Cukurova Medical Journal
ÇUKUROVA ÜNİVERSİTESİ TIP FAKÜLTESİ

Cukurova Med J 2025;50(3):899-907
DOI: 10.17826/cumj.1722027

RESEARCH

Matrigel and collagen I impact hepatocellular carcinoma cell behavior: a confluency-dependent study

Matrigel ve kolajen I hepatosit karsinomu hücre davranışını etkiler: konfluense bağımlı çalışma

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Abstract

Purpose: The tumor microenvironment significantly influences hepatocellular carcinoma (HCC) progression, with epithelial-mesenchymal transition (EMT) being a key driver of metastasis. This study explores the distinct roles of matrigel and type I collagen, two prominent ECM components, in modulating HepG2 liver cancer cell behavior.

Materials and Methods: HepG2 cells were cultured in type I collagen and Matrigel culture media, and the expression of YAP1, Na-K ATPase, and E-cadherin were investigated using a confocal microscope. We examine their impact on proliferation and the expression of, considering the influence of cell adherent.

Results: HepG2 cells showed the highest proliferation at post-confluent culture time in type 1 collagen medium. YAP1 expression was high in type 1 collagen medium at both culture times (and when they reached adherent, YAP1 localization was from the cytoplasm to the nucleus. Na-K ATPase expression was high in control cells non-adherent, while it increased in Matrigel adherent. E-cadherin showed a statistically significant decrease in type 1 collagen medium compared to control and its expression was found to be high in Matrigel cultured cells adherent.

Conclusion: These findings highlight the differential effects of extracellular matrix components on HCC cell proliferation and EMT-related protein expression, suggesting potential therapeutic targets within the tumor microenvironment.

Keywords:. Collagen type I, extracellular matrix, epithelial-mesenchymal transition, matrigel, YAP1

Öz

Amaç: Tümör mikroçevresi, hepatosellüler karsinom (HCC) ilerlemesini önemli ölçüde etkiler ve epitel-mezenkimal geçiş (EMT) metastazın temel itici gücüdür. Bu çalışma, iki önemli ECM bileşeni olan Matrigel ve Tip I kollajenin HepG2 karaciğer kanseri hücre davranışını düzenlemedeki farklı rollerini araştırmaktadır

Gereç ve Yöntem: HepG2 hücreleri tip I kollajen ve Matrigel kültür ortamında kültürlendi ve YAP1, Na-K ATPaz ve E-kadherin ifadesi konfokal mikroskop kullanılarak araştırıldı. Hücre birleşmesinin etkisini göz önünde bulundurarak bunların proliferasyon ve ifadesi üzerindeki etkileri araştırıldı.

Bulgular: HepG2 hücreler en yüksek proliferasyonu Tip I kolajen ortamda konfluent sonrası kültür zamanda göstermiştir. YAP1 ifadesi her iki kültür zamanında Tip I kolajenli ortamda yüksek çıkmış (ve tam konfluente ulaştıkları zamanda YAP1 lokalizasyonu sitoplazmadan çekirdeğe doğru olmuştur. Na-K ATPaz ekspresyonu, kontrol hücrelerde konfluent öncesi yüksekken, konfluent sonrası matrigel ortamda artış göstermiştir. E-kadherin, Tip I kolajen ortamda kontrol göre istatistiksel olarak azalma göstermiş, matrigel kültürlü hücrelerde konfluent sonrası dönemde ifadesi yüksek bulunmuştur.

Sonuç: Bu bulgular, ekstrasellülar matrix bileşenlerinin HCC hücre çoğalması ve EMT ile ilişkili protein ifadesi üzerindeki farklı etkilerini vurgulayarak, tümör mikroçevresi içinde potansiyel terapötik hedeflere işaret etmektedir.

Anahtar kelimeler: Ekstrasellüler matriks, epitel-mezankimal geçiş, matrigel, Tip I kolajen, YAP1

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Received: 18.06.2025 Accepted: 12.09.2025

INTRODUCTION

Hepatocellular carcinoma (HCC), the third leading cause of cancer deaths globally, is rapidly increasing in developed countries[1-3]. Epithelial-mesenchymal transition (EMT) is a critical process in the progression of this aggressive cancer. In cancers, EMT inducers are hypoxia, cytokines, and growth factors secreted by the tumor microenvironment, stroma crosstalk, metabolic changes, innate and adaptive immune responses, and treatment with antitumor drugs. Switch in gene expression from epithelial to mesenchymal phenotype is triggered by complex regulatory networks involving transcriptional control[4]. Also, during EMT, epithelial cells lose their characteristics and gain motility and invasiveness, becoming mesenchymal cells, which is pivotal in metastasis[5,6]. During the process of cell detachment from in situ, the downregulation or degradation of E-cadherin and the upregulation of N-cadherin destabilize adherens junctions and disrupt the intercellular junctions of MCSCs, promoting cell migration and invasion[7,8]. The activation of the "cadherin switch" can be used as a marker for the initiation of EMT. By regulating pro-apoptotic and anti-apoptotic genes, the "integrin switch" enables cells to make use of survival signals through overexpression or structural activation of integrins, and then changes the cell metabolism to prevent MCSCs from losing their nest due to apoptosis[9]. A significant reduction in E-cadherin, a key adhesion protein, is frequently observed in HCC, contributing to the metastatic phenotype and positioning E-cadherin as a potential therapeutic target. This loss disrupts cell-cell adhesion and may promote cancer spread[10-13].

Another protein, YAP, is known for its role in promoting cancer formation[14,15]. Previous studies have confirmed YAP's involvement in cancer development[16-18]. In addition, it has been reported that excessive secretion of the YAP protein causes liver enlargement and hepatocellular carcinoma[17]. Research conducted on mammals has revealed that many components of protein complexes related to cell-cell junctions regulate the Hippo pathway. The YAP protein, which is active in this pathway, is very important in the development of epithelial cell-cell connections[19].

Na-K ATPase consists of two subunits. Studies show that the NKAα1 subunit is abnormally overexpressed in cancers and is indicated as a potential target for anticancer drug development. It has been found that NKAα1 is also overexpressed in HCC, and that suppression of this gene leads to apoptosis by arresting the cell cycle in HCC cells[20].

Developing reliable *in vitro* models to understand liver cancer biology is essential. Microenvironmental factors can significantly affect cancer cell behavior, causing inconsistencies in research[16]. Collagen type I, one of the main components of liver extracellular matrix (ECM) has been found to cause disruption of E-cadherin-mediated cell-cell contacts and promote the proliferation of pancreatic carcinoma cells[21,22]. Matrigel is a thin extracellular matrix layers that play many different roles in the body. This solid basal membrane matrix is used both in cell culture and in vivo. Matrigel has been used in various studies with embryonic, normal, stem, or malignant cells[23].

Considering the profound impact of extracellular matrix (ECM) remodeling on oncogenic transformation, a comprehensive understanding of ECM-cancer cell interactions is essential for the development of novel therapeutic strategies. Previous studies have demonstrated that the growth and differentiation capacities of cells differ markedly between pre-confluent and confluent cultures across various cell types, including human skeletal muscle myoblasts, human hepatoma Huh7 cells, and human skin carcinoma cells[24,25].

In the present study, the effects of different ECM components- type I collagen and matrigel- on HCC HepG2 cells were investigated to cell confluence status. Specifically, the expression levels of key ECM-related proteins, including YAP1, E-cadherin, and Na-K ATPase, were analyzed via confocal microscopy in HepG2 cells cultured under varying microenvironmental conditions. Additionally, cell proliferation was quantitatively assessed to evaluate the influence of the extracellular matrix.

This study provides novel insights into how distinct ECM components differentially regulate HepG2 cell behavior in a confluency-dependent manner, advancing our understanding of ECM-mediated modulation of HCC progression. We hypothesized that type I collagen and Matrigel exert distinct effects on cell proliferation and the expression of YAP1, E-cadherin, and Na^+/K^+-ATPase, depending on HepG2 cell confluency.

MATERIALS AND METHODS

Cell culture and study design

This in vitro study was conducted in Maltepe University Cancer and Stem Cell Research Center. The human hepatocellular carcinoma (HepG2, ATCC, USA) cell line was maintained in Dulbecco's modified Eagle's medium (DMEM,) supplemented with L-glutamine, 10% (v/v) fetal bovine serum (FBS), and 1% antibiotics (10 mg/mL streptomycin and 10,000 U/mL penicillin) at 37°C in a humidified atmosphere of 5% CO_2. The cells were plated at a density of 5x10^4/cm² for all experiments in 24-well plates and $1x10^4$/cm² in 96-well plates. The following analyses were performed when the cells covered 60-80% of the culture dishes (pre-confluent) and when they covered the entire surface (confluent)[26]. Since this study is a cell culture study, ethics committee approval is not required.

Three experimental groups were established: Control: Cells were grown on untreated surfaces. Collagen I-Treated Group: Cells were grown on collagen type I (354249, Corning, Bedford, MA, USA) coated surfaces. Matrigel-Treated Group: Cells were grown on Matrigel Matrix Basement Membrane (356234, Corning, Bedford, MA, USA) coated surfaces.

Collagen type I and matrigel treatment

Cell culture dishes were coated with Collagen type I before seeding. Concentrations and application procedures were performed according to previous studies[27]. Matrigel application was performed according to the manufacturer's kit procedure.

Live cell imaging

HepG2 cells were observed under an inverted phase-contrast microscope (Primovert, Zeiss, Germany) to evaluate their morphology in different culture conditions during both non-adherent and adherent periods.

Cell proliferation assay

Proliferation rates were assessed using the CCK-8 (WST-8) assay kit (KTC011001, Abbkine, China) across six experimental groups. Absorbance at 450 nm was measured using a microplate reader (BioTek Synergy H1, USA). Proliferation rates were measured at 24th (pre-confluency) and 48th hours (confluency). To determine the cell viability for experimental groups, we calculated percent viability as follows: % viability = [(Optical density (OD) of the treated cell-OD of the blank)/(OD of control-OD of the blank)×100]. Data were represented as mean±standard error (SE) of three independent experiments made in three replicates.

H&E staining

Cells were fixed with 4% formaldehyde and stained with a Hematoxylin-Eosin Stain Kit (ScyTek Lab-73861, USA) for morphological evaluation. Stained samples were examined under a light microscope to assess nuclear structure and cytoplasmic changes.

Assessment of confocal microscopy

Coverslips were autoclaved and placed in 24-well plates. HepG2 cells were cultured on (i) untreated, (ii) collagen I-coated, or (iii) Matrigel-coated coverslips. Cells were fixed with 4% paraformaldehyde and washed with PBS, then incubated with primary antibodies for YAP (B-8) (sc-398182;1/100, Santa Cruz, Texas, USA) by single staining; Na-K ATPase (M7-PB-E9; 1/50, GenTex, CA, USA), and E-cadherin (RM, API3012AA, 1/50, CA, USA) by double staining for 1 hour at 37°C. After incubation, cells were washed with CelloIF solution, then incubated with secondary antibodies (Thermo Fisher Scientific) and counterstained with Hoechst 33342. All images were taken with a Zeiss confocal microscope (Zeiss, LSM700; Jena, Germany). For image acquisition, Zeiss, Zen 2012 software (version: Zen Black). The objective lens specifications: 20X oil, numerical aperture (N.A.):2.0. Images were captured with a photomultiplier tube (PMT) integrated with the microscope. Camera resolution: 2048x2048 pixels, time and space resolution: XY resolution: 512x512 pixels, Z resolution: 1 µm, T resolution: 30-second intervals. Image bit depth: 16-bit. Fluorescence filters used: 405 nm for DAPI, 488 nm for GFP, and 550 nm for Ds Red.

Statistical analysis

Statistical analyses were performed using the GraphPad Prism 9 analysis program (GraphPad Prism V. 9.01). Data are expressed as mean ± SEM. Statistical analysis was performed using two-way ANOVA. Dunnett's multiple comparison tests were used for the proliferation (CCK-8) rate of the experimental groups compared to the control group. Tukey's multiple comparison tests were used for

YAP1, Na-K ATPase, and E-Cadherin expression measurements under confocal microscopy. Significance was accepted at $p<0.05$.

RESULTS

Daily microscopic observations highlighted notable differences in HepG2 cell morphology depending on the culture surface (Figure 1A). Cells on untreated surfaces exhibited cluster formation. The cells are mostly round with only a few extensions, indicating poor adhesion and spreading. In contrast, cells on Type I collagen displayed a flattened, spread-out morphology with numerous extensions, indicative of strong adhesion. Meanwhile, cells cultured on Matrigel exhibited a more rounded appearance, suggesting reduced spreading.

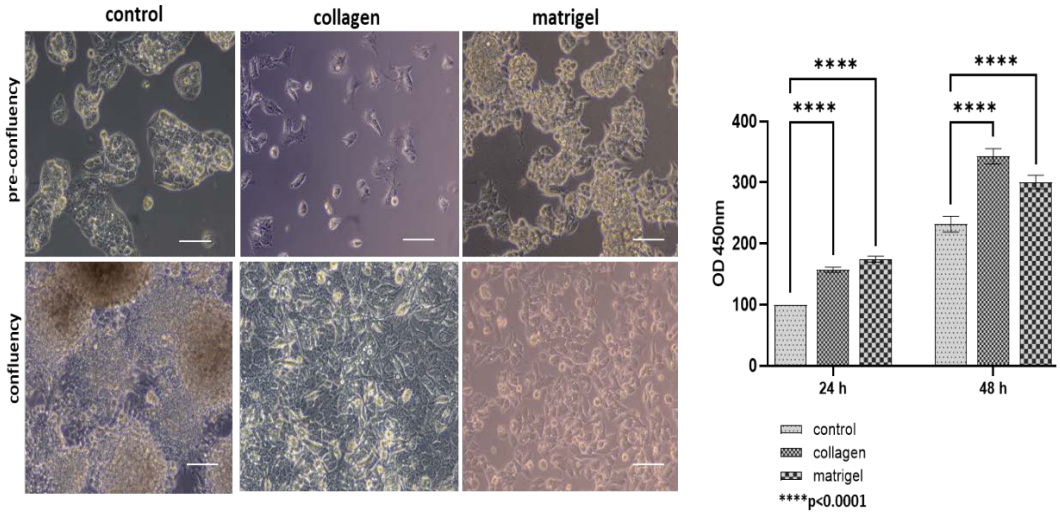

Figure 1. Phase-Contrast Microscope images and proliferation measurement.

Morphological images of hepG2 cells in different groups (A). Proliferation rates were measured using the CCK-8 assay at 24 (before confluency) and 48 hours (confluency) (B). ****p<0.0001. Scale bar, 40 µm.

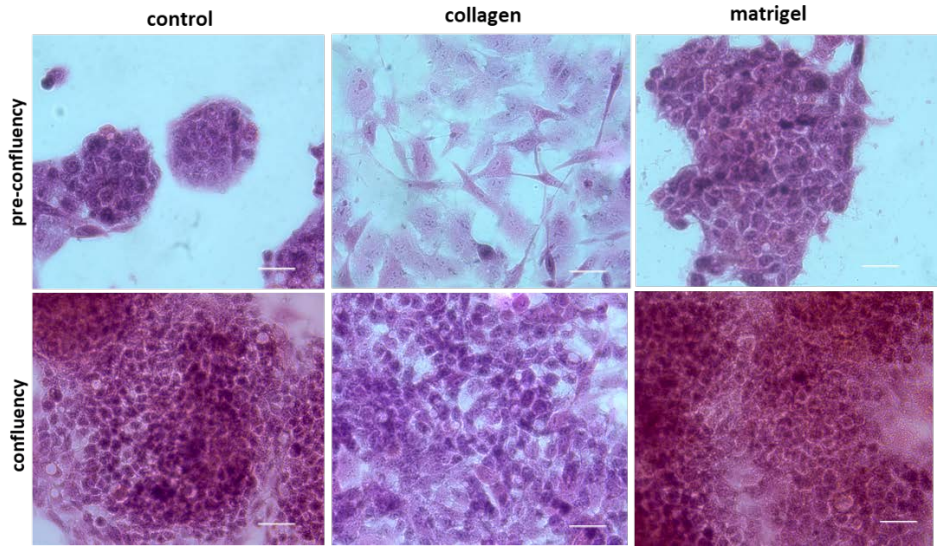

Figure 2. H&E staining images of HepG2 cells. Scale bar, 20 µm.

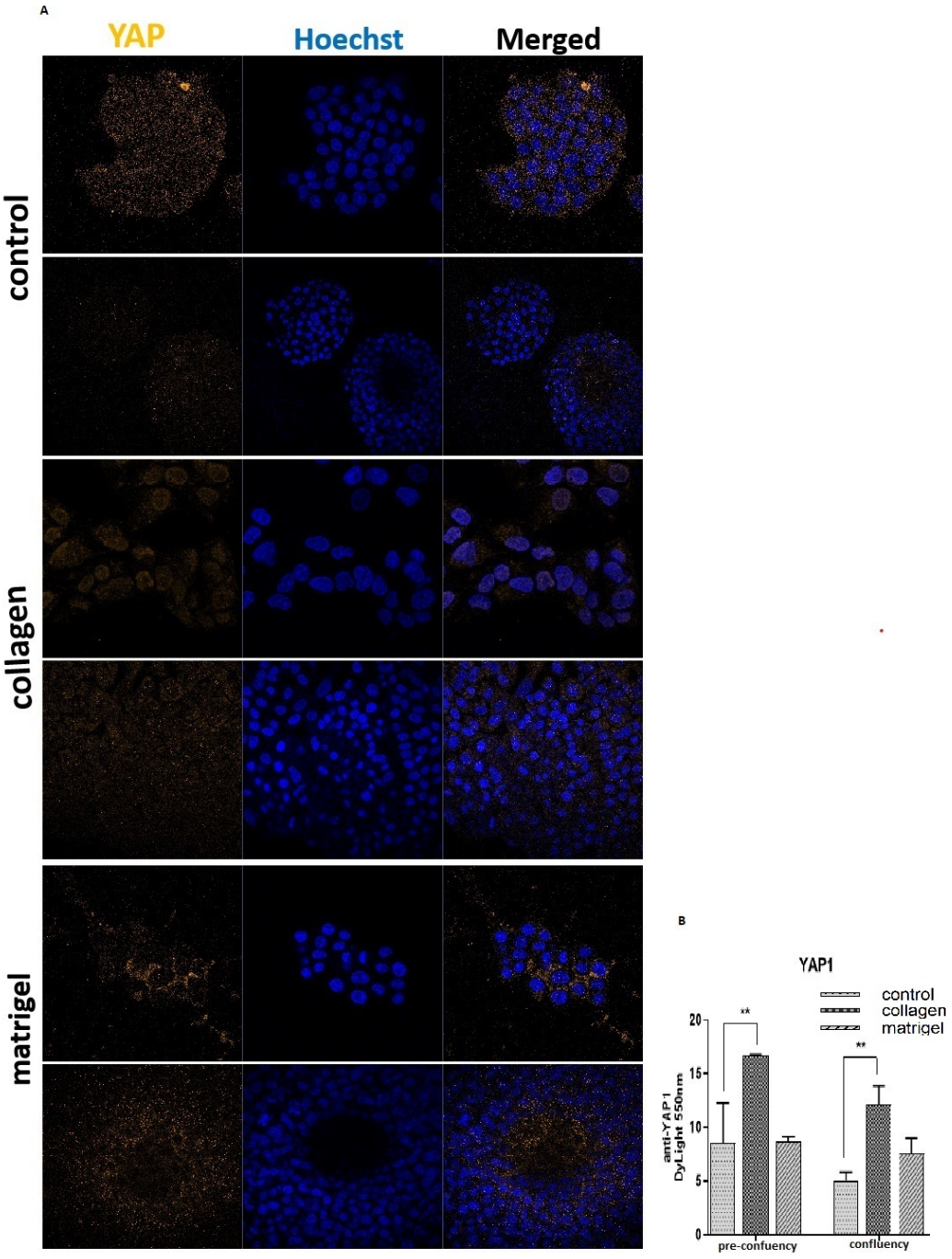

Figure 3. Confocal microscopic images of YAP1 in HepG2 cells. YAP1 localization and expression varied with culture conditions (A) and YAP1 expression levels were analyzed (B) using two-way ANOVA and Tukey's multiple comparison test, **p<0.0005 (b). Scale bar, 20 μm.

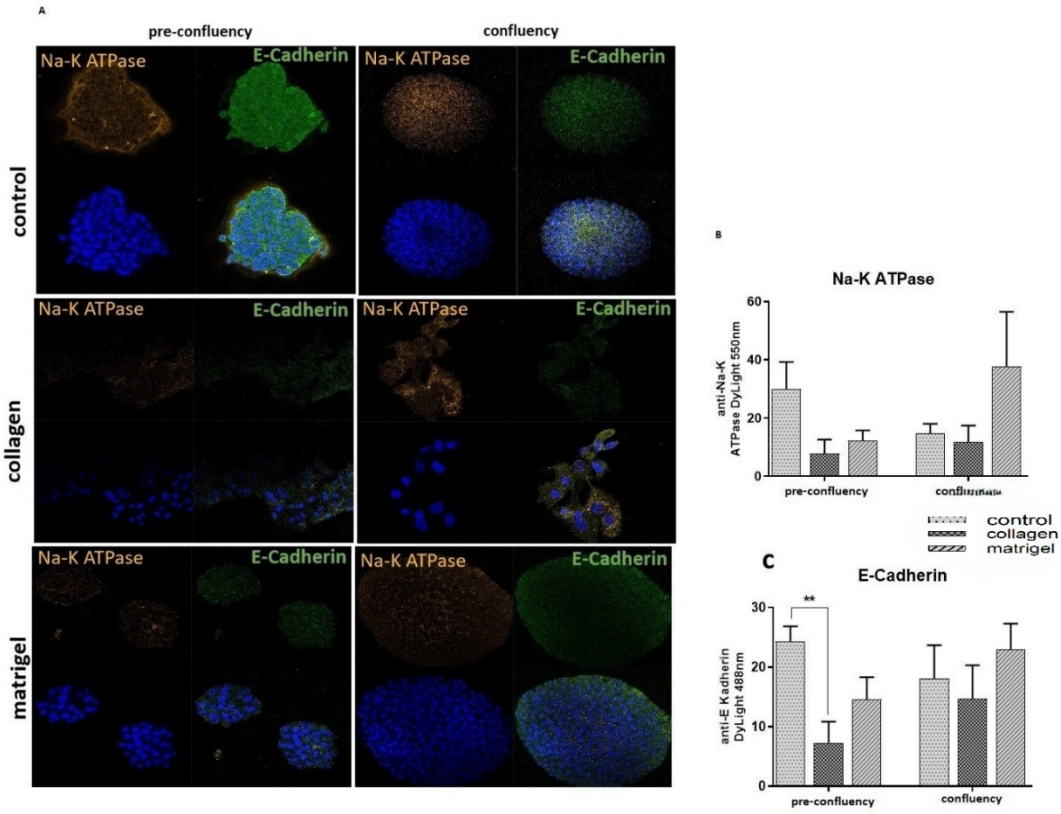

Figure 4. Confocal microscopic images of NA-K ATPase and E-Cadherin proteins in HepG2 cells (A). Comparison of Na-K ATPase (B) and E-Cadherin expression levels (C) in HepG2 cells growing on three different microenvironments. Na-K ATPase and E-Cadherin expression levels were analyzed using ANOVA, two-way analysis of variance Tukey's multiple comparison test.

The CCK-8 assay demonstrated a significant impact of the extracellular matrix on HepG2 proliferation (Figure 1B). By 48 hours (confluency), cells on collagen showed the highest proliferation rate, indicating that collagen supports sustained cell growth over time. Matrigel provided a significant early proliferation boost, with a higher rate observed at 24 hours (non-adherent) compared to the control group. Both matrigel and collagen cultures exhibited a statistically significant increase in proliferation compared to the control group ($p<0.0001$) at their respective peak times.

Hematoxylin and Eosin (H&E) staining revealed distinct morphologies across the different experimental conditions (Figure 2). HepG2 cells on untreated surfaces displayed clear nuclei and cytoplasm. Cells cultured on collagen type I showed dynamic changes in their spindle-shaped extensions and acidophilic cytoplasm. As cell confluency increased, intercellular adhesion intensified, and the spindle-shaped processes diminished. The culture conditions significantly influenced key adhesion molecules. YAP expression and localization differed across surfaces. In cells grown on collagen type I, YAP was predominantly nuclear in the non-adherent state but shifted to the cytoplasm after confluency. In contrast, YAP remained mostly cytoplasmic in the other groups. Confocal images confirmed these localization changes (Figure 3A). YAP1 expression was significantly higher in cells grown on collagen compared to the control group, both in the pre-confluency ($p=0.0015$) and confluency ($p=0.0044$) periods (Figure 3B).

Na-K ATPase protein expression was higher in the

control group before confluency but increased in cells grown on Matrigel after they became confluency. Collagen reduced Na-K ATPase expression in all groups (Figure 3A), although this difference was not statistically significant (Figure 3B).

E-Cadherin expression was significantly lower in cells grown on collagen compared to the control group during the pre-confluency ($p=0.0048$), suggesting delayed cell-cell adhesion. After confluency, E-Cadherin levels were highest in cells grown on Matrigel, but this difference was not statistically significant (Figure 4A). Differences in Na-K ATPase and E-cadherin expression across conditions were analyzed using ANOVA and Sidak's multiple comparison test (Figure 4B-C).

DISCUSSION

Liver cancer poses a significant global health challenge, necessitating the development of robust *in vitro* models for effective treatment strategies. This study provides novel insights into how the extracellular microenvironment (ECM), specifically matrigel and collagen type I, influences liver cancer cell behavior, addressing a critical need for more physiologically relevant *in vitro* systems. While previous research has explored the individual roles of these ECM components, our study uniquely investigates their comparative and confluency-dependent effects on key oncogenic drivers in HepG2 liver cancer cells.

Our findings reveal a striking increase in YAP expression in cells grown on type I collagen, consistent with clinical observations of YAP overexpression in HCC patients and its association with poorer tumor differentiation[15]. Critically, we demonstrate a dynamic shift in YAP localization from the nucleus (pre-confluency) to the cytoplasm (confluency) in collagen-cultured cells, revealing a novel level of regulation by type I collagen. This dynamic control of YAP activity by collagen is a significant contribution, as it suggests a mechanism by which the ECM can modulate cell proliferation and potentially contribute to aggressive HCC progression. Our findings support the established link between YAP activation and matrix remodeling, suggesting that collagen-induced YAP activation may contribute to a positive feedback loop of matrix stiffening and further YAP activation in liver cancer[17,18]. It has been reported that excessive secretion of the YAP protein causes liver enlargement and hepatocellular carcinoma[17].

Furthermore, we confirm and extend previous findings on the downregulation of E-cadherin by collagen type I, a crucial event in EMT and metastasis[12,28,29]. Our observation of reduced E-cadherin expression in collagen-cultured cells reinforces the concept that collagen promotes a more migratory and invasive phenotype in liver cancer.

Na-K ATPase consists of two subunits. Studies show that the NKAα1 subunit is abnormally overexpressed in cancers and is indicated as a potential target for anticancer drug development. It has been found that NKAα1 is also overexpressed in HCC, and that suppression of this gene leads to apoptosis by arresting the cell cycle in HCC cells[19].

Importantly, our study also sheds light on the role of Na, K-ATPase in liver cancer progression. We observed decreased expression of Na, K-ATPase in the non-adherent collagen group, suggesting a link between collagen and altered Na, K-ATPase activity. This finding is particularly relevant given the recent classification of Na, K-ATPase, alongside E-cadherin, as a potential biomarker for tumor staging and metastasis in other cancers, and the reported cytoplasmic localization of Na, K-ATPase in metastatic tissues[28-31]. Our study uniquely connects collagen to Na, K-ATPase regulation in liver cancer, opening new avenues for research into the role of this protein in HCC progression. Also, our observation that matrigel significantly enhanced liver cancer cell proliferation highlights the importance of carefully selecting ECM components for *in vitro* models.

This study provides novel insights into the dynamic regulation of YAP by type I collagen in liver cancer cells. The new findings in the study confirm previous findings on E-cadherin downregulation by collagen, linking it to a pro-migratory phenotype. The findings might demonstrate the possible link between collagen type I and Na,K-ATPase regulation in liver cancer, suggesting a potential role in metastasis. Finally, the findings underscore the importance of careful ECM selection in cancer research, as *in vitro* models can inadvertently affect cancer growth.

Despite these findings, this study has certain limitations. The experiments were conducted in vitro, which may not fully recapitulate the complexity of the in vivo tumor microenvironment. Moreover, only a limited number of ECM components and proteins were examined, leaving other potential regulatory

pathways unexplored. Further validation in animal models and patient-derived samples is required to confirm the translational relevance of these observations.

In conclusion, this study offers valuable and novel insights into the complex interplay between the extracellular matrix (ECM) and liver cancer cell behavior, addressing a critical need for more physiologically relevant *in vitro* models. Future research should investigate the specific signaling pathways mediating these effects to fully leverage the therapeutic potential of targeting the tumor microenvironment.

Author Contributions: Concept/Design : ZA; Data acquisition: -; Data analysis and interpretation: ZA, CD, GD; Drafting manuscript: ZA; Critical revision of manuscript: -; Final approval and accountability: ZA, CD, GD; Technical or material support: -; Supervision: -; Securing funding (if available): n/a.
Ethical Approval: The study is a cell culture study. Ethical committee permission is not required.
Peer-review: Externally peer-reviewed.
Conflict of Interest: Authors declared no conflict of interest.
Financial Disclosure: This study was supported by the Scientific and Technological Research Council of Turkey (TUBITAK).
No author has any direct or indirect financial interest that could influence the conduct or reporting of the submitted work.

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