A preclinical mouse model of invasive lobular breast cancer metastasis

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Abstract

Metastatic disease accounts for over 90% of cancer-related deaths, but the development of effective anti-metastatic agents has been hampered by the paucity of clinically relevant preclinical models of human metastatic disease. Here we report the development of a mouse model of spontaneous breast cancer metastasis which recapitulates key events in its formation and clinical course. Specifically, using the conditional \textit{K14cre;Cdh1^{1/2};Trp53^{1/2}} model of \textit{de novo} mammary tumor formation, we orthotopically transplanted invasive lobular carcinoma (mILC) fragments into mammary glands of wild-type syngeneic hosts. Once primary tumors were established in recipient mice, we mimicked the clinical course of treatment by performing a mastectomy. After surgery, recipient mice succumbed to widespread overt metastatic disease in lymph nodes, lungs and gastrointestinal tract. Genomic profiling of paired mammary tumors and distant metastases showed that our model provides a unique tool to further explore the biology of metastatic disease. Neoadjuvant and adjuvant intervention studies using standard-of-care chemotherapeutics demonstrated the value of this model in determining therapeutic agents that can target early and late-stage metastatic disease. In obtaining a more accurate preclinical model of metastatic lobular breast cancer, our work offers advances supporting the development of more effective treatment strategies for metastatic disease.
Introduction

Metastasis formation is a complex and dynamic process in which cancer cells escape the primary tumor and disseminate to secondary organs by successfully advancing through a sequence of several steps. After initial invasion of the extracellular matrix, cancer cells intravasate into blood and lymphatic vasculature, survive during transit and extravasate to colonize distant organs (1–3). Despite recent advances, many of the mechanisms by which cancer cells acquire the ability to overcome each of these successive barriers remain poorly understood. Furthermore, a growing body of evidence indicates that metastasis formation is influenced by a continuous cross-talk between cancer cells and their stromal environment (4). For example, organ-specific patterns of metastatic spread observed in distinct (sub)types of cancer strongly suggest that host factors play a critical role in the dissemination of cancer cells (5). This notion is further supported by the observation of chemokine-mediated trafficking of circulating tumor cells to distant sites (6). Recent studies also suggest that tumor-derived factors can facilitate metastatic colonization by recruiting bone marrow-derived hematopoietic progenitor cells to secondary sites, where these cells prime their environment to form a more hospitable and survival-permissive pre-metastatic niche (7–9).

To study metastasis formation in vivo, several mouse models of metastatic disease have been developed. Unfortunately, most of the currently available models only partially reflect the metastatic cascade. For example, experimental metastasis models based on intravenous injection of cancer cells do not recapitulate tumor cell invasion and intravasation, but only reflect homing of circulating tumor cells to an often limited set of secondary organs (10; 11). These issues are partially resolved in syngeneic or xenograft tumor transplantation models in which tumor cells derived from an established cancer cell line are transplanted subcutaneously or orthotopically into recipient mice. Xenograft metastasis models which carefully reflect cancer-cell intrinsic traits of parental human carcinomas are easily manipulated for mechanistic studies and have been particularly useful to evaluate therapeutic compounds targeting metastatic disease (12). However, in vitro maintained cancer cell lines fail to retain the cellular heterogeneity originally found in the parental tumor (13). Therefore, phenotypic variations in metastatic capacity that are present in spontaneous tumors are generally not recapitulated in cancer cell line-based metastasis models. Furthermore, xenograft metastasis models cannot be used to study the role of the adaptive immune system in disease progression and metastasis formation.

A third alternative to study metastasis formation in vivo is the employment of mouse models of de novo tumorigenesis. Utilizing these spontaneous mouse models to study metastatic dissemination offers several advantages over the previously described experimental systems (11). First, tumors derived from genetically engineered mouse (GEM) models often closely recapitulate...
the histopathological characteristics observed in human cancer. Furthermore, tissue-specific induction of mutations gives rise to orthotopic tumors in the context of a functional, immune competent microenvironment, thus recapitulating the cross-talk between an emerging tumor and its surroundings. Consequently, mouse models of de novo tumorigenesis are useful to study early stages of metastatic spread and to explore the role of the stromal microenvironment in disease progression. Nonetheless, studying advanced metastatic disease in GEM models is often hampered by the relatively low incidence of metastatic disease. Even if metastatic dissemination occurs, most animals will – unlike in human cancer – die from rapidly growing primary tumors that do not allow sufficient time for the emergence of advanced, clinically overt metastatic disease. Though these issues could be resolved by surgical resection of the primary tumor, this often proves unpractical as most animals develop multiple, asynchronously arising primary tumors (12).

To address this caveat, we set out to develop a novel, preclinical mouse model of spontaneous breast cancer metastasis by exploiting the well-characterized conditional K14cre;CdhlF/F;Trp53F/F mouse model of de novo mammary tumor formation (14). Our main aim was to design a clinically relevant mouse model that recapitulates invasive lobular breast cancer metastasis in humans and represents all major events of the metastatic cascade. In addition, metastatic disease should develop spontaneously in a variety of biologically relevant organs, at a high penetrance and within a reasonable, predictable time frame rendering it a suitable preclinical mouse model to study the biology of metastatic disease as well as to test novel therapeutic agents targeting metastatic disease.
Materials and Methods

Mice
The generation and characterization of K14cre;CdhlF/F;Trp53F/F mice – back-crossed onto the FVB/N background for this study – has previously been described in detail (14). Genotyping was performed by PCR analysis on tail tip DNA as described previously (14). Female FBV/N mice (aged 10-12 weeks) were bred at and obtained from the laboratory animal facility at the Netherlands Cancer Institute. Mice were kept in individually ventilated (intervention studies) and open cages (all other experiments) and food and water were provided ad libitum. Mouse handling and animal experimental procedures were approved by the institute’s Animal Ethics Committee and were performed in accordance with institutional guidelines and national ethical regulations.

Isolation of mammary donor tumors
In K14cre;CdhlF/F;Trp53F/F females, the onset of mammary tumor formation was monitored twice weekly by palpation starting at 4 months of age. Mammary tumor growth was measured using calipers. Once mammary tumors reached a size of ~10x10 mm, tumors were harvested and cut in small pieces (diameter ~1mm) while submerged in ice-cold PBS. Tumor fragments were collected by centrifugation at 1200 rpm for 5 min, resuspended in DMEM F12 containing 30% fetal calf serum and 10% dimethyl sulfoxide and stored at -150 °C till further use.

Orthotopic tumor transplantations
Based on immunohistochemical stainings, three K14cre;CdhlF/F;Trp53F/F derived mouse invasive lobular carcinomas (mILCs) – characterized by high cytokeratin 8 and absence of vimentin and E-cadherin expression – isolated from three independent mice were selected and used as donor tumors. Small tumor fragments (~ 1 mm in diameter) from these donor mILCs were orthotopically transplanted into the mammary fat pad of 10 week old wild-type syngeneic female recipients as described previously (15). Briefly, recipient animals were anesthetized by injecting a 7 ml/kg bolus of a 1:1:2 mixture of Hypnorm (Janssen Pharmaceutica, Goirle, The Netherlands) : Dormicum (Roche) : ddH2O intraperitoneally. After shaving and disinfection, a midline abdominal incision of 1cm was made at the level of the 4th nipple and a small pocket was created by puncturing the mammary fat pad using watchmaker’s forceps. A tumor fragment was inserted distal to the local lymph node, the mammary gland was repositioned, skin was stitched and buprenorfine 100 μg/kg was administered subcutaneously for postoperative pain relief.

Surgical resection of mammary tumors
The first occasion at which a tumor mass of ~ 2x2 mm was identified, was defined as the time of diagnosis. Tumor growth was measured twice weekly using calipers. Once recipient mammary tumors reached a size of ~ 15x15 mm, a mastectomy was performed. After induction of anesthesia and disinfection, a 2cm midline abdominal skin incision was made and tumor-supplying arteries were located and ligated. The mammary tumor including adjacent 4th and 5th mammary glands were separated from adherent tissues using forceps and soaked cotton swabs and the mammary tumor was excised and stored for further analysis. The skin was closed using stitches and buprenorphine 100 μg/kg was given for postoperative analgesia.

**Monitoring of metastatic disease**
Following mastectomy, all mice were monitored for disease progression and metastasis formation by palpation and daily observation of their physical health, appearance and behavior. Recipient animals were sacrificed when they developed clinical signs of distress caused by metastatic disease – i.e. respiratory distress (labored breathing as a result of lung metastases and pleural effusion leading to a reduced respiratory capacity), ascites, distended abdomen, rapid weight gain and severe anemia (associated with liver metastases) and palpable metastatic lesions in lymphoid organs – or suffered from locally relapsing tumors that reached a size of ~ 15x15 mm. Brain, lungs, liver, spleen, intestines, mesenterium, kidneys, adrenal glands, ovaries, uterus, mammary glands, left femur, sternum and tumor-draining and distant lymph nodes (mesenteric, renal & caudal) were collected and analyzed microscopically for the presence of metastatic foci. Macroscopically overt metastases were collected separately for further analysis.

**Histopathological and genomic characterization of mammary tumors and metastases**
Mammary tumors and metastases were characterized by histopathological, immunohistochemical and array comparative genomic hybridization (aCGH) analyses. Detailed methods are described in supplementary material.

**Neo-adjuvant and adjuvant chemotherapy treatments**
To study chemotherapy responses in mammary tumors and distant metastases, we generated a cohort of recipient mice transplanted with the same donor tumor (donor tumor 1). Tumor-bearing recipients were assigned to adjuvant or neo-adjuvant treatments with PBS (control), doxorubicin (5mg.kg⁻¹) or docetaxel (22mg.kg⁻¹) IV (tail vein injection) at maximum tolerable doses. Adjuvant and neo-adjuvant treatments were administered once weekly for a fixed period of 4 weeks. Neo-adjuvant intervention studies were initiated as soon as mammary tumors reached a size of 5x5 mm.
Following mastectomy at a tumor size of ~15x15mm, neo-adjuvant treated mice were monitored for disease progression as described previously. Adjuvant treated recipients underwent a mastectomy once the mammary tumor reached a size of 15x15 mm. Adjuvant treatments were initiated three days after mastectomy according to the same treatment schedule. Therapeutic profiles of mammary tumors and distant metastases were studied using mammary tumor growth (neo-adjuvant setting only) and metastasis-specific survival (both settings) as primary endpoints.

Statistical analysis
Array CGH data analyses were performed in R using the comparative module of the Kcsmart (16; 17) as implemented in the Bioconductor toolbox (version 2.8). All other data analyses were performed in GraphPad Prism version 5.01 (GraphPad Software Inc, La Jolla, CA, USA). Applied analyses are indicated in corresponding legends when appropriate.
Results

Transplantation of spontaneous K14cre;CdhlF/F;Trp53F/F derived mILCs results in outgrowth of clonally-related and phenotypically similar recipient mammary tumors

To develop a novel, preclinical mouse model of spontaneous breast cancer metastasis formation, we utilized the conditional K14cre;CdhlF/F;Trp53F/F mouse model of de novo mammary tumor formation previously described by Derksen et al (14). K14cre;CdhlF/F;Trp53F/F females spontaneously develop pleomorphic mouse invasive lobular carcinomas (mILCs) based on stochastic loss of E-cadherin and p53 in mammary epithelium. These spontaneous mILCs resemble human invasive lobular carcinomas with respect to their histopathological features as well as in their metastatic behavior(14; 18). Nonetheless, K14cre;CdhlF/F;Trp53F/F mice do not succumb to clinically overt metastatic disease, but die due to independent, asynchronously arising and rapidly growing primary tumors, thus hampering in depth analyses of metastatic disease in this spontaneous mouse model (12). To circumvent these limitations, we orthotopically transplanted small tumor fragments from three independent, spontaneous K14cre;CdhlF/F;Trp53F/F derived mILCs into mammary glands of wild-type syngeneic hosts (Fig. 1A&B). To prolong survival and allow sufficient time for disseminated cancer cells to establish metastases, we mimicked the clinical setting and surgically resected recipient mammary tumors that reached a size of ~ 15x15 mm (Fig. 1A&C). Following mastectomy, we closely monitored recipient mice for clinical signs and symptoms of metastatic disease (Fig. 1A).

To first explore whether recipient mammary tumors were phenotypically similar to their parental tumor, we characterized donor and recipient mammary tumors by means of morphological, immunohistochemical and array comparative genomic hybridization (aCGH) studies. Mammary donor tumors were morphologically classified as solid, moderately invasive, pleomorphic mILCs and uniformly expressed cytokeratin 8 (CK8), but did not express vimentin nor E-cadherin (Fig. 2A, upper panel). Consistent with these observations, recipient mammary tumors derived from donor tumor 1 were mostly classified as solid, pleomorphic mILCs and stained positive for CK8 while negative for vimentin and E-cadherin (Fig. 2A, middle panel and fig. S1A). The majority of recipient tumors derived from donor mILCs 2 and 3 displayed a more heterogeneous, biphasic morphology (Fig. 2A, lower panel and Fig. S1A). Though typical epithelial regions were still present in these tumors, areas with a mesenchymal or spindle-like cell morphology characterized by pleomorphic nuclei with densely packed chromatin and a small cytoplasm were also observed (Fig. 2A, lower panel). These findings were further confirmed by immunohistochemistry, which revealed sharply delineated regions of CK8+/vimentin- and CK8- /vimentin+ fields indicating a mixed composition of epithelial and mesenchymal-like components.
within recipient outgrowths (Fig. 2A, lower panel). The sharply delineated epithelial and mesenchymal-like areas suggest that these tumor cells originated from different, independent subclones of cancer cells that were present in the heterogeneous parental tumor. Like spontaneous donor tumors, recipient outgrowths were heavily infiltrated by T lymphocytes and macrophages (Fig. S1b) which have been shown to play a prominent role in breast cancer metastasis (19–21). Together, these findings indicate that transplanted K14cre;CdhlF/F;Trp53F/F derived mILC fragments give rise to recipient mammary tumors that closely resemble the histopathological characteristics of the pleiomorphic parental tumor.

To examine the genomic relationship between donor and recipient mammary tumors, we performed aCGH on recipient mammary tumors and their corresponding parental tumor. Genomic profiles of de novo K14cre;CdhlF/F;Trp53F/F donor tumors were highly conserved in transplanted recipient outgrowths (Fig. S2). Consistent with these observations, genomic profiles of recipient mammary tumors clustered according to their parental tumor (Fig. 2B). Together, these data indicate that transplantation of spontaneous K14cre;CdhlF/F;Trp53F/F derived mILCs leads to reconstitution of clonally-related recipient mammary tumors that conserve the genomic profile of the parental tumor.

**Surgical resection of mammary tumors results in widespread clinically overt metastatic disease in recipient mice**

To examine whether transplanted recipient mILCs maintain their capacity to disseminate and establish spontaneous metastases, we surgically resected recipient mammary tumors at a size of ~15x15 mm (Fig. 1A). Following mastectomy, 32/44 recipient mice succumbed to clinically overt metastatic disease in lungs (respiratory distress), liver (severe anemia, ascites accompanied by weight gain and a distended abdomen), spleen (palpable tumor mass) and/or tumor-draining or distant lymph nodes (tumor mass reaching a size of ~15x15 mm) (Fig. 3A). In addition, 12/44 recipient mice died due to locally relapsing tumors (Fig. 3A).

To further assess the extent and distribution of metastatic spread in our model, we microscopically analyzed organs isolated from recipient mice for the presence of metastatic foci. In 40/44 recipient mice, we observed metastatic foci in at least one organ. In 30/44 recipients, two or more organs were affected by metastatic disease (Fig. 3B). Consistent with our clinical findings, metastases were predominantly observed in lungs and tumor-draining lymph nodes, though liver, spleen and distant lymph nodes were also frequently affected (Table 1 and Fig. 3C). Furthermore, metastatic lesions were also observed in pancreas, mesenterium and peritoneum. This pattern of metastatic spread strongly correlates with the spectrum of organs affected in human ILC, as human...
ILCs are prone to metastasize to gastro-intestinal tract, ovaries and peritoneum (18). Together, these data show that recipient mILCs vigorously metastasize leading to widespread, clinically overt metastatic disease in a variety of organs.

**Metastatic dissemination occurs spontaneously and is not instigated by surgical manipulation of the primary tumor**

We aimed for a model in which metastatic dissemination occurs spontaneously. Yet, we could not exclude the possibility that metastatic disease in our model was inadvertently initiated by shedding cancer cells during surgical manipulation of the primary tumor. We reasoned that if metastatic dissemination was exclusively initiated by surgery-induced shedding of cancer cells, the occurrence of metastatic disease would be determined by the time of mastectomy. As a consequence, metastasis-specific survival after surgery would be similar for mice that undergo surgery at different time points in tumor development. Furthermore, surgery-induced shedding of cancer cells would be independent of the size of the resected primary tumor. To test these hypotheses, we performed a mastectomy at different time points during tumor development and surgically resected recipient tumors that reached a size of 5x5, 10x10 or 15x15 mm (Fig. 4A). Surgical resection of mammary tumors at a size of ≥10x10 mm led to metastatic disease in all animals, whereas mastectomy at a tumor size of 5x5 mm led to metastatic disease in only 55% of the animals (Fig. 4B). Interestingly, irrespective of the size of a resected tumor and the time of surgery, the interval between diagnosis of the primary tumor and the occurrence of clinically overt metastatic disease remained similar for mice that succumbed to metastatic disease (Fig. 4B). These data suggest that metastatic dissemination occurs around the time that a primary tumor reaches a size of ~5x5mm. To ensure that metastatic dissemination was not inadvertently initiated by shedding cancer cells during surgery, we reanalyzed these data and focused on the interval between surgery and the occurrence of metastatic disease. Metastasis-specific survival after surgery was inversely related to the time of surgery and the size of a resected tumor (Fig. 4C). Thus, these data suggest that metastatic dissemination in our model occurs spontaneously and is not initiated by surgery-induced shedding of cancer cells. However, these data do not exclude the possibility that surgical manipulation of the primary tumor contributes to metastatic dissemination of cancer cells.

**Metastatic foci in distant organs strongly resemble histopathological and genomic characteristics of the parental tumor**
To explore the relationship between recipient mammary tumors and their distant metastases, we characterized metastases by morphological, immunohistochemical and aCGH analyses and compared them to the parental recipient tumor. Metastatic foci were morphologically similar to epithelial regions within the corresponding recipient mammary tumor and expressed CK8, but not vimentin nor E-cadherin (Fig. 5A). These findings suggest that metastatic foci are either exclusively seeded by epithelial-like cancer cells or that both epithelial and mesenchymal-like cancer cells metastasize and eventually remain or transform to epithelial cells by a process known as mesenchymal-to-epithelial transition. Similar to parental recipient tumors, metastatic foci also showed abundant immune cell infiltrations (Fig. 5B).

To investigate the genomic relationship between recipient mammary tumors and their metastases, we performed aCGH and analyzed genomic profiles of paired primary tumors and distant metastases (Fig. S3). Unsupervised hierarchical clustering of genomic profiles revealed that local tumors and their distant metastases cluster according to the parental donor tumor (Fig. 5C). Within these clusters, neither recipient mammary tumors and their corresponding metastases nor site-specific lesions (i.e. mammary tumors, lymph node and lung metastases) could be separated (Fig. 5C). Thus, these data show that genomic profiles of clonally-related recipient tumors are highly conserved in regional and distant metastases and that few genomic alterations occur during transition from a primary tumor to a distant site. To more thoroughly examine potential site-specific alterations, we constructed so-called ‘delta-profiles’ and calculated the difference between the genomic profile of a recipient mammary tumor and its paired lymph node- or lung metastasis. Though we detected some differences, we did not observe recurrent site-specific alterations in genomic profiles of lymph node or lung metastases (Fig S4). Thus, these data show that recipient mammary tumors and distant metastases exhibit similar genomic profiles and that if copy number changes occurred, they did not recur in independent samples.

**Mammary tumors and distant metastases exhibit similar therapeutic profiles upon (neo-)adjuvant treatment with standard-of-care chemotherapeutics**

To study chemotherapy responses of clonally related mammary tumors and distant microscopic metastases, we generated a cohort of recipient mice transplanted with the same donor tumor. Tumor-bearing recipients were then assigned to adjuvant or neo-adjuvant treatments with PBS (control), doxorubicin or docetaxel. In both settings, treatments were administered once weekly for a fixed period of 4 weeks (Fig. 6A). Neo-adjuvant treatments initiated at a tumor size of 5x5mm resulted in marked stasis in tumor development. However, tumors rapidly regained growth after completion of the treatment (Fig. 6A&B). Consequently, neo-adjuvant treated animals that
underwent a mastectomy at a tumor size of 15x15mm eventually succumbed to metastatic disease (Fig. 6C&D). Likewise, adjuvant chemotherapy treatments targeting clinically undetectable microscopic metastases were initiated three days after mastectomy and led to an initial but temporary response resulting in a clear increase in metastasis-specific survival (Fig. 6C&D). Consistent with observations in human invasive lobular carcinoma (22), these data show that (neo-)adjuvant treatments with doxorubicin and docetaxel result in a survival benefit, but do not give rise to a durable, complete response. Furthermore, treatment-associated survival benefits for adjuvant and neo-adjuvant treated cohorts suggest that mammary tumors and distant metastases exhibit similar therapeutic profiles upon (neo-)adjuvant treatment with the standard-of-care chemotherapeutics doxorubicin and docetaxel.
Discussion

In this study, we have developed a preclinical mouse model of *de novo* breast cancer metastasis formation that recapitulates the key biological events of the metastatic cascade and closely mimics the clinical course of metastatic disease in humans. We utilized the well-characterized conditional *K14cre;CdhlF/F;Trp53F/F* mouse model of *de novo* mammary tumor formation which recapitulates several key features of human ILC (14). Exploiting these features, we orthotopically transplanted pleomorphic *K14cre;Cdhl1F/F;Trp53F/F* derived mILCs fragments into wild-type syngeneic recipient mice and found that donor and recipient mammary tumors showed similar histopathological and molecular traits. We then mimicked the clinical setting and surgically resected established recipient tumors. Thus, we were able to extend the life span of recipient animals, thereby allowing disseminated cancer cells to prosper and establish advanced distant metastases. As a result, recipient mice eventually succumbed to widespread clinically overt metastatic disease in lymph nodes, lungs and gastrointestinal tract. Extensive analysis of metastatic foci revealed that metastases maintained their mILC-like phenotype and that metastases were genomically hardly distinguishable from clonally-related recipient mammary tumors. (Neo-)adjuvant interventions studies with standard-of-care chemotherapeutics further revealed that clonally-related recipient tumors and distant metastases exhibited very similar therapeutic profiles.

Based on these results, we believe that our model provides a valuable tool to study metastatic dissemination in invasive lobular breast cancer and offers several advantages over most of the currently available metastasis models. First, metastatic dissemination in our model is not induced by intravenous injection of cancer cells, but occurs spontaneously by seeding cancer cells from orthotopically transplanted tumors. Thus, metastatic dissemination in this model more closely reflects the key biological events of the metastatic cascade. Furthermore, recipient mammary tumors in our model were not established by orthotopic transplantation of cancer cells derived from *in vitro* maintained cancer cell lines. Cancer cell line-based metastasis models have several advantages, as tumor cells are easily manipulated for mechanistic studies. Likewise, introduction of biomarkers for *in vivo* non-invasive imaging of disease progression is relatively straightforward. Yet, cell line-based metastasis models have their limitations, as *in vitro* maintained cancer cell lines fail to retain the cellular heterogeneity present in the parental tumor (13). Since this heterogeneity reflects a diverse composition of distinct subclones within a primary tumor, loss of biological variation could have important implications for metastatic behavior and therapy responses observed in these models (23). To circumvent these limitations, we orthotopically transplanted tumor fragments derived from *de novo* *K14cre;Cdhl1F/F;Trp53F/F* mILCs into wild-type hosts. Thus, we were able to reconstitute equally heterogeneous recipient mammary tumors. As a consequence,
recipient mammary tumors in our model are more likely to reflect the heterogeneity also observed in human cancer (24; 25). Though more realistic, it is important to note that this biological variety comes at the expense of experimental flexibility as tumors are more difficult to manipulate.

Second, by transplanting mILC fragments into syngeneic hosts, we were able to reconstitute mammary tumors in the context of a functional, immune-proficient microenvironment. Therefore, our model can be utilized to address the role of the immune system in breast cancer metastasis formation. This is essential, since accumulating evidence indicates that immune cells and their soluble mediators modulate the process of metastatic spread both at the level of the primary tumor as well as at distant sites (4; 26). Furthermore, since this system permits easy manipulation of the stromal compartment by transplanting tumor fragments into hosts with altered stromal traits, it can also be used to assess the functional involvement of other cancer-cell extrinsic factors.

Third, unlike in other models (27), metastatic disease in our model is not confined to a limited set of distant sites, but encompasses a variety of lymphoid and visceral organs. The common involvement of tumor-draining and distant lymph nodes suggests that metastatic spread in our model occurs at least partially by spontaneous lymphatic dissemination of cancer cells. In contrast to some other models, this pattern of metastatic dissemination arises spontaneously and does not require in vivo enrichment, selection and re-injection of cancer cells. Moreover, the distribution of organs affected by metastatic disease in our model is highly reminiscent to the metastatic spectrum observed in human invasive lobular breast cancer (18). Thus, based on these merits, our model presumably more closely reflects the biology of organ-specific metastatic colonization. Since various organs are often affected simultaneously, this model allows a careful, paired analysis of metastases arising in different anatomical locations as illustrated by our genomic studies. Extending these studies by an in-depth comparison of metastatic foci to their parental tumor paves the way to gain new insights into mechanisms regulating organ-specific metastasis formation.

Fourth, metastatic dissemination in our model led to clinically overt metastatic disease thus allowing us to determine metastasis-specific survival based on clinically defined endpoints. These clinically defined endpoints provide a more precise estimation of disease burden, as number, size and cumulative area of metastatic foci not necessarily correlate with the disturbance of organ function. For example, solely based on their critical location only few lung metastases might lead to a rapid deterioration in respiratory capacity. Likewise, pleural effusions commonly observed in lung metastases-bearing animals have a profound impact on respiratory capacity. Ultimately, these factors collectively result in organ failure leading to clinical signs of respiratory distress. As a result,
clinical signs of metastatic disease and related metastasis-specific survival more precisely reflect the disease burden as they incorporate all the afore mentioned factors.

Finally, given its penetrant and predictive metastatic phenotype, our model can also be used as a preclinical tool to test (novel) therapeutic agents targeting metastatic disease (27). As demonstrated by our chemotherapy intervention experiments, these studies can either be performed in an adjuvant or neo-adjuvant setting, thus allowing a careful and independent evaluation of therapeutic agents targeting the primary tumor and low-volume microscopic or advanced metastatic disease. (Neo-) adjuvant intervention studies in cohorts of mice transplanted with the same donor tumor can be combined to create a well-controlled experimental setting that allows a reproducible, pair-wise comparison of therapy efficacy in clonally-related mammary tumors and distant metastases. Observations in one cohort of recipient mice can subsequently be validated in a second cohort of mice transplanted with an independent donor tumor. If inter-tumor heterogeneity between independent K14cre;Cdh1F/F;Trp53F/F donor tumors gives rise to different responses, this approach can also be exploited to study the impact of naturally occurring donor-specific genomic aberrations on (organ-specific) metastasis formation and therapy response. It is however important to note that recipient mammary tumors in this model are derived from end-stage mammary donor tumors. Therefore, our model potentially underestimates the contribution of early disseminated cancer cells, which – based on their independent and potentially divergent somatic evolution – might have an impact on the observed therapeutic profiles (28). Another drawback of our system is the current lack of markers for in vivo noninvasive imaging of metastatic disease. However, this issue can be resolved by the introduction of bioluminescence or fluorescence imaging reporters in donor mice.

In conclusion, we successfully developed a preclinical mouse model of de novo breast cancer metastasis formation that maintains and exploits the unique features of the original K14cre;Cdh1F/F;Trp53F/F model, while simultaneously circumventing its limitations by performing a mastectomy to prevent premature tumor-associated loss of recipient mice. We believe that this model provides a valuable tool to study the biology of metastatic disease and to evaluate the efficacy of (novel) therapeutic agents targeting metastatic disease. Our experimental approach can be applied to similar mouse models of de novo tumorigenesis, thus yielding a broader availability of mouse models that faithfully recapitulate metastatic disease in humans. Together, these models are likely to provide new insights that will support the development of more effective treatment strategies and may therefore benefit many patients suffering from metastatic disease.
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References


### Tables

#### Table 1. Overview of recipient organs affected by metastatic disease

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<th></th>
<th>Recipients 1 (n=16)</th>
<th>Recipients 2 (n=14)</th>
<th>Recipients 3 (n=14)</th>
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* Tumor-draining lymph nodes
Legends

Figure 1. Overview of a preclinical mouse model of de novo breast cancer metastasis formation. (A) Small tumor fragments (~1x1 mm) derived from mouse invasive lobular carcinomas (mILCs) which spontaneously developed in K14cre;CdhlF/F;Trp53F/F mice (grey mice) are transplanted orthotopically into the 4th mammary gland of wild-type syngeneic recipient animals (white mice). Once recipient tumors reach a size of ~15x15 mm, mastectomy is performed. Following surgery, mice are monitored for clinical signs and symptoms of metastatic disease. (B) Tumor growth kinetics in recipient mice transplanted with three independent donor mILCs. Tumor growth is depicted as tumor size (mean mm² ± SEM) over time, starting from the time of diagnosis (day 0), i.e. the first occasion after transplantation at which a solid tumor mass of ~2x2 mm was identified (recipients 1, n=16; recipients 2, n=14; recipients 3, n=14). (C) Kaplan-Meier tumor latency curves of the same recipient animals as shown in (B) presenting the interval between diagnosis (day 0) and surgical resection of the primary tumor reaching a size of ~15x15 mm (defined as an event).

Figure 2. Recipient mice develop pleiomorphic mILCs that recapitulate the histopathological and molecular characteristics of the parental K14cre;CdhlF/F;Trp53F/F derived donor tumor. (A) Representative images of donor- (upper row) and recipient mammary tumors (middle & lower rows) characterized by histopathological and immunohistochemical stainings including cytokeratin 8 (CK8), vimentin and E-cadherin. The first two rows show typical mILCs characterized by positive CK8 staining, while negative for vimentin and E-cadherin. The lower panel shows a biphasic tumor composed of epithelial (CK8+) and mesenchymal (CK8-) areas. Note the normal mammary ducts, which serve as internal controls (a, arteriole; d, normal mammary duct; s, stroma; t, mammary tumor). Scale bar = 50 μm. (B) Heat map constructed by hierarchical clustering (average linkage) of aCGH profiles from three independent sets of paired donor and recipient mammary tumors. Using smoothed genomic profiles, the correlation distance (1-correlation) between all donor and recipient mammary tumors was calculated. (DR set, collection of mammary tumors consisting of one K14cre;CdhlF/F;Trp53F/F derived donor mILC (D, donor tumor; number refers to the donor) and 3-4 related recipient tumors (R, recipient tumor; number refers to the related donor; letter refers to an individual recipient)).

Figure 3. Recipient animals spontaneously develop widespread, clinically overt metastatic disease in various organs. (A) Kaplan-Meier metastasis-specific survival curves of recipient mice orthotopically transplanted with tumor fragments from three independent K14cre;CdhlF/F;Trp53F/F
derived mILCs. An event is defined as an animal that was sacrificed due to clinical signs of metastatic disease. Censored cases (n=12/44) indicate mice sacrificed due to locally relapsing tumors reaching a size of ~15x15 mm. In total, 13/16 (recipients 1), 8/14 (recipients 2) and 11/14 (recipients 3) recipient mice succumbed to clinically overt metastatic disease. (B) Organs collected from recipient mice were microscopically analyzed for the presence of metastatic foci. The number of organs affected by metastatic disease was quantified per animal (each depicted as one circle). (C) Representative low (upper panel) and high (lower panel) power microscopic images of organs most frequently affected by metastatic disease. Scale bar upper panel = 500 μm; scale bar lower panel = 50 μm.

**Figure 4. Metastatic dissemination is not instigated by surgical manipulation of mammary tumors.** Recipient mice transplanted with donor mILC 2 underwent a mastectomy once mammary tumors reached a size of 5x5, 10x10 or 15x15 mm (n=9, n=6 and n=8 per group, respectively). (A) Box plots representing the time (mean ± 95% confidence intervals) from diagnosis to surgical resection of the mammary tumor at the intended size. (B) Kaplan-Meier metastasis-specific survival curves of the same recipient mice as described in A. An event is defined as an animal that was sacrificed due to clinical signs of metastatic disease. Censored cases indicate mice that remained healthy till termination of the experiment. Animals that developed locally relapsing tumors were excluded from the analysis. (C) Kaplan-Meier metastasis-specific survival curves of the data presented in B, but t=0 was redefined as the time of surgery.

**Figure 5. Distant metastatic foci recapitulate the histopathological and molecular characteristics of the parental recipient mammary tumor.** (A) Histopathological and immunohistochemical characterization of metastatic foci. Representative images from lung metastases observed in a recipient transplanted with donor mILC 1 are shown. (B) Infiltration of metastatic foci by CD3+ T-lymphocytes and F4/80+ macrophages (brown staining). Scale bar = 50 μm. (C) Heat map constructed by hierarchical clustering (average linkage) of genomic profiles from ten sets (3-4 sets per donor) of recipient tumors and paired lymph node- and lung metastases. Using smoothed genomic profiles, the correlation distance (1-correlation) between recipient mammary tumors and metastases was calculated. (DR set, paired sets (indicated by lower case letters) of donor-related (indicated by numbers) recipient mammary tumors and their local and/or distant metastases. R, recipient tissue; ax. LN, axillary, tumor-draining lymph node metastasis; caud. LN, caudal lymph node metastasis; lung, lung metastasis; renal LN, renal lymph node metastasis; tumor, primary mammary tumor).
Figure 6. Clonally-related recipient tumors and distant metastases respond similarly to (neo-)adjuvant treatment with standard-of-care chemotherapeutics. (A) Schematic overview of (neo-)adjuvant chemotherapy treatments in tumor-bearing recipients transplanted with the same donor tumor. (Neo-)adjuvant treatments with PBS (control), doxorubicin or docetaxel were administered once weekly for a fixed period of 4 weeks. Neo-adjuvant treatments were initiated at a tumor size of 5x5mm, whereas adjuvant treatments were started three days after mastectomy. Mammary tumors were surgically resected at a size of ~15x15mm and mice were sacrificed once they developed clinical signs of metastatic disease. (B) Individual tumor growth curves of recipient mice that received neo-adjuvant treatment with PBS (black), doxorubicin (red) or docetaxel (blue)(n=7/treatment). (C&D) Kaplan-Meier metastasis-specific survival curves of recipient mice that underwent neo-adjuvant (n=7/treatment) or adjuvant treatment (n=10-11/treatment) with PBS (C&D), doxorubicin (C) or docetaxel (D). An event is defined as an animal that was sacrificed due to clinical signs of metastatic disease. Censored cases indicate mice sacrificed due to locally relapsing tumors reaching a size of ~15x15 mm. Statistical analyses were performed using the log-rank test to compare neo-adjuvant versus adjuvant doxorubicin and docetaxel treatments.
Figure 1

A

K14cre;CdhlF/F;Trp53F/F donor

Wild-type syngeneic recipient

Clinically overt metastatic disease

B

C

Recipients 1
Recipients 2
Recipients 3

Tumor size (mm²)

Time after diagnosis (days)

Tumor-bearing mice (%)

Time after diagnosis (days)
Figure 5

(A) H&E, Cytokeratin 8, Vimentin, E-cadherin

(B) CD3, F4/80

(C) Color Key

Value

−0.2 0.2 0.6 1.0

DR set 1
DR set 2
DR set 3

R3a ax. LN
R3a lung
R3c ax. LN
R3c lung
R3a tumor
R3b caud. LN
R3b tumor
R3b ax. LN
R3a caud. LN
R3b lung
R3c tumor
R3c renal LN
R1b ax. LN
R1c tumor
R1a lung
R1a tumor
R1b tumor
R1d lung
R1c lung
R1d ax. LN
R1d tumor
R1a lung
R1a renal LN
R2a tumor
R2a tumor
R2c tumor
R2b caud. LN
R2b lung
R2b lung
R2a ax. LN
Figure 6

A

5x5 mm

Mastectomy:

15x15 mm

↑ 7d

Neo-adjuvant

5x5 mm

Mastectomy:

15x15 mm

↑ 7d

Adjuvant

B

Tumor size (mm²)

Time after initiation treatment (days)

0 50 100 150

0 50 100 150

C

Metastasis-specific survival (%)

Time after diagnosis (days)

0 25 50 75 100

p=0.7673

D

Metastasis-specific survival (%)

Time after diagnosis (days)

0 25 50 75 100

p=0.6853

0 25 50 75 100

0 50 100 150

0 50 100 150

- Control
- Doxorubicin
- Docetaxel

- Control
- Doxorubicin
- Docetaxel

- Control
- Doxorubicin
- Docetaxel

- Control
- Doxorubicin
- Docetaxel

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A preclinical mouse model of invasive lobular breast cancer metastasis

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