Blueprints of disease: precision platforms for modelling breast cancer

Lutz, C.

CHAPTER 3

Rat models of hormone receptor-positive breast cancer

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Abstract

Hormone receptor-positive (HR^+) breast cancer (BC) is the most common type of breast cancer among women worldwide, accounting for 70-80% of all invasive cases. Patients with HR^+ BC are commonly treated with endocrine therapy but intrinsic or acquired resistance is a frequent problem, making HR^+ BC a focal point of intense research. Despite this, the malignancy still lacks adequate *in vitro* and *in vivo* models for the study of its initiation and progression as well as response and resistance to endocrine therapy. Since no mouse models are available that fully mimic the human disease, rat mammary tumour models could be further explored. Compared to mice, rats are more similar to humans in terms of mammary gland architecture, ductal origin of neoplastic lesions and hormone dependency status. Moreover, rats can develop spontaneous or induced mammary tumours that resemble human HR^+ BC. To date, six different types of rat models of HR^+ BC have been established. These include the spontaneous, carcinogen-induced, transplantation, hormone-induced, radiation-induced and genetically engineered rat mammary tumour models. Each model has distinct advantages, disadvantages and utility for studying HR^+ BC. This review provides a comprehensive overview of all published models to date.

Keywords
rat model, hormone receptor (HR), breast cancer (BC), mammary tumour

Introduction

Breast cancer (BC) remains the most frequently diagnosed malignancy in women globally, accounting for approximately 12% of all new annual cancer diagnoses [1,2]. It comprises a heterogeneous disease, with variable clinical outcomes and different histopathological and molecular features [3,4]. Breast tumours are traditionally classified into distinct molecular groups based on their oestrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression patterns [5-7]. Such expression levels can be detected by immunohistochemistry, and/or gene expression profiling, yielding three major BC subtypes, namely basal-like, HER2-enriched, and hormone receptor-positive (HR^+) tumours [3,6]. HR^+ tumours, which comprise 70-80% of all invasive cases, are characterised by the expression of oestrogen receptor (ER) and/or progesterone receptor (PR), as well as of many genes expressed by the luminal epithelial cells of the breast, often linked to ER activation [3,7]. HR^+ tumours encompass three molecular subtypes, known as luminal A, luminal B, and normal-like BCs. Compared to luminal A BC, luminal B tumours show higher proliferation (Ki67), greater histological grade, decreased differentiation, and a higher frequency of *TP53* and *PIK3CA* mutations [3].

Evaluation of prognosis and treatment options for the different molecular BC subtypes are based on a combination of clinical, pathological, and molecular methods [8,9]. Along with age, race and menopausal status, an important prognostic and clinical decision-making factor is ER status. Specifically, ER expression in breast tumours is, at least initially, associated with a favourable prognosis, given that patients with ER^+ BC are more likely to respond to hormonal therapy [10,11]. However, despite being the recommended treatment approach for ER^+ BC cases, endocrine therapy still fails to tackle the disease, as more than 50% of patients relapse after >5 years, frequently with more aggressive and metastatic disease [12,13]. This can be due to several reasons, including primary or secondary resistance to endocrine treatment, which are still poorly understood [12,13]. Since HR^+ tumours represent 70-80% of all BCs, most BC-related mortality can thus be attributed to ER^+ malignancies and their recurrence patterns [12,13].

To overcome such setbacks in the clinical management of luminal BC, accurate and appropriate modelling systems recapitulating human disease are pivotal. In the *in vitro* setting, efforts have been made toward the development of

HR$^+$ BC cell lines and organoids, although, especially for the latter, loss of HR expression and growth cessation after a few passages remain a challenge [14-18]. Moreover, such models present several drawbacks for the cancer research field, including the lack of a host organism and surrounding stromal and immune cells, and thus failure to recapitulate the tumour microenvironment *in vitro* [19,20]. The presence of genetic and epigenetic distinctions between the cell lines and parental tumours following long-term culturing also poses a challenge to the study of disease biology, making these *in vitro* models unreliable [21,22]. Such challenges can be overcome by the use of animal models, which not only enable the study of tumour-immune cell interactions, but also the investigation into tumour initiation, progression and metastasis [23].

Murine models in hormone receptor-positive breast cancer research

Mice have been instrumental for preclinical *in vivo* modelling of breast cancer. However, species-specific differences in systemic and mammary gland biology between mice and humans highlight the need for more accurate and representative models in breast cancer research. Compared to mice, rats are more similar to humans in terms of HR signalling and mammary gland architecture. Moreover, rats develop mammary carcinomas that are of ductal origin, HR$^+$ and responsive to oestrogen, just as the majority of human breast tumours. In the following section we discuss differences between humans, rats and mice, and their relevance for preclinical breast cancer modelling.

Mouse models

Mice, due to their small size, short generation times, and genetic modifiability, are frequently chosen for *in vivo* cancer modelling [24]. In the context of mouse models of HR$^+$ BC, transplantation models have been developed based on mouse mammary tumour cell lines allografted in syngeneic mice or on human BC cell lines or patient-derived tumour material xenografted into immunodeficient mice.

Human BC cell line-derived xenograft (CDX) models present the mainstay of modelling approaches, with MCF-7, T47D and ZR-75-1 being the most commonly engrafted lines [25-29]. Much research has been carried out to compare the molecular characteristics of these cell lines to HR$^+$ BC in patients [30-34]. Engraftment of human BC cell lines usually necessitates immunodeficient

mice [35], though recent studies have also utilized humanized models [36]. These CDX models have paved the way for molecular and pharmacological studies [28,29]. Fewer attempts have been made to establish mouse HR$^+$ mammary tumour cell lines for allograft studies [17,18,37,38]. Patient-derived tumour xenograft (PDX) models present an elegant alternative to established cell lines, evading the molecular alterations that are known to occur under long-term tissue culture conditions [39]. PDX models are propagated *in vivo* and recapitulate tumour heterogeneity whilst maintaining patient tumour features over successive generations of *in vivo* passaging in mice [39,40]. Large efforts were made to establish a sizable collection of well-characterised BC PDX models with regards to coverage of clinical subtypes, genomic, transcriptomic and proteomic features [41,42]. Of note, mouse transplantation models, especially PDXs, are still limited by the low engraftment rate of HR$^+$ BC, different protein expression profiles compared to the parental tumours [32], and the requirement for supplemental 17β-oestradiol (E2) given that endogenous mouse oestrogen levels are often unable to sustain xenograft growth [31,43]. To date, most transplantation models represent triple-negative BC and only few recapitulate HR$^+$ BC, and even fewer the luminal A subtype that is most commonly observed in the clinic [44] (**Figure 1**).

To better recapitulate *de novo* initiation and progression of HR$^+$ BC in immunoproficient animals, several strategies have been attempted to establish genetically engineered mouse models (GEMMs) of HR$^+$ BC. Initial models mostly relied on the use of the mouse mammary tumour virus (MMTV) promoter, which enabled the direct overexpression of ERα or viral oncoproteins, such as the polyomavirus middle T antigen, in the mouse mammary epithelium [45-48]. However, in these models, most mammary tumours are either ER-negative or lose their ER expression at later tumourigenic stages [46,49-51]. Indeed, the vast majority of GEMMs developed through genetic alterations in oestrogen signalling molecules or oncogenes have been reported to predominantly yield mammary tumours that are HR$^-$ or lose ER expression when exposed to endocrine therapy [52-54].

To date, only four GEMMs have been reported to develop HR$^+$ mammary tumours: the *Stat1*-knockout [55], BLG-*Cre;Kras*$^{(G12V)}$ [56], NRL-PRL [57], and Wap-*Cre;Pik3ca*$^{(H1047R)}$ [58] models. Though the *Stat1*-knockout, BLG-*Cre;Kras*$^{(G12V)}$, and NRL-PRL models develop HR$^+$ mammary tumours that are sensitive to hormonal perturbations such as ovariectomy and fulvestrant treatment [55,56], they all are hindered in their utilization potential by their driver mutations.

Rat

Mouse

Modeling of functional ER signaling	Yes	Limited
ER functionality recapitulates human biology	Yes [74, 75, 79, 81, 100]	Different functionality of ERβ, GATA3, FOXA1 [64-68]
Testing of current standard-of-care in ER⁺ BC	Endocrine therapy testing possible [137, 214, 219]	Unreliable for testing of endocrine therapy [43]
Availability of ER⁺ cell/ organoid resources	Several cell lines [103, 104] and organoids [101]	Limited number of cell lines [17, 18]
Engraftment of ER⁺ material in immunodeficient animals	Possible [104, 170]	Only possible with estrogen supplementation [43]

Figure 1: Comparison of rats and mice in the context of HR⁺ BC research, in terms of modelling of functional ER signalling and generated resources.

ER⁺ breast tumours in patients show only low or undetectable levels of STAT1 expression [55], and rarely carry activating mutations in *KRAS* [59], which have been reported to render ER⁺ tumours resistant to endocrine therapy [60-62]. The Wap-*Cre;Pik3ca*$^{(H1047R)}$ mouse model overcomes this limitation since *PIK3CA* is commonly mutated in human ER⁺ BCs [63]. Nonetheless, also the Wap-*Cre;Pik3ca*$^{(H1047R)}$ model requires continuous E2 supplementation prior to and after tumour onset [58], which contradicts the low physiological levels of E2 in post-menopausal patients [3].

HR signalling in human, rat and mouse

ERα signalling is different in mice compared to humans, possibly due to species-specific differences in pioneer factor usage [64]. In addition to the absence of the FOXA1 motif in the mouse Erα binding sites [65], further differences in HR signalling between mice and humans include the lack of Erα36 receptor expression in mice [66], and the distinct roles of growth factor amphiregulin (AREG) and insulin receptor substrates (IRS) in the

mouse mammary gland [67,68]. The lack of Erα36 receptor, a shorter isoform of Erα, responsible for PR regulation in BC [69] and the maintenance of ER$^+$ BC progenitor cells [70], has been linked to alterations in post-pubertal mouse mammary duct histology and epithelial cell proliferation [71]. Also, both AREG and IRS are pivotal for mammary epithelial cell proliferation, ductal formation and elongation, and overall mammary gland development in humans [72,73]. The lack of these factors in mice impairs their alveologenesis and mammary ductal development [67,68], underlining further differences in mammary gland biology between mice and humans. In contrast to mice, rats have been reported to display HR signalling pathways that are more comparable to those observed in humans and could thus represent a superior model for BC research.

Ovaries and the pituitary gland are two main sources of oestrogen production. While the pituitary glands of rats and humans express both ERα and ERβ [74,75], ERβ expression is absent in the mouse pituitary gland [76]. Given that ERβ overexpression has been shown to enhance oestrogen-induced prolactin gene expression [77], its expression in human and rat could indicate that the oestrogen regulation of prolactin is likely similar in these species, and distinct from the mouse. Indeed, prolactin signalling has been implicated in promoting ER$^+$ tumourigenesis in mice [78] and transgenic overexpression of rat prolactin ligand *rPrl* in the mouse mammary gland induced the formation of ER$^+$ mammary tumours [57].

Along the same lines, prolactin-mediated inhibition of lipolysis in adipose tissues is observed in both rats and humans, but not in mice [79], lending further support to the notion that the oestrogen-prolactin signalling axis is more strongly conserved between humans and rats, as compared to mice. This is especially relevant, as breast tumour aggressiveness has been linked to the presence of free fatty acids released by the tumour-surrounding adipocytes following lipolysis [80].

Finally, expression patterns of the pioneer factor GATA3 in normal mammary glands and carcinomas are comparable between rats and humans [81] and contrast with the low expression of GATA3 observed in mouse mammary epithelium [65]. Since GATA3 is an essential driver and a prognostic biomarker in ER$^+$ BC [82,83], the similar expression found in human and rat mammary tissues reinforces the relevance of rat models for studying ER$^+$ disease and -signalling interactions *in vivo* (**Figure 1**).

Mammary gland architecture differences between species

Tumours develop in an intricate interplay between cancer cells and their local tissue environment [84]. Mice are characterised by distinct differences in mammary gland architecture compared to humans [85]. Notably, mice do not develop terminal duct lobular units (TDLU) [85,86], the structure from which luminal tumours typically arise [87,88]. Furthermore, mice have low levels of serum oestradiol, as compared to both humans and rats [89-91]. The latter, coupled with the differences in oestrogen signalling and lack of mammary-specific growth factors could, at least in part, explain the architectural, histological and molecular differences observed between human and mouse mammary gland development and tumourigenesis. Indeed, mouse mammary tumours originate from the ductal stem instead of the TLDU and develop with distinct histopathological characteristics, which are more squamous and mesenchymal than those in humans [43,86], and commonly display gene expression profiles that differ significantly from human lesions [92,93]. Moreover, mice commonly present with ER^- and hormone-independent lesions, in contrast to both humans and rats [64,94,95].

Rats possess six pairs of mammary glands that develop lobuloalveolar structures resembling human TDLUs [96]. Compared to mice, drug pharmacokinetics in rats are more analogous to humans [97,98], and their larger body size facilitates (longitudinal) collection of tumour biopsies and blood, aiding the analysis of drug pharmacokinetics and pharmacodynamics [97-99]. Importantly, unlike most mouse models, rats reliably develop HR^+ and oestrogen-dependent mammary tumours across various models [100] and, thus far, have already enabled the generation of a larger number of ER^+ mammary tumour cell lines and organoid [101-104] (**Figure 2**).

Rat mammary tumour models can be categorised into six groups based on their induction methods: spontaneously occurring models, carcinogen-induced models, transplantation models, hormone-induced models, radiation-induced models, and genetically engineered rat models. In this review we discuss, in chronological order, each category of rat mammary tumour models and their (dis)advantages. In addition, we discuss the utility of the various rat mammary tumour models for preclinical studies as well as future research directions in modelling HR^+ BC in rats (**Figure 3, 4**).

	 Human	 Rat	 Mouse
Mammary gland architecture	 TDLU [85-88]	 Lobuloalveolar development post-puberty toward TDLUs [95, 96]	 Absence of TDLUs [85, 86]
Tumor origin	TDLU [87, 88]	TDLU [46, 108, 253]	Ductal [43, 86]
Serum estradiol levels	50-165 pg/mL [5, 10]	5-140 pg/mL [89-91]	5-35 pg/mL [91]
Tumor ER dependency	Dependent [3, 94]	Mostly dependent [94]	Mostly independent [94]
Frequency of ER⁺ tumors	 70-80% [3, 4, 6, 7, 93]	 70-80% [95, 100]	 Low [64, 92-95]
Histopathological presentation of tumors	Mostly adenocarcinomas [1, 4, 8, 86, 95]	Mostly adenocarcinomas [95, 99]	Many squamous and mesenchymal tumors [43, 86, 95]

Figure 2: Comparison of the different species, namely human, rat and mouse, in regards to their biology, mammary gland architecture and tumour features in the context of HR⁺ BC.

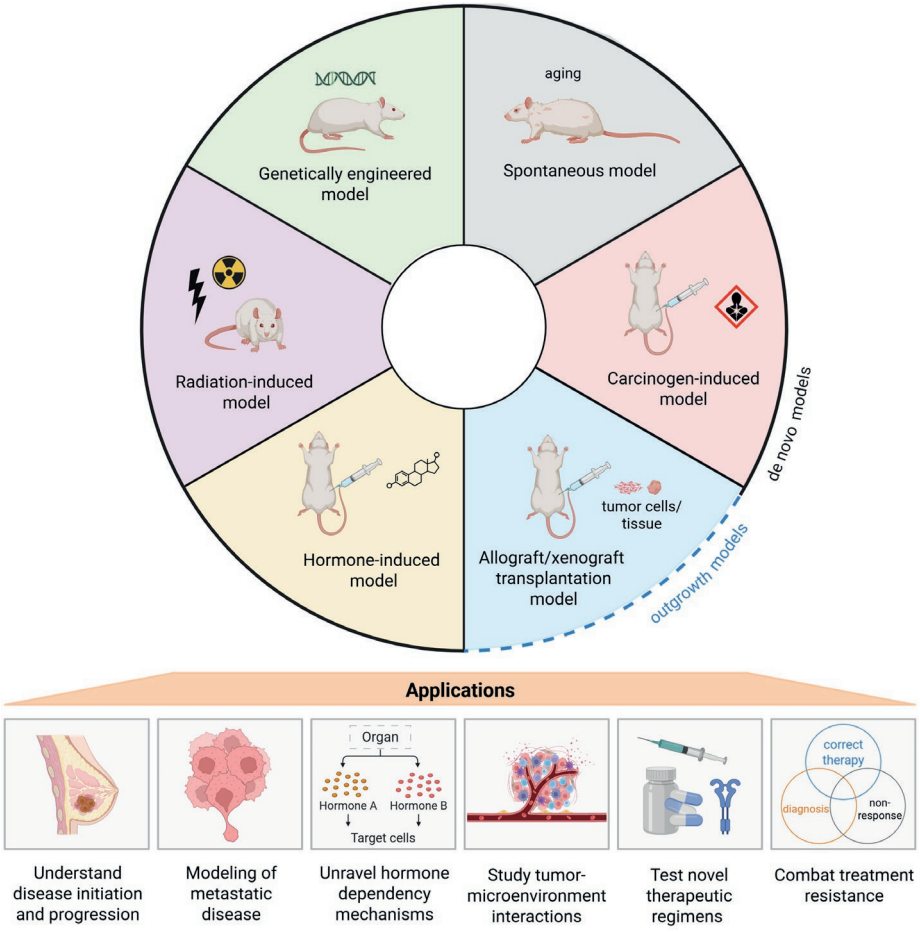


Figure 3: Overview of different rat tumour induction models, clockwise, in order of emergence, and their utility for BC research.

Spontaneous rat mammary tumour models

Background

Long before the ability to engineer cancer models, spontaneous mammary tumour systems were the mainstay BC models allowing the study of naturally occurring mammary lesions, which are infrequently observed in experimental animals, including aged female rats of different strains [24,105-107]. These include Sprague-Dawley (SD), Wistar, August, Albany-Hooded, Copenhagen, Lewis, and Fisher 344 rats, with the latter being particularly susceptible [108,109]. Since

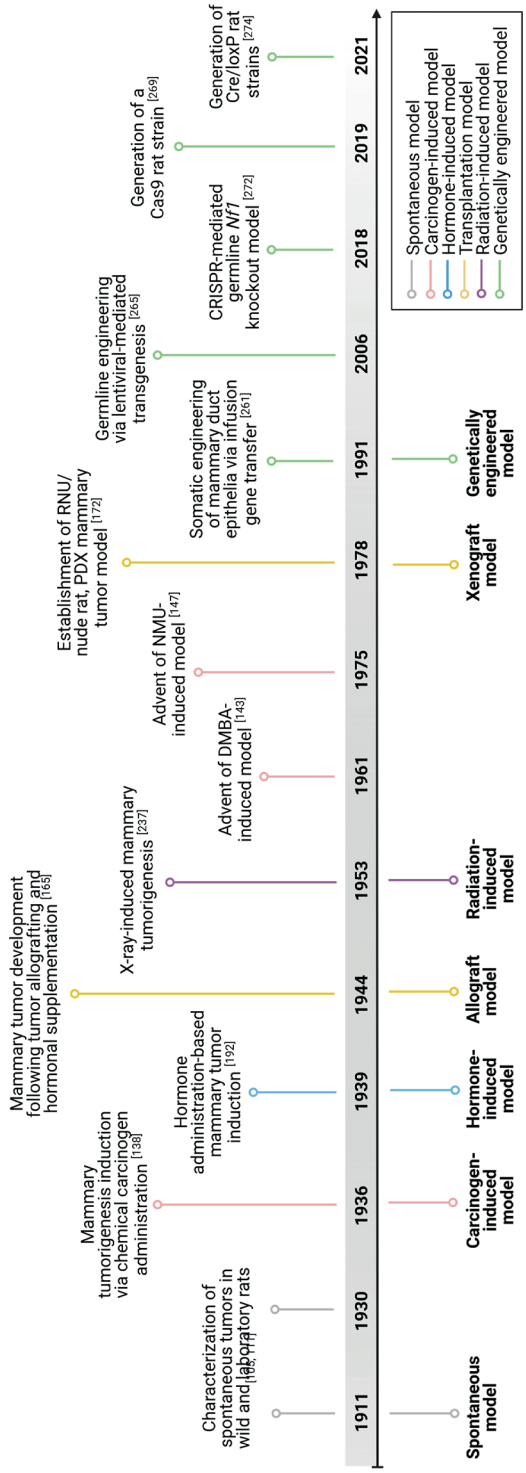


Figure 4: Notable advances in the generation of rat models of HR$^+$ BC over the past 113 years. Colours represent the different tumour induction models.

these spontaneous tumours are observed in non-genetically or -carcinogen treated animals, their development could recapitulate the human disease progression and tumour evolution more closely [24,102,110]. Historically, the occurrence of these tumours in wild rats has been described since the early 1900's, by Woolley and Wherry [111], though the first report in laboratory rats was only published in 1930 by Bullock and Curtis [105,106,112,113]. According to the authors, most spontaneous neoplasms in the rat mammary gland give rise to tumours of epithelial origin, of which the majority are benign growths [108,109,114].

A similar trend was demonstrated by Bagg and Hagopian [113] and Bryan *et al*. [112] who, in addition to observing a higher number of benign tumours in comparison to a lower incidence of malignant growth, shed light on the possible causes behind the neoplastic lesions. These include endocrine disturbances and a decline of the animal's fertility, alluding to the impact of age-related hormonal changes on mammary tumour incidence [108,109,114]. Particularly, the influence of age-associated endocrine perturbations was confirmed in later studies, in which ovariectomy before middle age onset and restoration of oestrous cycles in aged animals led to a decrease of spontaneous mammary tumour incidence [115,116].

In addition to age-related endocrine changes, other factors, such as caloric intake and environmental conditions, were shown to play a role in the development of spontaneous rat mammary tumours [117-119]. Regarding the former, previous studies indicated that, if fed a low-fat diet and presented with a reduction of body weight, rats are likely to exhibit a lower spontaneous mammary tumour incidence, in contrast with those given a high-fed diet and with a higher body weight [117,120]. Such an effect is also seen in the human setting, with a greater risk of ER^+ BCs being specifically linked to an elevated dietary fat consumption [120,121]. Moreover, environmental and housing conditions, such as social isolation, and deregulated light-dark cycles, and thus melatonin levels, have been suggested to influence the occurrence of spontaneous mammary tumours [110,118,119]. In patients, similar associations are observed, in particular concerning the cascade effect of sleep deprivation, light exposure at night and diminished melatonin levels in the elevated BC incidence figures [122,123].

Advantages

One of the main advantages of the spontaneous mammary tumour model concerns their HR expression and dependency status [124] (**Figure 5A**). As shown by Welsch *et al.* [125] and Meites [124], the incidence and development of spontaneous tumours is increased in the presence of higher prolactin levels, usually accompanied by a reduction in oestrogen secretion. Additionally, previous studies have also shown that hormonal withdrawals, such as ovariectomy and adrenalectomy, or treatment with ER antagonists, can hinder tumour growth and development in SD rats. On the other hand, hormonal supplementation, such as continuous administration of oestrogens and prolactin, enhances malignant and benign tumour formation, respectively, highlighting the hormone dependency in such spontaneously occurring tumours [108,126-128]. Cheung *et al.* [129] have also shown that, though limited, spontaneous carcinomas in Noble female rats display a positive ERβ cytoplasmic staining, as demonstrated by immunohistochemistry analysis. Another advantage of the model concerns the development of such naturally occurring tumours in genetically heterogeneous populations, similar to the human setting [24].

Disadvantages

Though in susceptible strains, such as SD and Wistar-related rats, spontaneous mammary tumour incidence is relatively high, most tumours display a benign outgrowth [108,114] (**Figure 5A**). These are usually fibroadenomas, fibromas and intraductal papillomas, with malignant disease, such as adenocarcinomas, being a rare occurrence [108,130]. Another disadvantage of this model comprises the long tumour development latencies of up to two years in susceptible strains, rendering it a time-consuming experimental model [24,108]. Moreover, since these tumours are heterogeneous, and animals display individual tumour growth variations, achieving a statistically significant number of tumour-bearing rats with uniform lesion growth can be challenging. Cha *et al.* [131] have also revealed that, in untreated conditions, the majority of premenopausal rats display detectable levels of *Hras* mutants, a mutation signature commonly seen in NMU-induced tumours. As subsequently confirmed by McKim *et al.* [132], such findings highlighted the role of *Hras* activating mutations in spontaneous tumours, deviating from the genetic makeup seen in human BC [131,133,134].

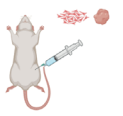
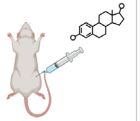
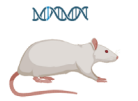
	A. aging  **Spontaneous model**	**B.**  **Carcinogen-induced model**	**C.**  **Transplantation model**	**D.**  **Hormone-induced model**	**E.**  **Radiation-induced model**	**F.**  **Genetically engineered model**
Advantages	Natural occurence Unperturbed lesion progression	Complete spectrum of lesion progression from hyperplasia and *in situ* lesions to metastasis Highly ER⁺ lesions Responsive to hormone alterations	Preservation of primary tumor samples Recapitulation of parental tumor features	Histopathologic and genetic makeup similar to the human counterpart	Tumors with similar genetic makeup to human lesions Well-studied mechanism-of-action	Spatiotemporal control Gene targeting at highly specific fashion Tailored recapitulation of molecular disease progression Immunoproficient models
Limitations	Long + varied tumour latencies Incomplete penetrance Mostly benign lesions	Mutational spectrum does not recapitulate human disease Development of non-mammary tumours Used carcinogens are no environmental risk factors for human disease	No *de novo* tumor development Often low engraftment rates Cell adaptation to long-term *in vitro* culture conditions Study of microenvironment not possible or severely hampered	Development of non-mammary tumours Long tumor latencies Study of hormone interaction hindered by perturbed hormone balance	Mostly benign lesions Long tumour latencies Radiation-induced late complications Off-target carcinogenic effects Study of microenvironmental and systemic factors not possible	Cost-inefficient Time-consuming Off-target mutation edits
Demonstrated clinical relevance	Description of genetic and hormonal components of mammary carcinogenesis [104, 111, 116, 126] Influence of aging on tumor formation [104, 117]	Preclinical model for PD-1/PD-L1 immunotherapy responses, in combination with TGFβ inhibition, for early early stage HR⁺ BC [101] Influence of environmental factors on ER⁺ BC [289-298]	Study of primary tumor biology and tumor growth dynamics [183, 184, 186]	Effects of diet, parity, and obesity on breast carcinogenesis [299-302] Influence of hormonal treatment or tamoxifen on tumor initiation [303-305]	Effect of systemic and local radiation on BC risk [235-237]	*In vivo* induction and recapitulation of relevant BC gene drivers [272]
Utilization potential	Limited			Metastatic disease modelling	Study DNA damage and its effect on BC initiation and progression	Targeted modelling of clinically relevant genetic aberrations Uncover potential treatment opportunities

Figure 5: Experimental advantages and limitations as well as unique clinical relevance and utilization potential of the different rat models of HR⁺ BC.

Carcinogen-induced rat mammary tumour models

Background

Following the first reports of spontaneous mammary tumours in rats, a second model based on the use of chemical carcinogens emerged. Such a carcinogen-induced rat mammary tumour model comprises the most heavily used and second oldest preclinical model to study human BC [135,136]. The system's generation entails the administration of a single or multiple repeated doses of a chemical carcinogen, usually delivered intravenously or intraperitoneally [137]. Deploying this method, carcinogenic tumourigenesis in the rat mammary gland was first achieved in the 1930s, when Dunning *et al*. described the development of sarcomas in various rat strains following subcutaneous injection of 1,2,5,6-dibenzanthracene or 3,4-benzopyrene [138]. Subsequently, in 1941 and 1949, Wilson *et al*. [139] and Shay *et al*. [140], respectively, reported the induction of mammary adenocarcinomas in albino rats following intraperitoneal administrations of the carcinogens 2-acetylaminofluorene (AAF) and methylcholanthrene (MCA). Their studies were the first to highlight the impact of the endocrine system on rat mammary cancer formation. Specifically, based on their experiments, non-ovariectomised female rats tended to be more susceptible to tumour development, in comparison to males and ovariectomised rats. AAF-induced tumours were also shown to be hindered by lactation, whereas gestation stimulated their growth [141,142].

Nevertheless, the model's popularity was only launched by Charles Huggins, who, in 1961, introduced an improved methodology in which mammary carcinomas in the rat were triggered by a single dose of 7,12-dimethylbenzanthracene (DMBA) [143]. Given its one-dose tumourigenic effects, Huggins's study enabled closer inspection of the initial and promoting stages of tumour development, alluding to its potential mammary gland ductal origin [142]. Moreover, similarly to other mammary tumour-inducing carcinogens, most DMBA-induced carcinomas are responsive to hormonal changes, as seen by a reduction in tumour growth once animals were ovariectomised, hypophysectomised or an increase in tumour growth once animals received hormone supplementations such as progesterone, oestradiol, or a combination thereof [144]. Interestingly, administration of progesterone alone was shown to enhance DMBA-induced tumour growth and incidence, alluding to the hormone's stimulating effect on tumour development, an event also seen in the clinical setting [144-146].

In light of the successful establishment of a chemically-induced rat mammary adenocarcinoma system *in vivo*, other carcinogens, such as N-methyl-N-nitrosourea (NMU), were also explored for the same purpose. Gullino *et al*. [147] were the first to document the induction of primary mammary tumours in different strains of female rats, including SD and Fisher 344, treated with NMU intravenously. In this study, NMU was injected three times, with an interval of four weeks between each dose, yielding a <70% tumour incidence in all strains tested. All tumours were histologically classified as adenocarcinomas or papillary carcinomas, displayed responsiveness to hormonal alterations via ovariectomy, and were highly metastatic, mainly toward bone marrow and spleen [147].

Given the different published models, the ideal carcinogenic agent and experimental conditions to study the human disease were further investigated in follow-up, comparative studies. Gusterson & Williams [148] reported that DMBA and NMU, the most commonly used carcinogens for inducing mammary tumours in rats to date, generate indistinguishable mammary tumours, with both agents failing to trigger local and distant invasion [149]. Gullino *et al*. 's claim regarding the NMU-induction tumour model's highly metastatic potential was also contradicted in Rose *et al*. [150] and Williams *et al*. [151] subsequent work, in which no metastasis was found in the autopsies. In terms of prognostic marker expression, Alvarado *et al*.'s [149] immunohistochemical analysis comparing tumours from the DMBA and NMU-induced models revealed that, while both groups yielded HR^+ tumours, NMU-induced lesions presented higher KI-67 and mitotic activity index scores. Such results could suggest that NMU trigger the formation of more aggressive mammary tumours, with similar immunohistochemical characteristics to the luminal B BC subtype [149,152].

Such differences in the rat mammary tumours induced by DMBA and NMU could be due to their distinct mechanisms of action [153]. Though both agents rely on DNA alkylation to prompt tumour development, unlike NMU, DMBA is considered an indirect alkylating agent, as it relies on the metabolic activation by hepatic cytochrome P450 enzymes. As a result, DMBA's carcinogenic effect is slower, leading to longer tumour latency periods [149,154]. Moreover, NMU-induced rat tumours were shown to display activating mutations in the *Hras* oncogene in over 85% of cases, whereas *Hras* mutations are rarely found in lesions induced by DMBA [133,134,155,156]. Since *HRAS* mutations are not frequently detected in human BCs, with rates as low as 1%, DMBA-induced rat tumours could thus be a better representation of the human disease [156,157].

Advantages

Despite their different metabolic effects on mammary tumour induction, DMBA and NMU share similar cellular targets within the rat mammary gland, namely the epithelial cells of the TDLU, as seen in the human setting [96,108,153]. This reflects the presence of mammary stem and progenitor cells that are prone to malignant tumourigenesis, as well as the TDLUs' high proliferative and low cell loss profile [137]. When given to rats of susceptible strains at the age of sexual maturity and full mammary gland development, DMBA and NMU can lead to a tumour incidence of up to 100% [100,136,137]. These lesions cover a wide range of mammary neoplasms, including benign fibroadenomas, fibromas, and intraductal proliferations. The latter can eventually develop into lesions resembling the human ductal carcinoma *in situ*, which could potentially develop further to HR^+ invasive carcinomas [109,153] (**Figure 5B**).

Given that over 70% of all invasive human BC cases are HR^+, the carcinogen-based induction of such tumours in rats offers many advantages to the disease *in vivo* modelling scene, presently consisting of a negligible amount of mouse models [43]. These include the possibility of exploring new therapeutic strategies for the management of primary BC. Moreover, with the model's ability to recapitulate the multistep malignant transformation process, including the potential development of invasive lesions from *in situ* carcinomas, it could also be used to study disease progression [109]. In the same context, the role of the tumour microenvironment in disease development and relapse could also be further explored, as enabled by the use of immunocompetent rats [43].

Furthermore, since this rat model presents hormone-sensitive tumours, fundamental and translational studies could be conducted to further understand the crosstalk between HR signalling and growth factors [43]. For instance, animal modelling-based investigations into the role of the cyclin D1/cyclin-dependent kinase (CDK) 4/6 pathway in BC pathogenesis were essential for the development of CDK 4/6 inhibitors, such as palbociclib [158,159]. However, comparably to first-line endocrine therapies, intrinsic and acquired resistance to CDK4/6 inhibition still occurs, with over 20% of patients initially failing to respond to the drug, while half of the responding patients relapse within 25 months [160]. This highlights the need for further research on drug resistance mechanisms and therapy efficacy, which could be achieved using the experimental carcinogen-induced tumour model.

Disadvantages

While the model yields HR$^+$ and -dependent mammary tumours, it still presents a few drawbacks in recapitulating BC *in vivo*. These include the distinct microscopic and macroscopic characteristics of the rat lesions, in comparison to the human counterpart. Specifically, the carcinogen-induced adenocarcinomas in the rat tend to be delineated, with predominant epithelial components. Moreover, rat mammary carcinomas often display a cribriform and papillary aspect with morphologically varying gland-like structures, which tends to differ from the ductal pattern seen in the human disease. Another contrasting feature of carcinogen-induced tumours in rats versus human BCs is its low metastasis incidence [161]. This was shown to be the case for both NMU and DMBA-induced lesions, even when they presented multifocal and displayed local aggressiveness [136,149,162]. The lack of metastasis in such tumours could be due to the simultaneous and equally proportional proliferation of luminal and basal epithelial cells in the rat mammary gland as suggested by Murad and von Haam [163]. Such proliferative behaviour is not seen in the human setting, as proliferation takes place mainly in epithelial cells [162]. Furthermore, the DMBA and NMU-induced rat mammary tumour model fails in accurately recapitulating BC *in vivo* due to the absence of these carcinogens in the human environment and organism [164]. Most importantly, the differing distinct genetic makeup between the human and the NMU-induced rat tumours, is a major drawback of the model [133] (**Figure 5B**).

Transplanted rat mammary tumour models

Background

In the context of the carcinogen-induced tumours in rats, mammary tumour induction experimentation was conducted, in which the induced tumours were successfully serially transplanted into other recipient rats [165,166]. A well-documented example of such allograft modelling derived from carcinogen-induced system concerns the 13762 adenocarcinoma line, obtained from a rat DMBA-induced mammary adenocarcinoma. The tumour line was maintained *in vivo* through serial passages in syngeneic female rats, as well as cryopreserved stocks [103,167]. Specifically in Neri *et al.*'s study [104], tumour pieces were expanded *in vivo* via subcutaneous implantation into the mammary fat pad of syngeneic female Fisher 344 rats, resulting in histologically similar tumours to the parental lesions. In a follow-up study, a similar trend could be observed when a 13762 clone was administered

as a single cell suspension into the mammary fat pad of syngeneic rats [168]. The generation of this novel, hormone-dependent BC model in rats was particularly relevant for the study of locally recurrent mammary tumours, but also metastatic formation [104,168].

Paralleling the advent of such carcinogen-derived allografting models, a rat BC xenotransplantation model was developed in 1987, by Vaupel *et al.* [169], using human tumour specimens. This occurred following the establishment of the RNU/nude rat model in 1978, an autosomal recessive *Foxn1* mutant characterised by a T cell deficiency, while displaying functional B and natural-killer cells [170-172]. In Vaupel *et al.*'s study, athymic RNU rats were successfully xenografted with human BC tissues obtained from different patients. Additionally, the RNU strain was deployed for xenotransplantation of small human breast tumour pieces successively passaged in nude mice which were administered via subcutaneous implantations into the flanks of recipient rats, yielding mammary medullary and squamous cell carcinomas [169].

Despite being the only rat strain able to successfully sustain human xenotransplants, RNU rats were still lagging behind immunodeficient mice, as the latter were still superior in terms of tumour engraftment efficiency [170,173,174]. To circumvent this, and in light of the CRISPR-Cas9 technology advances, novel immunocompromised rat models were developed, including the Sprague-Dawley *Rag2/Il2rg* double knockout (SRG) and the *Rag1/Rag2/Il2rg* triple knockout (SD-RG) strains [170,175,176]. Such severely immunodeficient inbred rats lacking mature T, B and NK cells have been shown to present not only a 100% engraftment rate of cancer cells, including the human BC cell line HCC1954, but also allow for patient-derived xenograft establishment and expansion [177]. In addition to its human cell line and patient-derived xenografting potential, the SRG rat strain can be applied for the establishment of mouse to rat cell xenografts, highlighting the model's versatility and promising role in the *in vivo* study of BC development [178].

Lastly, to better recapitulate human disease, immunodeficient rats have also been humanized, with the goal of investigating tumour immune microenvironment interactions and immunotherapy responses [179,180]. In the context of HR^+ BC, the Rag-/- Gamma chain-/- human signal regulatory protein alpha-positive (RRGS) rat strain, generated by Ménoret *et al.* [180], represented an important modelling tool for assessing antitumour immune responses *in vivo*. In this model, *Rag1* and *Il2rg* deficient rats express the human regulatory

protein SIRPα on their leukocytes, enabling them to circumvent macrophage-mediated xenograft rejection, leading to the successful engraftment and growth of a patient-derived, ER^+ and PR^+ breast carcinoma cell line [179,180]. Moreover, given the presence of human antitumour immune activities, as well as their inhibition when treated with antibody-based therapy, Ménoret *et al*. 's rat model could be further applied for the development of novel therapeutic approaches and regimens for the management of HR^+ BC.

Advantages

Though BC initiation and prevention could be investigated with the use of carcinogen-induced models, the understanding of disease progression, specially the *in situ* to invasive transition stage, was still limited [136,137]. Such a limitation could be overcome with the advent of transplantation syngeneic rat mammary tumour models, as demonstrated by Chan *et al*. 's study [181]. Their allograft BC model was developed by administering a subcutaneous injection of minced NMU-induced rat mammary neoplastic lesions, in suspension, into the mammary gland of the recipient rat, with the originating tumour then being serially transplanted for up to 5 generations. Investigation of tumour development was then enabled via the histopathological analysis of each transplant, highlighting the acquisition of a more invasive phenotype throughout the generations, in contrast to the parental *in situ* ductal carcinomas [181]. Furthermore, given that syngeneic models derived from carcinogen-induced tumours often generate highly metastatic tumours, as opposed to their carcinogen-induced origin, and similar to the human setting, they could be used to not only track the metastatic process, but also test new anti-metastatic agents [104,168,182].

Similarly, PDX and human cell line-derived xenograft rat models, given their direct derivation from human tumour samples, could also represent a valuable resource for drug response testing and understanding of disease progression [24,183]. Moreover, with the use of immunodeficient rats, PDXs enable BC *in vivo* modelling with tumour transplants likely to maintain the genomic features of the parental tumour, as seen in PDX BC mouse models [183] (**Figure 5C**). Alternatively, as a way to further faithfully recapitulate human BCs and their interaction with the immune microenvironment, humanized xenograft models could be applied to not only test immunotherapeutic drugs, but also explore the immune cell infiltration status of HR^+ tumours [184]. Since most BCs present as immune 'cold' tumours, characterised by a lymphocyte-depleted milieu, humanized models could thus enable the investigation into the boosting of immune cell infiltration in the tumours, consequently leading to immunotherapy responsiveness [184,185].

Disadvantages

Despite being a superior species in terms of mammary gland architecture and oestrogen-dependent, HR^+ mammary tumour formation, rats, when immunocompromised, do not perform better than nude mice when undergoing tumour xeno-transplantation [173,174] (**Figure 5C**). Specifically, athymic mice have been shown to exhibit a higher tumour establishment success rate, which could be due to a number of reasons [186]. These include the antitumour immune-dependent changes in the rat as it ages, directly affecting tumour growth and transplantability, as well as the ability of regaining immunity with time, which could lead to tumour development cessation [174]. Another disadvantage of cell-line derived transplantation models concerns their adaptation to long term *in vitro* growth conditions. As a result, genetic aberrations and histopathological differences could arise between the original breast tumour and the derived human cell line, rendering it inaccurate in terms of modelling the human disease setting [183,187].

Regarding the PDX rat tumour systems, a major limitation of the model is the required use of immunodeficient rat strains, and thus lack of immune cells in the tumour microenvironment. This hinders the study of natural human tumour development and perpetuation in the *in vivo* system, and potential application of immunotherapy for HR^+ tumours [183,188]. Additionally, engrafted cancer cells in PDX models tend to undergo clonal evolution within the tumours, heterogeneity loss, and a possible selection bias for transplantation [189,190]. Humanized xenograft rat models, an improved alternative to the immunocompromised ones, could also display a number of drawbacks, including the limited development of mature immune cells and the onset of graft-versus-host-disease, which could be lethal to the recipient animal as observed in mice [191].

Hormone-induced rat mammary tumour models

Background

In addition to playing a pivotal role in the establishment and characterization of the carcinogen-induced rat mammary tumour model, Geschickter was the first to describe rat mammary carcinogenesis following hormonal treatment [192,193]. His work revealed that the injection of different oestrogenic substances, including oestrone (E1), oestradiol and diethylstilboestrol, yield cancer formation as early as 25 days post-dosage. Most importantly, Geschickter's study highlighted that oestrogen-induced rat mammary carcinomas are

a result of physiological changes triggered by the hormones, rather than their carcinogenic nature [192,193]. This is in line with epidemiological and experimental evidence on the role of hormonal imbalance in BC development [194]. Specifically, previous studies have demonstrated a link between higher BC incidence and prolonged exposure to oestrogen due to, for instance, late menopause, obesity and long-term hormone replacement therapy [195-197]. Such oestrogen-related breast tumour initiation and progression is dependent on both ER-dependent and –independent mechanisms, which are responsible for the expression of ER-responsive genes, cell proliferation, as well as the production of tumourigenic, DNA-damaging oestrogen metabolites [198].

Regarding its administration mode, and similarly to what has been described in Geschickter's protocol, oestrogen induced mammary tumour development in rats can be promoted via subcutaneous pellet, silastic implants, or repeated intramuscular injections of the chemical dissolved in oil [199-202]. Among the different oestrogen variations, the naturally occurring form, namely E2, is the most frequently applied, though diethylstilbestrol, oestrone and 17α-ethinyloestradiol have also been used [137,203]. In addition to oestrogen, other hormones, such as testosterone and progesterone, were shown to contribute to breast carcinogenesis, and have thus been used to model the disease *in vivo* [203,204]. Interestingly, the administration of repeated progesterone doses to SD rats previously treated with MCA or DMBA resulted in enhanced mammary tumour formation, suggesting a synergistic effect of the combination of the two chemicals [200,205,206]. Combinations of E2 with progesterone and testosterone resulted in higher mammary tumour incidence, in contrast to either hormone alone [203,207,208].

To obtain such results, two different susceptible strains have mainly been used, namely the Noble (Nb) and August–Copenhagen–Irish (ACI) rats, two well-known models for hormone-inducible rat mammary carcinomas [203]. First described in the early 1940's, by Noble *et al*., the Noble rat model enabled a better understanding of mammary tumour progression in the presence or absence of a hormonal stimulus [209,210]. Their work was also crucial for the examination of the molecular mechanisms underlying hormone-induced mammary tumourigenesis, highlighting the role of different oncogenes, such as *Ccnd1* and *IgF2*, in tumour progression [211]. Similarly, the ACI rat model, initially established in 1997, is another unique rodent model that gained notoriety for being able to display a 100% mammary tumour incidence in the presence of continuous E2 supplementation at physiological levels. These

are similar to those observed during the human periovulatory phase of the menstrual cycle or pregnancy, underlying the relevance of E2 levels for elevated disease incidence observed in the human setting [202,203].

Advantages

The establishment of the Noble and ACI rat hormone-induced mammary carcinoma models offers a number of advantages in the BC research field, including the study of early tumour formation following hormonal treatment. As shown by Mense *et al.* [198], susceptible ACI rats can display mammary hyperplastic lobular units within 7 days of E2 exposure, with subsequent hyperplasia and ductal elongation within 15 days of treatment. As hormonal administration continues, luminal epithelial proliferative responses are triggered, eventually leading to the formation of ductal carcinomas *in situ* and invasive mammary carcinomas [203]. Though with a longer latency time, a similar tumourigenesis trend can be seen in the Noble rat model described by Xie *et al.* [207], with carcinoma lesions being fully developed after 5 to 6 months post hormonal treatment onset. Most importantly, such hormone-induced mammary carcinomas have been shown to express PR, ERα, ERβ, as well as the GATA binding protein 3, a transcription factor involved in the mammary luminal epithelium development [129,203,212]. Such molecular features are also present in the human luminal BC, suggesting that rat mammary tumours induced in this model are not only hormone-sensitive, but also resemble the human setting [203] (**Figure 5D**).

In addition to the expression of luminal BC markers, hormone-induced rat mammary tumours display similar genetic alterations to the ones observed in the human disease [129]. Specifically, Li *et al.* [213] have indicated that E2-induced mammary tumours in ACI rats, similarly to invasive human ductal BCs, exhibit high degrees of c-MYC overexpression and amplification and genome instability. The latter comprises high levels of aneuploidy, accompanied by non-random gain or loss patterns of specific chromosomes, a characteristic seen in approximately 85% of BCs [203,214,215]. Moreover, multiple quantitative trait locus analysis of the ACI rats in comparison to non-susceptible strains underlined the existence of oestrogen-induced mammary cancer (*Emca*) loci, which foster genetic determinants of E2-induced mammary tumour susceptibility. Notably, such *Emca* loci are orthologous to the genetic determinants of BC risk in humans, as demonstrated by genome wide studies [216-218]. Altogether, these data highlight the genetic similarities between the

lesions in the different species, thus enabling potential functional studies on hormone-dependent BC risk and incidence [218].

Another advantage of hormone-induced rat mammary tumour models concerns the possibility of impelling tumour growth regression via hormone administration cessation or in the presence of selective ER modulator drugs, such as tamoxifen [137,214,219]. As demonstrated by Harvell *et al.* [214], and unlike other rat mammary tumour models, E2-induced tumours in ACI rats completely regress following E2 implant removal, indicating their dependency on exogenous E2. The same could be observed in the Noble and Collip's [210] study, which described tumour growth recession also in the absence of progesterone. Their experiments also demonstrated that the novel hormone-induced tumour can appear and grow in a continuous manner [210]. The *in vivo* visualization of hormone-dependent tumour growth was essential for the development of anti-oestrogen drugs, eventually leading to the production and optimization of Fulvestrant, the first selective ER down-regulator (SERD) currently used as standard-of-care for HR^+ BCs patients [220-222].

Disadvantages

Despite its many advantages for the study of HR^+ BC *in vivo*, the use of hormone-induced rat mammary tumour models also presents drawbacks. These include the possible formation of primary lesions in hormone-independent organs, and the influence of the animal's age on the tumour latency [129,137] (**Figure 5D**). With regards to the former, previous studies on the carcinogenic role of endogenous oestrogens E1 and E2 and their products in two different rodent species revealed that tumour induction also takes place in the kidney, where the genesis of human hormonal cancers do not typically occur [223-225]. Such an event could be explained by the presence of specific mutations that lead to abnormal cell proliferation and cancer formation. In the case of diethylstilboestrol, these mutations arise from the product of a complex interaction between the hormone's catechol quinones and DNA, rather than oestrogen-receptor mediated cell proliferation reactions [224-226]. Thus, hormone-induced rat mammary tumours might be derived from the genotoxic effects of E1/E2 quinone metabolites, previously shown to trigger mutations responsible for initiating various human cancers [227].

Concerning the role of the animal's age on mammary tumour development, Geschickter and Byrnes [228] have previously demonstrated that younger rats on oestrogen supplementation tend to present a longer tumour latency period,

in comparison to older animals. For instance, one-month old rats displayed a tumour latency of 42 weeks, whereas 20-month old animals exhibited a latency of 13 weeks, indicating a possible protective effect of younger age against oestrogen inducible mammary carcinogenesis [137,228]. Given that older female rats tend to develop spontaneous mammary tumours, with their incidence proportionally rising as age increases, tumour development initially considered to be hormone-induced, could instead be attributed to the age status of the animal [102,107]. Though this can be counteracted with earlier experimental time points, the period for tumour latency and maintenance of physiological hormone levels, when giving continuous hormonal supplementation, can be at cost.

Radiation-induced rat mammary tumour models

Background

Ionizing radiation exposure has long been established as one of the environmental, etiological factors of human BC, with lesions being documented following doses as low as 0.1 to 0.5 Sievert [229-232]. While most knowledge on the impact of radiation on BC incidence are derived from epidemiological data on atomic bomb survivors, and patients exposed to diagnostic or therapeutic radiation, *in vivo* experimental models played a major role in the better understanding of radiation-induced breast carcinogenesis [231,233,234]. In particular, radiation-induced rat mammary tumour models were crucial for the study of dose-response relationships and the molecular mechanisms underlying tumour formation in the mammary tissue [235,236]. Following the publication of the first radiation-induced rat mammary tumour model in 1953, in which tumour development was induced in X-ray irradiated Sprague-Dawley rats, several other strains, radioactive agents, dosing and exposure time points have been tested, as well as combinatory studies of radiation- and hormone- or chemical-induced mammary tumour carcinogenesis [232,233,237-239].

Concerning the different radioactive carcinogens used to induce mammary tumours, sparsely ionizing, including X and γ-ray, and densely ionizing, such as neutron and carbon ions, compounds have been used, though γ-radiation is the most prevalent type [240-244]. Rats can be exposed to such agents via a single radiation dose, systemically delivered to the entire body or, though less frequently performed, a local dose, administered to a specific body part [108,235].

Regardless of the radiological agent and administration mode, irradiated rats appear to undergo a similar somatic mutational reaction to the carcinogen, leading to the development of corresponding mammary tumour identities [241,244]. Such tumours have been shown to develop within approximately 140 days to up to one year succeeding radiation exposure, with neoplasm incidence being directly proportional to the dose given [137,245].

Such radiation-induced mammary carcinogenesis in the rat is a result of two main molecular events, namely DNA damage, through double stranded breaks, and the generation of reactive oxygen and nitrogen species, both triggered by DNA and protein oxidation [137,246]. DNA damage consequently leads to mutations, copy number losses, deletions, chromosomal amplification, and an overall increased genomic instability, making cells prone to tumourigenesis [137]. Specifically, Loree *et al.*'s [236] study on irradiated rat mammary tissue has indicated the presence of genome-wide hypomethylation, accompanied by the down-regulation of the expression of DNA methyltransferases. Furthermore, their work highlighted the presence of altered cellular proliferation, apoptosis, and pro-survival signalling levels post-irradiation, with expression of cyclins D1 and D2, two known carcinogenesis markers, being notably elevated [236].

Among the different rat models used to study these radiation-induced carcinogenic effects, previous studies have demonstrated tumourigenesis susceptibility in at least 4 different strains, namely the Sprague-Dawley (SD), Wistar-related, including Wistar Albino Glaxo and Lewis, Copenhagen, and Long-Evans (LE) rats [233,235,237,245,247,248]. In particular, SD rats demonstrate elevated sensitivity to radiation, also at low doses, linked to a high incidence of mammary neoplasms [247,249]. A similar tumour burden trend was also observed in the LE strain which, like the SD one, scored a 56% tumour formation rate, in comparison to 5% in the Wistar-Lewis [247]. On the other hand, Shellabarger [250] has shown that the Lewis strain yields mammary adenocarcinomas in a comparable rate to SD rats, while not displaying a mammary fibroadenoma response, making it a valuable model for the study of adenocarcinoma formation. Moreover, rats of the Fischer F344 strain, when previously implanted with oestrogen pellets, have been shown to respond to X-ray exposure, yielding mammary carcinomas at a high incidence [251,252].

Advantages

In addition to being a well-studied carcinogen that elevates the risk of BC formation in the human setting, ionizing radiation enables modelling of HR^+ adenocarcinomas in rats. These tumours tend to display similar genetic alterations to those previously reported in human BCs, thus representing a relevant model to the study of luminal BCs *in vivo* [246] (**Figure 5E**). Specifically, a study performed by Moriyama *et al*. [244] revealed that both neutron and γ-radiation exposure results in an increased incidence of luminal mammary adenocarcinomas in SD rats, positive for ER and/or PR, while negative for HER2 in comparison to the non-irradiated control. Their study has also proposed the presence of focal copy-number losses in certain genes, including the tumour suppressor gene *Cdkn2a*, as a signature of radiation-induced rat mammary tumours [244]. Moreover, radiation exposure is shown to target mammary cells within the TDLU, resulting in a number of cellular alterations up to 8 weeks post-irradiation [253]. Early changes included persistent proliferation of TDLU cells, followed by the development of preneoplastic lesions, resembling the oncogenic process seen in human BC [96,253].

Disadvantages

The main disadvantage of radiation-based treatments is the high incidence of benign lesion growth, such as adenomas and fibroadenomas and the long latency periods [232]. In addition, and especially at high radiation doses, the model is hindered by the development of late complications, including vascular injury, formation of fibrotic tissue, necrosis and atrophy. In BC patients, radiation cardiotoxicity is a common side effect, as well as the formation of secondary malignancies, such as acute leukaemia [254] (**Figure 5E**). In fact, the presence of radiation-induced myeloid leukaemia in laboratory mice is observed since the 1930's, with its incidence increasing with age at which radiation exposure occurs [255,256]. Though the same has not been documented in rat models thus far, Huggins and Fukunishi [257] observed the occurrence of osteosarcomas, as well as of mesentery and intramuscular sarcomas in SD rats post-irradiation. Such off-target carcinogenic effects could hinder proper mammary tumour disease modelling, as the animals' movement could become compromised at an early time point [258]. Furthermore, given that animals are typically exposed to whole-body irradiation, their overall wellness and natural behavioural patterns might be compromised, leading to ethically deviant experimentation settings [259].

Genetically engineered rat mammary tumour models

Background

Unlike mouse models, in which genetic strategies have been widely used for various genome manipulations and functional studies, genetically engineered rat models for BC are rather scarce [260]. Such genetically manipulated models can be classified based on the type of mutations being induced, namely somatic and germline gene mutations. The first somatically engineered rat mammary tumour model dates back to 1991, entailing the administration of transgenic constructs containing a v-HA-ras-expressing viral vector into the mammary ductal epithelia giving rise to the infusion gene transfer model [261]. This model's administration is equivalent to the mouse mammary intraductal (MIND) method, initially used to mimic progression of ductal carcinoma *in situ* lesions *in vivo*, using cell lines or patient-derived tissue [262,263]. Such a tool enables specific anatomical targeting of the retroviral constructs, which only incorporate into the genome of proliferating mammary ductal epithelial cells [260]. In addition to retroviruses, lentivirus and adeno-associated virus (AAV) vectors can also be used to deliver genetic content and trigger *in situ* genome editing in rats, as seen in mouse models [100,264].

With respect to the germline models, lentiviral transgenic technology in rats has also been used, as highlighted by Dann *et al*., to generate new models with a stable and inheritable phenotype following depletions in a specific gene function [265]. The method enables targeted *in vivo* gene knockdowns through RNAi, and the successful delivery of shRNA-based vectors by lentiviruses [265,266]. Prior to that, other efforts have been conducted, but were hampered by several hindrances. These included the low efficacy and embryo survival in rats following pronuclear microinjection of a transgene into a fertilized oocyte, a technique typically used in transgenic mouse line production, and the lack of easily maintained rat embryonic stem (ES) cells, which could be genetically manipulated prior to host implantation [100,260,267]. The latter, an issue subsequently amended by an improved rat ES cell derivation and expansion protocol, is particularly relevant for the field given the method's ability to generate knock-ins, and conditional and inducible knockouts [266].

Moreover, *in vitro* genetic manipulations of spermatogonial cells significantly improved with the advent of the CRISPR/Cas9 technology, leading to targeted germline mutations in rats, opening doors to novel rat knockout transgenic

models targeting different genes of interest [100,268,269]. The CRISPR-Cas9 genome editing system has reshaped the mouse cancer BC modelling field by enabling somatic indel manipulation of tumour suppressor genes, or missense mutations in proto-oncogenes [270,271]. Specifically for rat HR$^+$ mammary tumours, Dischinger *et al.* [272] have demonstrated that CRISPR-mediated germline knockout of *Nf1*, a regulatory gene in the RAS pathway linked to increased luminal BC risk, leads to oestrogen-dependent, ER$^+$ mammary tumours in SD rats [272,273]. Dischinger *et al.*'s model is the first to describe the successful generation of germline knockout models using a CRISPR/sgRNA design injected into the pronuclei of fertilized rat zygotes.

Though no BC rat models with both germline and somatic engineering conditions have been established to date, the generation of a Cas9-tolerant rat strain, characterised by a Cre-recombinase dependent, CAG-promoter driven expression of Cas9 in the *Rosa26* locus, could offer new opportunities in the genetically engineered rat modelling scene [269]. Specifically, the establishment of such a germline Cas9-tolerant rat model could be applied to somatically model and study the loss-of-function mutational domain, an endeavour that has yet to be reported in rat models. Along the same lines, the subsequent establishment of a Cre-rat resource, which includes 10 tissue-specific, inducible Cre-rat lines, deploying the Cre-ERT2/loxP system, could enable the further exploitation of the species for the study of HR$^+$ BC [274].

Advantages

Genetically engineered rat mammary tumour models, though still limited in numbers, present a number of advantages for the HR$^+$ BC research field. These include the possibility of directly targeting the ductal structures of the rat mammary gland via, for instance, somatic engineering through the MIND methodology, and thus only genetically modifying the epithelial cells from which BC arise, and the ability to control timing of tumour initiation [261,262,275,276] (**Figure 5F**). The latter is especially relevant for viral-based genetic engineering systems, which could also present unique features, such as long-term and stable transgene expression, low immunogenicity and the ability to sustain inserts of up to 3500 bp [277,278]. Moreover, given that the spread of these viral infections is hampered by a defect in virus replication and the lack of a helper virus, it is possible to regulate the frequency of modified cells and viral integration through virus titration or hormonal stimulation of the mammary gland, respectively [261].

Gene editing employing homologous recombination in rat derived ES cells could also play an important role in the BC rat modelling field, as previous studies have revealed the method's suitability in achieving precise genetic modifications, including gene replacements and chromosomal rearrangements, which can lead to novel knockout model via germline transmission [266,279]. Such gene knockouts could also be induced or conditioned when combined with Cre/loxP systems, thus enabling temporal control and tissue targeted alterations in rat tumour suppressor genes [279]. Additionally, the combination of ES cells and Cas9-mediated gene editing has been shown to be a highly efficient genetic tool for the generation of compound gene mutant models [280]. This could be achieved by targeting various genes in one rat embryo via one RNA microinjection, as demonstrated by the authors. Though not yet applied for HR^+ BC rat modelling specifically, further optimization of such methods in the rat genome could provide a novel and potent platform for the study of human disease [279].

Moreover, genetically engineered models enable the targeted disruption of genes, and thus the possibility of recapitulating the disease genetic loci, and induction of specific overexpression, knockout or mutations in cells and tissues of interest [281-284]. As shown in Cas9-based base editor (BE) and prime editor (PE) mouse models, precise gene edits can also be achieved through somatic engineering [283-285]. Most importantly, and in light of the advent of immunotherapy, genetically engineered models are immunocompetent, thus making them valuable resources to the study of novel immunotherapeutic approaches, as well as the effects of certain genetic modifications on the tumour microenvironment [267].

Disadvantages

Despite its many advantages and possibilities for the BC *in vivo* modelling field, such rat genetically engineered models also exhibit limitations. For instance, viral-based methods are often limited by the vector packaging capacity, the inability to modify gene sequences, and the difficulty in attaining full gene ablation [265,266,286]. One of the major disadvantages of ES cell-mediated gene targeting is the establishment of germline-competent rat ES cell lines, as cell line injections into recipient blastocysts are required to generate chimeric animals. These are then extensively bred with the purpose of producing offspring with the manipulated ES cell genetic make-up. As a result, the method is considered to be laborious, expensive and time consuming [287] (**Figure 5F**). In the case of CRISPR-Cas9 gene editing systems,

off-target mutation edits could take place, as previously seen in cell lines and mouse models [283,288]. This could hinder the establishment of somatic modelling in rats though, given the limited number of research in the field, is yet to be seen.

Applications

The wide spectrum of rat BC models has been crucial for understanding BC initiation, progression, metastatic disease, has shed light on risk factors, and has enabled testing of various pharmacological compounds including hormonal therapies. The development of genetically engineered rat models will enable tailoring therapies to patient groups with specific mutational signatures, moving towards more personalized treatment avenues (**Figure 5A-F**).

Resource generation

Spontaneous mammary tumour models, given their naturally occurring incidence, have limited research applications but have been pivotal for developing rat tumour cell lines and studying the genetic and hormonal components of rat mammary carcinogenesis [102,103,248] (**Figure 5A**). Notably, carcinoma cell lines derived from spontaneous tumours were characterised as being ER^+ and oestrogen-dependent [102], demonstrating transplantability and retention of parental tumour growth rates and histological features upon engraftment into immunodeficient mice [23]. Following those pioneering studies, the advancement towards induced BC models has enabled further insights into BC risk factors, mechanisms of progression, and potential treatment opportunities.

Elucidating breast cancer risk factors

Risk factors of cancer progression have mainly been studied using rat models relying on carcinogens, hormones or radiation. DMBA– and NMU-induced rat mammary tumour models have significantly contributed to understanding tumour-modulating environmental factors [289-296] (**Figure 5B**). A carcinogen-induced model was also successfully deployed to show that weight gain prevention after menopause reduces the risk of obesity-associated tumour development [297]. Also the hormone-induced rat mammary cancer model has been instrumental in diet-gene interaction studies, pharmacological research on endocrine therapies, as well as the exploration of food antioxidants

[219,298,299]. For example, the hormone-induced ACI rat model has been crucial in investigating energy restriction diets and the effects of vitamin E supplementation on preventing and managing E2-induced mammary lesions [299,300]. Along those lines, studies employing radiation-induced BC models highlighted that parity and age of radiation exposure [301], and elevated insulin and leptin levels, leading to increased energy availability, promote mammary tumour development [302]. Furthermore, the ACI rat model allowed for evaluating the effects of tamoxifen on E2-metabolism mediated ROS production and DNA damage [219,303] (**Figure 5D**). Peterson *et al.* [304] using a radiation-induced BC model, found that rat carcinomas driven by the Her2/Neu pathway are more prone to tamoxifen chemoprevention failure, demonstrating a need for alternative therapeutic strategies.

Breast cancer initiation and progression

Mechanisms of cancer initiation have first been studied using the carcinogen NMU, offering initial insights into disease progression and the transition from *in situ* to invasive disease [136,137]. Following this, syngeneic animals generated from this carcinogen-induced model have shown to produce highly metastatic BCs, mimicking the human scenario and allowing the study of metastasis, a phenomenon rarely captured by other models [104,168,182]. In addition to Gullino *et al.*'s [147] observation of metastatic lesions to the bone marrow and spleens of rats with NMU-induced mammary tumours, lung and lymph node metastasis have been reported in oestrogen-induced mammary tumour models [193] and in transplantation models [104], highlighting the value of rat BC models for the study of metastatic disease (**Figure 5B-D**).

The advent of immunodeficient rat strains [175], together with naturally higher oestrogen levels in rats, presents an opportunity to establish PDX biobanks as previously done in mice, with the advantage to model ER^+ BC without exogenous hormone supplementation (**Figure 5C**). Genetic engineering has further paved the way for models recapitulating specific molecular BC subtypes and mutational signatures and for studying the impact of specific germline or somatic mutations in tumour suppressor genes or oncogenes associated with BC [264,272,305,306], such as the creation of rats with germline *Nf1* mutations using CRISPR/Cas9 gene editing [272] (**Figure 5F**).

Development and testing of breast cancer treatment strategies

Historically, clinical BC therapies have been developed using some of the earliest established rat models. DMBA-induced tumour models have facilitated pharmacological studies testing a range of drugs, such as letrozole, palbocyclib, lapatinib, tenofovir alone or in combination with doxorubicin, and sodium channel inhibitors [158,159,307-309]. Furthermore, NMU-induced models have been used to study BC prevention [310] as well as for nutritional and pharmacological studies focusing on hormone-related effects [311-313] and as a preclinical validation system for immunotherapy responses [101]. Importantly, allografting NMU-induced tumour pieces from inbred rats, such as Fischer 344, into syngeneic recipients has expanded the use of these models to test new anti-metastatic agents [104,168,182] (**Figure 5B**).

Tumour immunology studies

Whilst the rat immune system remains to be fully elucidated, it bears striking similarities with the human counterpart [314]. Rats may therefore present an opportunity to disentangle tumour-immune interactions and uncover immunotherapy treatment avenues in HR^+ BC. In fact, several studies have broken ground to combine rat modelling and tumour immunology. Rat mammary adenocarcinoma cell lines derived from carcinogen-induced tumours from inbred Fischer 344 rats were used to study the role of the immune system in tumour development and progression in rats [315-317]. These studies elucidated the natural killer (NK) cell activity against the MADB106 rat adenocarcinoma cell line transplanted in syngeneic rats, highlighting the tumouricidal interaction between NK cells and tumour cells. More recently, a study by Gil Del Alcazar *et al.* [101] has shed light on the role of the mammary tumour microenvironment in immune escape and responsiveness to immunotherapy using the NMU-induced rat mammary tumour model. These NMU-induced tumours displayed an evolution pattern within their microenvironment similar to the immune selection and editing that occurs in human cancers, rendering the model a useful platform to study tumour-immune interactions *in vivo* [101] (**Figure 5B**).

Discussion & future perspectives

Animal models have been shown to play a major role in the biological understanding of BC, enabling disease monitoring and the study of cancer initiation and progression *in vivo* [24]. In the context of HR$^+$ BC, the similarities between rat and human mammary tumourigenesis have made these rodents a promising species to model the disease, overcoming some of the challenges of modelling such tumours in mice [43,137]. Particularly, in contrast to mice, rats display TDLU-like structures as they are found in human breast anatomy, recapitulating the architectural makeup of the human compartment most commonly originating breast malignancies [87,88,96]. In addition to yielding tumours of ductal origin, rat models have been shown to develop HR$^+$ and oestrogen-dependent mammary tumours, with similar histopathological and hyperplastic characteristics to the human lesions [99,109]. Such features make rats useful models with potential for even greater utility to study HR$^+$ BC *in vivo*, as demonstrated by several modelling efforts and techniques reported to date.

Based on the tumour induction method, six distinct categories of rat mammary tumour models have been established to date, namely the carcinogen-induced, transplantation, hormone-induced, radiation-induced, genetically engineered, and spontaneous models. Though each model is based on distinct induction agents and carcinogenesis mechanisms, they have all been shown to induce HR$^+$ mammary tumours [124,147,181,203,244,272]. However, given that both spontaneous and carcinogen-induced rat mammary tumours carry genetic mutations that are rarely seen in human BCs, such as *Hras* mutations, the other modelling strategies could be superior at recapitulating the disease [131,132]. For instance, somatic mutations in radiation-induced tumours were found in signalling pathways also relevant to human breast cancer [242,244] whilst *Hras* and *Tp53* mutations were lacking [241]. Patient-derived tumours xenografted in immunodeficient mice have been shown to maintain not only the genetic features of the parental tumour, but also histopathological, epigenetic and transcriptomic characteristics [183]. Above all, the use of genetically engineered models enables precise, tissue-specific edits of (combinations of) driver genes of interest, thus ensuring an accurate genetic recapitulation of the human disease [21,281,282].

In addition to achieving a similar BC genetic makeup in the rat mammary tumours, other BC features could be recapitulated *in vivo* by combining the different models and exploiting their synergistic effects. Such experimental practice has previously been explored by Segaloff and Maxfield [318] and Broerse *et al.* [233], who investigated the combined effect of irradiation and oestrogen supplementation on rat mammary tumourigenesis. In both studies, the additive effect of radiation and hormones was demonstrated, as seen by an increase in mammary carcinoma incidence in the presence of both agents [233,318]. Furthermore, in the context of genetically modified models, previous studies in mice have highlighted a potential synergism between genetic engineering methods and induced hormonal disturbances, such as exogenous oestrogen supplementation and ovariectomy [319,320]. In particular, Dabrosin *et al.* reported an enhanced tumour growth rate in genetically engineered animals allografted with tumour cells and supplemented with oestradiol [319]. Though performed in mouse models, a similar approach could be employed in rats with the goal of establishing the ideal *in vivo* combinatorial conditions to recapitulate the human disease.

Though these models are promising tools to study and more reliably recapitulate HR^+ BC development and progression *in vivo*, they also display several limitations. As with most animal experimental models, animal welfare, ethical and legal concerns must be taken into account, as well as the need for skilled personnel, and adequate housing and husbandry conditions [321,322]. Moreover, *in vivo* experiments are often labor-intensive, cost-inefficient and time- consuming, resulting in models with long tumour latencies. In the case of rats, another drawback concerns the limited availability of resources and tools that can be applied to the species. For instance, while over 120,000 disease-related functional annotations have been made on human and mouse genes, less than 11,000 annotations can be found for rat genes [323]. Similarly, recent pioneering efforts into annotating the rat transcriptome have yet to achieve the unmet level of detail inherent to mouse and human transcriptomic atlases [324-328]. Along these lines, even though the concept of intrinsic molecular subtypes has revolutionized the clinical understanding and management of BC, rat mammary tumours are thus far almost always simply described as HR-positive or -negative without mentioning their relevance to the intrinsic subtype. It would be of great importance to place the historically generated rat models in the context of these intrinsic subtypes to better stratify model utility and applicability.

Notwithstanding these limitations, rat models are valuable research tools for studying human malignancies, including HR$^+$ BCs, and their further exploitation will offer exciting opportunities for the disease modelling field. In addition to the rat mammary tumour models described thus far, novel modelling strategies to be explored include the engraftment of rat-derived tumour organoids into syngeneic rats, and the use of prime editing technology. Concerning the former, and though allograft models have previously been established using tumour pieces or cell lines, the development of rat HR$^+$ organoid lines and their subsequent engraftment into syngeneic rats is still to be reported. Given that organoids can undergo genetic manipulation, including the introduction of reporter constructs and targeted genetic alterations, as well as fast expansion for drug screening prior to *in vivo* experimentation, their successful transplantation into rats could provide a new platform to model the disease. Due to the drastically divergent resources needed for experimental manipulation of *in vitro* and *in vivo* settings, organoid allograft modelling could represent a time- and cost-efficient alternative to studies entirely-executed *in vivo* [190].

Future advances in genetic modelling in rats could also be achieved through the use of prime editing tools, such as the recently-developed precise genome editing methods enabling both germline and somatic manipulation of cancer driver genes of interest with limited off-target effects [285,329]. As seen in mouse models, prime editing enables successful introduction of a broad spectrum of cancer-associated mutations, including transversions, multi-nucleotide substitutions and deletions with over 90% editing purity.

In conclusion, rat models display a number of advantages for the study of human HR$^+$ BCs, including the biological similarities to the human breast, the histopathological and morphological features displayed by the rat tumours, their ductal origin, and hormone dependency status [96,99,109,146,330]. The different rat models established to date display unique advantages and disadvantages, and enable a broad spectrum of different research applications. To further exploit the potential of rats in modelling HR$^+$ BC, the different models and tumour induction methods could be employed in combination, with ample opportunity to propel pre-clinical HR$^+$ BC research.

Contributions

R.N. and C.L. designed and wrote the manuscript under the guidance of H.A.M. and J.J. The literature search for this article was performed by R.N. and C.L. with the help of the Scientific Information Service of the Netherlands Cancer Institute. All authors critically revised and approved the final version of the manuscript.

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Competing interests

The authors have no competing interests to declare.

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