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Curcumin upregulates insulin-like growth factor binding protein-5 (IGFBP-5) and C/EBPα during oral cancer suppression

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Curcumin is a common food ingredient derived from the plant *Curcuma longa* and is a potent drug against tumorigenesis. Both insulin-like growth factor binding protein-5 (IGFBP-5) and CCAAT/enhancer-binding protein α (C/EBPα) are suppressors of head and neck carcinogenesis. We identified curcumin as an inducer of IGFBP-5 expression in multiple types of oral keratinocytes; furthermore, curcumin induces *IGFBP-5* promoter activity in SAS oral cancer cells. Promoter deletion mapping identified a region (nt −71 to nt −59 relative to the transcription start site) as containing a C/EBPα-binding element that is indispensable for curcumin-mediated IGFBP-5 upregulation. Chromatin immunoprecipitation assays revealed that *in vivo* binding of C/EBPα to this region was remarkably increased in the presence of curcumin. Curcumin increased nuclear C/EBPα expression and IGFBP-5 expression through p38 activation and this was abrogated by SB203580 treatment. Furthermore, MKK6 expression activated p38 and C/EBPα, increasing IGFBP-5 promoter activity and expression. Finally, curcumin-induced IGFBP-5 expression is associated with the suppression of xenograft tumorigenesis in mice due to oral cancer cells. We conclude that curcumin activates p38, which, in turn, activates the C/EBPα transactivator by interacting with binding elements in the *IGFBP-5* promoter. The consequential upregulation of C/EBPα and IGFBP-5 by curcumin is crucial to the suppression of oral carcinogenesis.

Key words: carcinoma, C/EBPα, curcumin, IGFBP-5, p38

Abbreviations: AP-1: activator protein-1; AP-2α: activator protein-2α; C/EBPα: CCAAT/enhancer-binding protein α; ChIP: chromatin immunoprecipitation; DMSO: dimethyl sulfoxide; FBS: fetal bovine serum; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GR: glucocorticoid receptor; HNSCC: head and neck squamous cell carcinoma; HPLC: high performance liquid chromatography; HSF: heat shock transcription factor; IGF: insulin-like growth factor; IGFBP-5: insulin-like growth factor binding protein-5; IGFR: insulin-like growth factor receptor; MAPK: mitogen-activated protein kinase; mAU: mili absorbance unit; NF-κB: nuclear factor kappa B; NHOK: normal human oral keratinocyte; OSCC: oral squamous cell carcinomas

Additional Supporting Information may be found in the online version of this article.

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Insulin-like growth factors (IGFs) that bind IGF receptors (IGFRs) with high affinity play important roles in regulating cell phenotypes, including proliferation, differentiation, migration and apoptosis. Various effects of IGFs and IGFRs during carcinogenesis have been recently identified and IGFRs would seem to be a therapeutic target.[1] Six IGF binding proteins (IGFBPs) have been identified in humans and these bind IGF with high affinity. The binding of IGFBPs to an IGF prolongs the half-life of IGF and also limits the bioavailability of free IGF to bind to IGFRs.[2] Downregulation of insulin-like growth factor binding protein-5 (IGFBP-5) is important in oral squamous cell carcinomas (OSCCs), particularly those showing poor differentiation.[3] Our *in vivo* study linked the downregulation of IGFBP-5 with an increase in tumorigenesis of OSCC cells.[4] IGFBP-5 was also downregulated in cervical carcinoma.[5] In addition, it was found to modulate tumor suppression by inhibiting angiogenesis,[6] and regulate the phenotype of fibroblasts.[7]

Diferuloylmethane (Curcumin), found in significant amounts in the Indian spice turmeric, is a polyphenol derived from the plant *Curcuma longa* and is a common food ingredient across the world.[8,9] Accumulated evidence has indicated that this polyphenol can be used to both prevent and treat cancer.[10,11] Curcumin exhibits cancer chemopreventive effects in a range of animal models of chemical carcinogenesis, including those resulting in head and neck squamous cell carcinoma (HNSCC) and OSCC.[12,13] This compound also has anti-oxidative effects, anti-inflammatory effects, anti-

angiogenesis and other properties; a number of mechanistic pathways have been proposed by which it might block the initiation and progression of cancer.[10,14] Curcumin regulates signaling elements such as mitogen-activated protein kinases (MAPK) members p38 and JNK; nuclear factor kappa B (NF-κB), signal transducer and activator of transcription 3 (stat 3) and others.[8–10,15–19] In HNSCC cells, liposome-encapsulated curcumin suppresses xenographic HNSCC growth by NF-κB inactivation.[19] Curcumin also abrogated the Stat 3 pathway in HNSCC cells.[18] CCAAT/enhancer binding proteins (C/EBPs) are members of the basic leucine zipper family of transcription factors, which include 6 isotypes in mammalian cells.[20] Curcumin modulates transcription factors including C/EBPs, NF-κB, activator protein-1 (AP-1) and others.[10,16–19,21,22]

Regulation of *IGFBP-5* gene expression by several factors has been described in multiple cell types.[23–27] The C/EBPα, glucocorticoid receptor (GR) and activator protein-2α (AP-2α) elements, which are upstream of the TATA box in *IGFBP-5* promoter, are critical for the transcription regulation of *IGFBP-5*.[24,28–30] C/EBPα is involved in the regulation of growth arrest associated with terminal differentiation as well as in the expression of genes associated with the differentiation in several cell types.[31] Evidence also indicated that C/EBPα is a suppressor of neoplasms including HNSCC.[32,33] C/EBPα is abundantly expressed in the epidermis,[34] and is a factor modulating G_1 checkpoint in keratinocytes.[35] Although medical use of curcumin may be limited to more superficial tissues such as skin or the oral mucosa due to the poor gastrointestinal absorption,[36] clinical trials have demonstrated that 8 g/day curcumin taken by mouth, which gave an average serum curcumin level of ~2 μM (~650 ng/ml), was not toxic to humans.[15] It was safe even when consumed at a dose of 12 g/day for 3 months.[15,16] Thereby, curcumin may have wide use in the prevention or treating of neoplasms. This study investigated the effects of curcumin on IGFBP-5 and C/EBPα and its relationship with OSCC suppression.

Material and Methods

Cell culture and reagents

SAS cell line is a poorly differentiated tongue squamous cell carcinoma cell.[37] It was grown in DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Biological Industries, Kibbutz Beit Haemek, Israel) at 37°C in a humidified atmosphere of 5% CO_2.[4] They were passaged at a density ratio of 1:8 when 80% confluence was reached. OECM-1 OSCC cell line was grown in RPMI medium (Invitrogen) containing 10% FBS (Biological Industries).[38] Normal human oral keratonicyes (NHOKs) were established from gingival epithelium and grown in keratinocyte serum free medium (K-SFM, Invitrogen).[39,40] OC3 OSCC cell line was grown in a combined K-SFM-DMEM medium (2:1, v/v) containing 3.3% FBS (Biological Industries).[41] SAS is a tumorigenic cell line on experimental animals and exhibits higher transfection efficiency than OECM-1 and OC3 cells.[4] It was used as the target cell in this study. Curcumin, dimethyl sulfoxide (DMSO), LY294002 and Ponceau S were purchased from Sigma-Aldrich (St. Louis, MO). SB203580 and SP600125 were purchased from Calbiochem (Darmstadt, Germany). Curcumin, LY294002, SB203580 and SP600125 were reconstituted in DMSO as 10–20 mM stocks.

Trypan blue dye exclusion assay

Cell viability was determined by 0.5% trypan blue (Biological Industries) excluding ability of cells.[41] Cells with intact membrane that expelled trypan blue were counted vital. All measurements were performed in triplicate to generate cell survival curves after curcumin treatment.

RT-PCR analysis

Total RNA was isolated using TRI reagent (Molecular Research Center, OH). One microgram total RNA was converted to cDNA using MMLV reverse transcriptase (Epicentre Tech., Madison, WI). A cDNA equivalent of 0.2 μg RNA was used to analyze mRNA expression. The primer sequences and optimized reaction conditions are described in Supporting Information Table S1. The amplicons were resolved on a 2% agarose gel and photographed by an imaging system (Viber Lourmat, Marne La Vallee, France). Quantitation of the signal density was performed using Bio-Profil® analysis program (Viber Lourmat). The mRNA expression levels of *IGFBP-5* and *C/EBPα* were normalized against the mRNA expression level of *GAPDH*.

Western blot analysis

Fifty micrograms of proteins from the whole cell lysate or nuclear extract isolated by the NucBuster™ Protein Extraction Kit (Novagen, Darmstadt, Germany) were resolved by electrophoresis on a 12.5% denaturing polyacrylamide gel followed previously used protocols.[38] The providers and the dilutions of the primary antibodies are presented in Supporting Information Table S2. Anti-mouse or anti-rabbit (Chemicon, Temecula, CA) secondary antibodies at 1:500 dilution were used. The signals were detected using a Western Lightning Chemiluminescence Reagent plus kit (Perkin-Elmer, Wellesley, MA) and Chemi-Smart 3000 image acquisition system (Viber Lourmat). Quantification was performed by normalizing the signals derived from the proteins being assayed against the signal from GAPDH.

Plasmids and stable cells

Full length (FL) promoter plasmid. The promoter region from −458 to +23 of IGFBP-5 was obtained by PCR amplification of human genomic DNA. This 481-bp amplicon was cloned into the *Sma I* site of the pGL3-basic vector (Promega, Madison, MI).

Δ3, Δ2 and Δ1 deleted promoter plasmids. Amplicons of Δ3, Δ2 and Δ1 deletion fragments were achieved by PCR reaction using FL DNA as template, the common downstream primer and individual upstream primer described in Supporting Information Table S3.[42] After digestion with *MluI* and *BglII* restriction enzymes, the products were cloned into pGL-3-basic vector (Promega).

Δ1 mutated promoter plasmids. Five Δ1 mutants, which contained the mutation at the predicted NF-κB, C/EBPα, GR, AP-2α and heat shock transcription factor (HSF) site, respectively, were obtained using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The primers used in mutagenesis are listed in Supporting Information Table S4. *EcoRI* restriction enzyme digestion sequence (GAATTC) was introduced to replace core sequence of original binding site in each mutant construct, which abrogated the transcription activity and allowed the detection of successful mutagenesis.

pC/EBPA-Luc reporter plasmid. The pC/EBPA-Luc construct (Panomics, Fremont, CA), containing 3 cis-acting DNA binding elements that are recognized by C/EBPα, was used to monitor and quantitate the binding activity of C/EBPα. Binding at these sites results in the expression of luciferase. pTA-luc (Panomics) was used as a control vector in this experiment.

pBabeMKK6. pBabeMKK6 retroviral plasmid for constitutive MKK6 expression (Cell Biolab, San Diego, CA) and pBabe control vector were transfected into packaging cells to harvest retroviruses. Stable SAS cells after viral infection and puromycin selection (1.5 μg/ml; Sigma-Aldrich) were designated SAS-pBabeMKK6 (for MKK6 expression) and SAS-pBabe (control).

shIGFBP-5i. shIGFBP-5i packed in lentiviruses was obtained from the RNA interference consortium (Academia Sinica, Taipei, Taiwan).[4] Stable SAS cells after viral infection and puromycin (1.5 μg/ml) selection were designated SAS-shIGFBP-5i (for the knockdown of IGFBP-5) and SAS-shLuci (control).

Promoter and reporter activity assay

Luciferase assays were carried out by cotransfecting cells with promoter plasmids or reporter plasmids together with pCMVβ-gal plasmid using the calcium phosphate precipitation technique.[4] Luciferase activity was assayed 36 hr later using a detection kit (Promega, Madison, WI) and analyzed by Victor counter (Perkin-Elmer). β-galactosidase activity was measured by optical density (OD_{420}) with spectrophotometer (Thermo Fisher Scientific, Pittsburg, PA) to normalize transfection efficiency.

EMSA (electrophoretic mobility-shift assay)

Nuclear extracts were collected using a NucBuster Protein Extraction Kit (Novagen). EMSA was performed by incubating 10 μg of nuclear extract with 1.5 ng γ^{32}P-end-labeled double-stranded oligonucleotides.[39,41] The oligonucleotides containing the C/EBPα and AP-2α recognition elements are listed in Supporting Information Table S5. The composition and specificity of binding was examined by competition with 50-fold excess of unlabeled consensus or mutant oligonucleotides. Protein-DNA complexes were resolved on a high ionic strength 4% polyacrylamide gel. The gel was dried after resolution and the signals from the radioactive bands were quantified by ImageQuant program (Amersham, Piscataway, NJ).

ChIP (chromatin immunoprecipitation) assay

ChIP assays were performed using a ChIP assay kit (Upstate, Lake Placid, NY). Briefly, cells were grown to 70% confluences in 100-mm dishes. The chromatin from formaldehyde-fixed cells was sonicated and immunoprecipitated using an antibody against C/EBPα (Supporting Information Table S2). The chromatin immunoprecipitate was analyzed by amplification of the nt −94 to +100 region of the *IGFBP-5* gene using PCR. This region contains the C/EBPα binding element between nt −71 to −59. The primers were forward, 5′-TGAGTTGGGTGTTGGGAAG-3′; and reverse, 5′-CGGAG-GAGGGGTAATGAAAAG-3′, and the amplicon was 194-bp in size. The amplicons were resolved on a 2% agarose gel and visualized by an imaging system (Viber Lourmat).

HPLC (high performance liquid chromatography)

Twelve-week-old female mice were kept in an environmentally controlled animal room with constant temperature and humidity, 12 hr dark–light cycle, and free access to standard laboratory food and water. They are fasted 12 hr before dosing. Administration of 500 mg/kg curcumin to rat by mouth intake rendered a peak plasma curcumin level occurring 20–60 min later.[43] Feeding of 500 mg/kg curcumin emulsified in 2% carboxymethyl cellulose (vehicle) was carried out on 6 mice using oral feeding tube (STF-173; Shinetech, Taipei, Taiwan). Two control mice were fed with vehicle. The heparinized blood was collected within an hour after feeding by heart puncture under anesthesia. Fifty microliter plasma sample was mixed with 100 μl acetonitrile containing 1.5 μg/ml 2-(4′-hydroxyazobene) benzic acid as an internal standard. After centrifugation, the supernatant was loaded to a HPLC machine system (Shimadzu, Kyoto, Japan) on Eclipse XDB-C_{18} reversed-phase column (4.6 × 150 mm, 5-μm particle size, Agilent, Santa Clara, CA) with the mobile phase composed of acetonitrile—10 mM monosodium phosphate (pH 3.5) (40:60, v/v) at a flow rate of 0.8 ml/min. The mobile phase was filtered through a 0.45-μm millipore membrane filter and then degassed by sonication before use. The detection wavelength was 425 nm and the run time was 27 min. Each fraction was collected according to the UV signal. All chemicals for HPLC assay were purchased from Sigma-Aldrich.

Tumorigenesis

Eight-week-old female NOD-SCID mice were used for the tumorigenesis assay.[4] Mice fed with 500 mg/kg/day curcumin in a pilot test showed no mortality or significant body weight

loss over a 2-week period. Stable SAS cells with lentiviral infection were injected (1×10^5 in 50-μl volume) subcutaneously into the flank of mice to induce xenografts. Daily feeding of either 500 mg/kg curcumin or the vehicles was performed for 25 days using oral feeding tube (Shinetech). The mice were also kept in an environmentally controlled animal room. Tumor volumes were calculated using the formula $0.5 \times a \times b^2$, where a and b are the long and short diameters of the tumors, respectively.[4] At the end of experiment, the mice were sacrificed under anesthesia, the tumors were resected and subjected to histopathological evaluation by Dr. Kuo-Wei Chang. The use of animals followed the approved animal use protocol of the National Yang-Ming University, Taiwan.

Statistics

Mann–Whitney test and ANOVA analysis were used for the statistical analysis. Results were considered to be statistically different when $p < 0.05$.

Results

Curcumin-induced IGFBP-5 expression in oral keratinocytes

Cells grew to ~70% confluence were treated with serially diluted curcumin for 24 hr. The viable cells at each dosage point were counted by trypan blue exclusion assay. The IC_{50} of SAS, OC3, OECM-1 cells and NHOKs were determined to be about 12, 8, 12 and 14–16 μM, respectively, from the survival curves (detailed analysis not shown). With 4 or 8 μM curcumin treatment for 24 hr, the *IGFBP-5* mRNA and IGFBP-5 protein increased in the SAS cells (Fig. 1*a*). The *IGFBP-5* mRNA expression in OC3 and OECM-1 cells was also increased on treatment with 4 or 8 μM curcumin (Fig. 1*b*). The expression of *IGFBP-5* mRNA and IGFBP-5 protein in NHOKs was also increased on treatment with 2–16 μM curcumin (Fig. 1*c*). IGFBP-5 expression in SAS cells and a representative NHOK cell was demonstrated in Figure 1*d*. Taken together, the findings suggest that curcumin upregulates IGFBP-5 in various oral keratinocytes.

C/EBPα mediated curcumin-induced transactivation of the IGFBP-5 promoter

To analyze the mechanisms whereby curcumin induces IGFBP-5 expression, SAS cells transfected with the FL promoter plasmid and a vector alone control were treated with curcumin and the cell lysates were collected to carry out luciferase assays. After normalization against β-galactosidase activity, the cells treated with 0–12 μM curcumin for 24 hr exhibited an induction of *IGFBP-5* promoter activity in a dose-dependent manner (Fig. 2*a*). A curcumin dose as low as 2 μM also induced *IGFBP-5* promoter activity. To stratify the major region of the *IGFBP-5* promoter that curcumin transactivate, a serial of 3 deletion constructs, Δ3, Δ2 and Δ1 plasmids, containing deleted *IGFBP-5* promoters, were generated (Fig. 2*b*, Lt). These reporters were transfected into SAS cells. Compared with the FL, the truncated promoters significantly decreased the reporter gene transactivation (Fig. 2*b*, Rt). However, the induction of transactivation of these deleted promoters by curcumin was similar to FL promoters. The results implied that the major region through which curcumin transactivation occurred is located between nt −83 and +23 in the shortest deletion Δ1. After analyzing the −83 to +23 region of the *IGFBP-5* promoter using F-MATCH (http://www.gene-regulation.com), potential binding fragments for the transcription factors NF-κB, C/EBPα, GR, AP-2α and HSF were identified (Fig. 2*c*, Lt). To further specify the curcumin transactivation sites, mutant plasmids of each site in Δ1 were generated by site-directed mutagenesis. It was noted that GR mutation resulted in an increase in reporter gene transactivation (Fig. 2*c*, Rt). Mutations in C/EBPα and AP-2α resulted in the decrease of basal promoter activity relative to parental Δ1. Following 12 μM curcumin treatment for 24 hr, C/EBPα mutation and AP-2α mutation, particularly C/EBPα mutation, exhibited a remarkable reduction in reporter gene transactivation compared to the parental Δ1. All the other mutants exhibited an induction of reporter gene transactivation by curcumin similar to the parental Δ1 (Fig. 2*c*, Rt). These results suggest mutations in either the C/EBPα or the AP-2α binding sequence, particularly in C/EBPα binding site, may suppress curcumin-induced *IGFBP-5* transactivation.

Curcumin-induced C/EBPα DNA binding activity in IGFBP-5 promoter

EMSA analysis was performed to gain insight into whether curcumin induced the binding activity of C/EBPα or AP-2α. Curcumin treatment increased the binding of C/EBPα to the *IGFBP-5* promoter in SAS cells in a dose-dependent manner (Fig. 3*a*, upper and lower). The binding was attenuated by competition with unlabeled consensus oligonucleotide. However, the binding of AP-2α to the *IGFBP-5* promoter was not enhanced by curcumin treatment in SAS cells (Fig. 3*a*, middle and lower). To reveal if C/EBPα expression underlies the increase of C/EBPα DNA binding, mRNA and protein expression of C/EBPα in curcumin-treated SAS cells was analyzed. Quantification of the results showed that 12 μM curcumin treatment for 24 hr significantly increased *C/EBPα* mRNA expression (Fig. 3*b*) and nuclear C/EBPα protein expression (Fig. 3*c*). ChIP assays were performed to investigate whether the induction by curcumin of IGFBP-5 expression was associated with the recruitment of C/EBPα to the *IGFBP-5* promoter *in vivo*. After treating with 8 or 12 μM curcumin for 24 hr, immunoprecipitation was performed using antibody against C/EBPα. The nucleotide sequence spanning −94 to +100 of *IGFBP-5*, which contains the C/EBPα binding site, was amplified by PCR. The results revealed that C/EBPα was not bound to *IGFBP-5* promoter in untreated cells. In contrast, either 8 or 12 μM curcumin treatment resulted in the binding of C/EBPα to the *IGFBP-5* promoter, which was pulled down by the antibody and then amplified by the PCR reaction (Fig. 3*d*). The *in vivo* induction effect of curcumin on the DNA binding of C/EBPα was further investigated using a reporter construct. SAS cells

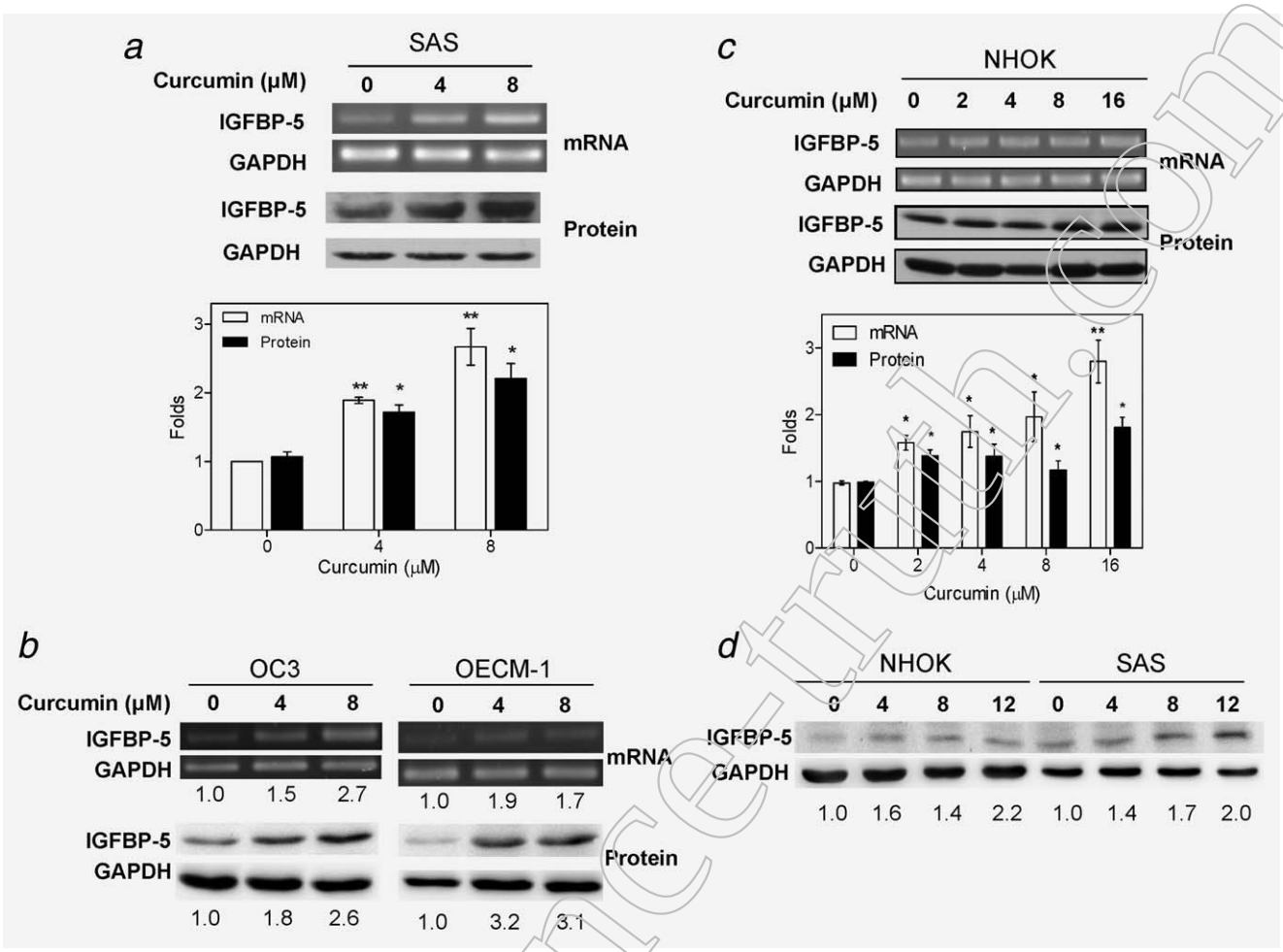

Figure 1. Curcumin-induced IGFBP-5 expression in oral keratinocytes. (*a*) SAS cells. SAS cells were treated with 4 or 8 μM curcumin for 24 hr. RT-PCR (upper) and Western blot analysis (middle) identified the upregulation of *IGFBP-5* mRNA and protein expression following treatment. Quantification (lower) showed an increase in *IGFBP-5* mRNA expression as well as the IGFBP-5 protein level in SAS cells. (*b*) OC3 cells (Lt) and OECM-1 cells (Rt). It showed the upregulation of *IGFBP-5* mRNA expression (upper) and protein expression (lower) following 4 or 8 μM curcumin treatment for 24 hr. (*c*) NHOK cell lines treated with 0–16 μM curcumin for 24 hr were subjected to RT-PCR (upper) and Western blot analysis (middle) to detect IGFBP-5 expression. Lower, quantification. Upregulation of *IGFBP-5* mRNA expression and protein can be seen for various doses of curcumin. (*d*) IGFBP-5 protein expression. The protein from a NHOK cell line and SAS cell line were run in the same gel for the comparison of IGFBP-5 expression level under different doses of curcumin treatment. The numbers below the pictures in (*b*, *d*) were the normalized values. Data shown were the mean ± SE from triplicate analysis in (*a*) and 7 distinctive NHOK cell lines in (*c*). *, $p < 0.05$; **, $p < 0.01$; Mann–Whitney analysis.

transfected with pC/EBPA-Luc reporter plasmid or pTA-Luc (control) were treated with curcumin for 24 hr and the cell lysates were analyzed. Untreated SAS cells showed only basal C/EBPα DNA binding activity when compared to cells transfected with the vector only; in contrast, the DNA binding activity of C/EBPα was significantly increased after 4 or 8 μM curcumin treatment (Fig. 3*e*).

Curcumin transactivated the IGFBP-5 promoter via the p38 pathway

To resolve the possible types of signaling activation involved in the transactivation of the *IGFBP-5* promoter, we pretreated SAS cells with the PI3K/AKT blocker LY294002, the p38 blocker SB203580 and the JNK blocker SP600125 for 1 hr and then analyzed the *IGFBP-5* promoter activity following 12 μM curcumin treatment for 24 hr.[9,44] Among the blockers, only 15 μM SB203580 was able to inhibit FL or Δ1 promoter activity (Fig. 4*a* and Supporting Information Fig. S1). SB203580 treatment reduced *IGFBP-5* mRNA on 12 μM curcumin induction for 24 hr (Fig. 4*b*). Furthermore, SB203580 also reduced curcumin-induced p38 activation, nuclear C/EBPα expression and IGFBP-5 protein expression (Fig. 4*c*). On activation of p38 in SAS cells by the expression of MKK6 in SAS-pBabeMKK6 cells, it was found that the cells exhibited an increased expression of C/EBPα and IGFBP-5 in relation to SAS-pBabe cells (Fig. 4*d*). IGFBP-5 Δ1 plasmid and control vector (pGL3-basic)

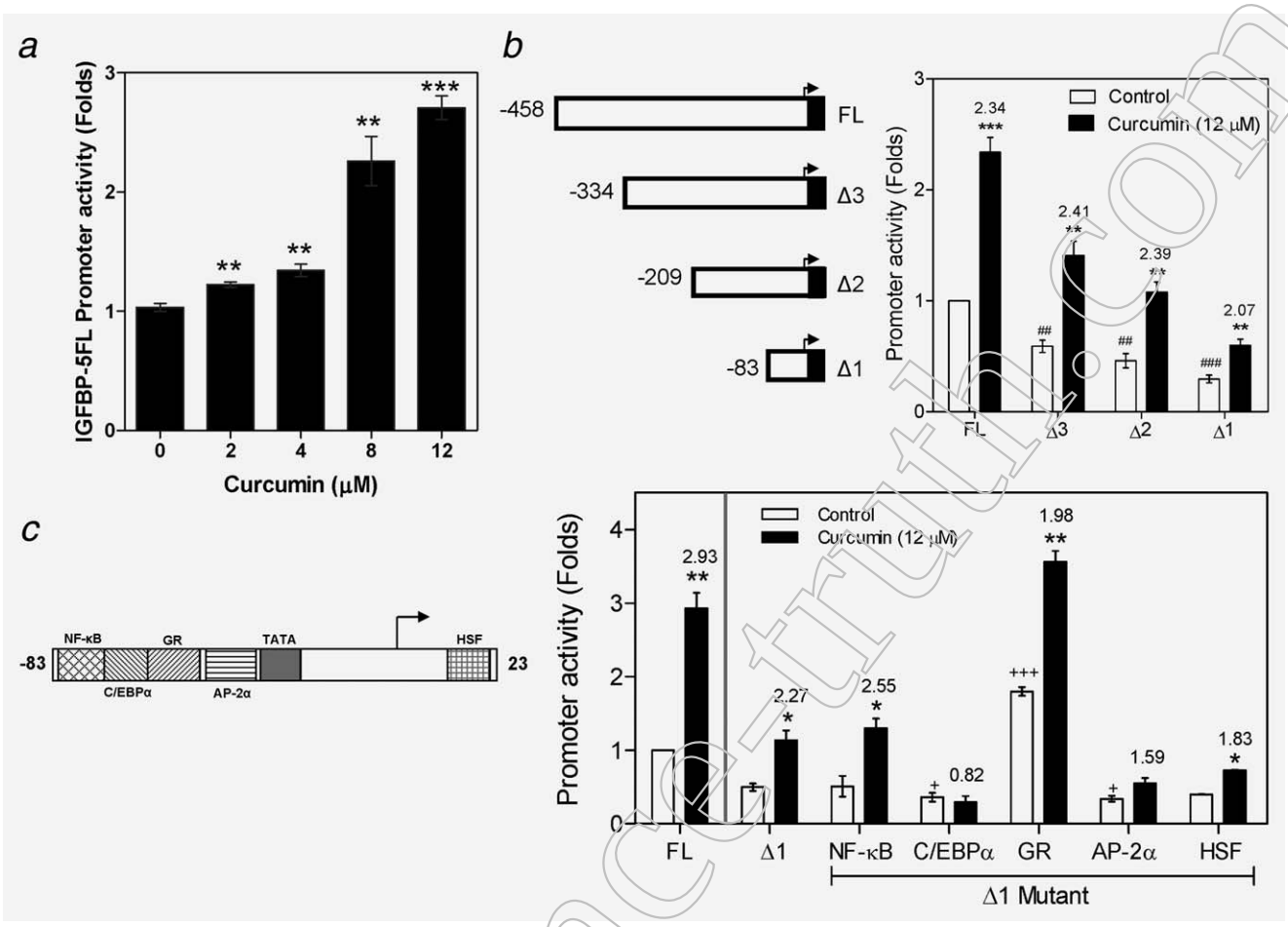

Figure 2. C/EBPα mediated curcumin-induced transactivation of the *IGFBP-5* promoter. (*a*) *IGFBP-5* promoter activity was assayed by detecting luciferase activity using SAS cells transiently transfected with FL and then treating with 0–12 μM curcumin for 24 hr. Fold activation was calculated by normalization the luciferase activity of reporter against the β-galactosidase activity exhibited by cotransfected pCMVβ-gal plasmid. Quantification showed that there was an increase in *IGFBP-5* promoter activity following treatment with various doses of curcumin. (*b*) Lt, diagram illustrating the various constructs used for the luciferase activity assay, which contained different deletions (Δ) of the *IGFBP-5* promoter. Rt, luciferase assays being carried out on SAS cells transfected with various reporter plasmids as indicated. There was a progressive decrease in basal *IGFBP-5* activity following consecutive promoter deletion. However, deletion of the region from −458 to −83 nucleotides did not remarkably change *IGFBP-5* promoter activity during induction by 12 μM curcumin. (*c*) Lt, diagram of Δ1 and the location of binding sites. Rt, quantification of promoter activity of the various constructs. Note the remarkable decrease in curcumin-induced *IGFBP-5* Δ1 promoter activity when C/EBPα and AP-2α elements were mutated. Data shown were mean ± SE from at least triplicate analysis. Numbers in (*b*, *c*), folds, comparison with controls. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$; comparison with control. ##, $p < 0.01$, ###, $p < 0.001$; comparison between FL and each Δ. +, $p < 0.05$, +++, $p < 0.001$; comparison between Δ1 and each Δ1 mutant; Mann–Whitney analysis.

were transfected to stable cells. Promoter activity assays indicated an increase of parental Δ1 activity in SAS-pBabeMKK6 cells relative to control (Fig. 4*e*). pC/EBPA-Luc plasmid and control vector (pTA-Luc) were also transfected to the stable cells. Reporter assays confirmed that there was an increase in the *in vivo* C/EBPα DNA binding affinity in the SAS-pBabeMKK6 cells relative to control (Fig. 4*e*).

Curcumin-induced IGFBP-5 expression reduced the tumorigenicity of SAS cells

The HPLC analysis was standardized and calibrated to generate an equation for plasma curcumin measurement (Supporting Information Fig. S2A, B). The sensitivity of HPLC analysis for detecting the plasma curcumin was determined to be 5 ng/ml by calibration. After feeding of 500 mg/kg curcumin for 20–60 min, 13 ng/ml and 9 ng/ml curcumin were detected in the plasma of 2 mice (Supporting Information Fig. S2C, D), while the plasma curcumin in remained mice was below the detectable range. The bioavailability of curcumin in mice seemed diverse and low.

To see the effect of curcumin-induced IGFBP-5 expression on tumorigenesis, stable cells SAS-shIGFBP-5i, which exhibits knockdown of IGFBP-5 and SAS-shLuci, which is a control, were generated. These stable cells had higher sensitivity to curcumin with an IC_{50} of 8 μM. Similar to parental cells, these subclones exhibited an upregulation of IGFBP-5 with

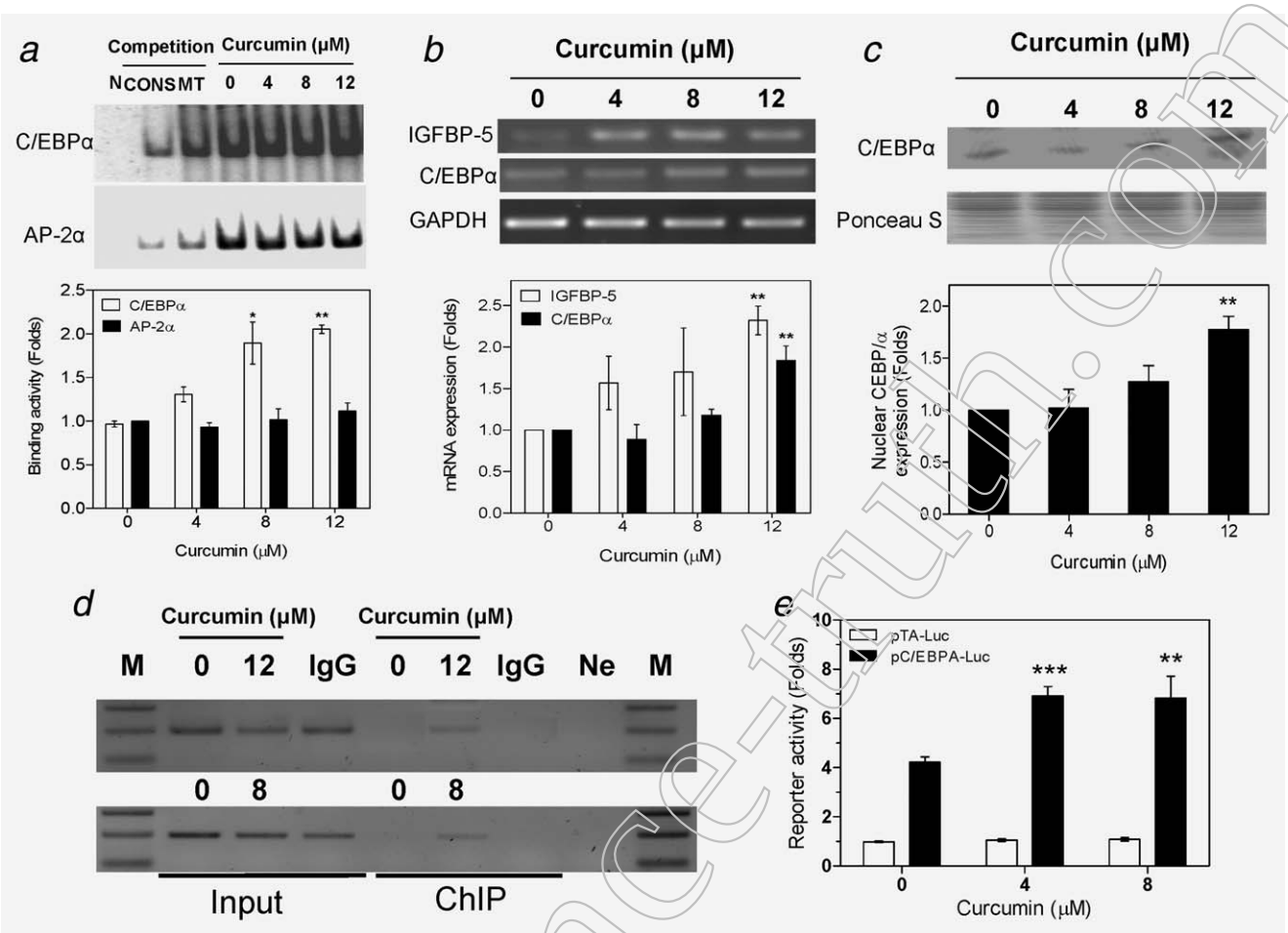

Figure 3. Curcumin-induced C/EBPα DNA binding activity of the *IGFBP-5* promoter *in vivo*. SAS cells were treated with 0–12 μM curcumin for 24 hr. (*a*) EMSA analysis. Upper and middle, electrophoresis for C/EBPα and AP-2α, respectively. Lower, quantification. It showed a progressive increase in C/EBPα binding activity other than AP-2α binding activity on curcumin treatment. The binding of C/EBPα was remarkably attenuated by competition with unlabeled consensus oligonucleotide and less affected by mutant oligunucleotide. The binding of AP-2α was also markedly attenuated by consensus oligonucleotide. N, no nuclear extracts; CONS, 50-fold unlabeled consensus oligonucleotide; MT, 50-fold unlabeled mutant oligonucleotide, respectively, were added to the mixture in 12 μM curcumin treatment. The sequences of oligonucleotides are in Supporting Information Table S5. (*b*) RT-PCR analysis. Upper, gel electrophoresis; lower, quantification. It showed a progressive increase in *IGFBP-5* and *C/EBPα* mRNA expression on curcumin treatment. (*c*) Western blot analysis using nuclear extract. Upper, gel electrophoresis; lower, quantification. The protein expression was normalized to the amount of protein stained by 0.1% Ponceau S. It showed a progressive increase in nuclear C/EBPα protein on curcumin treatment. (*d*) ChIP assay. The input represented PCR results from the chromatin pellets before immunoprecipitation. ChIP analysis revealed the precipitation of a C/EBPα and DNA complex by antibody following 12 μM (upper) or 8 μM (lower) curcumin treatment. IgG, preimmunized IgG control; Ne, negative control, M, molecular marker. The molecular weight of the upper, middle and lower band was 300-bp, 200-bp and 150-bp, respectively. (*e*) C/EBPα reporter activity assay. SAS cells were transfected with pC/EBPA-Luc or pTA-Luc plasmids for 8 hr, and then they were treated with curcumin for 24 hr. Luciferase assays were performed to show the increase in C/EBPα reporter activity following curcumin treatment. Data shown were the mean ± SE from triplicate or quadruplicate analysis. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; Mann–Whitney analysis.

8 μM curcumin treatment (Fig. 5*a*). Xenografts of SAS-shIGFBP-5i and SAS-shLuci were established by subcutaneous injection of cells to NOD-SCID mice. The mice were fed with 500 mg/kg curcumin or vehicle during the whole process of tumor induction. Analysis of the growth curve indicated an inhibitory effect of curcumin on the growth of SAS-shLuci tumors, while the inhibitory effect of curcumin was weaker for SAS-shIGFBP-5i tumors (Fig. 5*b*). In mice with or without curcumin feeding, knockdown of IGFBP-5 significantly increased the tumor growth. This was particularly eminent in mice with curcumin treatment. It would seem that the level of IGFBP-5 in the tumor cells contributed to tumor inhibition in xenografts treated with curcumin. The combined curcumin treatment and IGFBP-5 expression suppressed the tumorigenecity most significantly (Fig. 5*b*). Thus, on curcumin induction, the increased IGFBP-5 in SAS cells

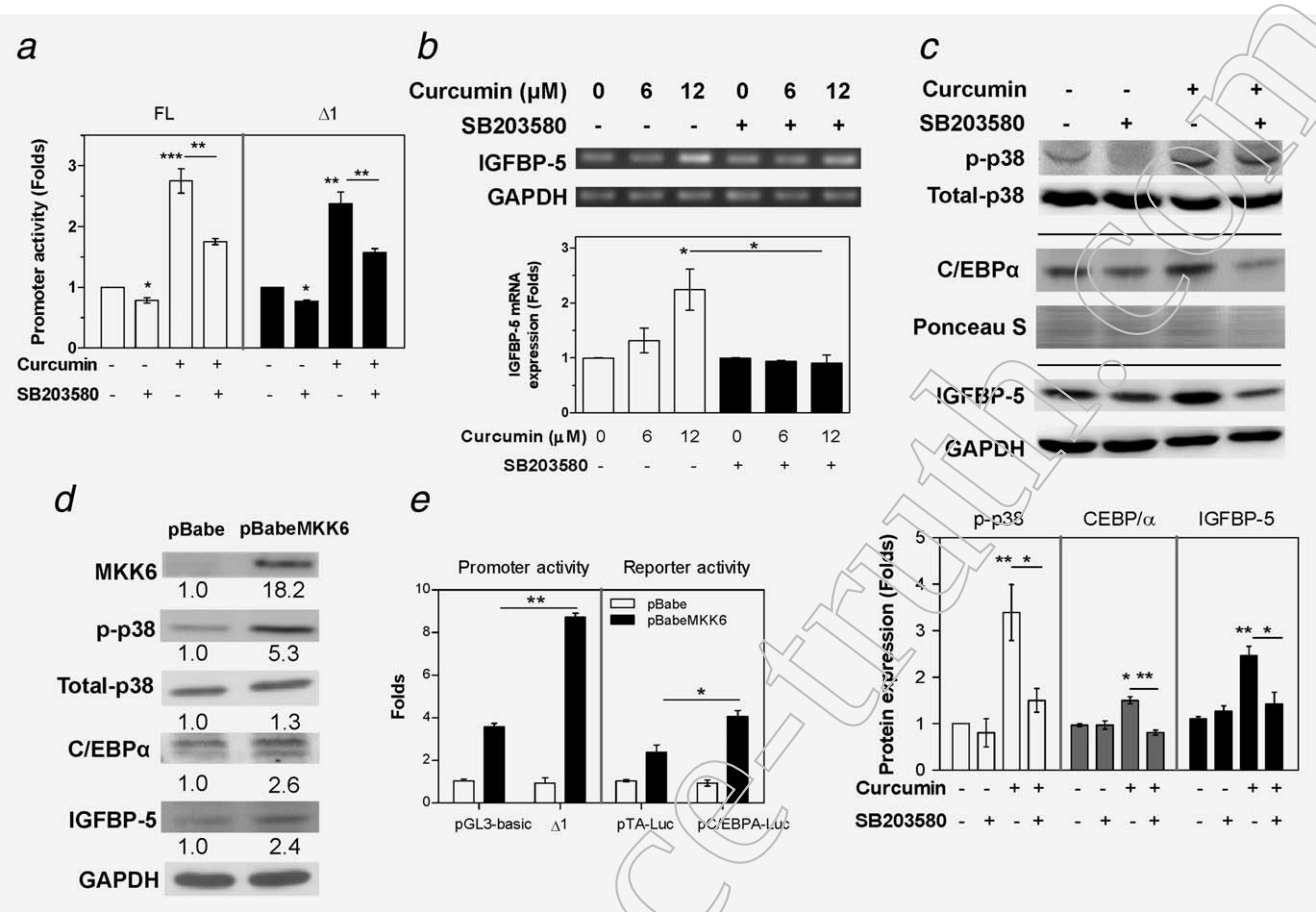

Figure 4. Curcumin increased IGFBP-5 and C/EBPα expression *via* the p38 pathway. SAS cells treated with curcumin and/or 15 μM SB203580 for 24 hr were subjected to analysis in (*a–c*). (*a*, *c*), 12 μM curcumin was used. (*a*) Promoter activity assay. Curcumin treatment transactivated FL and Δ1 by ~2- to 3-fold. This activation was attenuated by SB203580 pretreatment. (*b*) RT-PCR analysis. Upper, gel electrophoresis. Lower, quantification. It showed increase in *IGFBP-5* mRNA expression, which was abrogated by SB203580 pretreatment. (*c*) Western blot analysis. Upper, gel electrophoresis. Lower, quantification. It showed the activation of p38, and increased IGFBP-5 protein expression and nuclear C/EBPα protein expression on curcumin treatment, which was abrogated by SB203580 pretreatment. The nuclear C/EBPα protein level was normalized to the amount of protein stained by Ponceau S. Lines separated different individual analysis. (*d*, *e*) Analysis of SAS-pBabe cells and SAS-pBabeMKK6 cells. (*d*) Western blot analysis showed the expression of MKK6, activation of p38 and the upregulation of C/EBPα and IGFBP-5 expression in SAS-pBabeMKK6 compared to control. Numbers below pictures were the normalized values. (*e*) Lt, IGFBP-5 promoter activity assay. Stable cells were transfected with Δ1 plasmid or pGL3-basic vector together with pCMVβ-gal plasmid. The transactivation of promoter was revealed by normalized luciferase activity. Rt., C/EBPα reporter activity assay. Stable cells were transfected with pC/EBPA-Luc plasmid or pTA-luc vector together with pCMVβ-gal plasmid. The transactivation of promoter was revealed by normalized luciferase activity. Data shown were the mean ± SE from more than 3 distinctive experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; Mann–Whitney analysis.

mediated by curcumin seems to be associated with the suppression of oral tumorigenesis. There was no apparent histopathological difference being noted across tumors in different groups (Supporting Information Fig. S3). Figure 5*c* summarizes our thoughts on how curcumin regulates the p38, C/EBPα and IGFBP-5 cascade to give tumor suppression of OSCC.

Discussion

Evidence indicates that curcumin has cancer preventive or therapeutic effects.[12–14,19,45] We have defined that the suppressive effects of IGFBP-5 on the tumorigenesis of OSCC, which may be invaluable for neoplastic intervention.[4] Results from the present study further indicate that curcumin, can upregulate IGFBP-5 expression in multiple OSCC cell lines. We identified that the inhibitory effects of curcumin toward the tumorigenesis of SAS cells would seem to be mainly exerted by upregulating IGFBP-5. This study also revealed the upregulation of C/EBPα by curcumin, another tumor suppressor for HNSCC,[33] underlies the IGFBP-5 upregulation. Taken together, our findings provide novel molecular insights into curcumin-associated tumor inhibition.

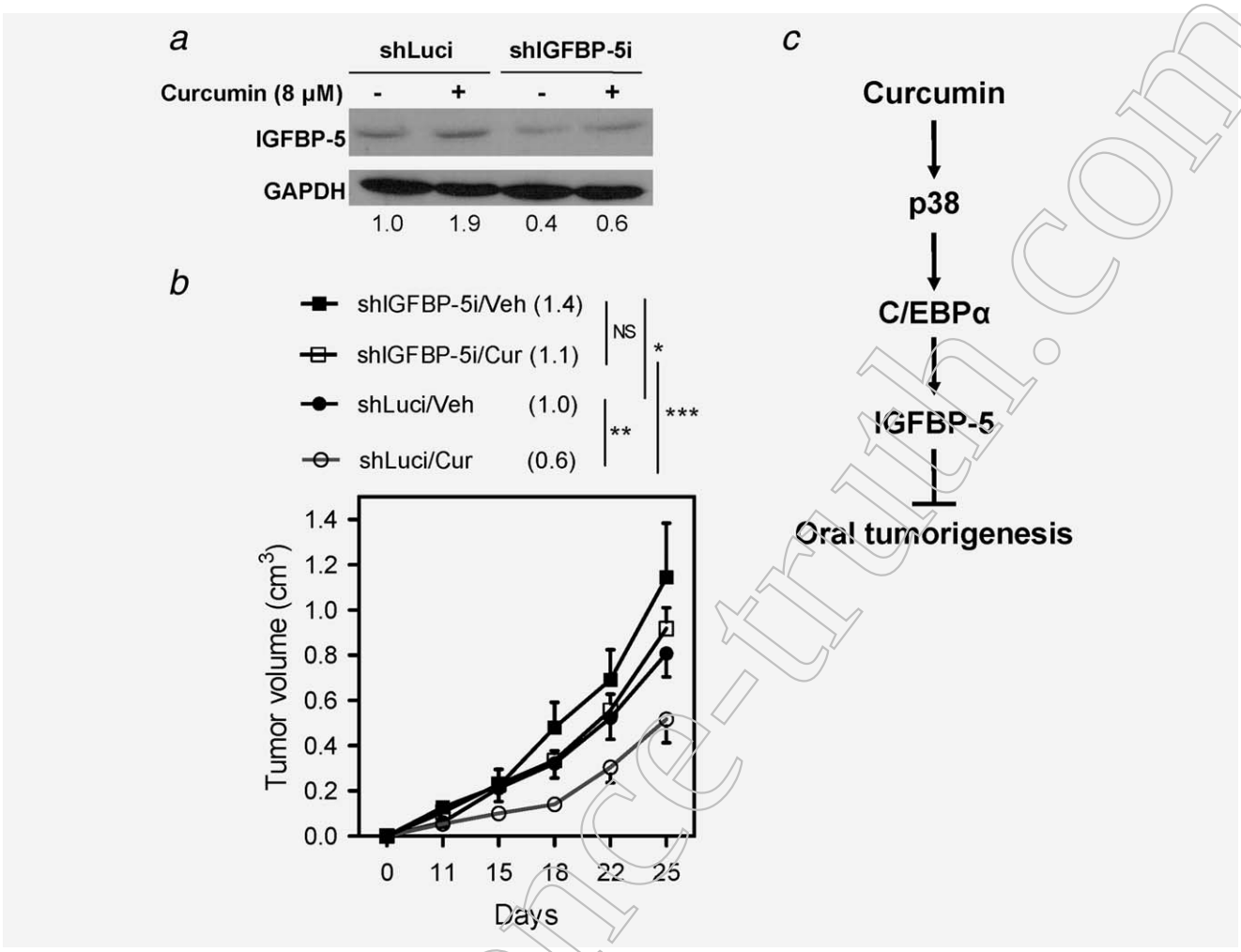

Figure 5. Association between curcumin-induced IGFBP-5 expression and the decrease of tumorigenesis in SAS cells. SAS-shLuci cells and SAS-shIGFBP-5i stable cells were generated. (*a*) Western blot analysis showed the decrease of IGFBP-5 expression for ~60% in SAS-shIGFBP-5i relative to the control. With 8 μM curcumin treatment, SAS-shLuci cells and SAS-shIGFBP-5i cells showed an increase in IGFBP-5 expression for ~90% and ~60%, respectively, relative to controls. Numbers below pictures were normalized values. (*b*) NOD-SCID mice were subcutaneously injected with 1×10^5 SAS-shIGFBP-5i and SAS-shLuci cells and fed with 500 mg/kg/day curcumin or vehicle for 25 days. The tumor volume was measured periodically. It revealed an effect of curcumin and IGFBP-5 on tumor suppression. Data shown were mean ± SE of 5–10 mice. Number within each parenthesis, mean tumor volume at Day 25. NS, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ANOVA analysis. (*c*) Diagram illustrating the molecular mechanism underlying curcumin-associated OSCC suppression identified in this study.

Curcumin acts as a regulator of transcription factors by activating a diversity of signaling pathway.[8–10,14,18,21,22] This study used promoter deletion analysis and luciferase assays to reveal that curcumin induced a marked increase of the *IGFBP-5* promoter activity. Further stratification has excluded the activation of NF-κB and GR may underlie IGFBP-5 up-regulation. The C/EBPα binding site, localized at nt −71 to −59 of the *IGFBP-5* promoter, was finally identified as the activation site that modulated by curcumin treatment. On *in vitro* EMSA analysis, as well as *in vivo* analysis including ChIP and reporter assays, the results of the promoter deletion mapping were confirmed. Our further dissection of the process identified an increase in nuclear C/EBPα expression as specifically underlying C/EBPα activation. Curcumin differentially activated MAPK or transcription factors in various kinds of cells under different treatment conditions.[9,16,17,21,22,46] In dermal keratinocytes, it suppresses p38δ and C/EBP, which are associated with apoptosis and a decrease in involucrin expression.[17] However, it induced C/EBPβ in prostate cancer cells.[22] In addition, curcumin activates p38 in hepatoma cells and ovarian cancer cells.[46,47] By screening multiple blockers for signaling activation, we identified a linkage between p38 activation and the increase in promoter activation of *IGFBP-5* in OSCC cells after curcumin treatment. The identification of GR for the suppression of *IGFBP-5* promoter activity is in concordance with a previous study.[28] AP-2α driven transactivation contributed to the high level of expression of IGFBP-5 in fibroblasts.[30] Although

mutation in the AP-2α element also slightly reduced curcumin-induced *IGFBP-5* promoter activity in this study, EMSA and expression experiments have suggested that there is only a minor impact of AP-2α on curcumin-induced *IGFBP-5* expression.

It is known that (−)-epigallocatechin-3-gallate, a green tea polyphenol, increases C/EBP protein level and increases complex formation at the C/EBP DNA binding site of the human involucrin promoter *via* the Ras, MEKK1, MEK3 and p38δ signaling cascade.[17] By pretreatment with a p38 blocker and the expression of MKK6 to activate p38, we have confirmed that there is a consequential relationship between p38 activation and the increase in C/EBPα DNA binding activity.[21,22] C/EBP activation is known to play a number of regulatory roles in the differentiation of epithelial cells including keratinocytes.[48,49] When overexpressed in hepatocytes, C/EBPα is also a strong inhibitor of cell growth.[50] It should be noted that IGFBP-5 expression is increased during retinoic acid-mediated differentiation of neuroblasts. Retinoid acid upregulates C/EBP and this increase in C/EBP could activate *IGFBP-5* through TATA binding.[23] Our previous studies have demonstrated that IGFBP-5 has an inhibitory role on the growth of OSCC cells.[4] Thus, signals that activate C/EBPα, which then causatively upregulate IGFBP-5, could therefore be important for the prevention or treatment of OSCC. Retinoid acid and 1α, 25(OH)2D3 upregulate IGFBP-5 and this hinders cell growth.[23,25–27] In future studies, it would be interesting to know the feasibility of using natural ingredients to activate IGFBP-5 during tumor therapy. Curcumin is known to upregulate the p53 and p21 tumor suppressors and to downregulate MDM2.[16] However, since the *p53* gene is mutated extensively in OSCC or HNSCC, the impact of p53 activation driven by curcumin on OSCC suppression is probably limited. In view of the fact that curcumin activates both C/EBPα and IGFBP-5, the clues from the present study substantiated the potential of curcumin as an anticancer drug for OSCC and that this activity would seem to involve the upregulation of tumor suppressors.

Bioavailability of curcumin in human through oral intakes was low.[16] However, this study demonstrated that 2 μM curcumin, a concentration of curcumin can be detected in human serum after oral intake,[15] induced *IGFBP-5* promoter activity. The bioavailability of curcumin in rat through oral feeding was also low.[43] Although this preliminary study detected plasma curcumin in mice by oral feeding of 500 mg/kg curcumin, the optimized doses, pharmacokinetics, and the sensitivity of assays need to be precisely defined. It was interesting that the plasma cucumin was also low in plasma of mice, while repeated curcumin intakes for a few weeks resulted in the tumor suppression. The combined curcumin treatment with the presence of IGFBP-5 expression rendered the most potent suppression. Both curcumin and IGFBP-5 were known to inhibit tumor cells *in vitro*.[4,19] Moreover, a low dose of curcumin inhibited the proliferation of keratinocyte when combining with UVA or light stimulation.[36] Both curcumin and IGFBP-5 could also inhibit vascularity in tumor xenografts.[6,14] The inhibition of tumor angiogenesis could be an additional factor underlying the suppression of xenographic tumors, even when the bioavailability of curcumin was low and the upregulation of IGFBP-5 was limited. Knockdown of IGFBP-5 eminently increased the tumorigenecity in control and curcumin-treated mice. The increase was particularly eminent in curcumin-treated mice, suggesting a potent anti-tumor effect for IGFBP-5 in curcumin-treated mice. The solitary effect of IGFBP-5 associated with stromal impairments could underlie the additional suppression.[7] Because that only the advanced tumors were available for tissue assay, we were unable to specify any histopathological change correlated with the discrepancies in tumorigenesis. The *in vivo* suppression activity associated with curcumin and IGFBP-5 requires further clarification. Liposome-encapsulated curcumin exhibited better solubility and oral absorbance,[19,43] the validation of curcumin regimen in combination with the modulation of IGFBP-5 expression may exert potent effects against OSCC.[19]

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