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Somatic base editing to model oncogenic drivers in breast cancer.

Human cancer is a disease of cooperating genetic events that is complex to model in vivo. A new study combines somatic base editing with a mouse model of breast cancer demonstrating the potential to rapidly investigate the function of disease specific point mutations.

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Today, a plethora of genetically engineered mouse models (GEMMs) exist that have introduced transgenes, specific mutations and gene knockouts whose expression can be triggered in both a spatial and time controlled manner ¹. These models have contributed a vast amount to our knowledge of homeostatic and disease processes, but the establishment and maintenance of GEMMs remains expensive and time consuming. Furthermore, the transmission of genetically altered alleles frequently involves surplus generation of mice with undesired genotypes. These considerations can render GEMMs impractical for budget and time-constrained projects.

For a number of years the Jonkers' lab have been leading the way in developing and refining mouse models of breast cancer to recapitulate the human disease. One such approach models *BRCA1* mutant breast cancer whereby conditional loss of *Brca1* and *Trp53* is driven specifically in the mammary epithelium by virtue of the whey acid protein Cre recombinase (WapCre). In this genetic context female mice develop mammary tumours with median survival of 198 days (range~100-260 days)². These tumours represent the triple negative breast cancer subtype as they lack expression of estrogen, progesterone and human epidermal growth factor receptors (ER, PR, HER2). The co-operative impact of MYC-overexpression in this model was elegantly demonstrated through addition of a germline MYC transgene that significantly accelerated disease onset². Importantly, this MYC- driven acceleration of *Brca1;Trp53* disease could also be achieved using somatic delivery of a lentivirus encoding MYC directly into the mammary gland by intraductal injection² (Fig 1A). Having already shown that intraductal delivery of small guide RNAs (sgRNAs) could effectively target germline encoded Cas9 nucleases to tumour suppressors such as *Pten* (Fig 1B), this somatic approach was used to introduce coincident MYC overexpression and PTEN loss (Fig 1C), which decreased latency of mammary tumorigenesis even further ^{2,3}. This methodology allowed the study of simultaneous somatic over-expression/loss of function specifically in the injected mammary gland, and in the context of pre-defined genetically engineered alleles. This work marked a technological advance in line with the 3R's approach but also reduced cost and time.

Whilst breast cancer is thought to be largely a disease of copy number alteration, pathogenic point mutations have also been identified as drivers of the disease ^{4,5}. Robust methods for studying the function of point mutations in mice include knock-in expression of mutant genes, which again entails significant cost and time. To circumvent this, and building on their previous work, Jonkers and colleagues have taken advantage of recently developed base editing enzymes to streamline the introduction of a range of somatic point mutations *in situ* ⁶. This involved generating a new transgenic mouse engineered to express a cytosine base editor, BE3 (developed by the Liu lab ⁷) in response to activation with Cre-recombinase ⁶. BE3 is a hybrid protein composed of *Streptococcus pyogenes* Cas9 nickase (SpCas9^{D10A}) fused with rat APOBEC1 cytosine deaminase and a uracil glycosylase inhibitor domain that can introduce targeted C-T base mutations when guided to genomic loci by specific sgRNAs ⁷. This transgene was inserted into the *Col1a* locus of *WapCre Brca1^{f/f};Trp53^{f/f}* ES cells using a GEMM-ESC approach ⁸ where the resultant mice have concomitant BE3 expression along with loss of BRCA1 and p53 in the mammary epithelium⁶.

To test the ability of BE3 to introduce nucleoside substitutions *in situ*, the authors focussed on the PI3-kinase/Akt pathway (normally antagonised by PTEN), which is frequently altered in breast cancer ⁹. Through this transgenic BE3 system oncogenic E17K missense mutations in AKT1 (Fig 1D) or missense mutations in PIK3CA (the catalytic subunit of PI3K), such as the hotspot mutations E542K and E545K (and the rarer E452K mutation), were introduced somatically by sgRNA intraductal delivery and all reduced the latency of *WapCre Brca1^{f/f};Trp53^{f/f}* Lenti-Myc tumour development. Target C-to-T conversion rates were ~60-80% in end-stage tumours and analysis by immunohistochemistry, western blot and RNA sequencing showed alteration in downstream pathways as expected by interference with AKT1/PI3K. Delivery of a Lentivirus encoding tandem *Pik3ca* and *Trp53* targeting sgRNA allowed editing at both loci and demonstrates the potential to rapidly model multiple co-operative events *in situ*. Importantly, as a control for off-target effects, introduction of a non-inactivating PIK3CA mutation - that should have neutral effect on PI3K activity - did not alter tumour latency. Presumably this is because this mutation provides no selective advantage and therefore this would be less well represented in the end stage tumour.

In theory, the modified Cas9 used here with nickase activity should avoid introduction of double strand breaks that can lead to indels and translocations. However, whilst attempting bi-allelic targeting of an sg*Pten*^{Q245} nonsense mutation the authors noted that indels were occurring and that *Pten*^{Q245} base edits never occurred at both alleles, instead either this edit was observed at one allele and a frame-shift indel at the other allele, or both alleles had the indel ⁶. To probe this further they addressed the impact of targeting a premature stop codon into the *Trp53* tumour suppressor gene in a context of *Trp53* heterozygosity. This revealed that when point mutations were targeted to tumour suppressors such as *Pten* or *Trp53*, unintended insertions and deletions at the targeted loci were actually selected for in this tumorigenic context. Other unintended mutations also occurred: bystander edits at local cytosines were

frequent in all models tested but in most cases this did not introduce a change in amino acid sequence. Furthermore, these bystander edits were not exclusively C-T with C-A and rarer C-G also being observed ⁶.

Although whole genome sequencing in 3T3 cells did not show potential pathogenic off-target effects of BE3 in the Annunziato study, BE3 editing at distal locations has been characterised in other systems and can occur in proto-oncogenes and tumour suppressors ^{10,11}. Whole genome sequencing is required to detect distant mutations and is a low-throughput process in heterogenous cell populations. Concerns about off-target effects are less relevant when modelling co-operative oncogenesis in the triple negative breast cancer model as *Brca1/Trp53* null tumours have defective DNA repair pathways with heavily mutated heterogenous genomes ². However, the prominence of unexpected indels when targeting tumour suppressors, such as PTEN and p53, in this study shows that unintended alterations that favour tumour development will be strongly selected for. It is possible that new oncogenic players relevant to triple negative breast cancer could be identified by enrichment for off-target editing in this model.

Progression in the field of base-editing is rampant, in the time it has taken to make the BE3 transgenic mouse and perform these studies further generations of base editors have been engineered that have fewer off target effects and also allow A-G conversion ^{12,13}. The work by Annunziato *et al.*, paves the way for incorporation of these newer base editors into *in situ* models of tumorigenesis. Furthermore, recently developed search and replace genome editing systems could also be developed for *in situ* use in GEMMs ¹⁴. Similarly, as future generations of RNA base editors that alter amino acid sequence without genome alteration are developed ¹⁵, precise, flexible and reversible modification may be achieved in pipelines based on the Jonkers format. This methodology is scalable and adaptable to many cell and tumour types through use of alternative Cre, GEMMs and sgRNA delivery method or site (Fig 1E). The advantage of orthotopic delivery into the mammary gland elegantly facilitates these approaches to model breast cancer; but it is exciting that with the advent of increased orthotopic delivery systems (eg colon ¹⁶) this should permit the wider application of base editing in modelling other tumour types.

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Fig. 1 | An expanding toolbox for modeling genetic aberrations in breast cancer. Schematic showing recent developments that combine germline encoded alterations in genetically engineered mouse models (GEMMs) with somatically altered genetics via mammary intraductal delivery of lentiviral vectors. This work creates a platform for future developments encompassing new types of nucleic acid editors and can be adapted for use in a variety of GEMMs to rapidly model the function of a range of genetic alterations in cancer. Future developments could include new cytosine base editor variants; adenine base editors; search-and-replace editing; RNA base editors; other cell type-specific Cre; inducible Cre; GEM that model other tumor types; and alternate delivery sites/systems.

■ Germline genetics
■ Somatic genetics

<i>WapCre</i> <i>Brca1^{fl/fl}</i> <i>Trp53^{fl/fl}</i> + Lenti-Myc ↓ Increased gene expression ²	<i>WapCre</i> <i>Cdh1^{fl/fl}</i> <i>Cas9</i> + Lenti-sg <i>Pten</i> ↓ Gene knockout ³	<i>WapCre</i> <i>Brca1^{fl/fl}</i> <i>Trp53^{fl/fl}</i> <i>Cas9</i> + Lenti-sg <i>Pten</i> ↓ Gene knockout and increased gene expression ²	<i>WapCre</i> <i>Brca1^{fl/fl}</i> <i>Trp53^{fl/fl}</i> <i>BE3</i> + Lenti-sgAkt ^{E17K} -Myc ↓ Point mutation C-T ⁶
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