hormones, cytokines and growth factors ("adipokines") to increase mammary cell proliferation and promote tumor angiogenesis. To date no studies have addressed the role of skeletal muscle in breast cancer however, despite the fact that skeletal muscle accounts for 30-50% of energy expenditure, plays a significant role in regulating insulin sensitivity and fatty acid and glucose metabolism, and is a source of cytokines and growth factors, including interleukins, Insulin-like Growth Factor (IGF) isoforms, IGF-binding proteins and myostatin.

To determine the effect of skeletal muscle mass on breast cancer, we sought to measure rates of chemically induced mammary tumorigenesis and progression in genetically hyper-muscular mice. The models we are using are mice bearing targeted deletion of the myostatin gene (Mstn-/- mice)[1] and mice expressing a truncated form of the myostatin receptor, the Activin Receptor Type IIB, specifically in skeletal muscle under control of the myosin light chain promoter [2]. The truncated receptor functions as a dominant negative, rendering the muscle resistant to signaling from myostatin and other ActRIIB ligands, including Activin and potentially GDF-11 [2]. Both strains of mice exhibit increased skeletal muscle mass and decreased adiposity versus wild-type controls. Reproduction and life span are grossly normal in these mice (unpublished data, T.Z.). Effects of either of these genetic manipulations on mammary tissue have not been characterized. Cows bearing mutations in the myostatin gene, however, are reported to produce less milk [3], suggesting that myostatin may influence mammary tissue, either directly or indirectly.

In order to induce mammary cancer in these mice, we chose to administer a combination of a tumor promoter, medroxyprogesterone acetate, and a carcinogen, dimethylbenz-a-anthracene, using a published protocol [4]. Unfortunately, we have experienced both high non-tumor associated mortality and low fertility (see below), slowing progress of this study and requiring us to seek a no-cost extension of the project.

By the completion of this study, projected to be in 12-18 months, tumor latency, size, stage and burden along with serum hormone/adipokine/myokine levels will be measured. Statistical analyses will be performed to identify relationships among genotypes, hormone/adipokine/myokine levels and rates of breast cancer initiation and progression.

B. BODY

The Tasks outlined in our statement of work were:

Task 1. To measure rates and progress of mammary tumorigenesis in genetically lean, hyper-muscular mice. (Months 1-12)

a) Myostatin null mice:
   i) Breed myostatin heterozygous mice to obtain the desired number and genotype: 56 female mstn+/+ and 56 female mstn -/- mice (Months 1-3).
   ii) Treat 40 mice per genotype with the carcinogen, DMBA, once a week for six weeks in the presence of medroxyprogesterone acetate (MPA) (Months 4-5).
   iii) Kill and necropsy mice upon gross evidence of tumor (Months 5-12).
   iv) Perform histopathological analsyis of tumors (Month 12).

b) Muscle-specific dominant-negative Activin Receptor Type IIB transgenic mice:
   i) Breed dnActRIIB+/o mice to obtain the desired number and genotype: 56 female dnActRIIB+/o and 56 female nullizygous mice (Months 1-3).
   ii) Treat 40 mice per genotype with the carcinogen, DMBA, once a week for six weeks in the presence of medroxyprogesterone acetate (MPA) (Months 4-5).
   iii) Kill and necropsy mice upon gross evidence of tumor (Months 5-12).
   iv) Perform histopathological analsyis of tumors (Month 12).

Task 2. To determine levels of hormones, adipokines, and myokines in normal and genetically hyper-muscular mice, with and without mammary carcinogenesis.

a) Serum collection and assay for potential mediators of mammary carcinogenesis, including hormones, adipokines and myokines (specifically estrogen, leptin, adiponectin, insulin, free and bound insulin-like growth factors, insulin-like growth factor binding proteins, interleukin-6, and growth hormone) in 8 young and 8 aged (1 year) wild-type and hypermuscular mice, for a total of 64 mice. Assays will be performed by a combination of ELISA and multiplex analyte profiling using Luminex technology (Months 3 and 12).

b) Serum collection and assay for potential mediators of mammary carcinogenesis, including hormones, adipokines and myokines (specifically estrogen, leptin, adiponectin, insulin, free and bound insulin-like growth factors, insulin-like growth factor binding proteins, interleukin-6, and growth hormone) in a subset of treated mice at sacrifice upon evidence of mammary tumorigenesis. Assays will be performed by a combination of ELISA and multiplex analyte profiling using Luminex technology (Months 3 through 12).

Task 3. To identify statistical relationships between myostatin genotype and breast cancer-related phenotype.

a) To compare/correlate rates of mammary tumor initiation and progression with myostatin and dnActRIIB genotype (Month 12).

b) To compare/correlate levels of serum hormones, adipokines and myokines with myostatin and dnActRIIB genotype (Month 12).

c) To compare/correlate levels of serum hormones, adipokines and myokines with mammary tumor incidence and stage (Month 12).

d) To compare/correlate levels of serum hormones, adipokines and myokines with mammary tumor incidence and stage and myostatin or dnActRIIB genotype (Month 12).

Progress Report

Task 1. To measure rates and progress of mammary tumorigenesis in genetically lean, hyper-muscular mice. (Months 1-12)

Low fertility/high pup mortality resulted in few mice for analysis:

In order to produce the numbers of mice indicated for the study, we set up cages containing triplet matings of one male and two females for each line. Mstn-/- mice have been backcrossed 10 generations onto C57BL6/J, thus rendering them >99% genetically identical to C57BL6/J. Thus we maintained the mstn-/- line by crossing mstn-/- males to mstn-/- females. A line of C57BL6/J mice were similarly bred to serve as controls. The transgenic MLC-dnActRIIB mice are on a mixed genetic background, thus mice hemizygous for the MLC-dnActRIIB transgene were crossed to C57BL6/J mice, with age-matched littermates serving as controls.

Although at least 12 triplet mating cages each were set up for the mstn-/- and MLC-dnActRIIB lines, few pups were born or survived to weaning. Interventions applied to increase fertility included switching the breeders to an enriched diet ("love mash"), providing both cotton and paper nestlets to promote nest building, adding plastic mouse "igloos" to the cages to provide a nesting environment, and moving the cages to various low-activity, low-vibration regions of the room. We also discovered that the room was routinely experiencing large temperature variations ranging from the low 60s to the high 80s during the day. We suspected that the temperature shifts might be impairing fertility or pup survival, consistent with published reports. Ultimately, working with the Division of Veterinary Resources and Physical Plant, we were able to resolve the temperature fluctuations and maintain the room in the range of 70-75F. This was resolved in mid-March, 2006.

Due to the low fertility rates of the transgenic and knockout lines, most of the female pups generated were put back into the breeding scheme. As a result, we did not attain the numbers of mice required and indicated in the original Statement of Work (40 mice per genotype). We will continue to breed the mice and subject them to the dosing regimen as described in the Statement of Work for the duration of the one-year No-Cost Extension.

The original model of MPA/DMBA mammary tumorigenesis resulted in high non-tumor related mortality, requiring refinement of the model:

Aldaz et al. described a method using medroxyprogesterone (MPA) to accelerate development and increase incidence of mouse tumors induced by dimethylbenzanthracene (DMBA)[4]. They describe implanting 2 subcutaneous pellets (standard release, no binder) of MPA, 20mg each, into 6 wk old female mice. Beginning

at nine weeks, DMBA was administered by gavage at a concentration of 1mg/dose in 0.1ml cottonseed oil at 9, 10, 12, and 13 weeks of age. Mice were palpated weekly to identify mammary tumor development.

Using this model, we experienced a nearly 100% mortality rate prior to the end of the DMBA administration, leaving no mice for analysis of mammary tumorigenesis. We ruled out technical issues such as gavage or carrier-associated injury/toxicity as the cause of death. MPA is also known as DepoProvera, a contraceptive in clinical use.

Improved MPA/DMBA model of mammary tumorigenesis:
In humans, DepoProvera is administered as an intramuscular injection every three months. To refine our model and reduce pain and distress, we substituted pellet implantation with subcutaneous injection of MPA, then titrated the DMBA dose in wild-type C57BL6/J mice to identify a dose with minimal toxicity. Ultimately we generated the following treatment scheme:

3 wks – tail cut and genotyping
4 wks – weaning and separation from parents
6 wks – s.c. injection of 20mg MPA
9 wks – gavage with 13.2 mg/kg DMBA in 0.1 ml cottonseed oil
10 wks – gavage with 13.2 mg/kg DMBA in 0.1 ml cottonseed oil
12 wks – gavage with 13.2 mg/kg DMBA in 0.1 ml cottonseed oil
13 wks – gavage with 13.2 mg/kg DMBA in 0.1 ml cottonseed oil

This dosing regimen had markedly increased survival, with 13 of 16 wild-type mice surviving to the end of the treatment schedule.

Task 2.To determine levels of hormones, adipokines, and myokines in normal and genetically hyper-muscular mice, with and without mammary carcinogenesis.

This task required collecting serum samples from mice of all the indicated strains and genotypes at 8wks and 1 year with no treatment, as well as at sacrifice after MPA/DMBA treatment. We have been collecting the necessary samples as the mice reach the desired age. Because the serum collection requires a terminal bleed, no mice have been sacrificed for the 8wk point yet, with those mice instead being used for breeding or MPA/DMBA treatment. Serum samples have been collected from one-year old mice from each strain and genotype. Sample from tumor bearing mice are being collected as the mice are analyzed. All of the samples are being kept at -80C in storage until all of the samples are available. The assays for serum adipokine and myokine levels will be performed once all samples are in hand, thereby minimize assay variations.

Task 3. To identify statistical relationships between myostatin genotype and breast cancer-related phenotype.

Obviously this Task requires completion of Tasks 1 and 2, which are still in progress. To date, we have subjected roughly 15 mice of each strain and genotype to the improved

MPA/DMBA protocol. To date, nearly 100% of the treated mice develop multiple, palpable mammary tumors, with a latency as short as 2 wks after the last DMBA injection. The number of mice analyzed to date is too small to present a valid analysis of differences among genotypes.

Table 1: Numbers of female mice of each strain and genotype subjected to this MPA/DMBA protocol to date.

Strain	Genotype	Subjected to MPA/DMBA protocol (n)
Mstn	-/-	14
Mstn	+/+	14
MLC-dnActRIIb	tg/o	16
MLC-dnActRIIb	o/o	15

Additional Considerations:

In view of the low fertility and precedent for low milk production in myostatin mutant cows, we have determined that a careful analysis of the baseline mammary phenotypes is required. Therefore, we are collecting mammary glands and making whole mount preparations from virgin, lactating, involuting and aged mice, particulary of the Mstn-/- and wild-type strains. These will be analyzed as part of this project.

C. KEY RESEARCH ACCOMPLISHMENTS

- □ Gained expertise in whole mount mammary gland preparations and histology.
- □ Established in our hands and refined the MPA/DMBA model to reduce non-tumor related toxicity.
- □ Resolved environmental control issues relating to low fertility to increase numbers of mice available for study.
- □ Banked blood and tissue specimens from all of the mice analyzed to date for analysis at end of study when all samples have been accumulated.
- □ Measured tumor latency, frequency, size and stage in a fraction of the mice initially proposed.

D. REPORTABLE OUTCOMES

No reportable outcomes to date.

E. CONCLUSIONS

To date, technical issues have hampered the progress of the project. We believe that the technical issues have been resolved, however, and are continuing the analysis proposed. We will ultimately determine whether hyper-muscularity and low adiposity confers a protective effect against mammary tumor incidence and progress in the mouse MPA/DMBA model. As well, we will describe the profile of serum adipokines and myokines in these models and their wild-type counterparts. We expect to have reportable results by within the next 12-18 months.

F. REFERENCES

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G. APPENDICES

None.