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Preclinical models to study obesity and breast cancer in females: Considerations, Caveats, and Tools.

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Abstract

Obesity increases the risk for breast cancer and is associated with poor outcomes for cancer patients. A variety of rodent models have been used to investigate these relationships; however, key differences in experimental approaches, as well as unique aspects of rodent physiology lead to variability in how these valuable models are implemented. We combine expertise in the development and implementation of preclinical models of obesity and breast cancer to disseminate effective practices for studies that integrate these fields. In this review, we share, based on our experience, key considerations for model selection, highlighting important technical nuances and tips for use of preclinical models in studies that integrate obesity with breast cancer risk and progression. We describe relevant mouse and rat paradigms, specifically highlighting differences in breast tumor subtypes, estrogen production, and strategies to manipulate hormone levels. We also outline options for diet composition and housing environments to promote obesity in female rodents. While we have applied our experience to understanding obesity-associated breast cancer, the experimental variables we incorporate have relevance to multiple fields that investigate women's health.

Keywords

obesity; adipose tissue; preclinical model; breast cancer; thermoneutrality; diet; ovariectomy; menopause; carcinogen; tumor subtype

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Introduction

Obesity has become a worldwide epidemic, with nearly 70% of adults in the United States and 52% worldwide identified as either overweight or obese (1–3). There are many negative health consequences associated with obesity, including an increased risk of breast cancer and poorer prognosis, particularly in postmenopausal women with estrogen dependent tumors (4–9). One limitation of research in this field has been the difficulty of developing preclinical models that accurately reflect the physiology of tumors that develop in humans. Thus, the goal of this paper is to provide insight into our experience in the development and implementation of rodent models that combine obesity and simulated menopause with the development of hormone-receptor (HR) positive mammary/breast tumors. The techniques that we describe for promoting excess adiposity can, however, be applied to any cancer that is associated with obesity, including endometrial, colon, pancreatic, and kidney cancers (10).

There are many research groups who have done tremendous work to optimize and improve preclinical models of breast cancer. Throughout this review we will touch on several such areas and direct the reader towards relevant publications that describe this work in detail. Our contribution to this field is incorporation of the relevant variable of obesity (increased adiposity) into these models. In the rat, we have been successful in establishing models with intact immune systems, while in mice we have done so in both immune-compromised and -competent animals.

Many of the tips, tools, considerations, and caveats that we describe can be applied to other existing models of breast cancer. In the current review, we will highlight key features and considerations as shown in Figure 1. We aim to provide examples based on our personal experience with these models and highlight considerations for reproducing and/or incorporation of these techniques for the development of new models. Together we hope that this will provide the basis for other investigators to further contribute to our knowledge of the complex relationship between obesity and breast cancer.

1. Models of Hormone Dependent Tumors

a. Breast Cancer Subtypes

It is well established that breast cancer is not one disease; rather, it occurs as distinct subtypes that display inter- and intra-tumoral heterogeneity. Clinically, tumors are analyzed and subtyped based on immunohistochemistry and/or in situ hybridization for expression of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) (11). Presence or absence of these receptors is currently used to dictate treatment. Over the past decade, scientists have used gene expression profiling to classify breast cancers by their molecular (intrinsic) subtypes, including normal-like, luminal A, luminal B, HER2-enriched, and basal (12, 13). Although not identical to the clinical subtypes, there are similarities between ER+/PR+ and luminal A/B tumors, HER2/ERBB2+ and HER2-enriched, and TN and basal cancers in terms of frequency, gene expression profiles, and prognoses (12, 13). The tumor subtypes and their association with obesity are as follows:

HER-2 / ERBB2 Positive (HER2+): Approximately 15% of breast tumors have amplification or overexpression of the human epidermal growth factor receptor 2 and are referred to as HER2+ (14). The HER2+ subtype is treated with targeted therapy that includes monoclonal antibodies or small molecule inhibitors and the 5-year survival for stage I HER2+ breast cancer is approximately 94% (14). Overall, most studies have failed to report an association between obesity and development of HER2+ tumors; however, a recent study has shown that bariatric surgery was associated with reduced HER2+ breast cancers (15) and emerging evidence suggests that obesity may be correlated with worse overall survival in patients with HER2+ metastatic disease (16). Thus, the link between obesity and HER2+ cancers warrants further investigation.

Triple Negative (TN): Tumors that lack ER, PR and HER2 are classified as triple negative (TN) (11, 14). These represent approximately 15% of all diagnoses and have a poorer disease free and overall survival compared with HR- and HER2+ tumors. For stage I TN tumors, the 5-year survival is approximately 85% (17). Because they lack the receptor targets that are present in the other subtypes, TN breast cancers are treated with chemotherapy (14). Obesity is associated with an increased risk for TN tumors in premenopausal women, but may not associate with overall- or disease-free survival in this population (6, 18).

Hormone Receptor Positive (HR+): Approximately 70% of tumors have >1% cells positive for the estrogen receptor (ER) and/or progesterone receptor (PR) and are referred to as ER/PR+ or hormone receptor (HR) positive (11, 14, 19). Obesity is associated with an increased risk for HR+ breast cancer, particularly after menopause (6). Patients with HR+ breast cancer are often treated with anti-estrogen therapies, such as the selective estrogen receptor modulator tamoxifen or one of several aromatase inhibitors. The prescribed treatment depends in part on the patient's age and menopausal status, as well as the stage and grade of the tumor. HR+ breast cancers have a favorable prognosis, with a 5-year disease-free survival for patients diagnosed with stage I tumors of 99% (14). However, the relatively long latency of tumor recurrence remains a clinical challenge (20). Women with obesity are more likely to experience HR+ cancer recurrence after treatment with the aromatase inhibitor anastrozole (21), suggesting the possibility that the excess estrogens associated with obesity may not be completely suppressed by standard doses (22, 23). However, clinical studies have found no added benefit to tumor response with high versus low aromatase inhibitor treatment (24, 25), and a recent study found that increasing the dose of letrozole, a more potent aromatase inhibitor than anastrozole (26), did not further reduce circulating estrogen levels in women with a body mass index (BMI) > 25 kg/m^2 (27). Together, these data indicate that factors instead of or in addition to estrogen may drive breast cancer growth in the context of obesity, which is an important consideration when designing preclinical models of this disease.

b. Modeling HR+ Tumors

One of the greatest limitations in developing preclinical models to study the interaction between obesity and breast cancer has been to model HR+ tumors in a postmenopausal setting. This is particularly important given the strong association between obesity and

breast cancer risk after menopause, where HR+ tumors are the predominant subtype. Despite this need, it has been notoriously difficult to generate models where grafted human tumors retain expression of the estrogen receptor (ER) after circulating estrogens are removed. Additionally, there are relatively few mouse models of mammary tumorigenesis that produce ER+ cancers (28). While we have not fully overcome all of these challenges, we have made significant strides in working to improve many aspects of these models, thus improving our ability to study the complex relationship between obesity and breast cancer in the postmenopausal setting.

The field of breast cancer research has a wide variety of model systems to use for scientific and preclinical studies. These include xenograft models that use patient-derived tumors (PDX) or established human cell lines, syngeneic mouse mammary tumor cell lines, genetically engineered/transgenic mice, and chemical carcinogen-induced models. Each system has benefits and limitations.

Chemical Carcinogen Models—The use of chemical carcinogens to induce tumor formation is not new; this approach was pioneered for development of mammary tumors by Dr. Charles Brenton Huggins (reviewed in (29)) and has been used for more than 70 years. One of the benefits of this approach is that it allows for induction of tumors in immune competent animals. The two most common carcinogens used to induce mammary tumors are 1-methyl-1-nitrosourea (MNU) and 7,12-Dimethylbenzathracene (DMBA). Work from Henry Thompson's lab has significantly expanded our knowledge regarding the use of carcinogens for tumor formation, and they have detailed the procedure for both MNU and DMBA elsewhere (30, 31). While some researchers have argued that carcinogen induces variability in tumor incidence (32), such studies have given multiple injections of MNU; administration of a single dose of MNU (either i.v. or i.p) has been shown to be highly specific and reproducible for induction of mammary carcinomas (33). Work by Thompson and colleagues has been instrumental in detailing the timing, doses, and administration of carcinogen needed to induce mammary tumor formation in a reproducible manner (30). By following these procedures, we have similarly found MNU to be a reliable and consistent means of inducing mammary tumor formation in rats (34–39). The tumors produced also have the benefit of reflecting clinical disease with respect to the proportion of tumors that are intraductal, the progression of histologic stages from hyperplasia to in situ to invasive, and hormone receptor positivity (40, 41). While we have not done so, MNU will also produce mammary tumors in mice; however, the dose of MNU needed will differ from that in the rat.

The technical aspects of preparing and administering MNU to rats are all described in detail in (30), and adhering to these guidelines will increase the specificity and reproducibility of tumor development. Briefly, key considerations critical to the success of this model, as well as our approach include:

a. Source and Handling of carcinogen

There are several reputable sources for MNU, and some that are less reliable. MNU is sensitive to temperature, light, and humidity. It should be stored at temperatures below −10°C, under conditions where exposure to light and

moisture are minimized. This includes shipping on dry ice. Ash Stevens (Detroit Michigan) was previously a preferred source for MNU (30), however, it is no longer producing this compound. Similarly, in recent years the chemical repository at the National Cancer Institute has not been able to provide sufficient volumes of carcinogen for studies. For the past several years, we have obtained MNU from MRI Global (Kansas City, Missouri) and found this to be a reliable source of the agent.

b. Preparation and Administration of MNU; Concentration of MNU

The dose of carcinogen will influence tumor incidence and multiplicity achieved. Studies generally use doses ranging from 10 to 50 mg/kg body weight (30, 42). 50 mg/kg body weight was reported to induce tumors in 100% of rats (41). Our unpublished data would suggest that higher doses may lead to an increased number of off-target effects.

We have consistently administered MNU at a dose of 50 mg/kg body weight, by i.p. injection. In our hands, and as reported by Thompson, this dose results in >80% of rats developing palpable tumors by 6 months (34–36, 38, 39). Although MNU can be delivered intravenously, subcutaneously, or intraperitoneally, the i.p. route has been reported to generate the most consistent tumor response (30). This increased reliability has been attributed to better accuracy in the delivery with this approach. We prepare MNU as described (30). For large cohorts of rats, we prepare several small vials of MNU which are solubilized in acidified saline (pH <5.0, final concentration 14 mg/mL) so that the powered carcinogen is dissolved immediately prior to injecting the animals. We ensure that MNU is used within 1h of addition of saline to the powered agent, although in most cases we prepare small enough batches that the solubilized MNU does not sit for longer than 40 minutes.

c. Age of Rat at Time of Administration

Early work by Huggins with DMBA found that the ideal window of sensitivity for carcinogen induction of mammary tumors was 50 to 65 days of age, and thus this range was often also used for MNU studies. This age range corresponds to the rapid ductal and alveolar epithelial proliferation taking place in the mammary gland during puberty. The carcinogenic insult to the mammary gland is diminished when delivered after 65 days of age (for both MNU and DMBA), thus carcinogen must be administered prior to this age.

Thompson and colleagues have performed dose-response studies with MNU, demonstrating no statistical differences in tumor incidence, number, or latency in rats injected with 50 mg MNU/kg body weight at 28, 35 or 42 days of age (42). Thus, when merging MNU-induced tumors with models of obesity, we have chosen to use the ~55 ± 2 days of age time frame, with a dose of 50mg/kg body weight, to ensure that almost all rats will develop a tumor, but will do so in a time frame that allows them to also develop diet-induced obesity before moving into the “menopausal” phase of our studies. In our experience, a single injection of

MNU given at this time does not alter weight gain or the development of obesity in these animals.

Transgenic and Syngeneic Models—Transgenic mouse models of breast cancer rely on mammary-specific promoters (e.g. mouse mammary tumor virus, MMTV; whey acidic protein, WAP) to drive expression of various oncogenes in the mammary ductal and alveolar epithelial cells (43, 44). Examples include (but are not limited to) the MMTV-Neu line, which expresses the rat HER2/ERBB2 proto-oncogene, the MMTV-Polyomavirus Middle T antigen (PyMT), which produces numerous rapidly growing tumors in young mice, and the MMTV-c-myc, which encodes a transcription factor that is often mutated in human breast cancer (43, 44). Mouse mammary tumor cell lines, such as the mouse-derived syngeneic transplant collection (MDST) (45) have been established that can be grafted orthotopically to study tumor progression. Syngeneic mammary tumor lines are established cancer cells, which precludes the investigation of the early stages of tumorigenesis in these models.

The use of transgenic mouse models and grafted syngeneic lines to study breast cancer has benefits such as the relatively short disease course compared to humans and the wide availability of molecular tools to interrogate DNA, RNA, and protein. Importantly, tumors grow in the presence of intact immune systems, which is critical for understanding many aspects of cancer biology (46). The variability in obesity predisposition among mouse strains should be considered when choosing a transgenic mouse model. While it is commonly thought that BALB/c mice are obesity-resistant, at least one recent study has shown that a high fat diet increased body weight, body fat, and circulating leptin levels in BALB/c mice, and this was associated with increased metastasis of 4T1 breast cancer cells in this model (47). Several transgenic breast cancer models are on the FVB background, and we have found that these females are also obesity-prone on a high fat diet using the methods described below (E.A. Wellberg and S.M. Anderson, unpublished data). A complete analysis of obesity predisposition in various transgenic mouse models of breast cancer would be a great benefit to the scientific community.

One main limitation of using mouse models to study human breast cancer is that, with few exceptions, most mouse mammary tumors lack ER either completely, or only express it very early in tumorigenesis (48), despite having luminal molecular signatures (49). Some exceptions to this include the prolactin transgenic mouse model (NRL-PRL), which produces tumors that express ER, as do cell lines derived from tumors in this line (50, 51). Another model, which conditionally expresses the SV40 large T Antigen (TAg) and ERα, under control of an MMTV-driven tetracycline-responsive transactivator produces mammary adenocarcinomas, salivary tumors, and lymphomas (52). The mammary tumors can be grafted into immune-compromised mice, where they grow larger in the presence versus absence of E_2 (52). A different group reported the generation of mammary cancer cell lines from a spontaneous tumor that formed in a STAT1-null mouse (53). Two lines from this mouse model, called SSM2 and SSM3, are reported to express ERα and PR, and to grow either in ovary-intact females or in the presence of supplemental E_2 (53). The SSM3 cells are sensitive to the ER downregulator, Fulvestrant, while the SSM2 cells are resistant (53). When implanted into a mouse from the same background as the original tumor donor, these cells may offer the ability to study ER+ breast cancer progression in the presence of a full

immune system. Another consideration when studying mammary development or breast cancer in rodents is that the morphology of the gland and surrounding stroma have key distinctions (54). Human terminal duct lobular units are typically more developed even before pregnancy than the mouse terminal end bud or side branches. In addition, human breasts can have loose intralobular stroma and dense interlobular stroma, while mouse mammary stroma has a predominance of adipocytes (54, 55). Finally, when using transgenic mouse models it should be noted that distant metastases are mainly found in the lungs, whereas humans develop metastases is lung, liver, bone, brain, and other tissues (56).

Human Cell Lines and Patient Derived Xenografts (PDX)—An alternative to transgenic or grafted mouse mammary tumor cells is the use of grafted established human breast cancer cells or patient-derived tumor tissues (57). There is a variety of human breast cancer cells that capture the clinical and molecular subtypes, including luminal/ER+ (MCF7, ZR75.1, T47D), HER2-enriched/HER2+ (BT474, SkBR3), and basal/TN (MDA-MB-231, BT20). Human breast cancer cells can be grown orthotopically in the inguinal mammary fat pads or as intraductal tumors (28, 58), which can support more relevant and sustained expression of ER (58). Basic and preclinical scientists have relied heavily upon these tools to elucidate mechanisms of breast cancer progression, metastasis, drug resistance, and to test the roles of individual genes and pharmacological therapies.

More recently, several institutions have in parallel developed banks of patient-derived breast cancer tissues that can be grafted into immune-compromised mice, referred to as patient-derived xenografts (PDX; reviewed in depth in (57, 59)). These PDX tumors are often propagated in NOD-SCID-IL2Rγ (NSG) mice, where small pieces of tumor (~1 mm × 2 mm) are implanted into the inguinal mammary fat pad using a 10-gauge trochar. Recently, novel cell lines have been derived from some PDX tumors, offering an even wider variety of resources for scientists to rigorously study breast cancer (60). The benefit of using these resources is that they recapitulate the heterogeneity found within and between human breast tumors, and notably, they represent the most commonly diagnosed subtype, HR+. The main limitation of using human tissues in mice is the risk of graft rejection. These cells and tumors will only grow in immune-compromised hosts, which limits their use in studies investigating the role of inflammation or immunity in the obesity or cancer process. One solution to overcome this limitation is the use of humanized mice. This can be accomplished by humanizing the mammary fat pad, where human stromal fibroblasts are injected along with human breast cancer epithelial cells, or by repopulating the immune system with human PBMCs (reviewed in (57)). While possible, this approach is expensive, and is not easily accomplished without assistance from those with significant experience with these techniques. Importantly, it is unclear how humanizing the immune system of mice will impact obesity predisposition and whole-body metabolism.

Each model system has benefits and limitations, but valuable information about breast cancer initiation, progression, and therapy response has been obtained using all of the paradigms described above. Overall, investigators must carefully consider the goals of their experiments and choose the approach and model system that allows these goals to be met.

2. Modeling Obesity in Rats and Mice

a. Rodent Models of Obesity

Defining and measuring obesity in rodents—There are several approaches to promote obesity in rodents; however, not all are amenable to studies that are combined with models of breast cancer. Importantly, there is not a standardized definition of obesity for rats and mice like there is for humans. To address this, work from the Hursting lab has attempted to characterize obesity criteria in mice that are reflective of the body mass index (BMI) cut points used in women. They generated female C57Bl/6 mice with a range of body fat percentages by feeding diets with varying fat content (up to 60%kCal fat) and energy densities (61). They then compared the % body fat of mice following 10 weeks on each diet to the % body fat of women within each BMI category, using data from the National Health and Nutrition Examination Survey (NHANES) III study (62). Using these criteria, they defined lean (calorie restricted), overweight (control diet-fed), and obese (DIO-fed) non-ovariectomized female mice as having percent body fat ranges of <26%, 26%−33%, and >37%, respectively. For mice OVX'd at 5-weeks of age and then fed the same diets, the percent body fat ranges for mice on the lean, overweight and obesity-inducing diets were <29%, 34%−40%, and >45% respectively (61). Our approach has been similar, although we have generally use the word "obesity" to describe an excess of adiposity, relative to a lean control group, and have used HF diets with slightly lower fat content (maximum of 46% kCal fat), which could affect adipose deposition. Further, while Hursting's group identified these % adiposity at the end of their study, we have generally used cut points earlier in a study time-course to prospectively categorize rodents as lean or obese before performing additional study interventions. Thus, in our studies, obesity has generally been identified as >20% body fat for adult female mice and rats prior to OVX. However, it can vary between cohorts and depending on the age at which body fat is measured. Adiposity then increases over time, with a significant increase in body fat deposition after OVX resulting in rodents than are upwards of 40% body fat. Excess adiposity is often accompanied by fasting insulin and/or glucose that are significantly higher than levels measured in LF fed controls; although, in animals that are all consuming the same HF diet, this difference in insulin and/or glucose may not always be apparent. The transdisciplinary field of obesity and cancer research would greatly benefit from adopting a standard approach to measuring and categorizing adiposity.

We most often measure body composition using quantitative magnetic resonance (qMR; EchoMRI, Houston TX), which is a rapid, non-invasive way to obtain fat mass, lean mass, as well as free and total body water content. While this is fast (<3 minutes per animal) and performed without anesthesia, it is limited in that it does not allow for measurements of regional fat distribution. Dual energy X-ray absorptiometry (DEXA) has the benefit that it allows for measures of regional adiposity and bone density; however, DEXA is time-intensive, requires placing animals under anesthesia, and requires more specialized equipment than available for many investigators. For investigators without either of these machines, total fat mass can be approximated by excising and weighing the subcutaneous (mammary), gonadal, retroperitoneal, and/or mesenteric fat pads at the time of sacrifice and

expressing them as a percent of total body mass. Further, this method can be used to supplement qMR data to gain insight into fat distribution at the end of a study.

Available obesity models—Preclinical models of obesity can be categorized as either 1) genetic models – single gene mutation, 2) diet-induced models, or 3) selective inbreeding of diet-induced models (37). At least seven single-gene mutations that lead to obesity have been identified in rodents, including those affecting leptin or the leptin receptor. These mutations give rise to monogenic forms of obesity with an extreme phenotype (63–65) and have proven valuable in understanding the mechanisms and contributions of specific factors involved in energy homeostasis and body weight regulation. Obesity in humans involves complex interactions between genes and the environment that converge to affect multiple tissues and cells in the body. Together these interactions favor the accumulation of excess adipose tissue, and obesity does not result from changes to a single gene. Thus, using a diet-induced approach to promote obesity may increase the physiological relevance of cancer studies and strengthen the ability to translate findings to humans.

Separating contributions of diet composition, adiposity, and metabolic dysfunction—Recent studies suggest that metabolic health may be an important contributor to obesity-associated cancer, with the highest risks for breast cancer diagnosis occurring in women who are classified as “metabolically unhealthy” and as obese based on BMI (8, 66, 67). In addition, a low dietary fat pattern associates with decreased incidence of death after breast cancer (68–71). Based on these observations, it may be desirable to dissociate the contributions of underlying metabolic dysfunction or diet composition from adiposity in preclinical cancer studies. Our approach has been to use semi-purified diets purchased from a reputable company, which supports reproducibility across studies and offers the advantage that investigational agents can be blended directly into the diet at the time of manufacturing, as needed. There are several stock diets available, or researchers can create custom diets tailored to individual study needs. Because there is considerable variability in the amount and source of fat, carbohydrate, and protein, the reproducibility of results within and across laboratories and institutions depends on investigators reporting the specific formulations and catalog numbers of all diets used. Regardless of the diet chosen, we encourage researchers to avoid the use of “chow” as a control. Chow provided by most animal facilities varies significantly across batches, institutions, and seasons, making a semi-purified low-fat diet a better choice to maintain lean rodents. The compositions of low, moderate, and high fat diets with and without sucrose that we have commonly used in our rodent studies are shown in Table 1.

b. Diet Selection

Mouse Studies—In mice, we have used diets with varying levels of fat and sugar to produce the physiological response(s) desired for our studies (72). Details of typical diets that we have used can be found in Table 1. Generally, we would expect that any diet containing at least 40% kcal from fat (such as the HF diet we use in rats; described below) should induce obesity in mice (regardless of the fat source), especially if combined with thermoneutral housing (described below). However, based on specific outcomes of interest in our ongoing studies, we have specifically used a high-fat high-sugar (HFHS) diet with

40% kCal fat with butter as the primary fat source, with additional ~30% kCal from sucrose (72). Milk fat (butter) provides a variety of fatty acids and also includes small amounts of cholesterol; however, it is likely that several fat sources, such as corn oil or lard, would result in a similar obesity phenotype. The low-fat diet we typically use contains 11% kCal from fat, with no added sucrose. We have also included a low-fat/high sucrose (LFHS) diet in preliminary studies (11% kCal fat; 30% kCal sucrose; data below) to separate the effects of sucrose-induced insulin resistance from the excess adiposity induced by high fat/high sucrose (HFHS). Studies performed using the HFHS diet, with LFLS as a control lead to reproducible fat gain in HFHS-fed compared to LFLS-fed females on the C57Bl/6 background, with ~50% of HFHS-fed mice achieving a higher body fat percentage than LF-fed mice irrespective of sucrose contents. In contrast, only ~20% of NSG females develop excess body fat on a HFHS diet at thermoneutrality (72).

Using these three diets, and also taking advantage of the distribution of body fat percentage across the HFHS-fed group, it is possible to generate several metabolic phenotypes that can be informative for mammary development and/or breast cancer studies. Mice fed either a LFLS or LFHS diet have similar body fat content of <20%, while HFHS-fed females average ~ 26% body fat (Figure 2A). The HFHS mice can be separated into two groups, one that is obesity-resistant (OR) and one that is obesity-prone (OP). The resulting body fat content is ~16% in the OR and ~39% in the OP (Figure 2B). Although the LFHS-fed and OR females maintain a body composition similar to LFLS-fed mice, their fasting insulin, glucose, and resulting HOMA-IR are elevated (Figures 2C–E). This allows the investigation of breast cancer in response to impaired glucose tolerance and hyperinsulinemia but in the absence of excess adiposity. In some cases, it may be desirable to keep the diet constant and investigate the effects of varying body fat. In the HFHS-fed group, the OR and OP phenotypes have different body fat contents, but have similarly elevated insulin, glucose, and HOMA-IR compared to LFLS-fed mice (Figure 2B–E).

Rat Studies: OR/OP Model—We take a similar, but slightly different approach in rat studies. The details of this approach (the OR/OP model of obesity) has evolved over the past two decades, based on early work in male rat; historical details and female-specific aspects of this model are reviewed elsewhere (37), and the timeline is summarized at the end of this review. Female Wistar rats are ordered to arrive at our facility at 5 weeks of age (±2d, with a known date of birth). Because the development of obesity requires that we limit physical activity, animals are individually housed in wire-bottom caging for the duration of the study. These cages also allow for the measurement of food intake and spill throughout the study. All rats are provided ad libitum access to a 46% fat diet (Research Diets, #12344; Table 1). Consumption of the HF diet begins as soon as possible upon arrival, subject to the facility and regulations on quarantine period. Our early studies in rats often relied on 60% fat in the diet; however, over the past decade we have shifted to using high fat diets that are more reflective of human consumption (40–50% kcal; Table 1) [34, 35, 37], with corn oil as the fat source. In our rat studies, we typically do not include sucrose as a portion of the carbohydrate component. By carefully selecting diets, we are able to consider and isolate variables that contribute to the obesity-cancer relationship, potentially strengthening the translational impact of cancer studies.

The response of Wistar rats to this obesogenic environment (HF diet + limited physical activity) is varied (Figure 3A–B). We take advantage of this variability to select rats that are resistant or prone to obesity (OR and OP, respectively; Figure 3B), and have found that they reliably mature into lean and obese adults. Unlike male rats, where this propensity for obesity can be easily identified as early as 5–6 weeks of age, selection of these phenotypes is more complex in females (37). In our experience, percent body fat at 14–18 weeks of age is a more reliable predictor of adult adiposity (37). Often, we select those in the bottom 30–40% as OR, and those in the top 30–40% as OP, although these ratios can be altered as needed based on study design. Further, we try to stratify based on natural breaks in the data, which sometimes results in slightly unbalanced groups. Figure 3C shows example data from a large study of approximately 300 rats. Selection of the OR and OP phenotype occurred using % body fat (qMR) at 14 weeks of age (Figure 3B). In this example, the bottom 36% and top 32% were selected as OR and OP, respectively, with the middle group moved to other studies. We then assessed body composition in these same animals 8 weeks later (~22 weeks of age). As shown in Figure 3C, obese animals had higher body weight, fat mass, and lean mass compared to their lean counterparts. Animals then underwent surgical ovariectomy (OVX; further described below), which induced significant weight gain in both the lean and obese groups (Figure 3D). This selection of lean and obese phenotypes and OVX-induced weight gain is reproducible, and we have seen similar results in several of our previously published studies of females using this model, both with (34–37, 39) and without tumors (73–75).

c. Housing Temperature

It can be challenging to promote obesity or fat gain in female mice, particularly if they have intact ovaries. This can be even more difficult in immune-compromised mice that lack T-cells, B-cells, and even NK cells and macrophages. To overcome these challenges, we use thermoneutrality. While the perfect housing temperature for mice is widely debated (76–80), and may depend on the sex, strain, age, or mass of the animal (80–82), it is clear that ambient vivarium temperatures cause chronic cold stress. While animal housing rooms with temperatures 20–23°C are comfortable for humans, mice housed at these temperatures can experience adaptive thermogenesis (83–86). The thermoneutral zone is the temperature range in which the metabolic rate is at resting levels and no adjustments (e.g. shivering, panting) are necessary to maintain a stable core body temperature (81). For rats, this was reported to be ~25°C–31°C (87) and for mice, it's reported to be ~26°C–34°C (80) with the recommendation of ~ 28°C–31°C based on robust metabolic phenotyping data (76, 77). We take two approaches to raise temperatures in our rats and mice; however, it should be noted that warming the mice is critical for the reproducible development of obesity in our studies, but it may not be necessary in rats. For the majority of our rat studies, juvenile Wistar females arrive at 5–6 weeks of age and are singly housed at ambient temperature (~23°C) in a standard vivarium. After an acclimation period of 2–3 days, females are placed on HFD (34, 37). MNU is administered at 55 ± 2 days of age, and rats are monitored weekly for palpable tumor formation. Once tumors reach a predefined size, rats are moved to a climate-controlled satellite room for anti-cancer interventions. The temperature of this room is maintained at 25–26°C; however, we have observed robust weight gain in female rats at standard vivarium temperatures.

To create the diet-induced obesity xenograft (DIOX) mouse model, we use the following approach. Female Rag1-null mice on the C57Bl/6 background (Jackson Labs #002216) arrive at ~5–6 weeks of age and are group housed (5 mice per cage) in a vivarium at ambient temperature. Mice are randomized based on body mass to either HFHS or LFLS diets and are rearranged so that they remain group housed. Mice remain on diet for ~2 weeks before they are moved to static cages placed upon warming blankets. To our knowledge, there are no published studies that suggest the timing of diet and warming influences adiposity development; however, our protocol relies on administering diet for a short time prior to commencing thermoneutrality.

When mice are approximately 8 weeks old, the cages are placed on blankets with warm circulating water. Figure 4A shows a typical cage set up. Cages are positioned such that half of the cage is directly on the blanket and half of the cage is directly on the rack. This allows the internal cage temperature to rise but gives the mice the option to choose the warmer or cooler side of the cage. It has been reported that mice do not prefer to spend all of their time at ~30°C and will choose a cooler temperature particularly at night when they are active (76, 77). Mice remain in cages on warming blankets for the duration of all tumor studies.

The warm water pump has four temperature settings: 10°C, 35°C, 38°C, and 42°C. In our studies, we select the highest setting (42°C) and also select continuous circulation. We used infrared thermometers to measure the temperature of the floors of cages housed on or off the warming blankets and the temperature of the blanket itself (Figure 4B) (72). We found the temperatures of the blankets, the floors of the cages housed on blankets, and the cages at ambient temperatures averaged 37.1°C, 29.4°C, and 23.7°C respectively (72) and Figure 4B. The average temperature of cages housed on warming blankets falls within the recommended temperature zone of ~28°C-30°C (76, 77). Within a few days of being placed on warming blankets, HFHS-fed but not LFLS-fed females begin to gain weight (Figure 4C, vertical line). In LFLS fed mice, warming does not affect body fat percentage (Figure 4D), nor does it affect fat or lean mass (Figure 4E–F). In contrast, warming increases body fat (Figure 4D–E) but does not increase lean mass in HFHS-fed mice (Figure 4F). Similar effects of warming are seen on body mass and composition in wild type C57Bl/6 females (Figure 5). HFHS-fed but not LFLS-fed gain more weight after warming compared to LFLS-fed mice (Figure 5A). Overall, compared to LFLS-fed females, HFHS-fed females housed for >6 weeks on warming blankets have a higher percentage of body fat (Figure 5B), nearly twice as much fat (Figure 5C), and slightly greater lean mass (Figure 5D).

We chose temperatures that supported robust and reproducible adipose gain in outbred Wistar rats (Figure 3 and [35, 53–55]), in immune compromised Rag1-null C57Bl/6 mice (Figure 4), and (72), and in wild type C57Bl/6 mice (Figure 5). We have applied the same methods to the NSG strain, which is widely used as a PDX recipient; however, only ~20% of mice are susceptible to obesity compared to ~50% of Rag1-null C57Bl/6 females (72). While the exact temperature at which mice should be housed is debated, one important consideration is whether the results of any preclinical study translate to humans (88). In our studies, we strive to confirm that the mechanisms we identify during obesity-associated breast cancer progression in rodents are also seen in breast cancer patients (35, 72), thus we feel the approaches to promote obesity are valid.

3. Modeling Menopause

a. Surgical ovariectomy (OVX)

Surgical ovariectomy (OVX) is a well-established approach to create a pseudo-postmenopausal state. While it is not a perfect reflection of the postmenopausal condition, it does have many correlates that make it an appropriate model for menopause. Like menopause in humans, OVX induces a transient, predictable period of weight gain and increase in adiposity (73, 89, 90) and in the context of breast cancer, we have also shown that OVX in the rat model causes the adverse effects of obesity on tumor promotion to emerge (34–36, 39). Using a surgical approach to model menopause allows the investigator to control the timing of menopause induction so that post-menopausal weight gain and tumor progression can be monitored in a time dependent fashion. While it may seem desirable to OVX rodents at an early age to shorten study timelines (and therefore cost), we highly recommend that investigators wait to perform this procedure until animals have reached maturity. We typically OVX mice when they are at least 12 weeks old, and rats when they are at least 18 weeks of age. These time point are used as they reflect the time at which weight gain generally begins to taper off, which suggests that animals are no longer depositing lean mass and are therefore considered fully mature (37)

b. Chemical Toxicant

An alternative approach to model menopause is the use of a chemical toxicant, such as 4-vinylcyclohexene diepoxide (VCD). VCD induces apoptosis of primordial follicles in the ovary without affecting other peripheral tissues, eventually leading to ovarian failure (menopause), as reviewed in (91, 92). While this approach more closely mimics the natural human progression through perimenopause into menopausal, is requires repeated daily injections of VCD and the loss of ovarian function is variable, occurring over weeks or months depending on the protocol used. This would make it difficult to follow timelines in studies investigating the menopause-induced changed in obesity, energy balance, and metabolism on tumor outcomes such as those of related to obesity and postmenopausal breast cancer; however, it may more accurately simulate the gradual loss of ovarian function observed in women. In our studies where we aim to isolate the metabolic changes that occur following estrogen loss, surgical ovariectomy provides a more appropriate and logistically feasible approach.

c. Estrogen supplementation & withdrawal for ER+ tumors

For many of these mouse models, the growth of estrogen-responsive tumors requires that animals be supplemented with estrogens, in the form of E_2 or conjugated estrogens, at least briefly for tumors to establish. Obviously, this becomes problematic when attempting to model the postmenopausal condition, where these hormone-responsive tumors grow in the absence of ovarian production of hormones.

Most human ER+ breast cancer cell lines or PDX tumors require supplemental E_2 to sustain initial growth in mice. This can be accomplished with several approaches. The most common is the subcutaneous pellet. E_2-containing pellets can be made by mixing powdered E_2 with cellulose at the desired ratio and packing the mixture into ~0.8 cm pieces of silastic

tubing that are sealed on both ends with adhesive. Beeswax hormone-containing pellets can similarly be made with the desired E_2 levels and implanted subcutaneously with a trochar, or pellets can be purchased (Innovative Research of America) and are available in a range of doses and delivery durations. One drawback of using pellets is the tendency for them to promote urine retention and potentially severe cystitis (93), but this may be avoided by reducing the levels of E_2 (94). Another approach to deliver E_2 is by diluting a stock solution into the drinking water (57, 95–97), which we now use in Rag1-null and wild type C57Bl/6 female mice. Supplementation of OVX'd C57Bl/6 female mice with 0.5 μM oral E_2 decreases gonadal fat mass (Figure 6A), increases uterine mass (Figure 6B), and increases the percent of mammary epithelial cells that express the ER target, PR (Figure 6C). While the pellets may result in circulating E_2 levels that decrease over time, the administration of oral E_2 achieves relatively constant circulating hormone levels in the animal. Wild type C57Bl/6 females supplemented with E_2 maintain a steady body mass over time, which we recently reported in a preprint (98), but HFHS-females were still heavier than LFLS-fed females. Likewise, HFHS-fed Rag1-null females supplemented with E_2 are heavier and have greater body fat than LFLS-females, and these parameters increase after E_2 withdrawal (72). For studies where it may be desirable to mimic the cyclical nature of hormone levels, such as that achieved by oral administration of hormone-replacement therapy in women, a novel approach using E_2 delivered in a bolus of hazelnut spread (such as Nutella) has been described (99).

4. General Study Timelines

General timelines, showing key features of both mouse and rat models are shown in Figure 7. Female mice (Figure 7A) are given semi-purified diets and housed at thermoneutrality for the duration of the study. Mature mice are OVX'd, supplemented with E_2 and implanted with tumors. Once tumors are established and reach the desired size, mice are either maintained on E_2 or E_2 is withdrawn. E_2 withdrawal associates with a period of weight gain and elevated insulin resistance, which we have shown is tumor promotional in some cases (72). Juvenile female rats (Figure 7B) are given semi-purified diet and tumors are initiated with MNU. Tumors emerge and grow as the animals mature, and rats are OVX'd based on a defined tumor burden. As we see in mice, the ~3 week period following OVX is characterized by weight gain and insulin resistance, and supports tumor progression particularly in obesity-prone (obese) females (35–37, 39).

Because tumors are carcinogen-induced, additional tumors will develop in many of the animals, allowing for the study of new tumor development in the postOVX period. Depending on the desired study outcome, rats can be maintained in the post-OVX period for up to 20 weeks, unless tumor burden requires an earlier endpoint.

5. Application to Other Paradigms

The approaches described in this paper have evolved through the efforts of numerous research teams and reflect our experiences with these models. Our studies have applied these techniques to breast cancer investigations; however, it is likely that similar approaches could be used to study mammary development and differentiation. We hope that by sharing some

of the nuances and intricacies of these techniques, other investigators will be able to apply these approaches to reproduce our findings and also to their own projects on breast cancer and mammary development, further expanding the toolbox available for future studies. As a research community, continuing to publish the details of these models and changes made as they evolve over time will strengthen our ability to answer important questions with increased rigor and reproducibility, which we hope will ultimately increase the clinical applicability of our findings.

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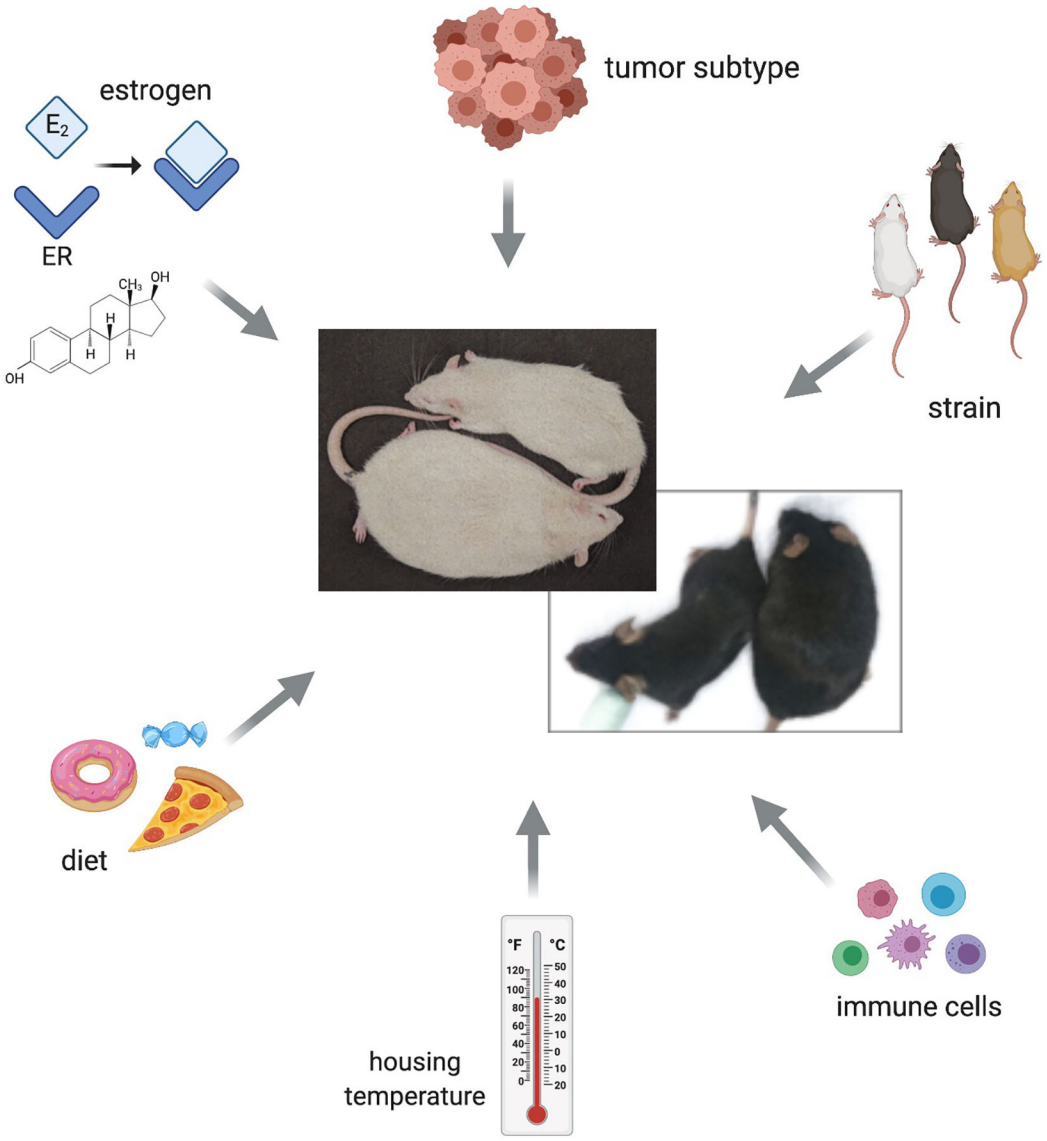

Figure 1. Considerations for preclinical modeling of obesity and breast cancer.

Each factor, including the presence of estrogen, tumor subtype, rodent strain, immune function, housing temperature, and diet influences and/or is influenced by obesity. Representative images of Lean and Obese female rats (top; white) and mice (bottom; black) derived from these models are shown.

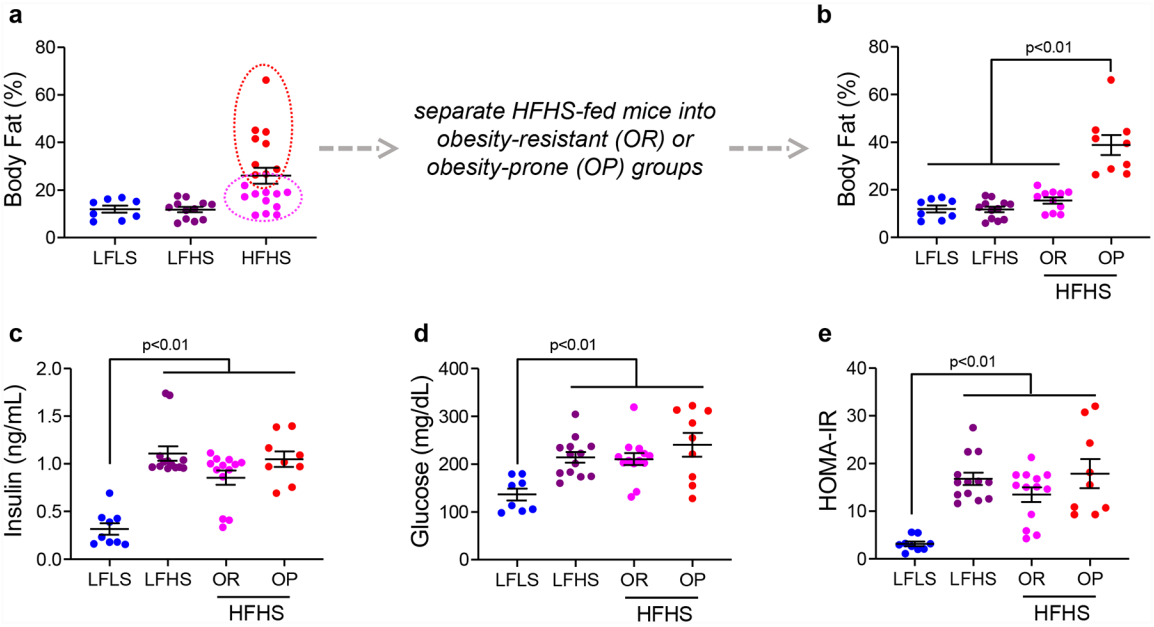

Figure 2. Diet-induced separation of obesity and metabolic dysfunction in mice.

(A) Body fat percentage measured by qMR in Rag1-null C57Bl/6 mice fed low-fat/low-sucrose (LFLS), low-fat/high-sucrose (LFHS), or high-fat/high-sucrose (HFHS) diets. Note the variability in body fat across the HFHS group. (B) Body fat percentage in LFLS, LFHS, and HFHS fed mice, showing the obesity resistant (OR) and obesity prone (OP) groups separately. (C) Fasting insulin and (D) glucose measured in mice on LFLS, LFHS, or HFHS diets. (E) HOMA-IR calculated from fasting insulin and glucose measures in mice on LFLS, LFHS, or HFHS diets. Bars represent mean and SEM. Data were analyzed by unpaired t-test.

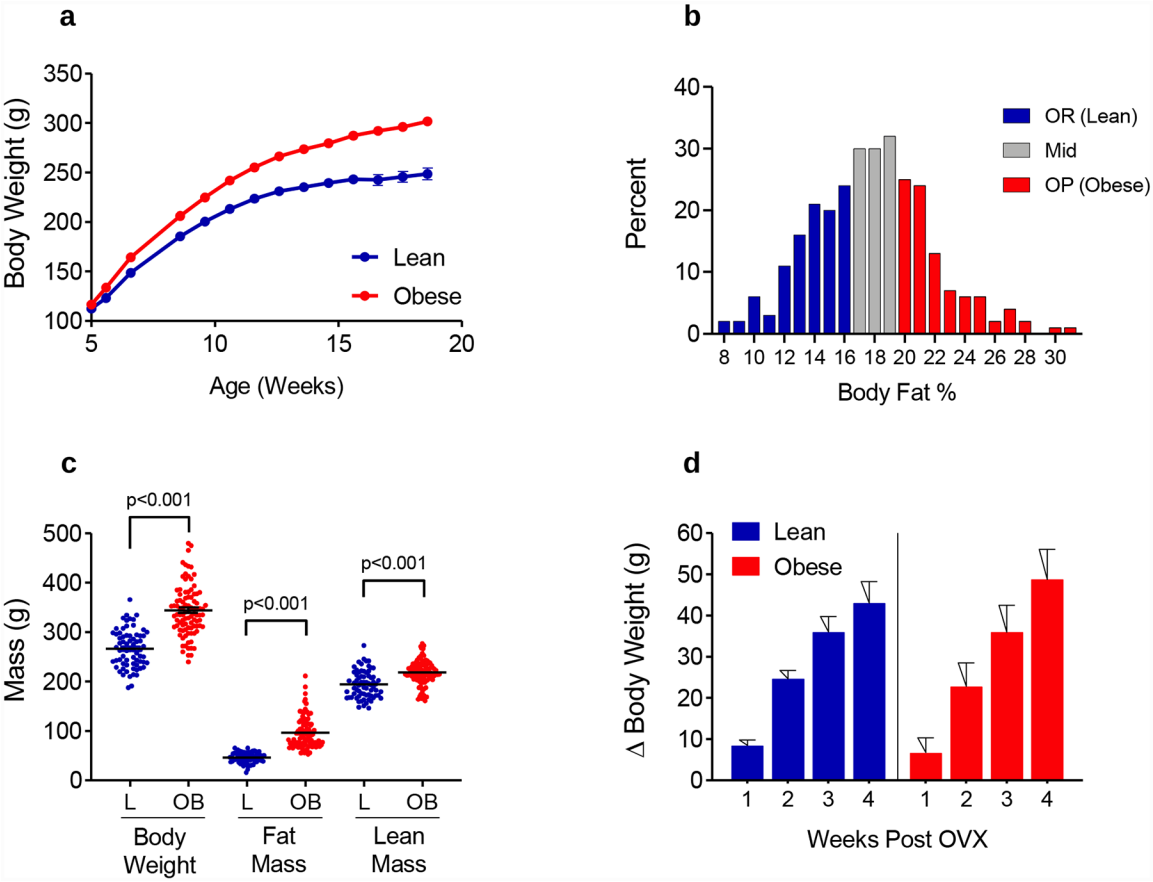

Figure 3. Diet-induced separation of obesity prone and resistant rats.

(A) Body weight for lean and obese rats all on the same HF diet (46% kcal fat) demonstrates the differential predisposition to obesity in the outbred Wistar strain. Graph represents weekly body weights from 288 rats (mean +/− SEM) based on classification as obesity resistant (OR) or obesity-prone (OP) using % body fat at 18 weeks of age. (B) Frequency distribution of % Body Fat at 18 weeks of age (N=288 rats). Color coding shows selection of OR (n=105 rats; 36%) and OP (n=91 rats; 32%) from the entire cohort; Middle Tertile animals (n=92 rats; 32%) were moved to other studies. (C) Body Composition in mature Lean and Obese Rats (mean = 22 weeks of age). (D) Loss of circulating hormones following OVX induces rapid weight gain over approximately 3–4 weeks in both lean and obese rats, with no difference in the cumulative weight gained between lean and obese groups. All data is presented as mean and SEM.

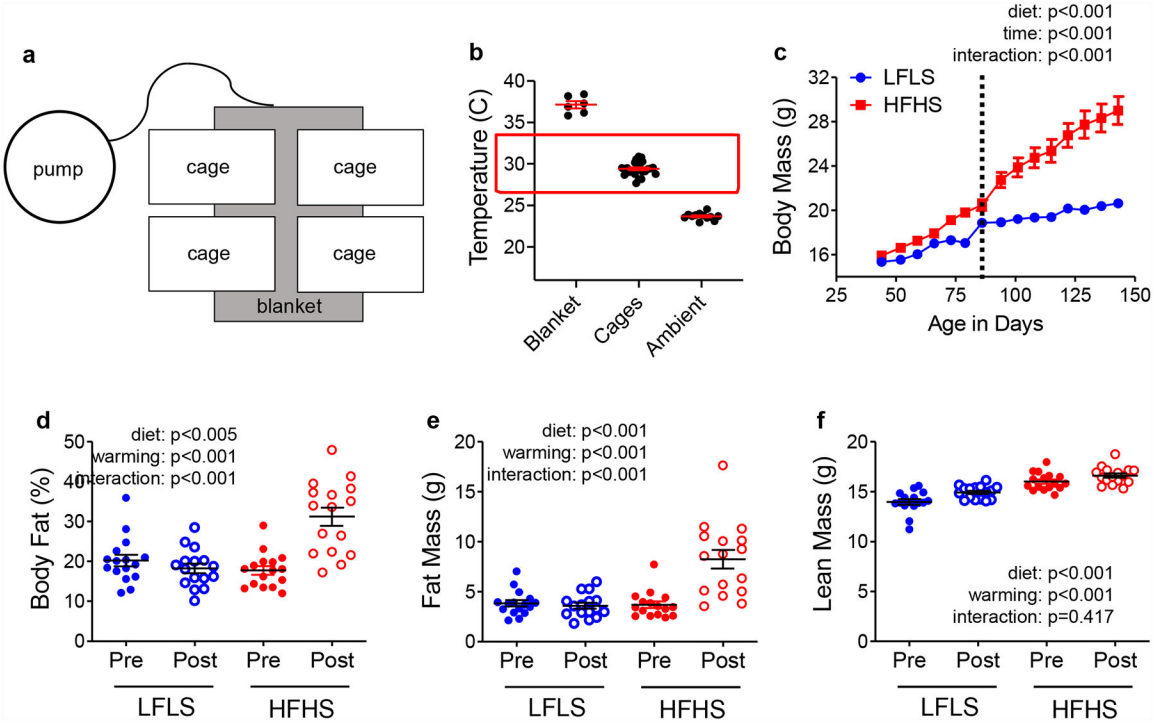

Figure 4. Effect of thermoneutral housing on body composition in Rag1-null C57Bl/6 female mice.

(A) diagram of cage placement on warm water blankets. Each cage is housed half on, and half off, the blanket. (B) Temperatures of the blanket surface, the bottom of the warmed cage, and the bottom of cages housed at ambient vivarium temperature, measured with an infrared thermometer. The red box indicates the mouse thermoneutral zone. (C) Body mass of LFLS and HFHS Rag1-null females. The vertical line indicates the initiation of thermoneutral housing; N=16 per group. (D) Body fat percentage, (E) Fat mass, and (F) lean mass measured before (pre) and after (post) warming with qMR in mice fed LFLS or HFHS diets; N=16 per group. Data were analyzed by 2-way ANOVA testing for main effects of diet or time (panel c) or diet and warming (panels d-f) and for an interaction.

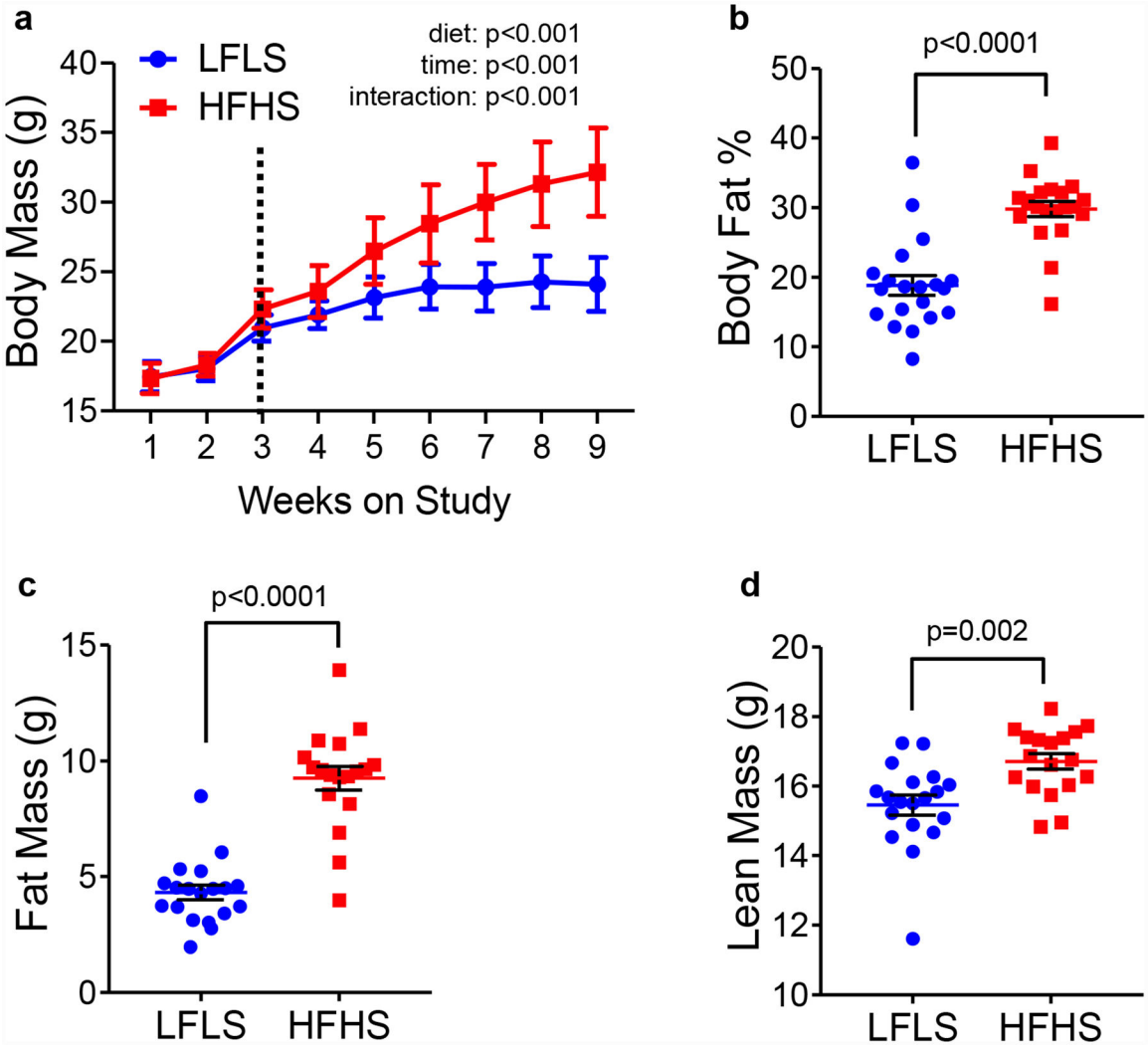

Figure 5. Effect of thermoneutral housing on body composition in wild type C57Bl/6 females.

(A) Body mass of LFLS and HFHS wild type C57Bl/6 females. The vertical line indicates the initiation of thermoneutral housing. (B) Body fat percentage in mice fed LFLS or HFHS diets measured after 9 weeks on study via qMR. Fat mass (C) and lean mass (D) in g are shown. N=19 LFLS, N=18 HFHS. Data were analyzed by 2-way ANOVA (panel a) testing for main effects of diet and time, or by unpaired t-tests (panels b-d).

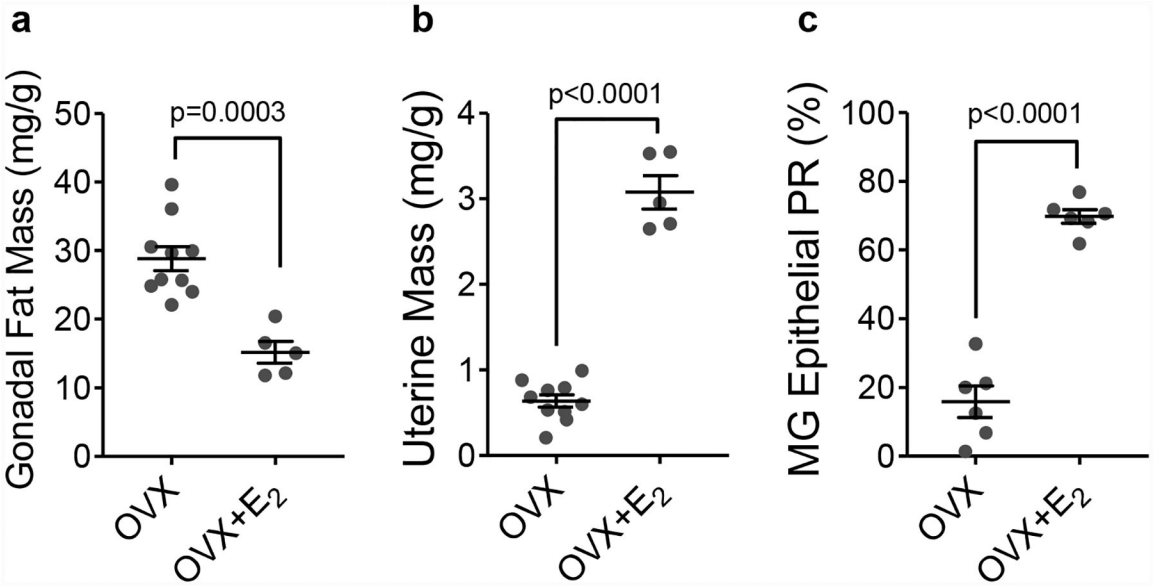

Figure 6. Effect of oral E2 administration in mice.

(A) Gonadal fat pad mass and (B) uterine mass expressed as mg per g body mass, and (C) percentage of mammary epithelial cells positive for the progesterone receptor (PR) by IHC in OVX mice or OVX mice supplemented with 0.5 μM E2 in drinking water. For *a* and *b*, N=10 OVX and N=5 OVX+E2. For *c* N=6 OVX and N=6 OVX+E2. Data were analyzed by unpaired t-test.

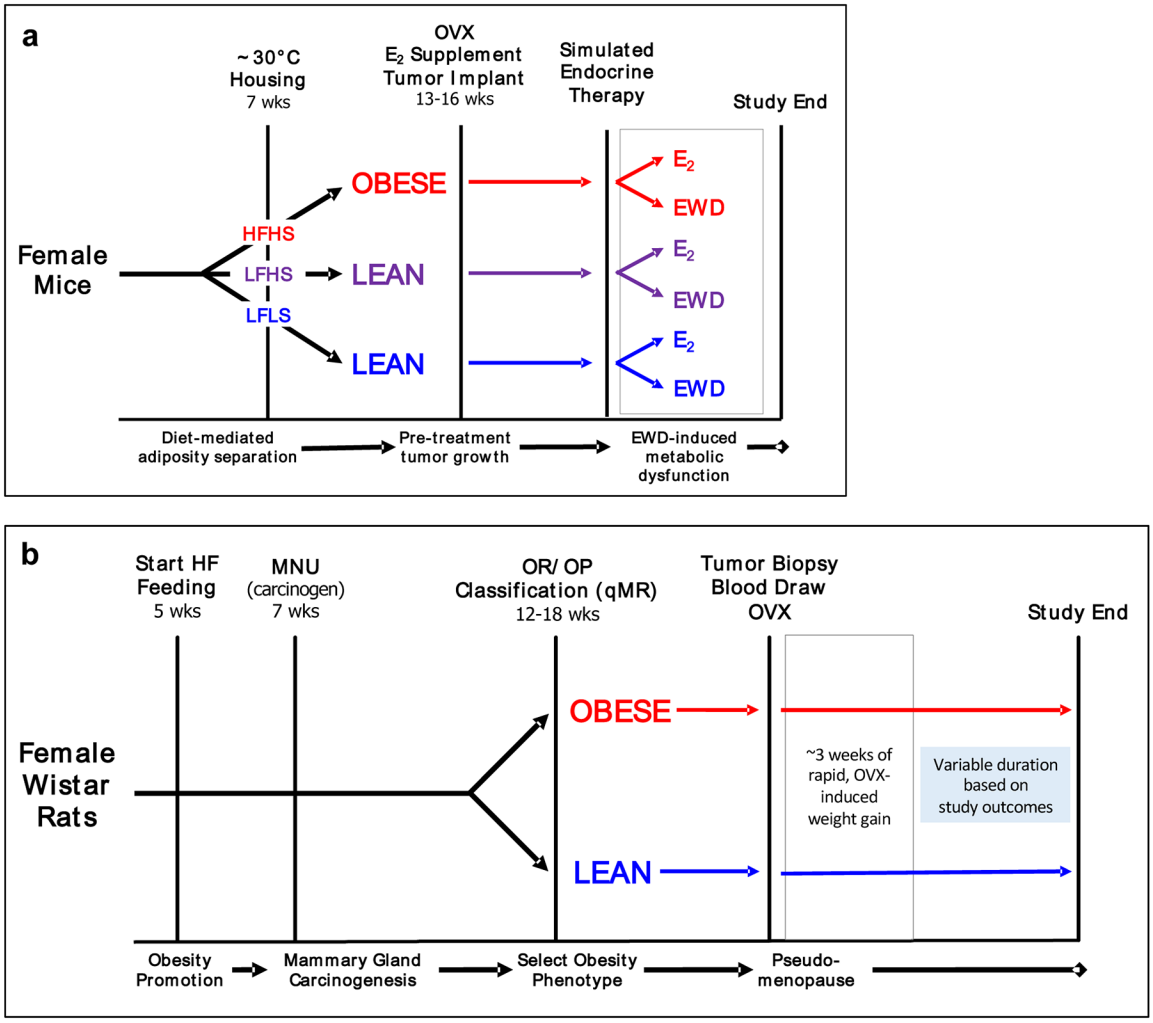

Figure 7. General Overview of Models.

Key features of studies done in (A) mice and (B) rats are shown. The grey box in each diagram represents the period following estrogen loss when weight gain and insulin resistance increase. OVX-ovariectomy; EWD-estrogen withdrawal; LFLS-low fat/low sucrose; LFHS-low fat/high sucrose; HFHS-high fat/high sucrose.

Table 1.

Diet numbers and composition used in mouse and rat obesity studies.

	MOUSE						RAT	
DIET	**Low Fat Low Sugar (LFLS)**		**Low Fat High Sugar (LFHS)**		**High Fat High Sugar (HFHS)**		**High-Fat (HF)**	
Product #	**D11092101**		**D19043002**		**D15031601**		**D12344**	
(Research Diets)	**g %**	**kcal %**	**g %**	**kcal %**	**g %**	**kcal %**	**g %**	**kcal %**
Protein	20.3	20.8	20.3	20.8	24.0	20.8	24.6	20.8
Carbohydrate	66.0	67.7	66.0	67.7	45.2	39.2	39.7	33.1
Fat	5.0	**11.5**	5.0	**11.5**	20.5	**40.0**	24.6	**46.1**
Total		100.0		100.0		100.0		100.0
kCal/gram	3.90		3.90		4.61		4.80	
Ingredient	**grams**	**kcal**	**grams**	**kcal**	**grams**	**kcal**	**grams**	**kcal**
Casein, Lactic	200	800	200	800	200	800	200	800
DL-Methionine	3	12	3	12	3	12	3	12
Corn Starch	531	2124	278	1110	-	-	163	650
Maltodextrin	119	476	80	320	80	320	150	600
Sucrose	-	-	**293**	**1170**	**293**	**1170**	-	-
Cellulose BW200	50	-	50	-	50	-	50	-
Milk Fat (Butter)	11	99	11	99	134	1209		
Corn Oil	39	351	39	351	39	351	200	1800
Minerals Mix S10001	35	-	35	-	35	-	35	-
Vitamin Mix V10001	10	40	10	40	10	40	10	40
Choline Bitartrate	2	-	2	-	2	-	2	-
Cholesterol	-	-	-	-	-	-	-	-
Total	1000.0	3902.0	1000.0	3902.0	845.8	3902.1	812.5	3902.0

Bold values indicate key differences in the fat and sucrose content of the diets, as described in the text