Identification of Dss1 as a 12-O-Tetradecanoylphorbol-13-acetate-responsive Gene Expressed in Keratinocyte Progenitor Cells, with Possible Involvement in Early Skin Tumorigenesis*

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This study identifies genes expressed early in 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced skin carcinogenesis in genetically initiated TgAC v-Ha-ras transgenic mice. Keratinocyte progenitor cells from TPA-treated TgAC mice were isolated with fluorescence-activated cell sorting and expression was analyzed using cDNA microarray technology. Eleven genes were identified whose expression changed significantly in response to carcinogen treatment. Deleted in split hand/split foot 1 (Dss1) is a gene associated with a heterogeneous limb developmental disorder called split hand/split foot malformation. cDNA microarray expression analysis showed that the mouse homologue of Dss1 is induced by TPA. Dss1 overexpression was detected by Northern blot analysis in early TPA-treated hyperplastic skins and in JB6 Cl 41-5a epidermal cells. Interestingly, Dss1 expression was also shown to be elevated in skin papillomas relative to normal skins, and further increased in squamous cell malignancies. Functional studies by ectopically constitutive expression of Dss1 in JB6 Cl 41-5a preneoplastic cells strongly increased focus formation and proliferation of these cells and enhanced efficiency of neoplastic transformation of the cells in soft agar. These results strongly suggest that Dss1 is a TPA-inducible gene that may play an important role in the early stages of skin carcinogenesis.

Skin carcinogenesis is a complex multistage process that progresses through distinct stages of initiation, promotion, progression, and malignancy (1–3). The TgAC mouse is a genetically modified (transgenic) form of the FVB/N mouse strain that carries a genomic copy of the v-Ha-ras gene fused to a fetal z-globin gene promoter (4). TgAC mice have already entered the initiation stage of cancer development and have a higher sensitivity to many types of environmentally inducible cancer than wild-type mice. TgAC mice develop hyperplasia in skin keratinocytes after exposure to tumor promoters such as TPA

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¶ The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; Dss1, Deleted in split hand/split foot 1; FACS, fluorescence-activated cell sorting; KSC, keratinocyte stem cell; SHFM1, split hand/split foot malformation; SMART, switching mechanism at the 5' end of DNA templates; TA, transit amplifying cell; RT, reverse transcription; MEM, minimal essential medium; FBS, fetal bovine serum; EGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; DAPI, 4,6-diamidino-2-phenylindole.
play important roles in skin tumor formation and metastasis. These genes include transin (19), c-myc and c-fos (20), mal1 (21), CD44 (22), urokinase plasminogen activator (23), MMP-9 (24), and serine protease BSSP (25). Many of these genes were identified using in vitro methods and cultured cell lines or two-step in vivo carcinogenesis with mouse skin as a target. These studies should be interpreted with some caution because the exact nature of the target cells may not be known with precision. This study identifies a novel gene that is induced in TPA-treated cells using a different approach than previous studies. The candidate integrin α6′/CD34′ keratinocyte progenitor cells were isolated from hyperplastic skin of TPA-treated animals and their gene expression analyzed using cDNA microarray. This method employs FACS, switching mechanism at the 5′ end of RNA templates (SMART) cDNA amplification, and mouse cDNA array technology. Eleven TPA-responsive genes were identified in the TgAC mouse; nine genes were significantly up-regulated by TPA, and two genes were remarkably down-regulated by TPA. Des1 was selected from the nine TPA-up-regulated genes for further characterization.

Des1 was originally identified on human chromosome 7q21.3-q22.1 as a gene deleted in patients with the heterogeneous limb developmental disorder SHFM1. Des1 encodes a 70-amino acid, highly acidic peptide (26). Up-regulation of Des1 was detected in TPA-treated mice using cDNA microarray and verified by semiquantitative RT-PCR, Northern blot, and in situ hybridization. Functional analysis of Des1 gene in TPA susceptible JB6 Cl 41-5a preneoplastic epidermal cells suggests that it is required for cell proliferation and neoplastic transformation.

MATERIALS AND METHODS

Animals and Cell Culture

Eight- to 10-week-old male homozygous Tg.AC mice were obtained from Taconic Laboratory of Animals and Services (Germantown, NY). Animal studies were carried out in compliance with NIH Guidelines for Humane Care and Use of Laboratory Animals. TPA-susceptible JB6 Cl 41-5a and TPA-resistant JB6 Cl 30-7b BALB/c mouse epidermal cell clonal variants, generated by Nancy Colburn et al. (27), were from American Type Culture Collections (Manassas, VA) and grown at 37 °C in a 5% CO2 atmosphere in Eagle’s minimal essential medium (Eagle’s MEM) supplemented with 5% heat-inactivated foetal bovine serum (FBS) containing 10% streptomycin sulfate (Invitrogen). Mouse fibroblast cells Rat-1 and monkey transformed kidney cells COS-1 were cultured in Dulbecco’s modified Eagle’s medium (DME) with 10% FBS, NIH/3T3 cells were maintained as described previously (28). Cell lines used in this study were free of mycoplasma infection.

Topical Treatment with TPA

Five micrograms of TPA (Sigma) in 200 μl of acetone were applied topically to groups of 5 homozygous male Tg.AC mice twice weekly for 2 weeks. Untreated control mice were sacrificed on day 1 (NS). Four dosing protocols were used as follows. Mice were dosed on day 1 and sacrificed on day 5 (designated as TPA1); mice were dosed on days 5 and sacrificed on day 5 (designated as TPA2); mice were dosed on days 1, 5, and 8; and sacrificed on day 12 (designated as TPA3); mice were dosed on days 1, 5, and 8 and sacrificed at least 48 h after the last dose (designated as TPA4). Papillomas and malignant tumors (one spindle cell tumor and two squamous cell carcinomas) were identified, removed, and characterized as described previously (7).

Gene Expression Profiling

AtlasMouse 1.2 MicroArray carrying 1176 cDNAs was obtained from Clontech (Palo Alto, CA). The keratinocytes were harvested from the dorsal skin of Tg.AC mice (29), and the integrin α6′/CD34′ keratinocyte progenitor cells were isolated using FACs (18). Total RNAs were extracted from TPA-treated or -untreated keratinocyte progenitor cells using StrataPrepTotal RNA Miniprep Kit (Stratagene, La Jolla, CA). Ten nanograms of total RNA was reverse-transcribed and amplified using the Atlas SMART™ system (Clontech). Five hundred nanograms of purified SMART cDNA were labeled for 30 min at 50 °C with [α-32P]dATP (10 μCi/μl; >2500 Ci/mmol; Amerham Biosciences) by using Klenow DNA polymerase (Clontech) and random hexamer priming. Array membrane was prehybridized with prewarmed ExpressHyb for 30 min with continuous agitation at 68 °C, and hybridized overnight with [α-32P]dATP-labeled probes (3.7 × 107 cpm/ml). The membrane was washed four times in 2× saline sodium citrate (SSC), 1% SDS for 30 min at 68 °C, and two times in 0.1× SSC, 0.5% SDS. Array membrane was scanned with a phosphorimagery (Typhoon 8600, Amerham Biosciences), and signals were quantified using ImageQuant 5.1 software (Amerham Biosciences).

Vector Constructions

The full-length Des1 cDNA was amplified by RT-PCR using Tg.AC mice skin total RNA. The Des1 forward and reverse primers were 5′-CAC GTCTG TGA AAA GAA GCA GCC-3′ and 5′-TGA TGT CT CTT CTT GGA GCC GTG CTG TT-3′, respectively. The PCR amplified Des1 cDNA was cloned into V5-His-tagged pcDNA3.1D/V5-His-TOPO™ mammalian expression vector (Invitrogen, Carlsbad, CA). Des1 cDNA fragment was inserted into retroviral vector by digesting pcDNA3.1D/Dl95-V5-His plasmid with HindIII and NotI and ligated in the HindIII-NotI sites of the pLNCXp (Clontech) using the LigateFast™ Rapid DNA Ligation System (Promega, San Luis, CA). A construct expressing Des1 sense or antisense RNA was generated as follows; pcDNA3.1D/Dl95-V5-His was digested with BamHI and XbaI, and the Des1 cDNA-fragmenting site was cloned into the BamHI- XbaI sites of the pT3/T7-U19 plasmid (Ambion, Austin, TX). The pEGFP-C3 vector (Clontech) was used to express the full-length Des1 protein fused to the C terminus of the enhanced green fluorescent protein (EGFP) in the JB6 Cl 41-5a epidermal cells. The pcDNA3.1D/Dl95-V5-His plasmid was digested sequentially with KpnI and ApeI and ligated into the KpnI-ApeI sites of the pEGFP-C3 plasmid using the LigateFast™ Rapid DNA Ligation System (Promega). The pEGFP-C3 plasmid expressing the native EGFP protein was used as a control. All the construct sequences were verified using an automated Applied Biosytems sequencer and the BigDye™ Terminator Kit (PerkinElmer Life Sciences, Foster City, CA). Plasmid DNAs were purified using purification kits from Qiagen (Stanford Valencia, CA) and were endotoxin-free when used for transfection in mammalian cells.

Cell Transduction

Cells were transfected with vector (mock), pcDNA3.1D/Dl95-V5-His, or pEGFP-C3/Dl95 plasmid DNA using LipofectAMINE PLUS™ reagents (Invitrogen) or by infection with virions packaged with ecdysone proneo plasmid virus. Cells were scored for packaging cells RetroPack™ PT-67 (Clontech). The cDNA for Dss1 was inserted into pLNCX, retroviral vector carry mouse Des1 gene driven by the cytomegalovirus promoter and neo gene driven by the long terminal repeat promoter. Plasmid-transfected and virus-infected cells were cultured for at least 2 weeks in medium containing 400 μg/ml Geneticin (G418) (Invitrogen). Cells were analyzed by Western blot or RT-PCR to confirm the expression of Des1.

RT-PCR Analysis

The single-stranded cDNA was prepared from total RNA using the Moloney murine leukemia virus reverse transcriptase SuperScript II (Invitrogen) with oligo(dT) primer and used as a template for PCR. The primers used for PCR were as follows: Des1 sense (5′-GAG GAG TTC CGT GAC GAG TAT CTC CCA-3′) and antisense (5′-ATG ATT GAG GGT CCC CC-3′) are from Genetologics Corp. (La Jolla, CA); pLNCX sequencing/PCR forward (2882–2906) (5′-ATCTTT GAG TGA GAA CCG TCA GAT C-3′) and reverse primers (3057–3032) (5′-ACC TAC AGG TGG GTT CTT TCA TCC CC-3′) are from Clontech; β2-microglobulin sense (5′-GAC TGG TCT CCT TAT ATC CGT G-3′) and antisense (5′-TTT GTT CGC TGC ATTA AAT TG-3′) are from Sigma; and β-actin sense and antisense primers were obtained from Clontech. PCR cycling was as follows: denaturation (94 °C, 45 s), annealing (58 °C, 45 s), and extension (72 °C, 2 min) for 30 cycles. Reaction was carried out in a PerkinElmer 9600 thermal cycler (PerkinElmer Life Sciences), and PCR products were analyzed using 2% agarose gels.

Northern Blot Analysis

Total RNA was prepared using a TRIzol reagent kit (Invitrogen) and digested with RNase-free DNase 1 (Ambion). Eight micrograms of isolated RNAs were separated electrophoretically on a 1% agarose gel containing glyoxal and transferred onto a BrightStar-Plus nylon membrane (Ambion). The membrane was UV-cross-linked and probed with...
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[Figure 1. a. Identification of genes differentially expressed between TPA-treated and -untreated skin integrin α6−CD34+ keratinocyte progenitor cells using a microarray analysis. [α-32P]UTP-labeled Dss1 antisense RNA (1 × 10⁶ cpm/ml). The riboprobe was prepared using in vitro Strip-EZ™ T7 RNA transcription kit (Ambion) with EcoRI-linearized T3/T7-U19-Dss1 as a template. Autoradiographs were developed using Amersham Biosciences hyperfilm™ MP at −80 °C. The integrity of total RNA is good, and the ratio of 28 and 18S ribosomal RNAs is ~2:1 in all samples. The signals were quantified using ImageQuant 5.1 software (Molecular Dynamics).

In Situ Hybridization

The in situ hybridization assay was performed as previously described (30). Briefly, the cutaneous tumors were removed from TgAC mice and fixed overnight in 10% neutral buffered formalin. The tissues were paraffin-embedded, and sections (6 μm) were cut onto SuperFrost plus microslide slides (Dugger, Vernon Hills, IL). The sections were deparaffinized and rehydrated by successive washes in xylene and graded alcohols to 2× SSC, then in 2× SSC, 0.5× SSC, 0.5× SSC, then applied with 20 μl of RNase (10 mg/ml). After several washes, the slides were dehydrated in graded alcohols and completely air-dried. The slides were then dipped into NTB-3 autoradiographic emulsion (Eastman Kodak), exposed for 10 days at room temperature in the dark, dried in a light-tight container, and developed in Kodak D19 fixer and developer. The sections were counterstained with hematoxylin, covered with coverslips, and photographed under dark-field illumination (model BX51, Olympus Optical Co., Tokyo, Japan).

Subcellular Localization

The eighty nanograms of different green fluorescent protein (GFP) constructs, pEGFP-C3 and pEGFP-C3/Dss1, were transiently transfected into the JB6 Cl 41-5a cells cultured at 37 °C in eight-well culture slides (Falcon, Bedford, MA) at a cell density of 2 × 10⁵ cells/well using LipofectAMINE PLUS™ reagents (Invitrogen) according to the protocol of the manufacturer. The GFP fluorescence was observed 48 h after transfection. Cells were washed twice with ice-cold 1× phosphate-buffered saline buffer (150 mM NaCl, 10 mM Na2HPO4, 10 mM KH2PO4, pH 7.4) and fixed in 2% paraformaldehyde in 1× phosphate-buffered saline for 10 min at room temperature. After washing five times for 2 min each, the cells were mounted with the Prolong antifade medium (Molecular Probes, Eugene, OR). For nuclear localization, a DNA-bound nucleic dye 4,6-diamidino-2-phenylindole (DAPI) (Vector, Burlingame, CA) was used. Cells were observed by fluorescence microscopy using a Leica DMRBE microscope (Wetzlar GmbH) equipped with a 63× objective and 100-watt mercury source. The images were taken with a Chroma GFP filter set for EGFP (excitation maximum 488 nm, emission maximum 507 nm), a DAPI filter set for chromatin (excitation maximum 350/364 nm, emission 400/505 nm), and a Spot RT cooled charge-coupled device (CCD) camera (Diagnostic Instruments, Inc., Sterling Heights, MI), and MetaMorph 5.0 software (Universal Imaging Corp., Downingtown, PA). Individual images were pseudocolored and overlaid.

For immunocytochemical analysis, cells (1 × 10⁵) were transiently transfected with 4 μg of pcDNA3.1/Dss1-V5-His in a 10-cm tissue culture plate using LipofectAMINE PLUS™ reagents (Invitrogen). Cells were incubated for 48 h, and 2 × 10⁵ cells were seeded on eight-well culture slides until cells attached. Cells were fixed with methanol for 10 min, probed with normal mouse IgG (negative control) or anti-V5 tag mouse monoclonal antibody for 30 min, and stained with amplified cDNA probes, prepared from TPA-treated (a) or -untreated (b) TgAC mouse skin integrin α6−CD34+ keratinocyte progenitor cells. Total RNAs (10 ng), were hybridized to separate Atlas™ Mouse 1.2 MicroArray according to the user manual (Clontech). The cDNAs in Atlas™ Mouse 1.2 MicroArray containing 1176 genes are printed in single spots. Results were quantified using ImageQuant 5.1 software. The remarkably similar array results were obtained in three independent experiments, one of which was shown. Dss1 gene is indicated by a circle. b, RT-PCR. Ten nanograms of total RNAs from TgAC mouse skin integrin α6−CD34+ keratinocyte progenitor cells treated or -untreated with TPA were assayed by semiquantitative RT-PCR using the Dss1-specific primers and housekeeping gene β2 microglobulin-specific primers, as described under "Materials and Methods." RT-PCR products were analyzed electrophoretically on 2% agarose gels.

[Figure 1. b. DNA ladder (Stratagene, La Jolla, CA) was used. Cells were observed by fluorescence microscopy using a Leica DMRBE microscope (Wetzlar GmbH) equipped with a 63× objective and 100-watt mercury source. The images were taken with a Chroma GFP filter set for EGFP (excitation maximum 488 nm, emission maximum 507 nm), a DAPI filter set for chromatin (excitation maximum 350/364 nm, emission 400/505 nm), and a Spot RT cooled charge-coupled device (CCD) camera (Diagnostic Instruments, Inc., Sterling Heights, MI), and MetaMorph 5.0 software (Universal Imaging Corp., Downingtown, PA). Individual images were pseudocolored and overlaid.
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**Western Blot Analysis**

Cells were lysed as described previously (32). Briefly, cells were lysed in lysis buffer consisting of 50 mm Tris-HCl (pH 7.5), 4 mm EDTA, 2 mm EGTA, 10 mm dithiothreitol, and 1 mm phenylmethylsulfonyl fluoride (Sigma). The whole-cell lysates were sonicated on ice and centrifuged at 100,000 × g for 1 h at 4 °C. Protein concentration was determined by Bradford assay (Bio-Rad). Proteins were separated electrophoretically on 10 or 12% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Amersham Biosciences). The membranes were probed with primary antibodies including anti-V5 mouse monoclonal antibody (1:5000) (Invitrogen) and anti-EGFP rabbit polyclonal antibody (1:2000) (Clontech). These antibodies were detected using horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:3000) (Amersham Biosciences) and enhanced chemiluminescence (ECL) (Amersham Biosciences). The membranes were stripped and rehybridized with an anti-α-tubulin mouse monoclonal antibody (1:1000) (Zymed Laboratories Inc., San Francisco, CA) for control to confirm loading efficiency.

**Transformation Assays**

*Focus-forming Activity—*Cells were seeded overnight at a density of 2 × 10⁴ cells/well in six-well plates. Cells were transfected with 1 μg of vector or pcDNA3.1/Dss1-V5-His and selected in medium containing 0.5% agar in Eagle’s MEM with 10% FBS and layered over 5 ml of medium containing 0.5% agar in Eagle’s MEM with 10% FBS. Cells were grown in 5% CO₂ atmosphere, and colonies with more than 8 cells were counted 18 days after seeding.

**RESULTS**

*In Vivo Gene Expression Profiles in Keratinocyte Progenitor Cells of TPA-treated TgAC Mice—*The goal of this study was to identify novel genes induced in the skin by TPA using an in vivo mouse model system. Gene expression profiles were determined using a mouse cDNA array spotted with 1176 genes. TgAC mice were treated with TPA, and keratinocyte progenitor cells carrying the cell surface markers, integrin α6 and CD34, were isolated by FACS. Control cells were harvested from animals not treated with TPA. The cDNA was prepared from 10 ng of RNase-free DNase 1-treated total RNA from keratinocyte progenitor cells by two independent methods: reverse transcription and PCR-based SMART amplification. The cDNA was labeled using Klenow-mediated incorporation of [α-3²P]dATP. The hybridization signals were quantified densitometrically using ImageQuant 5.1 software. The genes were characterized if their expression changed 2-fold or more in TPA-treated cells. Eleven genes were identified by gene expression profiling for which expression was up- or down-regulated by TPA (Fig. 1A). Nine genes were up-regulated and two genes were down-regulated by TPA (see Table I). Dss1 was induced 3.5-fold, and it was selected for further study. Dss1 expression was also verified in TPA-treated and untreated keratinocyte progenitor cells by semiquantitative RT-PCR. As shown in Fig. 1B, the result was consistent with the microarray experiment (i.e., 2–3-fold increase in Dss1 expression in cells exposed to TPA).

*Dss1 Is a TPA-responsive Gene Induced Early in Skin Tumorigenesis—*Previous studies demonstrated that chronic topical application of TPA to the skin of TgAC mice induces epidermal hyperplasia (30). Dss1 expression was analyzed in hyperplastic skin in TgAC mice exposed to various doses of TPA, as described under “Materials and Methods.” Dss1 expression increased in hyperplastic skin in a dose- and time-dependent manner (Fig. 2A). Similar results were obtained previously for TPA-induced expression of PCNA (30).

The dose response and kinetics of TPA-induced transcriptional activation of Dss1 were investigated using JB6 Cl 41-5a preneoplastic epithelial cells. Cells were grown in 5% FBS/Eagle’s MEM containing 0, 0.1, 1.0, and 100 ng/ml TPA; viable cells were harvested at 18 h, and total RNA was prepared for Northern blot analysis. Fig. 2B (a) shows that TPA induced Dss1 1.7-fold at 1.0 ng/ml, and maximal induction (∼2-fold) was reached at 10–100 ng/ml TPA. A kinetic analysis at 0, 1, 2, 4, 8, 12, 18, 24, and 36 h after treatment with 10 ng/ml TPA showed that Dss1 was induced 2.5-fold 1 h after TPA treatment and reached a maximal level of 3–5-fold 12–18 h after treatment. Dss1 expression appeared to decrease slightly 8 h after treatment and began to decline from the maximal level 18 h after treatment (Fig. 2B, b).

**Tissue Distribution of Dss1—*Tissue distribution of Dss1 mRNA was examined in TgAC adult mice using Northern blot analysis. Dss1 mRNA was transcribed in adult mouse tissues including heart, ovary, stomach, and skin. Dss1 was expressed at a higher level in heart than other tissues. In kidney, liver, lung, and spleen, Dss1 expression was barely detectable and Dss1 mRNA was not detected in brain and small intestine (Fig. 3). Similar results were observed in other strains of mice including BALB/c and C57BL/6 (data not shown).
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Subcellular Localization of Dss1—Tagging expressed proteins with the GFP from the jellyfish Aequorea victoria is a highly specific and sensitive technique for studying the intracellular dynamics of proteins and organelles (34, 35). We have constructed a vector encoding an EGFP-Dss1 fusion protein to directly examine the subcellular localization of Dss1 in epidermal cells. The plasmid DNA pEGFP-C3 or pEGFP-C3/Dss1, which expressed EGFP-Dss1 fusion protein, was transiently transfected into JB6 Cl 41-5a cells using the LipofectAMINE DNA transfection method. The pEGFP-C3 plasmid containing the cDNA encoding for EGFP alone was used as a control. After 48 h of transfection, cells were collected for preparation of the whole-cell lysates. As shown in Fig. 4A, Western blot analysis showed that EGFP-Dss1 fusion protein was efficiently expressed in JB6 Cl 41-5a cells using an anti-EGFP rabbit polyclonal antibody, when compared with mock EGFP control protein. Fig. 4B also showed the photographs obtained by fluorescence microscope. EGFP-Dss1 fusion protein had a diffuse and uniform green fluorescent distribution throughout the nucleus (I and IV), and was also detected in cytoplasm (IV). Just after taking the EGFP-Dss1 images, a DNA-bound fluorescent dye DAPI was added and the nucleus was stained into blue color (II and V). The light blue areas (III and VI) were obtained upon merging of the green (I and IV) and blue (II and V) images of identical cell. No cells were observed and exhibited a plasma membrane localization of Dss1. This distribution is similar to that seen in cells expressing GFP alone (data not shown). A similar expression pattern and distribution was also observed by immunocytochemical staining using anti-V5-tagged mouse monoclonal antibody to detect the V5-Dss1 native fusion protein (Fig. 4C, II). The negative control was probed with normal mouse IgG and showed the specificity of anti-V5-tagged mouse monoclonal antibody in immunocytochemical analysis (Fig. 4C, III).

Dss1 Overexpression Enhances Neoplastic Transformation in Preneoplastic Mouse Epidermal Cells—Dss1 was constitutively expressed in preneoplastic epidermal cells to determine whether increased expression of Dss1 potentially stimulates tumorigenesis in the skin. One plasmid construct, pcDNA3.1/Dss1-V5-His, was prepared to constitutively express Dss1 in TPA-induced skin tumors. As shown in Fig. 5A, Northern blot analysis confirmed that this construct expressed Dss1 efficiently in COS-1, JB6 Cl 30-7b, JB6 Cl 41-5a, Rat-1, and NIH/3T3 cells (Fig. 6A).

Constitutive expression of Ras family proteins and other oncogenic proteins increase foci-forming capability and decrease growth contact inhibition of normal untransformed cells

Fig. 2. A, overexpression of Dss1 mRNA in early TPA-induced hyperplastic skin tissues. The multiple doses of TPA treatment of TgAC mouse skins were extracted for total RNAs using TRIzol reagent (Invitrogen). Isolated RNA (8 μg) was separated electrophoretically on a 1% agarose gel containing glyoxal, transferred to the nylon membrane, and cross-linked by UV Stratalinker. The membrane was probed with [α-32P]UTP-labeled Dss1 (top panel) or GAPDH (lower panel) antisense RNA (1 × 106 cpm/ml). Autoradiographs were developed with Amersham Biosciences hyperfilm TM at –80 °C. RNA quantitation was determined by ImageQuant 5.1 software. The arrows indicate that the sizes in 0.5 and 1.4 kb are Dss1 and GAPDH, respectively. GAPDH was served as an internal control and attested to the equivalent amounts of total RNA loaded in each lane. B, TPA-induced transcriptional activation of Dss1 was in a dose- and time-dependent manner. TPA-susceptible JB6 Cl 41-5a epidermal cells were either treated for 18 h with indicated TPA concentrations (a) or exposed to 10 ng/ml TPA at indicated time points (b). Cells were harvested and total RNA was extracted using TRIzol reagent (Invitrogen). Eight micrograms of total RNAs were loaded in Northern blot analysis.

Fig. 3. Dss1 is expressed in a variety of TgAC mouse tissues. Tissue samples of the brain (B), heart (H), small intestine (SI), kidney (K), liver (Li), lung (Lu), ovary (O), spleen (Sp), stomach (St), and skin (Sk) were homogenized and extracted for total RNA using TRIzol reagent (Invitrogen). Mouse tissue total RNA (8 μg) Northern blot membrane was probed with [α-32P]UTP-labeled Dss1 (top panel) or GAPDH (lower panel) antisense RNA.

Subcellular Localization of Dss1—Tagging expressed proteins with the GFP from the jellyfish Aequorea victoria is a highly specific and sensitive technique for studying the intracellular dynamics of proteins and organelles (34, 35). We have constructed a vector encoding an EGFP-Dss1 fusion protein to directly examine the subcellular localization of Dss1 in epidermal cells. The plasmid DNA pEGFP-C3 or pEGFP-C3/Dss1, which expressed EGFP-Dss1 fusion protein, was transiently transfected into JB6 Cl 41-5a cells using the LipofectAMINE DNA transfection method. The pEGFP-C3 plasmid containing the cDNA encoding for EGFP alone was used as a control. After 48 h of transfection, cells were collected for preparation of the whole-cell lysates. As shown in Fig. 4A, Western blot analysis showed that EGFP-Dss1 fusion protein was efficiently expressed in JB6 Cl 41-5a cells using an anti-EGFP rabbit polyclonal antibody, when compared with mock EGFP control protein. Fig. 4B also showed the photographs obtained by fluorescence microscope. EGFP-Dss1 fusion protein had a diffuse and uniform green fluorescent distribution throughout the nucleus (I and IV), and was also detected in cytoplasm (IV). Just after taking the EGFP-Dss1 images, a DNA-bound fluorescent dye DAPI was added and the nucleus was stained into blue color (II and V). The light blue areas (III and VI) were obtained upon merging of the green (I and IV) and blue (II and V) images of identical cell. No cells were observed and exhibited a plasma membrane localization of Dss1. This distribution is similar to that seen in cells expressing GFP alone (data not shown). A similar expression pattern and distribution was also observed by immunocytochemical staining using anti-V5-tagged mouse monoclonal antibody to detect the V5-Dss1 native fusion protein (Fig. 4C, II). The negative control was probed with normal mouse IgG and showed the specificity of anti-V5-tagged mouse monoclonal antibody in immunocytochemical analysis (Fig. 4C, III).

Dss1 Overexpression in TPA-induced Skin Tumors—TPA induced an increase in Dss1 transcription level not only in in vitro keratinocyte progenitor cells and in early hyperplastic mouse skin, but also in in vitro JB6 Cl 41-5a cells. Dss1 expression was also examined in TPA-induced skin tumors. Interestingly, Dss1 RNA transcription was higher in TPA-mediated TgAC mouse skin tumors, including eight papillomas (2.5 ± 0.4-fold) and three malignant tumors (one spindle cell tumor and two squamous cell carcinomas) (6.2 ± 1.3-fold) than in normal skin (Fig. 5A). In addition, in situ hybridization assay was also employed to detect the expression of the Dss1 messenger RNA in TPA-induced skin tumors. As shown in Fig. 5B, Dss1-specific signals were overexpressed and localized in the squamous region of the papillomas (II) and malignancies (squamous cell carcinomas) (V), with some expression in the adjacent epidermis and hair follicles. However, normal-appearing skin adjacent to the papillomas and malignancies did not contain detectable Dss1 message (data not shown).

Dss1 Overexpression Enhances Neoplastic Transformation in Preneoplastic Mouse Epidermal Cells—Dss1 was constitutively expressed in preneoplastic epidermal cells to determine whether increased expression of Dss1 potentially stimulates tumorigenesis in the skin. One plasmid construct, pcDNA3.1/Dss1-V5-His, was prepared to constitutively express Dss1 in TPA-induced skin tumors. As shown in Fig. 5A, Northern blot analysis confirmed that this construct expressed Dss1 efficiently in COS-1, JB6 Cl 30-7b, JB6 Cl 41-5a, Rat-1, and NIH/3T3 cells (Fig. 6A).

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C3/Dss1 plasmid DNA using LipofectAMINE PLUS™ reagents well culture slides (Falcon) and transiently transfected with pEGFP-Dss1 that is indicated by (Molecular Probes). Fluorescence microscopies reveal the localization of paraformaldehyde, and mounted with the Prolong antifade medium (Invitrogen). Cells were incubated at 37°C.

Twenty micrograms of the whole-cell lysates from JB6 Cl 41-5a cells was probed with an anti-EGFP rabbit polyclonal antibody (anti-EGFP, Santa Cruz Biotechnology). Western blot analysis was also tested using a retroviro-based method to express Dss1. Dss1 and neoR were inserted into a bicistronic construct using an ecotropic retroviral vector pLNCX2 and transduced into JB6 Cl 41-5a cells. Cells were selected for G418 resistance for 14 days, and one drug-resistant pooled clone was identified (>100 colonies) (pLNCX2/Dss1-GR). Eight individual clones (designated as pLNCX2 and pLNCX2/Dss1-C1→-C7) were isolated that stably expressed Dss1 or the vector control. The stable Dss1-transduced clones produced a transcript of 443 bp detected by RT-PCR using a pair of pLNCX2 forward (2882–2906) and reverse (3057–3032) sequencing/PCR primers (Fig. 7A), indicating that Dss1 was successfully integrated and expressed. These clones expressed Dss1 mRNA at a variable level, but all stable clones expressed more Dss1 than control cells (1.4–3.2-fold; Fig. 7B). The vector pLNCX2-transduced cells with a band of 176 bp (Fig. 7A, lane 4) were served as a negative control and showed a low level expression of endogenous Dss1 (Fig. 7B, lane 2).

Constitutive Dss1 expression was also correlated with increased growth rate. As shown in Fig. 7C, growth curves of clones overexpressing Dss1 experienced an initial lag after plating, but grew at a significantly faster rate than control cells by 2 days after seeding. The growth rate was also enhanced in COS-1, JB6 Cl 30-7b, and NIH/3T3 cells overexpressing Dss1 (data not shown). In addition, cells stably overexpressing Dss1 have higher colony-forming efficiency than control cells. The colony-forming efficiency was 1.6–9-fold higher than control cells (Fig. 7D). These results indicate that cells that overexpressed Dss1 develop in vitro characteristics of typical of transformed cells.

**DISCUSSION**

This study uses a novel approach to identify Dss1 as a potentially important gene in early skin tumorigenesis in mice. The cDNA was amplified using a PCR-based SMART tech-
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Fig. 5. Elevated expression of Dss1 in neoplasms from TgAC mice. A, Northern blot analysis. Total RNAs were prepared from eight TgAC mice normal skin tissues (NS1–NS8) and tumors, including eight papillomas (P1–P8) and three malignancies (M1–M3) with one spindle cell tumor (M1) and two squamous cell carcinomas (M2 and M3). Normal skin tissues and tumors were homogenized and extracted for total RNAs using TRIzol reagent (Invitrogen). Total RNA (8 µg) Northern blot membrane was probed with [α-32P]UTP-labeled Dss1 (top panel) or GAPDH (lower panel) antisense RNA. B, in situ hybridization. To detect expression of the Dss1 message, in situ hybridization assay was performed on sections of TPA-induced skin tumors, including papilloma (I, II, and III) and squamous cell carcinoma (IV, V, and VI), using Dss1 sense (III and VI) and antisense (II and V) riboprobes. The silver grains indicate the signals in probe hybridization, and slides were counterstained with hematoxylin (I and IV). The photographs were taken under light field (I and IV) and dark field (II, III, V, and VI) conditions. Original magnification, ×100.

The gene expression profiles were generated using a mouse cDNA array membrane carrying 1176 genes. Expression was analyzed in keratinocyte progenitor cells from TPA-treated or control TgAC mice. Keratinocyte progenitor cells were isolated using FACS to select cells that express the progenitor cell markers, integrin α6 and CD34. This novel approach was highly effective and identified in vivo TPA-inducible effector genes that might lead to neoplastic transformation in skin. Eleven differentially expressed genes were identified (Fig. 1 and Table I); nine are up-regulated genes, such as those for galectin-7, nucleoside diphosphate kinase B (NDP kinase B), cytoskeletal epidermal keratin 14 (CK14), Dss1, DNA double-strand break repair RAD21 homolog, transcription termination factor 1 (TTF1), thymosin β4, calpactin I light chain, and 40 S ribosomal protein SA, and two are down-regulated genes like apolipoprotein E precursor and type I cytoskeletal keratin 15 (CK15). Dss1 is one of the most interesting identified genes and was selected for further characterization in this study. Here, our data have demonstrated that TPA was able to induce a high level of Dss1 expression in integrin α6–CD34+ keratinocyte progenitor cells (Fig. 1) and in early hyperplastic skins in vivo (Fig. 2A), and in JB6 Cl 41-5a preneoplastic epidermal cells in vivo (Fig. 2B). In addition, Dss1 is persistently overexpressed in TPA-induced skin tumors including papillomas and malignant tumors (spindle cell tumor and squamous cell carcinoma) (Fig. 5). Furthermore, constitutive expression of Dss1 could promote cell proliferation (Fig. 7C) and enhance the ability of preneoplastic epidermal cells, JB6 Cl 30-7b and JB6 Cl 41-5a, to grow in soft agar (Figs. 6C and 7D).

A previous report indicates that skin tumorigenesis may be initiated by cellular transformation of KSCs (36). Elevation of β-catenin levels enhances proliferative potential of keratinocytes, increases stem cell self-renewal, and decreases stem cell differentiation (37). In addition, activation of the Wnt signaling pathway stimulates carcinogenesis in epithelial cells (38). Trembus et al. in our laboratory showed that integrin α6 and CD34 were useful markers for hemopoietic stem and progenitor cells that are expressed in keratinocytes of the hair follicle bulge. Cells expressing integrin α6 and CD34 are quiescent and highly clonogenic progenitor cells (18). In this study, TgAC mouse was topically applied with multiple doses of TPA and dorsal skins were digested with trypsin and type IV collagenase. The candidate keratinocyte progenitor cells were isolated and enriched with anti-integrin α6 and anti-CD34 antibodies by FACS. Our results showed that the expression level of Dss1 was elevated in TPA-treated keratinocyte progenitor cells (Fig. 1) and is associated with the promotion stage of skin carcinogenesis in mice (Fig. 2A). In addition, Dss1 expression increases in a time- and dose-dependent manner (Fig. 2B) and occurs consistently in TPA-induced skin tumors, eight papillomas and three malignant tumors (one spindle cell tumor and two squamous cell carcinomas), with malignant tumors having the highest level of Dss1 (Fig. 5). These results indicate that Dss1 is a TPA-responsive gene that may be a useful marker for early skin tumorigenesis.

p63 is homologous to p53 and plays a role in limb, craniofacial, and epithelial development. In addition, p63 has been implicated in cell regeneration and stem cell division (39–41). Heterozygous germ line mutations in p63 cause ectrodactyly, ectodermal dysplasia, and facial clefts syndrome (42). p63 is also associated with proliferative potential in normal and neoplastic keratinocytes (43) as well as recently identified as a marker for keratinocyte stem cells (44). p63 and Dss1 are both involved in the autosomal dominant disease SHFM1 (26), which is a form of ectrodactyly characterized by deep median clefts, missing digits, and lobster claw-like appearance of the distal extremities (26, 45). Like p63, it would be of more interests to know whether Dss1 has the same biological functions with p63.

Our findings using immunocytochemical staining and GFP-protein fusion fluorescence analysis reveal that Dss1 is distributed in a uniform and diffuse pattern in the nucleus and is also detected in the cytoplasm (Fig. 4). Cells that exhibit a nuclear pattern of expression appear to be in a normal morphology. However, the cells that express EGFP in both the nuclear and cytoplasmic compartments seem to have a slightly different morphology, suggesting that they may be under stress or in an altered state of growth or differentiation. In contrast, a previous study using MCF7 breast cancer cells suggests that Dss1 is a
A nuclear protein that interacts directly with the protein product of breast cancer susceptibility gene Brca2 (46). It is possible that cytoplasmic Dss1 could be activated and transported into the nucleus where it could interact with nuclear proteins such as BRCA2. Further studies will be necessary before the significance of this unique cellular distribution can be fully understood.
A previous report indicated that Dss1 might be a transcription factor expressed during embryogenesis in regions of rapid cell growth such as limb bud, branchial arch, genital bud, and skin but not in regions of cell differentiation like digital condensations (26). Thus, it is possible that Dss1 promotes proliferation of these cells during embryogenesis. A recent study has established a direct link between BRCA2 and Dss1 using yeast two-hybrid systems, and also recognized the important growth roles controlled by Dss1-like protein in yeast. Loss of function of Dss1-like protein by deletion of Dss1 in Schizosaccharomyces pombe resulted in a defect in completion of cell division, eventually leading to an accumulation of cells with greater than 2×

FIG. 7. Constitutive expression of Dss1 remarkably enhances neoplastic transformation in Dss1-transduced JB6 Cl 41-5a epidermal cell stable clones. A, detection of the integrated Dss1 gene in JB6 Cl 41-5a Dss1 stable clones. RT-PCR was employed to detect the presence of Dss1 gene with a pair of PCR primers located on pLNCX, retroviral vector. The forward (2882–2906) and reverse primers (3057–3032) are 5′-AGC TGG TTT AGT GAA CCG TCA GAT C-3′ and 5′-ACC TAC AGG TGG GTG CTT TCA TTC CC-3′, respectively. Only the Dss1-transduced clones, JB6 Cl 41-5a/pLNCX/Dss1-GR, -C1→-C7, and positive control retroviral vector pLNCX/Dss1 showed a 443-bp band (top panel). The vector pLNCX-transduced cells with a band of 176 bp and water blank served as negative controls. β-Actin with a band of 540 bp served as an internal control (lower panel). B, a significant increase of Dss1 transcript in JB6 Cl 41-5a Dss1 stable clones. Northern blot analysis in Dss1-transduced cell clones, JB6 Cl 41-5a/pLNCX/Dss1-GR, -C1→-C7, and positive control plasmid pcDNA3.1/Dss1-V5-His showed a band of 0.5 kb in size. The vector pLNCX-transduced stable cell clone, in lane 2, showed a low level expression of endogenous Dss1 and served as a negative control. C, Dss1 stable clones demonstrate growth advantage in monolayer culture. Growth curves were generated for JB6 Cl 41-5a cells stably expressing mock or Dss1, as described under "Materials and Methods." Cells were counted in triplicate every other day for 8 days. D, anchorage-independent growth assay. One pLNCX-transduced stable cell clone and eight Dss1-transduced JB6 Cl 41-5a stable cell clones (pLNCX/Dss1-GR and pLNCX/Dss1-C1→-C7) were seeded at a density of 1 × 10⁴ in a 0.33% soft agar over a 0.5% agar bottom layer. Colony with greater than 8 cells was counted in triplicate at 18 days.
DNA contents (46). We have found that the elevated Dss1 expression in genetically modified JB6 Cl 41-5a individual stable clones, which were infected by pLNCX/Dss1 retroviral vector, produced by ecotropic packaging cells RetroPack™ PT-67, and selected by G418, significantly promoted cell proliferation under standard in vitro tissue culture conditions (Fig. 7C). This result was in good agreement with the levels of Dss1 transcription obtained in RNA Northern blot analysis (Fig. 7B). Similarly, an enhancement in the rate of cell growth was also observed in COS-1, JB6 Cl 30-7b, and NIH/3T3 cells that were stably transfected with pcDNA3.1/Dss1-V5-His plasmid DNA (data not shown). Conversely, we also found the rate of cell growth to be selectively inhibited in LipofectAMINE-pre-treated Dss1-overexpressing TgAC 43 skin malignant tumor cells by addition to the culture medium of a specific antisense oligonucleotide to block Dss1 synthesis. This treatment consistently showed a decrease of proliferation rate from 100% down to 25–30%, suggesting that more than 70% of growth inhibition was mediated by inactivation of Dss1-initiated cell cycle pathways.2 Cumulatively, these results demonstrate that Dss1 play a crucial role in regulating cell proliferation, although a Dss1 homologue SEM1 was also recently implicated in the differentiation of Scarabaeomyces cerevisiae (47). It raises a possibility that Dss1 has pleiotropic effects in a variety of cell types.

As seen in Fig. 6, Dss1 was able to successfully express in COS-1 cells, JB6 Cl 30-7b cells, JB6 Cl 41-5a cells, Rat-1, and NIH/3T3 cells (Fig. 6A). In addition, it appears to markedly increase focus-forming activity in epithelial cell lines (i.e. JB6 Cl 30-7b and JB6 Cl 41-5a) but not in fibroblast cell lines (i.e. NIH/3T3) (Fig. 6B). More importantly, overexpression of Dss1 increased colony-forming efficiency of JB6 Cl 41-5a and JB6 Cl 30-7b cells but not NIH/3T3 cells in soft agar (Fig. 6C). Thus, the ability of Dss1 to regulate cellular transformation may be specific for epithelial cells. To further confirm the functional roles played by Dss1 in enhancing neoplastic transformation, retroviral vector pLNCX/Dss1-infected JB6 Cl 41-5a individual stable clones were employed. We showed that stable integration of Dss1 full-length cDNA into JB6 Cl 41-5a cells (Fig. 7A) resulted in increased Dss1 mRNA levels (Fig. 7B) and acquisition of susceptibility to transformation in soft agar (Fig. 7D). Thus, elevated Dss1 expression was sufficient to enhance the transformation activity in JB6 Cl 41-5a epithelial cells, consistent with the results as described above. The transcriptional levels of Dss1 in one Dss1 pool stably clone (pLNCX/Dss1-GR) and seven individual stable clones (pLNCX/Dss1-Cl1→C7) were found to vary, but all were higher than that in control vector only clone (pLNCX) (Fig. 7B). The magnitude of enhancement of transformation was proportional to the transcription levels of Dss1 mRNA. Taken together, in addition to promoting cell proliferation, Dss1 strongly provided a crucial role in cellular transformation.

Previous studies indicated that activator protein-1 (AP-1) is required in an activated form for TPA-induced neoplastic transformation (27) and inhibition of AP-1 by a c-Jun transactivation domain deletion mutant (Tam67) or AP-1 transcription-repressing repressors block TPA-induced cell transformation in JB6 Cl 41-5a cells (48). Expression of Tam67 in transgenic mice blocked TPA-induced AP-1 activity and papilloma formation (49). Yang et al. (50) recently also indicated that Pdc4d is a novel transformation suppressor that inhibits AP-1 transactivation. It is interesting to speculate that TPA, AP-1, and Dss1 might coordinately regulate cell signaling and growth in epithelial cells.

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