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Large Animal Models of Breast Cancer

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Abstract

In this mini-review the status of animal modeling of BC will be reviewed, beginning with an overview of murine models, followed by a discussion of described and emerging large animal BC models.

Background: Breast Cancer Burden

The annual incidence of breast cancer (BC) in women (all ages and races) in the U.S. increased from 0.102% in 1980 to a peak of 0.142% in 1999, and then decreased slightly, plateauing at ~0.131% from 2011-2017.¹ As of 2017, a woman's lifetime risk of developing BC in the U.S. is 12.9%.¹ In 2021, the estimated number of new BC cases in the U.S. will be 281,550 (15.3% of all new cancer cases), with 43,600 estimated deaths (~20 per 100,000 in the general population, or 7% of all cancer deaths, or ~2% of all mortality in the U.S.).^{1,2} All-stages 5-year survival for BC has improved from 75% in 1975 to 90% in 2016,¹ secondary to earlier diagnosis and more efficacious therapy.³ However, survival with triple-negative breast cancer (TNBC; minimal/nil expression of the estrogen receptor, progesterone receptor, and epidermal growth factor receptor 2), which accounts for 10-20% of all BC,^{4,5} is 10, 20, and 30% lower at stages 2, 3, and 4, respectively, compared to non-TNBC.⁶ So, there remain a need for improved management of TNBC.

Current Status: Murine Modeling of BC

Commonly utilized murine BC models⁷⁻¹⁰ include cell-line derived xenograft (CDX), patient derived xenograft (PDX), humanized CDX and PDX, chemically induced, and genetically engineered murine (GEM) models.

Cell-line Derived Xenograft Models

Cell-line derived xenograft (CDX) murine models, which are based off the transplantation of human cell lines into immunocompromised animals, are simple, cost-effective, and useful for the assessment of breast cancer genetics and basic biological processes.^{11,12}

However, limitations associated with these models, including growth in the absence of a functioning host immune system, have made them poor predictors of clinically-relevant biologic behavior.^{11,12} They have reduced intra-tumoral heterogeneity which does not optimally represent a human breast tumor.¹¹ When compared to PDX and original patient tumors, cell lines have shown a loss of genetic expression profile and loss of tumor-specific genes,¹³ an alteration in growth and invasive properties, loss of specific cell populations, and selection of cell population.¹² They are frequently derived from highly aggressive malignant tumors or pleural effusions and thus are less useful in studying the early stages of disease.¹¹ The lack of a functional host immune system means that CDX tumors do not undergo any appreciative immunoediting,^{14,15} which is increasingly recognized as important during tumor growth and evolution. Overall, CDX models are useful for BC genetics and basic biology, but are poorly predictive of human response to test interventions.

Patient-Derived Xenograft Models

With the limitations of CDX, there has been significant growth in the use of patient-derived xenograft (PDX) murine models for translational cancer research. These models involve the transplantation of fragmented primary human tumor from surgical resection or biopsy into immune deficient mice. One of the main advantages of PDX models is that the grafted tumor remains biologically stable. Biologic stability is seen in relation to tumor architecture, gene expression, mutational status, growth kinetics, invasiveness, metastatic potential, and drug responsiveness.^{12,16,17} In breast cancer models, they have been shown to conserve ER, PR, and HER2 expression particularly when grafted directly into mammary ducts,^{18,19} and they have shown similar metastatic progression compared to human tumors.^{17,20} These features have made

PDX models useful for pre-clinical evaluation of drug therapy including new treatment targets, novel therapy combinations, treatment schedules, and even personalized drug therapy.¹²

However, PDX models are not without limitations. PDX models pose a slightly higher cost than CDX models and require access to fresh patient material.¹¹ The prolonged length of time it takes to generate tumors does not always match clinical or research needs.^{11,12,21} Despite early studies showing success with different breast cancer subtypes with orthotopic implantation,^{18,19} ideal location of implantation remains uncertain^{12,21} and engraftment failure rates remain high in hormone receptor positive tumors favoring more aggressive subtypes.^{12,17,22} Another concern is that by the third *in vivo* passage murine stroma replaces the human grafted stroma, which may result in changes in paracrine regulation as well as physical properties.^{23,24}

With PDX models favoring more aggressive subtypes, they have been used extensively to study triple negative breast cancer (TNBC) and metastasis. However, limitations have been apparent, including (i) the need to utilize immunodeficient mice which do not mimic the clinical effect of the immune system (immunoediting) on the development of metastasis,¹⁷ (ii) the prolonged time for studying metastasis,^{17,21} and (iii) the rarity of spontaneous metastasis development.²¹ Metastatic tumors do occur in PDX models, primarily in lungs and lymph nodes, but less so in the brain and bone (the latter are common sites seen in human breast cancer metastasis).¹¹ There is also high rates of tumor regrowth at primary implant sites which reduce survival time and failure of the metastatic sites to manifest the expected more aggressive phenotype when reimplanted (in contrast with what occurs with established cell lines).¹⁷ In summary, PDX models may be more predictive for preclinical drug evaluation, but ultimately are limited by the same issue afflicting the CDX model: a lack of a functional host immune system.

Humanized CDX and PDX models

A more recent development in murine cancer modeling is the humanized mouse model,²⁵ which has been useful for the study of the immune system's role in cancer. In brief, an immunocompromised mouse is engrafted with components of the human immune system, generating a functional human immune system somewhat similar to that observed in the originating patient, with the intention to create a more realistic tumor microenvironment.²⁶ There has been increased interest in these models for pre-clinical studies with immunotherapies and breast cancer, particularly TNBC and HER2+ cancers. Human CD34+ HSC-engrafted NSG mice harboring different types of PDX are now commercially available. Some researchers have described less expensive, in-house models via intravenous injection of hematopoietic stem cells into irradiated SCID mice, which have shown successful engraftment and metastasis.²⁷

Studies on breast cancer immunotherapy using humanized mice have successfully shown reduction of tumor growth,^{27,28} but they are not without limitations, including (i) a lack of GM-CSF in humanized mice, which is important for the differentiation and maturation of the myeloid lineage, and (ii) mismatching HLA typing between hNSG and PDX.²⁷ Some humanized murine models have shown xenograft-versus-host effect, in which mature human T cells attack their murine host, reducing the window in which this model can be used to study an implanted tumor.²⁶ So, while the lack of a functional immune system in conventional CDX/PDX models has been somewhat countered by introducing components of the human immune system in humanized mice, graft vs. host issues continue to define the utility of the humanized subjects.

Chemically Induced Murine Models

Although genetic models have provided insight into how particular mutations can induce tumorigenesis, these models may give an oversimplified view of how cancer develops. The occurrence of point mutations, changes in gene copy number, epigenetic modifications, and other genomic events can also be affected by environmental factors. Some human cancers develop secondary to chemical carcinogenesis; chemically induced models may be able to replicate the genetic events that occur during this process.²⁹ Polycyclic aromatic hydrocarbons such as 7,12-Dimethylbenz(a)-anthracene (DMBA) and methylcholanthrene (MC) compounds have been used in mice to induce breast tumors.³⁰⁻³⁴ These compounds can bind to DNA and cause errors in replication.³⁵ DMBA has been studied in knockout, hemizygous and SENCAR (SENSitivity to CARcinogenesis) mice, producing breast tumors after 3-34 weeks.³⁰⁻³³ MC has produced tumors after 7 months.³⁴ In addition to modeling genetic events of chemical carcinogenesis, the advantages of chemically induced models include (i) no need for immunodeficient subjects, (ii) autochthonous tumor development, and (iii) relatively low cost and ease of use. The disadvantages of chemically-induced models is that they are not genetically defined to the same extent as GEM models are, which can have implications on the investigator's ability to generalize from their data.

Genetically engineered murine models

There are several types of GEM models, including conventional, knockout, and conditional. Conventional GEM models are typically driven by mammary-specific promoters that direct expression of specific oncogenes (transgenes) which may not be specific to mammary epithelial cells. Common mammary promoters in use include the mouse mammary tumor virus long terminal repeat (MMTV-LTR), whey acidic protein (WAP), β -lactoglobulin (BLG) and the

5' flanking region of the C3 component of the rat prostate steroid binding protein.³⁶⁻³⁹ The first GEM model from the 1980s expressed the c-Myc oncogene under the control of the MMTV promoter, which resulted in mammary adenocarcinoma.⁴⁰ Since then, a number of transgenic mice have been created to study the role of proto-oncogenes in the development and progression of tumors. In addition to c-Myc, some of the oncogenes that have been overexpressed using the MMTV-LTR promoter include v-HRas, Wnt, PyMT, Neu, and ErbB2.⁴⁰⁻⁴⁵ Expression of these oncogenes resulted in mammary tumorigenesis and metastatic lesions.^{42,45,46}

GEM models were also generated to understand loss-of-function (knockout) of tumor suppressor genes. One of the most common knockout models involves the tumor suppressor Trp53.^{47,48} Mice that were homozygous for Trp53 null allele developed lymphomas and died at around 4-6 months of age.⁴⁸ In both the conventional and knockout models, the gene targets are not specific to a certain cell lineage. These models cannot recapitulate sporadic disease in which there is an accumulation of genetic events in a specific cell lineage. There also can be off-target effects in these models, and other types of cancers may arise in them. In addition, ~30% of the conventional knockout models are lethal in the perinatal period, which makes experimentation difficult.

To overcome these problems and prevent early embryonic death, conditional GEM models have been developed that allow for the activation of oncogenes and/or deletion of tumor suppressors, specifically targeted to the mammary gland (i.e., tissue-specific edits). These models utilize systems such as the Cre/loxP and Flp/rt systems for activation or deletion of genes.¹¹ These systems provide for controllable recombination events, such as removal of stop codon that subsequently permits transcription of a transgene.¹¹ Among the first two conditional GEM models created were WAP-Cre and MMTV-Cre^{49,50} These models have been able to

generate hereditary breast cancer models specifically by modeling the heterozygous mutations observed in the BRCA1/BRCA2 genes.⁵¹ GEM models that contained a conditional mutant BRCA1 allele and a disruption in Trp53 have accelerated mammary tumor development.⁵¹ Trp53 in combination with other genes has been extensively studied using conditional GEM models.⁵²

Tissue-specific genetic edits in conditional GEM models can also be induced at a specific time using the inducible Cre-ERT system or the tetracycline/ doxycycline promoter. In the Cre-ERT model the hormone-binding domain of the estrogen receptor (ERT) is fused to Cre-recombinase. Upon administration of tamoxifen (an estrogen analogue) Cre-recombinase is activated, leading to the recombination event marked by the loxP sites.⁵³ GEM models have become increasingly popular in BC and other cancer research, providing insight into disease initiation, progression, evaluation of drug delivery, therapeutic response and biomarkers in their natural microenvironment.⁷ GEM models typically have a normal immune system, tumor development is autochthonous, and by definition are genetically defined. The disadvantages of GEM models are the expense and complexity involved in their creation.

Small Non-Murine Models of BC

Rat BC Models

Rats have been considered a suitable animal model to study breast cancer due to their similarity with human mammary cancer in terms of histology, immunocytochemical markers and biological behavior of tumors.⁵⁴ The histologic characteristics of normal mammary luminal epithelium and myoepithelium is similar between rats and human.⁵⁵ Long term studies have shown that some rats can develop breast tumors spontaneously.⁵⁴ Use of a chemical carcinogen in rats can result in a shorter latency period to tumor development.⁵⁵⁻⁵⁹ Recently, 17 β -estradiol

(E2) was used to induce breast tumors in August Copenhagen Irish-rats by modulating estrogen mediated mechanisms in breast cancer development.⁵⁷

In xenograft-Matrigel® implantation experiments, younger rats have experienced greater tumor growth compared to older rats.^{60,61} A bone metastasis immunodeficient rat model has been developed in which human breast cancer cells (MDA-MB-231) were intra-arterially injected into a hindlimb artery.⁶² Genetically engineered rat models of breast cancer have been developed in which HER2 and TGF- α were overexpressed through the mouse mammary tumor virus (MMTV) promoter.⁶³ This model stochastically produced a variety of benign, hyperplastic and malignant lesions, including ductal carcinoma in situ and carcinoma within a year.

In a rat model with three copies of human HRAS proto-oncogene, induction of carcinogenesis with nitrosomethylurea resulted in large mammary tumors within 8 weeks.⁶⁴ Mammary carcinogenesis in rats was induced through injection of high-titer, Neu-containing, replication-defective retrovirus which produced hormonally-responsive *in situ* carcinomas within 15-days post infusion, and regressed spontaneously after 20-days post infusion.⁶⁵ Injection of human adenovirus type 9 (Ad9) also is known to induce estrogen-dependent mammary tumors in rats within 7-12 months.⁶⁶ Overall, however, use of rats in BC research has lagged far behind the broad use of mice.

Hamster BC Models

Similar to rats, nitrosomethylurea can induce mammary carcinoma in Syrian hamsters (*Mesocricetus auratus*), producing high-grade poorly-differentiated mammary adenocarcinomas.⁶⁷ Subcutaneous allogenic implantation of cell lines established from these primary tumors generated secondary tumors. Hamster models can be useful in studies on

oncolytic adenoviruses, a self-replicating cancer cell-killing virus. Oncolytic adenoviruses can replicate in immunocompetent hamsters, making the hamster model of cancer a suitable non-immunocompromised model to study therapeutic potential of these viruses.⁶⁸

Tree shrew BC Models

The tree shrew (*Tupaia belangeri chinensis*) can develop spontaneous mammary tumors, which are similar to human papillary tumors in terms of morphology and pathology.^{69,70} Chemical induction with DMBA plus medroxyprogesterone acetate (MPA) also produced breast cancer in tree shrews.⁷¹ Injection of a lentiviral vector with PyMT (polyomavirus middle T antigen, an oncogene that activates c-Src), into the mammary duct of tree shrews resulted in tumor development in all subjects by 7 weeks post-injection.⁷²

Feline BC Models

Feline mammary carcinomas (FMCs) are the third most common type of cancer in cats.⁷³ *In utero* implantation of allogeneic mammary cancer cell lines into fetal cats (*Felis catus*) produced tumor at the injection site, followed by widespread metastasis after 6-10 weeks.^{74,75} A nude mouse model of FMC demonstrated metastatic potential (bone, kidney, brain, lung and liver; i.e., common sites of metastasis in human breast cancer) after injection to the primary site.⁷⁶ Cancer stem cell-like populations in FMCs can form mammospheres (organoids) and are tumorigenic, radioresistant, and chemoresistant.^{77,78} Mammospheres can be used a model to study feline breast cancer. Morphological, histological, and molecular to similarities between FMCs and human breast cancer have been described and discussed.⁷⁹⁻⁸¹

Large Animal Models of BC

Justification for & Advantages of a Large Animal Model of BC

Breast cancer, and TNBC in particular, can be modeled with many of the above murine and other small animal models. However, none of the above models can overcome the limitation which drives the current proposal: inadequate subject size. Development of some diagnostic and interventional technologies require a human-sized model to understand how clinically relevant tumor size and tissue thickness affect the performance of the experimental technology, such as with three-dimensional scanning or with a tissue-ablation device. In addition, pharmacokinetic parameters (including absorption, distribution, metabolism, and excretion) can be vastly different between a 20 g mouse and a 70 kg patient.⁸² Testing the effect of an infused chemical entity (e.g., a novel anti-tumor agent) in a murine tumor model may result in an inaccurate conclusion secondary to these pharmacokinetic differences. As an example, a mouse vs. large subject scenario is utilized below.

With regard to drug distribution, tumor burden in a 20 gram mouse with a 1-cm tumor is ~400-fold greater (mass:mass) than in a 70 kg subject with a 2-cm tumor (Fig. 1). If the tumor acts as a sink for a candidate anti-tumor drug, then the tumor's ability to decrease the drug's plasma concentration would be much greater in the mouse. A consequence of this tumor sink effect⁸³ would be a gross underestimation of the drug's toxicity from murine data. Alternatively, if the drug penetrates poorly into dense tumor stroma,⁸⁴ then testing in a ~1 cm murine tumor may overestimate the drug's anti-tumor efficacy, as opposed to testing in a large (≥ 4 cm) tumor. These drug distribution issues in a murine model could be minimized with a large animal model (e.g., a 70 kg pig) with clinically-relevant tumor size. So, a primary justification for a large

animal BC model would be its ability to replicate human tumor dimension and human PK parameters.

Canine BC Models

Dog. Similar to cats, canine mammary tumors (CMTs) occur spontaneously and are the second most common cancer in dogs.⁸⁵ Development of CMTs is hormone dependent and showed dysregulated expression of BRCA1, BRCA2 and TP53,⁸⁶⁻⁸⁸ analogous to human BC. Transcriptional analysis of CMTs demonstrated pathways that are active in human cancer, including those involved with cell cycle regulation, apoptotic signaling, immune functions, endoplasmic reticulum stress, angiogenesis and cell migration.⁸⁹ Approximately 25% of the genetic alterations in metastatic CMTs were associated with human mammary cancer.⁹⁰ In addition, canine spontaneous mammary DCIS and invasive cancer shows similar histologic and molecular characteristics with DCIS and invasive cancer in humans.⁹¹ Spontaneous CMTs also can metastasize to lymph nodes and lung.⁹² Her2 overexpression in CMTs is controversial.⁹³ Benign tumors and mesenchymal tumors are more prevalent in CMTs; the latter are rare with human breast cancer.^{94,95}

Non-Human Primate BC Models

Mammary gland tissue from common marmoset and rhesus macaques have been used in mammosphere culture, and can be used as a *ex vivo* model to study breast cancer.^{96,97} However, due to the low incidence of spontaneous tumors, long incubation periods, and high costs, non-human primates have not been commonly used in breast cancer research.

Porcine BC Models

Swine have been used for decades as research subjects in diverse areas including transplantation, physiology, trauma, toxicology,^{98,99} and recently cancer.¹⁰⁰⁻¹⁰⁴ The porcine genome has been sequenced,¹⁰⁵ and annotation is ongoing.¹⁰⁶ Gene editing of pigs and creation of transgenic swine is now fairly common,^{102,103,107-110} some transgenics (such as the Oncopig¹⁰¹) are commercially available. With respect to breeding, the porcine gestation period (114 d) is relatively short (goat/sheep/cow = 150/152/283 d). In regards to pharmacokinetics, the pig has the most similarity to humans among studied mammals with respect to the cytochrome P450 enzymes.^{111,112} Similar pharmacokinetic behavior between humans and pigs has been reported for a number of compounds,¹¹³ and pig is recognized as a model for enabling determinations of *in vivo* kinetics and drug metabolism in general.^{113,114}

Investigators have transformed porcine mammary epithelial cells *in vitro* with SV40 large T antigen insertion¹¹⁵ or BRCA1 knockdown,¹¹⁶ with evidence of tumorigenicity in immunodeficient mice.¹¹⁵ In addition, a *BRCA1* haploinsufficient Yucatan minipig was generated with somatic cell nuclear transfer,¹¹⁷ but postnatal survival of cloned piglets was ≤ 18 days for reasons that were unclear (*BRCA1*^{+/-} mice are phenotypically normal¹¹⁸). As a proof of principle, our group has transformed porcine mammary epithelial cells with KRAS^{G12D} and p53^{R167H}, and produced tumors in xenografted nude mice. Our follow-up work in this area will focus on using porcine mammary epithelial cells transformed with *PIK3CA* mutants with *BRCA1* and *TP53* disruption¹¹⁹⁻¹²¹ to be utilized in an orthotopic porcine model of TNBC. To be clear, however, no porcine model of BC currently exists. The KRAS/p53 Oncopig¹⁰¹ is not a BC model, but rather a “generic” porcine tumor model, potentially allowing transformation of all cell types. Of note, KRAS mutation is relatively uncommon (1-2%) in BC.¹¹⁹⁻¹²¹ Given the recent

progress in porcine modeling of pancreatic cancer by multiple groups,^{101,104,122-128} it is conceivable that the best candidate for a large animal BC model will be the pig.

Disadvantages of Using Large Animal Cancer Models

In order to illustrate the potential disadvantages of using a large animal model of breast cancer, a comparison of porcine with murine models is described below. This does reveal a number of disadvantages with the porcine models, including:

1. **Costs.** Transgenic pigs are more expensive. Transgenic mice for BC research generally are 200-300 USD per subject (Jackson Laboratory), while transgenic pigs (e.g., the Oncopig) can be in the range of 1-2K USD. Per diem cost for pigs are generally ~10x the murine per diem at most institutions. Drug costs are higher in pigs because they require 100-1,000x the amount of drug used by mice. Labor costs are higher with pigs because of physical handling required each time a procedure is done.
2. **Husbandry.** More than 100-fold mice than pigs can be housed in the same space, which can limit experimental planning at most institutions. A Sinclair mini-pig can still reach up to 50 kg at 1.5 year, so the research facility has to accommodate animals of this size. The murine gestation period (20 d) is <20% of porcine gestation period, so crossbreeding is much quicker with mice. A relatively simple maneuver of placing a 20 g mouse under general anesthesia becomes more complicated when dealing with a 30-50 kg pig.
3. **Tools & Reagents.** The availability of antibodies, reagents, and other species-specific research tools is much greater in mice compared to pigs, though the availability for pigs has improved in the past decade.

4. **Subject Age.** In general, investigators only have access to young (<1 year) pigs; however, modeling epithelial tumors with young pigs may not be optimal.
5. **Research Community.** The number of investigators who utilize swine in cancer research is still relatively small, so other investigators may be reluctant to consider porcine experimentation.
6. **Social Issues.** Public reaction to swine use in research could be more negative compared to the reaction against rodent use. This possibility may demand more investigator effort devoted to public education.

Conclusion And Future Directions

The current landscape of animal modeling for breast cancer is dominated by murine models, which have developed into powerful and multi-faceted tools for the BC researcher. It would be difficult to improve on the utility that murine BC models have provided. However, there remain certain areas of research, such as device development and drug testing, which could benefit from the availability of a large animal model of BC. These BC models are still in their infancy, essentially at the point murine models were in the 1980's. While large animal BC models likely will never be able to match the proven utility and ease-of-use of murine models, the availability of validated large animal BC models could provide additional tools to the BC researcher that would address specific BC questions or BC-relevant technology development, such as those requiring a human sized subject for generation of relevant data.

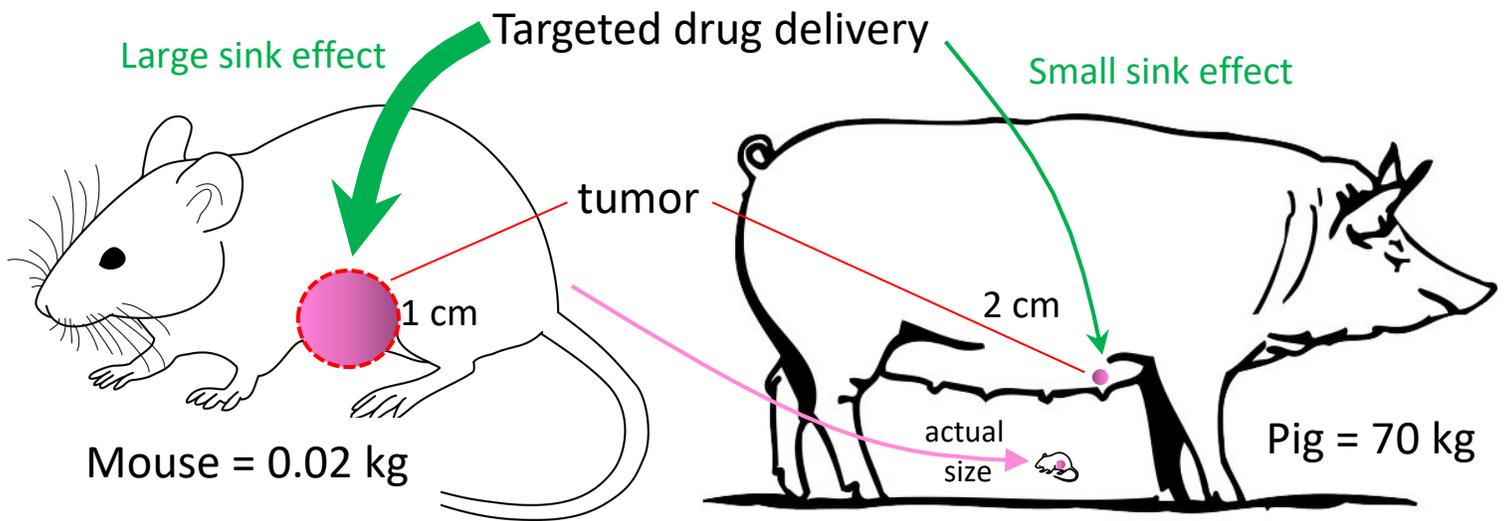


Fig. 1. Tumor Sink. In this example, murine tumor:body mass ratio is 400x the porcine ratio, even though murine tumor mass is only ~12% that of the porcine tumor. If a targeted drug preferentially concentrates in the tumor (i.e., tumor sink), then a given drug dose in the mouse may have a relatively high concentration in the tumor with respect to the plasma. The effect of tumor sink on plasma concentration at the same dose in the pig would be negligible because of the large body size. Thus, the same dose in the pig would produce a higher plasma concentration, possibly producing greater systemic toxicity than in the mouse.

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