Investigation of the role of tumor suppressor CYLD in the differentiation of breast epithelial cells into adipocytes

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Abstract

Metastatic breast cancer cells have been shown to transdifferentiate into growth arrested adipocytes. This capacity correlates with the plasticity of cells which have undergone epithelial-mesenchymal transition (EMT) and opens up new possibilities for therapeutic intervention. Inactivation or downregulation of the tumor suppressor CYLD induces EMT in mammary epithelial cells. Additionally, targeted inactivation of CYLD leads to lipid accumulation in mouse tissues. Based on these findings , we sought to investigate whether CYLD plays a role in the differentiation of breast cancer cells into adipocytes. For this purpose, we created CYLD –deficient breast cancer cell clones and subjected the generated clones to adipogenic stimuli in order to induce differentiation.

In order to generate the desired clones, we utilized the CRISPR/Cas9 system. Breast cancer cells were targeted with either one of three sgRNAs designed against sequences of the exons 4, 5 and 11 of the *CYLD* gene. After normal or impaired expression of the CYLD protein was verified, the cells were treated with adipogenic medium containing BMP2 and rosiglitazone for 10 days total. At the end of the induction, the cells were stained with Oil Red O and adipogenesis was assessed with bright-field microscopy and Oil Red O quantification.

The targeted inactivation of the *CYLD* gene was successful. The differentiation efficiency into adipocytes was limited in the CYLD expressing clones under the conditions that were selected based on the literature and the ablation of CYLD was not sufficient to induce differentiation of the CYLD null cells. Further studies will focus in the optimization of the differentiation protocol, as well as the analysis of differential gene expression between CYLD expressing and CYLD null cells, in order to elucidate the role of CYLD in mammary cell homeostasis.

Introduction

CYLD is a deubiquitinating enzyme functioning as a negative regulator of the NF-κB, JNK and p38 pathways through its catalytic activity[1]. The catalytic domain is located in the carboxyterminal region, while three CAP-Gly domains in the aminoterminal region facilitate the interaction between CYLD and other proteins and its cytoplasmic localization. CYLD has been initially identified as a tumor suppressor linked to the development of tumors of skin appendages[2], and has been later shown to be mutated or downregulated in various other types of cancer[3]. Furthermore, inactivation of CYLD has been shown to promote epithelial-mesenchymal transition in mammary epithelial cells[4].

Dysregulation of lipid homeostasis has been attributed to the loss of CYLD in mammal and insect models. In mouse model studies, it has been reported that liver-specific ablation of CYLD promotes non-alcoholic fatty liver disease[5], and mice lacking CYLD expression in epidermis develop sebaceous gland tumors[6]. In Drosophila, defective expression of the ortholog of human *CYLD* leads to impaired fat body formation and lipid accumulation[7].

Amongst malignancies that show promising therapeutic prospects through differentiation therapy, breast cancer cells have been shown to successfully transdifferentiate into growth arrested adipocytes[8]. This adipogenic capacity has been correlated with the plasticity that is displayed by cells that have undergone epithelial-mesenchymal transition.

Based on these findings, we decided to investigate whether CYLD inactivation is associated with increased adipogenic capacity in mammary epithelial cancer cells. We set three aims towards this end. First, to isolate single cell colonies derived from breast cancer cells targeted with the CRISPR/Cas9 system and characterize them with regard to the expression of CYLD, second, to induce differentiation of the CYLD expressing and CYLD-deficient generated clones and compare their differentiation capacity, and third, to study the differential gene expression between clones expressing or lacking CYLD in order to identify candidates via which CYLD could participate in the regulation of breast cancer cell differentiation.

Methods

Generation of CYLD-deficient and control clones

We utilized a lentiviral based approach in order to produce clones expressing (CYLD WT) or lacking expression of CYLD (CYLD KO). HEK293T cells were transfected with a mix consisting of the packaging vector pCMV-dR8.91, the envelope vector pCMV-VSV-G and four variants of the lentiviral vector lentiCRISPR v2, each containing a different sgRNA sequence, out of which 3 target the exons 4,5 and 11 of the human *CYLD* gene and one targets a sequence of the GFP gene. The transfection was carried out with a BES-$CaCl_2$ protocol. 48 hours post-transfection, the viral supernatant was collected and concentrated, and the cell lines of interest were infected in the presence of polybrene. After 24 hours, fresh medium was added to the infected cells, and after further 24 hours, the infected cell populations were subjected to selection in 0,5-2μg/ml puromycin (depending on the cell line) for 5 days. The resistant cells that emerged were plated in 96-well plates at a dilution of 1 cell per well. Single cell derived colonies were expanded and screened for CYLD expression. The levels of total CYLD were determined through western blot analysis. The β-actin protein was used as a loading control. Both antibodies against CYLD and β-actin were purchased from Santa Cruz Biotechnology.

Differentiation protocol

In order to induce adipogenic differentiation of CYLD WT and CYLD KO clones, the cells were seeded at a density of 20.000 per cm^2 in 96-well plates and incubated overnight at 37^{o}C 5% CO_2, and in the next day, adipogenic medium, as well as control medium lacking adipogenic factors, were added to the cells. The adipogenic medium consisted of 200ng/ml BMP2 for the first three days of the process, 200ng/ml BMP2 and 2μM rosiglitazone for the next four days and 2μM rosiglitazone for the remaining 3 days. On the tenth day since the initiation of the protocol, the cells were stained with Oil Red O. Cells were washed with PBS and fixed in 4% paraformaldehyde for 30 minutes. A wash with 60% isopropanol followed, and after that, 0,3% Oil Red O in 60% isopropanol was added in each well. After a 10 minute incubation, the cells were washed thoroughly and visualized by bright-field microscopy. Afterwards, Oil Red O was extracted from the cells during a 10 minute incubation in 100% isopropanol and the optical density of each sample was determined by phasmatophotometry at 500nm. Statistical analysis was carried out using Student's *t* test.

Results

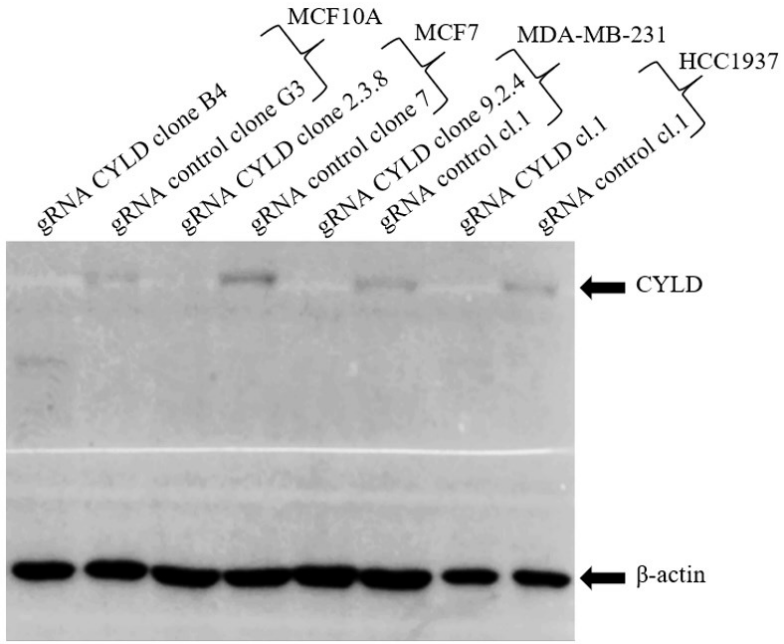

Figure 1. *Western blot analysis of single cell derived breast cancer cell clones*. Each pair of samples consists of one clone expressing a control sgRNA (gRNA control), displaying the physiological levels of CYLD in each particular cell line, and one clone expressing a sgRNA targeting CYLD (gRNA CYLD). The absence of CYLD expression in cells targeted with the appropriate sgRNAs is confirmed. β-actin represents the loading control.

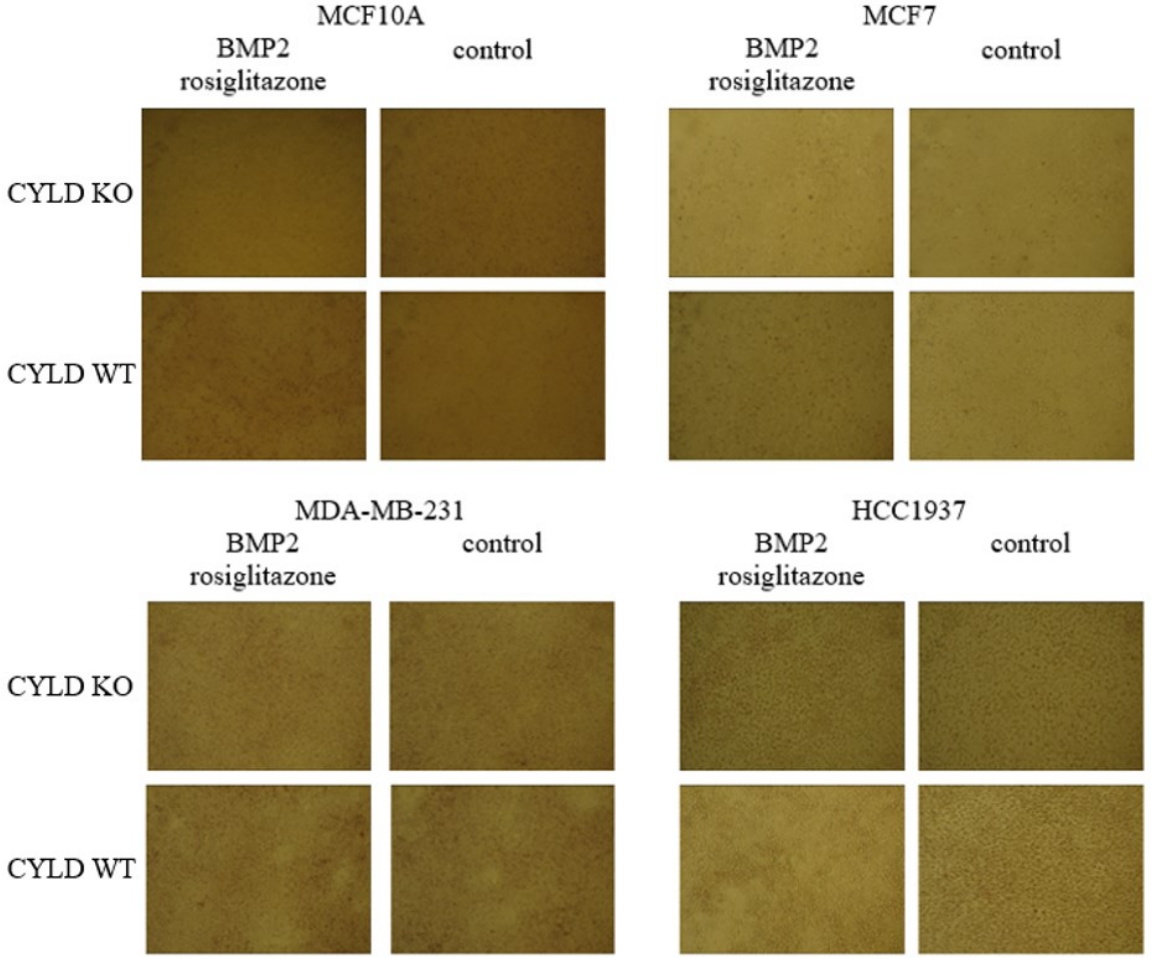

Figure 2. *Representative bright-field microscopy images of breast cancer clones at the endpoint of the applied adipogenesis protocol, after Oil Red O staining.* The differentiation efficiency is limited in both CYLD WT and CYLD KO clones. The inactivation of CYLD is not sufficient to induce adipogenic differentiation under the tested conditions. Scale: 80μm.

Results

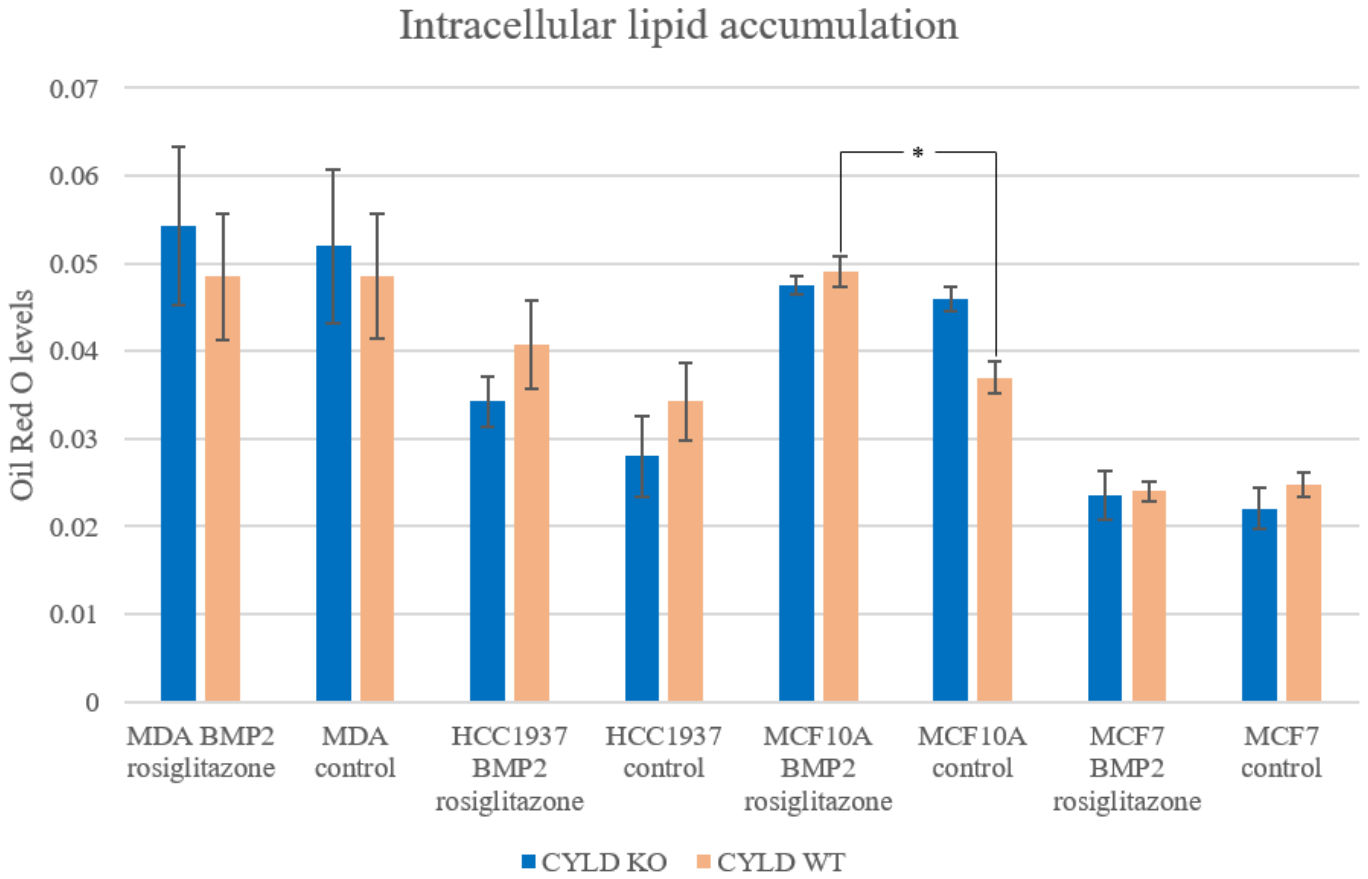

Figure 3. *Spectrophotometric assessment of intracellular lipid content based on eluted Oil Red O absorbance at 500nm.* Each pair represents a CYLD WT and CYLD KO clone derived from the same breast cancer cell line and exposed to control (control) or adipogenic culture conditions (BMP2 rosiglitazone). There were no statistically significant differences between the lipid content of cells cultured in the presence of adipogenic medium compared to cells of the same clone cultured in control medium, displaying that the differentiation efficiency was low under the attempted conditions. One exception was the MCF10A WT clone that had a statistically significant increase in its lipid content following adipogenic stimulation compared to the control cells (* p<0,01). In addition, a statistically significant higher lipid content was observed in MCF10A KO compared to MCF10A WT cells in non adipogenic conditions. Otherwise, there were no statistically significant differences between the levels of Oil Red O in CYLD WT and CYLD KO clones, showing that the absence of CYLD is not sufficient to increase the adipogenic capacity of the assayed clones. Average values (+/_SEM) from four independent are presented.

Conclussions

- A CRISPR mediated approach has been used successful to generate CYLD –deficient (CYLD KO) and control (CYLD WT) clones as tools to study the role of CYLD in breast cancer cell differentiation to adipocytes.
- The adipogenic capacity of CYLD KO and CYLD WT clones was limited under the experimental conditions that were chosen based on the literature. Further studies will be focused on the optimization of adipogenic conditions, potentially by incorporating alternative or additional adipogenic factors in the media. One such factor could be dexamethasone, since long-term treatment with it has reportedly been sufficient to induce differentiation of breast cancer cells into adipocytes[9].
- The inactivation of CYLD was not sufficient to induce adipogenic differentiation of the mammary epithelial cells under the conditions that were used.

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