SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

ARID1A mediates the antiproliferative effects of bexarotene and carvedilol combination treatment in normal and transformed breast cells

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1. INTRODUCTION AND THEORETICAL BACKGROUND

Breast cancer is among the most occurring abnormalities in women. It is the second cause of cancer-related death [1]. It can be treated by targeting certain growth regulators such as the estrogen receptor (ER) or the human epidermal growth signaling pathway (Her-2) [2]. However, at least 20% of breast cancer cases do not depend in their growth on these growth drivers. On the other hand, cells during the course of treatment could develop resistance to the drugs. Therefore, there is always a need to identify agents that could target other signaling pathways.

Despite the successful attempts in treating breast cancer, its increased incidence attenuates these attempts emphasizing the importance of risk assessment in order to prevent its development in people at high risk.

Clinical studies have demonstrated the feasibility of preventing ER-positive breast cancer development, using ER modulators, such as tamoxifen [3]. However, due to its lower tolerability and the fact that these agents are not effective in preventing ER-negative breast cancer, identifying compounds that could treat and/or prevent breast cancer development in a hormonal independent manner. Preclinical studies demonstrated that rexinoids, synthetic retinoids, suppress breast carcinogenesis in an ER-independent manner. However, when it comes to clinical usage even minor side effects should be taken into consideration [4]. Rexinoids usage was found to be associated with hypertriglyceridemia and hypothyroidism, emphasizing the importance of identifying agents that have synergistic effects in preventing breast cancer, giving a chance to decrease the dosage and thereby the associated side effects.

In our lab, the combination treatment of the non-selective beta blocker, carvedilol, enhanced the antiproliferative effects of the selective rexinoid, bexarotene, in normal but immortalized breast epithelial cells (HME-hTert) based on cell-based small

molecule high throughput screening. Moreover, the combination treatment decreased the growth of transformed breast cells. In our study, we are investigating the molecular mechanisms implicated behind the detected antiproliferative effects of bexarotene (Bex) and carvedilol (Carv) in normal and transformed breast cells.

2. AIMS OF OUR STUDY

Reverse phase proteomic array (RPPA) results showed induction in the protein levels of the chromatin remodeler ARID1A upon Beex+Carv treatment in normal cells but not in transformed cells. We hypothesise that upon the combined treatment ARID1A alters nucleosome organization and chromatin accessibility to modulate the expression of genes involved in cell transformation and proliferation regulation. To test our hypothesis we set the following specific aims; to identify:

1. *ARID1A genomic binding events upon Bex+Carv treatment in normal and transformed cells*
2. *Pathways that are affected by the change in ARID1A enrichment upon Bex+Carv treatment*
3. *The effect of ARID1A enrichment to its target regions on the expression of putative target genes upon Bex+Carv treatment*
4. *The impact of ARID1A knockdown on the detected effect of Bex+Carv treatment on target genes' regulation*
5. *The effect of Bex+Carv treatment on ARID1A targets' downstream signaling*

3. MATERIALS AND METHODS

3. 1Cell culture and treatments:

MCF-7 breast cancer cells were cultured in DMEM or phenol-red free DMEM media supplemented with 10% fetal bovine serum, and 100% penicillin/streptomycin/L-glutamine. HME-hTert mammary breast epithelial cells were maintained in MEBM medium supplemented with 50 µg/ml bovine pituitary extract, 5 µg/ml insulin, 10 ng/ml human recombinant epidermal growth factor, 0.5 µg/ml hydrocortisone, 30

µg/ml gentamicin, and 15 ng/ml amphotericin-B. Cells were maintained in a humidified atmosphere with 5% Co2 at 37ºC. Cells were treated at 30-40% confluence with bexarotene and/or carvedilol for different time points. The agents were dissolved in DMSO/Ethanol (50/50) which was used as a control. ARID1A knockdown was performed through transient transfection using a pool of siRNAs against ARID1A mRNA molecule. Transfection was performed for 2 or 3 days to study the effect on transcript or protein levels, respectively.

3. 2 Western Blotting:

Protein extracts were isolated from cells using RIPA lysis buffer (150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris pH8.0). To decrease viscosity cell lysate was sonicated for 5min in total (5 cycles, 30 sec on, 30 sec off). The supernatant was collected after 20 min centrifugation at 12000xg at 4ºC. 25-30µl total proteins were denatured and run on 6% SDS-polyacrylamide gel. Proteins were transferred into a PVDF membrane. Blots were blocked with 5% low-fat milk for 1 hour at RT, and then probed with primary antibody against the target protein overnight at 4ºC. Fluorophore probed secondary antibodies were used to generate a fluorescent signal that was detected using Odyssey® CLx imaging system (LI-COR).

3. 3Chromatin immunoprecipitation (ChIP):

Cells were crosslinked using 1% formaldehyde in PBS for 10 min at RT. Formaldehyde activity was quenched using 125mM glycine. Cells were washed with PBS and lysed in ChIP lysis buffer (1% Triton X-100, 0.1% SDS, 150mM NaCl, 1mM EDTA pH=8.0, 20mM Tris pH=8.0). Chromatin was sheared using sonication within the following conditions for 15 cycles (30 sec On, 30 sec Off). Sheared

chromatin was incubated with beads coated with non-specific antibodies to reduce background. The cleared chromatin was then divided into different IPs and incubated with beads coated with antibodies against specific targets, ARID1A, BRG1, or H3K27ac overnight at 4 ºC. DNA-Protein-Ab-beads complex was washed several times, with ChIP washing buffers. DNA was eluted using the elution buffer (0.1M NaHCO3, 1%SDS) for 15 min at 1,000 rpm at RT. DNA-Protein were de-crosslinked through overnight incubation with 0.2 M NaCl at 65°C. Eluted DNA was purified using the High Pure PCR Template Preparation Kit according to the manufacturer's instructions. DNA samples were subjected to the genomic core facility, university of Debrecen for sequencing or the signal at specific genomic regions was detected through qPCR.

3. 4 RNA isolation followed by reverse transcriptase-polymerase chain reaction:

RNA was isolated using trizol method or a NucleoSpin RNA isolation kit (MACHEREY-NAGEL, Ref: 740955.50) according to the manufacturer's instructions. 1 µl RNA was used for reverse transcription reaction; 50°C for 30 min followed by 5 min at 72°C. cDNA molecules were subjected to quantitative PCR reaction using primers against specific regions with the following program; initial denaturation at 95 °C for 2 min followed by 40 cycles of denaturation at 95 °C for 10 seconds then annealing and extension at 60 °C for 30 seconds for each cycle.

3. 5 Immunocytochemistry:

Cells were fixed using 4% formaldehyde in PBS. Cells' permeability was adjusted using permeabilization solution (0.5% Triton-x 100 in PBS) for 40 min at RT. Non-

protein containing spaces were blocked using (1% BSA/ 10% normal goat serum/ 0.3M glycine in 0.1% PBS-Tween) for 1 hour at RT. Cells were incubated with primary antibodies against specific targets overnight at 4ºC. Fluorescent secondary antibodies were added for 1 hour at RT. Protein-primary Ab- secondary Ab complex was crosslinked with 4% formaldehyde for 10 min at RT. Ammonium chloride was used to quencher formaldehyde effects. DAPI staining was used to stain nuclei. A fluorescent signal was detected using an inverted fluorescent microscope (LEICA DMi8).

3. 6 ChIP-Seq Data analysis:

ChIP-Seq datasets were analysed using two different platforms. H3K27ac ChIP-Seq analysis was performed using the Galaxy platform (https://usegalaxy.eu). Low-quality reads were removed using Tim Galore function. Reads were aligned to the reference genome (hg19) using Bowtie2 software. Peaks were called using MACS2 peaks calling software. Gene ontology analysis was performed using Genomic Regions Enrichment Annotations Tools (GREAT) v3.0.0. ARID1A ChIP-Seq analysis was performed on a published computational pipeline [5]. Raw reads were aligned to the reference genome (hg19) using BWA software. HMCan software was used for peak calling. Motif enrichment analysis and heatmaps production was performed using Homer software.

3. 7 Fluorescent images data analysis:

Fluorescent images were analysed using CellProfiler 4.2.1 software. Images related to one group of treatments were uploaded to the system. Reference stainings were used to identify the objects. DAPI was used for nuclei staining, and Tublin-β to stain the

whole cells. The fluorescent signal derived from target proteins was then measured after localizing the objects based on the reference dyes.

4. RESULTS:

4. 1 Bexarotene and carvedilol (Bex+Carv) combination treatment suppresses cell growth in normal and transformed breast cells:

Bex+Carv combination treatment was associated with a decrease in HME-hTert cell proliferation (preliminary data). Moreover, the combined treatment showed a superior effect in suppressing MCF-7 cell proliferation compared to the individual ones at relatively low doses.

4. 2 **ARID1A protein but not transcript levels were induced upon Bex+Carv treatment in HME-hTert cells**:

To investigate the molecular mechanism behind the antiproliferative effects of Bex+Carv Reverse phase proteomic array was performed (preliminary data) showing an induction in the protein levels of the chromatin remodeler ARID1A in HME-hTert cells. Here we validated the elevation of ARID1A protein levels using Western-blotting and immunostaining assays. ARID1A transcript levels showed no change after Bex+Carv treatment for different time points.

4. 3 **ARID1A genomic occupancy increased in MCF-7 cells upon Bex+Carv treatment**:

To investigate the effect of Bex+Carv on ARID1A genomic occupancy we performed chromatin immunoprecipitation followed by deep sequencing. ARID1A ChIP-Seq results showed that intronic and intergenic regions were occupied mainly by ARID1A. Around 17 thousand ARID1A binding events were gained upon Bex+Carv

treatment. DNA regions bound by ARID1A were identified to match FOXM1, GRHL2 and TRE transcription factor motifs.

4. 4 **ARID1A enrichment to regulatory elements assigned to genes involved in IGF-1 signaling pathway increased upon Bex+Carv treatment:**

The change in ARID1A occupancy upon Bex+Carv treatment affected genes involved in signaling pathways related to mammary epithelial cell proliferation and insulin-like growth factor signaling pathway based on gene ontology analysis. Further analysis showed that ARID1A was enriched to regulatory elements assigned to IGF-1R and IRS1 genes in MCF-7 cells upon Bex+Carv treatment. ARID1A enrichment was validated with ChIP-qPCR.

4. 5 **Bex+Carv treatment downregulates the protein expression levels of IGF-1R and IRS1 mediated by ARID1A actions:**

To examine the effect of ARID1A enrichment to its target regions on the expression of the associated genes, we measured the effect of Bex+Carv treatment on IGF-1R and IRS1 protein levels. The results showed a decrease in IGF-1R and IRS1 protein expression in MCF-7 cells which was abolished upon ARID1A knockdown.

4. 6 **ARID1A Knockdown enhances MCF-7 cell proliferation:**

To investigate the potential role of ARID1A on proliferation, we knock it down and assess its effect on IGF-1 pathway-related genes. The results showed an induction of IGF-1R protein expression levels in MCF-7 cells upon ARID1A knockdown, associated with an increase in cell counts.

4. 7 **ARID1A is recruited to regulatory elements assigned to genes involved in TGF-β signaling pathway, associated with a change in their expression upon Bex+Carv treatment in HME-hTert cells**:

ARID1A ChIP-Seq was performed in HME-hTert cells. Several target regions were identified. ARID1A and BRG1 ChIP-qPCR were performed to validate that the identified signal is coming from SWI/SNF. The results showed that upon Bex+Carv treatment ARID1A was enriched to regulatory elements assigned to genes involved in the transforming growth factor-beta signaling pathway. FOXQ1, KLF4, BMP6, and TGFBR2 were identified to be ARID1A putative target genes. ARID1A and/or BRG1 were detected +13 Kb, -14Kb, -27Kb to FOXQ1, KLF4, and BMP6 transcription start sites, respectively. ARID1A enrichment was associated with a decrease in the expression levels of FOXQ1 and KLF4, whereas induction of BMP6 transcript levels. TGFBR2 gene expression levels were downregulated upon Bex+Carv treatment associated with a change in ARID1A and/or BRG1 enrichment to its regulatory elements.

4. 8 **Bex+Carv treatment induces the expression of an epithelial marker but inhibits the expression of mesenchymal markers in HME-hTert cells mediated by ARID1A actions**

As ARID1A identified targets upon Bex+Carv treatment in HME-hTer cells were related to TGF-β, a known inducer of the epithelial to mesenchymal transition (EMT) process, we studied the effect of Bex+Carv treatment on the expression of epithelial and mesenchymal markers. The data showed an induction of the expression levels of E-cadherin, the epithelial marker, but a decrease in the gene and protein expression

levels of mesenchymal markers; fibronectin-1 and N-cadherin. The detected effects were abolished upon ARID1A knockdown.

5. DISCUSSION:

In this study, we identified ARID1A to play a critical role in the antiproliferative effects of bexarotene and carvedilol combination treatment in normal mammary immortalized breast epithelial cells (HME-hTert) and in ER-positive breast cancer cells (MCF-7). Based on other studies ARID1A was found to regulate the expression of cell cycle-related genes as part of its tumor suppression activity. In our study, we found that ARID1A is recruited to regulatory elements assigned to genes involved in the IGF-1 signaling pathway including IGF-1R and IRS1 associated with a decrease in their protein expression levels upon Bex+Carv treatment. IGF-1/IRS1 axis plays a role in regulating cell growth and proliferation [6], its activity is regulated by IGFBPs, which compete with IGF-1R protein to bind the IGF-1 molecule. Bexarotene positively regulates the expression of IGFBP6 participating in controlling the activity of IGF-1 signaling [7]. Here we found that the combined treatment of bexarotene and carvedilol combination treatment reduced the protein expression levels of IGF-1R and IRS1 in MCF-7 cells associated with a decrease in cell proliferation. In normal cells, we found that ARID1A is recruited to regulatory elements assigned to genes involved in transforming growth factor β, including FOXQ1, KLF4, BMP6 and TGFBR2,

FOXQ1 is a tumor-promoting gene that participates in the regulation of genes involved in cancer development. FOXQ1 negatively regulates the expression of the epithelial marker E-cadherin [8]. Here we found that upon Bex+Carv treatment ARID1A and BRG1 recruitment to FOXQ1 regulator element is associated with a suppression of its gene and protein expression levels. On the other hand, SWI/SNF studied subunits enrichment to regulatory regions assigned to the BMP6 gene was associated with an induction of its expression levels. BMP6 is a tumor suppressor [9] that positively regulates the expression of E-cadherin which was found to be induced upon the combined treatment. KLF4 is a transcription factor that has dual roles in regard to cell proliferation regulation [10]. It is considered a stem cell-like marker. In our model ARID1A and BRG1 were enriched to the KLF4 regulatory region associated with a decrease in their gene expression levels upon the combined treatment. As the identified ARID1A targets are related to TGF-β signaling, a potent regulator of the EMT process [11], we assessed the effect of Bex+Carv treatment on mesenchymal marker's expression, including fibronectin-1 and N-cadherin showing a decline in their gene and protein expression levels. The detected effects were reversed upon ARID1A knockdown. The results suggest that Bex+Carv treatment suppresses normal epithelial cell transformation through the actions of ARID1A.

6. SUMMARY:

The study showed that Bex+Carv treatment affects ARID1A occupancy to be enriched to the regulatory regions assigned to IGF-1R and IRS1 genes. The enrichment of ARID1A was associated with a downregulation of IGF-1R and IRS1 on the protein levels and MCF-7 cell proliferation **Figure 6-1.**

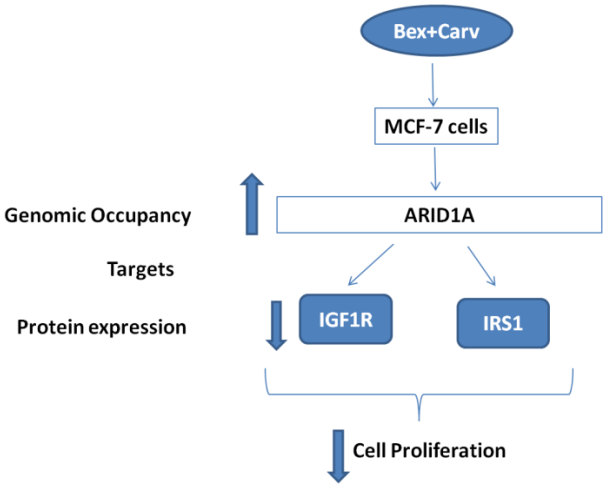

Figure 6-1: Schematic diagram showing a summary of the detected effects of Bex+Carv treatment on MCF-7 transformed cells mediated by ARID1A actions

Upon Bex+Carv treatment, ARID1A and BRG1 were recruited to regulatory elements assigned to genes involved to the TGF-Beta signaling pathway associated with an increase or a decrease in H3K27ac marks. The enrichment of SWI/SNF to its target regions alters the expression of corresponding genes; an increase in the case of Bmp6, or a decrease in KLF4, FOXQ1, and TGFBR2 transcript levels. Gene and protein expression levels of the mesenchymal markers fibronectin-1 and N-cadherin were downregulated whereas the expression level of E-cadherin was induced upon Bex+Carv treatment in HME-hTert cells **Figure 6-2.** The results revealed two mechanisms behind the antiproliferative and transformation effects of Bex+Carv treatment in normal and transformed breast cells mediated by ARID1A activity.

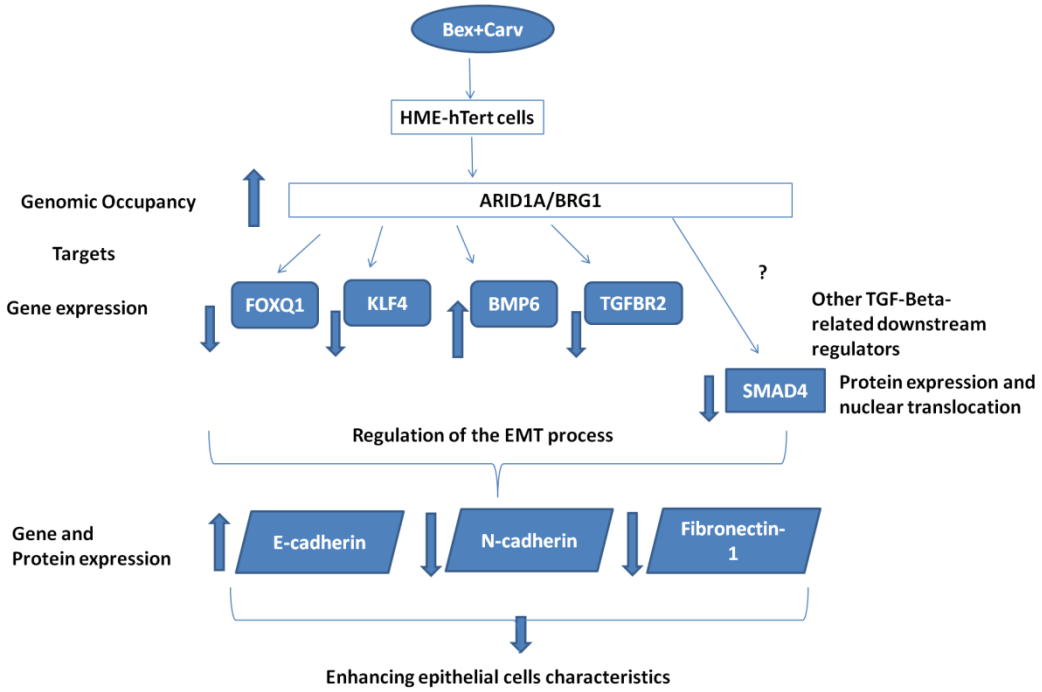

Figure 6-2: Schematic diagram showing a summary of the detected effects of Bex+Carv treatment on HME-hTert normal cells mediated by ARID1A and BRG1 actions

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   Cells. 11 (17), 1-15, 2022.
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