Mammary-specific inactivation of E-cadherin and p53 impairs functional gland development and leads to pleomorphic invasive lobular carcinoma in mice

Patrick W. B. Derksen1,2,3,*,‡, Tanya M. Braumuller1, Eline van der Burg1, Marten Hornsveld1, Elly Mesman4, Jelle Wesseling4, Paul Krimpenfort5 and Jos Jonkers1,‡

SUMMARY
Breast cancer is the most common malignancy in women of the Western world. Even though a large percentage of breast cancer patients show pathological complete remission after standard treatment regimes, approximately 30-40% are non-responsive and ultimately develop metastatic disease. To generate a good preclinical model of invasive breast cancer, we have taken a tissue-specific approach to somatically inactivate p53 and E-cadherin, the cardinal cell-cell adhesion receptor that is strongly associated with tumor invasiveness. In breast cancer, E-cadherin is found mutated or otherwise functionally silenced in invasive lobular carcinoma (ILC), which accounts for 10-15% of all breast cancers. We show that mammary-specific stochastic inactivation of conditional E-cadherin and p53 results in impaired mammary gland function during pregnancy through the induction of aneuploid resistance of mammary epithelium, resulting in loss of epithelial organization and a dysfunctional mammary gland. Moreover, combined inactivation of E-cadherin and p53 induced lactation-independent development of invasive and metastatic mammary carcinomas, which showed strong resemblance to human pleomorphic ILC. Dissemination patterns of mouse ILC mimic the human malignancy, showing metastasis to the gastrointestinal tract, peritoneum, lung, lymph nodes and bone. Our results confirm that loss of E-cadherin contributes to both mammary tumor initiation and metastasis, and establish a preclinical mouse model of human ILC that can be used for the development of novel intervention strategies to treat invasive breast cancer.

INTRODUCTION
Breast cancer affects a large number of females in the Western world, accounting for half a million deaths worldwide on an annual basis. Carcinoma of the breast is a heterogeneous disease based on pathological criteria, which is probably due to the multiplicity of genetic lesions that have accumulated during tumor development, resulting in distinct tumor types. The most frequently observed subtypes, invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC), are very distinct phenotypically as well as biochemically (Coradini et al., 2002; Korkola et al., 2003; Mathieu et al., 2004; Zhao et al., 2004; Stange et al., 2006). ILC is a subtype of breast cancer that accounts for 10-15% of all cases and has a greater tendency for multifocal development and bilateral presentation than other primary breast tumors (Newstead et al., 1992; Krecke and Gisvold, 1993; Helvie et al., 1993). Classical ILC is characterized by non-cohesive invasive cells that are arranged in trabecules without mass formation and calcification, a feature that hinders diagnosis using physical examination or mammography (Simpson et al., 2003). The vast majority of ILCs are mostly estrogen receptor (ER) positive and therefore responsive to endocrine therapy; however, they are mostly refractory to standard chemotherapy treatment once hormone receptor expression is lost (Gonzalez-Angulo et al., 2007), resulting in comparable survival rates for ILC and IDC (Molland et al., 2004; Tubiana-Hulin et al., 2006). Controversy still exists concerning the etiology of invasive breast cancer (IBC). Surprisingly, most clinicians do not regard lobular carcinoma in situ (LCIS) a precursor lesion for ILC, even though LCIS is regarded as a marker for progression to ipsilateral IBC, and 90% of LCIS-containing IBCs show a lobular phenotype (Wheeler et al., 1974; Rosen et al., 1978; Frykberg et al., 1987; Gump, 1993). Also, loss of E-cadherin, which we have postulated as the initiating and causal event in the development of mouse ILC (mILC) (Derksen et al., 2006), is a common feature of LCIS as well of the adjacent ILC cells (Vos et al., 1997).

E-cadherin is a key component of adherens junctions, structures that control the maintenance of epithelial integrity (Perez-Moreno et al., 2003). E-cadherin is a cell-cell adhesion molecule that functions as a scaffold in the formation of catenin-containing complexes that link E-cadherin to the actin and microtubule cytoskeleton (Hulskens et al., 1994; Takeichi, 1995; Perez-Moreno et al., 2003). In multiple types of cancer, loss of E-cadherin function through genetic or epigenetic mechanisms has been implicated in

1Division of Molecular Biology, Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands
2Department of Medical Oncology, University Medical Center Utrecht, 3508 AB Utrecht, The Netherlands
3Department of Pathology, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands
4Department of Pathology, Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands
5Division of Molecular Genetics, Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands
*Present address: Department of Pathology, UMC Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands
‡Authors for correspondence (p.w.b.derksen@umcutrecht.nl; j.jonkers@nki.nl)

Received 23 July 2010; Accepted 16 December 2010

© 2011. Published by The Company of Biologists Ltd
This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial Share Alike License (http://creativecommons.org/licenses/by-nc-sa/3.0), which permits unrestricted non-commercial use, distribution and reproduction in any medium provided that the original work is properly cited and all further distributions of the work or adaptation are subject to the same Creative Commons License terms.

Disease Models & Mechanisms 4, 347-358 (2011) doi:10.1242/dmm.006395
Disease Models & Mechanisms

RESEARCH ARTICLE

E-cadherin loss leads to pleomorphic mILC

progression and metastasis (Frixen et al., 1991; Vleminckx et al., 1991; Clepton-Jansen et al., 1994; Hulsken et al., 1994; Oda et al., 1994; Berx et al., 1995; Graff et al., 1995; Takeichi, 1995; Yoshiura et al., 1995; Savagner et al., 1997; Perl et al., 1998; Battle et al., 2000; Cano et al., 2000; Comijn et al., 2001; Fujita et al., 2003; Yang et al., 2004; Moody et al., 2005). Nonetheless, the molecular mechanisms that drive tumor development and progression upon loss of E-cadherin in breast cancer remain ill-defined.

To study all aspects of tumor pathology, mouse models are needed that mimic not only tumor phenotype, but also the initiating steps of human tumor development. In addition, it is important that mouse models reflect the events that are common in human ILC pathology such as lymphatic dissemination and subsequent distant metastasis, especially to bone. We have previously shown that tissue-specific inactivation of E-cadherin and p53 leads to the development of mILC (Derksen et al., 2006). While this mouse model mimics many aspects of human pathology, including metastatic dissemination, it is not as attractive as a preclinical model because of the severity of skin-related problems due to cytokeratin 14 (K14) promoter driven Cre recombinase (K14cre) expression in multiple epithelial tissues. In addition, the stochastic activity of K14cre in mammary gland epithelium precludes in vivo analysis of the direct consequences of E-cadherin loss, alone or in combination with loss of p53. In this study, we have developed a new mouse model for human ILC, based on mouse whey acidic protein (Wap) gene promoter driven Cre recombinase (Wcre) expression in the lumenal compartment of mammary epithelium (Fig. 1A). Based on Wap expression patterns (Hennighausen and Sippel, 1982), which is known to be expressed in the lumenal compartment of mammary epithelium (Fig. 1A). We examined the efficiency and tissue-specificity of Cre-mediated recombination by crossing Wcre founders with mice carrying the Rosa26-lacZ reporter (R26R mice) (Soriano, 1999). Bi-transgenic virgin and uniparous female littermates were analyzed for lacZ activity using X-gal-stained tissue sections and whole mount preparations. We started by analyzing the Wcre-mediated recombinase activity upon lactation. Examination of whole mount preparations from uniparous female Wcre;R26R mice showed robust β-galactosidase activity throughout the mammary gland (Fig. 1B, right panel). Histological analyses suggested lacZ expression in both the myoepithelial and luminal compartments of the uniparous mammary gland. In contrast to previously published results (Wagner et al., 1997), we could readily detect cells that had expressed Cre recombinase in mammary glands from virgin Wcre mice (Fig. 1B, left panel, and Fig. 1C). Examination of sectioned whole mounts revealed a weak and patchy staining pattern in 4-week-old virgin mammary glands, which was maintained throughout mammary gland development (Fig. 1C). The β-galactosidase staining patterns again suggested that Cre recombinase activity was not confined to luminal cells of the mammary gland but was also present in myoepithelial cells (Fig. 1C, bottom right panel). To substantiate this, we performed immunohistochemistry for cytokeratin (CK8), CK14 and smooth muscle actin (SMA) on sections from X-gal-stained Wcre;R26R virgin female mice. Unfortunately, CK14 immunohistochemistry did not yield interpretable results due to the incompatibility of the staining protocol with antigen retrieval (data not shown). However, although the majority of lacZ-positive (Cre-expressing) cells were coexpressing CK8, we could also detect lacZ positivity in CK8-negative cells (Fig. 1D, top panel). Moreover, we could detect activity of lacZ in SMA-positive cells (Fig. 1D, bottom panel), suggesting that Wcre is expressed in both luminal and myoepithelial cells in Wcre;R26R female mice.

Mammary-gland-specific conditional inactivation of E-cadherin is not tolerated

To study the effects of E-cadherin loss during mammary gland development and tumor formation, we crossed our Wcre transgenic animals to conditional E-cadherin knockout (CdhlF/F) mice (Derksen et al., 2006). Despite the extensive Cre-mediated recombination in the mammary gland of Wcre females (Fig. 1), we did not find morphological abnormalities in virgin, pregnant or parous Wcre;CdhlF/F mice (Fig. 2A, left panels). Although Wcre;CdhlF/F females lactated and were able to nurse their litters upon parturition, the produced quantity of milk and litters was markedly lower than in wild-type animals (data not shown). Upon histological examination, we did not observe abnormalities in mammary architecture, nor did we detect E-cadherin-negative ductal structures, indicating that – in agreement with previous studies (Boussadia et al., 2002; Derksen et al., 2006) – loss of E-cadherin is not tolerated in the mouse mammary gland (supplementary material Fig. S1). Moreover, Wcre-mediated loss of E-cadherin did not predispose mice to tumor formation, because none of the Wcre;CdhlF/F animals developed mammary carcinomas during their live span, even after the induction of multiple pregnancies, which is consistent with previously published studies using MMTVcre and K14cre conditional E-cadherin knockout mice (Boussadia et al., 2002; Derksen et al., 2006).

Developmental and lactational defects upon somatic inactivation of E-cadherin and p53

Mammary-specific loss of E-cadherin induces cell death by apoptosis (Boussadia et al., 2002). To investigate whether this apoptotic response to E-cadherin loss could be blocked by p53 inactivation, we introduced a conditional Trp53F/F allele (Jonkers et
E-cadherin loss leads to pleomorphic mILC

al., 2001) into the Wcre;Cdh1F/F mouse line. Next, we studied whether dual loss of E-cadherin and p53 would influence normal mammary gland development and function. To achieve effective Cre-mediated deletion of both Cdh1 and Trp53, we induced Wcre expression by mating and compared mammary gland morphology in Wcre;Cdh1F/F;Trp53F/F females with that in Wcre;Cdh1F/F and Cdh1F/F;Trp53F/F control mice. To this end, we examined carmine-stained whole mount preparations of fourth (inguinal) mammary glands, harvested at 14 and 17 days of pregnancy and at parturition (day 19.5). Mammary glands from virgin mice were used as a starting point. Whereas mammary glands from virgin Wcre;Cdh1F/F;Trp53F/F or Wcre;Cdh1F/F females showed no gross morphological abnormalities as compared with control female mice, Wcre;Cdh1F/F;Trp53F/F mammary glands harvested at day 14 of pregnancy displayed architectural abnormalities, showing severe ectasia (dilated ducts) and incomplete lobulo-alveolar development (Fig. 2). These effects became more pronounced as pregnancy progressed, with a filling of the mammary gland with nonfunctional tissue, resulting in complete disruption of the ductal structure at parturition (Fig. 2, bottom center panel). Wcre;Cdh1F/F and Cdh1F/F;Trp53F/F control animals showed no morphological mammary gland abnormalities during pregnancy (Fig. 2, left and right panels). Although Wcre;Cdh1F/F;Trp53F/F and Wcre;Cdh1F/F;Trp53F/F female mice produced healthy newborn offspring, all pups fostered by Wcre;Cdh1F/F;Trp53F/F dams died before weaning age due to starvation. Also, pups from Wcre;Cdh1F/F;Trp53F/F dams showed reduced survival rates due to the inhibition or absence of lactation. This phenotype could be rescued by fostering pups from Wcre;Cdh1F/F;Trp53F/+ female mice to wild-type recipient dams to normalize survival rates (data not shown). Surprisingly, we did not detect histological involution-induced apoptosis were observed after weening (data not shown). Surprisingly, we did not detect histological abnormalities in mammary glands from Wcre;Cdh1F/F mice (supplementary material Fig. S1 and data not shown). Normal ductal architecture was maintained throughout pregnancy, and E-
cadherin-negative ducts were not observed in these glands at either time point. These results show that loss of E-cadherin and p53 leads to aberrant lobulo-alveolar development during gestation. The developmental abnormalities are probably caused by loss of cell polarity and acquisition of anoikis resistance, resulting in loss of mammary gland organization and enhanced proliferative capacity of mammary epithelial cells, leading to a nonfunctional mammary gland.

E-cadherin loss collaborates with p53 loss in mammary tumorigenesis

We have previously shown that K14cre-mediated somatic inactivation of p53 in epithelial tissues results in the development of noninvasive adenocarcinoma and carcinosarcoma tumor types with a median latency of ~330 days (Liu et al., 2007). To target conditional loss of p53 to the mammary epithelial compartment, we crossed Trp53F/F conditional mutant mice (Liu et al., 2007) with our Wcre mice. All resulting Wcre;Trp53F/F female mice developed mammary tumors, with a median latency of 290 days (Fig. 4A). To study the tumor suppressor functions of E-cadherin, we crossed our Cdh1F conditional mice (Derksen et al., 2006) with the Wcre;Trp53F/F mouse model to produce cohorts of Wcre;Cdh1F/+;Trp53F/F and Wcre;Cdh1F/F;Trp53F/F female mice, which were mated once and monitored for subsequent tumor development. All mice were on a mixed genetic background of FVB/N and Ola129/sv (Derksen et al., 2006).

E-cadherin is not haploinsufficient for suppression of mammary tumor formation in these mice. By contrast, Wcre;Cdh1F/F;Trp53F/F mice developed mammary tumors with a significantly reduced tumor-free survival age (T50) of 194 days (Fig. 4A; P<0.0001). Tumor onset and progression in these mice were relatively uniform, with most tumors arising between 150 and 250 days. These findings demonstrate that combined inactivation of E-cadherin and p53 contribute synergistically to mammary tumor formation in these mice. Subsequent genetic analyses revealed uniform loss of both mutant Cdh1 alleles in tumors derived from Wcre;Cdh1F/F;Trp53F/F females (data not shown). As in the K14cre mILC model, we also detected uniform loss of the conditional and wild-type Trp53 alleles in mammary tumors from Wcre;Cdh1F/F;Trp53F/F, Wcre;Cdh1F/F;Trp53F/F and Wcre;Cdh1F/F;Trp53F/F females, indicating that loss of functional p53 is a prerequisite for mammary tumor formation in these mouse models (data not shown).

Lactation does not affect tumor onset, incidence, latency or metastasis formation in Wcre;Cdh1F/F;Trp53F/F mice

Because our analysis of Wcre;R26R mice also revealed Cre recombinase activity in virgin females (Fig. 1), we set out to investigate whether Wcre;Cdh1F/F;Trp53F/F and Wcre;Cdh1F/F;Trp53F/F female mice would develop mammary tumors in the absence of lactation. To this end, we produced ten female mice of each genotype and monitored tumor development in virgin animals. Interestingly, tumors arose in Wcre;Cdh1F/F;Trp53F/F virgin females with identical incidence and latency when compared with uniparous females (Fig. 4B). In line with this finding, tumor-free latency was also similar in virgin and parous Wcre;Cdh1F/F;Trp53F/F female mice (Fig. 4C). Moreover, the
tumor spectrum, invasiveness and metastatic dissemination were similar in virgin and parous Wcre;Cdh1 F/+;Trp53 F/F and Wcre;Cdh1 F/F;Trp53 F/F female mice (data not shown), indicating that Wcre expression levels in virgin female mice in the Wcre conditional E-cadherin and p53 mouse model (Wcre;Cdh1 F;Trp53 F) are sufficient to induce stochastic Cre-mediated loss of E-cadherin and p53. Loss of E-cadherin induces a shift from noninvasive adenocarcinoma to ILC with pleomorphic features Mammary tumors of Wcre;Trp53 F/F females were categorized into two groups and diagnosed as intermediate-grade adenocarcinoma (AC) or solid carcinoma/carcinosarcoma (SC/CS) tumor types, both characterized by expansive growth patterns. Tumors displayed benign noninvasive features and consisted of large epithelial cells forming solid nests or irregular glands. ACs showed membranous expression of E-cadherin and displayed a mixed but exclusive expression pattern of CK8 and CK14, but lacked expression of vimentin and SMA (Fig. 5A; supplementary material Table S1). SC/CS lesions were characterized by a metaplastic and biphasic histology that consisted of epithelial and mesenchymal elements (Fig. 5B-D), showing a heterogeneous and mutually exclusive expression pattern for CK8 and CK14, occasionally expressing vimentin and mostly lacking expression of E-cadherin (supplementary material Table S1). Expansive growth patterns were seen in the vast majority of tumors, which only sporadically metastasized to the lung (Table 1; supplementary material Tables S1 and S2).

Because most mammary tumors in Wcre;Trp53 F/F animals were neither invasive nor metastatic, we investigated the phenotypic consequences of loss of E-cadherin. Mammary-specific somatic loss of E-cadherin and p53 in Wcre;Cdh1 F/+;Trp53 F/F females resulted in a significant shift from expansive to invasive mammary tumors (P<0.0001; Table 1), which showed strong phenotypic similarities to human pleomorphic ILC (PILC). These tumors, which we have previously designated mILC (Derksen et al., 2006), developed with high incidence multifocally in several mammary glands (P<0.0001; Table 1). mILC cells grew in a non-cohesive manner in a trabecular fashion, were small in size, relatively pleomorphic in appearance and diagnosed as high grade (Fig. 5E,F). We could also detect signet ring cells, a typical occasional trait of human ILC (Fig. 5I). Like the adenocarcinomas from Wcre;Trp53 F/F females, lobular carcinomas showed a mixed CK8 and CK14 expression pattern, but did not express vimentin nor SMA (supplementary material Table S1), indicating that mILC cells display epithelial properties. Although most mILCs from Wcre;Cdh1 F/F;Trp53 F/F and Wcre;Cdh1 F/+;Trp53 F/F females were estrogen receptor (ER) negative, we occasionally found mILC lesions that weakly expressed ER. ER was mostly expressed by low-grade elements in mILC lesions, suggesting that ER expression is inversely correlated with tumor grade (supplementary material Table S1 and Fig. S4).

Wcre;Cdh1 F/F;Trp53 F/F and Wcre;Cdh1 F/+;Trp53 F/F females also developed SC/CS. Although displaying mILC components, these tumors predominantly exhibited a mixed epithelial and mesenchymal or spindle-shaped cell morphology, presenting large cells with pleomorphic nuclei, coarsely clumped chromatin and sparse cytoplasm. SC/CS tumor cells showed both expansive and invasive growth patterns, an exclusive and heterogeneous expression of CK8 and CK14, and predominantly expressed

![Fig. 3. Impaired lobulo-alveolar development upon dual inactivation of E-cadherin and p53. Comparative histochemistry during gestation of mammary glands from control (Cdh1 F/F;Trp53 F/F) and Wcre;Cdh1 F/+;Trp53 F/F animals. Lumenal cells were identified using CK8. Also shown are E-cadherin expression and proliferation by means of staining against incorporated BrdU. HE, hematoxylin and eosin. Original magnification: 100×.](image-url)
vimentin, but lacked expression of E-cadherin and SMA (Table 1; supplementary material Table S1).

**Loss of E-cadherin induces invasiveness and metastasis**

To explore whether the metastatic spread of mILC in the Wcre;Cdh1F/F;Trp53F/F model mimics the metastatic pattern of human ILC, we performed a detailed histological survey of various organs from tumor-bearing mice. Approximately 74% of the Wcre;Cdh1F/F;Trp53F/F females that presented mammary tumors of approximately 1 cm in diameter showed extensive local invasion and metastases to draining and distant lymph nodes (Fig. 6 and Table 1). Discohesive or loosely clustered mILC cells were detected in organs such as skin, lungs, liver, gastrointestinal tract, pancreas and spleen, or were diffusely disseminated throughout the peritoneal cavity (Fig. 6A-H), indicating that mILC in the Wcre;Cdh1F/F;Trp53F/F model recapitulates the histopathology and tumor biology of human ILC. In addition, several mice developed bone metastases, a feature that we have not observed in the K14cre;Cdh1F/F;Trp53F/F mouse model, exemplifying the additional value of the Wcre;Cdh1F/F;Trp53F/F model (Fig. 6I). mILC metastases showed a mixed and mutually exclusive expression pattern of CK8 and CK14, and displayed a cellular morphology similar to that of the primary tumor (Fig. 6; supplementary material Fig. S5).

From the primary tumors that developed in our mouse model, we isolated tumor cells, which were then cultured and subsequently transduced with luciferase-encoding lentiviruses to enable bioluminescence imaging. To further characterize the metastatic spectrum of mILC, we orthotopically transplanted recipient animals with approximately 10,000 luciferase-expressing Cdh1F/Δ;Trp53F/Δ (mILC) tumor cells. Using noninvasive bioluminescence imaging, we could image tumor growth and distant metastases, which developed approximately 5 weeks post transplantation (Fig. 7A; P<0.001). Metastases were detected in the peritoneal and thoracic cavity, contralateral mammary glands, lungs and bone (data not shown), illustrating that the full metastatic spectrum of human ILC is recapitulated upon orthotopic transplantation of mILC cell lines derived from the Wcre;Cdh1F/F;Trp53F/F model. By contrast, orthotopic transplantation of luciferase-marked Trp53F/Δ tumor cells showed a mixed growth pattern, depending on the cell line studied. Most E-cadherin-proficient tumor cells showed tumor growth, but did not metastasize (Fig. 7A). The difference in metastatic capacity between Trp53F/Δ and mILC tumor cells in situ correlated well with the acquisition of anchorage independence upon loss of E-cadherin (Fig. 7B), in analogy to tumor cells derived from the K14cre;Cdh1F/F;Trp53F/F mILC model (Derksen et al., 2006). In the absence of cell-matrix interactions, E-cadherin-expressing Trp53F/Δ tumor cells were not able to survive, whereas mILC cells readily survived and proliferated in an anchorage-independent fashion (Fig. 7B). Anoikis resistance was observed for cell lines derived from four independent mILC primary tumors, whereas cell lines from three independent Trp53F/Δ tumors showed anoikis induction under non-adherent culture conditions, indicating that loss of E-cadherin mediates survival of mammary tumor cells in the absence of cell-matrix interactions. In conclusion, in vitro anoikis resistance is a reliable prognosticator of in vivo metastatic dissemination, using cell lines derived from the Wcre;Cdh1F/F;Trp53F/F mouse model.
DISCUSSION
Research on breast cancer metastasis has greatly profited from recent advances in modeling metastatic disease in mice, using both transplantation techniques and genetic modification (Khanna and Hunter, 2005; Ottewell et al., 2006). Owing to the complex nature of the metastatic process, models that mimic both de novo tumor development and spontaneous metastasis formation are scarce, but nevertheless emerging. Here, we have used Cre-loxP-based conditional mutagenesis to develop a lactation-independent and mammary-gland-specific mouse model of human PILC. Wcre-mediated inactivation of E-cadherin and p53 in mice induced simultaneous development of multiple tumors in several mammary glands. This high penetrance implies that only one or a few additional hits are required to induce tumor formation of predisposed E-cadherin- and p53-deficient mammary progenitors. Wcre-mediated loss of E-cadherin and p53 induced numerous biphasic tumors in several mammary glands that displayed both epithelial and mesenchymal characteristics, suggesting that conditional inactivation based on Wap promoter activity occurs in a progenitor cell that is not yet fully committed to a distinct epithelial lineage. Although Wap is known to be transcribed during gestation and lactation (Pittius et al., 1988; Dale et al., 1992), we found that a substantial amount of Wcre mammary epithelial cells show Cre activity independent of gestation and lactation. Moreover, mammary glands from 4-week-old Wcre;R26R female mice already displayed β-galactosidase activity, indicating that the Wap promoter used in our studies might be activated by prolactin in young virgin animals at the onset of estrus (Topper and Freeman, 1980; Whittingham and Wood, 1983; Clarke et al., 1993). Nonetheless, the majority of mammary reconstitution assays that we have performed with mammary epithelial cells from 3-week-old Wcre;Cdh1F/F;Trp53F/F mice yielded phenotypically wild-type mammary glands (Eva J. Vlug and P. W. B. D., unpublished data), implying that the majority of mammary epithelial stem and/or progenitor cells in 3-week-old Wcre conditional mice have retained a non-switched (floxed) configuration and that the majority of Wcre-mediated recombination occurs upon the onset the first estrous cycle, which commences after approximately 28 days in mice (Caligioni, 2009). Because multiple copies of the Wcre transgene will have concatemerized during genomic integration, we envisage that physiological prolactin levels in the virgin animals might be sufficient to drive stochastic Wcre-mediated loss of E-cadherin and p53 in the absence of a lactational pulse. It is likely that dependency on E-cadherin function changes during differentiation of mammary stem and/or progenitor cells towards luminal and myoepithelial descendants. Cells that differentiate into luminal-type cells will become dependent on E-cadherin-based adherens junctions, and succumb to apoptosis upon loss of E-cadherin as a result of the absence of a redundant classical cadherin (Boussadia et al., 2002; Derksen et al., 2006). Following
this event, cells will be cleared from the fat-pad, resulting in the absence of E-cadherin-deficient glandular structures in MMTVcre;CdhlF/F and Wcre;CdhlF/F mice (Boussadia et al., 2002) (and this study). Myoepithelial cells do not express E-cadherin, and hence will not be influenced by Cdhl deletion, mainly because P-cadherin is the predominant classical cadherin that will maintain the junctional integrity in these cells (Radice et al., 1997). We therefore hypothesize that mammary stem and/or progenitor cells tolerate the absence of E-cadherin until differentiation into luminal descendants is commenced.

Our results show that loss of p53 confers resistance to the proapoptotic signals that are initiated upon E-cadherin inactivation, resulting in the survival of luminal cells in the absence of a functional adherens junction. In Wcre;CdhlF/F;Trp53F/+ female mice, inactivation of E-cadherin and p53 during gestation resulted in the accumulation of mostly CK8-positive epithelial cells and mesenchymal-type cells that fill the periductal environment, thereby inhibiting formation of a functional mammary gland. Interestingly, Wcre-mediated gene switching did not result in accelerated tumor development in parous females when compared with the incidence in virgin females or the tumor-free survival in our models, indicating that Cre recombinase activity during gestation and lactation is mostly restricted to differentiated cells that do not contribute to mammary tumor formation. By contrast, lactation-independent inactivation of p53 and E-cadherin in mammary stem and/or progenitor cells will give rise to transformed epithelial cell types that display enhanced survival and growth and, in addition, harbor stem-cell-like characteristics that might facilitate progression into carcinomas with a non-cohesive growth pattern typical for mouse and human ILC. In the rare event that the myoepithelial compartment is not affected by somatic inactivation of the mutant alleles and the integrity of the basement membrane remains unaffected, this might give rise to LCIS, as in the human situation. Nonetheless, most early mILC lesions show a disturbed myoepithelial and/or basement membrane architecture, which is most probably causal to their highly invasive character. Also, the stromal component that is abundantly present in the periductal regions might contribute to tumor cell invasiveness through deposition of extracellular matrix, as has been recently proposed (Levental et al., 2009).
E-cadherin loss leads to pleomorphic mILC.

Although human ILCs are mostly ER positive, we only detected sporadic ER-positive cells in low- or intermediate-grade mILC lesions that still display features of glandular and/or ductal architecture. High-grade invasive and metastatic mILC never showed ER expression, which is in line with previous observations that mouse mammary tumors in general show physiological differences compared with human breast cancer with respect to hormone receptor expression (Yoshidome et al., 2000; Jonkers et al., 2001; Derksen et al., 2006). We assume that the vast majority of the mammary tumors that form in Wcre;Cdh1F/F;Trp53F/F (mILC) cells (black bars) show anoikis resistance (P > 0.001). Error bars represent the standard deviation of triplicate measurements.

Fig. 7. mILC anoikis resistance correlates with in vivo metastasis. (A) mILC metastasis imaging in vivo. Bioluminescence imaging of recipient animals, which were orthotopically transplanted with luciferase-transduced Tpz3Δ/Δ cells (top panels) or mILC cells (bottom panels). The color bar represents bioluminescence intensity counts. Transplantations were performed using two different mILC and Tpz3Δ/Δ cell lines, in a minimum of five recipient animals. (B) Loss of E-cadherin induces anoikis resistance in the absence of p53. Tumor cell lines established from Wcre;Tpz3F/F (AC) and Wcre;Cdh1F/F;Tpz3F/F (mILC) female mice were plated onto non-coated low cluster wells and the percentage of cells expressing phosphatidylserine was determined using binding to FITC-conjugated annexin-V. Dead cells were detected using DNA binding to ToPro-3. In the presence of E-cadherin, tumor cells are not able to survive in the absence of anchorage (white bars), whereas E-cadherin-deficient Cdh1Δ/Δ;Tpz3Δ/Δ (mILC) cells (black bars) show anoikis resistance (P > 0.001). Error bars represent the standard deviation of triplicate measurements.

In conclusion, we have generated a novel Wcre transgenic mouse line that displays Cre recombinase activity in mammary stem and/or progenitor cells. Consequently, Wcre-driven somatic inactivation of E-cadherin and p53 results in mammary tumor formation with similar incidence and latency in both virgin and parous mice. Combined inactivation of p53 and E-cadherin leads to mILC, which is highly invasive and shows (lymph)angiogenic and diffuse dissemination with metastasis to the gastrointestinal tract and bone, similar to the human situation. Our mouse model therefore represents an excellent preclinical model to test novel intervention strategies for invasive and metastatic breast cancer.

METHODS

Generation of Wcre transgenic mice

We constructed the Wcre transgene from an expression cassette that includes a 4.5-kb genomic BamHI-Sall mouse Wap gene promoter fragment followed by a 0.65-kb rabbit β-globin intron and a 0.63-kb transcription termination/polyadenylation fragment derived from the human growth hormone gene. Cre coding sequences were inserted between the intron and the poly(A) fragment (Fig. 1A). Next, we separated the 7.8-kb transgene fragment from vector sequences, purified and injected it into the pronuclei of one-cell-stage embryos of FVB/N mice. Microinjected eggs were transferred at the two-cell stage into the oviducts of pseudopregnant recipient females. Heterozygous transgenic animals were identified using PCR analysis.

DNA analysis and genotyping

Genomic DNA isolation and Southern blot analysis were performed as described (Jonkers et al., 2001; Derksen et al., 2006). Detection of the Tpz3ΔF, Tpz3ΔF, Cdh1ΔF and Cdh1Δ alleles was done by PCR as described (Derksen et al., 2006). Transgenic Wcre animals were identified by PCR using primers 5′-AGCAGCCATCATG-3′ and 5′-CAGCACTGTTGCATCGACC-3′, yielding an amplification product of 432 bp.
Antibodies
Antibodies used were: mouse anti-E-cadherin (1:300; BD Biosciences), mouse anti β-catenin (1:150; BD Biosciences), rat anti-CK8 (Troma-1; 1:125; Developmental Studies Hybridoma Bank), rabbit anti-CK14 (1:10,000; BabCo), guinea pig anti-vimentin (1:400; RDI), rabbit anti-SMA (1:350; Lab Vision), mouse anti-BrdU (1:1000; DAKO), rabbit anti-ERα (1:1000; Santa Cruz Biotechnology). Secondary antibodies were: biotin-conjugated antimouse, anti-rat and anti-rabbit antibodies (DAKO), and biotin-conjugated anti-guinea pig (Jackson ImmunoResearch).

Stainings of whole mount preparations
Reconstituted mammary glands were dissected and stretched on a glass slide. The glands were fixed in a mixture of 6:3:1 methanol:1,1,1-trichloroethane:acetic acid (all Sigma) for 4 hours and processed as whole mounts, which were stained overnight with carmine aluminum staining solution [2 g/l carmine (Sigma), 5 g/l aluminum potassium sulphate dissolved in H2O]. After stepwise dehydration in 70%, 95% and 100% ethanol, the glands were cleared in xylene (Sigma) for 10 minutes before taking pictures. For the β-galactosidase staining, we fixed glands in 4% paraformaldehyde (Sigma), 2 mM MgCl2 (Merck) and 5 mM EGTA (Sigma), 2 mM MgCl2, 0.02% NP-40, 0.01% Na-deoxycholate in PBS, glands were stained overnight with X-Gal staining solution [5 mM ferro-cyanide (K4Fe(CN)6), 5 mM ferricyanide (K3Fe(CN)6), 2 mM MgCl2, 0.02% NP-40 and 1 mg/ml X-Gal (Biosolve)] at 37°C in the dark. After dehydration in ethanol, glands were cleared in xylene (Sigma) for 10 minutes. For determination of cellular proliferation, mice were injected intraperitoneally with 2 mg/ml BrdU (Sigma) 90 minutes prior to harvest of the mammary glands.

Histological analysis
Tissues were isolated, fixed and processed as described (Derksen et al., 2006).

Cell culture
Cells were isolated and cultured as described previously (Derksen et al., 2006).

Lentiviral production and transduction of cells
Lentiviral particles were produced by seeding 106 293T cells onto a 10 cm Petri dish and performing transient transfection after 24 hours with third-generation packaging constructs and a luciferase-encoding transfer vector (LV-luc) (Derksen et al., 2003). Supernatant containing lentiviral particles were harvested and concentrated tenfold by ultra centrifugation at 75,000 g for 4 hours. Tumor cells were infected for 16 hours in the presence of 4 μg/ml polybrene.

Orthotopic transplantations and bioluminescence imaging
3-week-old Rag2−/−:IL2Rγc−/− BALB/c female recipient mice (Gimeno et al., 2003) were anaesthetized with intraperitoneal injection of a mixture containing 25 μl fentanyl citrate/fluanisone (hypnorm; Janssen Pharmaceutica), 25 μl midazolam (dormicin; Roche) and 50 μl water. The fourth mammary gland was exposed and endogenous mammary epithelial tissue was removed. Next, approximately 10,000 luciferase-transduced tumor cells were injected in the cleared fat-pad using a 10 μl Hamilton syringe, after which the animals were sutured. After a recovery period of 2 weeks, mice were anesthetized with isofluorane (Janssen Pharmaceutica), injected intraperitoneally with 225 μg/g body weight n-luciferin (potassium salt; Biosynth AG) and imaged on an IVIS-200 bioluminescence imager (Xenogen). All animal experiments were performed in accordance with institutional guidelines and national regulations.

Anoikis assay
Cells were plated at a density of 75,000 cells per well in a six-well ultra-low cluster polystyrene culture dish (Corning). FITC-conjugated annexin-V (1:20; IQ Products) and ToPro-3 (1:2000; Molecular Probes) were added and annexin-V-positive apoptotic cells were analyzed by FACS as described (Derksen et al., 2003). Statistical significance was calculated using the Student’s t-test.

ACKNOWLEDGEMENTS
We thank Miranda van Amersfoort, Hermien Boerhout and Sabine Vishnudatt for technical support. Members of the Jonkers lab are acknowledged for reagents, help and fruitful discussions. We are also indebted to the animal facility and the animal pathology lab. Joost Vermaat is kindly acknowledged for the statistical analyses. This work was supported by grants from the Netherlands Organization for Scientific Research (ZonMw VIDI 917.36.347) and the Dutch Cancer Society (NKI 2002-2635 and NKI 2006-3486). P.W.B.D. was supported by grants from the Netherlands Organization for Scientific Research (ZonMw VENI 916.56.135 and VIDI 917.96.318).

E-cadherin loss leads to pleomorphic mILC

Clinical issue
Metastatic disease is the major cause of mortality in breast cancer patients. A hallmark of invasive and metastatic cells is inhibition of E-cadherin function. Invasive lobular carcinoma (ILC; the second most common type of primary breast cancer) is characterized by early loss of the epithelial cell-cell adhesion molecule E-cadherin. The classical form of ILC is characterized by non-cohesive and invasive cells but without mass formation, often resulting in a false-negative diagnosis using physical examination or mammography. Although most cases of ILC are estrogen receptor (ER) positive and therefore responsive to initial endocrine therapy, the disease is generally refractory to standard chemotherapy treatments once ER expression is lost at later stages.

Results
In this paper, the authors establish a new mammary-specific conditional knockout mouse model to show that combined stochastic inactivation of E-cadherin and p53 results in impaired mammary gland function. Phenotypically, mammary tumors in this model exhibit multiple features of human pleomorphic ILC, both in terms of their appearance and their metastatic behavior. The data show that somatic inactivation of E-cadherin renders mammary epithelial cells anoikis resistant in the context of p53 deficiency. Importantly, the female mice in this model develop highly invasive and metastatic mammary tumors for which the onset, incidence and metastasis are lactation independent.

Implications and future directions
The lactation-independent and mammary-gland-specific mouse model of human pleomorphic ILC presented in this paper provides a new tool to gain insight into the role of E-cadherin loss of function in mammary tumor initiation, progression and metastasis. The study also addresses how E-cadherin and p53 might cooperate in mammary tumor development. Given the similarities between mouse and human ILC, this model might ultimately contribute to the development of novel clinical intervention strategies for the treatment of metastatic breast cancer.

TRANSLATIONAL IMPACT

Disease Models & Mechanisms  •  DMM

356
E-cadherin loss leads to pleomorphic mILC

COMPETING INTERESTS
The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS
P.W.B.D. and J.J. designed the experiments. P.K. generated the Wcre transgenic mice. P.W.B.D., T.M.B., M.H. and E.v.d.B. performed the experiments. E.M. executed the immune histochemistry on tissue sections and J.W. diagnosed mouse pathology. P.W.B.D. wrote the paper with assistance from J.J.

SUPPLEMENTARY MATERIAL
Supplementary material for this article is available at http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.006395/-/DC1

REFERENCES
Alvarez, J. V., Perez, D. and Chodosh, L. A. (2006). MIL-cing the mouse mammary gland: A model for invasive lobular carcinoma. Cancer Cell 10, 347-349.
Arpino, G., Bardou, V. J., Clark, G. M. and Elledge, R. M. (2004). Infiltrating lobular carcinoma of the breast: tumor characteristics and clinical outcome. Breast Cancer Res. 6, R149-R156.
Batlle, E., Sancho, E., Franci, C., Domínguez, D., Monfar, M., Baulida, J. and Garcia-Diez, A. (2002). Apocrine differentiation in invasive lobular versus ductal breast carcinomas with in situ ductal and lobular apocrine carcinoma: case report. Pathol. Oncol. Res. 8, 151-152.
Khan, C. and Hunter, K. (2005). Modeling metastasis in vivo. Carcinogenesis 26, 513-523.
Korkola, J. E., DeVries, S., Fridyland, J., Hwang, E. S., Estep, A. L., Chen, Y. Y., Chew, K. L., Dairkee, S. H., Jensen, R. M. and Waldman, F. M. (2003). Differentiation of lobular versus ductal breast carcinomas by expression microarray analysis. Cancer Res. 63, 7167-7175.
Krecke, K. N. and Gisvold, J. J. (1993). Invasive lobular carcinoma of the breast: mammographic findings and extent of disease at diagnosis in 184 patients. AJR Am. J. Roentgenol. 161, 957-960.
Levental, K. R., Yu, H., Kass, L., Lakins, J. N., Egeblad, M., Erler, J. T., Fong, S. F., Ciszkai, K., Giaccia, A., Weninger, W. et al. (2009). Matrix crosslinking forces tumor progression by enhancing integrin signaling. Cell 139, 891-906.
Liu, X., Holstege, H., van der Gulden, H., Ten-Mulder, M., Zevenhoven, J., Velds, A., Kerkhoven, R. M., van Vliet, M. L., Wessels, L. F., Peterse, J. L. et al. (2007). Somatic loss of BRCA1 and p53 in mice induces mammary tumours with features of human BRCA1-mutated basallike breast cancer. Proc. Natl. Acad. Sci. USA 104, 12111-12116.
Marchetti, A., Buttitta, F., Pellegrini, S., Campani, D., Diella, F., Cecchetti, D., Callahan, R. and Bistocchi, M. (1993). P53 mutations and histological type of invasive breast carcinoma. Cancer Res. 53, 4665-4669.
Mathieu, M. C., Rouzier, R., Llombart-Cussac, A., Sideris, L., Koscielny, S., Travagli, J. P., Contesso, G., Delaloge, S. and Spielmann, M. (2004). The poor responsiveness of infiltrating lobular breast carcinomas to neoadjuvant chemotherapy can be explained by their biological profile. Eur. J. Cancer 40, 342-351.
Middleton, L. P., Palacios, D. M., Bryant, B. R., Krebs, P. I., Otis, C. N. and Merino, M. J. (2000). Pleomorphic lobular carcinoma: morphology, immunohistochemistry, and molecular analysis. Am. J. Surg. Pathol. 24, 1650-1656.
Mohsin, S. K., O’Connell, P., Allred, D. C. and Libby, A. L. (2003). Biomarker profile and genetic abnormalities in lobular carcinoma in situ. Breast Cancer Res. Treat. 90, 249-256.
Molland, J. G., Donnellan, M., Janu, N. C., Carmalt, H. L., Kennedy, C. W. and Gillett, D. J. (2004). Infiltrating lobular carcinoma-a comparison of diagnosis, management and outcome with infiltrating ductal carcinoma. Breast 13, 389-396.
Moody, S. E., Perez, D., Pan, T. C., Sarkisian, C. J., Portocarrero, C. P., Sterner, C. I., Notorfrancesco, K. L., Cardiff, R. D. and Chodosh, L. A. (2005). The transcriptional repressor Snail promotes mammary tumor recurrence. Cancer Cell 8, 197-209.
Newstead, G. M., Baute, P. B. and Toth, H. K. (1992). Invasive lobular and ductal carcinoma: mammographic findings and stage at diagnosis. Radiology 184, 623-627.
Oda, T., Kanai, Y., Oyama, T., Yoshiura, K., Shimoyama, Y., Birchmeier, W., Sugimura, T. and Hirohashi, S. (1994). E-cadherin gene mutations in human gastric carcinoma cell lines. Proc. Natl. Acad. Sci. USA 91, 1858-1862.
Orvieto, E., Maiorano, E., Bottiglieri, L., Maisonneuve, P., Rotmensch, N., Galimberti, V., Luini, A., Brenelli, F., Gatti, G. and Viale, G. (2008). Clinicopathologic characteristics of invasive lobular carcinoma of the breast: results of an analysis of 530 cases from a single institution. Cancer 113, 1511-1520.
E-cadherin loss leads to pleomorphic mLCC

Ottewell, P. D., Coleman, R. E. and Holen, I. (2006). From genetic abnormality to metastases: murine models of breast cancer and their use in the development of anticancer therapies. *Breast Cancer Res. Treat.* 96, 101-113.

Perez-Moreno, M., Jamora, C. and Fuchs, E. (2003). Sticky business: orchestrating cellular signals at adherens junctions. *Cell* 112, 535-548.

Perl, A. K., Wilgenbus, P., Dahl, U., Semb, H. and Christofori, G. (1998). A causal role for E-cadherin in the transition from adenoma to carcinoma. *Nature* 392, 190-193.

Pittius, C. W., Sankaran, L., Topper, Y. J. and Hennighausen, L. (1988). Comparison of the regulation of the whey acidic protein gene with that of a hybrid gene containing the whey acidic protein gene promoter in transgenic mice. *Mol. Endocrinol.* 2, 1027-1032.

Radice, G. L., Ferreira-Cornwell, M. C., Robinson, S. D., Rayburn, H., Chodosh, L. A., Perez-Moreno, M., Jamora, C. and Fuchs, E. (2003). Sticky business: orchestrating cellular signals at adherens junctions. *Cell* 112, 535-548.

Rosen, P. P., Lesser, M. L., Arroyo, C. D., Cranor, M., Borgen, P. and Norton, L. (2000). A new role for E-cadherin in the transition from adenoma to carcinoma. *Clin. Cancer Res.* 12, 345-352.

Stange, D. E., Radlwin, B., Schubert, F., Traub, F., Pich, A., Toedt, G., Mendrzyk, F., Lehmann, U., Eils, R., Kreipe, H. et al. (2006). High-resolution genomic profiling reveals association of chromosomal aberrations on 1q and 16p with histologic and genetic subgroups of invasive breast cancer. *Clin. Cancer Res.* 12, 345-352.

Topper, Y. J. and Freeman, C. S. (1980). Multiple hormone interactions in the developmental biology of the mammary gland. *Physiol. Rev.* 60, 1049-1106.

Tubiana-Hulin, M., Stevens, D., Lasry, S., Guinebretiere, J. M., Boult, L., Cohen-Solal, C., Cherel, P. and Rouesse, J. (2006). Response to neo-adjuvant chemotherapy in lobular and ductal breast carcinomas: a retrospective study on 860 patients from one institution. *Ann. Oncol.* 17, 1228-1233.

Vlieminckx, K., Vakaet, L., Jr, Mareel, M., Fiers, W. and van Roy, F. (1991). Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. *Cell* 66, 107-119.

Vos, C. B., Clearton-Jansen, A. M., Berx, G., de Leeuw, W. J., ter Haar, N. T., van Roy, F., Cornelisse, C. J., Peterse, J. L. and van de Vijver, M. J. (1997). E-cadherin inactivation in lobular carcinoma in situ of the breast: an early event in tumorigenesis. *Br. J. Cancer* 76, 1131-1133.

Wagner, K. U., Wall, R. J., St Onge, L., Gruss, P., Wynshaw-Boris, A., Garrett, L., Li, M., Furth, P. A. and Hennighausen, L. (1997). Cre-mediated gene deletion in the mammary gland: paradoxical loss of estrogen receptoralpha expression during tumor progression. *Cell* 66, 107-119.

Whittingham, D. G. and Wood, M. J. (1983). The Mouse in Biomedical Research, *Reproductive Physiology* (Foster, H. L., Small, J. D. and Fox, J. G., eds), pp. 137-164. New York: Academic Press.

Zhao, H., Langerod, A., Ji, Y., Nowels, K. W., Nesland, J. M., Tibshirani, R., Bukholm, I. K., Karesen, R., Botstein, D., Borresen-Dale, A. L. et al. (2004). Different gene expression patterns in invasive lobular and ductal carcinomas of the breast. *Mol. Biol. Cell* 15, 2525-2536.