Identification of cellular and genetic drivers of breast cancer heterogeneity in genetically engineered mouse tumour models

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Abstract

The heterogeneous nature of mammary tumours may arise from different initiating genetic lesions occurring in distinct cells of origin. Here, we generated mice in which Brca2, Pten and p53 were depleted in either basal mammary epithelial cells or luminal oestrogen receptor (ER)-negative cells. Basal cell-origin tumours displayed similar histological phenotypes, regardless of the depleted gene. In contrast, luminal ER-negative cells gave rise to diverse phenotypes, depending on the initiating lesions, including both ER-negative and, strikingly, ER-positive invasive ductal carcinomas. Molecular profiling demonstrated that luminal ER-negative cell-origin tumours resembled a range of the molecular subtypes of human breast cancer, including basal-like, luminal B and ‘normal-like’. Furthermore, a subset of these tumours resembled the ‘claudin-low’ tumour subtype. These findings demonstrate that not only do mammary tumour phenotypes depend on the interactions between cell of origin and driver genetic aberrations, but also multiple mammary tumour subtypes, including both ER-positive and -negative disease, can originate from a single epithelial cell type. This is a fundamental advance in our understanding of tumour aetiology.

Keywords: Brca2; Pten; p53; tumour heterogeneity; breast cancer molecular subtypes; basal-like

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Introduction

Breast cancer is a heterogeneous disease encompassing different histological and molecular subtypes, with distinct clinical behaviours [1–4]. The biological basis of this heterogeneity remains poorly understood; improving this understanding is key to better patient stratification. Although distinct molecular events occurring in different target cells may explain the variety of breast cancer phenotypes [5,6], there is not necessarily a direct correlation between tumour phenotype and its cell of origin. For instance, breast cancers of ‘basal-like’ subtype were proposed to arise from basal stem cells [7–10], but current models suggest that a substantial proportion, if not all, of these tumours derive from luminal-progenitor cells [11–13]. Disentangling the complex relationship between tumour-initiating genetic events, target cells and tumour phenotypes is ideally suited to studies using genetically engineered mouse models.

We previously demonstrated that when Brca1 and p53 loss were targeted to either basal or luminal ER-negative mammary (lumERneg) cells in mouse models, the balance of tumour phenotypes depended on the cell of origin. Although all tumours were molecularly classified as ‘basal-like’, histologically the basal cell-origin tumours were mostly adenomyoepitheliomas (AMEs), while the lumERneg cell-origin tumours were high-grade invasive...
ductal carcinomas of no special type (IDC-NSTs)[13]. It remains to be defined, however, whether the cell of origin is the prime determinant of tumour subtype, or whether initiating genetic hits also play a role in shaping phenotype, in addition to simply stimulating tumourigenesis.

To address this question, we generated conditional mouse models in which Brca2, p53 and/or Pten were deleted in distinct cell populations of the mouse mammary gland. To fully describe the tumours these animals developed, detailed histopathological, immunohistochemical and gene expression analyses were performed. We demonstrate that the relative contributions of cell of origin and molecular lesion to determining mammary tumour heterogeneity are context-dependent. The final tumour phenotype is the result of both interactions between the cell of origin and genetic aberrations, and epistatic interactions between genetic aberrations within a cancer.

Materials and methods

Tumour cohorts

The following genotypes were established and maintained until tumours developed: K14Cre:Brca2<sup>f</sup>/p53<sup>f</sup>, BlgCre:Brca2<sup>f</sup>/p53<sup>f</sup> virgin and parous; Pten<sup+v−</sup>, K14Cre:Pten<sup>f</sup>, BlgCre:Pten<sup>f</sup> virgin and parous; BlgCre:Pten<sup>f</sup>/p53<sup>f</sup>, BlgCre:Pten<sup>f</sup>/p53<sup>f</sup>. Parous mice went through two or three pregnancy cycles. Tumours were excised from humanely killed mice and half was fixed in 4% phosphate-buffered formalin (BIOS Europe Ltd, Skelmersdale, UK) overnight for paraffin embedding. The remainder was snap-frozen on dry ice for nucleic acid isolation.

Histology and immunohistochemistry

Haematoxylin and eosin (H&E) staining was performed using standard methods. Immunohistochemistry for ERα, p63, K14 and K18 and double p63/ER immunofluorescence were carried out as described [13,14]. Immunohistochemistry for PRA (hPRA7; ThermoScientific, UK) and PRB (αPR6; Abcam, Cambridge, UK) were performed using the ER protocol. Immunohistochemistry for human CLDN3 (Z323JM, Invitrogen-Life, Paisley, UK), CLDN4 (3E2C1, Invitrogen-Life), CDH1 (Zymed, CA, USA) and PTEN (6H2.1; Dako, Denmark) were performed as described [15,16].

Gene expression microarray analysis

Samples which underwent gene expression analysis were morphologically checked to be representative. Microarray hybridization was performed by UCL Genomics (UCL, London, UK), using the Affymetrix GeneChip Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA, USA). Data were read using the Affymetrix package in R (v 2.11.0) and annotated using Bioconductor 2.8. Arrays were normalized with the RMA method in Expression Console 1.1 and annotated with corresponding human orthologue annotation based upon the Mouse Genome Informatics database (http://www.informatics.jax.org/). Subgroup assignment was performed based upon nearest-centroid Spearman rank correlation over 0.1, as described [13,17], using published centroid data [18]. Meta-analysis of the mouse tumour signatures in human breast cancers is fully described in the online supplementary material (see Supplementary experimental procedures). MIAME-compliant data are available (ArrayExpress, E-MEXP-3663).

Results

To determine how different cells of origin interact with different initiating genetic lesions to drive tumour heterogeneity, we generated mouse cohorts carrying conditional alleles of Brca2, p53 and Pten together with either K14Cre or BlgCre, which preferentially target tumour formation to basal or lumER<sup>+</sup> cells, respectively [13]. Cohorts of virgin/parous BlgCre animals were established. For additional information about mouse cohorts, cells of origin of the tumours and full tumour details, see supplementary material (Supplementary experimental procedures and Tables S2, S3).

Cell of origin drives tumour phenotype in Brca2-deleted mammary tumours

All three cohorts of mice carrying conditional Brca2 and p53 alleles (K14Cre:Brca2<sup>2f</sup>/p53<sup>f</sup>, virgin BlgCre:Brca2<sup>2f</sup>/p53<sup>f</sup> and parous BlgCre:Brca2<sup>2f</sup>/p53<sup>f</sup>) developed mammary tumours. Median latency was significantly shorter (< 0.0001, log-rank test) in K14Cre:Brca2<sup>2f</sup>/p53<sup>f</sup> (197 ≤ range 47–243) days) compared to unfloxed exons (see supplementary material, Figure S1). Droplet digital PCR (ddPCR) demonstrated that tumours consistently had fewer copies of floxed Brca2 and p53 exons compared to unfloxed exons (see supplementary material, Figure S2). However, the presence of infiltrating immune cells (see supplementary material, Table S2) and likely contamination of tumour samples by other wild-type host cells meant that tumours rarely showed a floxed allele number which approached zero.

BlgCre:Brca2<sup>2f</sup>/p53<sup>f</sup> tumours were classified mainly as either IDC-NSTs (13/29, 44.8%; Figures 1B, 2A–F) or metaplastic spindle cell tumours (MSCTs; 14/29, 48.3%; Figure 2G–L). Tumours were high-grade, with pushing/mixed borders, a high nuclear pleomorphism, little or no tubule formation and a high mitotic index.
Figure 1. Brca2, Pten and Pten:p53-derived tumours have distinct features. (A) Survival curve for K14Cre:Brca2f/f:p53f/f (n = 29), virgin (n = 21) and parous (n = 18) BlgCre:Brca2f/f:p53f/f mice. Only data from animals developing mammary tumours are shown. K14Cre tumours had a significantly shorter latency (p < 0.0001; log rank test). (B) Relative abundance of histological phenotypes in Brca2 tumours arising in distinct cells of origin. IDC-NST, invasive ductal carcinoma of no special type; AME, adeno-myoepithelioma; DCIS, ductal carcinoma in situ; MSCT, metaplastic spindle cell tumour. (C–F) Mitotic index (number of mitotic figures in 10 high-power fields) and percentages of K14-, K18- and p63-positive cells within Brca2 tumours. Bars indicate median values; data points represent individual tumours and are coloured according to histological phenotype. (G) Survival curve for Pten+/− (n = 15), K14cre:Ptenf/f (n = 3), virgin (n = 43) and parous (n = 29) BlgCre:Ptenf/f; and Blgcre:Ptenf/f:p53f/f (n = 12) mice. Only data from animals developing malignant mammary tumours are shown. (H) Relative abundance of histological phenotypes in malignant Pten and Pten:p53 tumours. AME, adenomyoepithelioma; Encysted Papillary, encysted papillary carcinoma; Adenosquamous, metaplastic adenosquamous carcinoma; IDC-NST, invasive ductal carcinoma of no special type; MSCT, metaplastic spindle cell tumour. (I–L) Mitotic index and distribution of K14-, K18- and p63-positive cells within Pten and Pten:p53 tumours. Bars indicate median values; data points represent individual tumours and are coloured according to histological phenotype. Statistically significant differences in t-tests: *p < 0.05, **p < 0.01, ***p < 0.001
(MI; Figures 1C, 2B). Most tumours (25/29; 86%) were positive for keratin 14 and keratin 18 (K14 and K18, expressed in basal or luminal cells, respectively, in the normal mammary epithelium) and were weakly positive for p63. The majority (24/29) were also ER-negative (Figures 1D–F, 2C–F, 21–L). In contrast, histological analysis of K14Cre:Brca2\(^{2\theta}\),p53\(^{2\theta}\) tumours demonstrated that most tumours (7/11; 64%) were malignant AMEs (Figures 1B, 2M–R). All tumours were of high histological grade, with significantly higher MI (Figure 1C; \(p < 0.01\), K14Cre-vs-parous-BlgCre; \(p < 0.001\), K14Cre-vs-virgin-BlgCre, unpaired two-tailed \(t\)-test), and had multifocal necrosis. Tumours were K14/K18-positive, with a distinguishable distribution of K14- and K18-positive cells in abluminal and luminal cell layers, respectively, consistent with the AME diagnosis (Figures 1D, E, 2O, P). Compared with BlgCre tumours, K14Cre tumours had stronger p63 staining in significantly more (Figure 1F, \(p < 0.001\), unpaired two-tailed \(t\)-test) cells in each tumour (range 5–90%, predominantly in the abluminal cell layer; Figure 2Q). Of K14Cre tumours in which ER staining was determined, half (48/ 50%) were ER-negative (Figure 2R), three contained \(\leq 5\%\) ER-positive cells, but one had around 40% ER-positive cells. In both BlgCre and K14Cre Brca2 tumours, PR staining was concordant with ER staining, although typically fewer cells were PRA-positive than ER-positive, and fewer still were PRB-positive than PRA-positive. Thus, in some cases, weakly ER-positive tumours were PR-negative (see supplementary material, Table S2). Therefore, targeted deletion of Brca2 and p53 in basal or lumER\(^{\text{neg}}\) cells resulted in tumours with different latencies and histopathological features.

The tumour-initiating lesion determines the phenotype of luminal ER\(^{\text{neg}}\)-origin tumours

Next, we examined K14Cre:Pt\(\text{en}\)\(^{\theta}\), BlgCre:Pt\(\text{en}\)\(^{\theta}\) and BlgCre:Pt\(\text{en}\)\(^{\theta}\):p53\(^{2\theta}\)\&\(^{4\theta}\) mice as well as germline Pt\(\text{en}\) heterozygote mice (Pt\(\text{en}\)\(^{+/}\)). Due to a strong skin phenotype, only 14 K14Cre:Pt\(\text{en}\)\(^{\theta}\) mice could age older than 4 months old. From these, only five mammary tumours were obtained (from four mice). Mammary tumour latencies in K14Cre:Pt\(\text{en}\)\(^{\theta}\) (141–386 days), virgin BlgCre:Pt\(\text{en}\)\(^{\theta}\) [340 (range 128–711) days], parous BlgCre:Pt\(\text{en}\)\(^{\theta}\) [357 (range 245–771) days] and Pt\(\text{en}\)\(^{+/}\) mice [368 (range 100–434) days] were not significantly different (Figure 1G). However, BlgCre:Pt\(\text{en}\)\(^{\theta}\):p53\(^{2\theta}\)\&\(^{4\theta}\) mouse developed tumours [312 (range 139–361) days] significantly faster than parous BlgCre:Pt\(\text{en}\)\(^{\theta}\) mice (\(p < 0.05\), log rank test) and also faster, although not significantly, potentially due to the small sample size, than virgin BlgCre:Pt\(\text{en}\)\(^{\theta}\) mice (\(p = 0.078\), log rank test; Figure 1G).

All tumours had lower expression for Pt\(\text{en}\) floxed exon 4 compared with exon 6 (see supplementary material, Figure S3A, B), confirming recombination of the conditional allele during tumourigenesis. Expression of p53 exon 4 was higher in Pt\(\text{en}\)\(^{\theta}\) tumours, similar or lower in Pt\(\text{en}\)\(^{\theta}\):p53\(^{2\theta}\) tumours and always reduced in Pt\(\text{en}\)\(^{\theta}\):p53\(^{4\theta}\) tumours relative to control spleen (see supplementary material, Figure S3C). This is consistent with Pt\(\text{en}\) loss causing p53 induction in p53\(^{2\theta}\) mice and a dose-dependent reduction in this response following loss of one or two p53 alleles [19]. Again, ddPCR demonstrated that tumours from BlgCre:Pt\(\text{en}\)\(^{\theta}\):p53\(^{2\theta}\)\&\(^{4\theta}\) mice had fewer copies of floxed p53 exons compared to unfloxed exons (see supplementary material, Figure S2). The same caveats regarding infiltrating immune cells apply (see supplementary material, Table S2). For technical reasons, the ddPCR assay could not be performed on the floxed Pt\(\text{en}\) allele.

Pt\(\text{en}\) deletion generated both malignant and benign neoplasms regardless of the origin cell type (see supplementary material, Tables S2, S3). Benign tumours were classified as sclerosing adenosis and benign AMEs (Figure 3A–F), with 5/15 (33%) displaying papillary architecture (see supplementary material, Figure S4A–F). All showed strong K14, K18, p63 and ER staining (Figure 3C–F; see also supplementary material, Figure S4C–J).

In contrast to IDC-NSTs/MSCTs developing from lumER\(^{\text{neg}}\) cells in Brca2/p53 mice, malignant lumER\(^{\text{neg}}\)-origin Pt\(\text{en}\)\(^{\theta}\) tumours were AMEs (4/10; 40%) or metaplastic adenosquamous carcinomas (ASQCs; 4/10; 40%), with two additional tumours showing both elements (Figures 1H, 3G–R; see also supplementary material, Table S2). A subset displayed papillary architecture (see supplementary material, Figure S4L–Q). Tumours had pushing/mixed borders, central/multifocal necrosis and low/intermediate histological grades with intermediate nuclear pleomorphism, tubule formation and MI (Figure 1I). Metaplastic squamous cells were found in 8/10 (80%) tumours (5–75% cells; Figure 3N). All tumours were positive for K14, K18 and p63 (Figures 1J–L, 3I–K, 3O–Q). Remarkably, strong ER expression was seen in 7/8 (87.5%) of analysed malignant tumours (15–40% cells, Figure 3L, R; see also supplementary material, Figure S5). PRA expression was observed in 8/9 analysed tumours (1–30% cells; see supplementary material, Figure S5). Notably, both malignant Pt\(\text{en}\)\(^{+/}\) and K14Cre:Pt\(\text{en}\) tumours had similar phenotypes to lumER\(^{\text{neg}}\) cell-origin tumours (see supplementary material, Table S2).

Addition of p53 conditional alleles into the BlgCre:Pt\(\text{en}\) cohort increased the ratio of malignant:benign tumours, with 20/21 (95%) tumours being malignant (see supplementary material, Table S2). It also shifted the spectrum of histopathological phenotypes closer to that seen with BlgCre:Brca2\(^{2\theta}\),p53\(^{2\theta}\)\&\(^{4\theta}\) (Figure 1B, H), as half of all BlgCre:Pt\(\text{en}\)\(^{\theta}\):p53\(^{2\theta}\)\&\(^{4\theta}\) tumours were classified as either IDC-NSTs (5/20; Figure 4A–F) or MSCTs (6/20; Figure 4G–L). The remainder were diagnosed as malignant-AMEs (2/20, 10%; Figure 4M–R), ASQCs (4/20, 20%) or mixed tumours (3/20, 15%).

As opposed to the low/intermediate histological grades of Pt\(\text{en}\) tumours, BlgCre:Pt\(\text{en}\)\(^{\theta}\):p53\(^{2\theta}\)\&\(^{4\theta}\) carcinomas showed high histological grade, with high
nuclear pleomorphism, lack of tubule formation and high MI (Figure 1I). Tumours had mixed borders, with central/multifocal necrosis. Spindle (5–100%) and squamous (1–50%) metaplastic cells were seen in all tumours. All were K14/K18-positive (Figure 1J, K), but staining tended to be at low levels in IDC-NSTs (5–25%; Figure 4C, D), at very low levels in MSCTs (1–10%; Figure 4I, J) and at the highest levels in AMEs (40–60%; Figure 4O, P). p63 staining was positive in 18/20 (90%) tumours (1–80% cells) (Figures 1L, 4E, K, Q). Again, IDC-NSTs and MSCTs had few p63-positive cells and these were scattered through
Figure 3. *Pten* depletion in luminal progenitors generates benign and malignant tumours. (A–F) Features of BlgCre:*Pten* flox/flox benign AME: (A, B) low-power (A) and high-power (B) H&E-stained sections; (C) strong K14 staining in proliferating basal cells encasing the glandular structures and in spindle cells; (D) strong K18 staining in luminal cells; (E) strong p63 staining in basal cells; (F) strong ER staining in luminal cells. (G–L) Features of BlgCre:*Pten* flox/flox malignant AME: (G) low-power H&E-stained section showing multifocal necrosis and pushing borders; (H) high-power H&E-stained section showing gland-like structures and expansion of the abluminally located cells – note increased nuclear pleomorphism; (I) strong K14 staining in proliferating basal cells and in spindle cells; (J) strong K18 staining in luminal cells; (K) p63 and (L) ER staining; note the similarity of expression pattern of p63 and ER. (M–R) Features of a BlgCre:*Pten* flox/flox tumour, showing an adenosquamous carcinoma clone originating from an AME: (M) low-power H&E-stained section showing multifocal necrosis and mixed borders; (N) high-power H&E-stained section showing metaplastic squamous elements emerging from AME area; (O) K14 staining in neoplastic basal-like cells; (P) K18 staining in neoplastic luminal cells; (Q) strong p63 expression in epithelioid cells; (R) ER staining in neoplastic epithelioid cells. Bars = (A, G, M) 1.5 mm; (B–F, H–L and N–R) 100 μm. Inset boxes are magnified ×3. (See also supplementary material, Tables S2, S3, Figures S3, S4).
Figure 4. p53 loss alters tumour phenotypes in Pten knockout mice. (A–F) Features of a BlgCre:Ptenc/f:p53f/f IDC-NST: (A) low-power H&E-stained section showing central necrosis and mixed borders; (B) high-power H&E-stained section showing aberrant proliferation of highly pleomorphic neoplastic epithelioid cells; (C) K14 staining in neoplastic epithelioid cells; (D) K18 staining in neoplastic epithelioid cells; (E) p63 and (F) ER; note staining of epithelioid neoplastic cells. (G–L) Features of a BlgCre:Ptenc/f:p53−/− metaplastic spindle cell carcinoma: (G) low-power H&E-stained section showing multifocal necrosis and mixed borders; (H) high-power H&E-stained section showing abundant spindle cells; (I) lack of K14 expression in spindle cells; (J) K18 expression in small nests of epithelioid cells, no K18 staining in spindle cells; (K) p63; (L) tumour cells are negative for ER. (M–R) Features of a BlgCre:Ptenc/f:p53−/− malignant AME: (M) low-power H&E-stained section showing multifocal necrosis and pushing borders; (N) high-power H&E-stained section illustrating heterogeneous neoplastic populations; (O) K14 staining in abluminal cells; (P) K18 expression in pseudo-luminal cells; (Q) strong p63 expression in proliferating abluminal cells; (R) ER expression in cells located in the abluminal layer. Bars = (A, G, M) 1.5 mm; (B–F, H–L and N–R) 100 μm. Inset boxes are magnified ×3. (See also supplementary material, Tables S2, S3, Figures S5, S6)

the tumour, whereas AMEs had high levels of p63 staining organized into distinct abluminal epithelial layers and 'nests' of p63-positive neoplastic cells (Figure 4E, K, Q). ER staining was intense and frequent in tumour cells in 17/20 (85%) carcinomas (1–30% cells; see supplementary material, Figure S5) including IDC-NSTs (Figure 4F, R), but was either absent or expressed at low levels in MSCTs. Like the Brca2 cohorts, PR staining was concordant with ER staining, with fewer cells PRA-positive than ER-positive, and fewer still PRB-positive than PRA-positive (see supplementary material, Figure
Luminal ERneg-origin tumours display diverse molecular profiles determined by the initiating genetic lesion

We performed whole-transcriptome analysis of a subset of tumours from each genotype (including a previous collection of K14Cre:Brca1f/f:p53+/− and BlgCre:Brca1f/f:p53+/− tumours) [13], using the Affymetrix MouseChip Genome platform. Unsupervised hierarchical clustering showed that the tumours broadly clustered into three molecular groups (Figure 5). One included the Brca2:p53 and Pten:p53 tumours; the second group consisted of most of Pten-only tumours; the third group included Brca1:p53 and some Pten tumours. Pairwise SAM comparisons between groups delivered a list of significantly-associated genes, which were interrogated for GO terms and KEGG pathway analysis (see supplementary material, Table S4). The Brca2:p53/Pten:p53 group (group 1) and the Brca1:p53 group (group 3) genes were highly enriched for GO Bioprocess annotations associated with transcription, metabolism, biosynthesis and regulation of cell death. In contrast, the group 2 (Pten) genes were enriched for development, homeostasis, signalling and regulation of cell death bioprocesses and expressed genes involved in ‘response to hormone stimulus’ and ‘steroid metabolic process’. Pathway analysis showed a great similarity between all tumour groups (see supplementary material, Table S4), although with some differences. For instance, group 1 was enriched for genes associated with adhesion, junctional complexes and JAK–STAT signalling pathways, group 2 with genes associated with calcium signalling and vascular smooth muscle pathways, and group 3 with genes associated with the cell cycle and DNA replication pathways. Interestingly, genes for cysteine and methionine metabolism pathways were enriched in groups 1 and 3, while genes for glycine, serine, threonine and tyrosine metabolism pathways were enriched in group 2, suggesting fundamental differences in the metabolism of these tumour groups.

Importantly, these molecular clusters were determined by the initiating genetic lesion (Figure 5C), with expression profiles being consistent across tumours carrying the same initiating lesion. Tumours with different lesions were not randomly interspersed, neither did tumours cluster by Cre promoter. Thus, the tumour molecular profile was governed by its initiating genetic lesion, not by the cell to which those lesions were targeted.

Luminal ERneg cells generate basal-like, ‘normal breast-like’, luminal A and luminal B tumours

We next asked which human breast cancer molecular subtypes the mouse tumours of this analysis most closely resembled, using a single sample predictor gene set (SSP) [18] (Table 1, Figure 5E; see also supplementary material, Table S5). Consistent with their lack of ER expression, 9/13 (70%) Brca2:p53 mouse tumours classified as basal-like using the PAM50 gene set, irrespective of whether they were from the K14Cre or BlgCre cohorts. Of the Pten tumours, 17/21 (81%) tumours were categorized as ‘normal breast-like’, three tumours classified as luminal A and one as basal-like. Conversely, Pten:p53 tumours were classified as luminal B (4/10), ‘normal breast-like’ (3/10), luminal A (2/10) and one could not be assigned to any subtype. Differences in the proportions of the predominant subtypes within each genotype were highly significant ($p < 0.0001$, $\chi^2$ test) in pairwise genotype comparisons (Table 1).

PAM50 analysis is sensitive to sample cohort normalization issues [17]. We therefore interrogated different human breast tumour transcriptome datasets [1,3,18,21–23], including three enriched for BRCA1/2 mutation carriers [24–26], using mouse transcriptome signatures. We built three mouse molecular signatures based upon the top probes up- and down-regulated within each mouse group (Pten only; Brca1:p53 only; combined Brca2:p53/Pten:p53), identified by SAM pairwise comparisons. Signatures were applied to each sample from each dataset. Correlation heat maps for the mouse transcriptome signature in the human datasets (Figure 5F; see also supplementary material, Figure S8) confirmed, first, that the Brca1:p53 mouse signature was associated with the human basal-like subtype and with human BRCA1 breast cancers; second, that luminal A, normal breast-like and non-BRCA1/2 cancers were enriched in breast cancer samples with a gene signature similar to the Pten mouse tumours; and third, that the Brca2:p53/Pten:p53 signature was observed across the range of human breast cancer molecular subtypes. Notably, when testing human breast cancer datasets that included the claudin-low subtype, a particular enrichment for the Brca2:p53/Pten:p53 signature was
obtained in this group. The \(Brca2:p53/Pten:p53\) signature was not enriched in human \(BRCA2\) tumours; indeed, in one study [25] the association was with \(BRCA1\) tumours.

These results showed that tumours deriving from the same cell of origin, lum\(ER^{\neg}\) cells, not only had very different molecular features, depending on the initiating genetic lesions, but also spanned a broad range of human-equivalent molecular signatures. Hence, the ‘intrinsic subtype’ classification of a tumour does not necessarily reflect its cell of origin.

Luminal \(ER^{\neg}\) cells generate ‘claudin-low’ tumours

The claudin-low subtype is not distinguished by the PAM50 gene set. This subtype is characterized by
up-regulation of mesenchymal-associated genes and down-regulation of genes related to epithelial cell–cell junctions, particularly claudins CLDN3, −4 and −7 and CDH1 [22]. As a ‘mesenchymal-like’ appearance was typical of the MSCTs from our tumour cohorts, and enrichment for the Brca2:p53/ Pten:p53 signature, both tumour genotypes with high numbers of MSCTs, was observed in the claudin-low subtype in breast cancer datasets which included that group, we analysed expression of Cldn3, 4 and 7 and Cdh1 across the tumour panel categorized by histological phenotype. The results confirmed that MSCTs had significantly lower expression levels of these four genes compared to other tumour types (Figure 6), and indeed of the whole tumour panel categorized by histological phenotype [22] (see supplementary material, Figure S9). This demonstrates that the transcriptomic signature of MSCTs recapitulates that of claudin-low tumours, suggesting that this tumour type can also originate from lumERneg cells.

Human metaplastic tumours have variable PTEN expression but express low claudin levels

Using a pilot cohort of human breast cancers, including some very rare human AMEs, we examined whether there was an association between PTEN expression and the human histological phenotypes equivalent to those in our mouse cohorts. We found that staining of metaplastic tumours and IDC-NSTs was variable, but the few human AMEs we examined were very strongly PTEN-positive (see supplementary material, Table S6, Figure S10).

We also examined the same tumour group for expression of CLDN3, CLDN4 and CDH1. Unlike PTEN staining, these results were concordant with the mouse data, as human IDC-NSTs expressed high protein levels, but there was absence of expression in non-epithelial areas of spindle-cell carcinomas and metaplastic carcinomas with mesenchymal differentiation (see supplementary material, Table S6, Figure S10).

Discussion

Inter-tumour heterogeneity must arise from different (epi)genetic lesions occurring in different cells of origin. Here, we have applied histopathological and molecular pathology approaches to analyse tumours arising in genetically engineered mouse models from different initiating lesions in distinct cells of origin. We show that, in our model system, targeting tumour-initiating lesions to basal cells results primarily in adenomyoepitheliomas, whereas targeting lumERneg cells results in tumours with a range of histopathological features including metaplastic tumours and invasive ductal carcinomas [4]. Importantly, we have generated both ER-positive luminal-like and ER-negative basal-like tumours from this target population. We have also shown that the initial genetic lesion is the prime determinant of the molecular profile of the subsequent tumours arising from these cells. This suggests that, rather than being a truly stochastic process, the aetiology of tumour formation is largely deterministic and depends on the earliest events in carcinogenesis (ie the founder genetic/epigenetic events).

Germline mutations in human BRCA2 predispose to breast and ovarian cancers [27]. Although 66–93%
of BRCA2-associated human cancers are ER-positive in > 10% tumour cells [28,29], only 3/15 (20%) of the Brca2 IDC-NSTs described here were ER-positive (1–10% tumour cells). As the same cells of origin could generate ER-positive IDC-NSTs in the Pten:p53 model, this is not likely explained by a lack of potential to differentiate along this lineage. Moreover, most of the Brca2 mouse tumours had a molecular profile similar to human basal-like breast cancers (Table 1, Figure 5F) and did not resemble a typical human BRCA2 tumour profile (see supplementary material, Figure S8). It should be noted, however, that a subset (13–19%) of human BRCA2-mutated breast cancers have a basal-like molecular profile and are also ER-negative [30], which would be consistent with the similarity of the Brca2:p53/Pten:p53 signature to human BRCA1 tumours in data from one study [25]. It is possible that BtgCre:Brca2:p53 tumours model the basal-like subset of human BRCA2 breast cancers.

Loss of PTEN expression is recurrent in human breast cancers, in both basal and luminal subtypes [3]. In our study, targeting conditional depletion of Pten alone to mouse lumERneg-cells resulted in a different effect to Brca1/2:p53 loss, leading to the development of benign and malignant AMEs and ASQCs. Notably, AMEs were highly differentiated and ER-positive. In contrast, analysis of a pilot cohort of human breast cancers, including very rare human AMEs, found strong PTEN expression in these tumours. Larger numbers are required to confirm these findings, but they suggest that human AMEs are not associated with somatic PTEN loss, unlike in the mouse. Notably, however, breast tumours from germline PTEN loss-syndrome families are enriched for molecular apocrine differentiation, which is characterized by elevated levels of androgen signalling [31], and our mouse Pten tumour cohort also expressed high levels of the androgen receptor (see supplementary material, Table S4). The mouse tumour phenotypes were altered when conditional Pten:p53 alleles were combined, resulting in the development of MSCTs and ER-positive IDC-NSTs. In these tumours, the molecular changes observed (loss of claudins in MSCTs) were reflected in the equivalent human tumours.

Our findings show that a broad spectrum of tumour phenotypes can emerge from the lumERneg cell population, they suggest that p53 loss-of-function is a
prime driver of histopathological phenotype, and they demonstrate that, in contrast, cell of origin is not a strict driver of tumour phenotype. Our results are consistent with the notion that mammary tumour heterogeneity is a result of context-dependent interactions between cell of origin and early genetic hits. In the K14Cre basal-origin tumours we describe, the AME phenotype is the default tumour type, irrespective of the driving genetic lesion. Conversely, lumER\textsuperscript{neg} cells are able to generate a broad spectrum of tumour histological and molecular phenotypes, including highly aggressive ER/PR-negative and ER/PR-positive neoplasms. Since tumours had long latency periods, and additional genetic mutations must have arisen in all genetic backgrounds to permit tumour formation, the stability of histological phenotypes within each genetic background was notable. Either any additional genetic hits were stochastic and had little effect on overall tumour phenotype, or each cell of origin/genetic background combination developed a set of stereotypical lesions that contributed to the tumour phenotype. Future massively-parallel sequencing studies may lead to a deeper understanding of the mutational changes in these genetic backgrounds.

Interestingly, other groups described K14Cre-driven models (K14Cre:Brea\textsuperscript{f/f};p53\textsuperscript{f/f} and K14Cre:Ecad\textsuperscript{f/f};p53\textsuperscript{f/f}) [32,33], in which the predominant tumour phenotype was not an AME but rather a more typical luminal-like tumour. We have discussed this issue previously [13] but our current results support a model in which the Brea\textsuperscript{1} and Ecad alleles used by Liu and colleagues and Derksen and colleagues are dominant over the K14Cre cell of origin in driving tumour phenotype, in a way in which the alleles we have used are not.

Our study has important limitations. While the BlgCre transgene preferentially drives tumour formation in lumER\textsuperscript{neg} cells, we cannot definitively exclude that promoter ‘leakiness’ may, in a modest number of cases, result in tumours originating from other cell types, or that initial gene deletions may affect cell differentiation and thus alter the phenotype of the cell that finally transforms (see supplementary material, Supplementary experimental procedures); whereas equivalent mouse and human mammary epithelial cell types can be inferred (ie cells which are luminal or basal, ER-positive or ER-negative), the cell types in which allele recombination occurs in the mouse have not been directly mapped to human cell types; the mouse strains we have used, while mainly on a C57Bl6 background, are not pure-bred (see supplementary material, Supplementary experimental procedures) and there may be background strain-specific alleles linked to the conditional alleles which could affect tumour phenotypes; in our models, and in all current mouse models involving more than one conditional allele, it is not possible to control the order in which allele recombination occurs; and finally, we have not yet observed tumours that resemble sporadic human ER-positive IDC-NSTs with a luminal A molecular profile. We hypothesize that either lumER\textsuperscript{neg} progenitors will need to be targeted as the cell of origin for this tumour type, or that these tumours are simply too indolent to be modelled within the mouse lifespan. In general we note that, while mouse models are important as models of breast cancer, mice are not humans and caution must be exercised in extrapolating results between species, as is illustrated by the case of PTEN expression in AMEs.

Despite these limitations, this study does provide a fundamental advance in our understanding of the origins of mammary tumour heterogeneity. We provide multiple lines of evidence to demonstrate that the phenotype of a cancer is not a mere reflection of its cell of origin, calling into question conclusions about the histogenesis of malignancies derived from histopathological, immunophenotypical and transcriptomic analyses of fully developed tumours.

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Author contributions

LM and GM bred mouse lines, carried out post-mortem analyses, collected tumours, prepared RNA, carried out qPCR and bioinformatics analyses and assisted with experimental design, analysis of pathology and writing of manuscript; FAM carried out histopathological staining and assisted with its interpretation; HK genotyped mice, assisted with qPCR gene expression analysis and interpretation of qPCR data; DN-R and FM reviewed histopathological material; DR optimized and carried out double immunofluorescence staining for p63 and ER and assisted with its interpretation; KG optimized and carried out PRA and PRB staining and assisted with its interpretation; MA, MAL-G and JP carried out staining and analysis of human tissue samples; AM assisted with qPCR and bioinformatics analyses, collected tumours, prepared RNA, carried out double immunofluorescence staining for p63 and ER and assisted with its interpretation; JSR-F analysed mouse tumour pathology, advised on its interpretation and assisted with writing the manuscript; and MJS designed the study, assisted with analysis of tumour pathology and bioinformatic analysis and wrote the manuscript.

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SUPPLEMENTARY MATERIAL ON THE INTERNET

The following supplementary material may be found in the online version of this article:

Supplementary experimental procedures

Figure S1. Expression of Tp53 and Brca2 in Brca2\textsuperscript{-/-} p53\textsuperscript{-/-} mouse tumours

Figure S2. ddPCR analysis of conditional allele recombination in tumours

Figure S3. Expression of Pten and Tp53 in Pten\textsuperscript{-/-} and Pten\textsuperscript{-/-} p53\textsuperscript{-/-} mouse tumours

Figure S4. Papillary features in benign and malignant AMEs of Pten tumours

Figure S5. Features of benign Pten and Pten p53 tumours and comparison of ER staining in malignant Pten and Pten p53 tumours. Comparison of ER, PR-A and PR-B staining

Figure S6. Co-localization of p63 and ER in malignant but not benign adenomyoepitheliomas
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