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ORIGINAL ARTICLE

Squamous cell carcinoma growth in mice and in culture is regulated by c-Jun and its control of matrix metalloproteinase-2 and -9 expression

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Squamous cell carcinoma (SCC) is an invasive malignancy of epidermal keratinocytes. Surgical excision is currently the main treatment; however, this can cause scarring and disfigurement. There is accordingly, an acute need for alternative strategies to treat SCC. The transcription factor c-Jun is expressed in human SCC and another common form of invasive skin cancer, basal cell carcinoma together with the mitogenic marker-proliferating cell nuclear antigen. Here, we have employed DNAzymes (catalytic DNA molecules) targeting c-Jun (Dz13) to inhibit c-Jun expression in SCC cells. Dz13 inhibits SCC proliferation and suppresses solid SCC tumor growth and tumor angiogenesis in severe combined immunodeficient mice. We further demonstrate that Dz13 inhibits c-Jun, together with matrix metalloproteinase (MMP)-2 and MMP-9 expression in the tumors, consistent with DNAzyme inhibition of MMP-2 and MMP-9 gelatinolytic activity by zymography. Dz13 also suppressed the expression of vascular endothelial growth factor and fibroblast growth factor-2 in the tumors. These findings demonstrate that c-Jun regulates SCC growth and suggest that DNAzymes targeting this transcription factor may potentially be useful as inhibitors of cutaneous carcinoma.
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Introduction

Squamous cell carcinoma (SCC) is a malignancy of epidermal keratinocytes. SCC is a common form of invasive skin cancer (along with basal cell carcinoma (BCC)) in Caucasians, accounting for 20% of all cutaneous malignancies (Bernstein *et al*., 1996). SCC mostly arises on the sun-exposed skin of the middle-aged and elderly (Johnson *et al*., 1992). The incidence of SCC is not confined to the skin; it can occur in the mouth, esophagus, airways, uterine cervix and vulva. The primary cause of cutaneous SCC appears to be cumulative lifetime sun exposure, with both ultraviolet (UV) B and UVA being involved (Agar *et al*., 2004). Current treatment for SCC is surgical excision, but this can cause scarring and disfigurement, and can be cumbersome especially when many lesions are present. Radiotherapy is less commonly used but this can affect normal tissue and cause patient discomfort. There is, therefore, an acute need for alternative strategies to treat SCC, particularly as the population ages. BCCs are tumors derived from the epidermis that, unlike SCC, do not metastasize but are highly invasive and destructive.

The formation of new blood vessels from pre-existing vasculature (i.e., angiogenesis) underpins normal biologic processes such as wound healing and reproduction as well as many pathologic conditions, including sight-threatening ocular disorders (Ciardella *et al*., 2002), restenosis following angioplasty (Fuchs *et al*., 2001), and solid tumor growth and dissemination (Folkman, 1972). Angiogenesis is a complex multistep process that involves the proteolytic degradation of the basement membrane and surrounding extracellular matrix and microvascular endothelial cell proliferation, migration, tube formation and structural reorganization (Hanahan and Folkman, 1996). The efficacy of endogenous and synthetic inhibitors of angiogenesis is currently being evaluated in clinical trials (Kerbel and Folkman, 2002). The recent clinical success of the humanized anti-vascular endothelial growth factor (VEGF)-A monoclonal antibody Avastin (rhuMAb-VEGF) indicates that antiangiogenic therapy is viable in certain types of cancer (Ferrara, 2002). IMC-C225, an anti-epidermal growth factor receptor monoclonal antibody with antiangiogenic activity, has been used for the treatment of SCC of the head and neck (Herbst *et al*., 2001).

Immediate-early genes, like the transcription factor c-Jun, control the expression of multiple regulatory genes and are, by definition 'master-regulators'. Our understanding of the key transcription factors involved in the process of angiogenesis is limited at the present

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time. c-Jun is a member of the basic region-leucine zipper (bZIP) protein family that homodimerizes and heterodimerizes with other bZIP proteins to form the transcription factor, activating protein-1 (AP-1) (reviewed by Shaulian and Karin, 2001). c-Jun has been linked with cell proliferation, transformation and apoptosis. For example, skin tumor promotion is blocked in mice expressing a dominant-negative transactivation mutant of c-jun (Young *et al*., 1999). Microinjection of antibodies to c-Jun into Swiss 3T3 cells inhibits cell cycle progression (Kovary and Bravo, 1991). Compared with primary fibroblasts cultured from wild-type littermates, primary fibroblasts cultured from live heterozygous and homozygous mutant c-jun mouse embryos, which die *in utero* (Hilberg *et al*., 1993; Johnson *et al*., 1993), have greatly reduced growth rates in culture that cannot be overcome by the addition of mitogen (Johnson *et al*., 1993). c-Jun has also been implicated in apoptosis. For example, c-jun-null mouse embryo fibroblasts are resistant to apoptosis induced by UVC radiation (Shaulian *et al*., 2000). Recent studies by our group provided a direct link between c-Jun and the process of angiogenesis (Zhang *et al*., 2004). This was achieved using gene-specific catalytic DNA (Khachigian, 2000).

Insights into the function of a given gene product in a complex biological process such as angiogenesis may be obtained using gene-targeting strategies that employ DNA enzymes (DNAzymes). DNAzymes are synthetic, all-DNA-based catalysts that can be engineered to bind their complementary sequences in their target mRNA through Watson–Crick base pairing and cleave the mRNA at predetermined phosphodiester linkages (Khachigian, 2002). These catalysts have emerged as a potential new class of nucleic acid-based drugs because of their relative ease and low cost of synthesis and flexible rational design features. Gene specificity of a DNAzyme for an mRNA is determined by the sequence of deoxyribonucleotides in the hybridizing arms of the DNAzyme; the hybridizing arms are generally seven or more nucleotides long (Schubert *et al*., 2003). A 'general purpose' DNAzyme comprising a 15-nucleotide cation-dependent catalytic domain (designated '10–23') that cleaves the phosphodiester linkage between an unpaired purine and a paired pyrimidine in the target mRNA (Santoro and Joyce, 1997) was developed using a systematic *in vitro* selection strategy. DNAzymes do not rely on RNase H for destruction of the mRNA; these agents are stable in serum (Santiago *et al*., 1999; Dass *et al*., 2002) and can be produced at relative low cost. DNAzyme stability can be further increased, without compromising catalytic efficiency, by incorporation of structural modifications (such as base inversions, methylene bridges, etc.) into the molecule. We used DNAzymes targeting the immediate-early gene Egr-1 to suppress numerous vascular pathologic settings, such as intimal thickening after carotid artery injury in rats (Santiago *et al*., 1999; Lowe *et al*., 2002), in-stent restenosis after stenting coronary arteries in pigs (Lowe *et al*., 2001) and more recently, tumor angiogenesis (Fahmy *et al*., 2003). Here, we have inhibited SCC growth *in vitro* and *in vivo* using DNAzymes targeting c-Jun.

Results and discussion

c-Jun is expressed in human SCC and BCC

We performed immunohistochemistry on multiple sections of primary human SCC or BCC to determine the spatial pattern of c-Jun expression. Representative photomicrographs demonstrate that the antigen was strongly expressed in both skin cancer types (Figure 1), with comparatively weaker expression present in the normal overlying epidermis (Figure 1). c-Jun expression

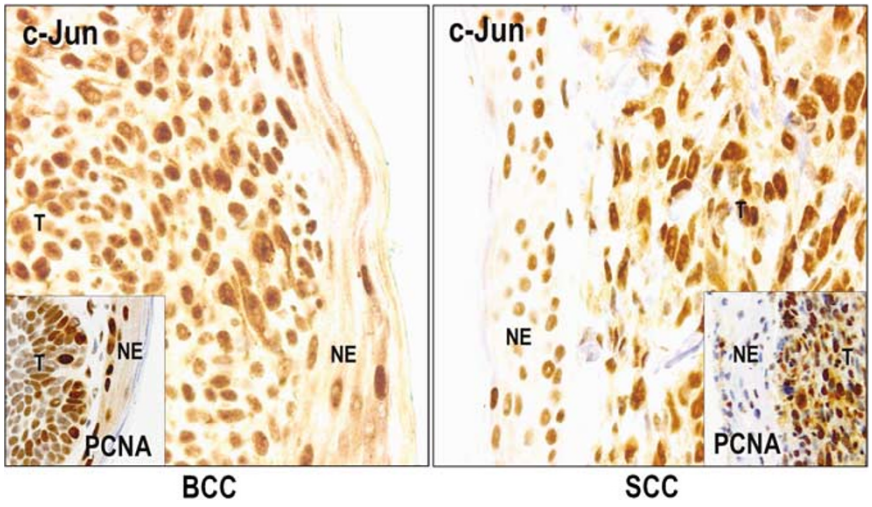

Figure 1 c-Jun is expressed in primary human SCC and BCC. Representative results from immunohistochemical analysis demonstrating c-Jun and PCNA expression in formalin-fixed paraffin-embedded SCC and BCC excised from humans. Immunostaining for each tumor type is representative of at least three independent human specimens. T = tumor; NE = normal overlying epidermis.

in these tumors strongly correlated with proliferating cell nuclear antigen (PCNA) staining indicating mitogenic activity (Figure 1). These data are supported by previous reports that have demonstrated that c-Jun is expressed in well-differentiated and moderately differentiated SCC (Karamouzis *et al*., 2004) and BCC (Ro, 1995). This led us to hypothesis that c-Jun may help drive skin carcinoma growth.

Dz13, but not Dz13scr inhibits c-Jun expression in SCC cells

To determine whether c-Jun is required for SCC growth, we transfected the T79 SCC cell line with Dz13 before stimulation of c-Jun expression with serum. These cells were rendered growth-quiescent by incubation in serum-free conditions for 24 h. Serum-inducible c-Jun protein expression after 2 h was inhibited by 0.2 and 0.4 μM Dz13 in a dose-dependent manner (Figure 2). Dz13scr, the scrambled-arm version of Dz13 which still retains the 15nt 10–23 catalytic domain (Santoro and Joyce, 1997), failed to suppress c-Jun (Figure 2). Dz13 also suppressed c-Jun levels in LK2 cells (Figure 2). The inability to grow BCC cells as lines in culture precluded *in vitro* investigation of Dz13 suppression of c-Jun expression and BCC growth.

Dz13 inhibits SCC proliferation

We hypothesized that as serum stimulates c-Jun expression in T79 SCC cells, Dz13 may negatively regulate proliferation induced by the mitogen in this cell type. Growth-quiescent T79 cells were exposed to serum for 3 days after transfection with or without Dz13 or Dz13scr. Dz13 inhibited serum-inducible T79 SCC proliferation in a dose-dependent manner at 0.2 and 0.4 μM (Figure 3a). To demonstrate that Dz13 inhibition of SCC cell growth was not confined to T79 cells, we performed proliferation assays using the LK2 SCC line. Serum stimulated LK2 growth within 2 days (Figure 3b), which was blocked by Dz13 in a dose-dependent manner at 0.2 and 0.4 μM (Figure 3b).

Dz13 inhibits solid SCC tumor growth and tumor angiogenesis in SCID mice

In order to determine whether Dz13 can influence the growth of solid SCC tumors, we assessed the effect of the DNAzyme on solid T79 SCC growth in immunocompromised severe combined immunodeficient (SCID) mice. Dz13 (50 μg) suppressed solid T79 SCC tumor growth 16 days after implantation (Figure 4a). Blinded quantitation of blood vessels in these tumors revealed Dz13 inhibition of tumor neovascularization (Figure 4b), whereas Dz13scr had no effect (Figure 4b).

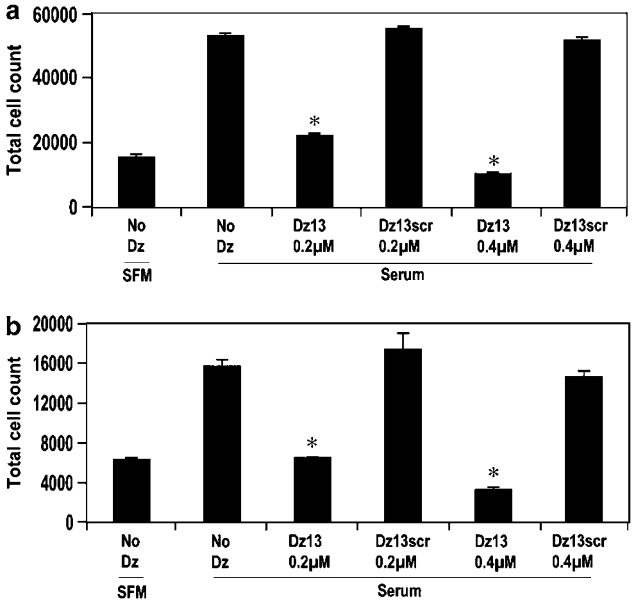

Figure 3 c-Jun DNAzyme suppresses SCC cell proliferation. Growth-quiescent (**a**) T79 SCC cells or (**b**) LK2 SCC cells were incubated with 10% serum for 2 days after prior transfection with 0.2 or 0.4 μM Dz13 using FuGENE6. The cells were resuspended using trypsin digestion and quantitated using a Coulter counter. The data are representative of at least two independent determinations performed in triplicate. * Indicates $P<0.05$ relative to control using Student's *t*-test.

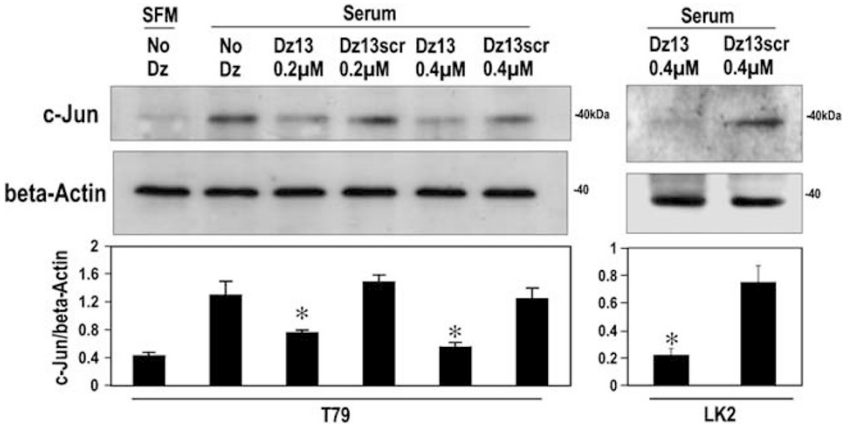

Figure 2 c-Jun DNAzyme suppresses c-Jun expression in SCC cells. Growth-quiescent T79 and LK2 SCC cells were incubated with 10% serum for 2 h after transfection with 0.2 or 0.4 μM Dz13 or Dz13scr, as indicated, using FuGENE6. Total cell lysates were prepared in RIPA buffer before Western blot analysis for c-Jun or β-actin and scanning densitometry. The data are representative of at least two independent determinations. * Indicates $P<0.05$ relative to control using Student's *t*-test.

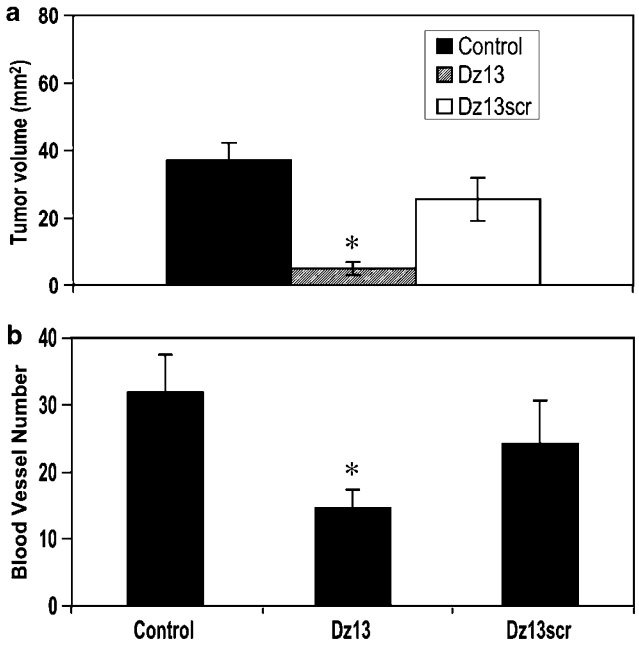

Figure 4 Dz13 inhibits T79 SCC growth as solid tumor xenografts in SCID mice. Six-week-old female SCID mice were injected subcutaneously in the dorsal mid-back region with a suspension of 5×10^5 T79 SCC cells in vehicle containing FuGENE6 with or without 50 μg Dz13 or Dz13scr. After 16 days, the mice were killed via cervical dislocation and their tumors were immediately removed and fixed in 10% formalin. The tumors were sectioned (6 μm) and stained with hematoxylin–eosin. (**a**) Tumor volume (in mm^3) was determined according to the formula: length $\times$ width $\times$ height $\times$ $\pi/6$. (**b**) Quantification of blood vessel density in three separate $\times$ 100 fields. $n = 5$ animals per group. * Indicates $P < 0.05$ relative to control using analysis of variance (ANOVA).

Dz13 inhibits c-Jun and MMP-9 expression in SCC tumors

To demonstrate that expression of c-Jun, the target of the DNAzyme, was indeed suppressed in the T79 SCC solid tumors, we performed immunohistochemical staining on cross-sections of paraffin-embedded formalin-fixed tissue. Dz13 suppressed c-Jun expression in these tumors (Figure 5a), whereas immunostaining in the Dz13scr cohort did not differ from the control (vehicle alone) group (Figure 5a). As matrix metalloproteinases, in particular MMP-9 (gelatinase B) are expressed and have strongly been implicated in SCC tumor invasion and progression (Kerkela and Saarialho-Kere, 2003), we immunostained for MMP-9 in these tumors. Dz13 suppressed MMP-9 staining in these tumors (Figure 5b). As MMP-9 is a c-Jun-dependent gene (Shin *et al*., 2002), these data suggest that Dz13 effects SCC solid tumor inhibition, at least in part, via its suppression of metalloproteinase.

Dz13 inhibits MMP-2 expression in SCC tumors

To provide additional evidence that Dz13 inhibits MMP-9 expression in the tumors, we assessed metalloproteinase activity in the supernatants of cultured T79 SCC cells by zymography. Transforming growth factor-β (TGF-β) stimulated MMP-9 activity (Figure 5c), which was inhibited by Dz13, but not Dz13scr (Figure 5c). Interestingly, Dz13 also suppressed MMP-2 activity (Figure 5c). Immunohistochemical analysis further revealed that Dz13 inhibits MMP-2 expression in the solid SCC tumors (Figure 5d). These findings are supported by our recent demonstration of Dz13 suppression of MMP-2 expression and activity in vascular endothelial cells (Zhang *et al*., 2004). Dz13 also reduced both VEGF-A (Figure 5e) and fibroblast growth factor (FGF)-2 (Figure 5f) expression in the tumors, whereas Dz13scr had no inhibitory effect. Dz13 suppression of the expression of these angiogenic growth factors is consistent with Figure 4b, showing lower blood vessel density in the active DNAzyme cohort.

In this paper, we have demonstrated that DNAzymes targeting the mRNA of the immediate-early gene c-Jun block c-Jun protein expression in SCC cells, inhibit SCC cell proliferation, and repress solid SCC tumor growth as xenografts. The DNAzyme inhibited both SCC cell growth and tumor angiogenesis. Dz13 blocked MMP-2 and MMP-9 expression *in vitro* and *in vivo,* both of which are capable of degrading type 4 collagen, a major component of basement membrane and are found activated in SCC patients (Patel *et al*., 2005). Although MMP-2 and MMP-9 are suppressed by Dz13, it is possible that c-Jun also regulates SCC growth through other MMPs. c-Jun was strongly expressed in human SCC and BCC, and expressed comparatively weakly in the overlying epidermis, possibly due to the 'field effect' of chronic UV exposure in these patients.

Our present findings using c-Jun DNAzymes are supported by previous studies linking c-Jun with SCC. Bowden *et al*. (1994) observed that a transactivation deletion mutant of c-Jun in two SCC cell lines (308 10Gy5 and PDV) blocked transactivation of AP-1-dependent reporter constructs driven by the c-jun, collagenase and stromelysin promoters, and compromised SCC formation following subcutaneous injection into athymic nude mice. Sato *et al*. (1997) demonstrated that a single dose of UVB (2 kJ/m^2), which can initiate SCC, applied to the backs of hairless mice transiently induced the expression of c-Jun, but not c-Ha-ras in the exposed area of the skin. Moreover, Malliri *et al*. (1998) found that epidermal growth factor-stimulated invasion of SCC-derived A431 cells was inhibited by a dominant-negative mutant of c-Jun, TAM67, suggesting a regulatory role for c-Jun in SCC invasion. As Dz13 can directly inhibit SCC cell growth, tumor vascularization and endothelial cell growth (Zhang *et al*., 2004), the *in vivo* efficacy of Dz13 as an inhibitor of solid SCC growth is likely to involve both direct and indirect suppression, via blockade of SCC proliferation and angiogenesis, respectively. Consistent with this, Dz13 inhibited the expression of two proangiogenic factors, VEGF-A and FGF-2 in the tumors. c-Jun DNAzymes may be useful as inhibitors of cutaneous SCC growth in the clinical setting. This could involve DNAzyme delivery via the intratumoral route as used in SCC patients treated locally with fluorouracil/epinephrine (Kraus *et al*., 1998), thereby avoiding surgery, systemic administration and associated risks.

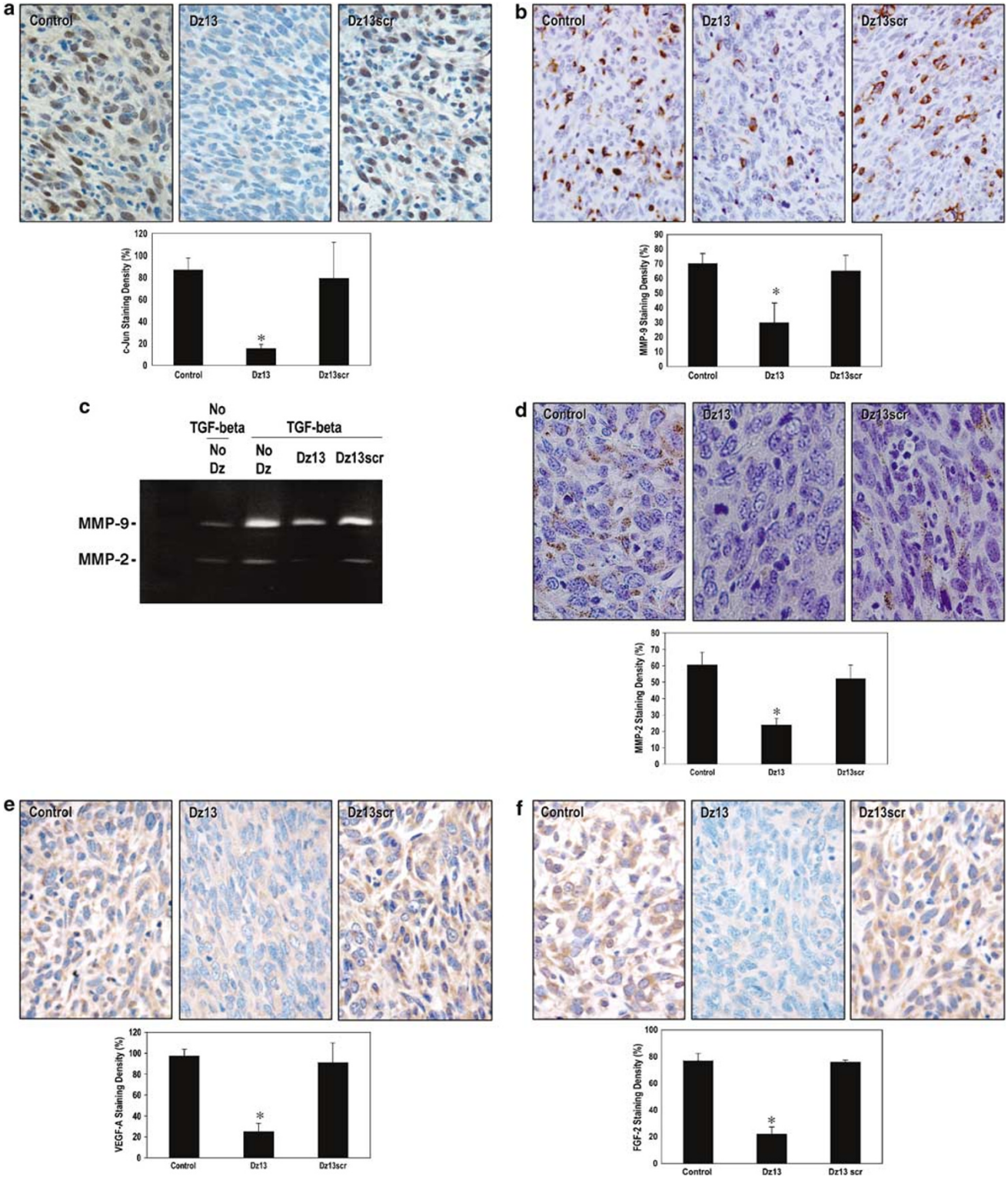

Figure 5 c-Jun, MMP-2 and MMP-9 expression is inhibited in solid T79 SCC tumors. Immunohistochemical analysis on formalin-fixed parrafin-embedded sections for (**a**) c-Jun and (**b**) MMP-9, (**d**) MMP-2, (**e**) VEGF-A and (**f**) FGF-2. (**c**) Zymographic assessment of MMP-9 and MMP-2 activity in the supernatants of TGF-β stimulated T79 SCC cells. Representative staining are shown in the figure. Staining density was quantified by counting the number of stained cells in three random fields per section (three separate sections/group) under $\times 400$ magnification. The y axis represents the number of cells staining minimally weakly expressed as a percentage of the total number of cells in that field. * Indicates $P<0.05$ relative to control using ANOVA.

Materials and methods

Source of primary human BCC and SCC, and immunohistochemical analysis

Six SCCs of the skin (two well differentiated, two moderately differentiated and two poorly differentiated) and four BCCs (one superficial, two solid and one cystic) were collected from patients undergoing surgical excision for treatment of the cancer. Immunohistochemistry using c-Jun and PCNA antibodies was performed on paraffin sections of the tumors using the streptavidin–biotin method and c-Jun rabbit polyclonal antibodies from Santa Cruz Biotechnology Inc. (CA, USA) and PCNA mouse monoclonal antibodies from DAKO (Denmark). The sections underwent microwave antigen retrieval. 3,3′-Diaminobenzene tetrahydrochloride was used as a chromogen for color development. Sections of human breast carcinoma served as positive controls. In the negative control, the primary antibody was replaced by phosphate-buffered saline (PBS) alone.

Cell culture, DNAzymes and transfection

T79 and LK2 SCC cell lines that arose in HRA:Skh-1 and C3H/HeN mice, respectively, exposed to chronic UV radiation (Halliday and Le, 2001) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, CA, USA) containing 2 mM glutamine, 5 U/ml penicillin–streptomycin and 10% fetal bovine serum (FBS) at 37°C, 5% CO_2. Dz13 and Dz13scr were synthesized by TriLink (San Diego, CA, USA) with a 3′-3′-linked inverted T. FuGENE6 reagent (Roche Diagnostics, Castle Hill, Sydney, Australia) was used to deliver Dz13 or Dz13scr, according to the manufacturer's instructions, 6 h after T79 and LK2 cell arrest in serum-free medium at subconfluence. Eighteen hours after first transfection, the cells were transfected again and the medium was then changed back to 10% serum-containing medium. The 'double-hit' transfection approach was used in order to maximize DNAzyme suppression of c-Jun expression. The first delivery would enable the DNAzyme to be in the cell before the induction of c-Jun mRNA.

Western blot analysis

Serum-starved T79 and LK2 SCC cells, seeded at a density of 7000 cells/100-mm Petri dish in 10 ml medium, were transfected twice with Dz13 or Dz13scr or vehicle alone at 60% confluence then incubated in 10% FBS for 2 h. The cells were harvested into radioimmunoprecipitation assay (RIPA) buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 5 mM ethylenediaminetetraacetic acid, 1% aprotinin, 2 mM phenylmethyl sulphonyl fluoride, followed by two freeze–thaw cycles and centrifugation at 8000 *g* in a microfuge (Sigma, MO, USA) for 20 min at 4°C to remove cell debris. Two and a half micrograms of protein was resolved on 12% polyacrylamide gels and transferred to polyvinylidene fluoride nylon membranes (Millipore, Bedford, MA, USA). The membranes were then probed with rabbit polyclonal c-Jun antibodies or β-actin (1:1000; 200 ng/ml; Santa Cruz Biotechnology, CA, USA) and incubated with horseradish peroxidase-conjugated swine-anti rabbit IgG (1:1000, DAKO, Denmark). The protein bands were visualized using the Western Lightning chemiluminescence kit (PerkinElmer Life Sciences, Boston, MA, USA).

Cell proliferation assay

Growth-quiescent T79 and LK2 cells, seeded in 96-well titre plates at a density of 2500 cells/200 μl well, were transfected with Dz13 or Dz13scr and incubated in 10 % serum for 2 days. The cells were harvested by trypsinization and resuspended in Isoton II (Coulter Electronics, Brookvale, NSW, Australia). Total cells suspensions were quantitated using a Coulter counter (Z series; Coulter Electronics).

T79 SCC solid tumor growth study and immunohistochemical analysis

Six-week-old female SCID mice (Animal Resource Centre, Perth, WA, Australia) were anesthetized by peritoneal injection with ketamil (60 μg/g) and ilium xylazil-20 (4 μg/g) (Troy Laboratories, Sydney, NSW, Australia) and injected subcutaneously in the dorsal mid-back region with 5×10^5 T79 cell suspension in 200 μl vehicle (50% Matrigel in PBS, pH 7.4) containing 2.5 μl FuGENE6 alone or with 50 μg of Dz13 or Dz13scr. Each group comprised five mice. At 16 days, the mice were killed via cervical dislocation and their tumors were immediately removed and fixed in 10% formalin. The tumors were sectioned (6 μm) and stained with hematoxylin–eosin. The number of blood vessels in three random $\times 100$ fields was determined in a blinded manner on two independent occasions. Tumor volumes (in mm^3) were determined according to the formula: length $\times$ width $\times$ height $\times \pi/6$. All animal procedures were approved by Animal Care and Ethics Committee of the University of New South Wales.

Sections of formalin-fixed and paraffin-embedded SCC tumor implanted in SCID mice as described above were used. Following deparaffinization and rehydration, the sections were incubated with rabbit polyclonal antibodies to c-Jun (1:200 dilution; Santa Cruz Biotechnology), MMP-9 (1:500 dilution; Sapphire Bioscience, Sydney, NSW, Australia), MMP-2 (1:20 dilution; Santa Cruz Biotechnology), VEGF-A (1:200 dilution, Santa Cruz Biotechnology) or FGF-2 (1:200 dilution, Santa Cruz Biotechnology). Immunoreactivity was indicated by incubating the sections with biotinylated secondary anti-rabbit antibodies and then Strepavidin (DAKO), further incubation with 3,3′-diaminobenzidine (Sigma), and counterstaining with Harris hematoxylin. Staining density was quantified by counting the number of stained cells in three random fields per section (three separate sections/group) under $\times 400$ magnification. The *y* axis represents the number of immunostained cells expressed as a percentage of the total number of cells in that field.

Gelatin zymography for MMP-2 and MMP-9 activity

Serum-starved T79 SCC cells were transfected twice with Dz13 or Dz13scr as described above. In the second transfection, cells were incubated with 10 ng/ml TGF-β (Promega, WI, USA) to induce MMP-2 and MMP-9 expression for 2 days in serum-free medium. The culture medium was harvested for measurement of MMP-2 and MMP-9 activity by zymography essentially as described (Zhang *et al*., 2004). Briefly, supernatant was electrophoresed on 10% polyacrylamide gels containing 1 mg/ml bovine type B gelatin (Sigma) at 4°C. After washing in 2.5% Triton X-100 for 30 min twice, gels were incubated in substrate buffer overnight at 37°C. Gels were stained in 0.2% Coomassie Blue R-250 and destained to reveal gelatinolytic activity.

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