Studying drug resistance in a *Brca1*-deficient mouse mammary tumor model

Thesis of PhD dissertation

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Budapest
2020

1. Introduction

Cancer is the second leading cause of death globally, accounting for an estimated 9.6 million deaths, or one in six deaths, in 2018. Breast cancer is the most common cancer in women worldwide, affecting approximately one in eight women. The breast cancer incidence rate has continued to slowly increase but the overall breast cancer death rate has decreased, attributed both to improvements in early detection (through screening) and targeted therapy.

In case of hormone- or growth factor-dependent breast cancers expressing ER (estrogen receptor), PR (progesterone receptor) and/or epidermal growth factor receptor 2 (HER2), targeted treatment options can significantly increase quality of life and survival. Conversely, triple negative breast cancer (TNBC), accounting for 15-20% of all breast cancer cases, carry a poor prognosis, as these tumors are insensitive to most available hormonal or targeted therapeutic agents. Furthermore, TNBC patients often carry mutations in genes involved in the maintenance of genomic integrity and DNA repair, like *BRCA* genes.

Germline mutations in *BRCA1* are responsible for a large proportion of inherited predispositions to breast and ovarian cancer. Women who carry mutations in *BRCA1* or *BRCA2* face 80% elevated lifetime risk to develop breast cancer by the age of 80.

In lack of targeted therapeutic options, chemotherapy remains the most effective treatment for TNBC. Clinical studies have shown that platinum-based neoadjuvant chemotherapy is highly effective in TNBC patients with *BRCA1* gene mutations and since 2018, patients relapsing after previous chemotherapy in the neoadjuvant, adjuvant, or metastatic setting are eligible to treatment with PARP inhibitors, but developing drug resistance still impedes successful cancer chemotherapy. Several different molecular mechanisms underlying drug resistance have been reported, but none of them resolved the problem of drug resistance in clinical treatment. Complex molecular and cellular processes like epithelial to mesenchymal transition (EMT), the interactions between the tumor and the tumor microenvironment, and the development of metastasis also play a role in cancer drug resistance. *BRCA1*- deficient TNBCs are aggressive subtype of breast cancer with limited therapeutic options, to study TNBCs we need better model systems.

In 2007. Lui et al. created a mouse model in which *Brca1* and *p53* deletions occur only in cytokeratin 14 (CK14) positive epithelial cells. The CK14-Cre driven somatic deletion of *Brca1* and *p53* resulted in solid carcinomas resembling high-grade IDC-NOS (invasive ductal carcinoma not otherwise specified) in humans. Significantly, these mammary tumors are highly proliferative, show ER-negativity and a high degree of genomic instability, similarly to human *BRCA1*-mutated hereditary breast cancers and sporadic basal-like breast cancers.

Over the years, the K14*cre;Brca1*$^{F/F}$*;p53*$^{F/F}$ mouse model of hereditary breast cancer has proved to be a useful tool to study tumor response and acquired therapy resistance of *BRCA1*-deficient breast cancers. *Brca1*$^{-/-}$*, p53*$^{-/-}$ tumors show initial response to therapy, but eventually all tumors acquire resistance to doxorubicin, docetaxel and olaparib but not to cisplatin.

In vitro tumor models are essential to study molecular mechanisms of tumor cell biology. Identification of genetic alterations through whole genome sequencing is an important tool for the characterization of tumor cells, however to analyze sequencing results, the use of isogenic cell lines is recommended. Furthermore, cell lines offer a cost-effective and relatively simple solution for additional genetic modifications, for example via lentiviral transduction, fluorescence protein expressing tumors, that have already undergone genetic modification (e.g., *Brca1* and *p53* deletions) can be established, without developing new genetically engineered mouse models (GEMMs).

My goal was to establish a model system which offer an opportunity to study *BRCA1*-deficient breast tumors, investigate the interactions between tumor and tumor microenvironment, and the molecular mechanisms of drug resistance.

2. Aims

Our overall aim was to study drug resistance mechanisms of *BRCA1*-deficient mammary tumors, using a clinically relevant mouse model of human *BRCA1*-associated hereditary breast cancer, in which *Brca1* and *p53* deletions occur only in $CK14^{+}$ epithelial cells. Our goal was to examine the efficacy of new therapeutic agents, investigate the mechanisms of developing drug resistance and establish a new model system to study drug resistance. The specific aims were the following:

1. Investigating the efficiency of pegylated liposomal doxorubicin (PLD) in a $Brca1^{-/-}; p53^{-/-}$ mouse mammary tumor model.
2. Establishment and characterization of a $Brca1^{-/-}; p53^{-/-}$ mouse mammary tumor cell line.
3. Studying molecular mechanisms of cisplatin resistance in $Brca1^{-/-}; p53^{-/-}$ mouse mammary tumor cell line.
4. Examining the cellular plasticity of *Brca1*-deficient tumor cells.
5. Genetic modification via lentiviral transduction of $Brca1^{-/-}; p53^{-/-}$ tumors.

3. Materials and methods

- Tumor pieces from K14*cre;Brca1*$^{F/F}$*;p53*$^{F/F}$ mice (a kind gift from Dr. Sven Rottenberg, University of Bern) were used for the animal experiments. Tumor pieces and tumor cells were orthotopically transplanted into the mammary fat pad of female wild-type FVB or GFP-expressing FVB ((FVB.Cg-Tg(CAG-EGFP)B5Nagy/J)) mice. Treatments with maximum tolerated dose (MTD) were started when the tumors reached 200 mm^3; repeated every 10 or 14 days. When the tumors reached 2000 mm^3, the animals were sacrificed.
- For the isolation of tumor cells, tumor tissue was cut into pieces, digested with collagenase and dispase in DMEM. Cells were cultured using primary culture medium (DMEM/F12 + 10% FBS + 10 % horse serum + 1% penicillin–streptomycin). 4T1, MDA-MB-231, MCF7 were cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium supplemented with 10% FBS, 5 mM glutamine, and 50 units/ml. All cell lines were cultivated at 37 °C with 5% CO_2.
- MSC (mesenchymal stem cell) and CST cells were differentiated into osteo- and adipogenic lineages by using lineage-specific differentiation culture media. To prove osteogenic differentiation, calcium deposition was stained with alizarin red S stain (Sigma-Aldrich). Cells were stained with oil red O (Sigma-Aldrich) to examine adipogenic differentiation.
- Genotyping was performed as using *Brca1*-deletion specific primers. Detection of *Brca1*-deleted allele with primers P1 and P4 yielded a 594-bp fragment while wild-type *Brca1* was detected by the product of P1-P2 primers (390bp). After the electrophoresis of PCR products on 1% agarose gel, the gel was stained with ethidium bromide.
- To evaluate growth rates and cell motility, cells were plated in 6- or 24-well culture plates. Images were taken every 6 or 12 hours using the JuLI Stage Real-Time Cell History Recorder (NanoEnTek).
- For RNA isolation, Direct-zol® MiniPrep kit (Zymo Research) was used according to the manufacturer's guidelines. cDNA samples were prepared from 500 ng total RNA using the Promega Reverse Transcription System Kit. The Pre-Developed aqMan® assay GAPDH (ThermoFisher) was used as endogenous

control in real-time qPCR experiments; for quantifying E-cadherin, vimentin, cytokeratin 8 and 14 mRNA levels the respective TaqMan® primers were used.

- To examine epithelial and mesenchymal cell markers and γH2AX foci, cells were seeded into 8-well μ-Slides, washed and fixed with 4% formaldehyde solution and then blocked. Next, samples were incubated overnight at 4°C with the relevant primary antibodies and after incubation, the cells were washed with PBS, and the secondary antibodies. Nuclei were labeled with DAPI. The absence or presence of the protein of interest was detected with a confocal microscope.
- To analyse cytokin levels, cell culture (CST and MSC) supernatants were tested using Mouse Inflammatory Cytokines Multi-Analyte ELISA Kit (Qiagen) according to the manufacturer's instructions.
- We determined the triplet base substitution spectra of heterozygous mutations in CST by whole genome sequencing data analysis; we examined copy number changes and mutational signatures based on COSMIC database.
- Viability was assessed using the PrestoBlue® assay (Life Technologies), according to the manufacturer's instructions. Viability of the cells was measured spectrophotometrically using an EnSpire microplate reader (Perkin Elmer). Data were normalized to untreated cells; curves were fitted by the Graph Pad Prism 5 software using the sigmoidal dose–response model. Curve fit statistics were used to determine mean concentrations of the selected drugs required to inhibit cell proliferation by 50% (IC_{50}).
- 2nd generation lentiviral vectors and packaging plasmids (pMD2.G and psPAX2) were obtained from Addgene. CST cells were transduced with GFP (pRRL-EF1-eGFP-WPRE) or mCherry (pRRL-EF1-mCherry-WPRE) expressing lentiviral supernatants prepared as follows: lentiviral particles were produced in HEK293T cells transfected by the calcium phosphate co-precipitation method. The multiplicity of Infection was determined by flow cytometry. Transduction of target CST cells was carried out on 6 well plates. After the transduction, cell lines were tested and sorted by flow cytometry.
- Sorting was performed using a FACSAria III cell sorter (BD Biosciences, San Jose, California, US) equipped with four lasers. Flow cytometry data was collected and analysis was performed using FACSDiva 8.02 software.

4. Results

Investigating the efficiency of pegylated liposomal doxorubicin (PLD) in a Brca1$^{-/-}$; p53$^{-/-}$ mouse mammary tumor model.

To study the therapeutic value of PLD in the *Brca1$^{-/-}$;p53$^{-/-}$* mammary breast cancer model, we orthotopically transplanted *Brca1$^{-/-}$;p53$^{-/-}$* tumor pieces into the fat pad of FVB mice. When the tumors reached 200 mm^3, tumors were treated with the MTD of either doxorubicin (DOX) or PLD. As compared to doxorubicin, treatment with the MTD of PLD resulted in 3-fold increase in median overall survival (49.5 vs 151.5 days). Treatment with the MTD of DOX induced resistance in all cases, however we detected resistance to PLD only in half of the cases. Increased survival could be explained by the delayed onset of drug resistance.

Favorable pharmacokinetics could explain the superior efficacy of PLD over DOX. Liposomal formulation of doxorubicin allowed a 60% increase of the MTD (8 mg/kg vs 5 mg/kg for PLD and DOX, respectively), which resulted in a 35-fold increase in the maximum peak doxorubicin concentration (31600 ± 6023 ng/µl vs 885.67 ± 240). Following intravenous injection of DOX, doxorubicin plasma levels decayed rapidly, whereas 7 days after treatment with a single dose of PLD, doxorubicin concentrations were still comparable to maximum levels observed in DOX-treated mice. Consequently, the AUC value was ~2600-fold higher for PLD as compared to DOX.

Establishment, characterization of a Brca1$^{-/-}$; p53$^{-/-}$ mouse mammary tumor cell line and studying molecular mechanisms of cisplatin resistance in Brca1$^{-/-}$; p53$^{-/-}$ mouse mammary tumor cell line.

We established primary cultures from *Brca1$^{-/-}$;p53$^{-/-}$* mammary tumors, designated as CST cell line. CST cells were further characterized in the context of epithelial (MCF7, 4T1) and mesenchymal (MDA-MB-231) breast cancer cell lines.

Genomic instability of CST cells was confirmed by whole genome sequencing, which revealed a very high number of single nucleotide variations (SNVs) compared to the FVB mouse genome, and frequent copy number changes indicating chromosomal instability. A deconstruction of the SNV spectrum into COSMIC mutational signatures derived from cancer sequences detected the presence of mutation signatures 3 and 8

associated with HR deficiency, as well as signature 18, which was described in different cancer types including breast cancer, in association with oxidative damage. In addition to single base substitution signatures, we also predicted the copy number status in 16 kbp bins, which revealed significant copy number changes and genome-wide chromosomal instability, corroborating our results of γ-H2AX quantification.

We isolated CST clones for sequencing. Tumors developing from cloned CST cells, following several cycles of treatment, became resistant to cisplatin, handing us a clinically relevant in vivo model to study the evolution of acquired cisplatin resistance. We have established primary cell lines from cisplatin sensitive and cisplatin resistant tumors and showed, that primary cells derived from cisplatin resistant tumors showed in vitro cisplatin resistance, suggesting that the mechanisms responsible for cisplatin resistance can be identified by the molecular analysis of the cell lines.

Examining the cellular plasticity of Brca1-deficient tumor cells.

To compare cancer cells to non-cancerous tissue derived from the same host, we isolated mesenchymal stem cells (MSC) from wild-type FVB mice. The established MSC cell line fulfills criteria commonly used for defining multipotent mouse mesenchymal stem/stromal cells, including adherence to plastic surface, specific cell-surface marker pattern and differentiation capability.

CST cells express vimentin, a prominent marker of the mesenchymal phenotype, whereas the expression of E-cadherin in CST cells is undetectable. Despite their mesenchymal phenotype, CST cells are of epithelial origin, as evidenced by the truncation of *Brca1* gene that occurred in the mammary epithelium of K14*cre;Brca1*$^{F/F}$*;p53*$^{F/F}$ mice. Furthermore, we showed that, in line with their stem cell properties, CST cells have the capacity to differentiate into adipo- and osteogenic lineages, and also express MSC markers. The plasticity of CST cells was also demonstrated by the detection of E-cadherin expressing epithelial and vimentin-positive mesenchymal cells in the same culture, resulting from spontaneous mesenchymal to epithelial transitions.

Genetic modification via lentiviral transduction of Brca1$^{-/-}$*; p53*$^{-/-}$ *tumors.*

Fluorescent protein expressing CST sublines were established by lentiviral transduction. We showed that stable expression of fluorescent proteins did not change the

phenotype of CST cells. The fluorescence of CST cells offers a tool to investigate tumor-stroma interactions. To allow efficient separation of tumor and stroma cells, CST-mCherry cells were orthotopically injected into GFP-positive FVB mice. When the tumors reached 200 mm^3, the animals were sacrificed. Tumors were removed, and the cells were sorted based on mCherry/GFP expression. In separate cultures the mCherry-positive CST cells preserved the characteristic mesenchymal morphology, while GFP-positive fibroblasts were larger, and exhibited a flat, polygonal, stellate-like morphology with formed lamellipodia.

To test the drug response pattern of the developing mCherry-CST tumors, when tumors reached 200 mm^3, mice were treated with the maximum tolerable dose (6mg/kg) of cisplatin with 2-week intervals. CST-derived tumors responded well to cisplatin, relapsing tumors remained sensitive to cisplatin, but the tumors were not eradicated.

In summary, we showed that the CST cell line established from *Brca1*$^{-/-}$*; p53*$^{-/-}$ mouse mammary tumors are suitable to study tumor formation, anticancer drug response and tumor-stroma interactions.

5. Conclusions

1. We showed that PLD treatment significantly increased the relapse-free and the overall survival of *Brca1*$^{-/-}$*; p53*$^{-/-}$ mouse mammary tumor bearing animals compared to doxorubicin, due to the delayed onset of resistance.

2. We established and characterized a *Brca1*$^{-/-}$*; p53*$^{-/-}$ mouse mammary tumor cell line, designated as CST. CST cells keep their tumorigenic potential and are suitable for testing new therapies.

3. We established a clinically relevant in vivo model for the study of acquired cisplatin resistance, which give us additional opportunity to study molecular mechanisms of cisplatin resistance

4. We verified the cellular plasticity and stem cell properties of CST cells. We showed that CST cells possess both epithelial tumor cell features and mesenchymal cell properties, and have the capacity to differentiate into bone and fat.

5. We genetically modified *Brca1*$^{-/-}$*,p53*$^{-/-}$ tumors via lentiviral transduction, and proved that *Brca1-KO* tumor cells (CST-mCh) are suitable to study tumor formation, anticancer drug response and tumor-stroma interactions.

6. Publications

Publications related to the thesis:

Lilla Hámori, Gyöngyi Kudlik, Kornélia Szebényi, Nóra Kucsma, Bálint Szeder, Ádám Póti, Ferenc Uher, György Várady, Dávid Szüts, József Tóvári and András Füredi, Gergely Szakács, *Establishment and Characterization of a Brca1-/-, P53-/- Mouse Mammary Tumor Cell Line.* International Journal of Molecular Sciences, 2020. **21** (4)
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Nagy, Edina Karai, Tímea Imre, Pál Szabó, Dávid Szüts, József Tóvári, Gergely Szakács: *Pegylated liposomal formulation of doxorubicin overcomes drug resistance in a genetically engineered mouse model of breast cancer.* Journal of Controlled Release, 2017 Sep 10;261:287-296
IF: 7,877

Further publications:

Bálint Szeder, Júlia Tárnoki-Zách, Dóra Lakatos, Virág Vas, Gyöngyi Kudlik, Balázs Merő, Kitti Koprivanacz, László Bányai, **Lilla Hámori**, Gergely Róna, András Czirók, András Füredi, László Buday; Absence of the *Tks4 Scaffold Protein Induces Epithelial-Mesenchymal Transition-Like Changes in Human Colon Cancer Cells*. Cells, 2019. **8** (11).
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