Obesity III
Obesogen Assays: Limitations, Strengths, and New Directions

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Overview of Obesogen Models

Established and Emerging Obesogenic Chemical Evaluation Models

*In vitro* models

*In vivo* models
Use of Models in Metabolic Health Toxicity Assessments

- Evaluating causal toxicity of chemicals relies on a combination of *in vitro* and *in vivo* models
  - Need for HTP, reliable *in vitro* models to accurately screen for and prioritize higher order testing
  - Need for reliable *in vivo* models that are cost-effective, have high translation to human health, and are well-validated

- Traditionally, MDC research has relied heavily on rodent-based cell and animal models (3T3-L1)
  - Models used have been broadening over time
    - Increasing use of MSCs and human cell models
    - Increasing use of fish models, particularly zebrafish
    - Increasing use of non-traditional models such as fruit fly, *c. elegans*
Potential Mechanisms of Metabolic Dysfunction

- Numerous potential mechanisms of metabolic disruption:
  - Adipose lineage commitment from MSCs
  - Adipocyte differentiation from precursor committed cells
    - Increased pre-adipocyte proliferation
    - Increased lipid uptake
  - Shifting energy balance to favor calorie storage
  - Altering basal metabolic rate
  - Altering hormonal control of appetite and satiety
  - Altering brain circuitry that controls food intake, energy expenditure

Heindel et al. 2017, Repro Tox
Adipocyte Differentiation Process

Adipocyte commitment
- Mesenchymal stem cell
- Other pathways: Myoblasts, Osteoblasts, Chondroblasts

Adipocyte differentiation
- Preadipose cell
  - Pref-1
  - A2COL6/pO2b4
  - LPLF A transport
  - PPARδ
- Preadipose cell
  - C/EBP βδ
  - IGF-1
  - PRAR γ2

- Immature adipose cell
  - C/EBP γ; GLUT4; β2 AR; β3 AR; ACC FAS; ME; ATP-citrate lyase; GPDH; HSL; ALBP; perilipin; apoE; low Km PDE; GPAT; LPAT; DGAT; SCDI

Emergence of very late markers and further triacylglycerol accumulation
- Growth arrest and emergence of early markers
- Growth reduction (clonal expansion) followed by emergence of late markers and triacylglycerol

Resemble brown/developing white adipose cell

Nagy et al. 2011, Mol Med
3T3-L1 Pre-adipocyte Adipogenesis Assay

- Swiss albino mouse embryonic fibroblast cell line – committed pre-adipocytes
- Extensively used over decades to evaluate adipogenesis
- Mechanisms of adipocyte differentiation well understood
- This assay has proven to be a reliable *in vitro* model for screening metabolic disruption *in vivo*.

**Differentiation cocktail:**
- 5% NCS -> FBS,
- 1 µg/mL insulin,
- 800 mM IBMX
Adipogenesis Assay Measures

- Triglyceride accumulation
  - AdipoRed - hydrophilic fluorescent dye
    - Partitions into lipid droplets in the cells

- Cell proliferation/cytotoxicity
  - NucBlue DNA dye (Hoechst 33342)
    - Partitions into nuclei and fluoresces upon binding DNA

Rosiglitazone (PPARγ agonist)
Diversity of Cell Model Utilization

*In vitro* models for metabolic disruption screening

**ADIPOCYTES**
- **Preadipocytes**
  - Proliferation
  - Adipogenesis
- **Mesenchymal Stem cells**
  - Characterization of Obesogens
- **Spheroid adipocyte model**
  - Adipose physiology

**LIVER**
- **HepaRG**
  - Aldolase B, CYP2E1, CYB3A4
  - CYP1A1, CYP1A2, CYP1B1
- **Primary Human Hepatocyte**
  - Drug metabolism
  - Liver enzyme induction
- **3D cell culture**
  - NASH model

**MUSCLE**
- **C2C12**
  - Insulin signaling
  - Mitochondrial function
  - Protein synthesis
- **L6**
  - IR expression
  - Glucose uptake
  - Insulin Resistance
- **Primary Myoblast**
  - Calcium signaling
  - Resting potential
Growing Reliance on MSCs, Human Cells

- Increasing commercial availability of human MSCs, human pre-adipocytes
  - Less reliance on donors, self-isolation
  - Can source from males/females, lean/obese, diabetic/non, subcutaneous/visceral, etc.

- Ability to examine the interplay of commitment across cell lineages (e.g., bone and adipose, muscle, etc.)

- Increasing utility of liver cell assays to examine TAFLD/NAFLD phenotypes, primary human hepatocytes (despite limitations) have increasing use in drug metabolism

- Limited but increasing evaluation of myogenic differentiation and ability of MDCs to suppress signaling/development
Examination of Adipocyte Lineage Commitment as More Novel Endpoint

**Commitment assays**

- **1 day**: Seed cells
- **2 days**: Test chemical treatments
- **7 days**: Confluency, Start of differentiation
- **7 days**: Adipocyte maintenance media, Assay cells

**Adipogenesis assays**

- **3 days**: Seed cells
- **7 days**: Confluency, Start of differentiation
- **7 days**: Adipocyte maintenance media, Assay cells

**Assay endpoints**:
- Triglyceride accumulation (Nile Red stain)
- Pre-adipocyte proliferation (Hoescht DNA stain)
- Adipocyte lineage commitment (comparison of triglyceride accumulation between exposure study designs)
 Increasing Diversity of *in vivo* Models

### In vivo models for metabolic disruption screening

<table>
<thead>
<tr>
<th>Models</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tbody>
<tr>
<td>Zebrafish</td>
<td>• Rapid development, ease of breeding, transparency</td>
<td>• Moderate flexibility</td>
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<td>• Metabolic organs/tissues are physiologically and anatomically similar to humans</td>
<td>• Moderate translational value</td>
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<td></td>
<td>• High-resolution fluorescent imaging of total body adipose</td>
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<td></td>
<td>• Ease of molecular manipulation, wealth of transgenic models</td>
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<tr>
<td>Medaka</td>
<td>• Genetic sex determination like humans</td>
<td>• Moderate flexibility</td>
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<tr>
<td></td>
<td>• Metabolic organs/tissues are physiologically and anatomically similar to humans</td>
<td>• Less characterization of adipose relative to zebrafish</td>
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<td></td>
<td>• Ease of molecular manipulation, small genome size, high diversity</td>
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<td>C. elegans</td>
<td>• Compounds that modulate fat storage and obesity can be identified</td>
<td>• Lower conservation of biological pathways with mammals</td>
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<td>• Food intake and energy expenditure can be measured easily</td>
<td>• Lack of specific organs and circulatory systems</td>
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<td>D. melanogaster</td>
<td>• Small size, short generation time, inexpensive and easy breeding</td>
<td>• Anatomically different from mammals</td>
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<td>• Several discrete organs perform the same as humans</td>
<td>• Lower conservation of many relevant biological pathways with mammals</td>
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<td>• Less regulations governing invertebrate animal use</td>
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<td>Rodents</td>
<td>• Well described model with clear translation to human outcomes</td>
<td>• Time consuming and expensive compared to above alternatives, but less so with larger animal models (e.g. porcine, bovine, ovine, and non-human primates).</td>
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<td>• Periconception, pregnancy, parental and offspring, short- and long-term, multi- and trans-generational outcomes can be assessed</td>
<td>• Ethical issues; regulatory push to reduce use of mammalian vertebrate animal models</td>
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<td></td>
<td>• Diverse housing materials readily available</td>
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<td>• Well-characterized &amp; customizable feed options readily available</td>
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<td>• Inbred and outbred models available to dissect role of genes, environment, and their interactions</td>
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Zebrafish as a Metabolic Model

- Measurable adipose/adipocytes appear as early as ~9-12 days of development in zebrafish, originally in the pancreatic and abdominal visceral depots.
- 34 anatomically/physiologically/molecularly distinct adipose depots throughout the body of the fish.
- Clear developmental timeline.
- Fish adipose tissue contains a heterogeneous cell population, including adipocyte progenitor cells – similar to mammals.
- Depots separated into subcutaneous, visceral, intramuscular adipose tissues, with characteristics similar to humans.
- Zebrafish do not have brown adipocyte tissue.

Minchin and Rawls, 2017 *Dis Mod & Mech*
Zebrafish as a Metabolic Model

- Molecular mechanisms underlying adipocyte and lipid depot development are highly conserved across vertebrates:
  - Genes associated with adipocyte differentiation (fabp, pparg, cebp), lipolysis (lipoprotein lipase), and endocrine function (leptin, adiponectin, adipsin)
  - Energy storage functions and morphology of adipose tissue

- Adipose depots respond to high fat challenge and food withdrawal as you would anticipate
  - Organisms utilize the adipose in times of food stress and pack on extra adipose with HFD

- Imaging of whole-animal adipose in mammals is limited, technically challenging, and generally low resolution, whereas imaging in fish is high-resolution and relatively easy

Minchin and Rawls, 2017 Dis Mod & Mech
**C. Elegans as a Metabolic Model**

- Small nematode living in temperature soil environments
- Main regulatory pathways of energy homeostasis shared with mammals
  - Lower conservation of many of these pathways and lack of specific organs
    - Lack PPARg, though express orthologs to PPARa and PPARd
    - No identifiable homolog for leptin
    - No cells specifically designed for lipid storage (i.e., adipocytes)
    - Store lipids primarily in intestinal and epidermal skin-like cells
- BPS, methylmercury, and other MDCs increase lipid deposition, similar to other *in vivo* MDC models
Drosophila melanogaster as a Metabolic Model

- Fruit fly model organism prized for rapid life cycle, large number of offspring per generation, and simpler genetics relative to most vertebrates
- Despite anatomical differences, lots of functional overlap with humans
  - Fat body covers many of the metabolic health functions of both liver and adipose tissue
- DEHP, methylmercury, PFAS have been described to increase weight/adiposity and/or signaling
The Future of Obesogen / MDC Screening

- Need for new/improved standardized testing methods to ID chemicals that disrupt metabolic health through diverse mechanisms.
  - Multiple large-scale EU efforts designed to help address this gap

- Improved understanding and validation of alternative / emerging *in vitro* and *in vivo* obesogen models.
  - Increasing use of other animal models, human *in vitro* models, and 3D/spheroid cell culture techniques

- Predictive modeling may offer some improved utility in screening the myriad chemicals in commerce for MDC properties
  - Need for reliable, reproducible ToxCast and other input data
  - Need for robust understanding of MIEs, contributory mechanisms
Questions?