RESEARCH ARTICLE

# Human amniotic epithelial cells (HAECS); a potential cell type for the hepatic disorders

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## Abstract

**Objective:** To isolate a homogenous population of human amniotic epithelial cells (hAECs) from the amniotic membrane of the human placenta and differentiate them into hepatic-like cells with the help of small molecules.

**Method:** hAECs were isolated by using the enzymatic digestion method and characterized for the presence of specific stem cell markers. In-vitro, hepatic differentiation of hAECs was carried out by using a combination of small molecules. Differentiated cells were observed under a live cell imaging microscope for morphological changes followed by gene and protein expression analysis by qPCR and immunocytochemistry respectively.

**Results:** The isolated hAECs attained characteristic cuboid epithelial shape and express stem cells marker. The hepatic differentiation method was optimized based on soluble chemical compounds supplied in the culture medium. The differentiated hAECs phenotypically acquire hepatic-like cell features and expressed hepatic markers as well as hepatic protein albumin at immature levels.

**Conclusion:** The isolated population of hAECs is highly proliferative. Moreover, hepatic markers expression in the isolated hAECs makes them an exclusive source for the treatment of chronic liver diseases.

**Keywords**: Amnion, Immunohistochemistry, Epithelial, Albumins, Liver, Digestion, Placenta

(JPMA 74: S-51 (Supple-2); 2024) **DOI: https://doi.org/10.47391/JPMA-DUHS-S11**

## Introduction

Acute and chronic liver diseases are the leading cause of mortality worldwide[1]. Although the liver has a remarkable regenerative capacity, interactions with drugs, toxic compounds, and viral pathogens could affect the regenerative potential and functionality of the liver [2-6].Continuous exposure could result in the end stage of liver pathologies and ultimately hepatic failure leading to complications including disturbance in the haematological system, renal dysfunction, impairment of the neurological system, and other metabolic abnormalities [2-3]. Orthotopic liver transplantation was used to manage end-stage liver diseases produced by chronic liver cirrhosis, and cancer and to recuperate severely impaired liver functions [4, 7].However, due to the severe shortage of donor liver organs, infection transmission, the probability of tissue/organ rejection, and other complications caused by long-term immuno-suppression, only limited patients could be treated with orthotopic liver transplantation (OLT) [1, 5, 8]. There is a growing set of healing choices that can support chronic or acute liver disease patients, including grafting; identified malfunctioned organs[9].existing donors, and cell-based therapies [1].

Cell-based therapies in patients with liver disease provide a hopeful alternative to solid organ transplantation [6, 10-11]. Cell transplantations have several benefits over liver transplantation since it offers a less invasive alternative and can be performed several times. Different types of cells have been exploited to produce donor-free and expandable sources of hepatocytes in liver tissue engineering[3].

Studies have shown the potential of stem cell-based therapies for Chronic Liver Diseases (CLD)[12-18]. It has been recognized that mesenchymal, embryonic, and induced pluripotent stem cells have therapeutic properties applicable to CLD. However, health, cost, accessibility, and ethical considerations hinder the clinical implementation of these candidates [10, 18]. These concerns have steered scientists to look for alternative sources of cells, one of the most promising of which are perinatal stem cells. [11, 19]. Extraembryonic tissues i.e., umbilical cord and fetal membrane are the rich sources of perinatal stem cells [12, 20]. Their exclusive origin is supposed to be the reason for the combined therapeutic and differential properties of mesenchymal stem cells and embryonic stem cells, respectively [21-22]. In addition, perinatal stem cells are immune-favoured and genetically stable, hence they do not form teratomas in animal models following

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transplantation[19, 21]. Moreover, perinatal stem cells are isolated from discarded material, making their collection as well as usage, less ethical. Such benefits together make perinatal stem cells a strong and more feasible source for clinical application than any other cell type [11, 19].

Human amniotic epithelial cells (hAECs) can be isolated relatively easily and do not involve a complicated laboratory setup[13, 23]. It is possible to separate approximately 100 million cells per placenta, they are fast-growing and can be cryopreserved for a long time. They possess targeted cell beneficial effects and can convert into clinically relevant cells. AE cells have some benefits over embryonic stem cells; stem cells isolated from other sources have the potential to form teratoma upon transplantation[14, 24]. whereas, hAECs have a stable karyotype and do not develop any tumours. They have reduced DNA damage, can bypass the immune system, and have the potential to inhibit inflammation[15, 25]. Compared to embryonic stem cells, hAECs have beneficial effects without any dangerous side effects, their low immunogenicity, and anti-inflammatory properties, have made them a promising source in regenerative medicine.

In addition, hAECs can undergo hepatic differentiation by using growth factors, transcription factors, micro RNAs, and small molecules, as these are the tools for differentiation and maturation. Among them, small molecules are the cost-effective chemical tools including molecules responsible for epigenetic alteration such as DNA methylation, histone acetylation, and deacetylation. Inhibitors of histone deacetylation promote hepatic differentiation and maturation. Small molecules are used as a substitute for growth factors and transcription factors in hepatic differentiation and maturation consequently having low cost and enhanced yield[16, 26]. This cost-effective study was designed to investigate the potential for the cells from which we have previously isolated a non-controversial, and safe source of stem cells i.e., human amniotic epithelial cells (hAECs) for the differentiation of hepatocytes to provide great improvement opportunities for in-vitro hepatocytes differentiation and to augment the recovery of degenerated liver tissue upon transplantation.

## Materials and Methods

Approval was taken from ERC Ziauddin University with reference code: 1050419QKANA. This experimental in-vitro study was conducted on the human placenta (amniotic epithelial cells) from 2019-2020 at Ziauddin University. Purposive sampling was used to collect a total of 20-25 human placentas to retrieve amniotic epithelial cells. Every experiment was conducted in triplicate.

### Collection of Human Amniotic Membrane

Placenta was collected after taking written and informed consent from the patients, from Ziauddin Hospital. Patients with maternal diabetes, hypertension, thyroid abnormalities, placenta Previa, placenta abruption, and abnormality in the foetus detected on scanning during the antenatal period were not included in the study.

The placenta was collected from patients with age 20-30 years having healthy full-term pregnancies, with a preference for term elective Caesarean sections. The amniotic membrane was manually separated from the chorion layer, starting from the reflected edge of the foetal membranes towards the umbilical cord. A 7 cm long piece of amnion was cut and placed in a 50 ml of falcon tube which has 20 ml of PBS to remove large blood clots. The amnion was transferred into another 50 ml falcon containing 20 ml PBS, to wash off the remaining blood and debris. The process was repeated thrice and then the sample was shifted to the stem cell culture lab.

### hAECs Isolation by Enzymatic Digestion

The human amniotic epithelial cells were isolated in class II biosafety cabinets according to the following optimized protocol. After washing with sterile PBS, amnion was digested with trypsin (Thermo Scientific, Life Technologies, USA), and the plates were incubated at 37°C with 5% CO2 for 30 minutes. After incubation, the gel was homogenized in a DMEM medium using pipetting and then. The medium containing hAECs was transferred into three 25 cm2 cell culture flasks in DMEM containing 10% foetal bovine serum (FBS) (Thermo Scientific, Life Technologies, USA). Penicillin/streptomycin (Thermo Scientific, Life Technologies, USA), sodium pyruvate (Thermo Scientific, Life Technologies, USA), and L-glutamine (Thermo Scientific, Life Technologies, USA), incubated at 37°C in a 5% CO2 incubator. The medium was changed after every three days till cells become 70% confluent.

### Characterization of the Isolated hAECs

**Gene Expression Analysis:** The isolated hAECs were characterized for the presence of stem cell (CD73, CD90, CD105), epithelial (CD133), and pluripotency (Oct 4) markers by q-PCR. Total RNA was isolated from the cells by using Trizol (Thermo Scientific) reagent. Following trypsinization, the cell pellet was re-suspended in 1mL of Trizol reagent. After that chloroform (200 µL/mL of Trizol-1:5 volume) was added and vortexed for 15 seconds followed by incubation at room temperature for 15-20 minutes. Centrifugation was carried out for phase separation at 12000 g for 10-15 minutes. The upper aqueous phase containing RNA was carefully transferred into a 1.5 ml Eppendorf tube, in which 0.5 ml chilled

isopropanol was added for RNA Precipitation followed by incubation at room temperature for 30 minutes and centrifugation at 12000 g for 10-15 minutes. For the rehydration of RNA, 1 ml of 70% ethanol (Merck) was added, and centrifugation was carried out to get the desired RNA sample. The RNA pellet was air-dried and suspended in 20 µL of RNase-free water. The concentration of isolated RNA was quantified by using Multi Scan Sky Spectrophotometer. cDNA was synthesized by using the "Revert Aid First Strand cDNA Synthesis Kit" (Thermo Scientific) according to the manual's instructions. For the preparation of cDNA 1 µg RNA, 1 µL Oligo dT Primer, was mixed with nuclease-free water to make a total solution of 12 µL. Then the mixture was centrifuged and incubated at 65°C for 5 minutes. Afterward, 4 µL 5X reaction buffer, 2 µL dNTPs (10mM), 1µL RNase out, and 1µL of Revert Aid (200 U/µL) were also added before the second incubation at 42°C for an hour. The reaction was terminated by heating at 70°C for 5 minutes and the product was stored immediately at -20°C. The primers used for q-PCR are enlisted in Table 1. To make a 20 µL reaction, 0.8 µL of cDNA was added to 9.2 µL SYBR green master mix (Thermo Scientific) with 10 µl primer. After completion of the 40 PCR cycles of denaturation, annealing, and extension, CT values were acquired. GAPDH expression was used as an internal control.

**Protein Expression Analysis:** The cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100, washed with PBS for 15 minutes, and incubated with the primary antibodies overnight against CD90, Vimentin, and CK18 (Cloud-Clone Corp). After washing with PBS, the cells were incubated with Alexa fluor 596 donkey anti-rabbit secondary antibody. Stained cells were observed under a fluorescent microscope after washing with PBS.

**Differentiation Induction**

**Cytotoxicity Analysis by MTT Assay:** MTT assay measures the redox activity of living cells. In-vitro cytotoxicity of drugs (Valproic acid) was carried out by MTT (3- [4, 5- dimethylthiazol-2-y1] tetrazolium bromide) colorimetric assay. MTT is reduced by the activity of mitochondrial dehydrogenases in living cells. The colour of the dye changed to purple, and the intensity of the colour is directly proportional to the number of viable cells. A cell suspension containing 30,000 cells/ml was added to the 96-well plate, followed by 24 hours of incubation so that cells become adhered to the surface of the plate. After attachment, the media was aspirated, and cells were treated with different concentrations (1 mM, 5 mM, and 10 mM) of Valproic acid in triplicates. After 48 hours of incubation, the media was aspirated, and the MTT dye was added to the wells containing treated cells and the cells of the control wells. The plate was then incubated for 4 hours. After that, MTT was removed and 100 µl of DMSO was added to each well to dissolve MTT crystals. After 30 minutes of incubation at room temperature, the plate was read in a spectrophotometer at 540 nm.

**Treatment with the Compound:** Before differentiation induction, cells were cultured in an incomplete IMDM (Gibco, Life Technologies, USA) medium for 24 hours. After the incubation period, the cells were treated with 1 mmol Valproic acid (VPA) for three days. On the fourth day, 2 µmol salvianolic acid B2 and 2 µmol dexamethasone were added to the medium which was changed every third day for 2 weeks.

**Analysis of Treated Cells**

**Morphological Analysis:** Human amniotic epithelial cells were analyzed for morphological changes after 24 hours of treatment with different concentrations of Valproic acid (1 mmol, 5 mmol, and 10 mmol), as well as after 2 weeks following differentiation induction protocol under a live cell imaging microscope. Morphological changes were noted in the treated cells and compared to untreated human amniotic epithelial cells.

**Gene Expression Analysis:** Expression of hepatic genes ALB, HNF3β, and pluripotency gene i.e., Oct4., were analyzed in human amniotic epithelial cells and treated cells (T1) by q-PCR as mentioned previously.

**Protein Expression Analysis:** The cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100, washed with PBS for 15 minutes, and incubated with the primary antibodies overnight against albumin (Thermo Scientific, Life Technologies, USA) and actin (Thermo Scientific, Life Technologies, USA). After washing with PBS, the cells were incubated with Alexa fluor 596 donkey anti-rabbit secondary antibody. Stained cells were observed under a fluorescent microscope after washing with PBS.

**Statistical analysis**

For the analysis of data SPSS (version 20) was used. All statistical numeric values were presented as mean± S.E of the mean (SEM). Quantitative data was analyzed by using an independent sample t-test. The significant difference between the groups was considered significant at a set P-value $< 0.05$.

## Results

**Gene Expression Analysis by RT-PCR:** The isolated hAECs showed positive expression of stem cell (CD73, CD90, CD105), epithelial (CD133), and pluripotency (Oct 4) specific genes (Figure 1a).

**Protein ExpressionAnalysis by Immunocytochemistry**: The isolated hAECs showed positive expression of stem cell (CD90, Vimentin) and epithelial (CK18) specific proteins. Actin expression was used for the positive control (Figure. 1b).

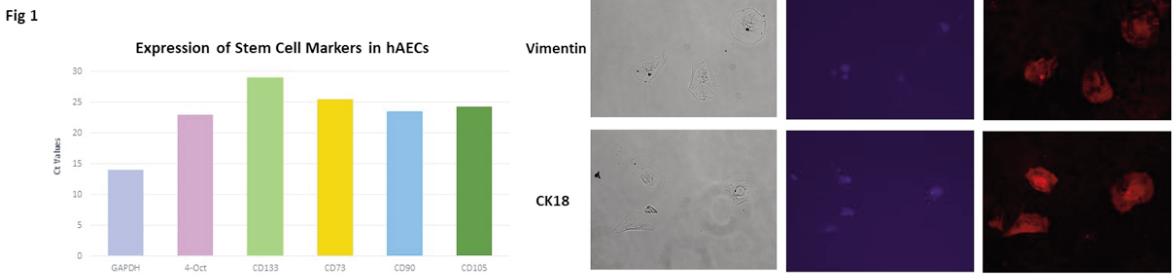


**Figure-1:** Characterization of hAECs by gene and protein analysis:
(a) Graphical presentation of the stem cell (CD73, CD90, and CD105), epithelial (CD133), and pluripotency marker (Oct 4) expression in hAECs. (b) hAECs were shown to be positive for Actin, CD90, vimentin, and CK18. Alexa Fluor 546 donkey anti-rabbit secondary antibody was used for detection. 40× magnification images were captured by an inverted fluorescent microscope.

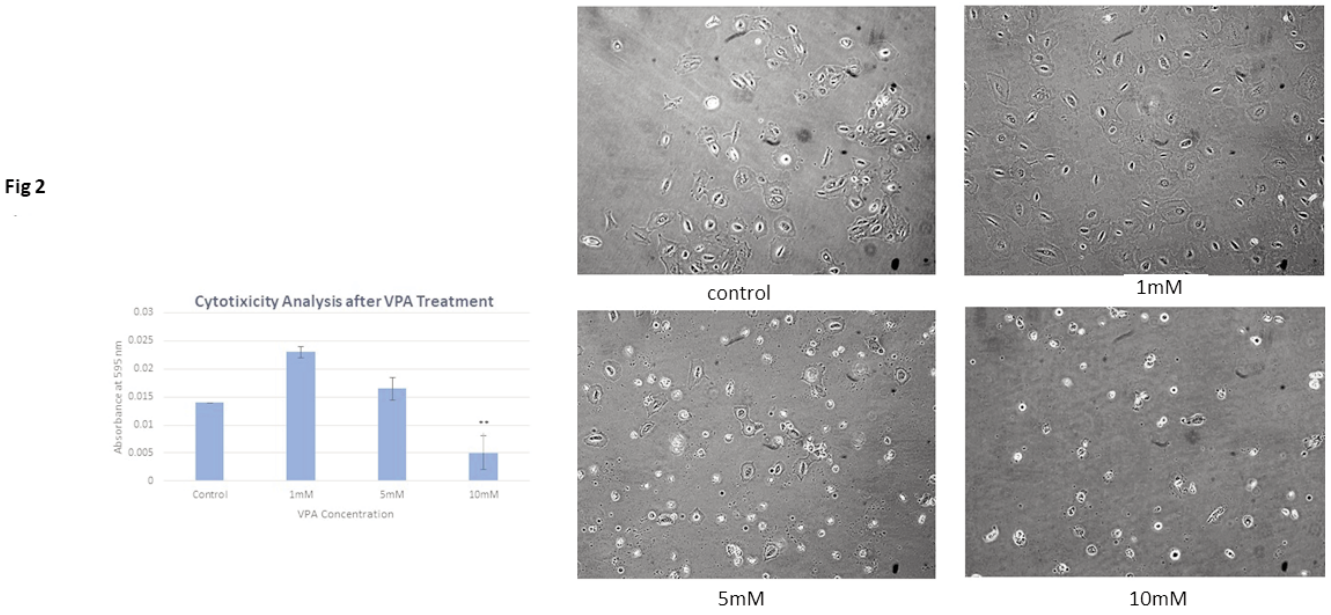


**Figure-2:** Cytotoxic effects of valproic acid following 48 h treatment and Morphological analysis of HAECs after 24 hours VPA treatment
(a) the graph shows the dose-dependent growth inhibitory effect of the drug on hAECs. A significant difference was observed in the cell growth inhibition between untreated and hAECs 10 mM Valproic Acid treated cells and is indicated as **P<0.01 (b) Morphology of the HAECs cells grown in the presence of different concentrations of valproic acid in untreated HAECs, 1 mM VPA, 5 mM VPA and 10 mM VPA. Images were taken at 10× magnification under a live cell imaging microscope.

**Cytotoxicity Analysis by MTT Assay**: We analyzed the cytotoxic effect of valproic acid at different concentrations (1 mmol, 5 mmol, and 10 mmol) on hAECs by MTT assay after 48 hours of incubation. It was observed that there is no significant death in the cells treated with 1 mM and 5 mM Valproic acid. Whereas its increased concentration i.e., 10 mM was found to be significantly (P-value <0.001) cytotoxic for the cells. (Figure 2a). The morphological changes in the cells were also observed after treatment with different concentrations of Valproic acid. Cells treated with 5 mM appeared shrunk and round in shape as compared to the cells treated with 1 mM Valproic acid which showed similar morphology to the control. However, most of the cells detached from the flask and died with 10 mM Valproic acid treatment (Figure 2b).

### Analysis Of Treated Cells

**Morphological Analysis:** The treated groups have shown altered morphology of the human amniotic epithelial cells after 2 weeks of culture in the hepatic induction media. Phenotypically, untreated hAECs (i) showed cobblestone-like morphology as compared to the cells treated with (ii) 1 mM VPA that revealed polygonal shape with prominent nuclei, scant cytoplasm, and enriched cytoplasmic granules. Moreover, the size of the cells was found to be increased as compared to the untreated controls (Figure 3a).

**Gene Expression Analysis by qPCR:** Hepatic-specific genes i.e., Albumin, HNF3β, and pluripotency gene i.e., Oct4 expression were analyzed in both untreated and treated human amniotic epithelial cells by qPCR. The expression of the genes was normalized with the expression of the housekeeping gene (GAPDH). The treated hAECs showed significantly increased expression of hepatic specific gene albumin ($p<0.001$) while no significant change was observed in the expression of HNF3β and Oct4 as compared to the untreated hAECs (Figure 3b).

**ProteinExpression Analysis by Immunocytochemistry**: It was observed that the hepatic-specific protein albumin showed positive expression in untreated as well as treated hAECs (Figure 3c). The quantification of the expression was analyzed by Image J software. Graphical representation of albumin expression is displaying higher albumin expression in untreated hAECs as compared to the treated cells (Figure 3d).

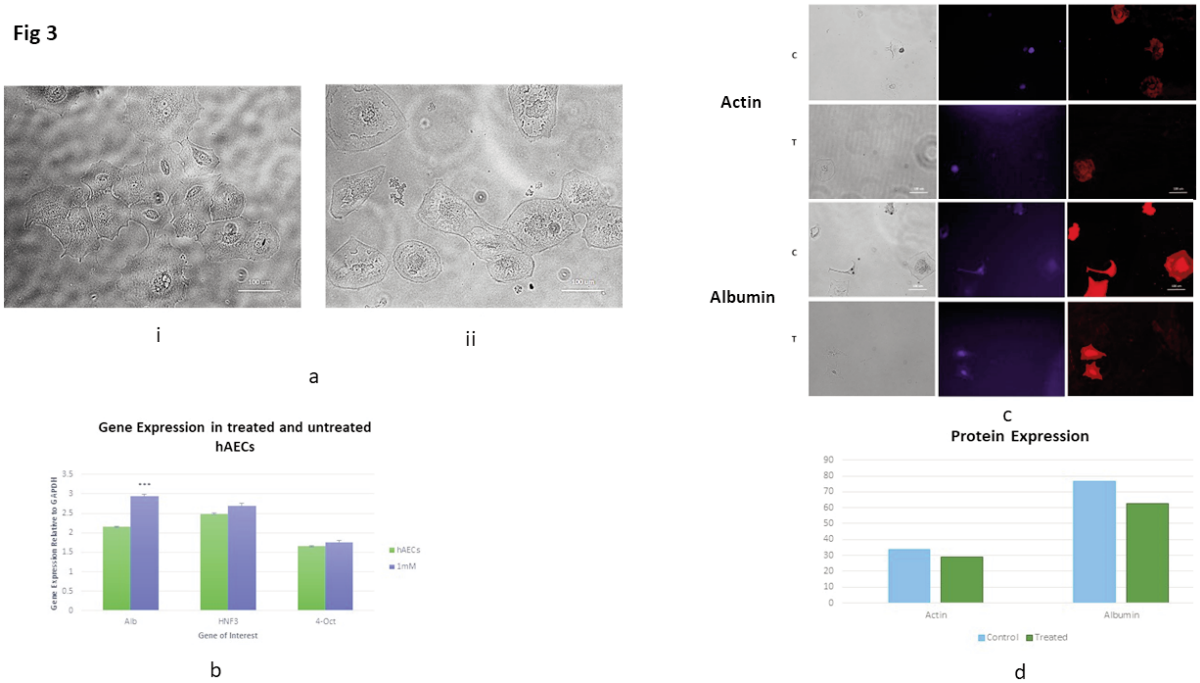


**Figure-3:** Morphology, gene & protein expression analysis of treated & untreated hAECs after 2 weeks:
(a) HAECs cells grown in the presence of differentiated media with different combinations of valproic acid (i) 1 mM VPA, salvianolic acid B2, and dexamethasone resulted in altered morphology of the human amniotic epithelial cells as compared to the (ii) untreated controls. Images were taken at 40× magnification under a live cell imaging microscope. (b)Graphical presentation of the relative expressions of Albumin, HNF3B, and Oct 4 in untreated hAECs cells (control) and treated cells (1 mM VPA). (c) Immunocytochemical analysis of actin and albumin in untreated hAECs cells (control) and treated cells (1mM VPA). (d) Graphical presentation of the quantification of actin (positive control) and albumin (hepatic protein) in untreated hAECs cells (control) and treated cells (1 mM VPA). Higher expression of albumin is presented in untreated hAECs as compared to the treated cells.

## Discussion

Cell transplantation has shown a lot of promise, and the progress made in preclinical and clinical studies over the past several decades offers a growing basis for its use in treating a variety of liver disorders [13] and provides a hopeful alternative to solid organ transplantation [6]. Cell transplantation has several benefits over liver transplantation since it offers a less invasive alternative and can be performed several times. Different types of cells have been exploited to produce donor-free and expandable sources of hepatocytes in liver tissue engineering [3].

Human amniotic epithelial cells (hAECs) have some benefits over embryonic stem cells; stem cells isolated from other sources have the potential to form teratoma upon transplantation [14] Whereas, hAECs have a stable karyotype and do not develop any tumours. They have reduced DNA damage can bypass the immune system and have the potential to inhibit inflammation. Compared to embryonic stem cells, hAECs cells have beneficial effects without any dangerous side effects, their low immunogenicity and anti-inflammatory properties, have made them a promising source in regenerative medicine. Their smaller size, relative to hepatocytes, offers practical advantages for injection and grafting [23].

For cellular therapy stem cells must be differentiated into the desired cell type [18]. There are two main approaches to differentiating a stem cell, the first one is direct reprogramming and the second is indirect reprogramming. These strategies, aim to attain certain objectives of availing a healthy and full-fledged cell having clinical purposes [19]. Cellular transformation can be done step by step by using different growth factors, transfection of the key regulatory transcription factors, and by using small molecules. In vitro, the addition of growth factors is responsible for cellular expansion but solely cannot transform cell morphology [16]. Transforming growth factors, fibroblast growth factor and activin also contribute to the proliferation and differentiation of hepatocytes [20]. Activation of transcription factors via signalling pathway is required for committed cell destiny along with regulation of the mature state of the cell. Transcription factors facilitate the maturation of hepatic cells as well as the differentiation of non-hepatic cells to hepatic-like cells [16]. Several groups documented the generation of hepatocytes-like cells (HLCs), from different types of cells by using stepwise protocols and a combination of growth factors. HNF4α, FOXA1, GATA4, and HNF1a combinations were used to turn embryonic or adult fibroblasts of the mouse into induced hepatocytes (iHep) [8]. More recently, overexpression of FOXA3, HNF1α, and HNF4α has produced human iHeps from fibroblasts. After iHeps transplantation, the acute liver failure mice model sustained survival and restored liver function, showing positive differentiation of non-hepatic cells into hepatocytes [21].

The chemical agents present in nature can be used in maintaining and regulating cell destiny as they are more potent, productive, safe, and provides a cost-effective and practical foundation for in vitro hepatic differentiation, with tremendous clinical potential [22-23]. When dental pulp derived MSCs (MSC-DP) were cultured in the presence of HGF, dexamethasone, oncostatin, and hydrogen sulfide, they changed their shape into hepatic-like cells and synthesized albumin and IGF-1 [24]. In another research, CHIR99021, a small molecule Glycogen synthase kinase (GSK) inhibitor, was used instead of growth factor alone with DMSO and dexamethasone for hepatocyte maturation [25]. Valproic acid morphologically and functionally converted Bone marrow and UCMSCs into HLCs in a study [26]. The wnt/β-catenin pathway plays an important role in cell proliferation, cell specification fate, and apoptosis and thus is vital in hepatic regeneration [21]. Hepatic differentiation of hESCs (human embryonic stem cells) by regulation of the WNT/β catenin pathway [22] and inhibition of the Notch pathway also contributes to hepatic differentiation [23].

In this study, we used a combination of a small molecule that works as the activator and inhibitor of the key signaling pathways used in the development of hepatocytes. hAECs were cultured first in the presence of histone deacetylase inhibitor (Valproic acid) for 3 days after that the cells were grown in the presence of Wnt pathway activators (Salvionic acid & Dexamethasone) till day 14, for the differentiation of hAECs into hepatic like cells. We first optimize the working concentration of VPA by treating the cells with different concentrations of the VPA (1 mM, 5 mM, and 10 mM). Increased cell proliferation was observed after treatment with 1 mM VPA whereas no significant change was observed n 5 mM VPA-treated cells. However, significant cell death was observed in the 10 mM VPA group. 1 mM VPA-treated cells were further cultured in the presence of Salvianolic acid B and dexamethasone for two weeks. Following treatment rounded undifferentiated human amniotic epithelial cells were morphologically differentiated into polygonal hepatic-like cells. Lin et al observed that differentiated AECs i.e., hepatic-like cells proliferated abundantly and became more polygonal and granular, accumulating more cell organelles such as mitochondria, and endoplasmic reticulum. Formation of bile canaliculi, a pathognomonic hallmark of differentiated hepatocytes

that was visible between cells, under electron microscopy. Undifferentiated AECs, on the other hand, lacked these cell organelles, and canaliculi[25]. Under a phase contrast microscope, the produced HLCs (hepatic-like cells) had a polygonal form with prominent nuclei, sparse cytoplasm, and abundant cytoplasmic granules and some of the HLCs are binucleated which are similar to the features of normal human hepatocytes in culture[26]. In this study, we observed amniotic epithelial stem cells phenotypically differentiated into hepatic-like cells that showed cuboidal to polygonal shape with prominent nuclei, scant cytoplasm, and some cells were binucleated also enriched cytoplasmic granules under live cell imaging microscope. These findings revealed that when hAECs were treated with small molecules, they lost their amniotic epithelial stem cell phenotype and acquired a hepatocytic phenotype in terms of cell shape. Hepatic genes i.e., Albumin, HNF3β, and pluripotency gene i.e., Oct4 expression were analyzed in both untreated and treated human amniotic epithelial cells. Treated cells showed significantly increased expression of hepatocytes-specific marker albumin while no significant change in the expression of HNF3β, and Oct4 were observed as compared to the untreated hAECs. Protein expression was analyzed in the treated and untreated cells by immunocytochemistry. It was observed that the albumin is expressed in untreated as well as treated hAECs.

Hepatic differentiation of the stem cells method was currently optimized based on soluble chemical compounds supplied in the culture medium, and the HLCs produced showed altered phenotypes that were polygonal in shape and some were binucleated but differentiated cells (HLCs) demonstrated immature features on the level of the gene. Moreover, our results showed that human amniotic epithelial cells are smaller in size as compared to the treated cells and expressed high levels of hepatic cell markers which implies its significant potential for regenerative purposes, especially for hepatic disorders.

## Conclusion

The isolated population of hAECs is highly proliferative. Moreover, hepatic markers expression in the isolated hAECs makes them an exclusive source for the treatment of chronic liver diseases.

**Limitation**: Sample size was not calculated for the Amniotic membrane collection

**Disclaimer:** This article is from the MPhil Thesis of Dr. Qurat ul Ain Khan

**Conflict of Interest:** None.

**Funding disclosure** SRGP funding by HEC.

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