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RESEARCH ARTICLE

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Promotion effects of mono-2-ethyhexyl phthalate (MEHP) on migration and invasion of human melanoma cells via activation of TGF-β signals

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Malignant melanoma is one of the most leading forms of skin cancer associated with a low patient survival rate. There is an urgent need to illustrate risk factors that can trigger the motility of melanoma cancer cells. Our present study revealed that mono-(2-ethylhexyl)phthalate (MEHP) exposure can significantly increase the in vitro migration and invasion of WM983A and A375 cells. Among the tested cytokines, MEHP can increase the expression of transforming growth factor β (TGF-β). Inhibition of TGF-β via its neutralization antibody can attenuate MEHP-induced cell migration and invasion. Further, upregulation of TGF-β mediated MEHP-induced activation of Smad signals and upregulation of Snail in melanoma cells. Blocking the TGF-β/Smad signal pathway can attenuate MEHP-induced cell migration. Estrogen receptor α (ERα) was essential for MEHP-induced expression of TGF-β. In addition, MEHP can increase the expression of ERα in melanoma cells. Collectively, our study found that MEHP can stimulate the progression of melanoma via TGF-β signals.

Significance: Mono-(2-ethylhexyl)phthalate (MEHP) is the active and most toxic metabolite of di(2-ethylhexyl)phthalate (DEHP). Our present study revealed that MEHP can trigger the in vitro migration and invasion of melanoma cells via upregulation of TGF-β/Snail signals. It revealed that daily exposure to MEHP might be a risk factor for melanoma patients.

KEYWORDS

MEHP, melanoma, migration, invasion, TGF-β

1 | INTRODUCTION

As the most aggressive skin cancer, melanoma can metastasize through the lymphatic and blood vessels.[1] Melanoma is difficulty to cure due to the high potential of malignancy and is responsible for 80% of skin cancer deaths.[2] The high capability of cell invasion is associated with an epithelial to mesenchymal transition (EMT)–like phenotype, which is characterized by the variation of cell-cell adhesion molecules of the cadherins family.[3] During the last half century, the incidence of melanoma increased dramatically.[4] Targeted treatment can achieve an impressive improvement of survival of melanoma patients.[5] However, the disease progresses toward metastasis limited the range of therapeutic options.[6] Therefore, the illustration of risk factors promoting the metastasis of melanoma cells is important for prevention and treatment of melanoma.

Increasing evidences suggested that sex hormones have direct correlation with the progression of melanoma.[7] Epidemiological data suggested that the incidence of melanoma was nearly twofold higher in males than in females.[8] In females, the incidence rate of women aged 20 to 45 was higher than that of the age greater 45 years.[8] Further, the melanoma neoplasms are thicker during pregnancy than those diagnosed outside of pregnancy.[9] In a melanoma fish model (*Xiphophorus couchianus*) exposure to irradiation, a twofold lower incidence of melanoma in females was observed than that in males.[10] Diethylstilbestrol

treatment can enhance melanomagenesis in mouse B16 melanoma cells.[11] All these data suggested that melanoma is a hormone-related cancer, and its progression can be regulated by estrogenic signals.

Various cytokines such as transforming growth factor β (TGF-β) can govern morphogenesis and the progression of melanoma.[12] It has been revealed that TGF-β promotes amoeboid features of melanoma such as cell rounding, membrane blebbing, high levels of contractility, and increased invasion.[13] In addition, TGF-β can promote melanoma cell pseudo-EMT through upregulation of matrix metalloproteinase MMP9 and downregulation of the epithelial marker E-cadherin.[14] Overexpression of SMAD7, an inhibitory SMAD that interferes with TGF-β receptor function, can reduce the metastasis of melanoma cells.[15] These data suggested that cytokines such as TGF-β may critical for the development and progression of melanoma.

Recently, more and more studies suggested that environmental endocrine disrupting chemicals (EDCs), which have similar structure of estrogen, can trigger the progression of hormone-related or not related cancers.[16,17] Di(2-ethylhexyl)phthalate (DEHP) has been widely used in polyvinyl chloride medical materials.[18] Its active and most toxic metabolite, mono-(2-ethylhexyl)phthalate (MEHP), has been shown to promote the progression of various cancers such as cervical cancer[19] and Wilm's cancer.[20] DEHP can trigger the growth of melanoma via differential effect on M1-and M2-polarized macrophages in mouse model.[21] However, the potential effect of MEHP on the progression of melanoma has not been investigated.

Our present study found that MEHP treatment can increase the migration and invasion of melanoma cells via upregulation of TGF-β and induction of EMT phenotype. Inhibition of TGF-β/Smad signal pathway can attenuate MEHP-induced cell migration. Further, estrogen receptor α (ERα) was involved in MEHP-induced progression of melanoma cells.

2 | MATERIALS AND METHODS

2.1 | Reagents and materials

All chemicals including MEHP were obtained from Sigma Chemical Co (St Louis, Missouri). The inhibitors including SB431542, ICI 182780, and G15 were purchased from Selleck Chemicals (Houston, Texas). All compounds were solubilized in dimethyl sulfoxide (DMSO) with the final DMSO concentrations less than 0.5%. The neutralization antibody against TGF-β was purchased from R&D Systems. Fetal bovine serum (FBS) and medium were bought from Gibco (Grand Island, New York).

2.2 | Cell culture and treatment

Human melanoma cancer cell lines WM983A and A375 were cultivated in HEPES-buffered, 2mM L-glutamin containing RPMI1640-medium (Gibco/Life Technologies, Darmstadt, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units of penicillin, and 100 mg/mL of streptomycin (#P433, Sigma), at 37°C with 5% CO_2. The MEHP work solutions (100μM) were made immediately prior to the treatment. Control cells were treated with 0.5% DMSO only.

2.3 | Assessment of cell proliferation

The effects of MEHP on the proliferation of melanoma cells were tested by use of the cell counting kit-8 (CCK-8). Cells were plated into 96-well plates for 12 hours prior exposed to various concentrations of MEHP for another 48 hours. At the end of the experiments, CCK solution (10 μL per well) was added into the 96-well plates, incubated for 4 hours at 37°C, and measured by a microplate reader at 490 nm. Each measurement was carried out in triplicate.

2.4 | In vitro migration and invasion assay

Cell migration effect was evaluated by use of wound-healing test according to the previous study.[22] Cells were cultured in 6-cm culture and grown to almost confluent cell monolayer. After scratched with a pipette tip to generate the cell-free zone, cells were washed three times, cultured with medium containing MEHP, and counted using a light microscope with 200× magnification. The relative cell migration was calculated with the formula of the previous study.[22]

Effects of MEHP on invasion of melanoma cells were tested by 100 μg/cm^2 of Matrigel (Becton Dickinson, Bedford, Massachusetts) coated cell culture inserts (Corning Costar Corporation, Cambridge, MA) with 8-μm pores. Cells (6×10^4) in the upper compartment in 100 μL of DMEM serum-free were treated with or without MEHP, and then 600-μL full medium was placed into the lower compartment of the chamber. After incubation for 48 hours, invaded cells were fixed and stained with crystal violet solution and counted using light microscopy (at 200× magnification).

2.5 | Western blot analysis

Cells were cultured with or without MEHP prior lysed using 50-μL RIPA lysis buffer (Thermo Scientific, Rockford, Illinois) containing protease inhibitors (Roche Applied Science, Penzberg, Germany). The protein concentration was tested by the Lowry protein assay using DC protein assay kit (Bio-Rad, Hercules, California). Then 30-μg total protein was separated by 4% to 12% bis-Tris gradient gel (Novex, Invitrogen) and transferred to polyvinylidene fluoride membrane (Invitrogen) with NuPage transfer buffer (Invitrogen). After incubated with primary antibody and horseradish peroxidase-conjugated secondary antibodies, membrane was visualized by use of ECL substrate (Amersham Bioscience, Piscataway, New Jersey) with a Kodak Gel Logic 100 imaging system (Kodak, Rochester, New York). GAPDH was used as the loading control for normalization. The antibodies used in the present study were E-Cad (Bioworld, 3195S, 1:500), p-Smad2 (Santa Cruz, sc-11769, 1:500), Smad2 (Proteintech, 10231-1-AP, 1:500), H2A.X (Proteintech, 10856-1-AP, 1:500), Snail (Bioworld, BS1853, 1:500), Zeb1(Abcam, 124512, 1:1000), Twist (Abcam, 175430, 1:1000), ERα (Santa Cruz, sc-8002, 1:500), and GAPDH (BOSTER, BM3876, 1:1000).

2.6 | Quantitative real-time PCR (qRT-PCR)

Total RNAs were harvested with the RNeasy Mini Kit (Qiagen, Valencia, California). Genomic DNA was digested using RNase-free DNase (Qiagen, Valencia, California). Then the cDNA was synthesized using Superscript III (Invitrogen, Carlsbad, California), 500 ng of total RNA, and 1 μL of random primer. The qRT-PCR was performed to assess the expression of targeted genes by use of iQ SYBR Green Supermix (BioRad) and a Bio-Rad CFX Connect Real-Time PCR Detection System. The primers were TGF-β, 5′-GGC CAG ATC CTG TCC AAG C-3′ (forward) and 5′-GTG GGT TTC CAC CAT TAG CAC-3′ (reverse); interleukin-8 (IL-8), 5′-TTT TGC CAA GGA GTG CTA AAG A-3′ (forward) and 5′-AAC CCT CTG CAC CCA GTT TTC-3′ (reverse); IL-32, 5′-TGG CGG CTT ATT ATG AGG AGC-3′ (forward) and 5′-CTC GGC ACC GTA ATC CAT CT-3′ (reverse); estrogen receptor (ERα), 5′-CCC ACT CAA CAG CGT GTC TC-3′ (forward) and 5′-CGT CGA TTA TCT GAA TTT GGC CT-3′ (reverse); GAPDH, 5′-GAC TCA TGA CCA CAG TCC ATG C-3′ (forward) and 5′-AGA GGC AGG GAT GAT GTT CTG-3′ (reverse). The data were analysed by use of the comparative cycle threshold method ($2^{-\Delta\Delta CT}$). GPADH was used as the loading control for normalization. Experiments were run in triplicate with consistency.

2.7 | Enzyme-linked immunosorbant assay (ELISA)

The expression of TGF-β was measured by use of ELISA kit purchased from R&D Systems according to the manufacturer's protocol. Assays were run in triplicate with consistency.

2.8 | Statistical analysis

All data were expressed as means ± standard deviation (SD) of at least three independent experiments. The Student *t* test was used to analyse differences between two treatment groups by use of SPSS 13.0 software (SPSS, Inc, Chicago, Illinois). $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | MEHP triggers cell motility of melanoma cells

It has been revealed that MEHP less than 25μM had no significant effect on the cell proliferation.[23] We further checked the potential effects of nanomolar concentrations of MEHP on proliferation and

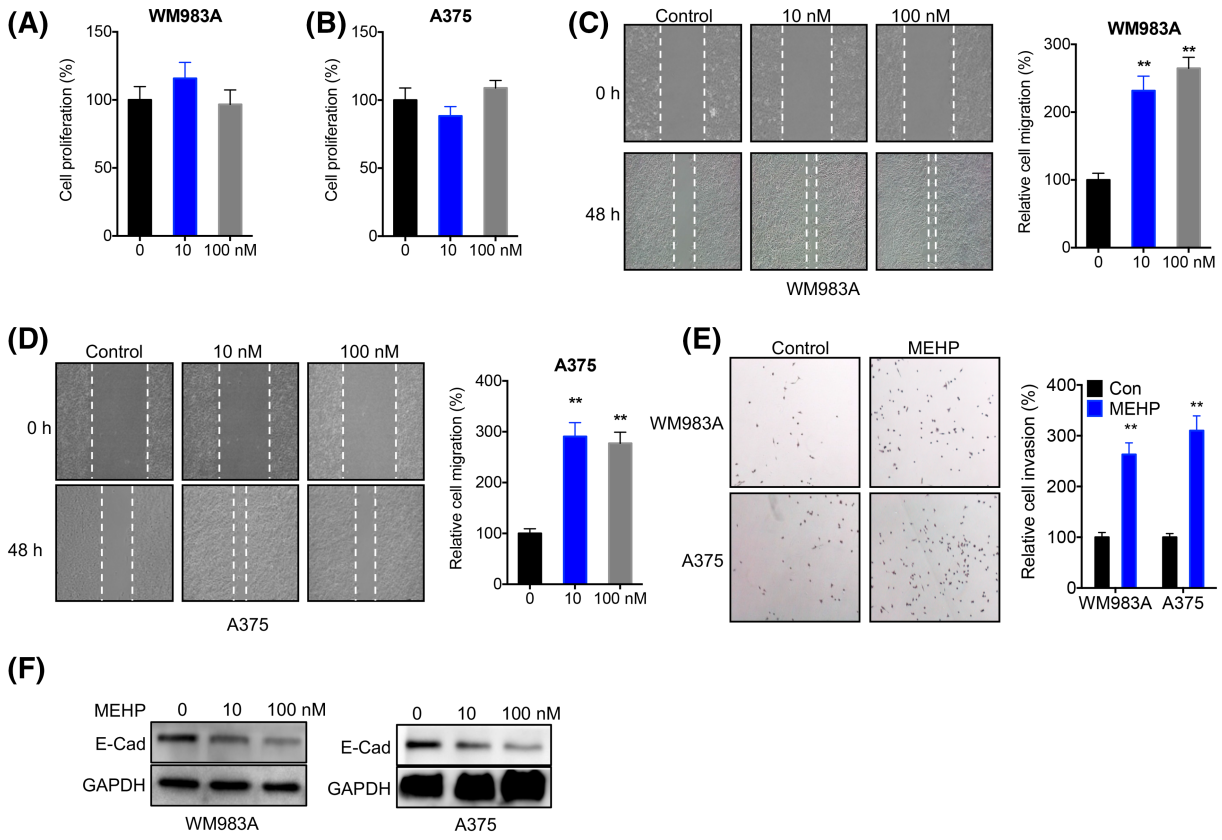

FIGURE 1 MEHP triggers the cell motility of melanoma cells. (A) WM983A or (B) A375 cells were exposed to increasing concentrations of MEHP for 48 hours, and cell viability was measured by use of CCK-8 kit; (C) WM983A or (D) A375 cells were exposed to increasing concentrations of MEHP for 48 hours, and cell migration was tested by wound healing assay; (E) Cells were treated with or without 10nM MEHP for 48 hours, and cell invasion was tested by Transwell analysis; (F) cells were treated with MEHP for 48 hours, and the expression of E-Cad was tested by western blot analysis. Data are presented as means ± SD of three independent experiments. $^{**}P < .01$ compared with control

migration of melanoma cells. Our data showed that both 10nM and 100nM MEHP had no significant effect on proliferation of WM983A or A375 cells (Figure 1A,B). However, both 10nM and 100nM MEHP can significantly promote the in vitro wound healing of both WM983A (Figure 1C) and A375 (Figure 1D) cells. Consistently, Transwell assay indicated that 10nM MEHP treatment can increase the invasion of both WM983A and A375 cells (Figure 1E). In addition, MEHP treatment can decrease the expression of E-Cad in WM983A and A375 cells (Figure 1F). All these data suggested that MEHP can trigger the migration and invasion of melanoma cells.

3.2 | MEHP increases the expression of TGF-β in melanoma cells

Previous studies indicated that various cytokines such as TGF-β,[13] IL-8,[24] and IL-32[25] can increase the cell motility of melanoma. We found that MEHP treatment significantly increased the mRNA expression of TGF-β in both WM983A (Figure 2A) and A375 (Figure 2B) cells, while had no effect on the expression of IL-8 or IL-32. Further, ELISA confirmed that MEHP increased the expression of TGF-β in both WM983A (Figure 2C) and A375 (Figure 2D) cells. Further, MEHP can increase the expression of TGF-β in WM983A cells via both concentration-dependent (Figure 2E) and time-dependent (Figure 2F) manners.

3.3 | Upregulation of TGF-β is essential for MEHP-induced migration of melanoma cells

TGF-β has been widely used to induce EMT of cancer cells.[26] In addition, the high capability of melanoma cell invasion is associated with EMT phenotype.[27] We then investigate whether TGF-β was involved in MEHP-induced migration and migration of melanoma cells. Our data showed that the neutralization antibody of TGF-β (anti-TGF-β) can increase inhibit the migration of WM983A cells and attenuate MEHP-induced cell migration (Figure 3A). Further, anti-TGF-β also blocked MEHP-induced invasion of WM983A cells (Figure 3B) and downregulation of E-Cad (Figure 3C). These results suggested that the upregulation of TGF-β was essential for MEHP-increased cell motility of melanoma cells.

3.4 | MEHP activates TGF-β/SMAD signals to trigger cell motility

TGF-β can activate Smad pathway in various cancer cells to trigger cell motility.[28] Considering that MEHP can increase the expression of TGF-β in melanoma cells, we further investigated whether Smad2 was involved in MEHP-induced cell migration. Our data showed that MEHP treatment can increase the phosphorylation of both Smad2 in both WM983A (Figure 4A) and A375 (Figure 4B) cells. However, the

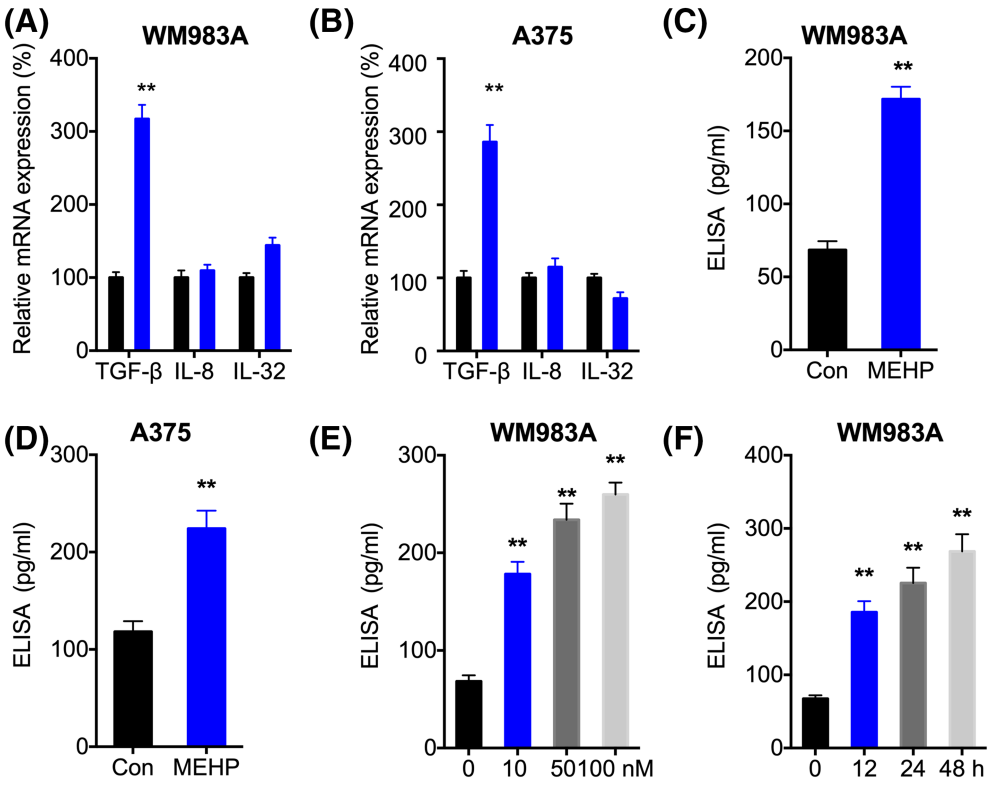

FIGURE 2 MEHP increases the expression of TGF-β in melanoma cells. (A) WM983A or (B) A375 cells were exposed to 10nM MEHP for 48 hours, and the expression of cytokines was measured by qRT-PCR; (C) WM983A or (D) A375 cells were exposed to 10nM MEHP for 48 hours, and the expression of TGF-β was checked by ELISA; (E) WM983A cells were treated with increasing concentration of MEHP for 48 hours, and the expression of TGF-β was checked by ELISA; (F) WM983A cells were treated with 10nM MEHP for the increasing time periods, and the expression of TGF-β was checked by ELISA. Data are presented as means ± SD of three independent experiments. $^{**}P < .01$ compared with control

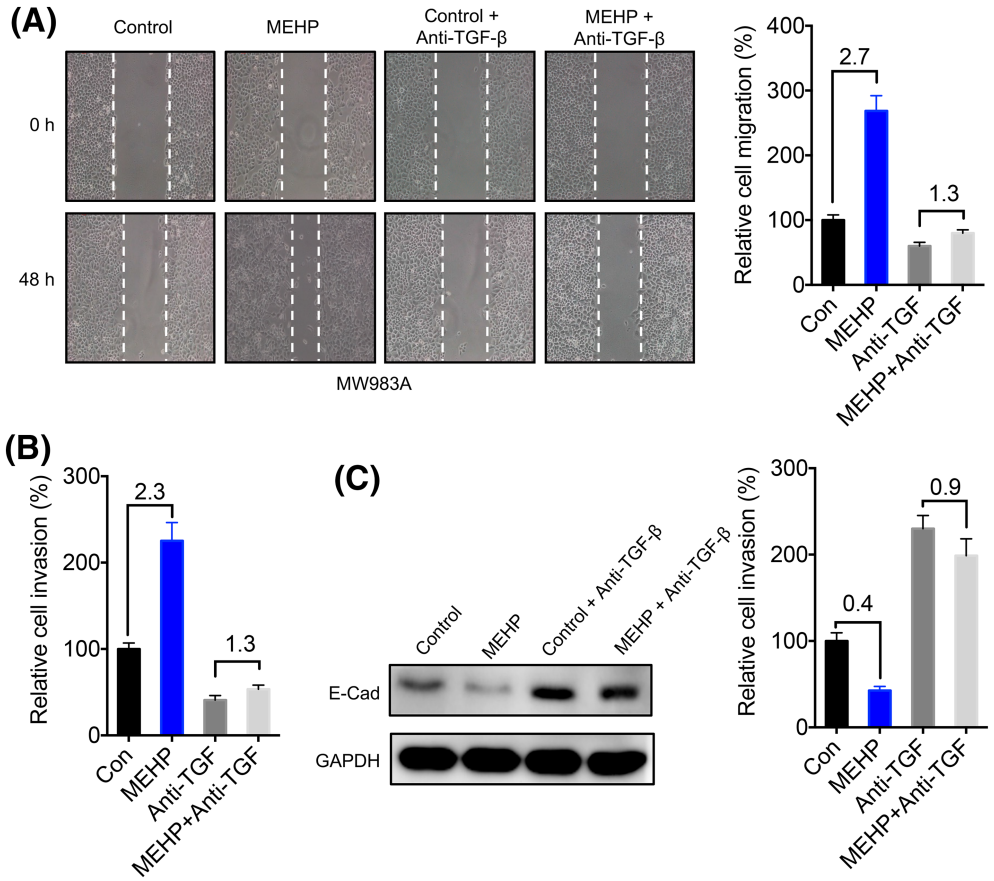

FIGURE 3 Upregulation of TGF-β is essential for MEHP-induced migration of melanoma cells. WM983A cells were pretreated with or without 100 ng/mL of anti-TGF-β for 90 minutes and then further treated with or without 10nM MEHP for 48 hours: (A) The cell migration was tested by wound healing assay; (B) the cell invasion was tested by Transwell; and (C) the expression of E-Cad was tested by western blot analysis. Data are presented as means ± SD of three independent experiments

anti-TGF-β can attenuate MEHP-induced phosphorylation of Smad2. Further, MEHP increased the nuclear localization of Smad2 in WM983A cells (Figure 4C). To test whether Samd2 signal was involved in MEHP-induced cell migration, we pretreated cells with SB431542, the inhibitor of Smad2/3 signal pathway. Our data showed that SB431542 attenuated MEHP decreased expression of E-Cad (Figure 4D) and invasion of WM983A cells (Figure 4E). These results indicated that MEHP activates TGF-β/SMAD signals to trigger cell motility.

3.5 | MEHP increases the expression of Snail in melanoma cells via TGF-β/SMAD signals

The transcription factors (TFs) such as Zeb1, Snail, and Twist can regulate the EMT of cancer cells and promote the progression of melanoma.[29,30] We then investigated the potential effect of MEHP on the expression of EMT-TFs. The data showed that MEHP treatment increased the expression of Snail in WM983A cells (Figure 5A). Consistently, MEHP increased the expression of Snail in A375 cells (Figure 5 B). Both anti-TGF-β (Figure 5C) and SB431542 (Figure 5D) can attenuate MEHP-induced expression of Snail in WM983 cells. It indicated that MEHP increases the expression of Snail in melanoma cells via TGF-β/SMAD signals.

3.6 | MEHP increases the expression of TGF-β via ERα

It has been reported that both ERα and G-protein coupled estrogen receptor (GPER) are involved in the EDCs-induced biological functions.[31] We found that the inhibitor of ERα (ICI 182 780), but not GPER (G15), can abolish MEHP-induced upregulation of TGF-β in both WM983A (Figure 6A) and A375 (Figure 6B) cells. Further, MEHP treatment can increase the nuclear translocation of ERα in WM983A cells (Figure 6C). In addition, MEHP can increase the mRNA (Figure 6D) and protein (Figure 6E) expression of ERα in WM983A cells. These data suggested that MEHP increases the expression of TGF-β via ERα.

4 | DISCUSSION

Although various studies indicated that estrogenic signals are critical for the progression of melanoma, only few investigations concerning the potential risks of EDCs on metastasis of melanoma cells. One

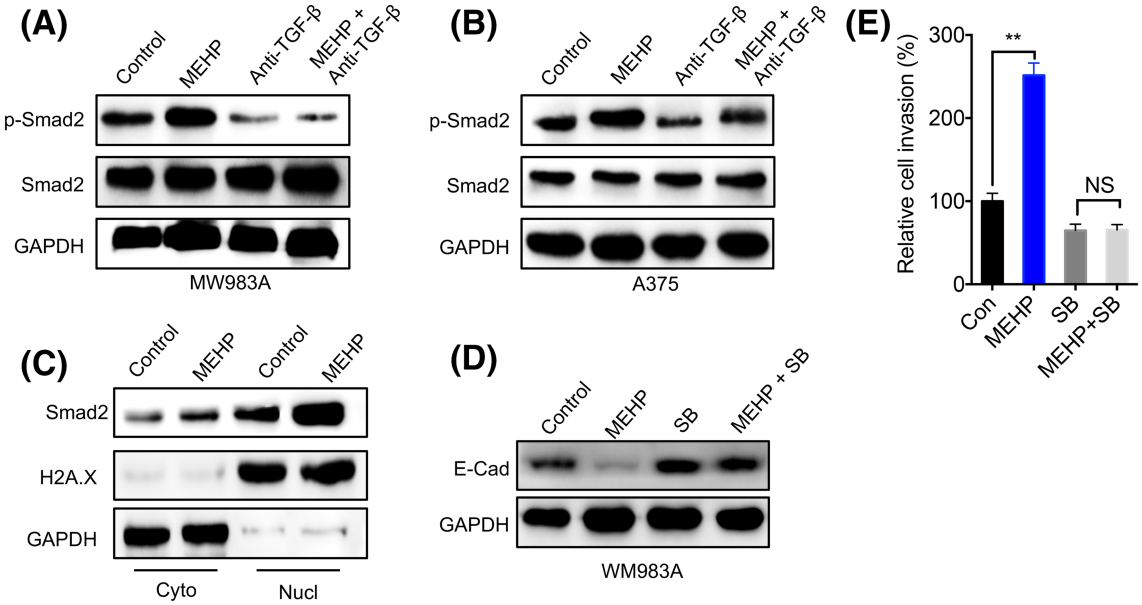

FIGURE 4 MEHP activates TGF-β/SMAD signals to trigger cell motility. (A) WM983A or (B) A375 cells were pretreated with or without 100 ng/mL of anti-TGF-β for 90 minutes and then further treated with or without 10nM MEHP for 1 hour, the expression of p-Smad2 and Smad2 was checked; (C) WM983A cells were pretreated with or without 100 ng/mL anti-TGF-β for 90 min and then further treated with or without 10nM MEHP for 12 hours, the subcellular localization of Smad2 was checked; WM983A cells were pretreated with or without 10μM SB431542 (SB) and then further treated with or without 10nM MEHP for 48 hours, (D) the expression of E-Cad was measured by western blot analysis, and (E) the cell invasion was checked by Transwell analysis. Data are presented as means ± SD of three independent experiments. $^{**}P < .01$ compared with control. NS, no significant

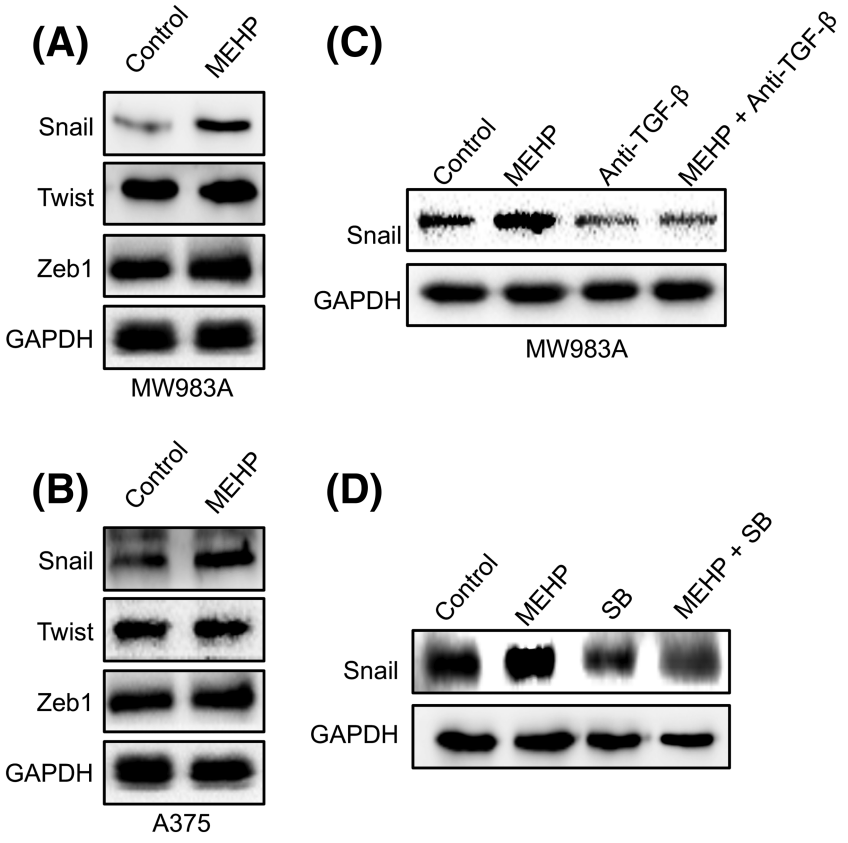

FIGURE 5 MEHP increases the expression of Snail in melanoma cells via TGF-β/SMAD signals. (A) WM983A or (B) A375 cells were treated with or without 10nM MEHP for 24 hours, the expression of EMT-TFs was checked; (C) WM983A cells were pretreated with or without 100 ng/mL of anti-TGF-β for 90 min and then further treated with or without 10nM MEHP for 24 hours, the expression of Snail was checked; (D) WM983A cells were pretreated with or without 10μM SB431542 (SB) and then further treated with or without 10nM MEHP for 24 hours, the expression of Snail was checked

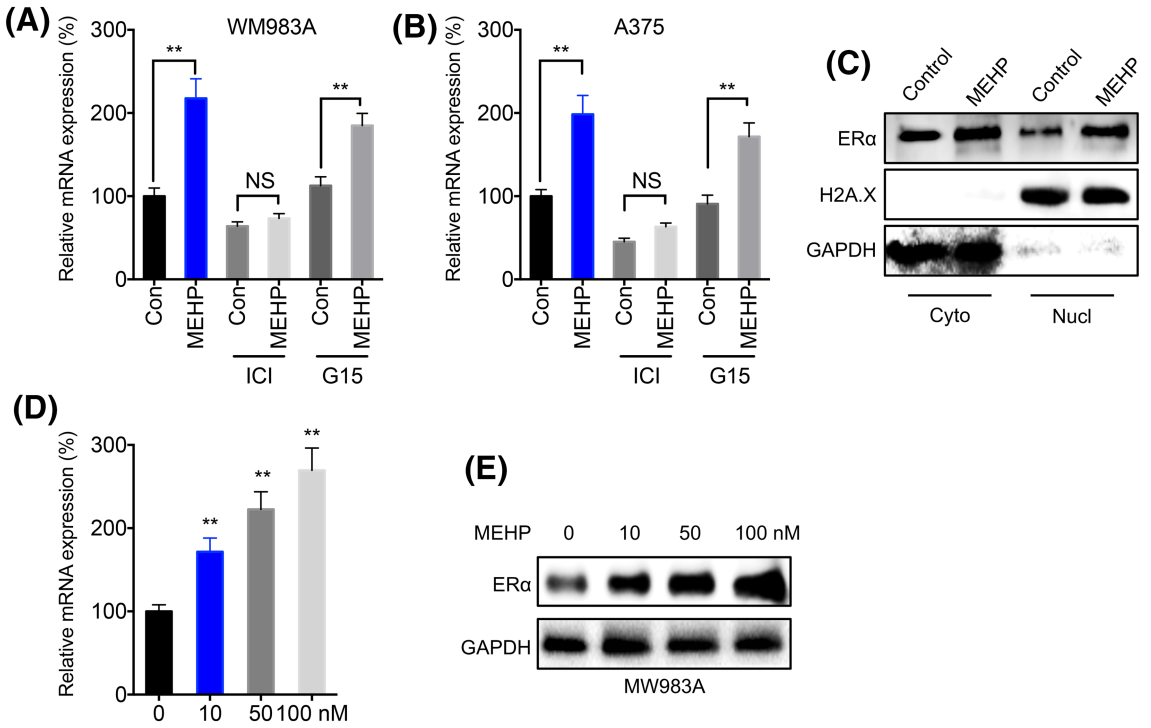

FIGURE 6 MEHP increases the expression of TGF-β via ERα. (A) WM983A or (B) A375 cells were pretreated with 10μM ICI182780 (inhibitor of ERα) and G15 (GPER inhibitor) for 90 minutes and then further treated with or without 10nM MEHP for 24 hours, the mRNA expression of TGF-β was measured by qRT-PCR; MW983A cells were treated with increasing concentrations of MEHP for 24 hours, the (C) mRNA and (D) protein expression of ERα was measured. Data are presented as means ± SD of three independent experiments. $^{**}P < .01$ compared with control. NS, no significant

recent study investigated the potential association between uveal melanoma and EDCs including bisphenol A (BPA), chlorophenol, polyaromatic hydrocarbons (PAH), and heavy metals. The results showed that occupational exposure to EDC was not associated with an increased risk for uveal melanoma.[32] Our present study found that nanomolar concentrations of MEHP can increase the migration and invasion of melanoma cells. Consistently, MEHP can increase the migration and invasion of various cancer cells including Wilms' tumour[20] and testicular embryonal carcinoma.[22] Melanoma is a highly aggressive tumour and has very poor prognosis due to the metastasis.[33] Due to the occurrence of the invasion and the metastasis is the key factor for melanoma to be life-threatening,[34] the in vivo effect of MEHP on the metastasis of melanoma should be further investigated.

Our data showed that the activation of TGF-β/Smad signal pathway is essential for MEHP-induced migration and invasion of melanoma cells. MEHP can increase the expression of TGF-β in melanoma cells. TGF-β–regulated signal is crucial for EMT and metastatic dissemination of melanoma cells.[35] In addition, TGF-β is a potent regulator of the contractile actomyosin cytoskeleton in melanoma.[13] The essential role of TGF-β in MEHP-induced cell migration was confirmed in the present study that anti-TGF-β can abolish MEHP-induced cell migration and the regulation of E-Cad and Snail. Consistently, TGF-β is essential for BPA-, octylphenol-, and nonylphenol-induced proliferation of uterine leiomyoma cells.[36] In addition, TGF-β signals were also involved in nonylphenol- and BPA-induced cell growth of ovarian cancer cells,[37] while fluorene-9-bisphenol can repress TGF-β signalling pathway to inhibit EMT of endometrial cancer cells. MEHP can activate the phosphorylation of Smad via TGF-β; further, the inhibitor of Smad receptor can attenuate MEHP-induced cell migration. Smad proteins are critical downstream mediators of TGF-β and important for melanoma development.[38] Further, SMAD cascade is activated via an autocrine fashion in human melanoma cell lines.[39] It has been reported that Smad protein can crosstalk with other signalling pathways such as Wnt, Notch, Hippo, Hedgehog (Hh), and mitogen-activated protein kinase (MAPK).[38] Whether MEHP can regulate the activation of these signal pathways via Smad needs further investigations.

ERα, while not GPER, was involved in MEHP-induced expression of TGF-β in melanoma cells. The observations of influence of estrogen on melanoma cells are controversial.[40] There is a significant difference between the expression of ER in male versus female origin melanomas.[41] Markedly decreased expression of ERβ has been observed in the metastatic phase of the melanoma.[42] Activation of GPER can inhibit progression of melanoma and improve response to immunotherapy.[43] In the present study, we found that only inhibitor of ERα can abolish MEHP-induced expression of TGF-β. Interestingly, MEHP can also increase the expression of ERα in melanoma cells, while the mechanisms need more studies.

Collectively, our present study found that MEHP can increase the migration and invasion of melanoma cells via upregulation of TGF-β and then activation of Smad signals. ERα is essential for MEHP-

induced expression of TGF-β. Although further studies concerning the in vivo effects of MEHP on metastasis are needed, our results suggested that exposure to EDCs such as MEHP might be potential risk factors for the development of melanoma.

AVAILABILITY OF DATA AND MATERIAL

All data and materials are available.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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