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HPV16 E5 expression induces switching from FGFR2b to FGFR2c and epithelial-mesenchymal transition

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The E5 oncoprotein of the human papillomavirus type 16 (HPV16 E5) deregulates epithelial homeostasis through the modulation of receptor tyrosine kinases and their signaling. Accordingly, the fibroblast growth factor receptor 2b (FGFR2b/KGFR), epithelial splicing transcript variant of the FGFR2, is down-modulated by the viral protein expression, leading to impairment of keratinocyte differentiation. Here, we report that, in cell models of transfected human keratinocytes as well as in cervical epithelial cells containing episomal HPV16, the down-regulation of FGFR2b induced by 16E5 is associated with the aberrant expression of the mesenchymal FGFR2c isoform as a consequence of splicing switch: in fact, quantitative RT-PCR analysis showed that this molecular event is transcriptionally regulated by the epithelial splicing regulatory proteins 1 and 2 (ESRP1 and ESRP2) and is able to produce effects synergistic with those caused by TGF β treatment. Immunofluorescence analysis revealed that this altered FGFR2 splicing leads to changes in the specificity for the ligands FGFs and in the cellular response, triggering epithelial-mesenchymal transition (EMT). Through 16E5 or FGFR2 silencing as well as inhibition of FGFR2 activity we demonstrated the direct role of the viral protein in the receptor isoform switching and EMT, suggesting that these early molecular events during HPV infection might represent additional mechanisms driving cervical transformation and tumor progression.

The fibroblast growth factor receptors (FGFRs) are receptor tyrosine kinases (RTKs) expressed on different tissues and involved in the control of cellular key processes such as growth, differentiation, migration and survival.^{1,2} The receptor extracellular domain is characterized by three immunoglobulin (Ig) loops (IgI-III) and the Ig like-domains II and III form the high affinity binding site for their ligands. Tissue specific alternative splicing of the IgIII domain in FGFR1–3 generates IIIb and IIIc isoforms and is responsible for ligand selection. While the FGFRIIIb isoforms are mostly expressed in epithelial tissues, the FGFRIIIc isoforms are generally present on mesenchymal cells: since also the ligand expression patterns are characterized by tissue specificity, the FGF signaling is tightly controlled in a paracrine and context-

dependent manner and is crucial for the epithelial-stromal interaction and homeostasis.

FGF/FGFR signaling may play oncogenic roles in tumorigenesis; however, through the triggering of cell differentiation, receptor activation might lead to opposite tumor suppressive outcome.^{3,4} In fact, deregulated and out-of-context oncogenic FGFR signaling is frequently associated with carcinogenesis and altered paracrine or autocrine loops of receptor activation are often established in tumor progression. Interestingly, the switching from the FGFRIIIb to the FGFRIIIc isoforms is involved in epithelial-mesenchymal transition (EMT) and cancer progression.^{5,6} However, reduced FGFR expression and loss-of function mutations are also frequently involved in the tumorigenic process. These opposite roles have been reported mostly in the case of FGFR2, whose alternative splicing generates the epithelial FGFR2IIIb isoform (FGFR2b, also named keratinocyte growth factor receptor KGFR), which appears to play an unusual and unique role acting as a tumor suppressor *in vitro* and *in vivo*.^{7,8} and the mesenchymal FGFR2IIIc (FGFR2c), which exerts oncogenic activity in different types of cancer.^{1,3,4}

The infection with high-risk genotypes of human papillomavirus (HPV), such as the HPV16 and HPV18 viruses, is known to be a major risk factor for cervical cancer and appears to be responsible for its progression.⁹ Among the different viral products, the E5 oncoprotein of the HPV16 is involved in cervical carcinogenesis through cooperation with the other two viral oncogenes E6 and E7 and transforms epithelial cells by deregulating cell growth, survival and differentiation through the modulation of RTKs and of their signalling.^{10,11} Consistent with this latter assumption, we have recently proposed the existence of a functional crosstalk

Key words: HPV16 E5, FGFR, epithelial-mesenchymal transition, human keratinocytes

*D.R. and F.B. contributed equally to this work

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What's new?

Of the fibroblast growth factor receptor (FGFR) pathways implicated in cancer, those involving the epithelial isoform FGFR2b, which is potentially tumor suppressive, and the mesenchymal isoform FGFR2c, which is potentially oncogenic, are of special interest. This study shows that a splicing switch from FGFR2b to FGFR2c occurs in the presence of the E5 oncoprotein of human papillomavirus (HPV) type 16, which downregulates FGFR2b. The switch leads to alterations in ligand specificity and cellular response, which appear to be associated with epithelial-mesenchymal transition. The findings suggest that this switching drives HPV-associated cervical transformation and tumor progression.

among the HPV16 E5 protein and the FGFR2b/KGFR: in fact, we have shown that 16E5 expression induces alteration of the signaling and endocytic traffic as well as down-modulation of the receptor^{12,13} and this molecular interplay might be crucial in perturbing the cell proliferation/differentiation and in driving tumorigenesis. Therefore, with the aim to further clarify the molecular events underlying this transforming interplay, we analyzed here if the FGFR2b/KGFR down-modulation induced by HPV16 E5 expression, observed in our previous studies, would be only one of the consequences of a more complex mechanism of FGF/FGFR2 signaling deregulation involving switching from FGFR2b to FGFR2c isoforms, changes in the specificity for the ligands FGFs and in the cellular response as well as triggering of EMT.

Material and Methods**Cells and treatments**

The human keratinocyte cell line HaCaT¹⁴ was cultured in Dulbecco's DMEM, supplemented with 10% fetal bovine serum plus antibiotics. HaCaT cells stably transfected with the construct pMSG 16E5 (HaCaT pMSG E5) or with the empty vector (HaCaT pMSG)¹⁵ were cultured as reported above and were treated with 1 μ M dexamethasone (Dex) for different times (6, 12 and 24 hr) to induce 16E5 expression. The human cervical keratinocyte cell line W12 initiated from a low-grade cervical lesion,¹⁶ which retains \sim 100 to 200 copies of the HPV16 episomes per cell,^{16–18} was cultured as previously described¹⁶ and used at the passage 6 (W12p6). Primary cultures of human keratinocytes and human fibroblasts derived from healthy skin (HKs and HF, respectively) were obtained from patients attending the Dermatology Unit of the Sant'Andrea Hospital of Rome; all patients were extensively informed and their consent for the investigation was given and collected in written form in accordance with guidelines approved by the management of the Sant'Andrea Hospital. Primary cells were isolated and cultured as previously described.^{19,20}

HaCaT cells were transiently transfected with pCI-neo expression vector containing 16E5-HA²¹ (HaCaT E5) or with the pCI-neo empty vector (HaCaT pCI-neo). Alternatively, HaCaT cells were cotransfected with the above pCI-neo vectors and with pMXs-IRES-blast2 Esrp1-FF retroviral expression construct (kindly provided by Dr. Russ Carstens, University of Pennsylvania School of Medicine, Philadelphia,

PA; HaCaT ESRP1) or with empty vectors (HaCaT EVs). W12p6 cells were transfected with pMXs-IRES-blast2 Esrp1-FF (W12p6 ESRP1) or with empty vector (W12p6 EV). For transfections, jetPEITM DNA Transfection Reagent (Polyplus-transfection, New York, NY) or Fugene HD (Promega, Madison, WI) were used according to manufacturer's instructions.

For RNA interference and *FGFR2* or *16E5* silencing, HaCaT cells were transfected with Bek small interfering RNA (FGFR2 siRNA) (Santa Cruz Biotechnology, Santa Cruz, CA) or with an unrelated siRNA as a control (control siRNA), while W12p6 cells were transfected with the E5 siRNA sequence (5'-TGGTATTACTATTGTGGATAA-3')²² or the control sequence (5'-AATTCTCCGAACGTGTCACGT-3') (Qiagen, Valencia, CA), using Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

For growth factors stimulation, cells were serum starved or incubated with 20 ng/ml KGF (Upstate Biotechnology, Lake Placid, NY) or with 20 ng/ml FGF2 (PeproTech, London, UK) for 24 hr at 37°C or with 10 ng/ml TGF- β 1 (PeproTech) for 48 hr at 37°C.

For inhibition of FGFR2b and FGFR2c-specific tyrosine kinase activity, cells were preincubated with the specific FGFR2 tyrosine kinase inhibitor SU5402 25 μ M (Calbiochem, Nottingham, UK) for 1 hr before treatments with growth factors.

Microinjection

Microinjection was performed with an Eppendorf microinjector (Eppendorf, Hamburg, Germany) and an inverted microscope (Zeiss, Oberkochen, Germany). A mixture of 100 nM Bek siRNA (Santa Cruz) 100 ng/ μ l pCI-neo 16E5-HA and 1 mg/ml rabbit IgG (Cappel Research Products, Durham, NC) in PBS were microinjected in the cytoplasm of HaCaT cells to simultaneously induce RNA interference and consequent FGFR2b/FGFR2c silencing and 16E5 overexpression. Unrelated siRNA was microinjected as negative control.

Immunofluorescence

HaCaT keratinocytes and W12p6 cells, grown on coverslips, were fixed with 4% paraformaldehyde in PBS for 30 min at 25°C followed by treatment with 0.1 M glycine for 20 min at 25°C and with 0.1% Triton X-100 for additional 5 min at 25°C to allow permeabilization. Cells were then incubated

for 1 hr at 25°C with the following primary mouse monoclonal antibodies: anti-HA (1:50 in PBS; Covance, Berkeley, CA), anti-E cadherin (1:50 in PBS; Dako, Carpinteria, CA), anti-vimentin (1:50 in PBS; Dako) or anti-β4 integrin (1:50 in PBS; Santa Cruz). The primary antibodies were visualized using goat anti-mouse IgG-FITC (1:50 in PBS; Cappel) for 30 min at 25°C. Goat anti-rabbit IgG-Texas Red (1:100 in PBS; Jackson ImmunoResearch Laboratories, West Grove, PA) was used to identify microinjected cells. Actin cytoskeleton was visualized using TRITC-phalloidin (1:100 in PBS; Sigma-Aldrich, Saint Louis, MO). Nuclei were stained with DAPI (1:1000 in PBS; Sigma). Coverslips were finally mounted with mowiol (Sigma) for observation. Fluorescence signals were analyzed by scanning cells in a series of sequential sections with an Apo-Tome System (Zeiss); image analysis was performed by the Axiovision software (Zeiss) and 3D reconstruction of a selection of three central optical sections was shown in each figure. Quantitative analysis of the percentage of cells expressing the different epithelial or mesenchymal markers was assessed counting for each sample a total of 100 cells, randomly observed in 10 microscopic fields from three different experiments. To discriminate between positive and negative cells, the fluorescence signal intensity was analyzed using the KS300 3.0 Image Processing System (Zeiss) and the cut-off value was selected for both unstimulated or growth factor-stimulated samples. Results have been expressed as mean values ± standard deviation (SD). *p* values were calculated using Student's *t* test and significance level has been defined as *p* < 0.05.

Western blot analysis

HaCaT cells were lysed in a buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 5 mM EGTA, supplemented with protease inhibitors (10 μg/ml aprotinin, 1 mM PMSF and 10 μg/ml leupeptin) and phosphatase inhibitors (1 mM sodium orthovanadate, 20 mM sodium pyrophosphate and 0.5 M NaF); 50 μg of total protein were resolved under reducing conditions by 12% SDS-PAGE and transferred to reinforced nitrocellulose (BA-S 83, Schleider and Schuell, Keene, NH). The membranes were blocked with 5% non fat dry milk in PBS 0.1% Tween 20 and incubated with anti-HA monoclonal antibody (Covance) or anti-Bek (C-17, Santa Cruz) polyclonal antibodies followed by enhanced chemiluminescence detection (ECL, Amersham, Alington Heights, IL). The membranes were rehydrated and probed again with anti-actin (Sigma) monoclonal antibody, to estimate the protein equal loading.

Primers

Oligonucleotide primers for target genes and for the housekeeping gene were chosen with the assistance of the Oligo 5.0 computer program (National Biosciences, Plymouth, MN) and purchased from Invitrogen. The following primers were used: for *FGFR2b/KGFR* target gene: 5'-CGTGGAAAA GAACGGCAGTAAATA-3 (sense), 5'-GAACTATTTATCCC CGAGTGCTTG-3' (anti-sense); for *FGFR2c* target gene: 5'-

TGAGGACGCTGGGGAATATACG-3 (sense), 5'- TAGTCT GGGGAAGCTGTAATCTCCT -3' (anti-sense); for *HPV 16E5* gene 5'-CGCTGCTTTTGTCTGTGTCT-3' (sense), 5'-GCGTGCATGTGTATGTATTAATAAAA-3' (antisense); for *ESRP1* target gene 5'- GGCTCGGATGAGAAGGAGTT-3' (sense), 5'-GCACTTCGTGCAACTGTCC-3' (antisense); for *ESRP2* target gene 5'-GCTGTTATCCTCCATCTACTCAA AG-3' (sense), 5'- GTCCACCACATCAGCCTTG-3' (anti-sense); for the 18S rRNA housekeeping gene: 5'-AACCAAC CCGGTCAGCCCCT-3' (sense), 5'-TTCGAATGGGTGCGTCG CCGC-3' (antisense). For each primer pair, we performed no-template control and no-reverse-transcriptase control (RT negative) assays, which produced negligible signals.

RNA extraction and cDNA synthesis

RNA was extracted using the TRIzol method (Invitrogen) according to manufacturer's instructions and eluted with 0.1% diethylpyrocarbonate-treated water. Each sample was treated with DNAase I (Invitrogen). Total RNA concentration was quantitated by spectrophotometry; 1 μg of total RNA was used to reverse transcription using iScript™ cDNA synthesis kit (Bio-Rad) according to manufacturer's instructions.

PCR amplification and real-time quantitation

Real-time PCR was performed using the iCycler Real-Time Detection System (iQ5 Bio-Rad) with optimized PCR conditions. The reaction was carried out in 96-well plate using iQ SYBR Green Supermix (Bio-Rad) adding forward and reverse primers for each gene and 1 μl of diluted template cDNA to a final reaction volume of 15 μl. All assays included a negative control and were replicated three times. The thermal cycling program was performed as described.¹² Real-time quantitation was performed with the help of the iCycler IQ optical system software version 3.0a (Bio-Rad Laboratories), according to the manufacturer's manual. Results are reported as mean ± standard error (SE) from three different experiments in triplicate. Student's *t* test was performed and significance levels have been defined as *p* < 0.05.

Results

HPV16 E5 down-modulates FGFR2b and induces FGFR2c expression in human keratinocytes

Since deregulated and out-of-context signaling of the FGFRs appear to play a role in carcinogenesis and because we have recently demonstrated that FGFR2b/KGFR is down-modulated at both transcript and protein levels by the expression of the oncogenic HPV16 E5 product,^{12,13} we investigated here if the decreased expression of the FGFR2b epithelial splicing isoform induced by the viral protein would correspond to an aberrant expression of the FGFR2c mesenchymal splicing variant. To this aim, we used the human keratinocyte HaCaT cell line, spontaneously immortalized from a primary culture of keratinocytes¹⁴ and previously used as a powerful cellular model in numerous studies including ours,^{12,13} on the effects of HPV16 E5.

First, the specificity of the primers, designed to discriminate among the epithelial FGFR2b and mesenchymal FGFR2c isoforms, was tested not only on the HaCaT cells, but also on skin-derived primary cultured keratinocytes (HKs) and fibroblasts (HFs). The mRNA transcript levels of FGFR2b and FGFR2c were quantitated by real-time relative RT-PCR

using 18S rRNA as housekeeping gene: in accordance with the tissue specificity of the two splice variants, the FGFR2b appeared expressed exclusively on HKs and HaCaT keratinocytes (Fig. 1a, left panels), while the FGFR2c isoform is present only on the HFs (Fig. 1a, right panels). Then, to evaluate the effects of 16E5 on the expression of the two receptor

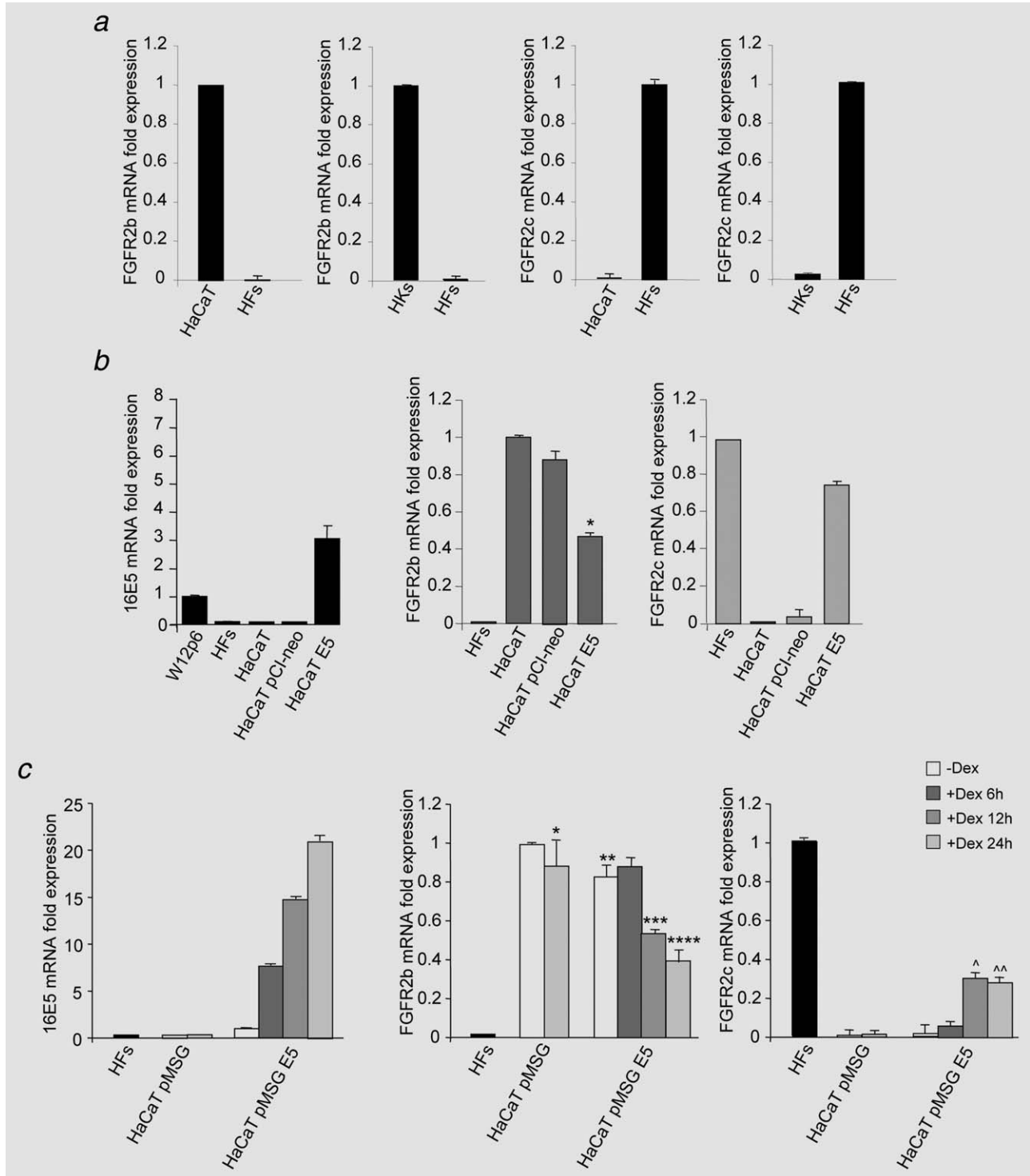


Figure 1.

splicing products, pre-confluent HaCaT cells were transiently transfected with pCI-neo E5-HA expression vector²¹ (HaCaT E5) or with the empty vector alone (HaCaT pCI-neo) as previously described.^{12,13} The mRNA transcript levels of 16E5, FGFR2b and FGFR2c were quantitated as above and the 16E5 mRNA expression levels were normalized with respect to the levels of the viral protein transcript in the HPV16-positive cervical epithelial cell line W12¹⁶ at the passage 6 (W12p6) as previously reported.^{13,23} The results showed that, as expected,^{12,13} the expression of 16E5 in the HaCaT transfected cells led to a clear decrease of FGFR2b expression (Fig. 1b, central panel). In contrast, new and high expression the FGFR2c was found in the HaCaT E5 cells (Fig. 1b, right panel), suggesting that the viral protein is able to interfere with the correct transcription and splicing of the FGFR2 gene which occurs in the physiological epithelial context.

To confirm the effects of 16E5 on FGFR2b/2c differential expression also in a cell model of stable transfection, in which the expression of the viral protein would be under the control of an inducible promoter, we performed the real-time RT-PCR analysis as above using HaCaT cells stably transfected with the construct pMSG 16E5 (HaCaT pMSG E5),¹⁵ in which the expression of the viral protein was progressively induced, by treatment with Dex 1 μ M, in a time-dependent manner (6, 12 and 24 hr of treatment, Fig. 1c, left panel). HaCaT pMSG cells were used as negative control. The results showed that, while the transcript levels of FGFR2b progressively decreased (Fig. 1c, central panel) upon treatment with Dex at 12 and 24 hr, concomitant with the increasing amounts of 16E5 mRNA (Fig. 1c, left panel), the FGFR2c levels appeared clearly increased at 12 hr of Dex treatment and reaching the maximum of induction at that time point (Fig. 1c, right panel), suggesting that 16E5 might be directly responsible for an altered splicing.

16E5 triggers FGFR2b to FGFR2c isoform switch through down-modulation of ESRPs

It is known that the epithelial splicing regulatory proteins 1 and 2 (ESRP1 and ESRP2) control alternative splicing events of a number of genes,²⁴ including the FGFR2.⁵ Therefore, to demonstrate that the changes in FGFR2b and FGFR2c expression, occurring in the presence of 16E5, are the conse-

quence of a ESRP-dependent switch of the mutually exclusive splice variants, we evaluated by real-time RT-PCR as above the transcript levels of ESRP1 and ESRP2 in the HaCaT cells transfected with E5 transiently (HaCaT E5, Fig. 2a) or in a stable inducible manner (HaCaT pMSG E5, Fig. 2b). In both experimental models, the mRNA levels of either ESRP1 or ESRP2 appeared drastically decreased in cells expressing E5 compared with control cells transfected with the empty vectors pCI-neo or pMSG (Figs. 2a and 2b), indicating that the down-modulation of the ESRPs might represent the major molecular mechanism involved in the isoform switch. Interestingly, consistent with the peak of FGFR2c induction in HaCaT pMSG E5 cells after 12 hr of treatment with Dex (Fig. 1c), both ESRP1 and 2 reached their maximal mRNA expression after 12 hr Dex (Fig. 2b), providing a further suggestion of the ESRP involvement in the process.

To confirm that the receptor switch triggered by E5 is directly regulated by ESRPs, we overexpressed ESRP1 in the HaCaT E5 cells by transient cotransfection (HaCaT E5/ESRP1) as described in Material and Methods. Control cells (HaCaT EVs) were cotransfected with the two empty vectors (pCI-neo and pMXs-IRES-*blast2*). In accordance with the suggested major role of ESRPs in regulating the splicing event induced by 16E5, the restored expression of ESRP1 (Fig. 2c, left panel) was able to contrast the effect of the viral protein, leading to a significant increase in the mRNA expression of the FGFR2b variant (Fig. 2c, central panel) and a decrease in that of FGFR2c compared with HaCaT E5 cells (Fig. 2c, right panel).

Because the tissue-specific isoform splicing of FGFRs is triggered by TGF β for driving EMT,^{6,25} we wondered if the expression of E5 would amplify the effects of TGF β 1 treatment on the FGFR2 switch and ESRP down-modulation. To this aim, HaCaT E5 and HaCaT pCI-neo cells were treated with TGF β 1 for 48h and then analyzed by real time RT-PCR as above: incubation with the growth factor of the control cells, transfected with the empty vector pCI-neo, caused a decreasing of FGFR2b and increasing of the FGFR2c expression (Fig. 2d), as a result of the splicing switch consequent to the expected down-modulation of ESRP1 and ESRP2 induced by the treatment (Fig. 2e). Consistent with our hypothesis,

Figure 1. HPV16 E5 changes the expression of FGFR2b and FGFR2c mRNA transcripts. (a) FGFR2b and FGFR2c transcript levels were quantitated by real-time relative RT-PCR in HaCaT cells and in primary human epidermal keratinocytes (HKs) or dermal fibroblasts (HFs), confirming the tissue specific expression of FGFR2b on the epithelial cells and that of FGFR2c on the fibroblasts. (b) The 16E5 mRNA (left panel), FGFR2b mRNA (central panel) and FGFR2c mRNA (right panel) were quantitated in HaCaT cells transfected with pCI-neo E5-HA expression vector (HaCaT E5) compared with the empty vector alone (HaCaT pCI-neo). The 16E5 mRNA expression levels were normalized with respect to the levels of the viral protein mRNA in the subclone W12p6 of the HPV16-positive cervical epithelial cell line W12 (left panel). In HaCaT E5 cells, the decreased expression of the FGFR2b epithelial isoform corresponds to the appearance of the FGFR2c mesenchymal variant. (c) HaCaT pMSG E5 cells and HaCaT pMSG used as negative control were treated with Dex for different times (6, 12 and 24 hr). The 16E5 (left panel), FGFR2b (central panel) and FGFR2c (right panel) transcript levels were estimated by real-time RT-PCR: in HaCaT pMSG E5 cells, the increasing expression of the viral protein upon Dex treatment leads to a decrease of FGFR2b and appearance of FGFR2c clearly detectable at the 12 h time point. Results are expressed as mean values \pm SE. Student's t test was performed as reported in Material and Methods and significance level has been defined as follows: (b) * $p < 0.05$ vs HaCaT pCI-neo cells; (c) * NS vs the corresponding Dex-untreated cells, **, ^ $p < 0.05$ vs the corresponding corresponding HaCaT pMSG cells treated with Dex for 6 hr, ***, ^ $p < 0.05$ vs the corresponding cells treated with Dex for 6 hr, ****, ^^, ^^ NS vs the corresponding cells treated with Dex for 12 hr.

these molecular events triggered by TGFβ1 were magnified in cells expressing 16E5 (Figs. 2d and 2e), suggesting a synergistic effect produced by the viral protein in conjunction with

the growth factor stimulation. 16E5 promotes EMT through FGFR2b to FGFR2c switch and it changes the cell response to FGFR2 ligands.

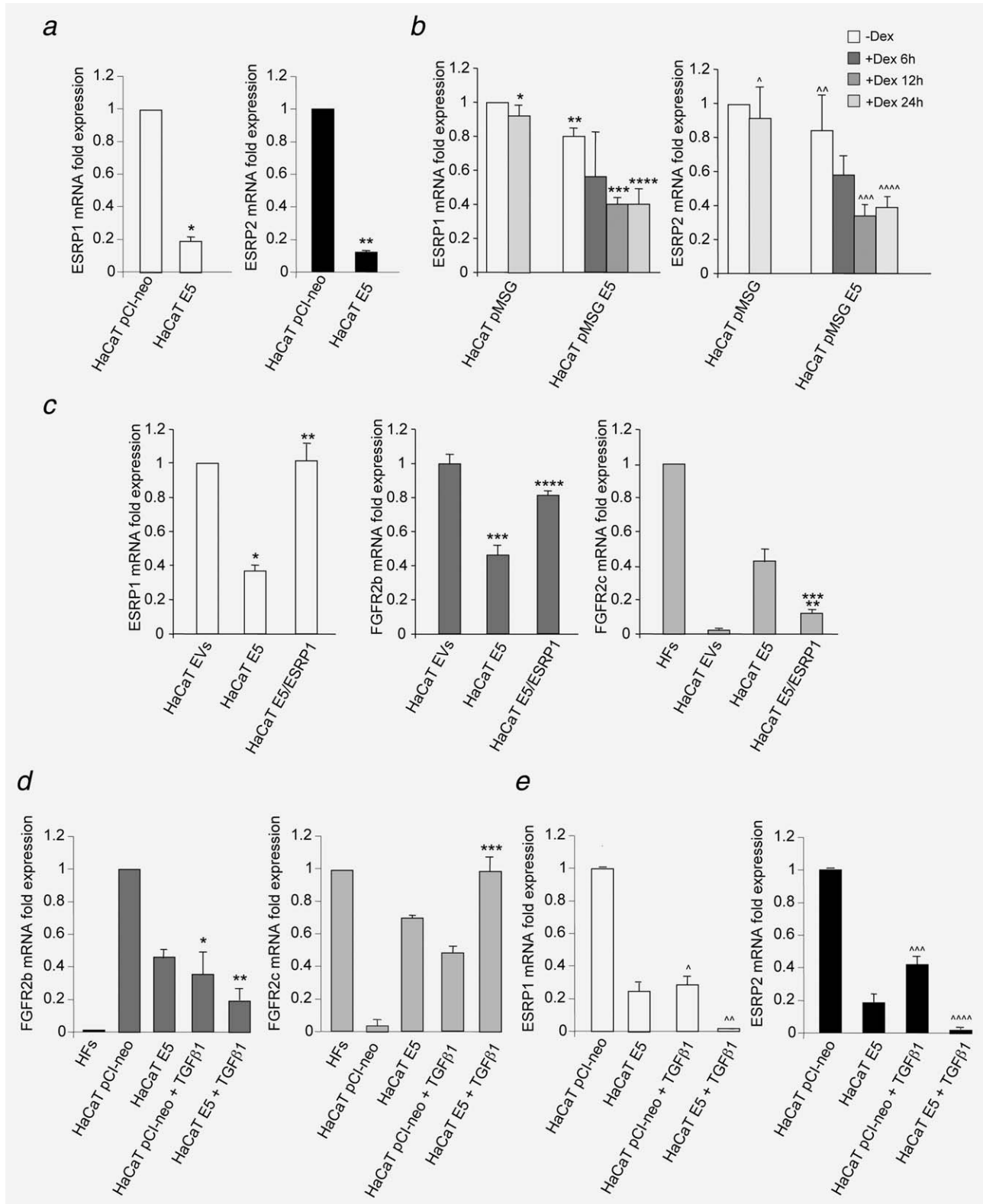


Figure 2.

Since it has been shown that switching from the FGFR2b to the FGFR2c isoforms is involved in EMT and cancer progression^{5,6} and that this appears to be a consequence of a change in sensitivity of the transitioning cells to the FGFs, from the epithelial specific KGF/FGF7 to the mesenchymal specific FGF2,⁶ we wondered whether also the E5 viral protein, similarly to TGF β , might be able to promote EMT through this molecular event. Therefore, we first analyzed if the presence of E5 in the HaCaT keratinocytes would be associated with morphological features and actin cytoskeletal reorganization reminiscent of an ongoing EMT process: in agreement with our hypothesis, a clear shift from the polygonal epithelial versus the spindle mesenchymal morphology was observed in a portion of the HaCaT E5 cells, positively stained with anti-HA monoclonal antibody to visualize the viral protein (Fig. 3a). Double immunofluorescence labeling with TRITC-conjugated phalloidin, which binds to filamentous actin, revealed the appearance of stress fibers, common characteristic of fibroblast-like cells, in the more elongated E5-positive HaCaT keratinocytes (Fig. 3a, inset in the central panel), suggesting that HPV16E5, similarly to the 16E6 and 16E7 viral products,^{26,27} may cause the EMT process.

To demonstrate the possible direct role played by FGFR2 in the EMT promotion induced by 16E5, we analyzed the effects of the receptor depletion. To this aim, HaCaT cells were transfected with small interfering RNA for FGFR2/Bek (FGFR2 siRNA) or with an unrelated siRNA used as control and western blot analysis using anti-Bek polyclonal antibodies, which recognize both receptor splicing variants, confirmed the efficient depletion of the FGFR2 protein in FGFR2 siRNA-transfected cells (Fig. 3b). The equal loading was assessed with anti-actin antibody. Then, we performed coinjection of E5 cDNA, to express the viral protein, with FGFR2 siRNA, to obtain receptor silencing and mouse IgG to identify the microinjected cells. In parallel, microinjection with E5 cDNA and the unrelated siRNA was performed. Immuno-

fluorescence analysis to evaluate the EMT process, assessed by the decrease of the plasma membrane epithelial marker E-cadherin and the appearance of the cytoplasmic mesenchymal marker vimentin, showed that unstimulated cells expressing the E5 (Fig. 3c, E5 cDNA/control siRNA, -GFs) maintained the epithelial phenotype observed also in control cells transfected with the empty pCI-neo vector (Fig. 3c, left panel) or in the uninjected cells surrounding the injected ones, which indicates that, in the absence of growth factor stimulation, the expression of 16E5 alone is not able to induce EMT. Therefore, to ascertain whether the shift in sensitivity to the growth factors specific for the two FGFR2 isoforms would play the major role in the process, we treated the coinjected cells with the FGFR2b ligand KGF/FGF7 or with the FGFR2c ligand FGF2: while the stimulation with KGF did not cause relevant changes in cell morphology as well as in EMT markers (Fig. 3c, central panels), treatment with FGF2 induced the elongated mesenchymal morphology associated with a clear decrease in E-cadherin and appearance of vimentin signal (Fig. 3c, central panels). Consistent with the above hypothesis that the FGFR2b/2c switch is crucial in the E5-promoted EMT process, depletion of the FGFR2 (Fig. 3c, right panel, E5 cDNA/FGFR2 siRNA) abolished the effects observed upon FGF2 treatment, demonstrating that the transition is a direct consequence of the switching from FGFR2b to FGFR2c.

The viral protein effects are exerted also in the context of cervical epithelial cells containing episomal HPV16

To better establish the contribution of E5-induced FGFR2 altered splicing in the context of cervical carcinogenesis and to further clarify the complex epithelial FGF-network of autocrine and paracrine interactions occurring *in vivo* and the role of the stromal microenvironment in this network, we used the well established *in vitro* model of cervical W12 cells,¹⁶ containing episomal HPV16, grown at early passage (W12p6) as above (Fig. 1b). To this aim, W12p6 cells were

Figure 2. 16E5 induces FGFR2b/FGFR2c switching through down-regulation of ESRPs. (a) ESRP1 and ESRP2 transcript levels were quantitated by real-time relative RT-PCR in HaCaT pCI-neo and HaCaT E5 cells: a clear decrease of both ESRP1 and ESRP2 mRNA is evident in cells expressing the viral protein. (b) ESRP1 and ESRP2 transcription is down-regulated also in HaCaT pMSG E5 cells after the induction of the 16E5 expression following Dex treatment: the peak of the ESRP down-modulation is reached after 12 h of treatment, concomitant with the maximal induction of FGFR2c shown in Figure 1c. (c) Overexpression of ESRP1 in HaCaT E5 cells by transient cotransfection (HaCaT E5/ESRP1), able to restore the ESRP1 expression, leads to a significant enhancement of FGFR2b transcription and decrease of that of FGFR2c also in the presence of 16E5, compared with HaCaT E5 cells transfected to the viral protein alone (HaCaT E5). ESRP1 and FGFR2b mRNA values were normalized respect to the levels in HaCaT cells transfected with the two empty vectors (HaCaT EVs), while those of FGFR2c were normalized respect to HFs. (d,e) HaCaT E5 and HaCaT pCI-neo were treated with TGF β 1 10 ng/ml for 48 hr at 37°C to trigger FGFR switching and the mRNA levels of FGFR2b and FGFR2c (d) or of ESRP1 and ESRP2 (e) were quantitated by real time RT-PCR as above. TGF β 1 treatment induces FGFR2b/FGFR2c isoform switch (d) and down-modulation of ESRPs transcription (e). The presence of the viral protein in HaCaT E5 cells treated with the growth factor produces a synergistic effect (d,e). Results are expressed as mean values \pm SE. Student's t test was performed as reported in Material and Methods and significance level has been defined as follows: (a) *, ** $p < 0.001$ vs HaCaT pCI-neo cells; (b) *, ^ NS vs the corresponding Dex-untreated cells, ** $p < 0.01$ vs the corresponding Dex-untreated HaCaT pMSG cells, *** $p < 0.01$ vs the corresponding Dex-untreated cells, ****, ^^^, ^^^ NS vs the corresponding cells treated with Dex for 12 hr, ^^ $p < 0.05$ vs the corresponding Dex-untreated HaCaT pMSG cells, ^^^ $p < 0.05$ vs the corresponding Dex-untreated cells; (c) *, *** $p < 0.05$ vs HaCaT EVs cells, **, ****, ***** $p < 0.05$ vs HaCaT E5 cells; (d) * $p < 0.05$ vs the corresponding TGF β 1-untreated cells, ** $p < 0.01$ vs the corresponding HaCaT pCI-neo cells, *** $p < 0.05$ vs the corresponding HaCaT pCI-neo cells; (e) ^ $p < 0.001$ vs the corresponding TGF β 1-untreated cells, ^^ $p < 0.05$ vs the corresponding HaCaT pCI-neo cells, ^^^ $p < 0.01$ vs the corresponding TGF β 1-untreated cells, ^^^^ $p < 0.01$ vs the corresponding HaCaT pCI-neo cells.

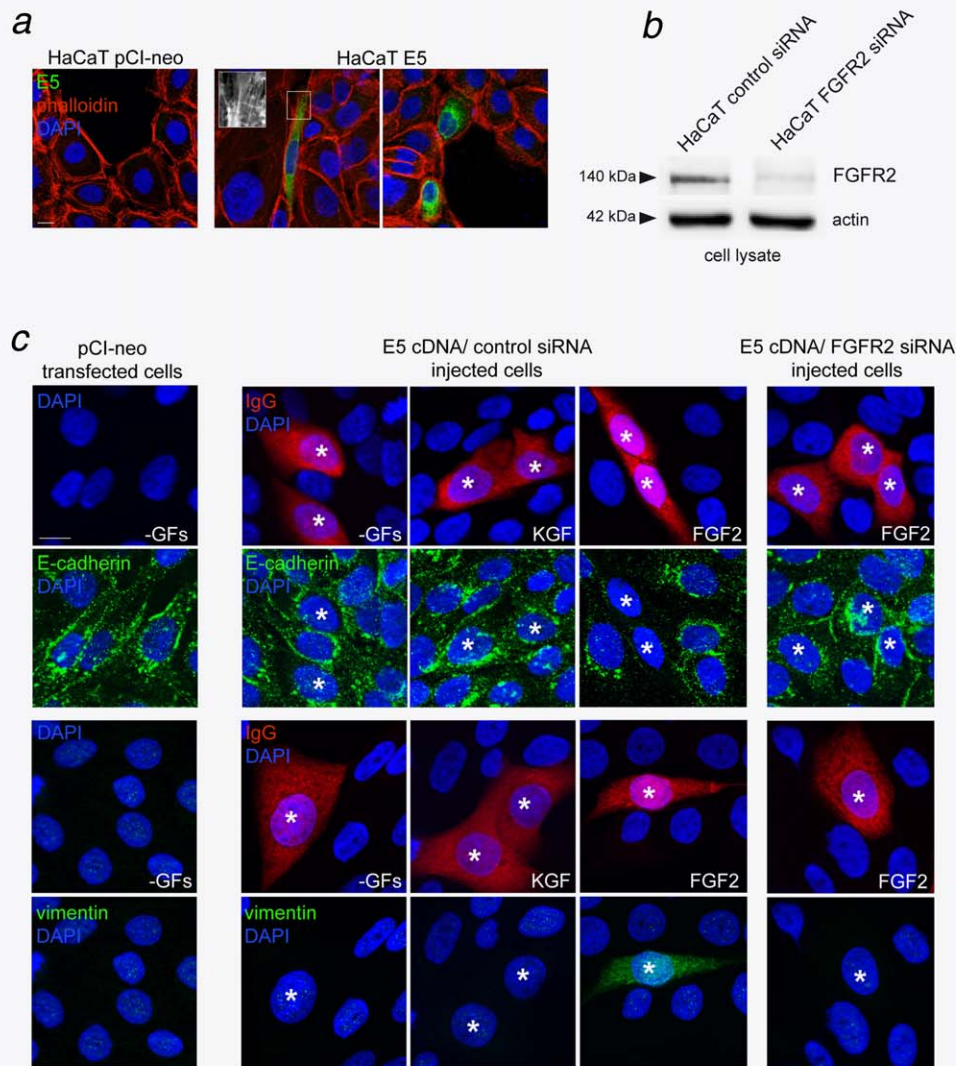


Figure 3. 16E5 promotes EMT through FGFR2 isoform switch and modifies the response to FGFs. (a) HaCaT pCI-neo and HaCaT E5 cells, grown in serum, were fixed and double immunofluorescence was performed using anti-HA monoclonal antibody, to visualize the 16E5 protein and TRITC-conjugated phalloidin, to analyze the actin cytoskeleton organization. Cell nuclei were visualized by DAPI. A portion of HaCaT E5 cells, positively stained for the viral protein (green), shows spindle mesenchymal morphology (HaCaT E5, left panel) and actin reorganization (red) with appearance of stress fibers (inset), while others (HaCaT E5, right panel) seem to maintain the polygonal epithelial shape. Bar: 10 μ m. (b) HaCaT cells were transfected with small interfering RNA for FGFR2/Bek (FGFR2 siRNA) to obtain receptor silencing or with an unrelated siRNA used as control. Western blot analysis using anti-Bek polyclonal antibodies, recognizing both FGFR2b/2c proteins, confirms that the receptor expression, shown by the specific 140 kDa band corresponding to the molecular weight of FGFR2, is efficiently down-modulated in FGFR2 siRNA-transfected cells. The equal loading was assessed with anti-actin antibody. (c) HaCaT cells were coinjected with FGFR2 siRNA, E5 cDNA and mouse IgG to identify the injected cells. Coinjection of E5 cDNA and an unrelated siRNA was performed as control. After injection, cells were serum starved and then treated with the FGFR2b ligand KGF/FGF7 or with the FGFR2c ligand FGF2 before fixation. Alternatively, cells were kept in serum-free medium (-GFs) and then fixed. Immunofluorescence analysis to evaluate the EMT process was performed with anti-Ecadherin or anti-vimentin monoclonal antibodies and injected cells were visualized with anti-IgG polyclonal antibodies. Cell nuclei were visualized by DAPI. The pattern of staining observed in injected cells was analyzed in comparison with untreated control pCI-neo transfected cells (left panels) and with the uninjected cells surrounding the injected ones. In injected cells expressing E5 (asterisks in E5 cDNA/control siRNA), either unstimulated (-GFs) or treated with KGF, the signal corresponding to the plasma membrane epithelial marker E-cadherin appears unmodified respect to control pCI-neo transfected or uninjected cells and no staining for the cytoplasmic mesenchymal marker vimentin is visible. In contrast, treatment with FGF2 induces a clear decrease in E-cadherin staining and appearance of vimentin signal associated with an elongated mesenchymal morphology. Depletion of the FGFR2 (E5 cDNA/FGFR2 siRNA, right panels) is able to abolish the effects observed upon FGF2 treatment, since the injected cells maintain both the epithelial shape and the E-cadherin signal and are negative for the vimentin EMT marker. Bar: 10 μ m. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

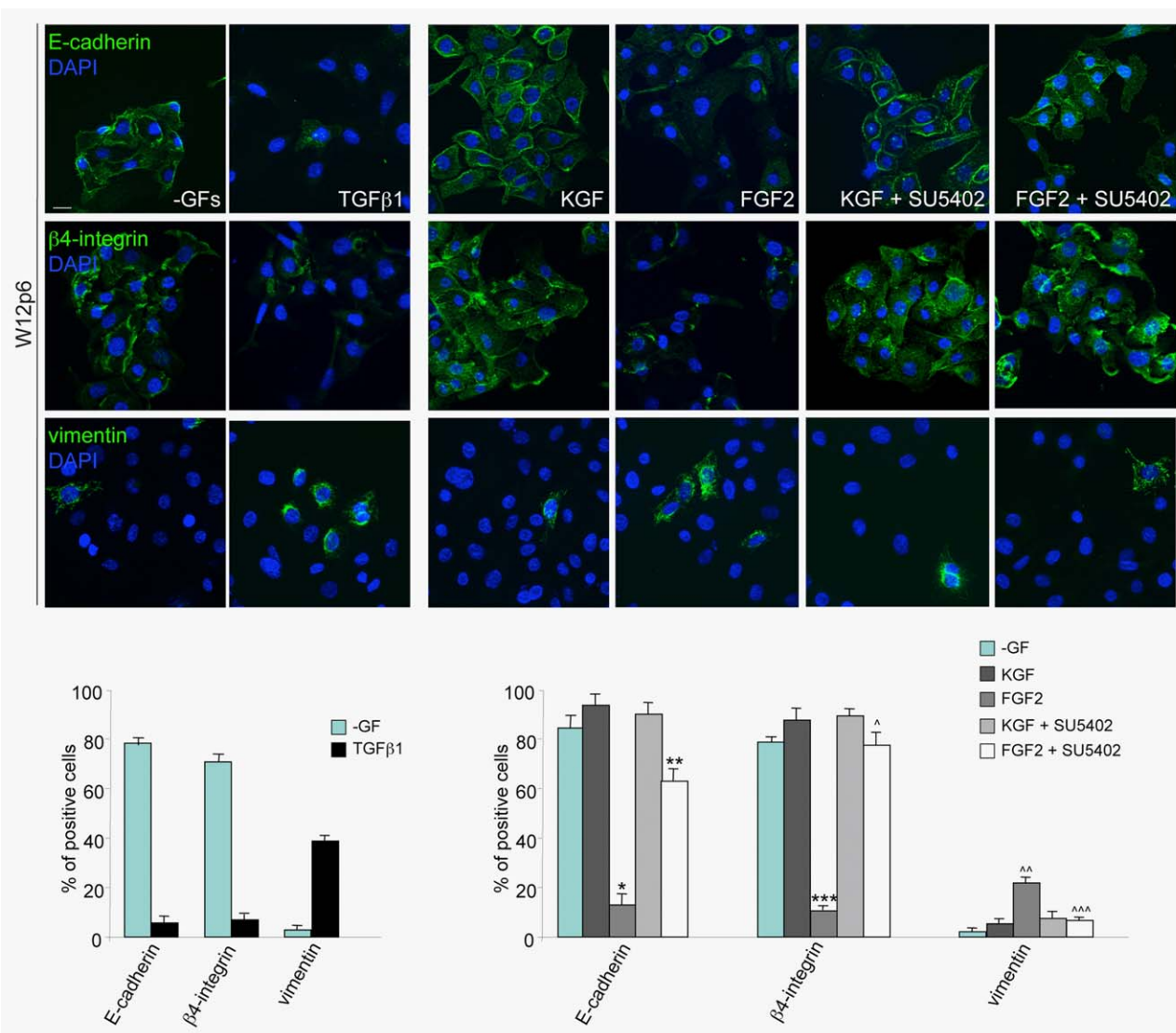


Figure 4. FGF2 triggers EMT in cervical epithelial cells expressing HPV16 E5. W12p6 cells, containing episomal HPV16 and expressing 16E5 as shown in Figure 1b, were treated with TGFβ1, as positive control of EMT induction, or with KGF/FGF7 or FGF2 ligands to activate FGFR2b and FGFR2c, respectively. Quantitative immunofluorescence analysis, using antibodies directed against the epithelial markers E-cadherin and β4-integrin or recognizing the mesenchymal marker vimentin, was performed as described in Material and Methods to evaluate the percentages of cells positive for the EMT markers. Similarly, to TGFβ1 stimulation, FGF2 treatment induces a drastic decrease in the percentage of cells showing the plasma membrane staining for the epithelial markers as well as the appearance in a significant percentage of cells of the cytosolic and filamentous signal of the mesenchymal marker vimentin. In contrast, KGF/FGF7 treatment does not modify the pattern of staining observed in unstimulated (-GFs) cells. The FGF2-induced effects are abolished in the presence of the specific FGFR tyrosine kinase inhibitor SU5402. The quantitative analysis was performed and the cut-off of the signal intensity was selected as described in Material and Methods. Results are expressed as mean values ± SD. Student's t test was performed as reported in Material and Methods and significance level has been defined as follows: *, ***, ^^ $p < 0.001$ vs the corresponding untreated cells, **, ^, ^^ $p < 0.001$ vs the corresponding FGF2-treated cells. Bar: 10 μm [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

treated with TGFβ1, as positive control of EMT induction, or with KGF/FGF7 or FGF2 ligands to activate FGFR2b and FGFR2c, respectively. Quantitative immunofluorescence analysis, performed to evaluate the percentages of positivity for the EMT markers as described in Material and Methods, revealed that the decrease of epithelial markers (E-cadherin and β4-integrin), as well as the appearance of the mesenchymal marker vimentin, in response to FGF2 was comparable

to that obtained upon TGFβ1 stimulation (Fig. 4), confirming the EMT promotion in the pathological context of cervical cells expressing HPV16 E5. This effect was abolished in the presence of the specific FGFR tyrosine kinase inhibitor SU5402 (Fig. 4, right panel), indicating that receptor activation and signaling are required for the promotion of EMT.

To confirm also in this cellular model of cervical carcinogenesis that the molecular mechanism of FGFR2 splicing is

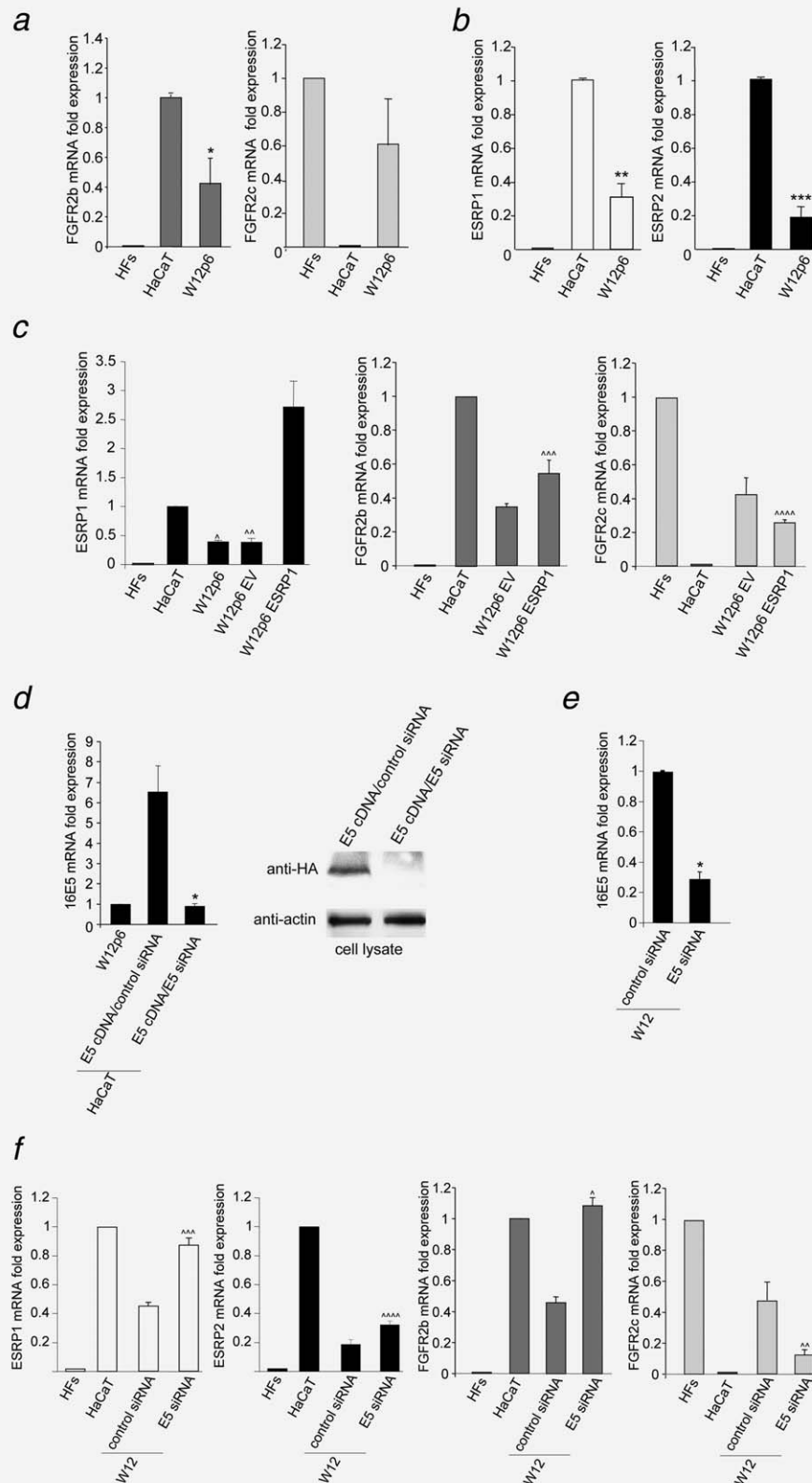


Figure 5.

controlled by ESRPs and that E5 expression down-modulates them inducing switching from FGFR2b to FGFR2c, RT-PCR was performed as above on W12p6 cells compared with HaCaT keratinocytes and human dermal fibroblasts (HFs): concomitant with the decreased transcript levels of FGFR2b and a nonphysiological expression of FGFR2c (Fig. 5a), also the ESRP1 and ESRP2 mRNAs were much lower respect to the HaCaT keratinocytes (Fig. 5b). In addition, the overexpression of ESRP1, performed by transient transfection with pMXs-IRES-blast2 Esrp1-FF (W12p6 ESRP1) or with empty vector (W12p6 EV), was able to induce a significant increase in the mRNA expression of the FGFR2b variant (Fig. 5c, central panel) and a decrease in that of FGFR2c (Fig. 5c, right panel) in W12p6 ESRP1 cells respect to W12p6 EV control cells, providing a further evidence in favor of the key role played by the ESRPs in the splicing event also in this cellular model.

Finally, to demonstrate the direct role exerted by 16E5 on the receptor isoform switching, we analyzed the effects of the depletion of the viral protein in W12p6 cells using small interfering RNA for 16E5 (E5 siRNA) or control siRNA as described in Material and Methods. We first performed experiments on HaCaT cells by cotransfection of E5-HA cDNA and E5 siRNA to verify the efficiency of 16E5 silencing through real-time RT-PCR and western blot analysis using anti-HA monoclonal antibody, confirming the efficient depletion of the 16E5 protein in E5 siRNA-transfected cells (Fig. 5d). The equal loading was assessed with anti-actin antibody (Fig. 5d). Then we transfected W12p6 cells with the E5 siRNA or with the control siRNA and we evaluated the E5 gene expression silencing at the transcript level by real-time RT-PCR (Fig. 5e). In agreement with our working hypothesis that 16E5 expression induces FGFR2b/2c switch, depletion of the viral protein led to an increase of the mRNA levels of either ESRP1 or ESRP2 (Fig. 5f, left panels) and to a restored expression of FGFR2b associated with a decrease of the FGFR2c isoform (Fig. 5f, right panels).

Discussion

In light of our previous results proposing the functional crosstalk among the viral protein HPV16 E5 and the growth

factor receptor FGFR2b/KGFR and their opposite roles played in epithelial carcinogenesis,^{12,13} the present study was focused on the possible dysregulated FGFR2 splicing and consequent EMT triggering which may occur in early stages of HPV infection: our results, confirming our starting hypothesis, suggest that these molecular events would represent an additional mechanism of cervical transformation and tumor progression. In addition, we showed that 16E5 is a powerful tool for perturbing the epithelial homeostasis controlled by growth factor receptors: in fact, using the cellular models expressing this viral product, our study might contribute to the ongoing definition of the role of FGF/FGFR tumor-suppressive or oncogenic signaling as well as of that played by the FGF-network of autocrine and paracrine interactions between the epithelial context and the stromal microenvironment.

We demonstrated here that the changes in the expression of the mutually exclusive splice variants FGFR2b and FGFR2c, occurring in the presence of 16E5, are the consequence of a ESRP-dependent switch, since the restored expression of ESRP1 is able to contrast the effect of the viral protein. These findings provide a further evidence in favor of the proposed major role played by the ESRPs in the FGFR2 switch⁵ and indicate that oncogenic viral products, such as the HPV16 E5, may act on this molecular mechanism to alter the ligand-dependent receptor signaling and the epithelial-stromal interactions for cell transformation. Consistent with this expectation, we showed also that the effects exerted by the viral protein are synergistic with those observed in response to TGF β , which is known to trigger the isoform splicing of FGFRs for driving EMT^{6,25} and which appears to be involved in cervical carcinogenesis.^{28–30} Because we have recently reported that the expression of 16E5 down-modulates the TGF β signaling pathway,²³ further work will be addressed to analyze in detail the FGF and TGF β crosstalk in the unexplored context of the HPV-associated early carcinogenesis and its relationship with the inflammatory microenvironment.

In addition to the evident synergistic and interplaying roles played by 16E5 and TGF β in FGFR2 switch and EMT induction, our results appear to suggest that also other

Figure 5. FGFR2b to FGFR2c switch and down-regulation of ESRPs induced by 16E5 in the W12p6 cervical model of HPV early carcinogenesis. (a,b) The transcript levels of FGFR2b and FGFR2c (a) and ESRP1 and ESRP2 (b) were quantitated by real-time relative RT-PCR in W12p6 and in HaCaT cells and primary human dermal fibroblasts (HFs). A significant decrease of FGFR2b mRNA and a nonphysiological expression of FGFR2c (a) are found in W12p6, together with a down-regulation of both ESRP1 and ESRP2 transcript levels (b). (c) Overexpression of ESRP1 in W12p6 cells by transient transfection (W12p6 ESRP1) induces a significant increase of the FGFR2b mRNA and a decrease in that of FGFR2c compared to W12p6 EV control cells. (d–f) Silencing of 16E5 in HaCaT E5 cells (d) and in W12p6 cells (e, f) using E5 siRNA or control siRNA: the western blot analysis was performed using anti-HA monoclonal antibody and the equal loading was assessed with anti-actin antibody (d). The efficient depletion of 16E5 at both transcript and protein levels is evident in HaCaT E5 cells (d) as well as at the mRNA level in W12p6 cells (e). The transcript levels of FGFR2b and FGFR2c and ESRP1 and ESRP2 (f), quantitated by real-time relative RT-PCR as above, show that the depletion of the viral protein by E5 siRNA leads to an increase of ESRP1 or ESRP2 mRNA (f) and to a restored expression of FGFR2b associated with a decrease of the FGFR2c isoform (f) compared with W12p6 control siRNA cells. Results are expressed as mean values \pm SE. Student's t test was performed as reported in Material and Methods and significance level has been defined as follows: (a,b) *, **, ***, $p < 0.05$ vs HaCaT cells; (c) ^ and ^^ $p < 0,05$ vs HaCaT cells; ^^^, ^^^^^ $p < 0,05$ vs W12p6 EV cells; (d,e) * $p < 0,01$ vs the corresponding E5 cDNA/ control siRNA or control siRNA cells; (f) ^ $p < 0.01$, ^^ and ^^^^^ $p < 0,05$ and ^^^ $p < 0,005$ vs W12p6 control siRNA.

mediators of epithelial homeostasis, able to trigger EMT in keratinocytes and regulated in the presence of 16E5,³¹ might participate to the process induced by the viral protein; therefore, because the 16E5 functional activity, leading to perturbation of cell growth and behavior,³² is mainly exerted on differentiating cells,^{13,33} future work will be focused in comparing the effects of the viral product in undifferentiated versus differentiating keratinocytes to understand if 16E5 could differently modulate the EMT at different stages of keratinocyte differentiation.

Finally, although EMT might result also from HPV16 E6 and E7 expression,^{26,27} our results indicate that HPV16 E5,

whose expression is known to precede those of E6 and E7, would play a major role in the EMT associated with the viral infection, since FGFR2 inhibition in W12p6 cells specifically blocks the process. In this scenario of early molecular drivers of viral oncogenesis, 16E5 would change the cell response to the microenvironmental growth factors and in turn would induce EMT through FGFR2 switch and nonphysiological expression of the FGFR2c isoform.

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