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Case study

# Long-term multi-modal recording reveals unpredictable non-genetic adaptation routes in dormant breast cancer cells

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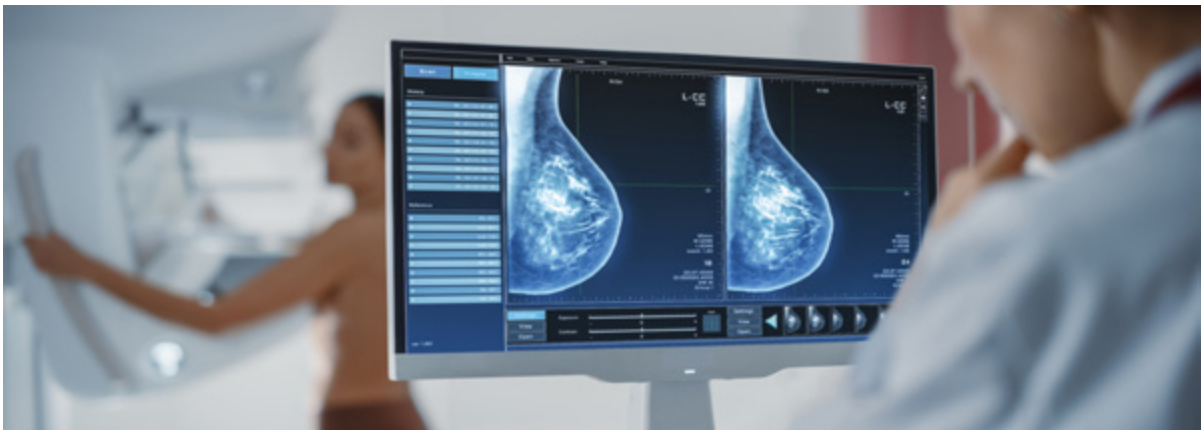

**Research objective:**

To decipher the mechanisms of adaptation to endocrine therapy in hormone dependent breast cancer (HDBC). To establish whether there are genetic or phenotypic aberrations involved in the awakening of dormant HDBC cells after treatment with adjuvant endocrine therapies.

**Sample collection:**

HDBC model cell lines MCF-7 and T47D cells were grown without passaging in High Yield PERformance Flask (HYPERflask) cell culture vessels and maintained either in 100nM 4-Hydroxytamoxifen or phenol red-free DMEM supplemented with 10% charcoal stripped FCS (oestrogen deprivation to mimic aromatase inhibitor treatment). Cells were monitored twice a week for apparent growth changes and harvested upon awakening. The untreated arm of TRADITIOM was maintained in oestradiol-supplemented media and underwent serial passaging. Untreated cells were collected at the corresponding collection points of the treated arm.

Patient biopsies were collected at diagnosis, during treatment, and at tumour progression and local relapse. Extraction of DNA from fresh frozen samples was carried out using DNeasy Blood and Tissue kit. Extraction from FFPE samples was conducted using GeneRead DNA FFPE Kit.

**Sequencing strategy:**

Genomic barcode sequencing was carried out on libraries prepared from PCR products with the barcode cassette using NovaSeq6000 platform (150 bp-paired end). Whole genome sequencing (WGS) was carried out and samples were prepared using a PCR-free library protocol and sequenced on Illumina NovaSeq 6000 using 150 bp paired-end sequencing with 30X coverage. FFPE and fresh frozen patient samples were sequenced using the NovaSeq S2 50bp paired end protocol with 100X coverage. RNA sequencing samples were prepared using a polyA enrichment protocol and sequenced on Illumina NovaSeq 6000 using 150 bp paired-end sequencing. scRNAseq libraries were prepared following chromium Single Cell 3' Reagent Kits User Guide (v3.1 Chemistry) from 10X genomics and sequenced on Illumina NovaSeq 6000 using 150 bp paired-end sequencing with 300M reads per sample.

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**Background:**

Hormone dependent breast cancers (HDBCs) are routinely treated with adjuvant endocrine therapies (ETs) such as tamoxifen or aromatase inhibitors (AI) to abrogate oestrogen signalling. This treatment induces dormancy in cancer cells, a state characterised by cell cycle arrest and inhibition of replicative processes. These cells can reawaken from dormancy to facilitate relapse and metastasis. It has been previously shown that in 60% of relapse cases, there is no underlying genetic mutation in known cancer drivers that could explain drug resistance (Razavi et al., 2018). Even with this extensive research, whether these mutations exist before dormancy or are acquired *de novo* before awakening remains unclear. Due to the downregulation of DNA replication in dormant cells, which is required for Darwinian evolution and the acquirement of mutations, the hypothesis of this study is that there are non-genetic factors responsible for the reawakening of dormant cells.

**Results:**

*Samples from relapsed patients did not exhibit de novo mutations in breast cancer drivers*

A patient with bilateral metastatic HDBC undergoing long-term primary ET (with no surgery) responded to therapy in 6 months, followed by 12 months of stable minimal residual disease (putative dormancy). After 48 months however, both tumours progressed and awakened whilst under therapy. The patient opted for surgery at this point and samples were analysed using WGS. Focusing on known pathogenic single-nucleotide variants (SNVs) in breast cancer drivers did not identify any SNVs compatible with progression. Analysis of 4 other cases with similar clinical history, even with less stringent mutational filters, did not identify *de novo* breast cancer drivers at the time of tumour awakening. The results from these experiments supported the hypothesis that tumour awakening may be caused by non-genetic factors and led to the establishment of the TRADITIOM study for the *in vitro* analysis of awakening events.

*TRADITIOM study*

TRADITIOM (**TR**acking **A**daptation, **D**ormancy and awaken**I**ng with mul**ti**-**OM**ics) was developed as a long-term trackable *in vitro* study to analyse the role of non-genetic and genetic adaptive processes involved in tumour awakening. 100,000 differentially barcoded MCF7 HDBC cells were expanded to a founder population (POT) of 90 million cells, resulting in differential representation of the 100,000 barcodes across the population. The low multiplicity of infection (MOI) of the system ensured that each cell only contained one barcode. 56 trackable HYPERflasks were set up, each containing 2 million cells from the founder population, with a 90-95% overlap in barcode composition, demonstrating the highly comparable starting conditions across all HYPERflasks. Cells within these carbon copy HYPERflasks were exposed to either aromatase inhibitor (AI) or tamoxifen (TAM) and not subjected to passaging for the 6-month duration of the experiment. Remaining cells were left untreated (UT) and serially passaged throughout the experiment or collected as time zero (T0) to determine the initial overlap between barcode composition in the different carbon copies. The design of this experiment reduced the impact of confounding factors that are often associated with lineage tracing studies, such as cell passaging and short-term follow up. The untreated arm of the experiment was passaged to explore the changes passaging can induce in the genotype and phenotype of cells.

*Awakening occurs in an asynchronous fashion*

Each carbon copy cell population treated with ET exhibited an initial growth phase followed by a decrease in total cell number, leading to cell dormancy after 30 days. This dormant state persisted for 90 days in the AI treatment arm and 60 days in the TAM treatment arm. Despite the homogeneous starting populations within the HYPERflasks, cells were shown to awaken in an asynchronous manner across both treatment arms. To further assess the adaptive trajectories, cells were passaged for an extra 30 days and their response to ET conditions recorded. These cells were named Terminal End Point (TEP), reflecting relapses in the clinical setting. Carbon copies within both the TAM arm and AI arm had radically different responses to continued treatment exposure, suggesting that the phenotypes associated with awakening are also highly divergent.

*Awakening occurs in distinct lineages and is not driven by recurrent de novo genetic hits*

3500, 4000 and 15000 surviving barcodes were represented in awakening cells across untreated, AI and TAM arms, respectively, suggesting that awakening is not mediated by a small set of resistant cells, but many different cell lineages can contribute to it over time. As the cells approached TEP, 1 or 2 "winner" barcodes swept through the AI and TAM treatment populations . 11 of the 13 carbon copies that awakened and reached TEP had individual unique winner barcodes, demonstrating that awakening is not driven by a pre-existent clone. Analysis of known breast cancer drivers and non-coding regions using WGS provided no evidence of a recurrent *de novo* genetic incident in awakening samples. Together these results support the hypothesis that awakening is not mediated by a genetic event and is stochastic in nature. WGS also ruled out a common pre-existing genetic driver across carbon copies.

*"Once lineages have passed the dormancy bottleneck, given sufficient time they all have a chance of awakening."*

Barcode analysis of cells subsampled from the founder population (POT ) and T0 (time zero-initial) populations indicate that initial barcode frequency may influence the likelihood of entering dormancy, however once dormancy is reached, barcode frequency is not a reliable predictor of awakening. Also, the runner-up barcode, present at the second highest frequency at awakening, may outcompete the winner barcode during awakening to TEP transition. This could have significant implications for the metastatic cascade theory, which until now has been thought to be a process of increasingly frequent genetic events in late-stage disease contributing to metastasis.

*The phenotypic heterogeneity of awakened cells indicated divergence*

When cells enter dormancy, their transcriptional profiles were found to be predictable and homogenous across the treatment arms. Gene set enrichment analysis of dormant cells showed downregulation of metabolic activities, the G2M checkpoint and MYC signalling. Dormant cells also activate pro-inflammatory pathways, namely NF-kB and IL-6. When cells awaken and move towards TEP, their transcriptional profiles begin to diverge significantly. Many of the hallmarks of dormancy are reversed and the phenotypes observed are highly heterogenous and unpredictable across awakening lineages and their respective TEPs.

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*scRNAseq analysis revealed cell plasticity and co-existence of dormant and awakening lineages at relapse*

Traditiom Live Single Cell (LSC) combined live imaging, lineage tracing and single cell RNA-seq. Cells were imaged twice a week from the onset of therapy and monitored for awakening events. Cells were harvested at the onset of treatment, dormancy and awakening and analysed by scRNA-seq. Spatial analysis of TRADITIOM LSC showed that awakening events happen in a localised fashion in each individual flask and that individual lineages attempted awakening several times before the final event (failed awakenings). Traditiom LSC confirmed the asynchronous awakening dynamic in response to ET and unique barcode swipes in each individual replicate. The transcriptome of the awakening lineages diverged at awakening, showing that winning lineages acquire alternative phenotypic profiles distinct from dormant ones. Cell state lineage tracing clearly showed that winner and non-winner lineages are indistinguishable before treatment onset. These data support the notion that awakening lineages do not evolve from a common pre-existent epigenetic ancestor. Finally, TRADITIOM LSC revealed a constant level of plasticity in the system, with a subpopulation of the winning lineage returning to or maintaining the dormant cell state.

The dormant epigenome is characterised by the acquisition of repressive histone marks that are lost at awakening in a process similar to epigenetic decay. Further analysis was done to assess the epigenetic state of cells at dormancy and awakening. It was found that dormant cells gained histone methylation markers associated with a heterochromatin state, a repressive state of chromatin that blocks access of transcriptional machinery. These changes are reversed in awakening cells, suggesting that this epigenetic decay may be involved in the transition from dormancy to awakening and be responsible for the apparent stochastic manner by which awakening occurs.

**Discussion & Conclusions**

The findings of this study highlight the absence of a common genetic trigger for the awakening of hormone-dependent breast cancer cells both *in vitro* and *in vivo*, and hint at the responsibility of epigenetic decay in triggering this stochastic event. It proposes the “pop-corn” model for the evolution of HDBC cells in response to therapy. ET induced a widespread non-genetic cell state creating a micro-disseminated dormant pool. Long latency of relapse points against the selection of a pre-existent genetic clone, and the loss of replicative mutational processes in the dormant pool strongly constrains the emergence of *de novo* drivers. Nevertheless, dormancy is unstable, which predicts that all dormant cells will eventually “pop” or awake given infinite time. During awakening, cell plasticity pushes individual awakening cells toward divergent phenotypes, so therapeutically targeting the phenotype of the first awakening cell will have little effect on subsequent awakening clones. These findings offer a new possible therapeutic opportunity for the treatment of HDBC by which targeting cells that enter dormancy, when cell heterogeneity is at its minimum, might prevent relapse.

**Future work**

The TRADITIOM study showed the importance of developing strategies to target cells entering dormancy and future work involves finding actionable targets that can be used to impair the fitness of dormant cells to prevent relapse. For this purpose, work on targeting the dormant epigenetic landscape is currently on-going.