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Original paper

CRISPR/Cas9 gene editing in Huh7 and Hepa RG cell lines

LILIANA PASLARU[1*#], PETRUTA ALEXANDRU[2*], SANDA M. CRETOIU[3,*], SIMONA O. DIMA[1], IRINEL POPESCU[1]

[1]Center of Excellence in Translational Medicine, Fundeni Clinical Institute, 022328, Bucharest, Romania
[2]Department of Molecular Cell Biology, Institute of Biochemistry of the Romanian Academy, 060031, Bucharest, Romania
[3]Department of Cell and Molecular Biology and Histology, Carol Davila University of Medicine and Pharmacy, 050474 Bucharest, Romania

**authors have equal contributions*

Abstract

Objectives. Mutations and overexpression of β-catenin are associated with many cancers including hepatocellular carcinomas (HCC). Activation of β-catenin signaling may result in resistance to chemotherapeutic agents. The aim of this study was to generate, by CRISPR/Cas9 gene editing technology, a viable cellular model usable to study the detailed mechanisms of HCC and to find target molecules for the development of novel therapeutic drugs. **Methods and results.** Human Huh7 and HepaRG cells were transfected with CRISPR/CAS β-catenin KO plasmid (h) plus β-catenin HDR plasmid (h). Edited clones were validated by fluorescent microscopy and Western Blot analysis and were further cultured. Additionally, the differential response of parental and KO cells to antitumoral drugs was tested. To our knowledge, this is the first report using CRISPR/Cas9 technology on human HCC cell lines to evaluate the correlation between β-catenin expression and antitumoral drug effects. **Conclusion.** Our results suggest that β-catenin is possibly involved in chemotherapy resistance, since the Huh7 β-catenin^{KO} cells appeared to be more sensitive compared to Huh7^{WT}.

Keywords CRISPR/cas9, Genome editing, Liver cancer, Wnt/β-catenin signaling pathway, Hepatocellular carcinoma (HCC)

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✉ *Corresponding author: LILIANA PASLARU, Center of Excellence in Translational Medicine, Fundeni Clinical Institute, 022328, Bucharest, Romania
E-mail: lilianaliviapaslaru@yahoo.fr Petruta Alexandru alexpety@yahoo.com, Sanda M. Cretoiu sanda@cretoiu.ro, Simona O. Dima dima.simona@gmail.com, Irinel Popescu prof.irinelpopescu@gmail.com

Introduction

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer, the fifth most common cancer and the third cause of cancer-related death worldwide (El-Serag, 2007). Its rates of incidence and mortality have been markedly increasing over the last decades and is more often in males than females (2.4:1) (Ferlay, 2015). HCC is an aggressive cancer with a poor prognosis that occurs most often in people with chronic liver diseases, such as cirrhosis caused by hepatitis B or hepatitis C infection, alcohol abuse or other toxic compounds (Balogh, 2016). HCC therapies depend upon the tumor features, hepatic reserve, presence or absence of extrahepatic metastasis or vascular invasion and include resection, liver transplantation, percutaneous ablation, and systemic chemotherapy (Balogh, 2016).

The multidisciplinary approach of HCC over the course of several decades, along with fundamental and clinical research, coupled with numerous promising therapy trials, have failed to reduce the alarming incidence of liver cancer. Current pharmacologic anti-cancer solutions are limited in practice by elevated and nonspecific cytotoxicity, as well as by the cells gaining resistance against chemotherapy (Arruebo, 2011). Because standard chemotherapy has not been effective, researchers have been looking for a targeted therapy or/and combinatorial treatments (Hu, 2012). In targeted therapy, drugs seek out specific receptors and proteins unique to cancer cells; its goal is to interfere with genes or proteins involved in tumor growth to block the spread of the disease (Carrington, 2015). Once attached to target cancer cells, the drugs work by either killing the cells or helping other therapies (e.g. chemotherapy), to identify and target cancer cells (Padma, 2015).

The CRISPR (clustered regularly interspaced short palindromic repeats) and CRISPR-associated (Cas) genes were initially discovered in the 1980s in *E. coli* (Ishino, 1987). CRISPR is a family of DNA sequences derived from DNA fragments from viruses that infected bacteria and which play a key role in the antiviral defense system of prokaryotes (Barrangou, 2015). CRISPR/Cas9 (prokaryotic type II) is a gene/genome editing tool, already successfully applied in animal models (e.g. mouse) (Yin, 2014) and human cell lines (Cong, 2013; Mali, 2013a; Sander, 2014; Mali, 2013b). Nowadays, we can consider the CRISPR/Cas 9 system as important mechanisms for adaptive immunization, biogenesis of small interfering RNAs, and sequence-specific targeting and cleavage of viral and plasmid DNA (Rath, 2015; Strich, 2018). The system offers sequence-specific direct editing of DNA and therefore, this method can achieve complete loss-of-function compared to RNAi-based approaches (Xue, 2014).

One of the pathogenic mechanisms that prevails in hepatocarcinogenesis is due to the mutations that occur in one or more oncogenes or in tumor suppressor genes. The main alterations reside in cellular signaling pathways that regulate tumor cell proliferation, differentiation, angiogenesis, invasion and metastasis which are nowadays seen as possible therapeutic targets (Whittaker, 2010). Among these signaling pathways aberrant activation of the canonical Wnt/β-catenin signaling pathway is frequently mutated in liver cancer and was described as having a role in HCC tumorigenesis (Liu, 2016). β-catenin is also a component of the cadherin complex, which controls cell-cell adhesion and influences cell migration (Nelson, 2004). β-catenin is a transcription factor, and a key component in the WNT signaling pathway and is frequently mutated in liver cancer (Khalaf, 2018). In cancer, cadherins control the equilibrium between promotion and suppression of invasion. The E-cadherin/β-catenin complex is actively involved in epithelial to mesenchymal (EMT) and mesenchymal to epithelial (MET) transitions and consequently contributing to tissue/organ fibrosis (including liver fibrosis), cancer development (Tian, 2011). Similar to E cadherin, the cytoplasmic part of N-cadherin forms a complex with several molecules, such as catenins (p120 catenin, β-catenin and α-catenin) which are possible regulators of cadherin functions (Shapiro, 2009). In most malignancies, E-cadherin is downregulated and functions as an invasion suppressor while N-cadherin is frequently upregulated, stimulates migration and invasion of cells and functions as an invasion promoter. However, the multiple functions of cadherins can be different depending on the cellular context (Mrozik, 2018).

The aim of our study was to generate a cellular liver cancer model, for defining and understanding the mechanism of action of different antitumoral drugs (in monotherapy or combinations). To realize this model, genetically modified tumoral liver cells were obtained by CRISPR/Cas 9 gene editing technology. In the present study, one targeted the Ctnnb1(h) gene which encodes β-catenin, a multifactorial protein having important roles in the organism. This is the first report of genomic editing of Huh7 an HepaRG human cell lines, where successful co-transfection of the CRISPR/Cas9 KO plasmid and HDR plasmid were visualized by detection of the red fluorescent protein (RFP) via fluorescent microscopy. Additionally, the differential response of parental and KO cells to some antitumoral drugs was tested.

Material and methods

Nowadays, there are three types of CRISPR mechanisms, of which we used type II depicted in the diagram (Figure 1). Gene editing was performed using the *Santa Cruz β-catenin CRISPR/Cas* knockout system (reagents and method).

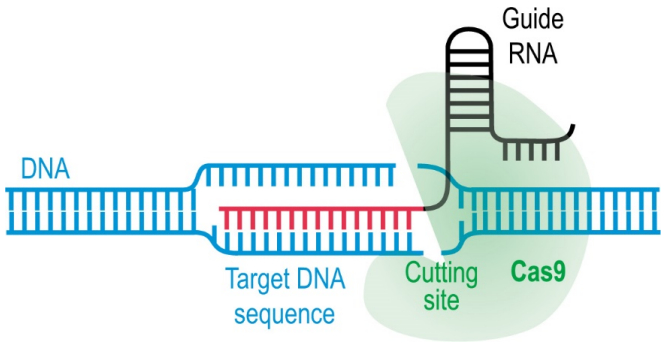

Figure 1. Editing a gene using the Crispr/Cas 9 technique. A created genetic sequence called guide RNA must match the DNA that is intended to be modified. This sequence is added to a cell together with Cas9, acting like molecular scissors which cut the DNA. The guide RNA home in on the targeted DNA, the scissors leaves the scene and a edited DNA is obtained after enzymes repair the cuts.

1. Cell cultures

Huh7 human HCC cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco/Life Technologies Ltd., Paisley, UK) and HepaRG cells were grown in William's E medium (Gibco) supplemented with 10% fetal bovine serum. For both cultures 50 units/ml penicillin, 50 µg/ml streptomycin, 2 mM Glutamax (Invitrogen Molecular Probes, Eugene, OR, USA), 5 µg/ml insulin and $5x10^5$M hydrocortisone hemisuccinate were used, in a humidified atmosphere containing 5% CO_2 at 37^0C. For CRISPR/Cas gene editing technique, the cells (1.5x 10^5 - 2.5x 10^5) were cultured (in 6 well plate) in 3 ml of antibiotic-free growth medium per well, 24 hours (to a 40–80% confluency).

2. CRISPR/Cas9 KO Plasmid and HDR Plasmid Transfection

Prior to transfection, the media was replaced with fresh antibiotic-free growth medium. 300 µl Plasmid DNA/UltraCruz® Transfection Reagent Complex containing: CRISPR/CAS β-catenin KO plasmid (h) sc-400038, and β-catenin HDR plasmid (h)sc-400038-HDR (Santa Cruz Biotechnology, Inc., USA) was added to the cells. Cells were incubated for 24–72 hours under standard conditions. No media replacement is necessary during the first 24 hours post-transfection, but fresh media was added as needed 24–72 hours post-transfection. After incubation, successful transfection of CRISPR/Cas9 KO Plasmid may be visually confirmed by detection of the green fluorescent protein (GFP) via fluorescent microscopy. Successful co-transfection of the CRISPR/Cas9 KO Plasmid and HDR Plasmid, may be visually confirmed by detection of the red fluorescent protein (RFP) via fluorescent microscopy. Confirmation was also obtained by Western blot using β-catenin Antibody (E-5) (Santa Cruz Biotechnology, Inc., USA sc-7963).

3. Puromycin Selection

The cells co-transfected with CRISPR/Cas9 KO Plasmid and HDR Plasmid, were selected with media containing the antibiotic puromycin (Santa Cruz Biotechnology, Inc., USA, sc-108071). Prior to treatment we established the optimal concentration of puromycin (working solution ranges from 1–10 µg/ml) for the target cell line and then the lowest concentration that kills 100% of non-transfected cells in 3–5 days from the start of puromycin selection was used. After 2 days of puromycin treatment, the media was aspirate and replaced and after 4 days the selected cells were cultured.

4. Western Blot Analysis

All cell lines were lysed on ice with lysis buffer supplemented with protease inhibitors for 20 min and centrifuged at 14000xg for 10 min. Total protein concentration was measured using BCA Protein Assay (Fermentas, Thermo Fisher Scientific, MA) and 10µg of total protein were separated by SDS-PAGE and then transferred onto a PVDF membrane (Millipore), incubated with primary antibodies (β-catenin, Santa Cruz) overnight at 4^0C and after washing, the membranes were incubated with HRP-conjugated rabbit anti-mouse secondary antibody (1/10000, Santa Cruz) at room temperature for 1h. Immunoreactive bands were visualized by chemiluminescence using the ECL detection system (Fermentas) and then were exposed to photographic films. Calnexin and actin were used as a loading control.

5. Immunofluorescence and Confocal Microscopy

Huh7 cells were fixed in 4% PFA and permeabilized in PBS with 1% BSA and 0,2% Triton, nonspecific binding site were blocked with PBS containing 1% BSA for 1 h. Incubation with primary antibodies against N-cadherin, E-cadherin (Cell Signaling Technology, BioZyme SRL) and β-catenin (Santa Cruz) was done for

30 minutes at room temperature, following the manufacturer's recommendations and with appropriate controls. Cells were washed and incubated with specific secondary antibodies conjugated with AlexaFluor 488 and AlexaFluor 647 and samples were examined with an LSM710 Zeiss confocal microscope and the image were analyzed by Zen2010 software (Carl Zeiss Instruments, Jena, Germany).

6. Antitumoral drugs assay

Cells were seeded onto 24 well plates and treated or not with different concentrations of oxaliplatin or doxorubicin (Accord Healthcare) for 48h. The cells were visualized using microscopic imaging (10x objective), harvested and counted using a Countess Automated Cell Counter (Thermo Fisher Scientific).

Results

In order to obtain genetically modified cells, in our experiments we used a β-catenin CRISPR /Cas9 knockout system (targeting Ctnnb1 (h)gene). Huh7 and HepaRG, cell lines, were transfected with CRISPR/CAS β-catenin KO plasmid (h) plus β-catenin HDR plasmid (h). The transfected cells were selected by puromycin treatment and were further cultured.

1. CRISPR/ Cas9 mediated β Catenin knockout

The efficacy and the efficiency of our CRISPR/ Cas9 editing experiment was evaluated by fluorescence imaging (Figure 2). The successful transfection of CRISPR/Cas9 KO Plasmid presence was evaluated and it was visually confirmed by detection of the green fluorescent protein (GFP). The successful co-transfection of the CRISPR/Cas9 KO Plasmid and HDR Plasmid, was visually confirmed by detection of the red fluorescent protein (RFP) via fluorescent microscopy.

Additionally, western blotting analysis of β-catenin expression in HuH 7 and HepaRG cells confirmed that the knockout of *Ctnnb1 (h)*gene, (which encodes β-catenin), using the CRISPR/Cas9 technology, was successful in both cell lines (Figure 3).

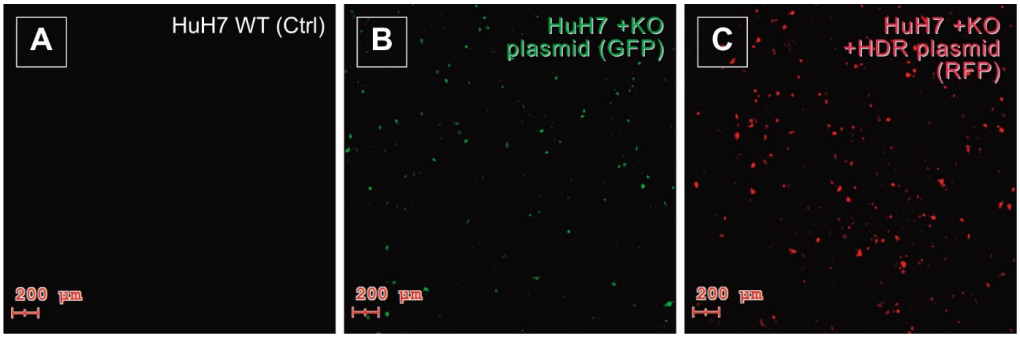

Figure 2. Fluorescence imaging of knockout of β-catenin by CRISPR/Cas9 system in Huh7 cell line. **A)** Parental WT (wild type) Huh 7 cells. **B)** β Catenin KO plasmid transfected Huh 7 cells evidenced by green fluorescent protein (GFP). **C)** β Catenin KO plasmid plus HDR plasmid co-transfected Huh 7 cells evidenced by red fluorescent protein (RFP).

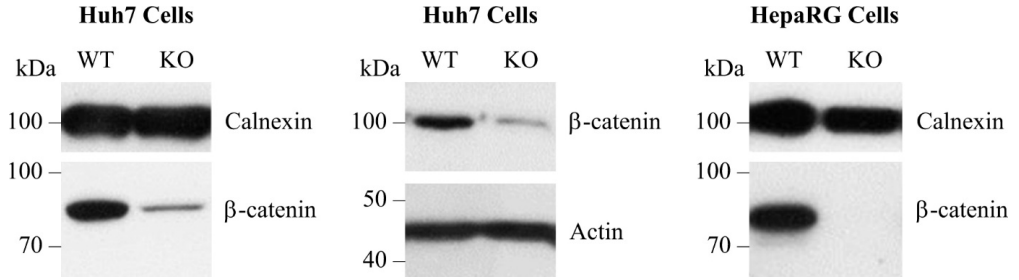

Figure 3. Knockout of β-catenin by CRISPR/Cas9 system in Huh7 and HepaRG cell lines - Western blot analysis. $2x10^5$ cells were seeded onto 6 well plates for 48h, lysed and samples analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with anti-β-catenin antibody. Calnexin and actin were used as a loading control.

2. Structural and functional differences between the parental cells lines and the KO ones

β Catenin/Cadherin relationship

Considering the structural and functional relationship in β-Catenin/E-cadherin and N-cadherin complexes, cytological examination of cultures by immunofluorescence microscopy was performed. The cells were double stained for β-catenin and for E-cadherin or N-cadherin. We demonstrated the distribution of N-Cadherin or E-Cadherin relative to that of β-catenin in Huh7 parental and KO cells. The merged images in parental WT cells, evidentiated co-localization of β-catenin and E-cadherin or N-cadherin. Our results confirmed the strong correlation between the expression of β-Catenin and cadherins: the knockout of β-Catenin considerably impaired the cadherins expression (Figures 4 & 5).

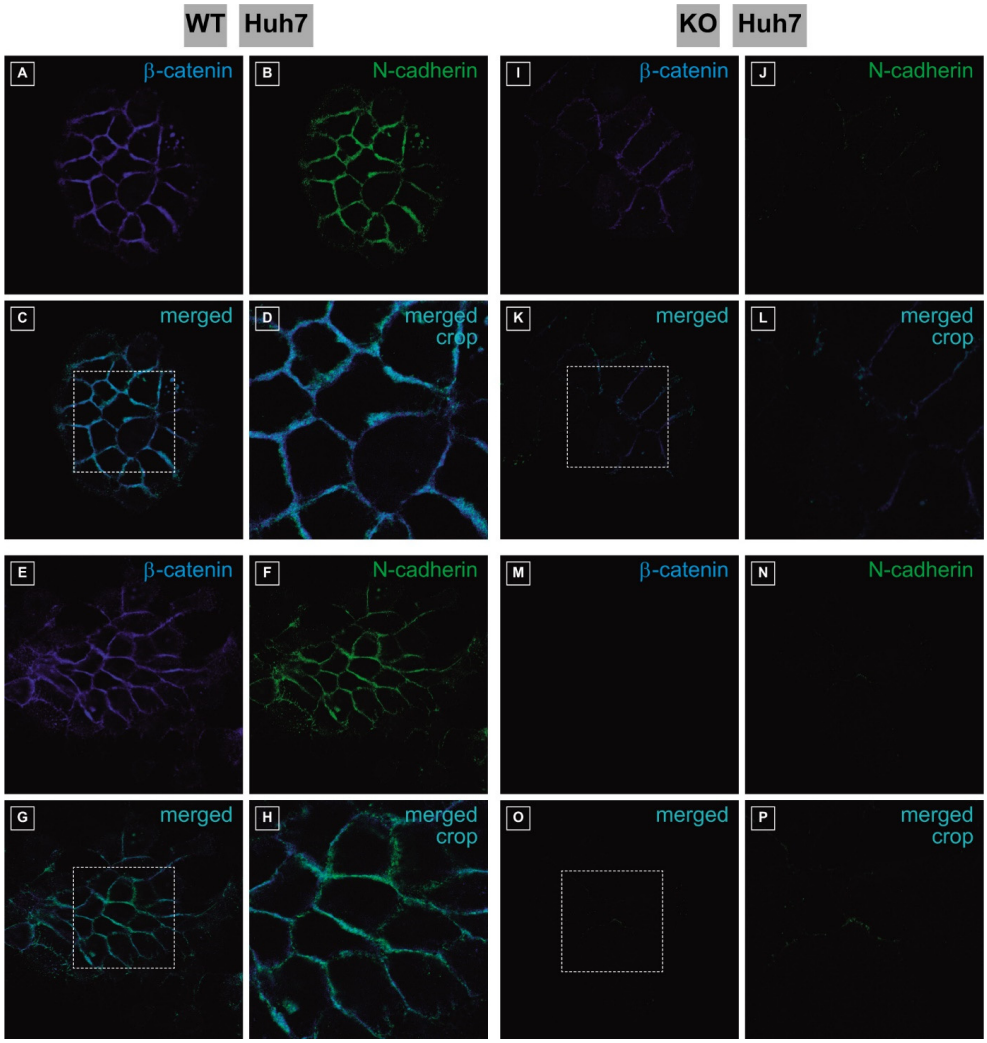

Figure 4. Representative images of parental Huh7^{WT} and Huh7^{KO} cell lines with N-cadherin, and β-catenin membrane expression. The Huh7^{WT} cells were fixed and stained for β-catenin (green) (**A, E**) and N-cadherin (**B, F**) Original magnification 40x. Colocalization of β-catenin with N-cadherin is depicted in **C, D** (40x), **G, H** (digital zoom of the selected area). The expression of N-cadherin in Huh7^{KO} cells is altered by β-catenin knock-out (**I-P**).

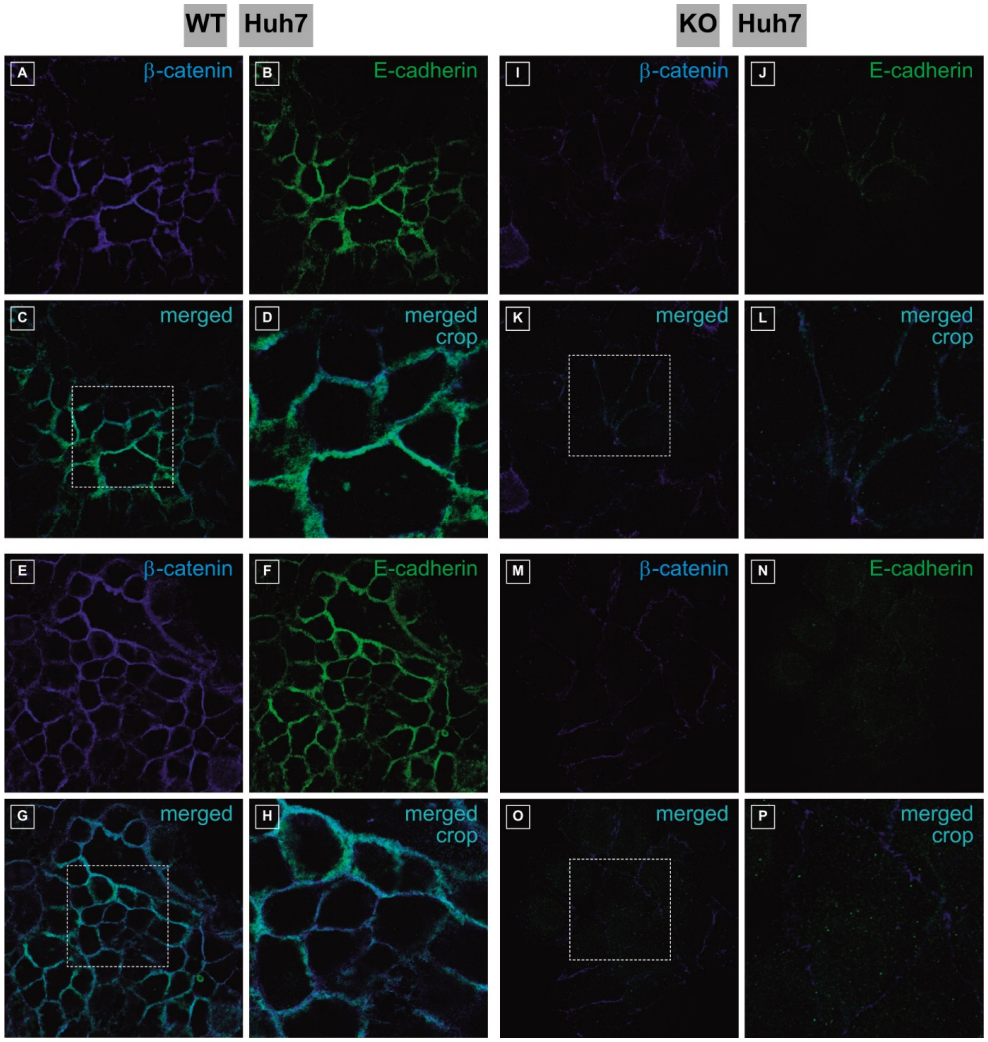

Figure 5. Representative images of parental Huh7^{WT} and Huh7^{KO} cell lines with N-cadherin, and β-catenin membrane expression. The Huh7^{WT} cells were fixed and stained for β-catenin (green) (**A, E**) and N-cadherin (**B, F**) Original magnification 40x. Colocalization of β-catenin with N-cadherin is depicted in **C, D** (40x), **G, H** (digital zoom of the selected area). The expression of N-cadherin in Huh7^{KO} cells is altered by β-catenin knock-out (**I-P**).

3. Response to anti tumoral drugs

The response to a specific drug can be affected by inherited or induced genetic modifications. To determine if antitumoral drugs exhibit different effects on β-catenin^{KO} cells versus parental tumoral cells, cells were incubated with progressive concentrations of oxaliplatin or doxorubicin for 48 hours.

The antitumoral drugs treatment of Huh7^{WT} and Huh7 β-catenin^{KO} cells presented a dose dependent response in both WT and KO cells, and induced the inhibition of their proliferation. However, KO cells presented a higher sensitivity to antitumoral treatment comparing to WT ones, more evident for oxaliplatin, confirming / demonstrating the role of β-catenin in cancer cell proliferation (Figure 6 &7). The effect of oxaliplatin was higher in KO cells at concentrations between 15µg/ml-25µg/ml, while the effects of doxorubicin were noticed at concentrations between 0.4µg/ml and 0.6-08 µg/ml.

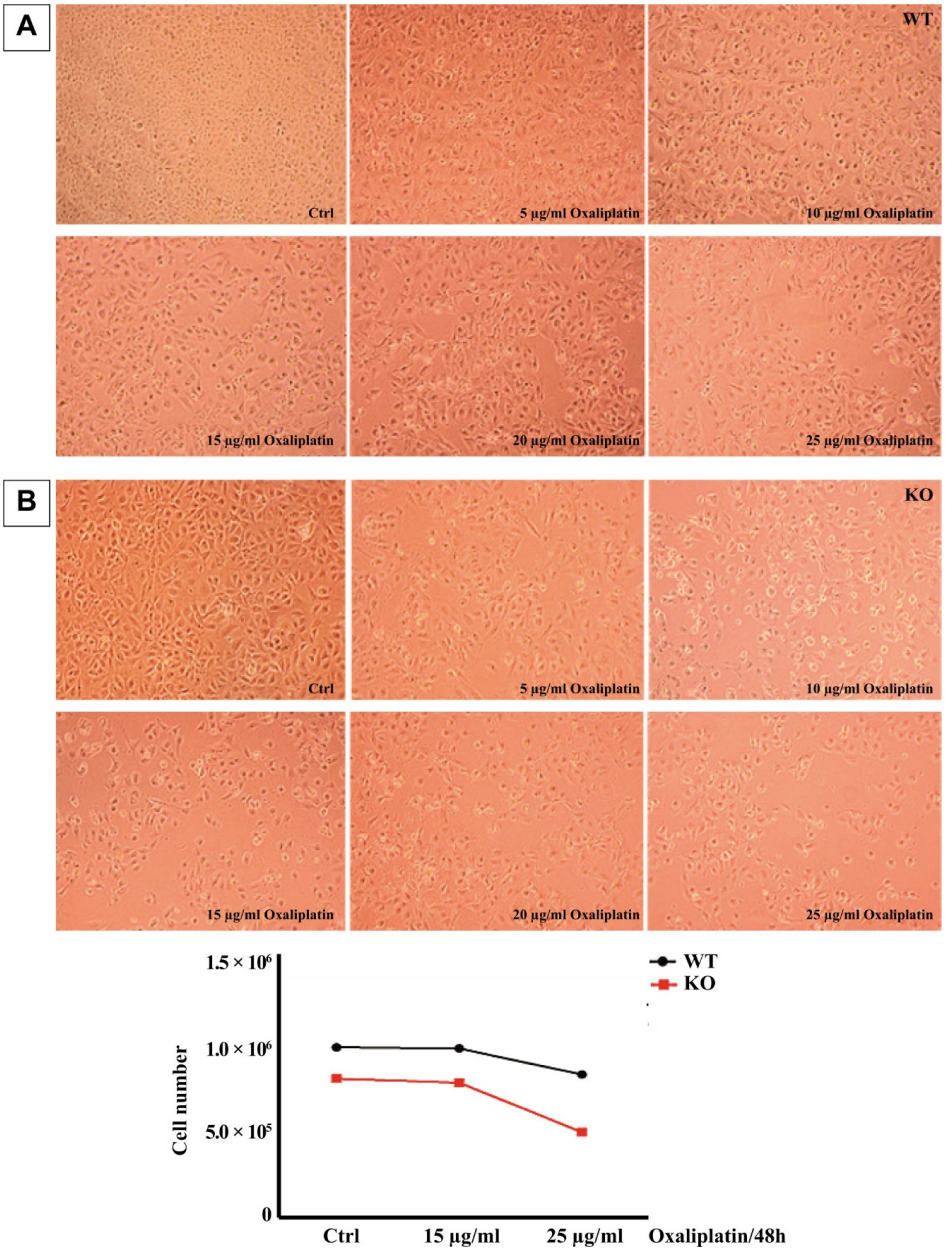

Figure 6. Effect of oxaliplatin treatment on cell proliferation in Huh7^{WT}and Huh7 β-catenin^{KO} cell lines. Cells were seeded onto 24 well plates and treated or not with oxaliplatin for 48 h. The cells were visualized using microscopic imaging (original magnification 10x) (**A**), harvested and counted using a Countess Automated Cell Counter (**B**). (WT – parental cells, KO – gene edited cells)

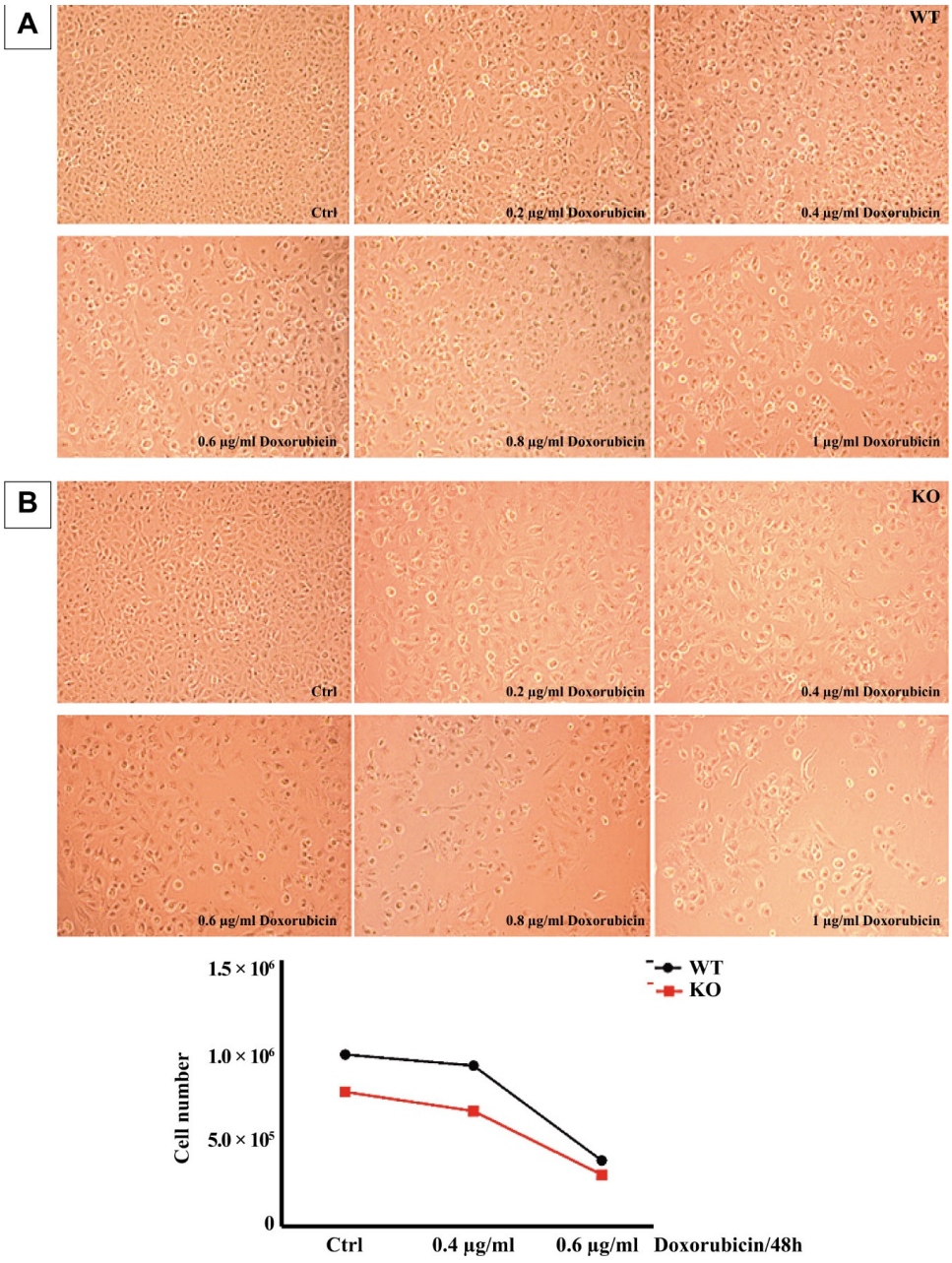

Figure 7. Effect of doxorubicin treatment on cell proliferation in Huh7^{WT} and Huh7 β-catenin^{KO} cell lines. Cells were seeded onto 24 well plates and treated or not with doxorubicin for 48 h. The cells were visualized using microscopic imaging (original magnification 10x) (**A**), harvested and counted using a Countess Automated Cell Counter (**B**) (WT – parental cells, KO – gene edited cells).

Results obtained on HepaRG cells, after treating the WT and KO cultures with the same concentrations of oxaliplatin and doxorubicin as the Huh 7 cells, were different. In HepaRG cells there is no difference in the sensitivity to doxorubicin treatment. However, the HepaRG KO cells seemed to be more sensitive for oxaliplatin (15-25 µg/ml) (Figures 8 & 9).

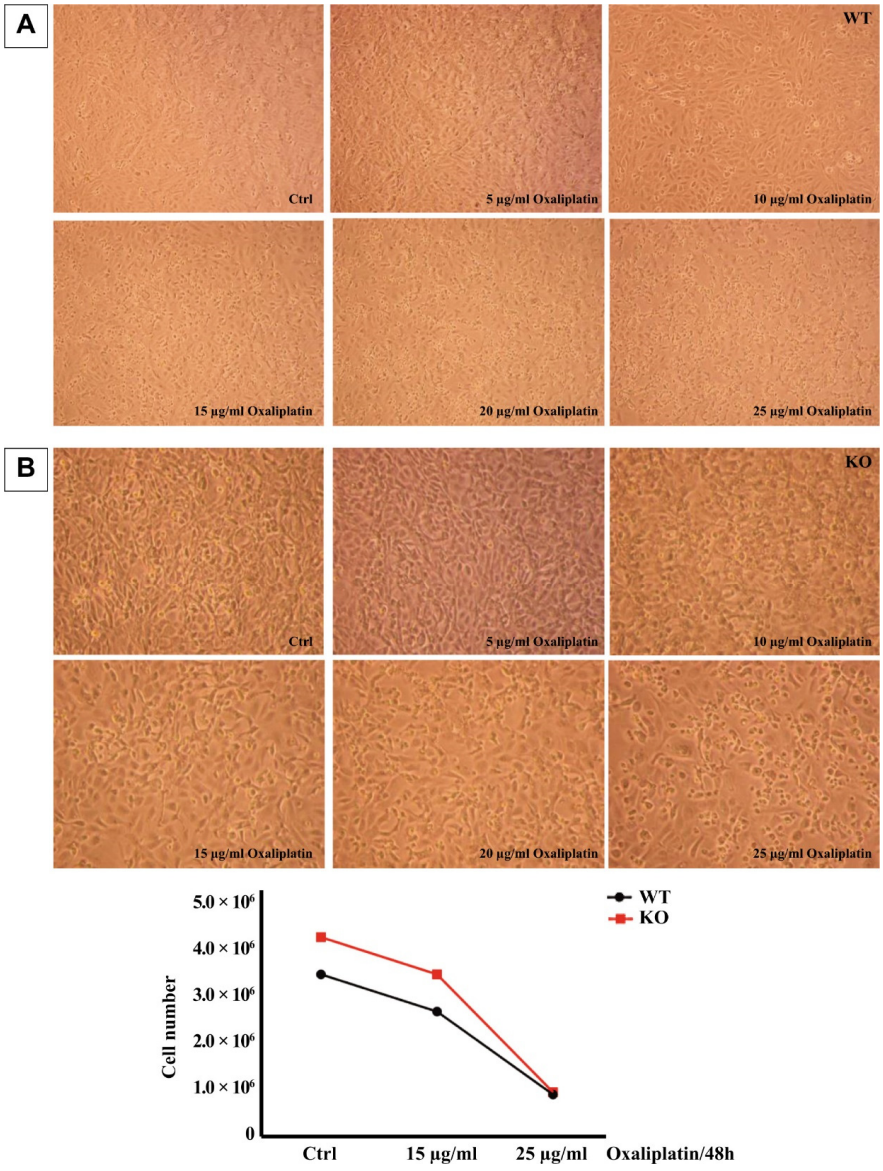

Figure 8. Effect of oxaliplatin treatment on cell proliferation in HepaRG^{WT} and HepaRG^{KO} cell lines. Cells were seeded onto 24 well plates and treated or not with oxaliplatin for 48 h. The cells were visualized using microscopic imaging (original magnification 10x) (**A**), harvested and counted using a Countess Automated Cell Counter (**B**) (WT – parental cells, KO – gene edited cells).

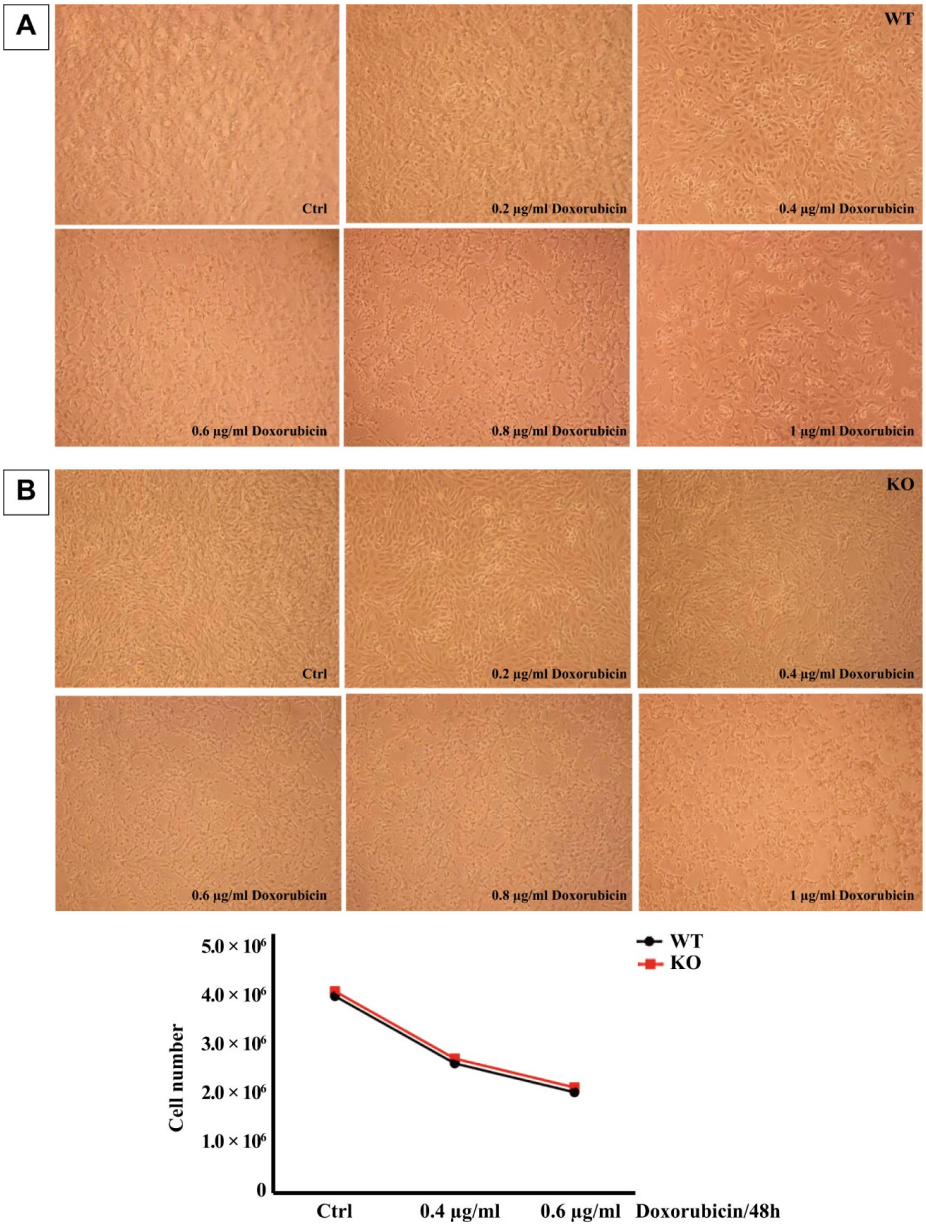

Figure 9. Effect of doxorubicin treatment on cell proliferation in HepaRG^{WT} and HepaRG^{KO} cell lines. Cells were seeded onto 24 well plates and treated or not with doxorubicin for 48 h. The cells were visualized using microscopic imaging (original magnification 10x) (**A**), harvested and counted using a Countess Automated Cell Counter (**B**) (WT – parental cells, KO – gene edited cells).

Discussion

For targeted and systematically study of molecular alterations in cancer, a series of disease cellular models were developed. In this models, development of genome engineering technologies allowed to perform precise and specific modifications of DNA sequences (Goodspeed, 2016). CRISPR-Cas9 gene editing in mammalian cells has rapidly changed the landscape of cancer research by facilitating the engineering of normal and cancer genomes (Sánchez-Rivera, 2015). Since knockout models are an important tool for investigating gene functions *in vivo* and *in vitro*, we imagined such an *in vitro* model to investigate the effects of β-catenin in hepatocytes and its involvement in proliferation and tumorigenesis. *Ctnnb1* gene knockout was obtained in Huh7 HCC and in HepaRG cell lines by CRISPR-Cas9 technology. Because of permanent genome alterations, tumor cells have defects in regulatory circuits that govern normal cell proliferation and homeostasis (Hanahan, 2011). Multiple genetic and epigenetic modifications induce cancer development, progression and antitumoral drugs resistance (Sarkar, 2013). Many studies demonstrated that abnormal activation of Wnt/β-catenin signaling can be detected in approximately 50%-70% of HCC and that possibly, genetic and/or epigenetic aberration of *Ctnnb1* (and its protein degradation regulators) are the major cause of Wnt signaling overactivation (Ding, 2015). Numerous studies revealed the efficiency of genome editing in a variety of cells and organisms via CRISPR-Cas9-mediated technology (Doudna, 2014).

The response to therapy is one of the most important in the evolution of HCC. Therefore, identifying the molecular pathways that would favor the action of chemotherapeutic drugs is essential. Found to be frequently upregulated in HCC, the Wnt/β-catenin pathway seems to be involved in tumorigenesis, drug resistance and tumor metastasis. Therefore, β-catenin has emerged as an alternative target in HCC (Vilchez, 2016). However, the involvement of β-catenin pathway in drug chemoresistance is contradicted by other studies. For example, Rosa et al., found no difference in the expression of β-catenin in pre- and post-neoadjuvant chemotherapy in triple-negative breast cancers specimens, but the importance of the study is hampered by the small number of cases (Rosa, 2015). Recently, Dong et al., showed that long-term activation of the nuclear xenobiotic receptor CAR and β-catenin induces HCC and created "a new model for tumor prevention and treatment studies with direct relevance to human HCC" (Dong, 2015). In this view there are no models created on hepatic cell lines, with direct involvement of the β-catenin pathway, and more so, one that involves editing the genome for the *Ctnnb1*gene. We assessed the effects of oxaliplatin and doxorubicin treatment on cell viability on Huh7^{WT} and Huh7 β-catenin^{KO} cell lines and compared them with the effects obtained on HepaRG cells, WT and KO, by counting with a Countess Automated Cell Counter. Our study shows a differential sensitivity to antitumoral drugs observed between Huh7 HCC cell line and HepaRG cell line. While in Huh7 cells we found that antitumoral drugs are more efficient on KO cells, there are no differences found between HepaRG^{WT} and HepaRG^{KO} This situation might be explained by the characteristic of HepaRG line which acts more like human primary hepatocytes and are not able to develop tumor after transplantation in nude mice (Cerec, 2007). Furthermore, the behavior of HepaRG cells in culture is determined by the plated cell density. Thus, they can be differentiated cells at high cell density, or undergo transdifferentiation into hepatocytic and biliary lineages at low density (Cerec, 2007; Klein, 2015). Our results, which are still in a preliminary phase, suggest that β-catenin is possibly involved in chemotherapy resistance, since the chemotherapeutic drugs were more efficient on Huh7 β-catenin^{KO} cell lines comparative with Huh7^{WT}. However, more complex studies are needed before reaching firm conclusions about the role of β-catenin in cytostatic sensitivity.

Conclusion

In order to develop a liver cancer model to study the causes of Wnt/β-catenin signaling aberration, we generated β-catenin KO tumoral cells, using the CRISPR/Cas9 gene editing technology. Here we describe a novel one step approach to modify *Ctnnb1* gene in human Huh7 and HepaRG cell lines having as purpose tracking the effects of cytostatic drugs on β-catenin KO lines. The CRISPR-Cas9 system represents a versatile and innovative tool in personalized medicine as a conduit between the bench and the bedside. This study is a preliminary one, which will lead to the possibility of its extension from the *in vitro* model to animal experimental models *in vivo*. In the future, the application of this technology will allow for the complete identification of the protein domains that support the multiplication and metastasis of cancer cells and which can be used for developing effective chemotherapeutic agents against HCC.

Acknowledgements

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