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Examining Maternal Obesity Effects on Oogenesis, Follicular Development, and Embryonic Myogenesis

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Introduction

The CDC estimates that more than one-third of U.S. adults (approximately 78.6 million) are obese. Despite national efforts to combat this with diet and exercise, the number of obese adults and children continues to climb. It is critical to emphasize that obesity can lead to lifelong, chronic complications and health risks including hypertension, heart disease, and diabetes.

Diet and obesity also have important impacts on the reproductive health of female including the ability to become pregnant (fertility) and risk for complications during pregnancy. Furthermore, for women who do become pregnant, obesity can affect the development of the embryo and fetus, with studies showing impaired physiological function of important organ systems (e.g. cardiovascular, metabolic organs, and brain) in the otherwise viable offspring.

THE OBJECTIVES OF THIS STUDY INCLUDED:

- Determining if consumption of dietary exosomes, which contain miRNAs that can regulate gene expression in target tissues, regulates the activation and growth of ovarian follicles.
- Developing tools to identify changes in skeletal muscle development in offspring from obese compared to lean mothers.

Ovary Collection and Follicle Quantification

C57/BL6 fed ExoPlus or ExoMinus diet for 4 weeks

↓

Ovary collection

↓

Fixed with 4% paraformaldehyde and paraffin embedded

↓

Sectioning and hemotoxylin and eosin staining

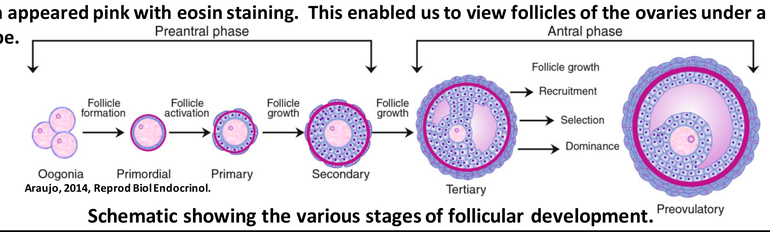
↓

Quantification and comparison of follicle count between diet types

MicroRNA (miRNA) are small-non coding RNAs which regulate mRNA degradation and translation. Recent reports show that miRNAs are found in bovine milk enclosed in extracellular vesicles. These vesicles (exosomes) protect the miRNA resulting in their absorption. Interestingly female mice fed a diet depleted of these vesicles had reduced pregnancy rates and litter sizes. To see if this was due to abnormal follicle/oocyte growth, we collected ovaries from C57BL/6 female mice fed a diet that included bovine milk containing the exosomes (ExoPlus, control) or diet depleted of exosomes (ExoMinus).

Next, ovaries from animals of the two diet groups were collected and isolated. Ovary samples were first dehydrated using successively more concentrated ethanol washes followed by addition of xylene to remove the ethanol in preparation for embedding. After the samples were embedded in paraffin, partitioned into 5 µm sections, and were placed onto slides.

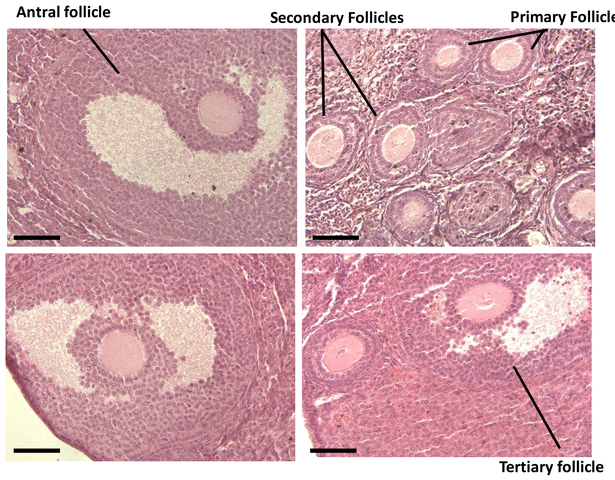
Slides were then rehydrated by exposure to xylene, then to successively less concentrated ethanol washes, and finally to water. They were then stained with hemotoxylin and eosin techniques. Hemotoxylin stains nucleic acid complexes a purple color, while the extracellular matrix and cytoplasm appeared pink with eosin staining. This enabled us to view follicles of the ovaries under a microscope.



Araujo, 2014, Reprod Biol Endocrinol.

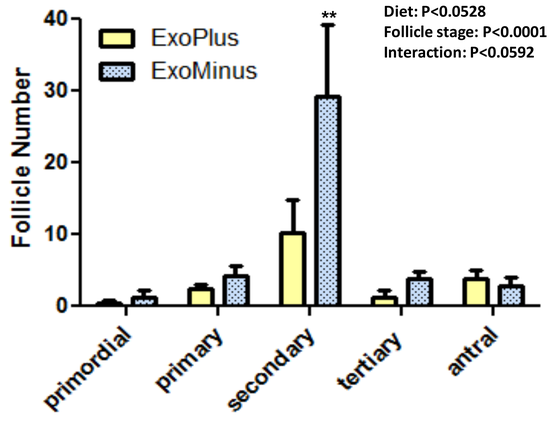
Schematic showing the various stages of follicular development.

More follicles progressed to the secondary stage in mice receiving bovine milk depleted of exosomes compared to mice receiving bovine milk containing exosomes



To determine if the difference in diet (exoplus containing normal dietary amounts of miRNA and exominus being depleted of these miRNAs), follicles from the primordial stage to antral stage were identified and counted. These images were taken of the H&E ovary sections and are representative of what was observed under the microscope when counting the follicles.

Approximately 10-12 ovary slides per animal (each slide containing 9 tissue-section samples) were collected and organized according to the order in which they were cut in. Slides were then selected for counting based on those that contained a more complete cross-section of the ovary, one per animal. It is important to note that he entire oocyte nucleus had to be observed in order to control for duplicate counting. Follicles were then counted once and categorized based on physical indicators of their developmental stage and totaled accordingly.

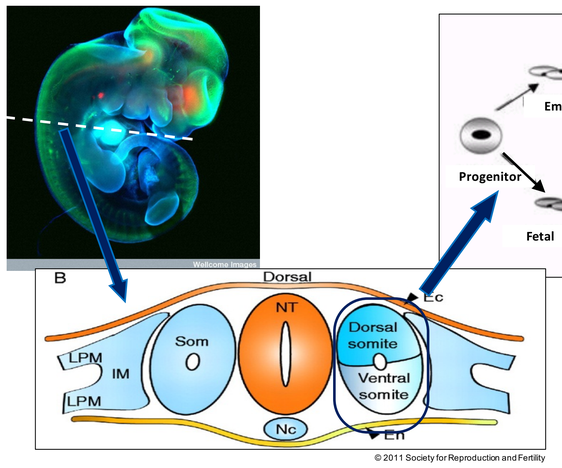


Diet: P<0.0528
Follicle stage: P<0.0001
Interaction: P<0.0592

To determine if the difference in diet (exoplus containing normal dietary amounts of miRNA and exominus being depleted of these miRNAs), follicles from the primordial stage to antral stage were identified and counted.

The average number (± SEM) of each follicle stage (primordial, primary, secondary, tertiary, and antral) in the ovaries from each experimental group (ExoPlus, ExoMinus) were analyzed using two-way ANOVA. This analysis showed a significant effect of follicle stage as well as a tendency for an effect of diet on the number of follicles counted. There was also a tendency for an interaction between diet and follicle stage (P<0.0592). Bonferroni post-test showed that this interaction was due to a significant increased in the number of follicles at the secondary stage in ovaries from ExoMinus compared to ExoPlus fed mice.

Children of obese mothers are at increased risk of metabolic disease which may be due to a difference in the development of skeletal muscle

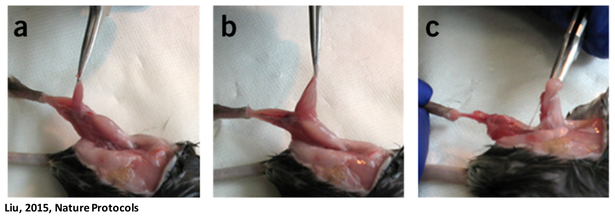


Maltin, 2008, Organogenesis

Skeletal muscle develops from progenitor cells adjacent to the neural tube (NT). Progenitors are activated to grow based on signals from surrounding cells. These external cues and the stage of development (embryonic vs. fetal) regulate transcription factor (*Pax7, MyoD, etc*) expression and ultimately the number and types of fibers that develop.

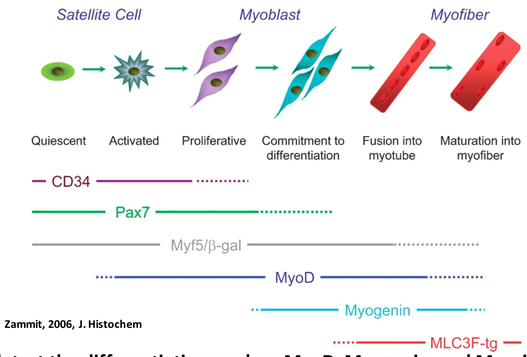
Maternal obesity can impact levels of these transcription factors and therefore the type of skeletal muscle fibers that develop. In previous studies, we showed increased expression of desmin in embryos from obese mothers indicative of excessive differentiation of progenitor and proliferating cells. It is our hypothesis that this increase in skeletal muscle differentitation during an early timepoint in development may result in an overabundance of Type I (slow oxidative fibers) and thereby change the metabolism of the offspring. Alternatively, the increased differentiation may reduce the population of progenitor cells available for activation later in development and result in muscle fibers with reduced diameter and capacity for hypertrophy. Finally, we hypothesize that offspring from obese mothers may have a reduced population of progenitor cells important for renewal of skeletal muscle fibers during adult life. Together, these changes in skeletal muscle physiology would have profound effects on metabolism and potentially predispose affected offspring for type II Diabetes.

Immunofluorescent Staining of Mouse Hind limb Skeletal Muscle with Markers of Progenitor, Proliferating, Differentiating, and Mature Cells

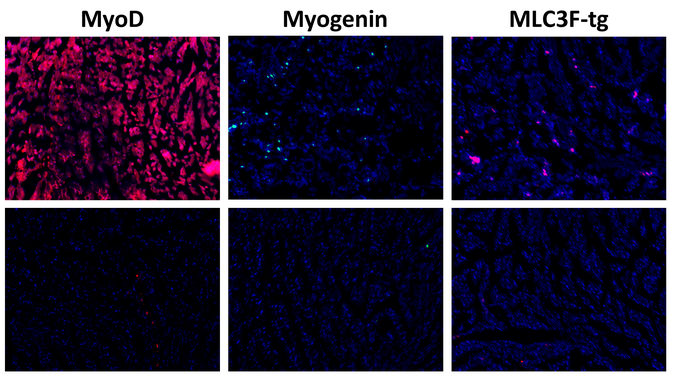


Liu, 2015, Nature Protocols

- The objective of this project was to determine if muscle progenitor and fiber number are indeed decreased in offspring from obese compared to lean mothers. To achieve this objective we first needed to validate the detection of important markers of skeletal muscle progenitors, myoblasts, and myofibers.
- Hind limb muscles were collected from *CD1* mouse pups at 5-days of age.
- The tissue was cryopreserved, embedded, and cryosectioned (10 µm sections).



Zammit, 2006, J. Histochem



To detect the differentiation markers MyoD, Myogenin, and Myosin (MLC3F-tg) immunofluorescence was performed using the hindlimb sections described above. Tissue samples were exposed initially to a primary antibody specific for the target marker and then hybridized to a secondary antibody containing a fluorochrome. The samples were then imaged using fluorescent microscopy. It is important to note that currently the conditions of Pax 7 are still being worked on for the continuation of this study. The purpose of the negative control was to allow differentiation between non-specific background signaling with other cellular components and signaling produced by the targeted markers. The results indicate that location and differentiation of these biomarkers from other cellular components was successfully achieved.

Conclusions and Future Directions

Although pregnancy rates and litter size are decreased in females that consume an ExoMinus compared to ExoPlus diet, there was increased activation of follicle growth in the ExoMInus versus Exoplus fed mice. It may be that this increased activation results in increased atresia of follicles and depletion of the ovarian reserve since there is no difference in the number of antral follicles between the two experimental groups. This would have the potential to cause premature ovarian failure and could contribute to decreased pregnancy rates. Future studies will determine the impact of the ExoMinus diet on ovulation rate and early embryonic development.

We have successfully identified antibodies against the proteins MyoD, Myogenin, and MLC3F-tg. In future studies we will continue to examine how maternal obesity affects the expression of these skeletal muscle markers of progenitor cell activation, proliferation, and differentiation in order to identify differences in the number of cells at each developmental stage in the hind limb. To profile the expression of these markers in the hindlimb will be collected from mice ages 5 days to 120 days (3 months) that had a lean vs. obese mother.

We will also investigate any changes in muscle type (oxidative versus glycolytic muscle fiber types) by sectioning and differentially staining the sections microscopically analyzing the density and sizes of the muscle. Satellite cells will also be isolated and incubated with varying concentrations of insulin, catecholamines, and cytokines to determine the proliferative capacity to each respective hormone. Then, these cells will be stained and analyzed.

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