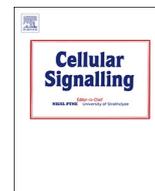




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## Tumor-specific inhibitory action of decorin on different hepatoma cell lines

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## ABSTRACT

**Background:** In spite of therapeutic approaches, liver cancer is still one of the deadliest type of tumor in which tumor microenvironment may play an active role in the outcome of the disease. Decorin, a small leucine-rich proteoglycan is not only responsible for assembly and maintenance of the integrity of the extracellular matrix, but a natural inhibitor of cell surface receptors, thus it exerts antitumorigenic effects. Here we addressed the question whether this effect of decorin is independent of the tumor phenotypes including differentiation, proliferation and invasion.

**Method:** Four hepatoma cell lines HepG2, Hep3B, HuH7 and HLE, possessing different molecular backgrounds, were selected to investigate. After proliferation tests, pRTK arrays, WB analyses, and immunofluorescent examinations were performed on decorin treated and control cells for comparison.

**Results:** Significant growth inhibitory potential of decorin on three out of four hepatoma cell lines was proven, however the mode of its action was different. Induction of p21<sup>WAF1/CIP1</sup>, increased inactivation of c-myc and  $\beta$ -catenin, and decrease of EGFR, GSK3 $\beta$  and ERK1/2 phosphorylation levels were observed in HepG2 cells, pathways already well-described in literature. However, in the p53 deficient Hep3B and HuH7, InsR and IGF-1R were the main receptors transmitting signals. In harmony with its receptor status, Hep3B cells displayed high level of activated AKT. As the cell line is retinoblastoma mutant, ATR/Chk1/Wee1 system might hinder the cell cycle in G2/M phase via phosphorylation of CDK1. In Huh7 cells, all RTKs were inhibited by decorin followed by downregulation of AKT. Furthermore, HuH7 cell line responded with concentration-dependent ERK activation and increased phospho-c-myc level. Decorin had only a non-significant effect on the proliferation rate of HLE cell line. However, it responded with a significant decrease of pAKT, c-myc and  $\beta$ -catenin activity. In this special cell line, the inhibition of TGF $\beta$  may be the first step of the protective effect of decorin.

**Conclusions:** Based on our results decorin may be a candidate therapeutic agent in the battle against liver cancer, but several questions need to be answered. It is certain that decorin is capable to exert its suppressor effect in hepatoma cells without respect to their phenotype and molecular background.

## List of abbreviations

BSA	bovine serum albumin
CDC25A	cell division cycle 25 homolog A
CDK	cyclin dependent kinase
DAPI	4',6-diamidino-2-phenylindole
DCN	decorin
DEN	diethylnitrosamine
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor

ELISA	enzyme-linked immunosorbent assay
EMT	epithelial–mesenchymal transition
ErbB2	Receptor tyrosine-protein kinase erbB-2; erythroblastic oncogene B
ERK 1/2	extracellular signal-regulated kinase 1/2
FBS	fetal bovine serum
GAG	glycosaminoglycan
GSK3	glycogen synthase kinase 3
HCC	hepatocellular carcinoma
HGF	hepatocyte growth factor
IC	immunocytochemistry
IGF-1R	insulin-like growth factor 1 receptor
InsR	insulin receptor
IRS	Insulin receptor substrate
MAPK	mitogen-activated protein kinase
Met	hepatocyte growth factor receptor

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MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PKB	protein kinase B
PVDF	polyvinylidene difluoride
Rb	retinoblastoma protein
RTK	receptor tyrosine kinase
SLRP	small leucine rich proteoglycan
TAA	thioacetamide
TBS	tris buffered saline
TGFβ	transforming growth factor beta
TP53	p53 protein gene
VEGFR-2	vascular endothelial growth factor receptor 2
WB	Western blot
WNT	proto-oncogene protein Wnt

## 1. Introduction

Hepatocellular carcinomas (HCC), representing the vast majority of the primary liver cancers, are the second leader cause of death among malignant tumors worldwide (782,000 cases and 745,000 deaths annually) [1]. The development of HCC depends on multiple factors, such as etiology and/or severity of other underlying diseases, lifestyle of patients, behavioral and environmental risk factors [2]. Influenced by these circumstances, HCC progression is a complex process which involves several molecular regulatory pathways of hepatoma cells, and the actual conditions of the tumor microenvironment. In most of the cases human HCCs develop from cirrhotic liver. Within years chronic inflammation and hepatocyte regeneration, together with the oncogenic action of hepatitis viruses and action of inflammatory cytokines push cirrhotic liver toward the development of liver cancer [3].

TGF-β is one of the major profibrotic cytokines involved in development of liver cirrhosis by stimulating nonparenchymal liver cells, especially hepatic stellate cells, and portal fibroblasts, for synthesis of excess extracellular matrix (ECM) [4]. Increasing amount of ECM in cirrhosis results in elevated levels of connective tissue proteins and proteoglycans, including decorin.

Decorin is a small leucine rich proteoglycan (SLRP), which as a member of extracellular matrix is responsible for maintaining the structural integrity of collagens [5]. Decorin binds to collagens, therefore has a spacer function between matrix fibers ensured by its glycosaminoglycan (GAG) side chain. It can be found in a variety of connective tissues, including skin, tendon, cartilage, and bone. In the healthy liver, the amount of decorin is quite low, its interstitial deposition is correlated with the increase of other connective tissue elements during fibrogenesis [6,7].

Researches of the past decades show that decorin has an inhibitory effect on tumor progression. Overexpression of decorin inhibits the proliferation of colorectal cancer cells [8], ovarium carcinoma cells [9] and breast cancer cells [10] in vitro. Furthermore, it prevents tumor cell-mediated angiogenesis [11] and effectively blocks metastatic spreading of breast cancer [12]. Several studies hypothesize that decorin may have a role in tumorigenesis of various other organs such as pancreas [13] or larynx [14] and these are only a few examples out many data, reported in recent years. Decorin was reported to suppress proliferation of HepG2 hepatoma cell line through p21<sup>WAF1/CIP1</sup> upregulation [15], and our previous investigations showed that decorin deficiency promotes hepatic carcinogenesis in vivo [16].

The best known mechanism of decorin antiproliferative action is its capability to bind to and inhibit cell surface tyrosine kinase receptors. Decorin core protein effectively binds to EGFR [17], IGF-IR [18], ErbB2 [19], Met (HGF receptor) [20] and VEGFR-2 [21]. Furthermore, decorin can interact with the natural ligand PDGF rather than to the receptor itself hindering downstream signaling pathways [22]. Decorin is also able to bind and inhibit TGF-β1, which leads to attenuated connective tissue production and hindered fibrogenesis [23]. Via these interactions decorin can prevent the binding of a particular ligand to its receptor, it can activate the receptors similarly to their ligands, and/or

can cause caveolin-mediated receptor internalization [24]. However it happens; decorin binding provokes intracellular signals which has an impact on cell proliferation [25–27].

When surgical removal cannot be taken into account, the management of hepatocellular cancer is not resolved. Therefore, better understanding of the molecular mechanisms driving HCC progression together with seeking for new approaches in its treatment has an urging importance.

Accordingly, by using recombinant decorin, we focused our research on human hepatoma cell lines HepG2, Hep3B, HuH7 and HLE to gain more information about the mechanism as this proteoglycan hinders their progression. We addressed the question if decorin has the potential to inhibit the proliferation of these hepatoma cell lines without respect of their phenotype. Furthermore, we aimed to explore the underlying molecular mechanisms leading to the observed effects of decorin.

## 2. Material and methods

### 2.1. Production of human recombinant decorin

For proteoglycan production a pCMV expression vector (Thermo Fisher Scientific, Waltham, MA, USA) construct containing the human decorin cDNA sequence was created (Szilák Laboratories, Bioinformatics & Molecule-design Ltd. Szeged, Hungary). Next, CHO-S (Chinese hamster ovary) cells (Gibco/Thermo Fisher) were transfected by the expression vector using electroporation by Neon™ transfection system, following manufacturer's recommendations (Invitrogen/Thermo Fisher). Successfully transfected cells were selected by adding 500 µg/ml of G418 solution to cell culture medium (Sigma) and human recombinant decorin production was conducted following manufacturer's recommendations (Gibco/Thermo Fisher). The recombinant proteoglycan was isolated as previously described [28]. After chromatographic purification the samples were dialyzed against distilled water and the proteoglycan content was checked by dimethyl methylene blue (DMB, Sigma-Aldrich, St. Luis, MO, USA) staining. Finally, salts were added to the samples to convert the distilled water to 1 × phosphate buffered saline (PBS), and the final decorin concentration was adjusted to 1000 µg/ml. These stock solutions were stored at –20 °C until use.

### 2.2. Tissue culture and reagents

HepG2 and Hep3B cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA), HuH-7 and HLE were acquired from the Japanese Collection of Research Bioresources Cell Bank (Osaka, JP). Cells were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Luis, MO, USA) with 1000 mg/l (5.5 mmol/l) glucose concentration, supplemented with 10% [v/v] fetal bovine serum (Sigma), and 1% [v/v] (Penicillin/Streptomycin (Sigma) in an atmosphere containing 5% CO<sub>2</sub> at 37 °C. Decorin was dissolved in sterile PBS and added to culture medium at final concentrations of 50 or 100 µg/ml (DCN50 and DCN100 groups) after 24 h of serum deprivation. Cultivation of cells is detailed in the particular assays.

### 2.3. Proliferation assay

Cell proliferation was estimated using a 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay (Sigma) measuring metabolic activity. Cells were plated in 96-well flat bottom tissue culture plate, containing 100 µl of DMEM (supplemented with 10% FBS) tissue culture medium, at a concentration of  $2 \times 10^3$  cells/well for HepG2 and HLE, and of  $5 \times 10^3$  for Hep3B and HuH7 hepatoma cell lines. After adhesion of cells, medium was changed to serum free DMEM for 24 h. The experiment was performed in serum deprived conditions for 0, 24, 48 and 72 h at 0, 50 and 100 µg/ml decorin concentration. Each well contained 180 µl medium and additional 20 µl PBS with the

appropriate amount of decorin. Twenty  $\mu\text{l}$  MTT solution was added per well at the endpoints. After 4 h incubation, formazan crystals were dissolved in 150  $\mu\text{l}$  of DMSO. Plates were shaken for 1 min and optical density was read on a multi-well scanning spectrophotometer (Labsystems Multiskan MS) at 570 nm.

#### 2.4. Enzyme-linked immunosorbent assay (ELISA)

TGF- $\beta$ 1 content in culture medium was assessed using Solid Phase Sandwich ELISA kit (Quantikine R&D Systems, Minneapolis, MN, USA, Cat. No.: DB100B). Fifty  $\mu\text{l}$  of medium was added to the microplate wells, and each sample was tested in triplicates. The procedure was performed according to the manufacturer's recommendation.

#### 2.5. Phospho-RTK array and Western blot

Cells grown in T25 cell culture flasks were harvested at 15 min and 48 h time points by using 1.0 ml lysis buffer (20 mM TRIS pH 7.5, 2 mM EDTA, 150 mM NaCl, 1% Triton-X100, 0.5% Protease Inhibitor Cocktail (Sigma) 2 mM  $\text{Na}_3\text{VO}_4$ , 10 mM NaF). After sonication and incubation for 30 min on ice, samples were centrifuged at 15,000g for 5 min. Supernatants were kept and protein concentrations were measured by Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). The activities of phospho-receptor tyrosine kinases (Phospho-RTKs) were assessed by their relative levels of phosphorylation using the Proteome Profiler Human Phospho-RTK Array Kit (Cat. no.: ARY001B, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Two hundred  $\mu\text{g}$  of total proteins was added to each membrane. Signals were developed by incubating the membrane in SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce/Thermo Fisher Scientific, Waltham, MA, USA), and visualized on a Kodak Image Station 4000MM Digital Imaging System.

For Western blot, 20  $\mu\text{g}$  of total proteins were mixed with loading buffer containing 1.5 v/v%  $\beta$ -mercaptoethanol and incubated at 99 °C for 5 min. Denatured samples were loaded onto a 10% polyacrylamide gel and were run for 30 min at 200 V on a Mini Protean vertical electrophoresis equipment (Bio-Rad). Proteins were transferred to a PVDF membrane (Millipore, Billerica, MA, USA) by blotting for 16 h at 75 mA. Ponceau staining was applied to determine blotting efficiency. Membranes were blocked with 5 w/v% non-fat dry milk (Bio-Rad) in TBS for 1 h followed by incubation with the primary antibodies at 4 °C for 16 h. Beta-actin immunostaining served as loading control. Membranes were washed 5 times with TBS containing 0.05 v/v% Tween-20, then were incubated with appropriate secondary antibodies for 1 h. Signals were detected by SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce/Thermo Fisher Scientific) and visualized by Kodak Image Station 4000MM Digital Imaging System. Western blot analyses were performed 3 independent times. The density of the bands was measured by the Kodak Image Station. For antibody specifications and dilutions applied see Supplementary Table 1.

#### 2.6. Immunocytochemistry

Cells were cultured in 6-well plates (with seeding density of  $1.3 \times 10^5$  cells/well for HepG2 and HLE, and  $2.15 \times 10^5$  cells/well for Hep3B and HuH7), each well contained a sterilized glass coverslip. After 24 h of serum starvation the cells were exposed to decorin for 48 h (in the same conditions as it was previously described). Cells were fixed in ice-cold methanol for 10 min. Coverslips were washed with phosphate buffered saline with 0.05% Tween 20 (PBST), and aspecific binding sites were blocked with 5% bovine serum albumin (BSA) dissolved in PBS. Cells were incubated overnight at 4 °C with primary antibody (anti-CTNBN1; HPA029159; Sigma) at a dilution of 1:100. Thereafter, cells were washed and incubated with secondary antibody (AlexaFluor 488; Invitrogen/Thermo Fisher Scientific) and DAPI for one hour. After the last washing procedure, fluorescent mounting media

(Dako, Glostrup, Denmark) was applied, and epifluorescent microscope (Nikon Eclipse E600) was used for evaluation.

#### 2.7. Statistical analysis

All statistical analyses were performed using GraphPad Prism 7.00 software (Graphpad Software Inc., La Jolla, CA, USA). Data were tested for normal distribution using the omnibus normality test of D'Agostino & Pearson. Significance of changes were tested using a nonparametric test (the Mann–Whitney  $U$  test) or the Student's  $t$ -test, depending on the distribution of the data. The independent experimental sets were compared for reproducibility. Only reproducible significant changes were considered as significant. Significance was declared at the standard  $p < 0.05$  level.

### 3. Results

#### 3.1. Proliferation inhibitory action of decorin is different in the hepatoma cell lines

To capture the effect of decorin on cell proliferation MTT assay was performed on each cell line. As Fig. 1. shows, proliferation rate of 3 out of 4 hepatoma cell lines were significantly inhibited upon decorin exposure.

The growth inhibition of Hep3B appeared 24 h after decorin exposure and was sustained during the entire experiment. Similar effect was observed on HepG2 cells, however decreased efficiency of decorin was seen at 48 h. The Huh7 cell line reacted to exogenous decorin only after 48 h, but the inhibitory effect lasted until the end of the experiment. In contrast, HLE cells showed only a slight decrease in proliferation rate after 72 h, but the difference did not reach statistical significance.

#### 3.2. Decorin has an impact on TGF- $\beta$ 1 secretion

As TGF- $\beta$ 1 is a known binding partner of decorin, we studied the secreted amounts of the growth factor by ELISA from cell culture supernatants. Fig. 2. shows the levels of TGF- $\beta$ 1 after a 48-h exposure to 50 and 100  $\mu\text{g}/\text{ml}$  decorin normalized to untreated controls. In HepG2 cell line, a significant decrease in the growth factor level was measured in DCN50 and DCN100 groups by 49% and 21% respectively ( $p < 0.05$ ). TGF- $\beta$ 1 secretion was significantly hindered in Hep3B (29% and 36% in DCN 50 and DCN100 groups in ( $p < 0.01$ ,  $p < 0.05$ )) and HLE cells as well; 68% and 53% in treated groups ( $p < 0.05$ ). However, no significant change in secreted TGF- $\beta$ 1 amount was detected in HuH7 cells upon decorin exposure.

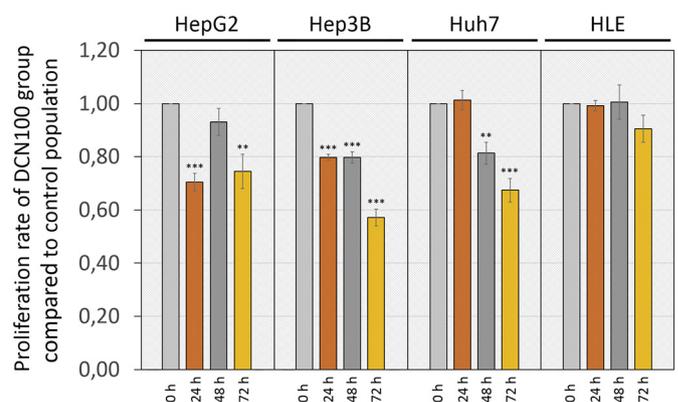


Fig. 1. Proliferation rate of hepatoma cell lines after decorin treatment. (DCN100 = 100  $\mu\text{g}/\text{ml}$  decorin in cell culture media) compared and normalized to control cells. Data are expressed as mean of normalized values  $\pm$  SD. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

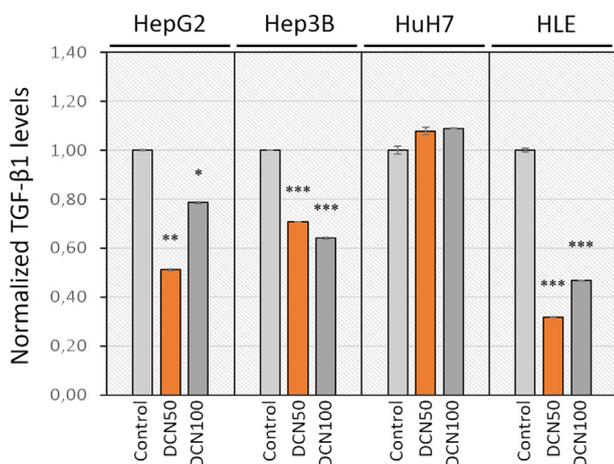


Fig. 2. TGF-β1 production of HepG2, Hep3B, HuH7 and HLE hepatoma cell lines exposed to decorin. Diagram shows normalized TGF-β1 levels after 48 h of decorin treatment from media of hepatoma cell lines. \*p < 0.05; \*\* p < 0.01.

3.3. Different signaling pathways are provoked by decorin in hepatoma cell lines

Next, we aimed to unravel the signaling processes provoked by decorin in each particular cell line. To this end, tyrosine kinase receptors and their major signaling pathways implicated in the proliferation of hepatomas (MAPK, WNT, PKB/Akt) has been studied. In addition, expression of representative proteins of cell cycle have been detected.

3.3.1. The “conventional” HepG2

After an early activation of EGFR receptor upon 15 min decorin treatment, (Fig. S1), EGFR was the only detectable phosphorylated RTK in HepG2 cells (Fig. 3) at the 48 h time-point. Its phosphorylation

decreased upon decorin exposure in a dose dependent manner (Fig. 3). Cells exposed to 50 (DCN50) or 100 μg/ml (DCN100) decorin displayed a decrease of the receptor by 48% and 84% respectively relative to the control cell population (p < 0.001, Fig. 3).

Decreased EGFR phosphorylation went parallel with reduced activation of ERK1 (30% in DCN50 and 22% in DCN100 groups relative to control [p < 0.001 and non-significant]), and ERK2 proteins (79% and 64% in DCN50 and DCN100 [p < 0.001 in both groups]). Furthermore, levels of the cyclin-dependent kinase inhibitor p21<sup>WAF1/CIP1</sup> and p27<sup>KIP1</sup> were elevated. We observed a 4.8-fold and a 3.7-fold increase in p21<sup>WAF1/CIP1</sup> protein in DCN50 and DCN100 groups respectively (p < 0.01 in both groups). In case of p27<sup>KIP1</sup>, a 1.74-fold and a 1.5-fold elevation was measured in DCN50 and DCN100 groups after 48 h of treatment (p < 0.01 and p < 0.05) (Fig. 4.). No considerable change in Akt phosphorylation was found.

Regarding other signaling pathway proteins, we focused on the phosphorylation of c-myc, GSK3α/β and β-catenin (Fig. 4.). In HepG2 cells, decorin exposure resulted in reduced phosphorylation of GSK3α/β, and elevated levels of phospho-β-catenin. Phosphorylation of GSK3α was decreased by 15% and 44% in DCN50 and DCN100 groups, relative to control (p < 0.05 and p < 0.01), while phospho-GSK3β amount was reduced by 39% and 50% (p < 0.001 in both groups). As a result, we observed elevated phosphorylation of β-catenin (2.1-fold and 2.4-fold in DCN50 and DCN100 groups vs. control, p < 0.01 in both groups), and c-myc (2.5-fold and 4.7-fold in DCN50 and DCN100 groups vs. control; p < 0.05 and p < 0.01) (Fig. 4.).

In harmony with these results β-catenin vanished from the nuclei of HepG2 cells (Fig. 5) showing immunolocalization on the cell membrane after 48 h of decorin treatment.

3.3.2. The “restriction point deficient” Hep3B

The Hep3B cell line bears a deleterious mutation in genes coding for p53 and retinoblastoma proteins resulting in failure in cell cycle blockade in G1 phase. Regarding TK receptors, changes in EGFR, InsR

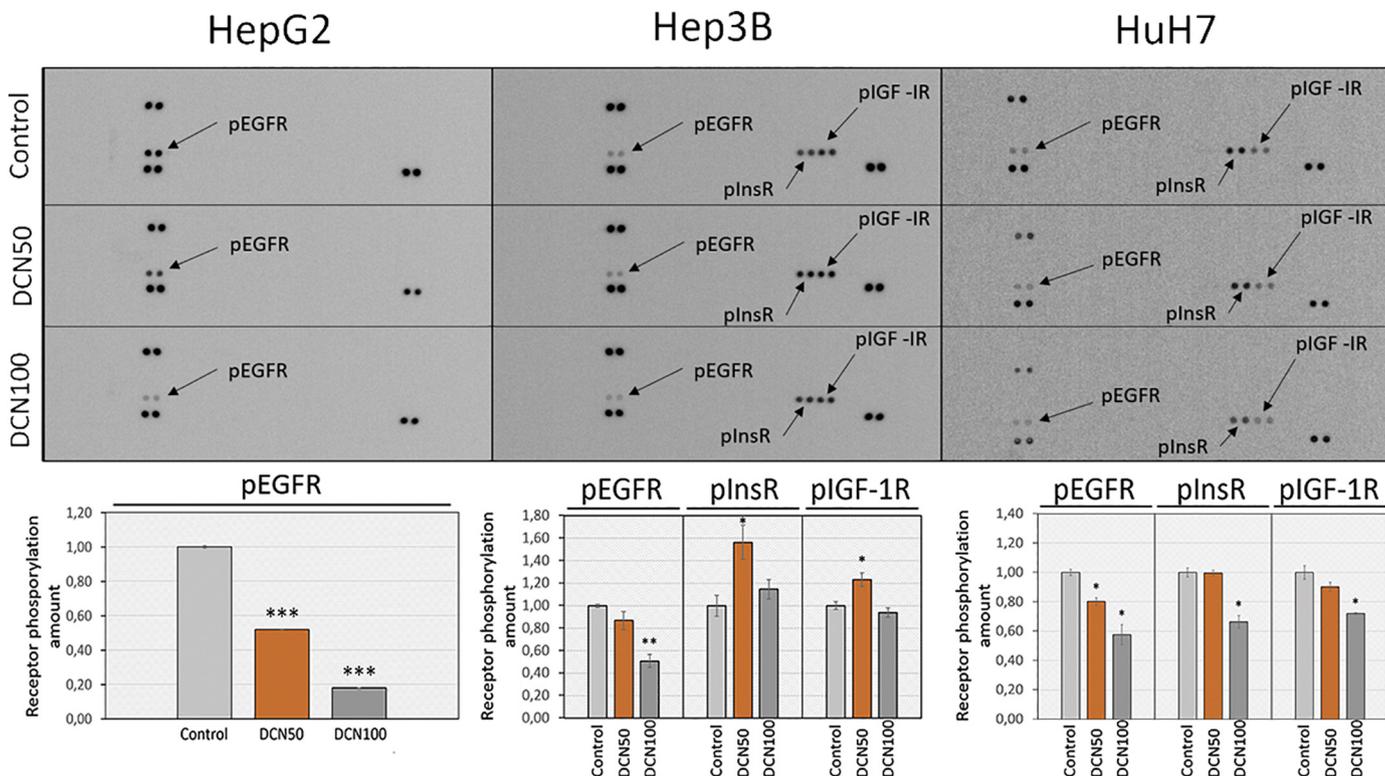


Fig. 3. Activity of receptor tyrosine kinases in HepG2, Hep3B and HuH7 cell lines after 48 h of decorin treatment. Bar charts show the levels of detected phospho-receptors in decorin treated groups compared to the untreated controls in each cell line \*p < 0.05; \*\* p < 0.01; \*\*\* p < 0.001.

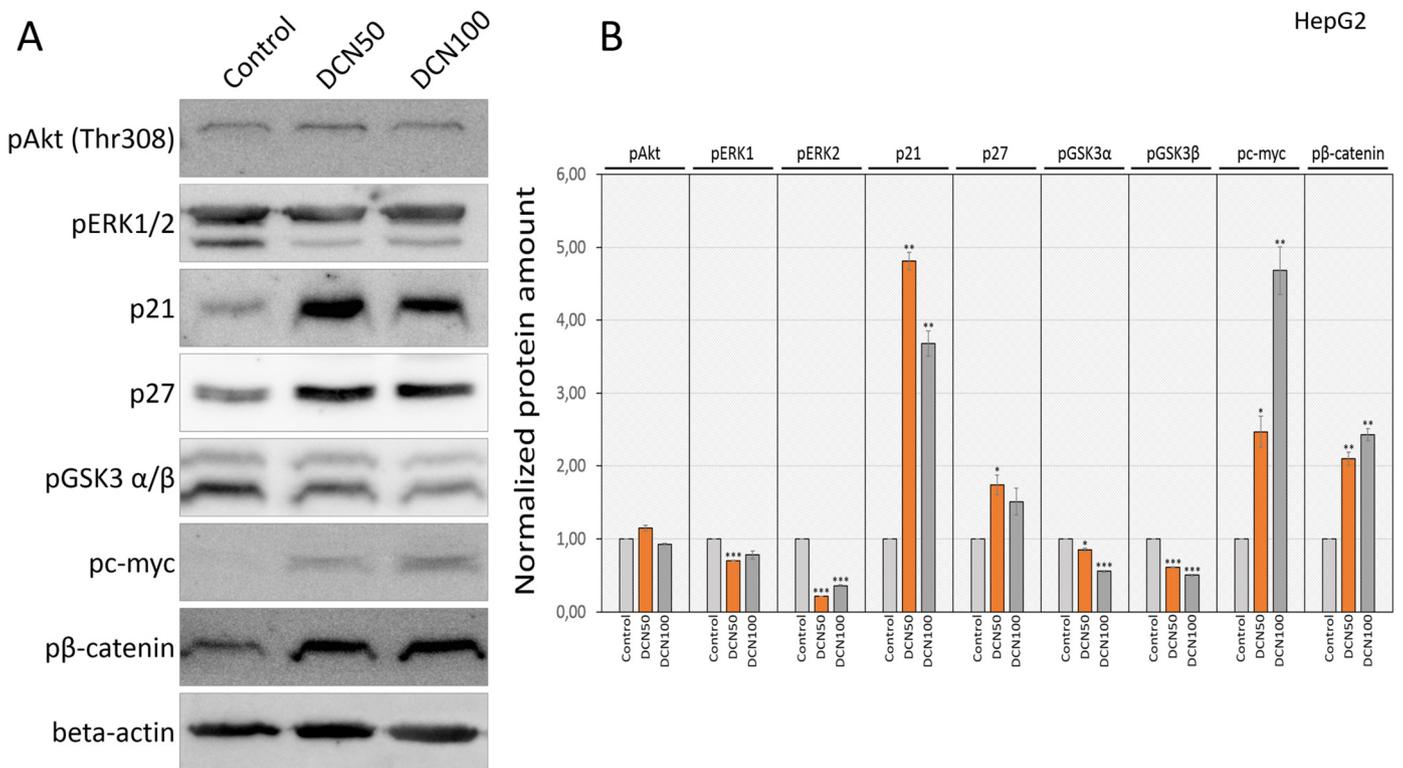


Fig. 4. Western blot analysis of various signaling proteins in HepG2 cells after 48 h of decorin treatment. Beta-actin was used as loading control (A). Bar charts represent the relative amount of investigated proteins in DCN50 and DCN100 treated cells (B). Data are shown as mean of normalized values  $\pm$  SD. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

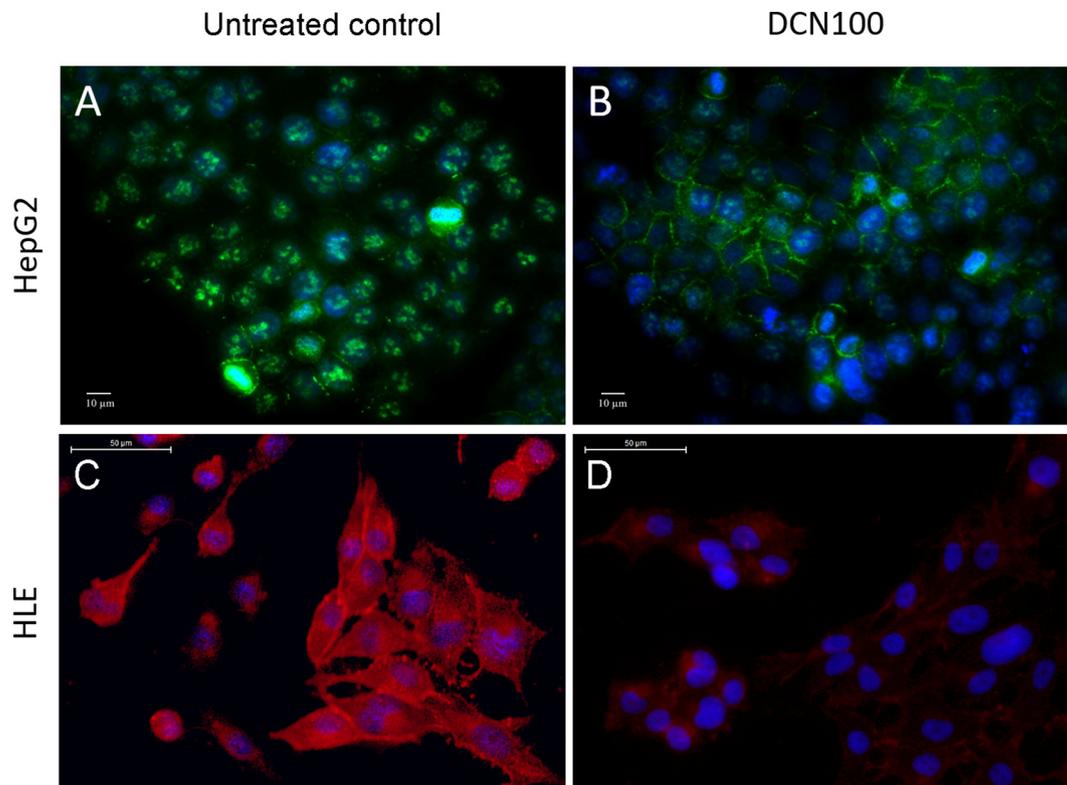
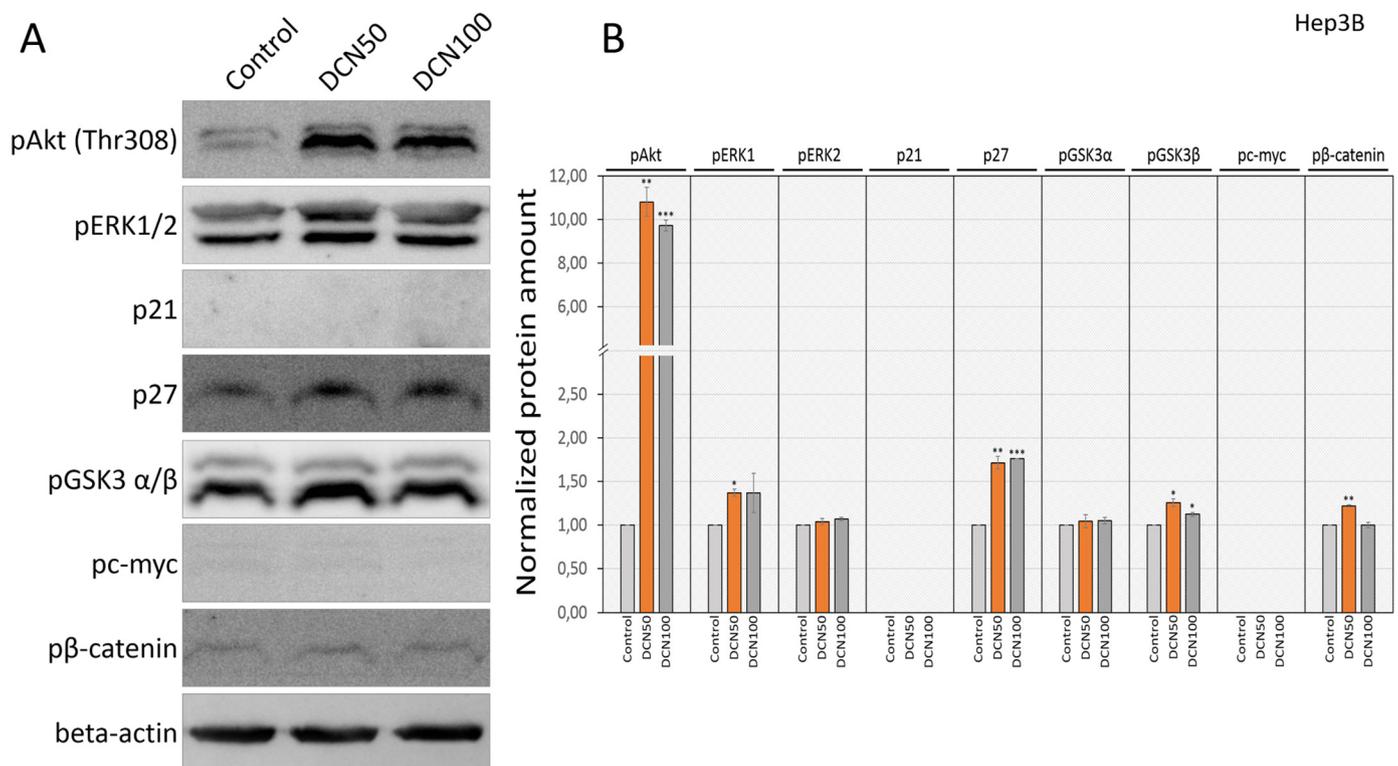


Fig. 5. Representative images of immunocytochemical analysis of  $\beta$ -catenin localization in control (A) and decorin treated (B) HepG2 cells, as well as in control HLE cells (C) and after decorin exposure (F). Scale bar = 10  $\mu$ m (A, B) and 50  $\mu$ m (C, D).



**Fig. 6.** Western blot analysis of various signaling proteins in Hep3B cells after 48 h of decorin treatment. Beta-actin was used as loading control (A). Bar charts represent the relative amount of investigated proteins in DCN50 and DCN100 treated cells (B). Data are shown as mean of normalized values  $\pm$  SD. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

and IGF-IR phosphorylation were detected upon decorin exposure (Fig. 3). At 15 min, inhibition of EGFR by decorin was observed in Hep3B cells, and no change in InsR and IGF-1R levels were detected (Fig. S1). After 48 h, similarly to HepG2, the active EGFR level was reduced by 13% and 49% in DCN50 and DCN100 groups relative to control cells (non-significant in DCN50 and  $p < 0.01$  in DCN100); however we found enhanced activation of InsR and IGF-IR in Hep3B cells. While pInsR increased with 1.56-fold in DCN50 group compared to untreated control ( $p < 0.05$ ), the elevation was not significant in DCN100 group. For pIGF-IR, the pattern was quite similar, 1.23-fold increase was detected in DCN50 vs. control ( $p < 0.05$ ), however the change in DCN100 group did not reach statistical significance (Fig. 3).

In parallel with IGF and insulin receptor activation we found considerable elevation in AKT level, phosphorylated on threonine 308 residue (Fig. 6.). A 10.8 and a 9.7-fold increase was observed in DCN50 and DCN100 groups compared to untreated control Hep3B cells ( $p < 0.01$  and  $p < 0.001$  respectively). While a 37% increase of pERK1 was detected in both treated populations; ( $p < 0.05$  in DCN50 group and non-significant in DCN100 group), we found no significant change in the phosphorylation of ERK2. We did not detect p21<sup>WAF1/CIP1</sup> in Hep3B cell line either in untreated, or in decorin exposed cells. However, the amount of p27<sup>KIP1</sup> protein was significantly increased after 48 h of decorin exposure; 71% and 76% elevations were observed in DCN50 and DCN100 groups ( $p < 0.01$  and  $p < 0.001$ ). While phosphorylation of GSK3 $\alpha$  showed no alternations after decorin treatment compared to the control cells, minor elevations were detected in case of phospho-GSK3 $\beta$  levels after treatment (26% and 12% in DCN50 and DCN100 groups;  $p < 0.05$  in both cases). We could not detect phospho-c-myc in Hep3B cells and there was no significant change in phospho- $\beta$ -catenin levels (Fig. 6).

As Hep3B cells are TP53 and RB mutant, we needed to look for a cell cycle regulator beyond G1 phase. We found a marked induction of CDK1 phosphorylation in these cells. We observed 2.42-fold elevation in DCN50 group, and a 2.04-fold increase in DCN100 population

( $p < 0.01$  in both groups) (Fig. 7.). In parallel with the increased phospho-CDK1 level, a 24% decrease in phosphatase Cdc25A and a 35% elevation in Wee1 kinase mRNA level were detected in DCN50 group.

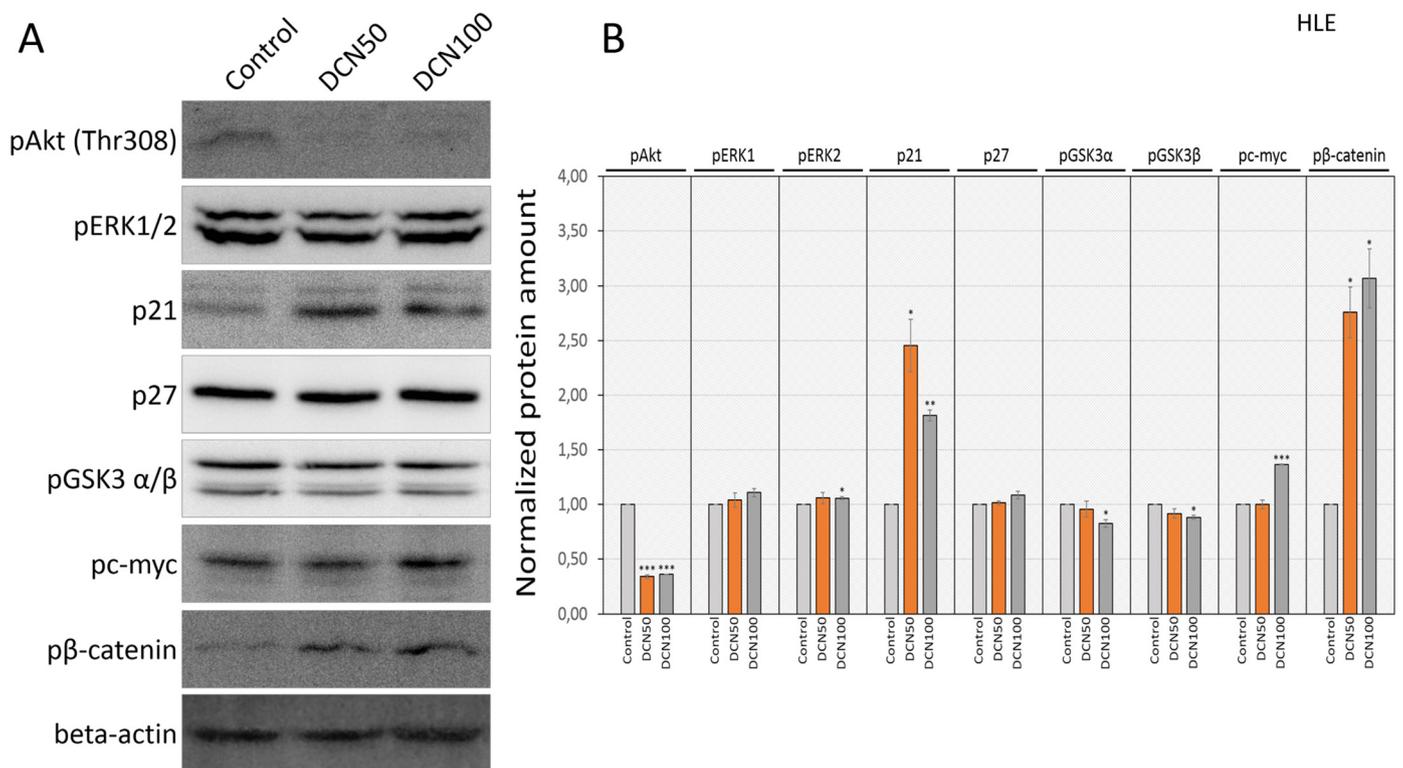
### 3.3.3. The “p53 mutant” HuH7

The HuH7 cell line carries a missense mutation in p53 gene resulting in the overexpression of the mutant protein with oncogenic properties. After 15 min, decorin was able to decrease the level of active EGFR, and induced InsR and IGF-1R phosphorylation. Minute amounts of active FGFR and RYK were also seen on the membranes (Fig. S1). After 48 h, the HuH7 cells displayed active EGFR, InsR and IGF-IR similarly to Hep3B. EGFR phosphorylation was significantly decreased after decorin exposure by 20% in DCN50 group and by 42% in DCN100 group ( $p < 0.05$  in both cases). InsR and IGF-IR phosphorylation were also altered, although we could not detect significant change in the DCN50 group of HuH7 cells. However, phospho-InsR level was decreased by 34% and IGF-1R phosphorylation was lessened by 28% in DCN100 group ( $p < 0.05$  in both cases, Fig. 3.).

Akt phosphorylation was extensively inhibited after 48 h of decorin exposure in HuH7 cells. 44% loss of phosphorylated Akt was seen in DCN50, and 82% in DCN100 group ( $p < 0.001$  and  $p < 0.001$ ). In contrast to our previous findings, we detected massive ERK activation. A 2.8-fold and a 3.9-fold elevation were found in pERK1 levels in DCN50 and DCN100 groups relative to control cells ( $p < 0.001$  and  $p < 0.01$ ), while a 12.7-fold and a 19.8-fold increase was observed in case of ERK2 ( $p < 0.001$  in both cases).

We could not detect p21<sup>WAF1/CIP1</sup> in HuH7 cells. P27<sup>KIP1</sup> displayed no major alternation after decorin treatment. GSK3 phosphorylation displayed a very similar pattern to that of pInsR and pIGF-IR. Phospho-GSK3 $\alpha$  level did not increase significantly in DCN50 group, while it lessened by 61% in DCN100 group ( $p < 0.01$ ). In parallel, phospho-GSK3 $\beta$  level elevated by 32% in DCN50 group, while it decreased by 62% in DCN100 group ( $p < 0.05$  and  $p < 0.001$ ) of HuH7 cells. We





**Fig. 9.** Western blot analysis of various signaling proteins in HLE cells after 48 h of decrin treatment. Beta-actin was used as loading control (A). Bar charts represent the relative amount of investigated proteins in DCN50 and DCN100 treated cells (B). Data are shown as mean of normalized values  $\pm$  SD. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

found intensive upregulation of c-myc phosphorylation after decrin exposure. A 3.17 and a 2.92-fold increase was detected in DCN50, and in DCN100 groups respectively, compared to the untreated control cells ( $p < 0.001$  and  $p < 0.01$ ). We could not detect phosphorylated  $\beta$ -catenin in HuH7 cell line (Fig. 8.).

### 3.3.4. The “fibroblast-like” HLE

HLE cells share several features with mesenchymal cells such as the ability to migrate and being vimentin positive. After 15 min, active EGFR was detected in HLE cells, but the proteoglycan did not alter their levels. Trace amounts of RYK, ErbB3 and Axl were also detected (Fig. S1). Interestingly, no phosphorylated RTK was detected in the cell line after 48 h. Despite of this, we found considerable downregulation of Akt phosphorylation, 66% and 64% decrease in DCN50 and DCN100 groups relative to the control population of HLE cells ( $p < 0.001$  in both cases). However, no major changes were observed in pERK1 and pERK2 levels. While the protein level of p27<sup>KIP1</sup> did not alter upon decrin exposure, the p21<sup>WAF1/CIP1</sup> amount showed significant upregulation (2.45 and 1.82-fold increase in DCN50 and DCN100 group, respectively;  $p < 0.05$  and  $p < 0.01$ ). The phospho-GSK3 protein levels slightly decreased. GSK3 $\alpha$  phosphorylation lessened by 4% and 17% in decrin-exposed groups ( $p < 0.05$  in DCN100 group), while phospho-GSK3 $\beta$  decreased by 8% and 12% in DCN50 and DCN100 groups compared to untreated HLE cells ( $p < 0.05$  in DCN100 group). While phospho-c-myc amount showed no change at all in DCN50 group relative to control, we found a 37% increase in DCN100 group ( $p < 0.001$ ). Finally, considerable upregulation in  $\beta$ -catenin phosphorylation was observed in HLE cells; 2.8 and a 3.1-fold elevation in DCN50 and DCN100 groups were detected compared to control population ( $p < 0.05$  in both cases) (Fig. 9.). In parallel disappearance of the protein from the nucleus and the cytoplasm was also observed by immunostaining after decrin treatment (Fig. 5C, D).

## 4. Discussion

Previously we proved the protective role of decrin against thioacetamide (TA) and diethylnitrosamine (DEN) induced hepatocarcinogenesis [16], however it was a further question whether the proteoglycan shows efficacy against human hepatomas, as well. The question is all the more important, as management of liver cancers other than surgery fails to provide improvement for decades [29].

The inhibitory effect of human recombinant decrin on HepG2 cells through p21<sup>WAF1/CIP1</sup> [15] and p57<sup>KIP2</sup> upregulation was previously reported [30], therefore HepG2 was applied as an internal control in our experiments. Our results obtained on this cell line were in a full agreement with the literature data. Decrin exposure was able to diminish the proliferation rate of HepG2 cells, although its rate of inhibition slowed down at 48 h. The other two differentiated hepatoma cell lines, Hep3B and Huh7, showed stepwise decrease in proliferation starting at 24 and 48 h respectively. However, decrin did not inhibit the proliferation of HLE cells. Detailed analysis of signaling pathways revealed that decrin initiates different molecular responses in our hepatoma cell lines even if their antiproliferative response is identical. For HepG2 cells, activation of EGFR provides stimulus for continuous proliferation, and decrin initiates its effect through downregulation of EGFR signaling [31]. Accordingly, we observed a concentration-dependent inhibition of EGFR, with decreased phospho-ERK1/2, and elevated p27<sup>KIP1</sup> and p21<sup>WAF1/CIP1</sup> amounts in HepG2 cells. Furthermore, decrease in degradative phosphorylation of GSK3 $\alpha/\beta$  was noticed which could be related to the signaling events described above, as downregulation of ERK1/2 activity can result in reactivation of GSK3 $\beta$  [32]. This leads to phosphorylation and subsequent proteasomal degradation of  $\beta$ -catenin and c-Myc [33]. As an effect of decrin the nuclear localization of  $\beta$ -catenin decreased, and the protein mainly localized in the cell membrane of HepG2 cells (Fig. 10).

However, neither Hep3B, nor HuH7 cells use EGFR as primary signaling receptor. In contrast, basic activities of insulin receptor (InsR)



studies are needed to reveal underlying signaling processes. However, this newly discovered impact of proteoglycan on G2/M cell cycle processes further reinforces the tumor suppressor ability of decorin.

In the HuH7 cell line, decorin administration reduced the amount of phospho-InsR and phospho-IGF-1R, as expected. In addition, we witnessed a concentration dependent decrease in the phosphorylation of EGFR after decorin exposure, similarly to that of HepG2 and Hep3B cells. In parallel with the inhibition of receptors, a dose-dependent lessening of pAkt amount was observed. As a consequence, the phosphorylation of GSK-3 decreased accompanied by elevated phospho-c-Myc level. Although we found no detectable change in levels of p21<sup>WAF1/CIP1</sup> or p27<sup>KIP1</sup>, c-Myc deactivation might cause alternations in cell cycle regulation, such as induction of p15<sup>INK4B</sup> or p16<sup>INK4A</sup>, or inhibition of Cyclin A2, Cyclin D2 and Cyclin E1, all are specific partner genes of c-Myc [43]. The most remarkable change, we observed in these experiments was the massive ERK activation (Fig. 10). Similar phenomenon was described previously in HuH7 cell after cisplatin exposure [44]. This ERK phosphorylation ultimately leads to apoptosis, thus, activated ERK1/2 can act as tumor suppressor molecules in HuH7 cells [45]. Furthermore, it was proposed that ERK1/2 may initiate either stimulatory or inhibitory effect on proliferation and the level of its activity determines that which of the opposite actions will prevail [46]. Additional investigations are needed to clarify the exact mechanisms of signal transduction after decorin treatment in HuH7 hepatoma cell line.

Among the investigated HCC cell lines HLE is considered as a non-differentiated one [47], as supported by its strong vimentin positivity. This might be the reason why we could not find any activated EGFR even on the surface of untreated control cells. Furthermore, neither of the examined RTKs was active in HLE cells. In harmony with that HLE cells showed no considerable change in ERK1/2 phosphorylation. In the light of this, it becomes more understandable, that we found only a minor suppression of cell proliferation by MTT assay. On the other hand, 48 h after decorin exposure, > 50% decrease of pAkt<sup>Thr308</sup> was observed together with significant downregulation of pAkt<sup>Thr308</sup> [48]. As HLE already went through the process of EMT [49], where Akt activation plays crucial role, we may say that in this case decorin interferes with EMT, and exerts modest antiproliferative action. The slight GSK3 $\beta$  activation which could explain the inactivating phosphorylation of  $\beta$ -catenin and c-Myc (Fig. 10.) with subsequent p21<sup>WAF1/CIP1</sup> induction were insufficient to block or slow down the proliferation rate of HLE cells after 72 h of decorin exposure. Since many mesenchymal cells can internalize the decorin [50,51], it is conceivable that mesenchymal-type HLE also possesses a decorin-specific endocytosis receptor.

Further evidence for the mesenchymal behavior of HLE cells is their ability to migrate, which is enhanced by TGF $\beta$  treatment [52]. On the other hand, Dzieran and coworkers described, that HLE cell line is insensitive to the cytostatic effect of growth factor, which can explain the result of our MTT assay. The bipolar behavior of TGF $\beta$  is a well-known phenomenon in liver tumors, where the implication of caveolin is recently described [53]. When autocrine TGF $\beta$  production starts in tumor cells, it triggers EMT and contributes to the tumor invasion. Except for HuH7, decorin significantly reduced TGF $\beta$  levels in each hepatoma cell line. Since our studies were performed in a serum free environment, the TGF $\beta$  found in the medium derived from tumor cells, however, we do not know whether the growth factor produced by the hepatomas became activated. It is certain that by reducing the TGF $\beta$  level, decorin may inhibit EMT and, if cells have already undergone EMT, it can hinder the tumor promoting activity of the cytokine.

In summary, our results demonstrate that the impact of decorin is highly cell-type specific reflecting on the reality of cancer biology. However, in vitro experiments have their limitations, e.g. unable to capture interactions between the tumor microenvironment and hepatoma cells, to extrapolate in vitro doses to in vivo, to detect the consequences, adverse effects of long-term treatment. To gain further insights to the molecular mechanisms behind antitumor actions of decorin, additional in vivo studies are needed.

## 5. Conclusions

Decorin proved to be an effective tumor suppressor on hepatocellular carcinoma cell lines in vitro. Proliferation rate of each cell line was hindered, although the least differentiated HLE showed only a modest reaction in MTT assays. All the cell lines responded to decorin exposure at molecular level, however they activated different signaling pathways according to their origin and differentiation state. Upon decorin exposure HepG2 followed the previously described canonical reactions, such as EGFR dephosphorylation, and p21<sup>WAF1/CIP1</sup> activation, but other cell lines reacted differently. Decorin modulated the insulin receptor phosphorylation in Hep3B and HuH7 cells, which receptor was not described as an interacting partner of decorin before. We found G2/M phase cell cycle blockade in Hep3B, which is also a novel way how decorin exerts its antiproliferative effect. In HLE decorin action is accomplished via TGF $\beta$ 1 pathway which seems to have weak influence on proliferation.

We can conclude that action of decorin highly depends on the molecular background of the hepatomas, despite the same tissue of origin or similar morphology.

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## Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Competing interests

Not applicable.

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## Authors' contributions

ZH conducted the in vitro experiments and performed immunofluorescent and biochemical examinations. ZH and KB analyzed and interpreted the research data, and were major contributors in writing the manuscript. AR performed additional experiments, and helped in edition of figures. LS designed and provided the decorin expression vector. TD conducted short-term in vitro experiments, IK supervised the manuscript and finalized the background and discussion sections. All authors read and approved the final manuscript.

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