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The role of HIF-1 α in the TGF- β 2-mediated epithelial-to-mesenchymal transition of human lens epithelial cells

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Abstract

Human lens epithelial cells (HLE) undergo mesenchymal transition and become fibrotic during posterior capsule opacification (PCO), which is a frequent complication after cataract surgery. TGF- β 2 has been implicated in this fibrosis. Previous studies have focused on the role of hypoxia-inducible factor-1 α (HIF-1 α) in fibrotic diseases, but the role of HIF-1 α in the TGF- β 2-mediated fibrosis in HLE is not known. TGF- β 2 treatment (10 ng/mL, 48 h) increased the HIF-1 α levels along with the EMT markers in cultured human lens epithelial cells (FHL124 cells). The increase in HIF-1 α corresponded to an increase in VEGF-A in the culture medium. However, exogenous addition of VEGF-A (up to 10 ng/mL) did not alter the EMT marker levels in HLE. Addition of a prolyl hydroxylase inhibitor, dimethylxalylglycine (DMOG, up to 10 μ M), enhanced the levels of HIF-1 α , and secreted VEGF-A but did not alter the EMT marker levels. However, treatment of cells with a HIF-1 α translational inhibitor, KC7F2, significantly reduced the TGF- β 2-mediated EMT response. This was accompanied by a reduction in the ERK phosphorylation and nuclear translocation of Snail and Slug. Together, these data suggest that HIF-1 α is important for the TGF- β 2-mediated EMT of human lens epithelial cells.

Keywords

epithelial-to-mesenchymal transition; HIF-1 α ; KC7F2; lens epithelial cells; TGF- β 2; VEGF-A

1 | INTRODUCTION

Cataracts are the major blinding disease in the world. The number of people who suffered from cataracts in the US in 2010 was approximately 24 million, and this is expected to increase to nearly 40 million by 2030 (<https://nei.nih.gov/eyedata/cataract>). Worldwide,

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AUTHORS' CONTRIBUTION

RHN and RBN conceived, designed, and analyzed all experiments. RBN performed all experiments. The authors reviewed the results and approved the final version of the manuscript.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

visual impairment from cataracts was nearly 95 million in 2014,¹ and this is expected to significantly increase in the coming years.

The lens is avascular and abundant in proteins. The single layer of anterior epithelial cells adherent to capsule is the principal metabolic source for the lens. The rest of the lens is formed of terminally differentiated and elongated fiber cells, which are rich in proteins known as crystallins. During cataract surgery, a circular portion of the anterior capsule of the lens is excised (capsulorhexis), which allows surgeons to access the cataractous fiber material, emulsify, and suction it out of the lens, leaving behind a hollow bag of lens capsule.² An intraocular lens (IOL) is implanted in the capsular bag. This procedure results in restoration of vision to a high degree in most patients. However, cataract surgery is not foolproof; attempts are made during surgery to remove the epithelial cells that adhere to the anterior capsule adjacent to the capsulorhexis region. Despite best efforts (polishing), some epithelial cells remain adherent to the capsule. These cells proliferate and migrate to the cell-free posterior capsule and transdifferentiate into myofibroblast-like cells in a process known as epithelial-to-mesenchymal transition (EMT).³ During EMT, epithelial cells secrete excessive extracellular matrix proteins such as collagen I and fibronectin and lose their apical-basal polarity through degradation of the transmembrane protein, E-cadherin. This leads to loss of cell-to-cell adhesion, and eventually the cells lose their epithelial cell morphology. During this process, the cells synthesize α -smooth muscle actin (α -SMA), and vimentin along with a host of other proteins that are typically upregulated in cells with a mesenchymal phenotype.³ In addition, integrins that play an important role in cell proliferation and migration⁴ are upregulated during EMT (reviewed in⁵). The excessive synthesis of extracellular matrix and α -SMA leads to wrinkling of the posterior capsule along with fibrous tissue formation that results in a blockage of the visual axis. This process is known as posterior capsule opacification (PCO) or secondary cataract formation.⁶

Improvement in the design and material composition of IOL have reduced the incidence of PCO. However, soft IOLs, which are being used for multifocal vision, generally increase the rate of PCO. In general, PCO occurs in nearly 30–40% of elderly cataract patients within 2–5 years after surgery^{7,8} and requires clearing of the visual axis by treatment with a ND-Yag laser (posterior capsulotomy). While the posterior capsulotomy restores vision again, it can result in other complications, such as endophthalmitis, retinal detachment, elevation of intraocular pressure, cystoid macular edema, and IOL subluxation.⁹ Thus, better therapies are required to prevent PCO, which in turn requires a better understanding of mechanisms involved in EMT.

Transforming growth factor beta (TGF- β) is generally considered to be the initiating cytokine for this process. This protein has three isoforms: 1, 2, and 3. Among these, TGF- β 2 is the dominant isoform in the eye.^{10,11} Previous studies have shown that TGF- β 2 plays an important role in the EMT of lens epithelial cells.^{12,13} TGF- β 2 induces EMT through the classical canonical (Smad signaling),¹⁴ and non-canonical (extracellular signal-regulated kinases [ERK], mitogen-activated protein kinases (MAPK) and phosphoinositide 3-kinase [PI3K]) pathways.¹⁵ Smad signaling is accompanied by the activation of transcription factors such as Snail and Slug.^{16,17} Previous studies have also shown that histone

deacetylases, sprouty protein, aldose reductase, and β -catenin/CBP play important roles in TGF- β 2-mediated EMT.^{17–20}

HIF is a heterodimer consisting of an α - and a β -subunit, which are highly conserved and expressed at basal levels in cells. Under normoxic conditions, the prolyl hydroxylase (PHD) that is present in the cytosol of cells hydroxylates proline residues (at positions 402 and 564) in HIF-1 α , marking it for degradation via the ubiquitin E3 ligase complex.²¹ However, under hypoxic conditions, PHD is inhibited, which leads to the retention of HIF-1 α , and subsequent nuclear translocation. HIF-1 α thus translocated binds to the hypoxia-responsive element (HRE) in DNA and initiates transcription of various genes that play roles in angiogenesis, cell proliferation/survival, apoptosis, erythropoiesis, etc.^{22,23} In addition, HIF-1 α has been shown to promote EMT in various types of tumors and fibrotic diseases.^{24–26} VEGF is one of the target genes of HIF-1 α ²⁷ and has been implicated in EMT.^{28,29} VEGF binding to its receptor leads to autophosphorylation of specific tyrosine residues in the cytoplasmic domain and such autophosphorylation plays an important role in cell proliferation and migration.³⁰

The lens is situated in a hypoxic environment in the eye. The oxygen partial pressure (pO₂) at the surface of the lens is 2.9 mm Hg, which is lower than 24.1 mm Hg at the surface of the eye.³¹ However, after cataract surgery the pO₂ at the surface of the lens increases to ~11 mm Hg.³² After cataract surgery, because of the higher pO₂, HIF-1 α should degrade. However, TGF- β 2, which is elevated in the aqueous humor after cataract surgery (reviewed in¹³), has been shown to upregulate HIF-1 α in cells.^{33,34} As, TGF- β 2 is the major cytokine for the EMT of lens epithelial cells, we reasoned that HIF-1 α might be involved in the EMT of lens epithelial cells. In this study, we tested the role of HIF-1 α in the TGF- β 2-mediated EMT of lens epithelial cells.

2 | MATERIALS AND METHODS

2.1 | Treatment of FHL124 cells with TGF- β 2

FHL124 cells were obtained from Dr. Michael Wormstone, University of East Anglia, UK (originally from Prof. John Reddan, Oakland University, MI) and were authenticated by the analysis of short tandem repeats.³⁵ Cells were cultured in MEM containing 5% FBS and gentamycin/L-glutamate (1:100) and treated with human recombinant TGF- β 2 (10 ng/mL, Sigma-Aldrich, St. Louis, MO) in serum-free medium for 4 h (for the signaling experiments), or 16 h (PHD-2), 24 h (for PHD-2, qPCR, and immunocytochemistry) or 48 h (for PHD-2 and EMT marker assessments).

2.2 | Treatment of FHL124 cells with VEGF-A or dimethyloxalyglycine (DMOG) or overexpression of HIF-1 α

To study the effect of externally added VEGF-A, human recombinant VEGF-A (PeproTech, Rocky Hill, NJ) was used at selected concentrations (0–10 ng/mL) for 24 h (in pVEGFR2 analyses) or 48 h (in EMT marker analyses) in serum-free medium. The cells were treated with DMOG (Santa Cruz Biotechnology, Dallas, TX, 0–10 μ M) for 48 h to increase the levels of HIF-1 α in cells. To overexpress HIF-1 α , the cells were transfected with either an

empty vector (Kind gift from Dr. Niklaus Mueller, Department of Ophthalmology, University of Colorado Denver) or a HIF-1 α plasmid (Addgene, Cambridge, MA) using Lipofectamine LTX and Plus Reagent (Life Technologies, Grand Island, NY) according to the manufacturers' instructions. After the above procedures (4 h), the cells were treated with TGF- β 2 (10 ng/mL) for either 4 h (for HIF-1 α measurement) or 20 h (EMT markers measurements).

2.3 | Treatment with a HIF-1 α translational inhibitor

To study the effect of TGF- β 2 under reduced levels of HIF-1 α , the cells were treated with various concentrations (0–200 nM) of a HIF-1 α translational inhibitor, KC7F2 (Tocris, Minneapolis, MN) for 3.5 h and then treated with or without TGF- β 2, as above.

2.4 | Measurement of HIF-1 α mRNA levels

FHL124 cell were treated with or without TGF- β 2 (10 ng/mL) for 24 h and total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA) and cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen). qPCR was performed with SYBR green using a CFX Connect Real-time PCR System (Bio-Rad laboratories, Hercules, CA) with the following primers, HIF-1 α - FP: 5'-CCAGAACCTACTGC - TAATGC-3' and RP:5'-TTATGTATGTGGGTAGGAGATG-3'. GAPDH-FP: 5'-GTCAGTGGTGGACCTGACCT-3' and RP: 5'-TGCTGTAGCCAAATTCGTTG-3'.

2.5 | Western blotting

Mammalian Protein Extraction Reagent (M-PER—Thermo Scientific, Waltham, MA) that contained a protease and phosphatase inhibitor cocktail (Sigma-Aldrich) was diluted 1:100 for use in preparing the total cell lysates. The cytosolic and nuclear fractions were prepared using NucBuster protein extraction reagent (Millipore, Billerica, MA). For VEGFR2, cell lysate was prepared using 1 \times RIPA buffer (Cell Signaling, Danvers, MA). We used lysates from human umbilical vein endothelial cells and human retinal endothelial cells as positive controls for Western blots in Supplementary Figure S2A and S2B. These protein extracts (10–30 μ g) were used for SDS-PAGE (12%) or gradient gel (4–20%, Bio-Rad, Hercules, CA) and Western blotting. After electrophoretic transfer, membranes were blocked with 5% blocking-grade non-fat dry milk in TBST, and the membranes were incubated overnight at 4°C with primary antibodies against the following proteins: α -SMA (Sigma-Aldrich, Cat # A5228, diluted 1:5000), β -actin (Cat # 4970L, diluted 1:5000), HIF-1 α (Cat # 3716S), PHD-2 (Cat #3293S), VEGFR2 (phosphorylated Y951 Cat # 4991T and total Cat # 9698T), E-cadherin (Cat #3195S), α V Integrin (Cat # 4711S), β 1 Integrin (Cat # 4706S), ERK (phosphorylated Cat # 4370S and total Cat # 4694S), Snail (Cat # 3879S), Slug (Cat # 9585S), and histone (H3) (Cat # 9715S, diluted 1:5000) (from Cell Signaling and diluted 1:1000 unless noted otherwise). Appropriate HRP-conjugated secondary antibodies (diluted 1:5000, Cell Signaling) were used, and the protein bands were detected using the SuperSignal West Pico or Femto Kits (Pierce Chemical, Dallas, TX).

2.6 | Immunocytochemistry

FHL124 cells were cultured on chamber slides (Lab-Tek II, NY) and treated with KC7F2 (200 nM) for 3.5 h, followed by TGF- β 2 for 24–48 h. After treatment, the cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and stained for f-actin as described below. The cells were treated with 0.1% Triton X-100 for 5 min at room temperature, blocked with 5% normal goat serum for 1 h, and then incubated with Texas Red-conjugated phalloidin (1:40 dilution, Life Technologies) at 37°C for 1 h. For HIF-1 α , the cells were permeabilized with ice-cold 80% methanol for 20 min at –20°C, blocked with 5% normal goat serum for 1 h, and incubated overnight at 4°C with an antibody for HIF-1 α (Sigma-Aldrich, Cat # H6536, 1:50 dilution). This was followed by 1 h of incubation with Alexa Fluor 488-goat anti-mouse IgG (1:250 dilution, Life Technologies) at 37°C. Cells that were stained with Alexa Fluor 488-goat anti-mouse IgG alone served as the negative control. The slides were mounted with DAPI/Vectashield for observations of the nuclei. Images were taken with a fluorescence microscope (Nikon Eclipse 80i, Nikon Corporation, Tokyo, Japan) using Nikon NIS software. Mean fluorescence intensity of HIF-1 α was calculated using ImageJ software (NIH).

2.7 | ELISA for VEGF-A

FHL124 cells were treated with TGF- β 2 as described above, and the media was collected after culturing cells for 48 h. This was used immediately for analysis of VEGF-A or stored at 4°C for 2–3 days prior to the ELISA assay. The VEGF-A levels were measured using a VEGF Human ELISA Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

2.8 | Statistical analysis

The data are presented as the means \pm SD of the specific number of experiments indicated in the figure legends. The data were analyzed with GraphPad Prism software (Version 7) by either one-way ANOVA or Student's *t*-test. The differences were considered significant at *P* < 0.05.

3 | RESULTS

3.1 | TGF- β 2 upregulates HIF-1 α and VEGF-A during EMT of FHL124 cells

First, we determined the effect of TGF- β 2 on HIF-1 α in FHL124 cells. Treatment of the cells with TGF- β 2 for 24 h caused an increase in the levels of HIF-1 α , as evident from the immunocytochemical data (Figure 1A, left four panels), which showed an increase in fluorescence intensity (Figure 1B). The negative control (secondary antibody alone, Figure 1A, right four panels) showed negligible staining. We were not able to show the upregulation of HIF-1 α protein by Western blotting because the results were inconsistent, possibly due to extremely low levels of HIF-1 α , but we found an increase in HIF-1 α mRNA (Figure 1C) upon TGF- β 2 treatment. However, TGF- β 2 treatment had no effect on the PHD-2 protein content, determined by Western blotting (Supplementary Figure S1A and S1B). TGF- β 2 treatment increased the VEGF-A levels in the conditioned media (Figure 1D). Western blotting for p-VEGFR2 (Y951) showed very faint bands in FHL124 cells and the levels were

unaltered by the TGF- β 2 treatment (Supplementary Figure S2A). We also determined the effect of TGF- β 2 on the EMT-associated proteins α -SMA, α V integrin, and β 1 integrin by Western blotting and found that they were all significantly upregulated in the TGF- β 2-treated cells compared to the untreated control cells (Figure 1E–G).

3.2 | Exogenous VEGF-A does not promote the TGF- β 2-mediated EMT of FHL124 cells

As we found higher levels of VEGF-A in the conditioned media after TGF- β 2 treatment, we sought to determine whether there was an association between externally added VEGF-A and the EMT response. Exogenous VEGF-A did not significantly alter the TGF- β 2-mediated effects on α -SMA, α V integrin, and β 1 integrin compared with untreated cells (Figure 2A–C). Similar to TGF- β 2 treatment, VEGF-A treatment had no effect on the phosphorylation of VEGFR2 (Y951) (Supplementary Figure S2B).

3.3 | Upregulation of HIF-1 α with a PHD inhibitor (DMOG) did not induce an EMT response in FHL124 cells

We observed a concentration-dependent increase in HIF-1 α (Figure 3A, up to 6 μ M) and VEGF-A (Figure 3B, in the media, up to 10 μ M) following treatment with DMOG for 48 h. However, this agent had no significant effect on the levels of EMT markers, α -SMA, α V integrin, and β 1 integrin, compared with the untreated cells (Figure 3C–E).

3.4 | A HIF-1 α translational inhibitor (KC7F2) inhibited TGF- β 2-mediated EMT in FHL124 cells

Next, we investigated whether a reduction in the levels of HIF-1 α would negatively affect the TGF- β 2-mediated EMT. We used KC7F2, a translational inhibitor of HIF-1 α . Cells that had been treated with TGF- β 2 for 24 h showed an increase in HIF-1 α staining (Figure 4A). KC7F2 treatment reduced the levels of HIF-1 α in cells (Figure 4A, left panels). The HIF-1 α fluorescence intensity was reduced in KC7F2 + TGF- β 2 treated cells compared to the TGF- β 2 alone treated cells (Figure 4B). The specificity of the HIF-1 α staining was confirmed in negative control experiments in which the HIF-1 α antibody was omitted during immunohistochemistry; those experiments showed no significant staining for HIF-1 α (Figure 4A, right panels). Our attempts to demonstrate a reduction in HIF-1 α at the protein level by Western blotting were not successful due to inconsistent results that were possibly the result of the extremely low levels of HIF-1 α . We did not check the mRNA levels of HIF-1 α since KC7F2 is a translation inhibitor.

As expected, TGF- β 2 treatment increased the VEGF-A levels in the media, but KC7F2 (at 200 nM) significantly reduced the levels (Figure 4C). We found concentration-dependent decreases in the levels of α -SMA, α V integrin, and β 1 integrin with increasing concentrations of KC7F2 compared to the cells treated with TGF- β 2 alone (Figure 4D–F). TGF- β 2 treatment showed a significant reduction ($P < 0.05$) in the E-cadherin levels and the treatment with KC7F2 reversed this effect (Supplementary Figure S3). We also investigated the cytoskeletal remodeling (F-actin) by immunofluorescence. We found an increase in the F-actin staining after the cells were treated with TGF- β 2 compared to control cells (Figure 4G). Such a staining was reduced by KC7F2 treatment (Figure 4G).

3.5 | KC7F2 inhibited the TGF- β 2-mediated signaling in FHL124 cells

We further verified the effect of KC7F2 on the TGF- β 2-mediated phosphorylation and nuclear translocation of signaling molecules. The cells that were treated with TGF- β 2 showed increases in p44/42 MAPK (ERK) phosphorylation and the nuclear translocation of the transcription factors Snail and Slug, which were reduced by the KC7F2 treatment (Figure 5A–C). The TGF- β 2-treated cells showed increases in the phosphorylation and nuclear translocation of Smad2, but there were no significant differences between the TGF- β 2-treated and TGF- β 2 + KC7F2-treated groups (Figure 5D).

3.6 | Overexpression of HIF-1 α had no effect on the TGF- β 2-mediated EMT of FHL124 cells

Cells transfected with a HIF-1 α plasmid showed significantly higher levels of HIF-1 α after 8 h of transfection (Figure 6A). Interestingly, we did not see any significant difference in the TGF- β 2-mediated EMT response between the empty vector and HIF-1 α transfected cells (Figure 6B–D).

4 | DISCUSSION

The objective of this study was to determine the role of HIF-1 α in the TGF- β 2-mediated EMT of lens epithelial cells. This was based on numerous studies on other cell types that have shown that HIF-1 α plays an important role in EMT.^{29,36–38} The eye lens exists in a hypoxic environment³¹ and therefore should favor HIF-1 α activation in its epithelial cells. In fact, a previous study showed such activation in mouse lens epithelial cells.³⁹ Introduction of oxygen during cataract surgery would be expected to decrease the HIF-1 α levels in lens epithelial cells and inhibit their EMT. However, TGF- β 2 levels are upregulated in the anterior eye after cataract surgery,⁴⁰ which could upregulate HIF-1 α in epithelial cells. Thus, whether TGF- β 2 opposes the effects of normoxia and initiates an EMT effect through HIF-1 α upregulation (independent of oxygen) was not known.

Our study showed that TGF- β 2 upregulates the HIF-1 α levels in FHL124 cells. The higher VEGF-A levels in the conditioned media further confirmed that HIF-1 α was activated in response to TGF- β 2. Previous studies have shown HIF-1 α stabilization through PHD inhibition in response to TGF- β 1 in the hepatoma cell line HepG2, fibrosarcoma HT1080 cells,³³ and renal tubular cells.⁴¹ The observed HIF-1 α up-regulation in HLE does not appear to be due to changes in PHD-2 levels, as we found the levels to be unaltered by TGF- β 2 treatment. However, it could be due the observed increase in HIF-1 α mRNA levels.

Higher levels of HIF-1 α upon DMOG treatment did not elicit an EMT response. This result suggested that upregulation of HIF-1 α alone is not a critical factor for the EMT of FHL124 cells. Similarly, externally added VEGF-A failed to induce the EMT response without TGF- β 2. One recent study reported that VEGF-A released from lens epithelial cells, through an autocrine mechanism promotes cell survival, growth and myofibroblast formation,⁴² and showed that those changes were inhibited by a small molecule inhibitor for the VEGF-A receptor. Our results showed phosphorylation of VEGFR2 at tyrosine residue (Y951) was not altered after TGF- β 2 or VEGF-A treatment. This could explain why neither the

upregulated VEGF-A (after TGF- β 2 treatment) nor exogenous VEGF-A was able to induce/promote EMT in FHL124 cells.

Previous studies have shown that KC7F2 suppresses the key regulators of HIF-1 α protein synthesis⁴³ and thereby reduces the HIF-1 α protein content.⁴⁴ Similar to these findings, we observed that KC7F2 reduced the levels of the HIF-1 α protein in TGF- β 2-treated cells. What was striking in our study was the reduction in the TGF- β 2-mediated EMT when the HIF-1 α levels were reduced by the treatment with KC7F2. This implied that HIF-1 α is important for the TGF- β 2-mediated EMT of lens epithelial cells. One previous study suggested that HIF-1 α augments the TGF- β -mediated EMT by promoting the activation of latent TGF- β 1.⁴⁵ However, this was unlikely in our study because we used the active form of TGF- β 2 to induce EMT.

The above observation that HIF-1 α is important for the TGF- β 2-mediated EMT begs the question as to what signaling pathway of TGF- β 2 might be affected by very low levels of HIF-1 α . Our results suggested that subnormal HIF-1 α levels suppressed the non-canonical signaling arm of TGF- β 2. This corroborates several other reports that have shown ERK activation under hypoxic conditions.^{46–49} Additionally, low levels of HIF-1 α also decreased the Slug and Snail levels. These transcription factors are essential for the downregulation of E-cadherin during EMT. Similar observations have been made in other cells. For example, treatment with a HIF-1 α siRNA inhibited the epidermal growth factor-mediated upregulation of Snail and Slug in ovarian cancer cell lines.⁵⁰ When HIF-1 α was overexpressed, there was no further increase in the TGF- β 2-mediated EMT of FHL124 cells. This indicated that HIF-1 α upregulated by TGF- β 2 alone was sufficient for an EMT response. Based on these results, we propose a scheme (Figure 7) in which we hypothesize that HIF-1 α promotes TGF- β 2 mediated EMT through the non-canonical pathway. However, we cannot rule out the participation of the canonical pathway, as Snail and Slug (which are Smad-dependent) are downregulated when the HIF-1 α expression was inhibited by KC7F2.

In conclusion, our study showed that HIF-1 α is important for the TGF- β 2-mediated EMT in lens epithelial cells and suggested that HIF-1 α could be a target for the prevention of PCO.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

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|------------|---------------------------------|
| PCO | posterior capsule opacification |
| HLE | human lens epithelial cells |

| | |
|---------------------------------|---|
| EMT | epithelial-to-mesenchymal transition |
| α-SMA | alpha smooth muscle actin |
| FBS | fetal bovine serum |
| MEM | minimum essential medium |
| HIF-1α | hypoxia-inducible factor-1 α |
| VEGF-A | vascular endothelial growth factor-A |
| VEGFR2 | vascular endothelial growth factor receptor 2 |
| DMOG | dimethylxalylglycine |
| TGF-β2 | transforming growth factor-beta 2 |
| IOL | intraocular lens |
| ERK | extracellular signal-regulated kinases |
| MAPK | mitogen-activated protein kinases |
| PI3 K | phosphoinositide 3-kinase |
| PHD | prolyl hydroxylase |
| PHD-2 | prolyl hydroxylase-2 |
| HRE | hypoxia-responsive element |
| pO₂ | oxygen partial pressure |
| M-PER | mammalian protein extraction reagent |
| KC7F2 | N,N'-(dithiodi-2,1-ethanediy)bis[2,5-dichlorobenzenesulfonamide |
| ECM | extracellular matrix |
| DAPI | 4',6-diamidino-2-phenylindole dihydrochloride |

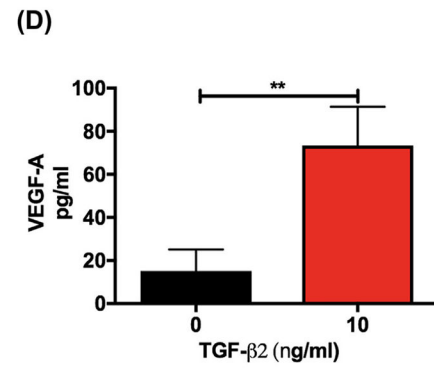
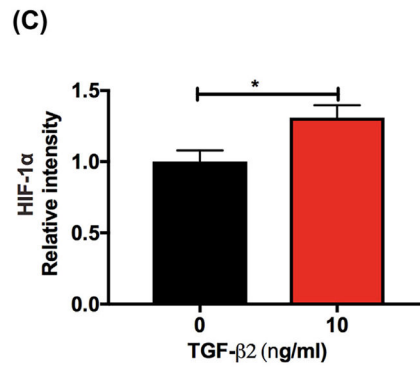
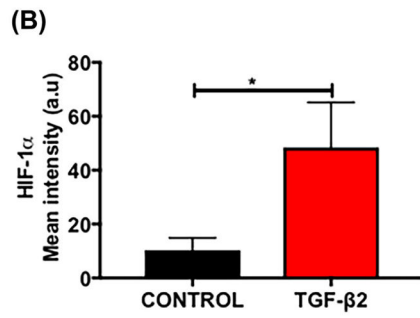
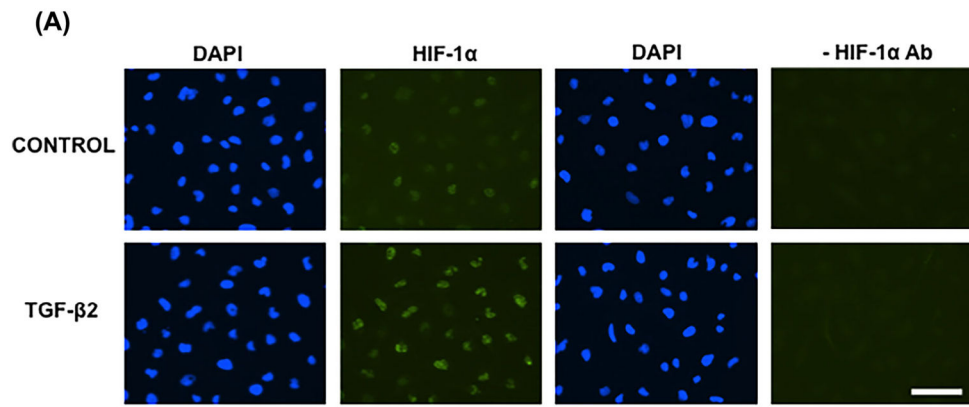
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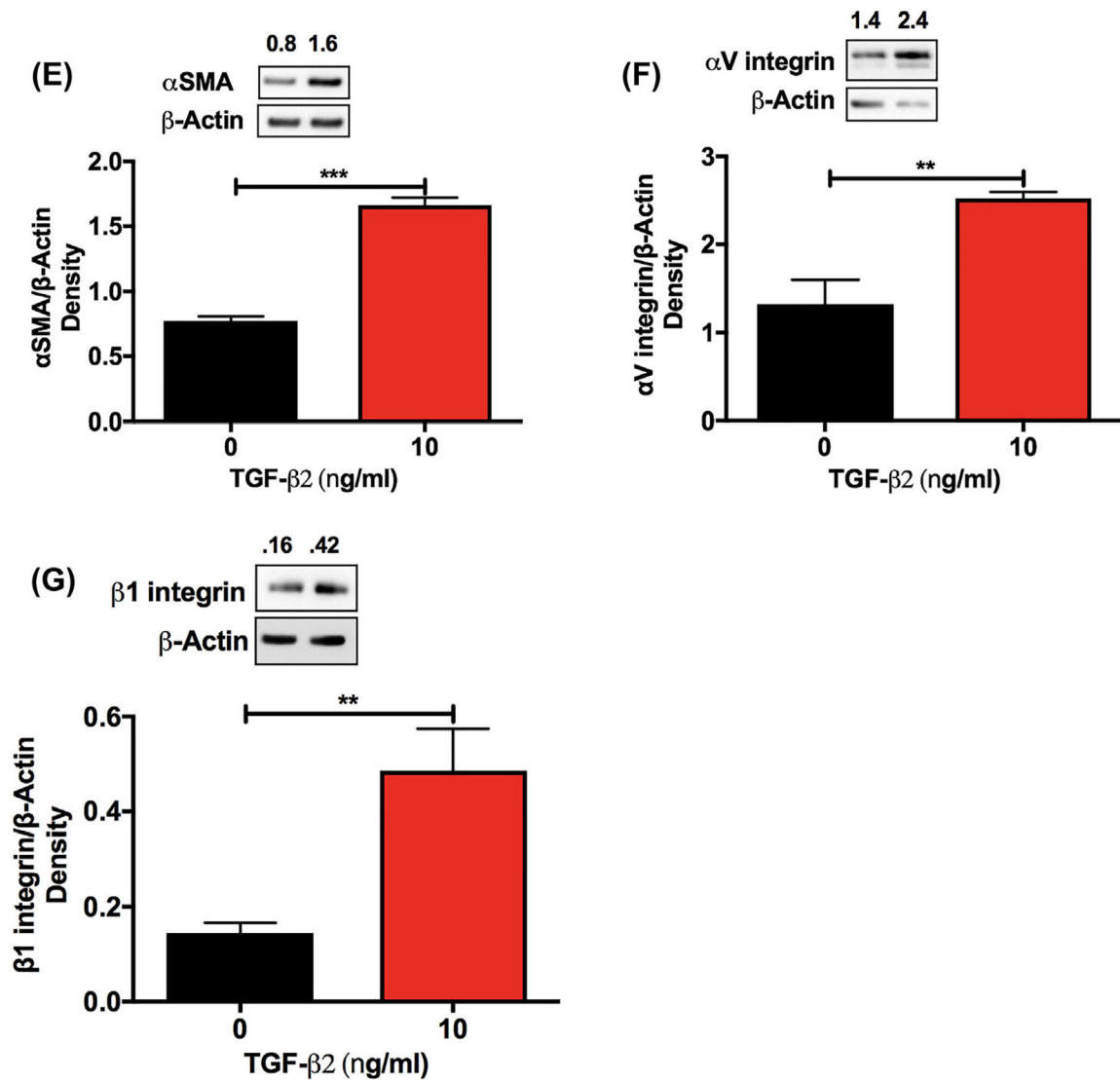
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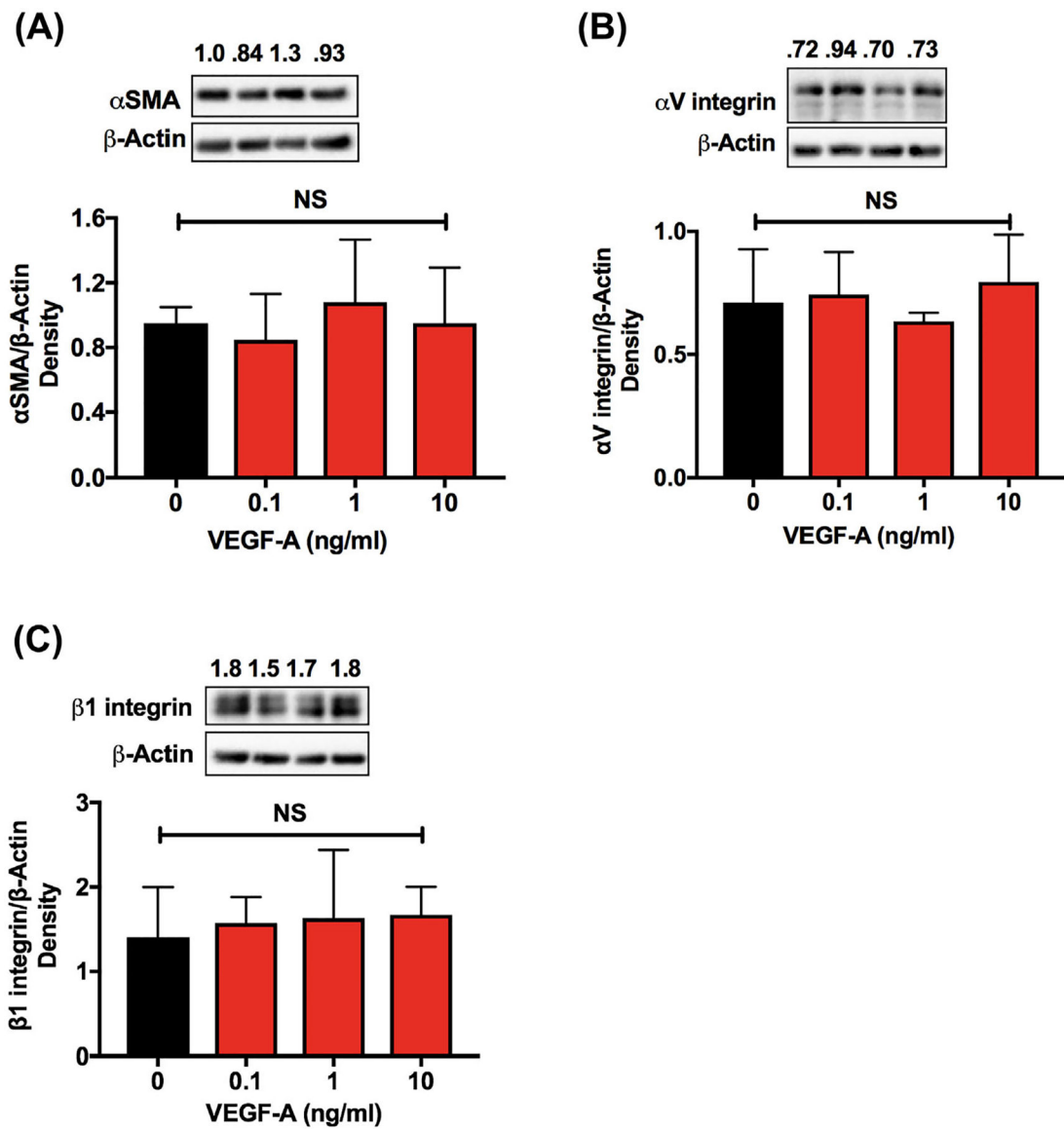
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**FIGURE 1.**

TGF-β2 upregulates HIF-1α and VEGF-A during EMT in human lens epithelial cells. The cells were treated with human recombinant TGF-β2 (10 ng/mL) for 24–48 h. HIF-1α (A) was detected using an antibody against HIF-1α and Alexa Fluor 488-goat anti-mouse IgG (left four panels). Mean fluorescence intensity of HIF-1α was calculated using ImageJ software (B). In the negative controls, the HIF-1α antibody was omitted (right four panels). The images are shown at 20× magnification. Scale bar = 100 μm. HIF-1α mRNA was measured by qPCR (C). The VEGF-A levels in the conditioned media were measured by an ELISA (D). The levels of the EMT-associated proteins α-SMA, αV integrin, and β1 integrin were measured by Western blotting (E-G). The densitometric analyses from triplicate assays (means ± SD) are shown in the bar graphs. * $P < 0.05$, ** $P < 0.005$ and *** $P < 0.0005$

**FIGURE 2.**

Exogenous VEGF-A does not induce EMT in human lens epithelial cells. FHL124 cells were treated with human recombinant VEGF-A for 48 h. The VEGF-A-mediated induction of the α -SMA, α V integrin, and β 1 integrin proteins was measured by Western blotting (A-C). The densitometric analyses from triplicate assays (means \pm SD) are shown in the bar graphs. NS = not significant

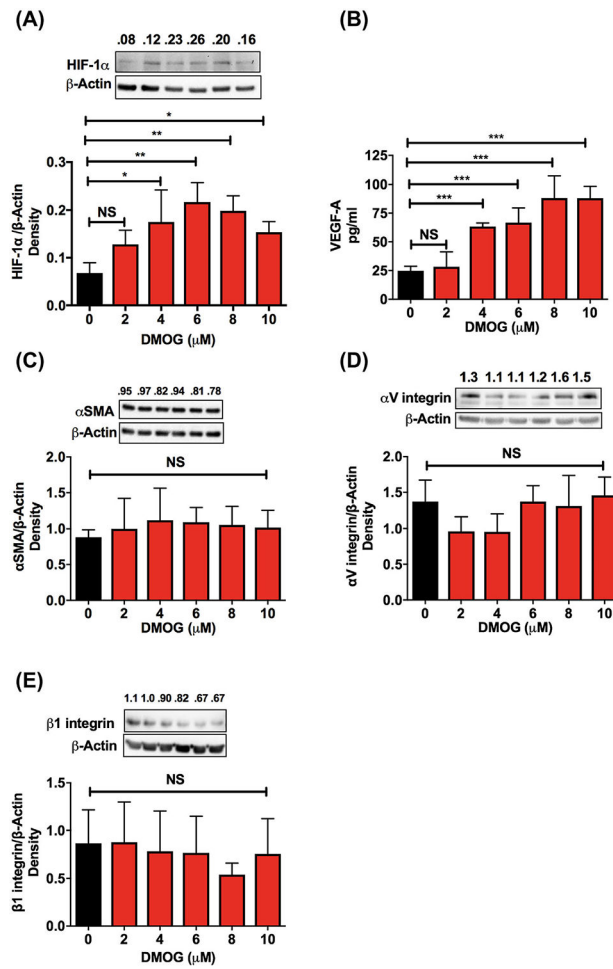
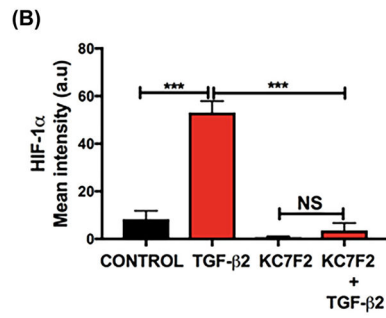
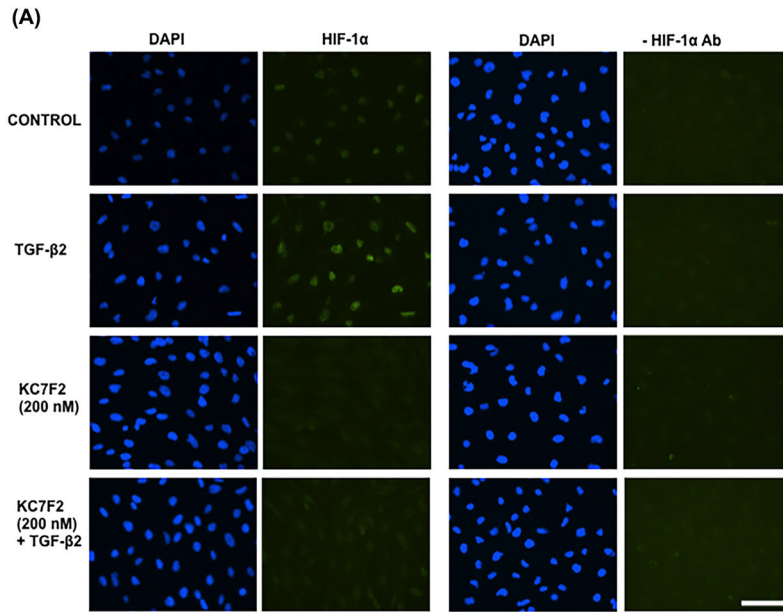


FIGURE 3.

The upregulation of HIF-1α by DMOG had no effect on the EMT-associated proteins in human lens epithelial cells. FHL124 cells were treated with DMOG (0–10 μM) β1 integrin were measured by Western blotting (C-E). The densitometric analyses from triplicate assays (means ± SD) are shown in the bar graphs. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$ and NS = not significant



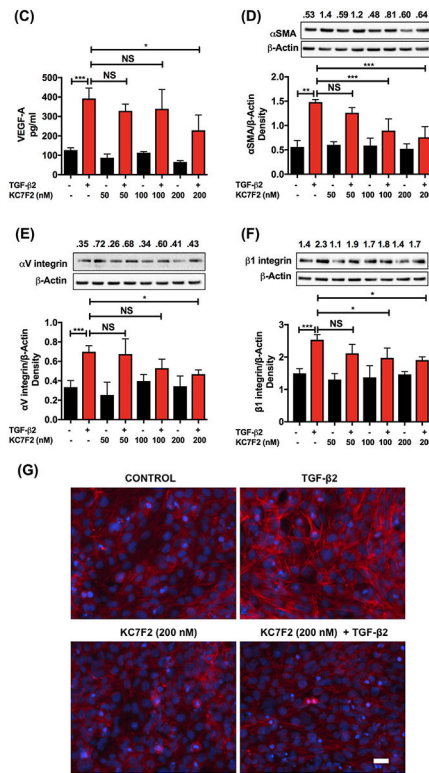
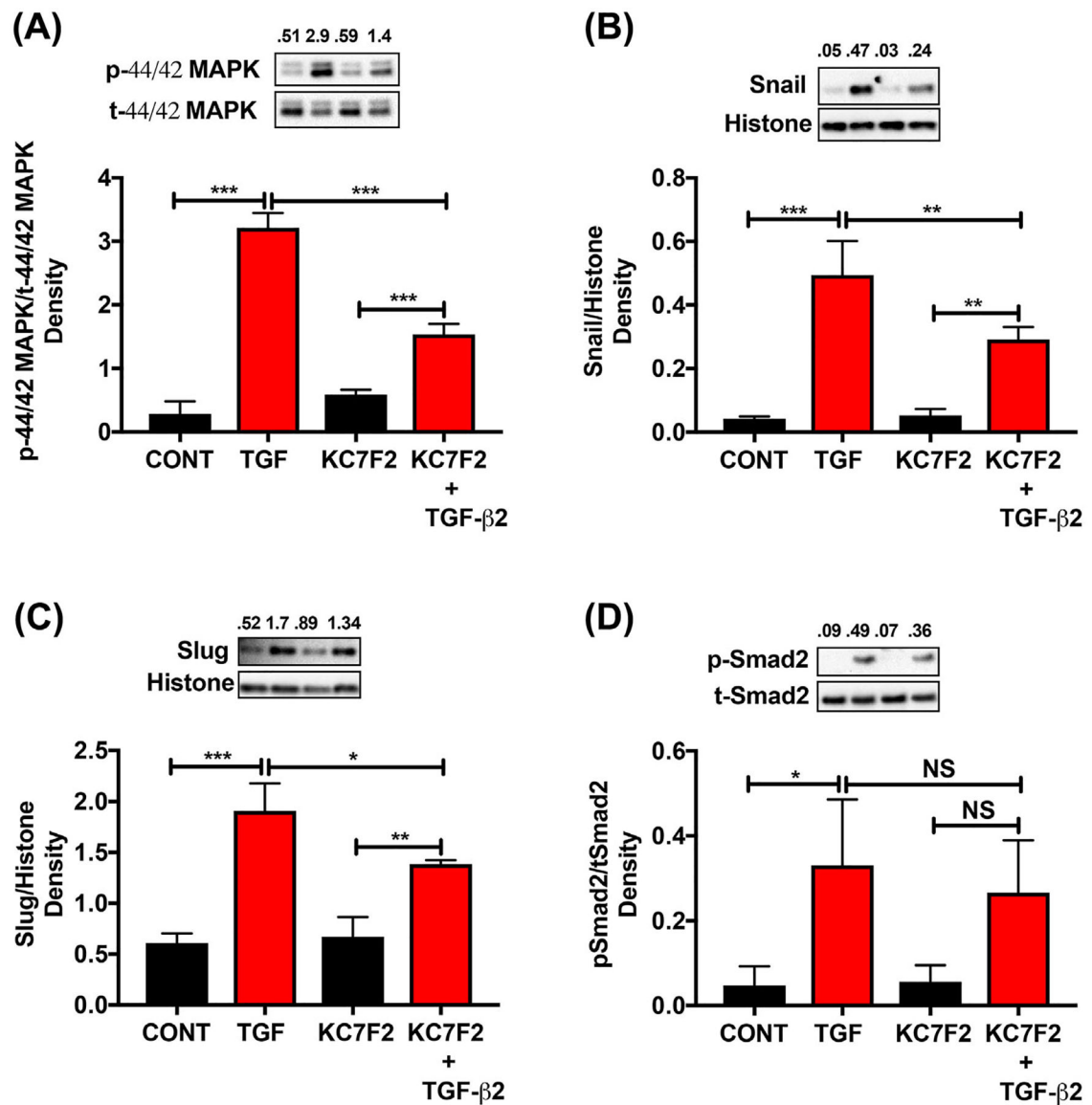
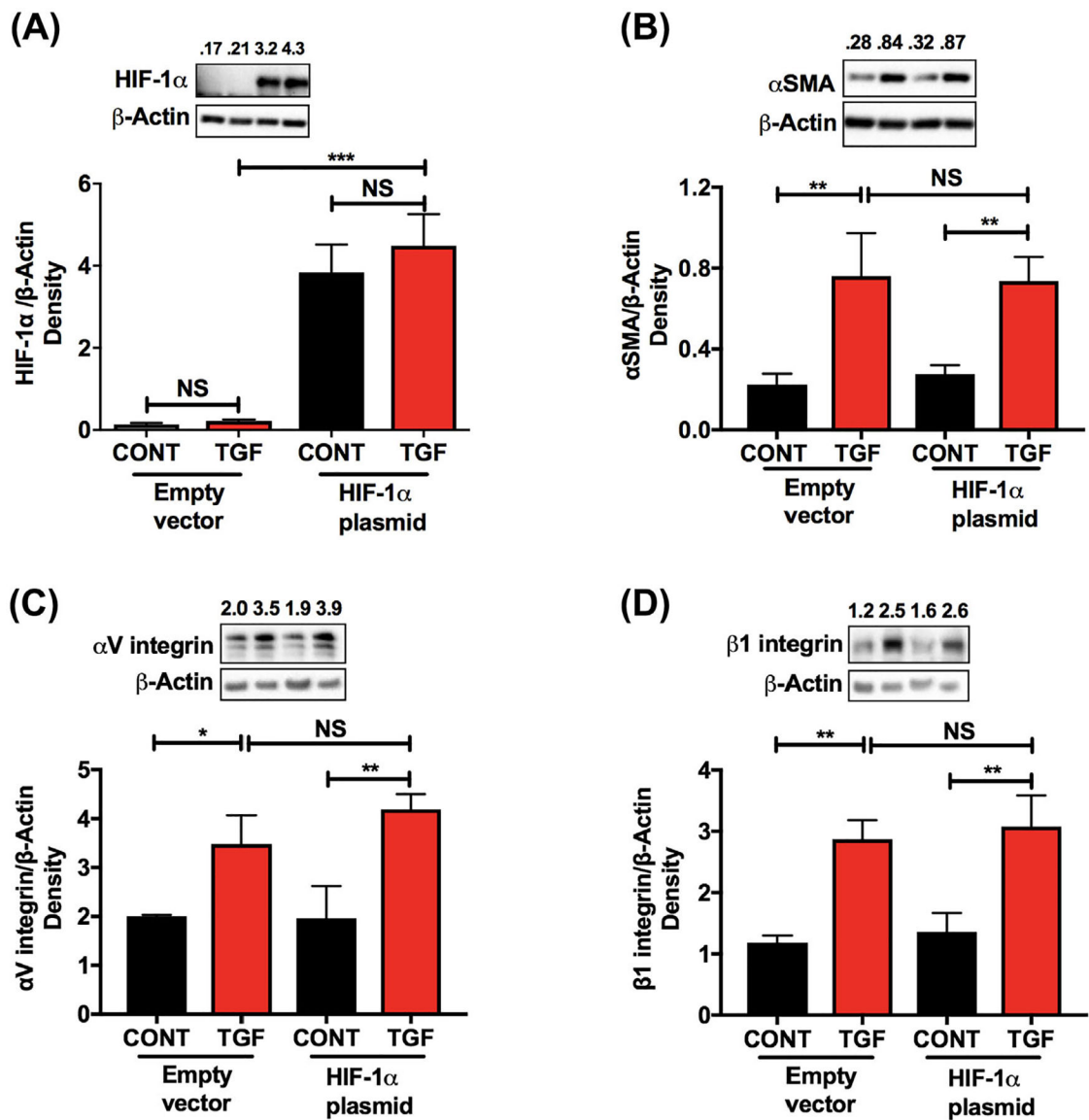


FIGURE 4.

Effect of a HIF-1α translational inhibitor on the TGF-β2-mediated EMT and cytoskeleton remodeling in human lens epithelial cells. FHL124 cells were treated with KC7F2 (0–200 nM) for 3.5 h and then with TGF-β2 (10 ng/mL) for 24–48 h. The HIF-1α levels (A) were measured by immunofluorescence (left panels) and mean fluorescence intensity of HIF-1α (B). The HIF-1α primary antibody was omitted in the negative controls (right panels). The VEGF-A levels were measured by an ELISA (C). The protein levels of α-SMA, αV integrin, and β1 integrin were measured by Western blotting (D-F). Cytoskeletal remodeling (F-actin) was assessed using Texas Red-conjugated phalloidin (G). All images are shown at 20 × magnification. Scale bar = 100 μm. The densitometric analyses from triplicate assays (means ± SD) are shown in the bar graphs. **P* < 0.05, ***P* < 0.005, ****P* < 0.0005 and NS = not significant

**FIGURE 5.**

Effect of a HIF-1 α translational inhibitor on TGF- β 2-mediated signaling in human lens epithelial cells. FHL124 cells were treated with KC7F2 and TGF- β 2 as described in Methods. The levels of p44/42 MAPK (A) in the cytosolic fraction and Snail (B), Slug (C), and pSmad2 (D) in the nuclear fraction were measured by Western blotting. The densitometric analyses from triplicate assays (means \pm SD) are shown in the bar graphs. * P < 0.05, ** P < 0.005, *** P < 0.0005 and NS = not significant. CONT = Control and TGF = TGF- β 2 treated

**FIGURE 6.**

Overexpression of HIF-1 α had no effect on the TGF- β 2-mediated EMT in human lens epithelial cells. FHL124 cells were transfected with either the empty vector or the HIF-1 α plasmid and treated with or without TGF- β 2 (10 ng/mL) for 4–20 h as described in the methods. The protein content of HIF-1 α was measured by Western blotting (A). The levels of α -SMA, α V integrin, and β 1 integrin were measured by Western blotting (B–D). The densitometric analyses from triplicate assays (means \pm SD) are shown in the bar graphs. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$ and NS = not significant. CONT = Control and TGF = TGF- β 2 treated

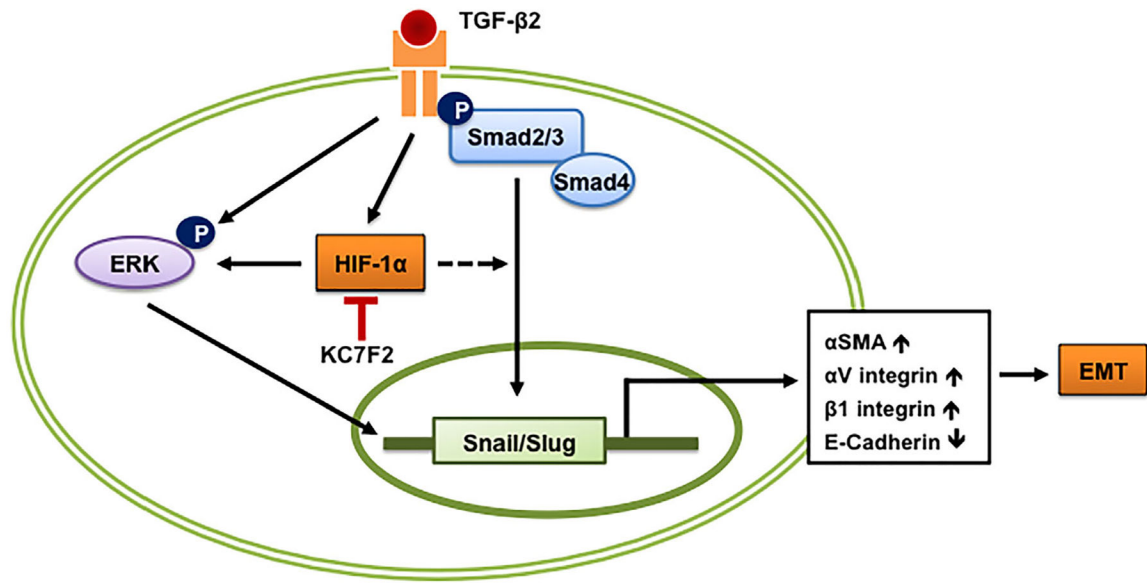


FIGURE 7.
Proposed scheme on the role of HIF-1α in TGF-β2-induced EMT of human lens epithelial cells