

## Original Article

**Running Title:** Effects of Collagen Type I on Apoptotic Gene Expression in HepG2 Cells

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### Collagen Type I Induces a Balance in the Expression of Anti- and Pro-Apoptotic Genes in Hepatocellular Carcinoma Cells

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#### Abstract

**Background:** The importance of extracellular matrix (ECM) components in the progression of hepatocellular carcinoma (HCC) has been shown in many studies. Although restoring or activating apoptosis in tumors is an active area of cancer research, little is known regarding the effects of collagen type I, the main ECM component in the liver, on apoptosis of HCC cells. Here, we investigated the apoptotic profiles of HCC cells in a microenvironment with collagen type I.

**Method:** In this in vitro study, we assessed the effects of collagen type I on HepG2 cells in pre-confluent and confluent states. We determined the mRNA levels of 25 genes, which are the key players of apoptosis. Flow cytometry-based apoptosis detection was performed by use of Annexin V/PI staining. Confocal laser scanning microscopy was used to assess P53 immunofluorescence in the cells.

**Results:** The microenvironment with collagen type I and the confluency state of HepG2 cells affected the expression of 13 genes involved in apoptosis. We observed no significant change in the number of cells undergoing apoptosis depending on the confluency state or the presence of collagen type I. P53 immunofluorescence demonstrated no significant changes.

**Conclusion:** We propose an apoptotic balance concerning overall cell survival which might be caused by the counteraction of positive and negative mediators of apoptosis. This study might provide data for the involvement of collagen type I in apoptotic responses of HCC, and contribute to a better understanding of cancer microenvironment.

**Keywords:** Cancer, Cell death, Extracellular matrix, HepG2, Microenvironment

## Introduction

The dynamic nature of the transformed cells depends on the complex network of molecular interactions that involves external structural determinants besides intrinsic signals.<sup>1</sup> Among alterations reported in hepatocellular carcinoma (HCC), patients are the cancer-associated microenvironment components including the extracellular matrix (ECM).<sup>2,3</sup> Increased matrix stiffness has been shown to promote HCC progression.<sup>4,5</sup> Fibril-forming collagen types I and III are found in higher levels in HCC in comparison to the other collagens.<sup>6</sup> Enhanced matrix cross-linking by increased deposition of collagen type I was also demonstrated for ovarian tumor and glioblastoma.<sup>7,8</sup>

The involvement of ECM proteins in the regulation of apoptosis is an active area of cancer research. Collagen type I was proposed to induce resistance to apoptosis by upregulating the anti-apoptotic MCL-1 protein in pancreatic cancer cells.<sup>9</sup> Acellularised ECM containing collagen type I and fibronectin reduced the number of apoptotic esophageal cancer cells in the presence of drugs compared to cells grown on plastic.<sup>10</sup> In cervical cancer cells, a negative correlation was evident between collagen type I alpha 1 chain expression level and radiation-induced apoptosis.<sup>11</sup>

Considering the enormous effects of ECM remodeling on oncogenic transformation, a better understanding of the ECM/cancer cell interactions becomes critical for the development of new treatment strategies. In this work, the effect of collagen type I on apoptosis was studied on the HCC HepG2 cells depending on the confluency of the cells. Growth and differentiation potentials of cells were observed to change extensively in pre-confluent and confluent cultures in different types of cells including human skeletal muscle myoblasts, human hepatoma

Huh7 cells, and human skin carcinoma cells.<sup>12-14</sup>

We analyzed the following markers to identify if collagen type I affects apoptosis of HepG2 cells: (i) mRNA analysis of 25 genes involved in apoptosis, (ii) Annexin V binding assay demonstrating the apoptotic cells, (iii) P53 immunofluorescence. To the best of our knowledge, this study is the first approach to examine the effects of collagen type I, the most important collagen type in the ECM in the liver, on the HCC cell line.

## Materials and Methods

### *Cell culture*

The present *in vitro* study employed the human HCC (HepG2, ATCC, USA) cell line. HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Pan Biotech, Germany) supplemented with L-glutamine, 10% (v/v) heat-inactivated fetal bovine serum (FBS; Pan Biotech, Germany), 100 IU/ml penicillin, and 100 µg/ml streptomycin (Pan Biotech, Germany) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in a Panasonic incubator. All cells were passaged with 0.25% (v/v) trypsin (Pan Biotech, Germany).

To minimize the inconsistency due to different passage numbers, all experiments were planned as summarized in figure 1. The cells were seeded into either un-coated or collagen type I coated *in vitro* culture plates. The analysis was performed within two different periods: (i) when the cells were 60 to 70% confluent (pre-confluency state) and (ii) on the first day when they reached a 100% confluency (confluency state).

### *Collagen type I treatment*

2 mL collagen type I high concentration (rat tail, the concentration range of 8-11 mg/ml; Corning, USA) was mixed with 31 mL distilled H<sub>2</sub>O and left to adhere to culture surfaces overnight at 37°C. The surface was

washed with PBS to remove excess collagen.

### ***Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)***

Expression patterns of 25 genes involved in apoptosis of liver cancer were determined with the RT<sup>2</sup> Profiler™ PCR Array Human Liver Cancer (Qiagen, Germany). These genes are reported to be the most relevant genes to liver cancer apoptosis. Total RNA was extracted using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions and quantified by use of a Take3 micro-volume plate in a BioTek microplate reader (USA). Through the use of RT<sup>2</sup> First Strand Kit (Qiagen), a 500 ng high-quality total RNA was then reverse transcribed. The cDNA was mixed with RT<sup>2</sup> SYBR Green Mastermix (Qiagen) and subsequently loaded into a RT<sup>2</sup> Profiler™ PCR Array Human Liver Cancer which contains primers for housekeeping genes in addition to 84 genes of interest. The manufacturer's instructions were strictly followed. Real-time PCR (qPCR) reaction was performed in a LightCycler® 96 System (Roche Life Science). Delta delta Ct ( $2^{-\Delta\Delta Ct}$ ) relative quantitation method was employed for quantitation of qPCR products.<sup>15</sup>

All data were normalized to an average of two housekeeping genes, beta-2-microglobulin (*B2M*) and ribosomal protein lateral stalk subunit P0 (*RPLP0*) which showed minimal variation among samples. Bar graph and heatmap were generated using GraphPad Prism V.7.04 (USA).

### ***Quantitation of apoptosis using flow cytometry***

Cell death was assessed with Annexin V/Propidium iodide (PI) apoptosis detection assay according to the manufacturer's instructions (BD Pharmingen, BD Biosciences, USA). HepG2 cells were briefly trypsinized, washed twice in PBS, and resuspended in 1X Binding Buffer (0.01 M HEPES/NaOH (pH 7.4), 0.14 M NaCl, 2.5

mM CaCl<sub>2</sub>) at a concentration of  $1 \times 10^6$  cells/mL. Annexin V-APC and PI were added to 100  $\mu$ L of cell suspension and cells were incubated for 15 min at RT in the dark under gentle continuous agitation. Suspension volume was completed to 500  $\mu$ L with Binding Buffer and flow cytometric analysis was carried out in a BD AccuriC6 Plus flow cytometer (BD Biosciences). Twenty-thousand events were collected for each sample. Cells incubated in binding buffer yet not stained, cells stained with only Annexin V-APC, and cells stained only with PI were used to set up the quadrants for analysis. Annexin V demonstrates a high affinity for externalized phosphatidylserine, an early apoptotic feature, whereas PI is a vital stain and discriminates between viable and dead cells. This enables to distinguish between viable cells (Annexin V-/PI-), early apoptotic cells (Annexin V+/PI-), and late apoptotic/dead cells (Annexin V+/PI+).

### ***Assessment of apoptosis by immunofluorescence microscopy***

Coverslips were autoclaved and placed into the wells of a 24-well plate. HepG2 cells were grown on either non-treated (uncoated) or collagen type I coated coverslips. At both pre-confluency or confluency states, the cells were washed twice with PBS and fixed in Kwik-Diff™ Reagent 1, Fixative (Thermo Fisher Scientific, USA) for 20 min. Fixed cells were washed three times with PBS, blocked in 2% bovine serum albumin for 10 min at RT, and immunostained with primary P53 mouse monoclonal antibody (Biocare Medical, USA) overnight at 4°C. We washed the cells three times and probed with secondary goat anti-mouse IgG (H+L) antibody DyLight® 488 conjugate (Thermo Fisher Scientific) for 1 h at 37°C. The cells were counter-stained with Hoechst 33258 and observed under the Zeiss LSM 700 confocal laser scanning microscope (Germany).

### ***Statistical analysis***

Apoptosis and microscopy data are expressed as mean  $\pm$  standard error of the means (SEM). Statistical evaluation was performed by one-way ANOVA test using GraphPad Prism V.8.01 and post-hoc Tukey analyses were carried out to find groups whose mean differences were significant.

## Results

### **Expression profiles of apoptotic genes**

Differential mRNA levels for 25 apoptotic genes, namely *BAX*, *BCL2*, *BCL2L1* (*BCLXL*), *BID*, *BIRC2* (*c-IAP1*), *BIRC5*, *CASP8* (*FLICE*), *CFLAR* (*Casper*), *DLC1*, *E2F1*, *EP300*, *FADD*, *FAS* (*TNFRSF6*), *FHIT*, *GADD45B*, *GSTP1*, *MCL1*, *MSH2*, *PYCARD* (*TMS1*, *ASC*), *RUNX3*, *SOCS3*, *TERT*, *TNFRSF10B* (*DR5*), *TNFSF10* (*TRAIL*), and *WT1* were assessed with and without collagen type I in pre-confluent and confluent states. Cells grown in uncoated plates and harvested at pre-confluent state (pre-confluent-uncoated group) constituted the control group for analysis. Significant changes at the expression of 13 genes that showed at least a two-fold change in the expression in comparison to the control group are shown in figure 2a. Changes were also demonstrated in a heat map (Figure 2b). Red and green colors represent up- and downregulated genes, respectively. Among these, only *FHIT* and detoxification enzyme encoding *GSTP1* were found to be upregulated (2.1- and 2.6-fold, respectively) in pre-confluency in response to collagen type I (pre-confluent-coated with collagen type I). Anti-apoptotic genes *BCL-2*, *BCL-XL*, *MCL1*, tumor suppressor genes *DLC-1*, *FHIT*, positive mediator *GADD45B*, catalytic subunit of telomerase *TERT*, and tumor necrosis factor receptor *TNFRSF10B* were upregulated at least a two-fold in the presence of collagen type I in confluent cells (confluent-coated with collagen type I group). In the same group, a dramatic decrease in Wilms' tumor 1 gene (*WT1*)

expression was observed (91%) (confluent-coated with collagen type I group). *TERT* gene expression was also lower in this group (51%).

More than two-fold increases in the expression of *TNFRSF10B* and apoptosis inducer *TNFSF10* (*TRAIL*) were evident in the confluent group grown on uncoated plates (confluent-uncoated group). On the other hand, apoptosis inducer *FAS* and pro-apoptotic *BID* were downregulated at 55% and 60% in the same group (confluent-uncoated group).

Interestingly, these findings indicated a counteraction of positive mediators of apoptosis in response to negative mediators under the influence of collagen type I in both pre-confluency and confluency groups. An analogous pattern was also observed for the confluent-uncoated group when compared to the control group.

### **Flow cytometry-based apoptosis detection**

Annexin V/PI apoptosis detection assay was performed to evaluate the influence of collagen type I on cell survival and apoptosis rates in cells in pre-confluent and confluent groups (Fig. 3a, b, c, d, and e). One-way ANOVA test was performed and no significant change was found among the groups (Fig. 3e). *F* and *p* values for each group are as following:  $F_{(3,7)}=2.771$ ,  $P=0.1205$  for live cells;  $F_{(3,7)}=3.776$ ,  $P=0.0671$  for non-apoptotic dead cells;  $F_{(3,7)}=1.687$ ,  $P=0.2463$  for late apoptotic/dead cells;  $F_{(3,7)}=0.111$ ,  $P=0.9504$  for early apoptotic cells.

Cells in early apoptosis (Annexin V+/PI-) were found to be in minority in all samples. Although type I collagen decreased the apoptosis slightly, the differences were not statistically significant.

### **Immunofluorescence staining: expression of P53**

The four groups of HepG2 cells were stained with Hoechst 33258 and P53 antibody to determine the effect of culture

conditions on P53 expression (Fig. 4a, b, c, and d). One-way ANOVA test did not show any significant difference in fluorescence intensity of P53 among groups [ $F_{(3,8)}=3.392$ ,  $P=0.0742$ ]. Additionally, no morphological changes characteristic to apoptotic cells were detected. This result supports the proposed apoptotic balance of the cells.

## Discussion

This study focuses on the effect of collagen type I on apoptotic profiles of liver HCC cells, HepG2 cell line. Cell density and intercellular signaling may affect the behavior of the cancer cells. Because of the transformation of the cells from “proliferation” to “differentiation” state due to the intra- and inter-cellular signaling just after confluency. As this might result in changes in the behavior of the cells including apoptosis, both culture conditions were analyzed.

Differential expressions of 25 apoptotic genes which are thought to have important roles in HCC apoptosis were investigated in this study. Among them, 13 genes showed at least a two-fold change in expression in comparison to the control group (pre-confluent-uncoated group). Despite the differential expressions, a balance due to a possible counteraction of positive and negative mediators was demonstrated. In higher cell density, tumor necrosis factor receptor *TNFRSF10B* and apoptosis inducer *TNFSF10* were upregulated, while apoptosis inducer *FAS* and pro-apoptotic *BID* were downregulated. The observed alterations are associated with both extrinsic and intrinsic pathways of apoptosis. Human leukemic HL-60 cells were shown to spontaneously undergo apoptosis in the course of their proliferation due to the increase of cell density. The authors advised a mechanism of cell auto-regulation due to the inappropriately crowded condition.<sup>16</sup> Nevertheless, in this study, besides the

observed balance in apoptotic expression profiles, no significant change in Annexin V/PI staining and apoptotic behavior was found. Similar apoptosis patterns in both pre-confluency and confluency states might suggest that the cells in proliferating and differentiation stages exhibit a similar survival pattern under classical culture conditions.

Under the influence of collagen type I, the cells in the pre-confluent state revealed a different apoptotic gene expression profile. Tumor suppressor gene *FHIT*, detoxification enzyme encoding *GSTP1*, Wilms' tumor 1 gene (*WT1*), and catalytic subunit of telomerase *TERT* were found to be regulated. Among them, *WT1* displayed around 90% downregulation in comparison to the control group. *WT1* was demonstrated to be expressed in several HCC cell lines, besides the tumor tissue in 42% of patients with HCC<sup>17</sup> and associated with poor prognosis.<sup>18</sup> *WT1* in Sertoli cells was proposed to maintain the testicular cord integrity via regulating the collagen type IV protein levels.<sup>19</sup> Thus, the presence of collagen type I in the microenvironment might explain the dramatic decrease of *WT1* in pre-confluent-coated with collagen type I group. In addition to *WT1*, *TERT* gene expression was also lower whereas the expressions of *FHIT* and *GSTP1* were higher. The overall effect of the altered genes (positive and negative mediators) together with the flow cytometry results points to stability concerning cellular apoptosis. No significant change was found in cellular apoptosis by Annexin V/PI staining.

The most extensive changes in apoptotic gene expression were seen in the confluent group grown on collagen type I coated surface. A simultaneous upregulation of several positive and negative mediators was demonstrated. Anti-apoptotic genes *BCL-2*, *BCL-XL*, *MCL1*, tumor suppressor genes

*DLC-1*, *FHIT*, positive mediator *GADD45B*, catalytic subunit of telomerase *TERT*, and tumor necrosis factor receptor *TNFRSF10B* were observed to have at least a two-fold increased expression in comparison to the pre-confluent-uncoated group. Concordant with the simultaneous upregulation of several positive and negative mediators, no change was noticed in the apoptotic behavior of the same cells. Similarly, the expression of tumor suppressor P53 did not demonstrate any significant changes. A counteraction of positive mediators of apoptosis in response to negative mediators might explain the steady-state of the cells concerning cellular apoptosis.

It has been established that increased expression and accumulation of collagen type I is associated with malignancies particularly with a high risk of metastasis.<sup>20</sup> Higher levels of collagen type I have been found in HCC in comparison to the other collagens and are thought to contribute to a continuous matrix remodeling and changes in ECM stiffening, and eventually to a tumorigenic microenvironment.<sup>6</sup> The role of collagen I in the progression of HCC has been illustrated in several studies. HCC cells cultured on collagen type I had a higher proliferation rate than those on collagen type IV and fibronectin.<sup>21</sup> Zhang et al. cultured heat-exposed HCC cells in different ECM gels and proposed that collagen type I dramatically enhanced the aggressive progression of residual HCC cells.<sup>22</sup> On the level of gene expression, a comprehensive analysis of the ECM transcriptome in HCC demonstrated the upregulation of *COL1A1* and *COL1A2* encoding for collagen type I polypeptide chains.<sup>23</sup>

The tumor microenvironment can control cancer progression at many levels including regulation of gene expression at multiple stages.<sup>24</sup> Among the major pathways implicated in HCC are the

RAS/RAF/MEK/ERK, PI3K/AKT/mTOR, HGF/MET, FGF, IGF, JAK/STAT, P53, and TGF- $\beta$  signaling pathways, the regulation of which eventually leads to HCC progression through increased proliferation, differentiation, and reduced apoptosis.<sup>2</sup> The involvement of genes, that control the two main pathways of apoptosis, the extrinsic and the intrinsic pathways, has been investigated extensively in the development of HCC.<sup>25-27</sup> However, the impact of ECM components on cellular apoptosis remains largely unknown.

Recent studies have provided evidence that collagen type I inhibits apoptosis and confers a survival advantage. *COL1A1* was proposed to play a crucial role in the prevention of apoptosis induced by radiation in cervical cancer cells.<sup>11</sup> Another study demonstrated that collagen type I inhibited apoptosis in HeLa cells induced by exogenous hydroxyl radicals.<sup>28</sup> Furthermore, collagen type I was observed to reduce apoptosis of AsPC-1 pancreatic cancer cells in response to 5-fluorouracil by upregulating *MCL-1*.<sup>9</sup> The protective effect of collagen was also shown in leukemic cells.<sup>29</sup>

Our study also demonstrated higher levels of *MCL-1* in response to collagen type I in the growth environment. Interestingly, no effect on cellular apoptosis was evident. This might be related to the absence of a drug that triggered apoptosis. A similar result was presented by Senthebane et al. in esophageal cancer cells.<sup>10</sup> The authors pointed out that the cell cycle profiles and cell proliferation on decellularised ECM rich in collagen type I and fibronectin compared to cells on grown on plastic revealed no significant differences in the absence of any drugs. They only observed decreased cell proliferation on plastic in the presence of 5-fluorouracil, epirubicin, and cisplatin. Besides, the authors showed reduced drug-induced apoptosis of cells on decellularised ECM in comparison to cells plated on

plastic.<sup>10</sup> Apoptosis-inducing drugs or another type of external stress might cause the reregulation of positive and negative mediators of apoptosis, and hence a different apoptotic behavior and cell survival pattern are generated. Additionally, the cells in our work were grown on collagen type I for a maximum duration of one week. An increase in culture duration might cause a different apoptotic response. It is also important to note that hepatocellular death can be apoptotic, necroptotic, or necrotic.<sup>30</sup> Therefore, the response might be in another way of cell death than apoptosis.

Cell culture studies have some limitations regarding their inability to mimic the surrounding cellular microenvironment *in vivo*, which is required to understand the physiological cell behavior. Although they are more homogenous in comparison to primary cultures, commercial cell lines might be associated with variations in genotype and phenotype over time, which would be a limitation to the studies with cell lines.

### Conclusion

To our knowledge, the current study evaluated the effects of collagen type I on the expression of 25 genes related to apoptosis and HCC for the first time. Apoptotic features of liver cancer cells at pre-confluency and confluency states were also compared. The results illustrated changes in the expression of pro- and anti-apoptosis genes. No changes in cellular apoptosis were evident.

This work suggests potential players of apoptotic responses that are differentially expressed depending on the cell densities and presence of collagen type I. Future studies are required to further evaluate the effect of collagen type I in 3D-environment on cellular apoptosis. Nevertheless, this study might be a starting point to explore the role of microenvironments in modulating the

apoptotic behavior of HCC cells and to better understand the microenvironments in cancer.

### Acknowledgement

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### Conflict of Interest

The authors have no competing interests.

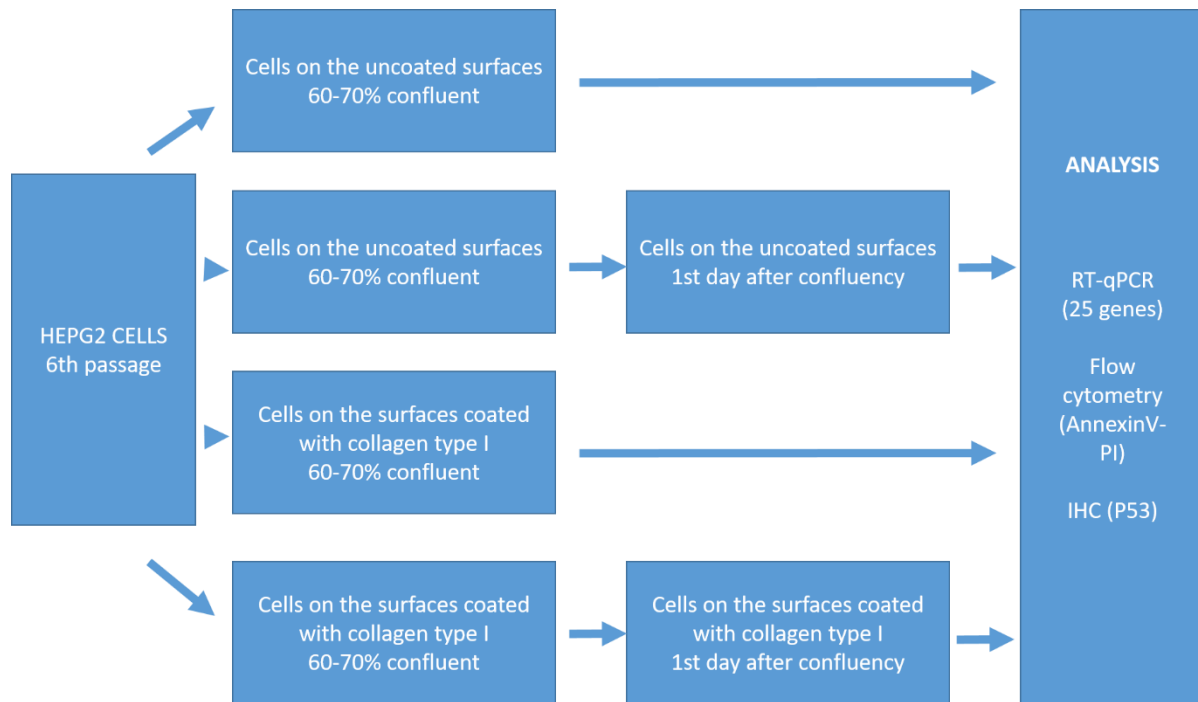
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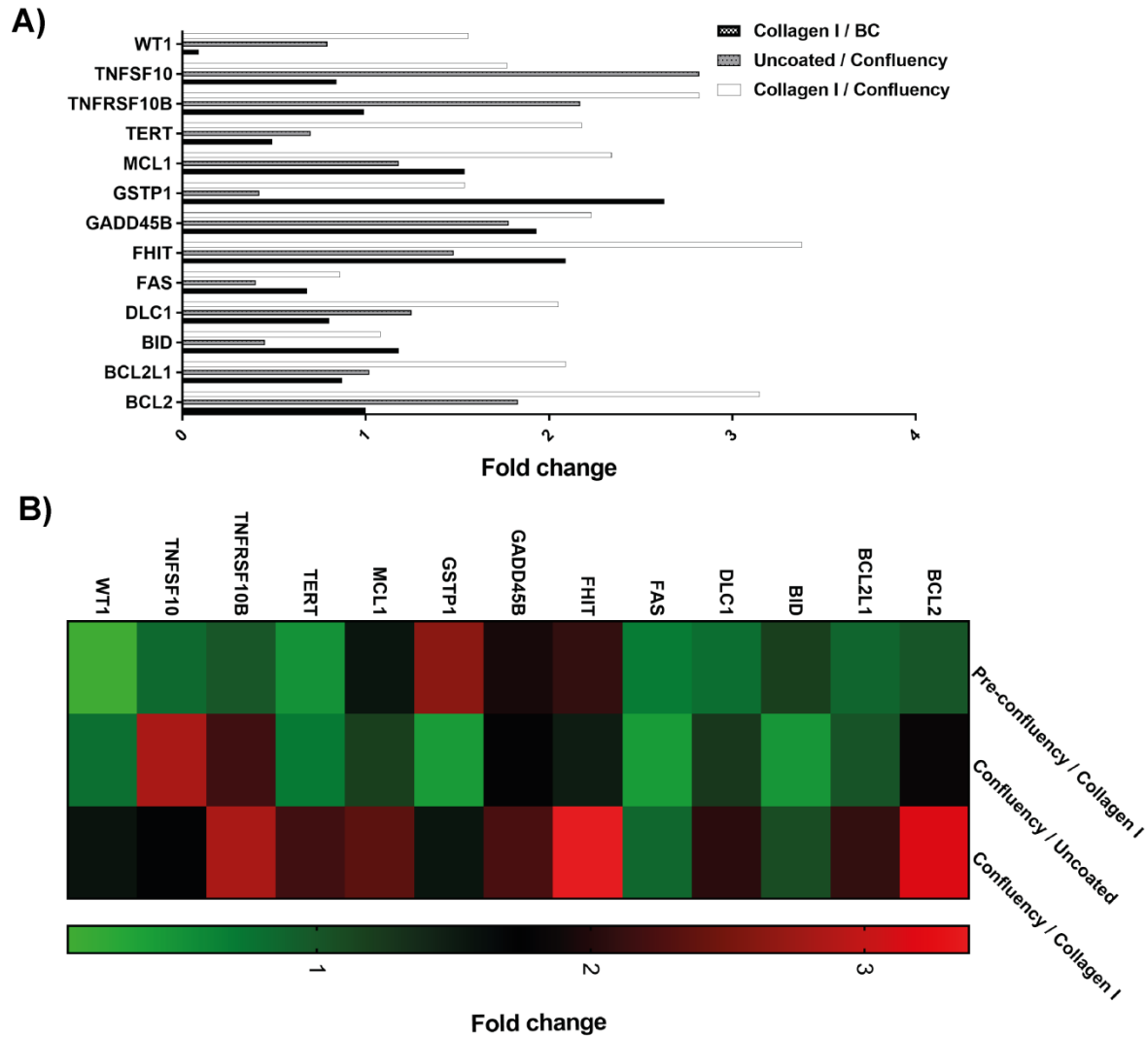
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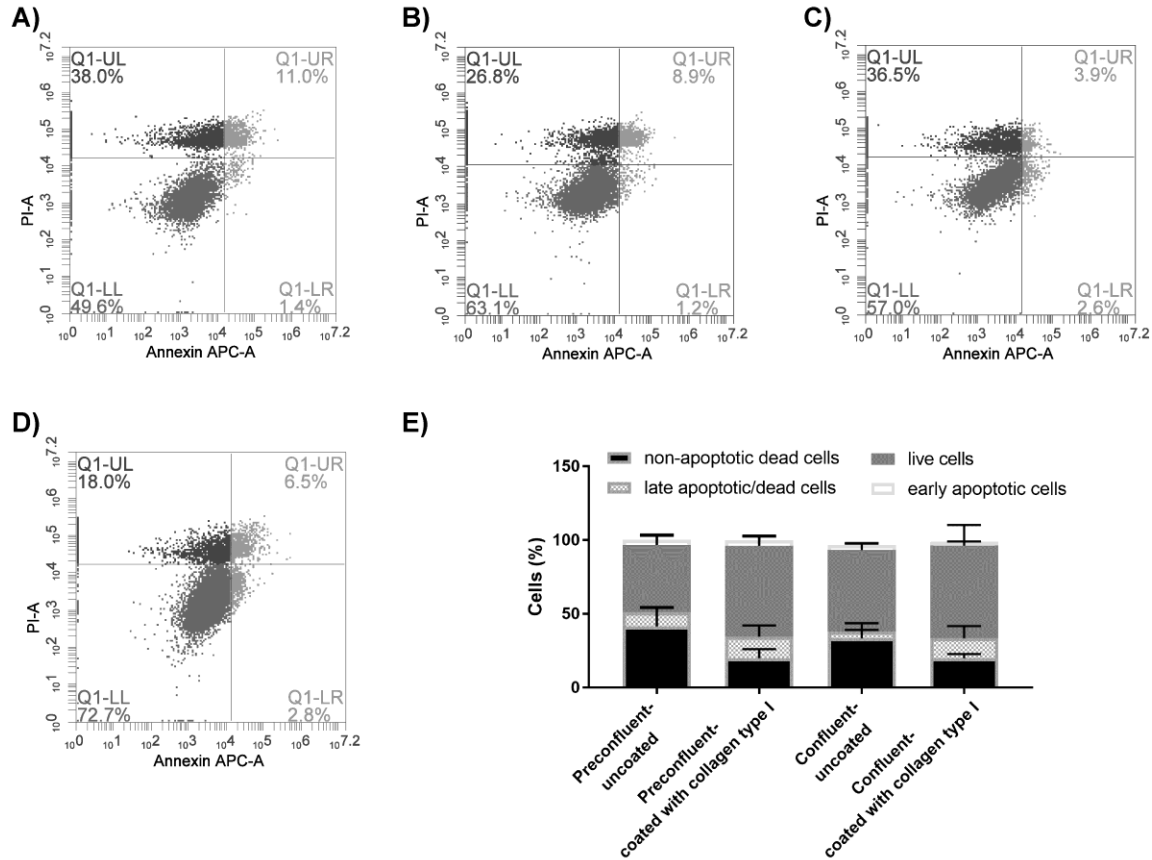
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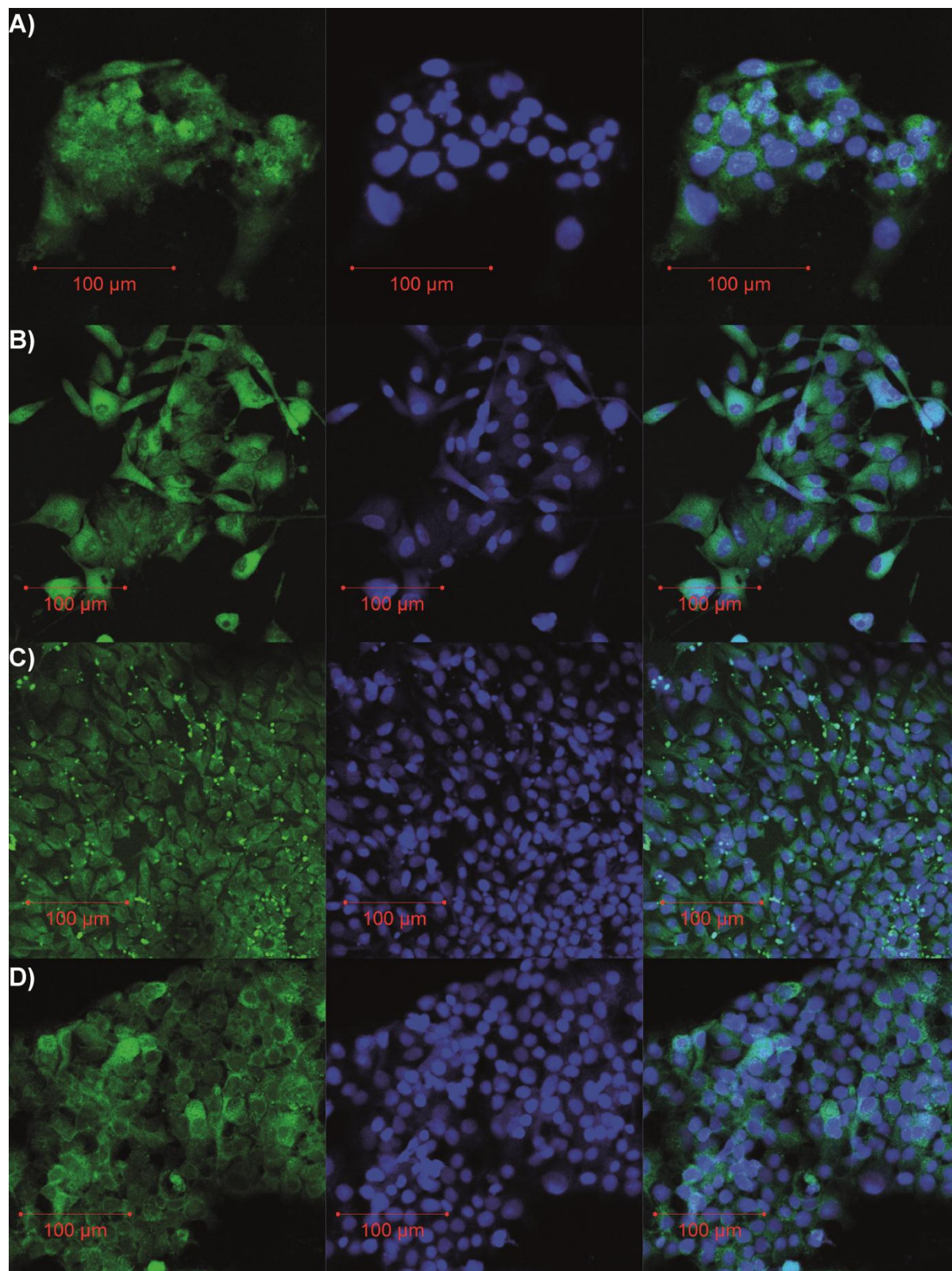
**Figure 1.** This Scheme demonstrates the experimental design.

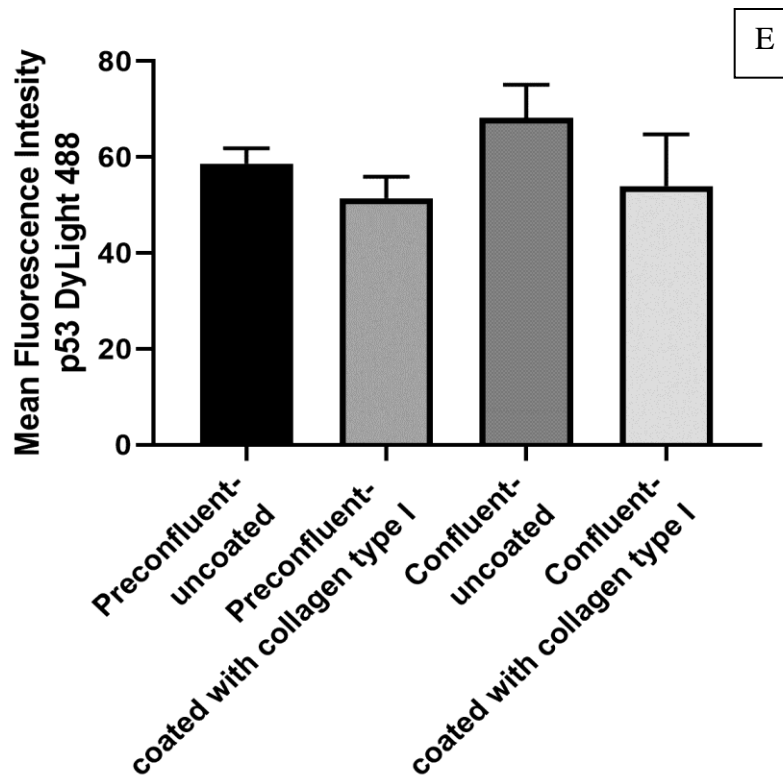


**Figure 2.** Bar graph (A) heat map (B) demonstrating the genes that changed at least a two-fold. Delta delta Ct ( $2^{-\Delta\Delta CT}$ ) relative quantitation method was used for the calculation of fold changes. The red and green colors in the heat map represent increasing or decreasing genes respectively in three groups against the control group.



**Figure 3.** Annexin V-APC/PI double staining analysis of apoptosis in HepG2 cells in four groups: (A) pre-confluent-uncoated (B) pre-confluent-coated with collagen type I (C) confluent-uncoated (D) confluent-coated with collagen type I. Dot plots for flow cytometric analysis are shown. (E) The mean values of three independent experiments were plotted as a bar graph to demonstrate the percentages of living, early apoptotic, and late apoptotic/dead and non-apoptotic /dead cells. One-way ANOVA test was performed and no significant change was found among the groups.





**Figure 4.** Confocal microscopic images of HepG2 cells labeled with the P-53 antibody. DyLight® 488 was used as the secondary antibody for the groups (A) pre-confluent-uncoated,(B) pre-confluent-coated with collagen type I,(C)confluent-uncoated, (D) confluent-coated with collagen type I. Nuclei were counterstained blue with Hoechst 33258. Images merged (right). No morphological changes characteristic to apoptotic cells were detected. (E)Bar graph demonstrates the mean fluorescence intensity of P53 as mean  $\pm$  SEM. One-way ANOVA test showed no significant change among groups ( $F_{(3,8)}=3.392$ ,  $P=0.0742$ ).