

Autocrine/Paracrine Secreted Frizzled-related Protein 2 Induces Cellular Resistance to Apoptosis

A POSSIBLE MECHANISM OF MAMMARY TUMORIGENESIS*

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Jia-Lin Lee, Chung-Tien Lin, Ling-Ling Chueh, and Ching-Jin Chang^{‡§}

From the Department and Graduate Institute of Veterinary Medicine, College of Bioresources and Agriculture, National Taiwan University and the [‡]Institute of Biological Chemistry, Academia Sinica, Taipei 106, Taiwan

Abnormal regulation of apoptosis and cell proliferation is thought to be involved in tumor formation. The secreted Frizzled-related protein 2 (SFRP2) was detected in primary culture of canine mammary gland tumors but not in normal mammary tissues. Thus, to elucidate the role of SFRP2 in mammary tumorigenesis, we overexpressed SFRP2 in mammary gland tumor and MCF7 cells. The results indicated that SFRP2 is secreted and incorporated into the extracellular matrix (ECM) of the tumor and normal cells. In an attempt to understand the molecular basis underlying the interaction between SFRP2 and ECM, co-immunoprecipitation and cell adhesion assays were carried out. SFRP2 was found to be associated with the fibronectin-integrin protein complex and could promote cell adhesion. DNA fragmentation and caspase 3 activity analyses showed that the susceptibility of the cells to UV-induced apoptosis decreased in the context of SFRP2 overexpression. Upon disruption of the fibronectin-integrin connection, the antiapoptosis activity of SFRP2 was decreased. Moreover, SFRP2 was found to induce tumorous transformation in normal mammary epithelial cells and to inhibit apoptosis in a modified paracrine model. Collectively, our results emphasize the relevance of SFRP2 and ECM in mammary tumorigenesis and provide further insight into the mechanism of SFRP2 action.

mal mammary gland development (4). The results of some studies suggested that cells derived from human cancers have a decreased capacity in undergoing apoptosis in response to various physiological stimuli (2, 5). Thus, deregulation of apoptosis, with the potential consequence of cell survival and drug resistance enhancement, may lead to the development of breast cancer (6).

Wnt/Frizzled-mediated β -catenin/Tcf transcription is an important survival pathway (7). In recent studies, Wnt signaling has been demonstrated to be involved in the specification of cell fate, polarity and proliferation, tissue patterning, apoptosis, and onset of neoplasia (8, 9). Existing evidence indicates that the Frizzled family of integral membrane proteins acts as Wnt receptors. Greater complexity in the Wnt signaling has emerged with the discovery of a family of secreted Frizzled-related proteins (SFRPs). Seven SFRPs have been identified to date (10). They are secreted glycoproteins containing an N-terminal domain that is typically ~30–50% identical to the putative Wnt-binding site of the Frizzled receptor (11, 12) and a C-terminal heparin-binding domain with weak homology to netrins (proteins involved in axonal guidance) (13, 14). Both domains are cysteine-rich, having 10 and 6 cysteine residues in the Frizzled-like and heparin-binding domains, respectively.

In our study on the tumorigenesis of canine mammary gland tumors (MGTs), we have found that a member of the SFRP family, SFRP2, is highly induced in canine MGTs (15). SFRP2 can serve as a modulator of Wnt signaling and is involved in the development and progression of cancer (16). During development, it was found that *SFRP2* is expressed in spatially restricted patterns in the telencephalon, diencephalon, spinal cord, and ventral thalamus and that their expression is reduced in *Pax-6* mutants but not in *Dlx-1/Dlx-2* mutants (17). Interestingly *SFRP2* is abundantly expressed in quiescent uterine stromal cells but is down-regulated by estrogens (18). During disease or cancer progression, up-regulation of SFRP2 in the retinas of retinitis pigmentosa may reflect an antiapoptotic response (19). Overexpression of transfected SFRP2 in breast adenocarcinoma cells increased their resistance to apoptotic signals and was associated with increased intracellular levels of β -catenin (12). SFRP2 overexpression could inhibit glioma cell motility and was associated with down-regulation of matrix metalloproteinase-2 activity (20).

The exact role of SFRP2 in mammary tumorigenesis is currently unknown. SFRPs are processed through the secretory pathway but remain associated with cells. In the presence of heparin, SFRP can be released into the medium (21). In this study, we aimed to test the possible interaction of SFRP2 with the ECM. We demonstrated that their interaction can transduce an antiapoptotic signal in mammary cancer cells. More-

Elucidating the molecular mechanisms of signals by which cell-ECM¹ interaction promotes cell growth and survival is critical to understanding the biology of cancer. Such studies will undoubtedly provide important new insights into the cellular factors involved in tumorigenesis. Moreover homeostasis in normal tissue is regulated by a balance between proliferative activity and cell loss by apoptosis (1, 2). Dislodging cell-cell contacts and morphological changes pertaining to imbalance in the cell-cell adhesion complex are some of the features of the apoptotic cells (3). The process of apoptosis is integral to nor-

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[‡] To whom requests for reprints should be addressed: Inst. of Biological Chemistry, Academia Sinica, No. 1, Section 4, Roosevelt Rd., Taipei 106, Taiwan. Tel.: 886-2-23630261 (ext. 4131); Fax: 886-2-2363-5038; E-mail: chingjin@gate.sinica.edu.tw.

[§] The abbreviations used are: ECM, extracellular matrix; FN, fibronectin; MGT, mammary gland tumor; SFRP, secreted Frizzled-related protein; CMV, cytomegalovirus; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium.

over, SFRP2 was also found to induce normal mammary epithelial cell tumorous transformation and to inhibit apoptosis in the modified paracrine model.

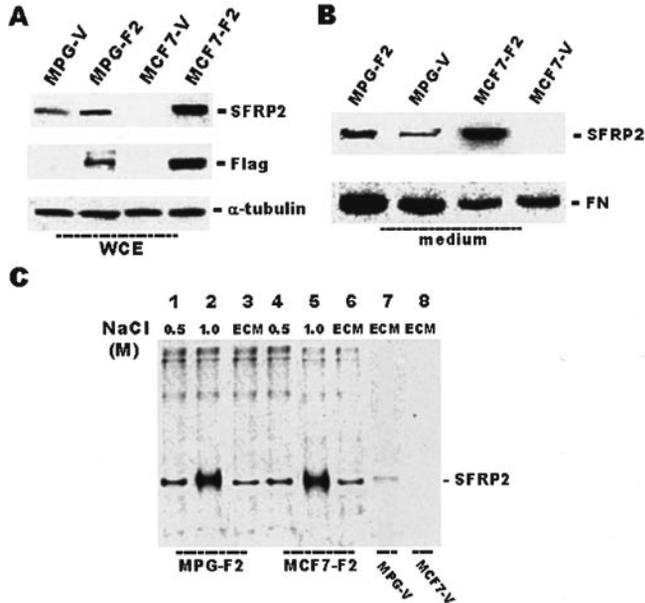


FIG. 1. SFRP2 is incorporated into the ECM. *A*, expression of the endogenous or exogenous SFRP2. MPG and MCF7 cells were transfected with expression vectors for SFRP2 proteins (MPG-F2 and MCF7-F2 cells) or the vector backbone (MPG-V and MCF7-V cells) and selected by G418. Expression of the SFRP2 proteins was determined by Western blot analysis. *B*, expression of the SFRP2 and FN proteins in cultured medium was determined by Western blot analysis. Proteins were prepared from the cultured media of stable cell lines described in *A*. *C*, the confluent cell layers were released from the culture dishes by incubating with PBS containing 5 mM EDTA. The proteins in lanes 1, 2, 4, and 5 were eluted from the cell-free ECM at room temperature for 10 min with 0.5 and 1 M NaCl, respectively. After eluting with 1 M NaCl, the insoluble ECM components were then extracted with 1× SDS Laemmli buffer (lanes 3 and 6). The insoluble ECM components in lanes 7 and 8 were extracted with 1× SDS Laemmli buffer without prior elution with any reagents. Lanes 7 and 8 were the ECM derived from MPG and MCF7 cells transfected with the expression vector alone. WCE, whole cell extracts.

MATERIALS AND METHODS

Primary Culture from Canine MGTs—Surgically resected specimens were collected from the freshly excised specimens. Primary culture was prepared by a mechanical technique instead of enzymatic digestion as described previously (22). After agar and dilution cloning, purified cancer cells were plated on a 25T flask and incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air. Cancer cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone, Logan, Utah). The cells were subcultured at subconfluency by harvesting with 0.05% trypsin, EDTA. The cells of primary culture tested positive for cytokeratin 8 and 18, ensuring that they were not fibroblasts. Primary culture from canine MGTs was termed "MPG," while that from canine normal mammary tissue was termed "NMG" (22).

Expression Plasmids and Antibodies—The canine homologue of SFRP2 cDNA was first isolated and cloned from a specifically expressed gene population of a canine subtractive retinal cDNA library (23, 24). Full-length canine SFRP2 cDNA was subcloned into the mammalian expression vector pCMV-Taq4 (Stratagene, La Jolla, CA) where C-terminal FLAG-tagged SFRP2 is under control of the CMV promoter and can be selected by neomycin resistance. The rabbit polyclonal anti-SFRP2 antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The mouse monoclonal anti-FN and the goat polyclonal anti-integrin $\alpha_5\beta_1$ antibodies were purchased from Chemicon International, Inc. (Temecula, CA).

Cell Culture and Stably Transfected Cells Lines—MPG and MCF-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The cells were transfected using the LipofectAMINE Plus reagent (Invitrogen) according to the manufacturer's instructions. After selection in G418 (1.0 mg/ml), the transfected cells were cloned by limited dilution. The clones expressing FLAG-tagged SFRP2 were expanded and maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and G418 (200 μ g/ml).

Preparation of Secreted Proteins, ECM Components, and ECM-associated Proteins—The media from cell culture were concentrated by centrifuging at 7,500 \times g for 15 min using Vivaspine6 concentrators (VIVA Science, Hannover, Germany). For experiments of adhesion assay, ECM materials derived from the cultured cells were extracted overnight at 4 °C in 2 M urea and 0.05 M Tris-HCl (pH 7.4). The soluble materials were dialyzed against PBS and used to coat plates. To determine the affinity of the ECM association of SFRP2, the confluent cell layers were released from the culture dishes by incubating with PBS containing 5 mM EDTA. The remaining ECM components were extracted with 0.5 or 1 M NaCl at room temperature for 10 min and 0.05 or 0.1 mg/ml soluble heparin at 37 °C for 30 min. The ECM components remaining on the culture dishes were washed and extracted with 1×

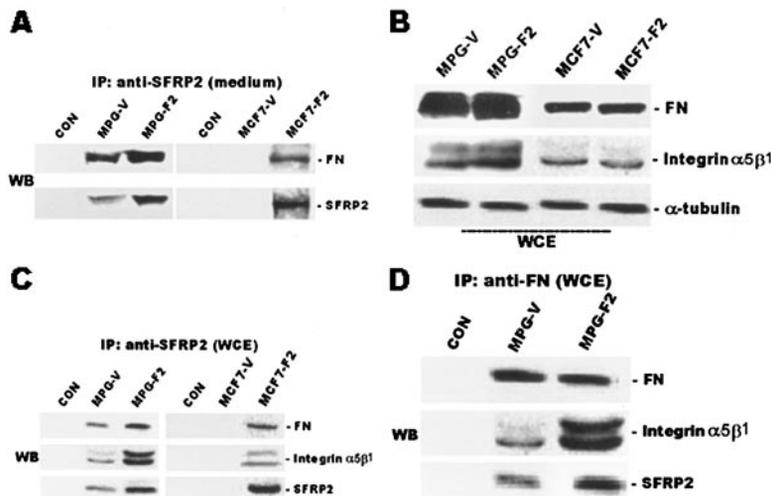


FIG. 2. SFRP2 is associated with FN and enhances integrin $\alpha_5\beta_1$ -FN binding affinity. *A*, immunoprecipitation of the SFRP2 proteins in cultured medium. Proteins were prepared as in Fig. 1*B*. SFRP2 was immunoprecipitated with anti-SFRP2 antibody as indicated. SFRP2 and FN were detected by Western blot analysis. The control lane (CON) represents the immunoprecipitation performed with only protein A beads and the SFRP2 overexpression medium. *B*, expressions of SFRP2, integrin $\alpha_5\beta_1$, and FN proteins of whole cell extracts (WCE) were determined by Western blot analysis. Whole cell extracts were prepared from the same cells as in Fig. 1*A*. *C*, immunoprecipitation of the SFRP2 proteins from whole cell extracts was done as in *A*. To solubilize FN that is not extractable by routine immunoprecipitation lysis buffer, the cells were preincubated with soluble FN for 1 h. *D*, immunoprecipitation of the FN proteins from whole cell extracts was done as in *C*. WB, Western blot; IP, immunoprecipitation.

SDS Laemmli sample buffer with 5% β -mercaptoethanol. The details were performed essentially as described previously (25).

Protein Preparation, Immunoprecipitation, and Immunoblotting—Cells were grown to ~80% confluence, washed with PBS, and scraped into 100 μ l of lysis buffer. For experiments with blocking antibody (integrin $\alpha_5\beta_1$, 0.1 mg/ml) or soluble heparin (0.1 mg/ml, Sigma), cells were preincubated at 37 °C for 30 min. The details of immunoprecipitation and immunoblotting were performed essentially as described previously (26). To avoid difficult extraction of insoluble FN by routine immunoprecipitation lysis buffer, the cells were preincubated with soluble FN (Invitrogen) for 1 h. Whole cell extracts were prepared by lysing the cells with buffer containing 25 mM HEPES (pH 7.6), 0.3 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5% Nonidet P-40, and 0.5 mM dithiothreitol. Insoluble material (cell debris) was discarded by centrifugation at 13,000 $\times g$ for 20 min at 4 °C. Lysates were divided to ensure that each aliquot was derived from an equal number of cells. For immunoprecipitation analysis, 1 mg of whole cell extracts was precleared with preimmune serum and protein A-Sepharose in 0.5 ml of immunoprecipitation buffer (25 mM HEPES (pH 7.6), 0.25 M NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 0.5 mM dithiothreitol, 6% glycerol) at 4 °C for 2 h. The precleared supernatants were incubated with 5 μ g of anti-SFRP2 or anti-FN antibody and protein A-Sepharose at 4 °C for 90 min. After extensive washes, the protein complex was dissolved in SDS loading buffer and subjected to SDS-PAGE. The separated polypeptides were blotted onto a Hybond-C membrane (Amersham Biosciences) and probed with anti-SFRP2, anti-FN, or anti-integrin $\alpha_5\beta_1$ antibody. The results were detected with an enhanced chemiluminescence kit (Amersham Biosciences).

Colony-forming Efficiency Assay—First 1.0 $\times 10^3$ cells in 2 ml of the standard culture medium containing 20% fetal bovine serum with 0.3% agar were laid on a 1-ml basal layer of 1% agar in 35-mm Petri dishes. After 1 week, 0.4 ml of fresh medium was added, and colony formation was determined on Day 14. The details were performed essentially as described previously (27).

Proliferation Assay—Cell proliferation was assessed using a standard MTT assay (Promega, Madison, WI). Typically 10³ cells were plated into each well in 96-well plates. Details of the assay were performed according to the manufacturer's instructions. The premixed, optimized dye solution was added to each well. After a 4-h incubation, the solubilization/stop solution was then added to the culture wells to resolve the formazan product. Relative concentrations of formazan were determined by quantifying the optical absorbance at 570 nm using a control wavelength of 690 nm in an automatic plate reader.

UV Irradiation and DNA Laddering Assay—For UV irradiation, cells were plated on a 60-mm culture dish in growth media. The medium was reduced to 1 ml/dish, and culture dishes were uncovered in a UV cross-linker (model HL-2000, UVP Laboratory Products, Upland, CA) as described previously (28). For experiments with blocking antibody (integrin $\alpha_5\beta_1$, 0.1 mg/ml) or heparin (0.1 mg/ml), cells were preincubated at 37 °C for 30 min. UV irradiation was carried out with the doses of 100 and 200 J/m². Following irradiation, 3 ml of growth medium were added, and the cells were incubated at 37 °C in a CO₂ incubator. Apoptosis was determined by DNA fragmentation assays. After 24 h of incubation, the medium of cells was collected and added to the scraped cells to allow centrifugation of both adhering and floating cells. The details of DNA laddering assays were essentially as described previously (29).

Caspase 3 Assay—Caspase 3 activity assays were performed using the Caspase 3 Colorimetric Assay kit from Chemicon International, Inc.. Following counting, 10⁶ cells were pelleted and subsequently lysed in 50 μ l of lysis buffer. Whole supernatant following sedimentation was incubated with 50 μ l of reaction buffer supplemented with 20 μ l of deionized H₂O and 10 μ l of the caspase 3 colorimetric substrate, Ac-DEVD-p-nitroanilide. Following 2 h of incubation at 37 °C, caspase 3 protease activity was measured using a spectrophotometer at 405 nm. Experiments were performed within the linear range of the assay, and absorbance reading was normalized by the protein concentration of each lysate using a protein assay kit (Bio-Rad).

Adhesion Assay—Cells were grown to ~80% confluence, washed with PBS, and scraped into PBS. Microtiter plates (96-well) were precoated with ECM materials derived from the cultured cells or the adhesive protein 10 ng/ μ l FN (Invitrogen) and subsequently blocked with 10 mg/ml bovine serum albumin (BSA) to prevent nonspecific cell adhesion. The details of the assay were essentially as described previously (30). For the experiment with Mn²⁺, 2 mM MnCl₂ was added into the cell suspension before the adhesion assays. To estimate the reference value for 100% attachment, cells were seeded on plates precoated with ECM materials or 20 ng/ μ l FN and incubated for 3 h at 37 °C in a 5% CO₂ atmosphere.

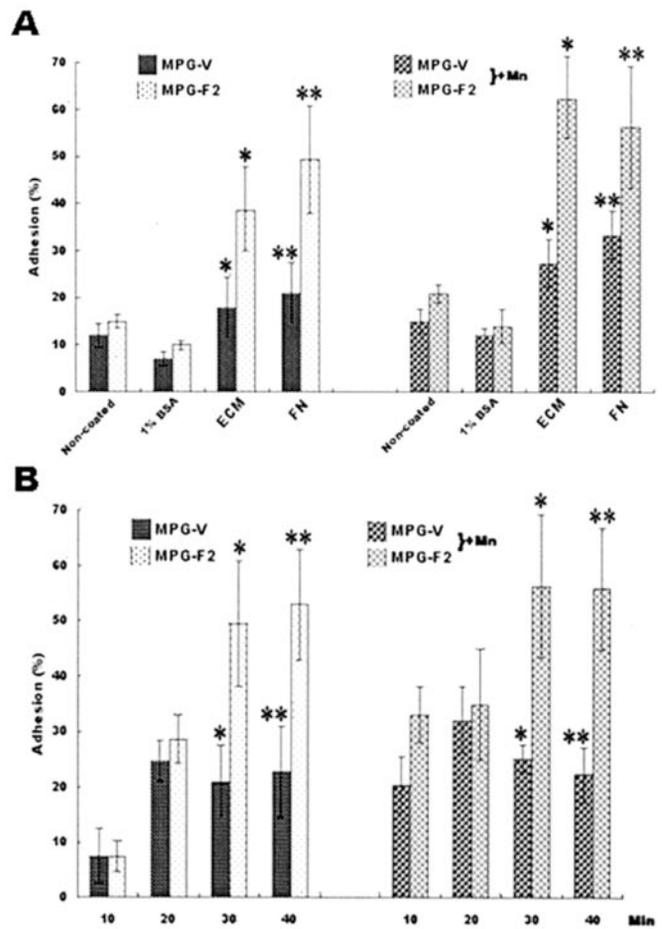


FIG. 3. SFRP2 can promote cell adhesion. Results of the adhesion assay are shown. Cells (as in Fig. 1A) were incubated without (left) or with (right) 2 mM MnCl₂ for 30 min. A, cells were replated on non-coated, 1% bovine serum albumin (BSA), whole ECM extracts derived from MPG cells, or FN for 30 min, respectively. B, cells were replated on FN and allowed to adhere for 10, 20, 30, or 40 min. The percentage of adhesion was calculated as described under "Materials and Methods." Data from three separate experiments are shown as means \pm S.E. * and **, $p < 0.05$.

Statistical Analysis—Data are expressed as means from three independent experiments. Statistical analysis was performed by Student's *t* test. $p < 0.05$ was considered statistically significant.

RESULTS

SFRP2 Is Incorporated into the ECM—We have cloned canine SFRP2 from retina and characterized its expression in canine MGTs (15, 23). To further study the role of SFRP2 in mammary tumorigenesis, vector encoding C-terminally FLAG-tagged SFRP2 was constructed and delivered into canine and human mammary cancer cell lines. The canine cell line MPG was isolated in our laboratory, and it has been characterized as being of epithelial origin (22). After being selected by G418, the stable clones of MPG-F2 and MCF7-F2 were generated, and the expression of SFRP2 was analyzed by Western blotting. Fig. 1A shows that MPG cells have a basal expression of SFRP2, and a dose-dependent expression of ectopic SFRP2-FLAG protein was observed in both MPG and MCF7 cells. Furthermore the presence of SFRP2 protein can be detected in the concentrated media from the stably expressed SFRP2 cells (Fig. 1B). Thus, consistent with previous reports, SFRP2 is indeed a secreted protein. Therefore, we conclude that SFRP2 is present in both cell lysates and culture medium in our overexpressed system.

In our previous study, the immunohistochemical staining result of canine MGT tissues showed that SFRP2 existed in the

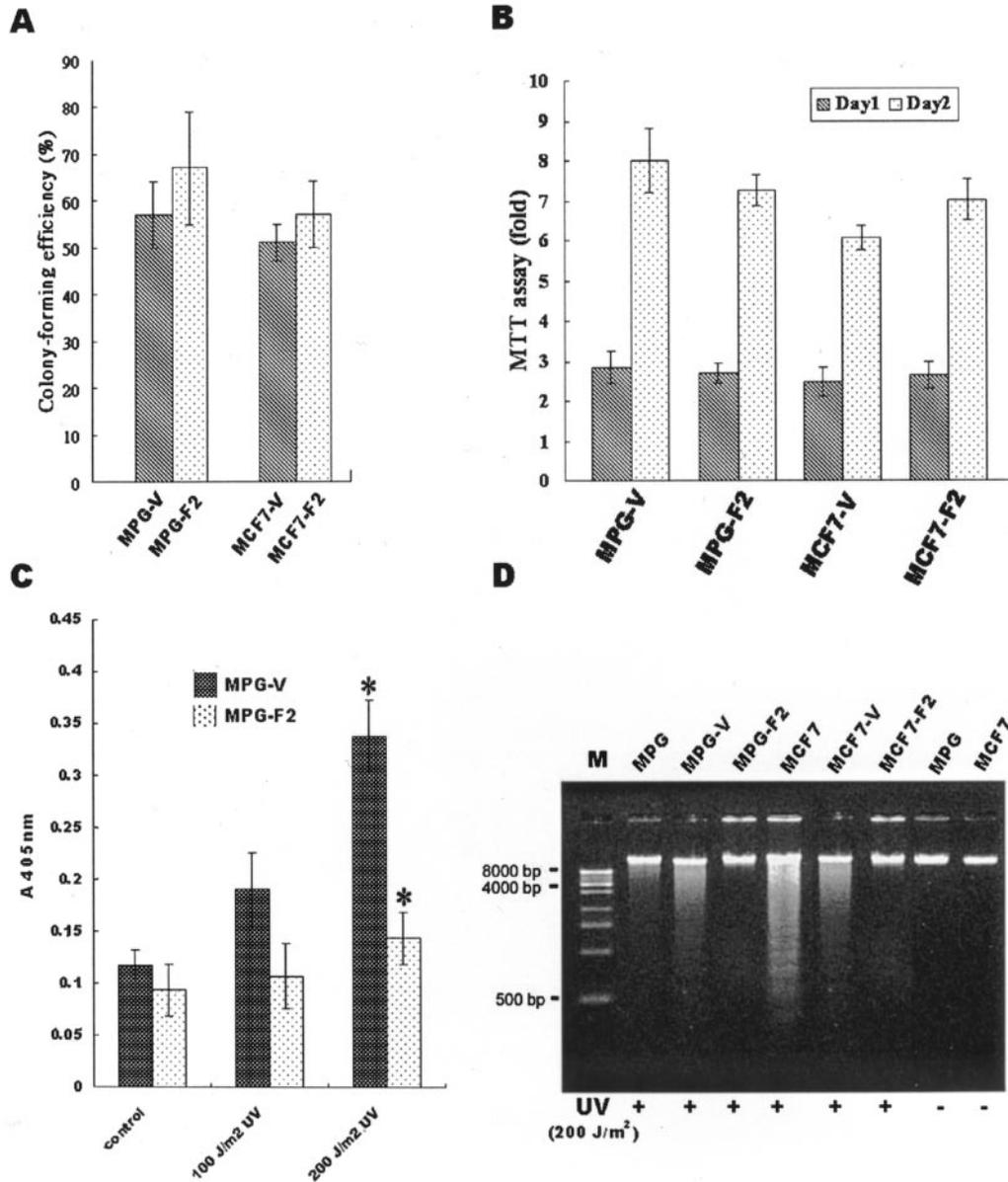


FIG. 4. Overexpression of SFRP2 results in decrease of sensitivity to UV-induced apoptosis. *A*, colony-forming efficiency assay. Colony-forming efficiency was expressed as the ratio of the number of colonies at Day 14 to the original number of cells per dish at the time of suspension in soft agar. *B*, proliferation assay. Proliferation rate was detected by MTT assay. Data are shown as -fold increases and are normalized at Day 0 (1.0×10^3 cells, fold = 1). Results are represented as the means \pm S.E. of three independent experiments. *C* and *D*, apoptotic analysis of the MPG and MCF7 transfectants. After selection by G418, the cells were irradiated with UV at 100 and 200 J/m² in *C* and 200 J/m² in *D*. The cells were analyzed for cell death detection by enzyme-linked immunosorbent assay (in *C*) and DNA fragmentation by DNA laddering (in *D*) 24 h after irradiation. Results are represented as the means \pm S.E. of three independent experiments. *, $p < 0.05$. Lane *M*, molecular weight markers.

extracellular compartment of tumor cells and in connective tissues (15). One possible reason is that SFRP2 may be associated with the ECM. To further address this issue, the ECM association of SFRP2 was examined by extracting the cell-free ECM deposited on the culture dishes by SFRP2-expressing MPG cells with different concentrations of NaCl. The eluted protein and the insoluble materials after extraction were subjected to Western blot analysis. As shown in Fig. 1C, most of the ECM-incorporated SFRP2 was eluted from ECM with 1.0 M NaCl; a fair amount of SFRP2 remained in the ECM after 1.0 M NaCl extraction, indicating a strong ECM association and implicating a gradual incorporation process of SFRP2 into the ECM.

SFRP2 Is Associated with FN and Enhances Integrin $\alpha_5\beta_1$ -FN Binding Affinity—In an attempt to identify the ECM component(s) that binds to SFRP2 *in vivo*, immunohistochem-

ical staining was performed. After screening 50 canine MGT tissues, the results showed that SFRP2 and FN shared identical expression profiles (data not shown). To assess the possibility of an interaction between SFRP2 and FN, the immunoprecipitated SFRP2 complexes from culture media were subjected to Western blotting using anti-FN antibody. Fig. 2A shows that the SFRP2 antibody could co-immunoprecipitate FN from MPG and MCF7 cell culture media.

FN has been reported to interact with integrin receptor (31, 32). To investigate the signaling pathways of SFRP2, we further examined whether integrin receptor is present in the SFRP2 protein complexes. Expression of integrin $\alpha_5\beta_1$ in both MPG and MCF7 cells was confirmed (Fig. 2B, the slow migrating band is α_5 , and the other is β_1). Co-immunoprecipitation experiments using the anti-SFRP2 antibody revealed that FN as well as integrin $\alpha_5\beta_1$ receptor associate with SFRP2 in MPG

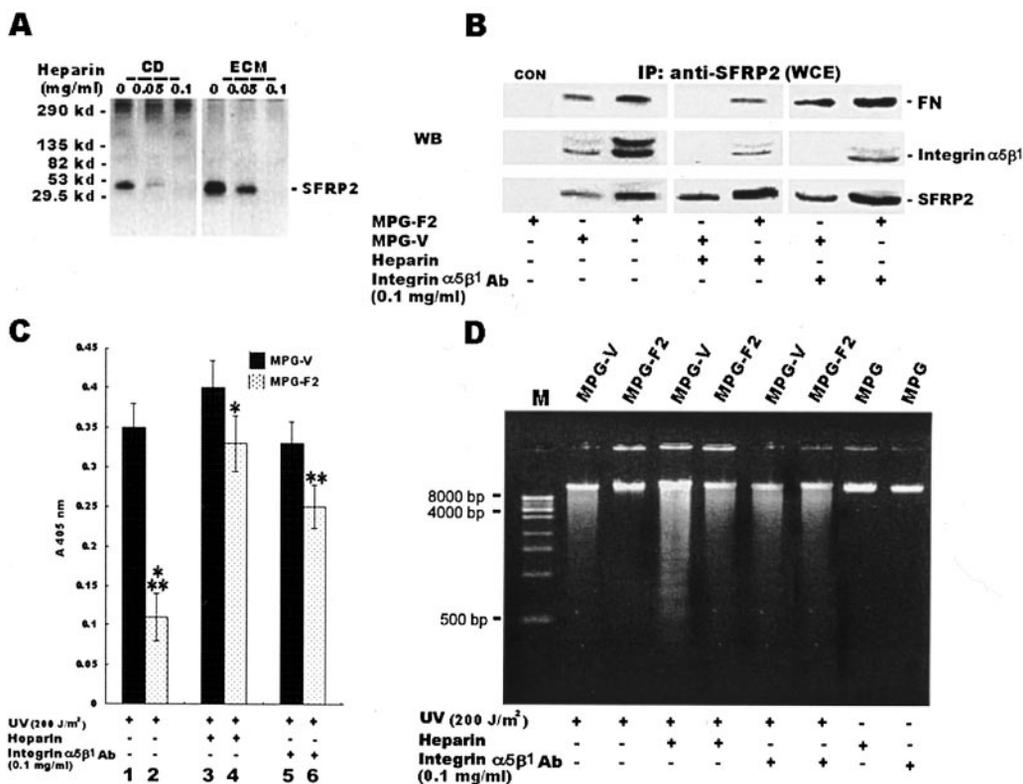


FIG. 5. SFRP2-FN-integrin $\alpha_5\beta_1$ interaction is required to reduce cell susceptibility to UV-induced apoptosis. A, the MPG-F2 cells stably expressing SFRP2 were incubated in serum-free medium containing 0, 0.05, or 0.1 mg/ml soluble heparin at 37 °C. Cell debris (CD) and cell-free ECM extracted with 1× SDS Laemmli buffer were collected after 30 min. B, immunoprecipitation of the SFRP2 proteins. MPG-F2 and MPG-V cells were incubated with 0.1 mg/ml heparin or integrin $\alpha_5\beta_1$ antibody at 37 °C for 30 min. Experimental steps were the same as those for Fig. 2C. The control lane (CON) showed mock immunoprecipitation. C and D, apoptotic analysis. The same cells as in B were irradiated with UV at 200 J/m². The cells were analyzed for cell death detection by enzyme-linked immunosorbent assay (in C) and DNA fragmentation by DNA laddering (in D) 24 h after irradiation. Results are represented as the means \pm S.E. of three independent experiments. *, $p < 0.05$. IP, immunoprecipitation; Ab, antibody; WB, Western blot; WCE, whole cell extracts. Lane M, molecular weight markers.

and MCF7 cells (Fig. 2C). To confirm the interaction between SFRP2 and FN, a reciprocal co-immunoprecipitation assay was performed using anti-FN antibody. Interestingly, although the amounts of precipitated FN were nearly identical in the two cell clones, we observed that the amounts of precipitated integrin receptors were in direct proportion to those of precipitated SFRP2 (Fig. 2D). Taken together, these results demonstrate that SFRP2 is an interacting component of the FN-integrin receptor protein complexes and could enhance the formation of FN-integrin receptor complexes.

Expression of SFRP2 Could Promote Cell Adhesion—With the knowledge that the interaction of FN and integrin receptor promotes cell adhesion, we compared the level of cell adhesion in the presence or absence of SFRP2. As shown in Fig. 3A, MPG cells overexpressing SFRP2 displayed a stronger adherence to dishes coated with FN and whole ECM extracts derived from MPG cells than the control cells. Moreover SFRP2 could further promote cell adhesion in the presence of Mn²⁺, an integrin β_1 receptor stimulator (33), especially on dishes coated with whole ECM extracts. It is possible that, in addition to the integrin $\alpha_5\beta_1$ -FN pathway, the SFRP2 may promote cell adhesion through other pathways. Fig. 3B shows that overexpression of SFRP2 in MPG cells led to a significant acceleration of cell adhesion to FN at 30 min after plating. Thus, according to the results of the co-immunoprecipitation and adhesion assay, we can conclude that the stimulatory effect of SFRP2 on cell adhesion is mediated through enhancing integrin $\alpha_5\beta_1$ -FN binding.

Overexpression of SFRP2 Results in Diminished Sensitivity to UV-induced Apoptosis—The biological consequences of SFRP2 expression on mammary tumorigenesis were evaluated by colony-forming efficiency, MTT, and apoptosis assays. The

results revealed that colony-forming efficiency (Fig. 4A) and cell proliferation rate (Fig. 4B) of MPG and MCF7 cells were not significantly altered by the expression of SFRP2. To further examine the apoptosis activity of SFRP2, the stable cell lines described in Fig. 1A were exposed to UV and subjected to caspase 3 activity and DNA fragmentation analysis. Fig. 4C shows that the caspase 3 activity in MPG cells was decreased in the presence of SFRP2 after UV irradiation. Additionally overexpression of SFRP2 in MPG and MCF7 cells could inhibit DNA fragmentation caused by UV (Fig. 4D). We also tried to induce apoptosis by using tumor necrosis factor α or serum starvation in MPG cells, but the cells seemed to show resistance to these treatments. Thus, the UV was used as the primary means to trigger apoptosis in the following experiments. Taken together, these results demonstrated that canine SFRP2, acting in an autocrine manner, has antiapoptosis activity in mammary cancer cells.

SFRP2-FN-Integrin $\alpha_5\beta_1$ Interaction Is Required to Reduce Cell Susceptibility to UV-induced Apoptosis—We have provided evidence demonstrating that SFRP2 interacts with FN and that expression of SFRP2 could protect cell from UV-induced apoptosis. To examine whether the interaction of SFRP2 with FN is essential for its antiapoptosis activity, we used inhibitors to interrupt the interaction between SFRP2 and FN. Heparin has been reported to release Frizzled-related protein from the cell surface (21). As shown in Fig. 5A, in the absence of soluble heparin, SFRP2 was associated abundantly with the cell debris and ECM. Conversely, in the presence of soluble heparin (0.1 mg/ml), most of the SFRP2 was released from cell debris and ECM. Anti-integrin $\alpha_5\beta_1$ antibody was also used to block the interaction between ECM and integrin $\alpha_5\beta_1$. An immunopre-

cipitation assay using anti-SFRP2 antibody was done in the presence of heparin or anti-integrin $\alpha_5\beta_1$ antibody. As shown in Fig. 5B, the level of FN co-immunoprecipitated with SFRP2 was decreased in the presence of heparin. In addition, anti-integrin $\alpha_5\beta_1$ antibody appeared to block the association of integrin with the SFRP2-FN complexes as revealed by the co-immunoprecipitation experiment. Moreover results of the caspase 3 activity and DNA fragmentation analysis indicated that the antiapoptosis effect of SFRP2 was abrogated in the presence of heparin or anti-integrin $\alpha_5\beta_1$ antibody (Fig. 5, C and D). Collectively the results from these inhibitor studies demonstrated that proper SFRP2-FN interaction is required to suppress cell susceptibility to UV-induced apoptosis.

Paracrine Activity of SFRP2 on Neighboring Normal Cells—Our previous immunohistochemical staining results of canine MGT tissues showed that the extracellular SFRP2 is especially prominent in the border between tumorous and normal mammary tissues (15). For further characterization of the mechanism of SFRP2 action, we next investigated whether secreted SFRP2 associates with FN and acts as a paracrine factor. In the paracrine model, we chose NMG cells, which were isolated from canine normal mammary tissues in our laboratory (22). As shown in Fig. 6A, NMG cells expressed FN and integrin $\alpha_5\beta_1$ but lacked SFRP2 expression. To assay the paracrine activity of SFRP2, the media from MPG-F2 cells stably expressing SFRP2 were added to the NMG cells. Similar to the results in Fig. 1C, SFRP2 was expectedly found to be associated with the ECM of treated NMGs and extractable by NaCl (Fig. 6B). Furthermore SFRP2-treated NMG cells also displayed an increased adherence to dishes coated with FN and whole ECM extracts derived from MPG cells as compared with cells treated in the control media (Fig. 6C).

Paracrine SFRP2 Induces Tumorous Transformation and Inhibits Apoptosis in the Neighboring Normal Cells—To further probe the potential link of SFRP2 to regulation of cell growth, we performed a colony formation assay and found that paracrine SFRP2 had a stimulatory effect on the formation efficiency (Fig. 7A). Surprisingly the cell proliferation rate of NMG cells was not significantly altered by paracrine SFRP2 (Fig. 7B). In agreement with the above results (Fig. 4C), in the presence of SFRP2, the UV irradiation-induced caspase 3 activity and DNA fragmentation in the NMG cells were diminished (Fig. 7, C and D).

Taken together, the findings presented here suggest that SFRP2, acting as either an autocrine or paracrine factor, promotes cell survival by inducing a resistance to apoptosis signals. Functional interaction between SFRP2 and ECM may thus be a possible mechanism of mammary tumorigenesis.

DISCUSSION

Despite the well established role of Wnt signaling in oncogenesis (34), the involvement of the SFRP family in cancer is still under investigation. In recent years, several reports have described the different expression patterns of SFRPs in various cancers. For example, SFRP1 is down-regulated in breast cancer (35) but is overexpressed in uterine leiomyoma (36). frpHE (SFRP4) is up-regulated in the stroma of endometrial and breast carcinomas (37). Moreover SFRP1 and SFRP2 are produced by the majority of the long term and *ex vivo* malignant glioma cell lines (20). Our previous studies have also demonstrated that canine SFRP2 was expressed in MGTs but absent in normal mammary tissues (15). In this study, we showed that autocrine/paracrine SFRP2 could decrease cell sensitivity to UV-induced apoptosis in canine and human mammary cancer cells. This result is consistent with the observation that the mouse homologue of SFRP2 could enhance the cell viability of MCF7 cells in response to the treatment with tumor necrosis

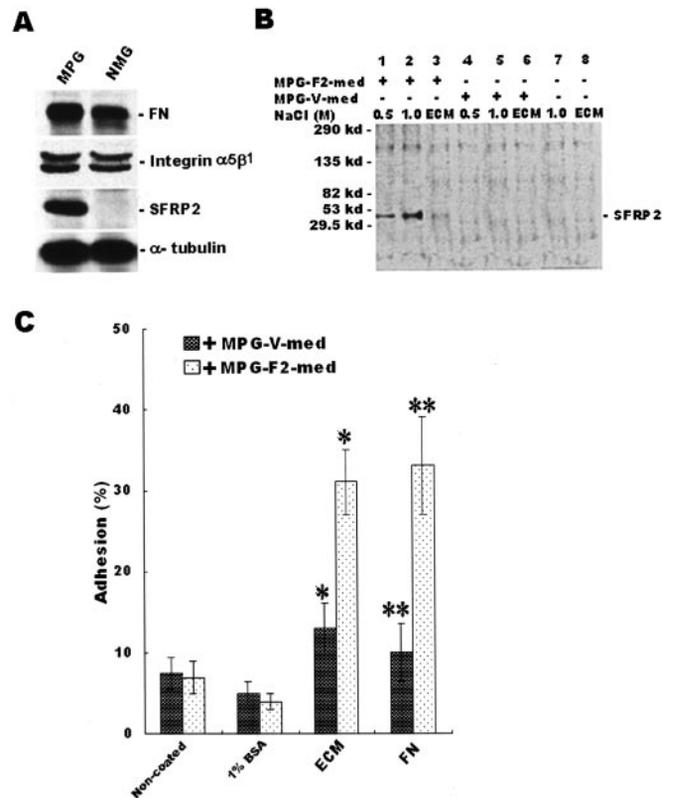


Fig. 6. Paracrine activity of SFRP2 on the neighboring normal cells. A, expressions of SFRP2, integrin $\alpha_5\beta_1$, and FN proteins were determined by Western blot analysis on whole cell extracts. B, after incubating with the medium from MPG-F2 (lanes 1–3) or MPG-V cells (lanes 4–6) at 37 °C for 1 h, the confluent NMG cell layers were released from the culture dishes by incubating with PBS containing 5 mM EDTA. The proteins in lanes 1, 2, 4, and 5 were eluted from the cell-free ECM at room temperature for 10 min with 0.5 and 1 M NaCl, respectively. After eluting with 1 M NaCl, the insoluble ECM components were then extracted with 1× SDS Laemmli buffer (lanes 3 and 6). Lanes 7 and 8 were the protein derived from NMG cells without prior incubation with any medium. C, adhesion assay. After incubating with the medium from MPG-F2 or MPG-V cells at 37 °C for 1 h, NMG cells were replated on non-coated, 1% bovine serum albumin (BSA), whole ECM extracts derived from MPG cells, or FN for 30 min, respectively. The percentage of adhesion was calculated as described under “Materials and Methods.” Data from three separate experiments are shown as means \pm S.E. * and **, $p < 0.05$. med, medium.

factor or ceramide (12). The antiapoptotic activity of SFRP2 offers a clue that it may be involved in either the formation or progression of mammary tumors. More importantly, investigating new drugs that potentially restore the apoptotic defense mechanism will possess substantial therapeutic benefit for treatment of mammary tumors.

SFRP2 is a secreted glycoprotein. Functional characterization of this protein is our main research focus. The progression of mammary gland tumors is a complex multistep process resulting from the alteration of controlling cell proliferation and differentiation, thus leading to abnormal cellular growth. In addition, several recent *in vitro* and *in vivo* studies have demonstrated that ECM proteins, including FN, may be important paracrine factors influencing mammary gland growth, morphogenesis, and lactation (38). The signaling of transmembrane receptor integrin $\alpha_5\beta_1$ -mediated cell adhesion to FN is required for initiating mitogenesis (39, 40). In this study, the physical interaction of SFRP2 with FN-integrin protein complex was demonstrated by co-immunoprecipitation, and it was further found that cell adhesion could be promoted in the presence of SFRP2. Although we cannot rule out the existence of other signaling pathways by which SFRP2 mediates its antiapoptotic

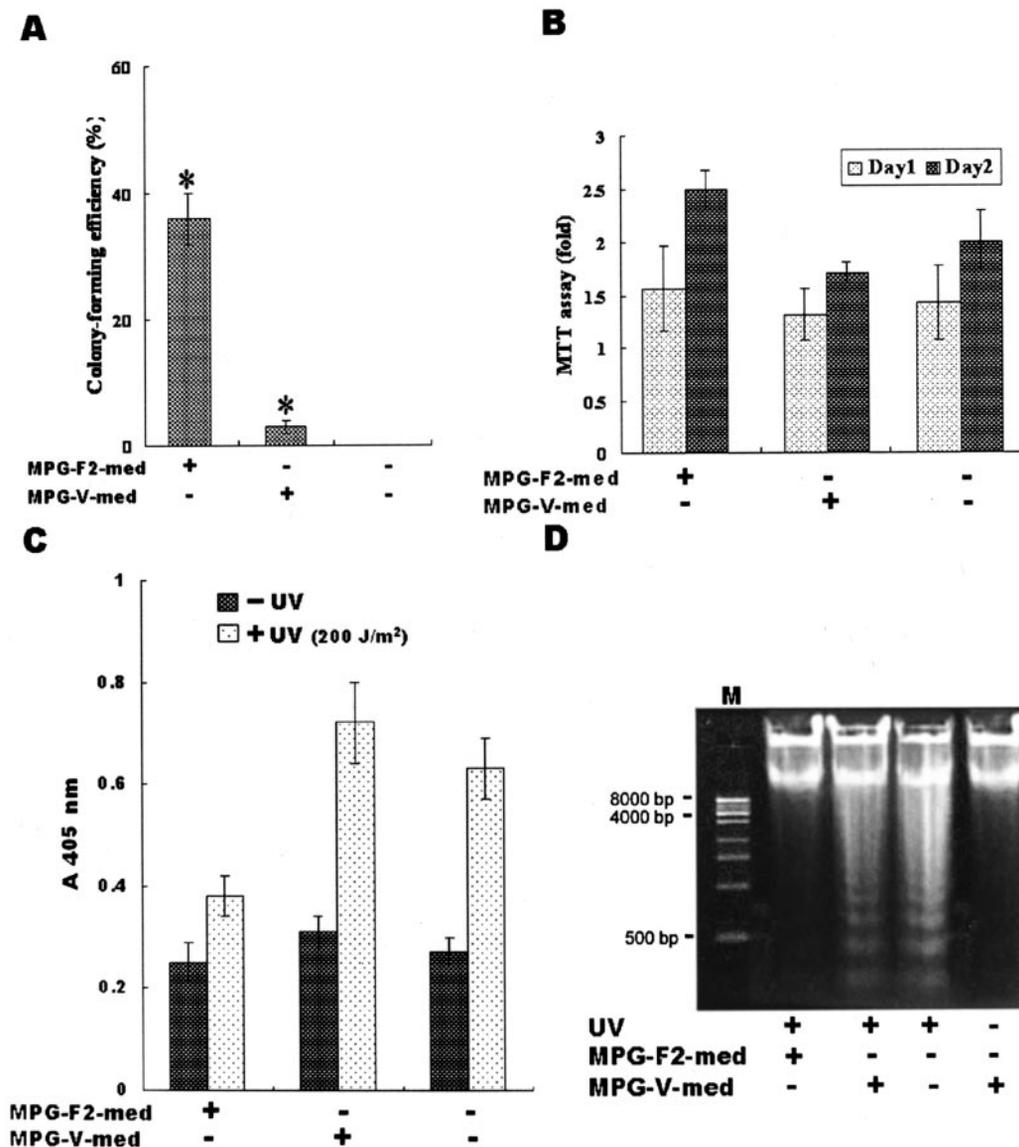


FIG. 7. Paracrine SFRP2 induces tumorous transformation and inhibits apoptosis in neighboring normal cells. NMG cells were incubated with the medium (refreshed every 12 h) derived from MPG-F2 or MPG-V culture at 37 °C for 24 h. **A**, colony-forming efficiency assay. Colony-forming efficiency was expressed as the ratio of the number of colonies at Day 14 to the original number of cells per dish at the time of suspension in soft agar. **B**, proliferation assay. Proliferation rate was detected by MTT assay. Data are shown as -fold increases and are normalized at Day 0 (1.0×10^3 cells, fold = 1). Results are represented as the means \pm S.E. of three independent experiments. **C** and **D**, apoptotic analysis. The cells were irradiated with UV at 200 J/m². The cells were analyzed for cell death detection by enzyme-linked immunosorbent assay (in **C**) and DNA fragmentation by DNA laddering (in **D**) 24 h after irradiation. Results are represented as the means \pm S.E. of three independent experiments. *, $p < 0.05$. med, medium. Lane M, molecular weight markers.

effect, our results clearly showed that, when the interaction of SFRP2 with FN-integrin complex was blocked, the protective effect of SFRP2 on UV-induced apoptosis was dramatically diminished. Therefore, our results link FN to mammary tumorigenesis as part of the SFRP2/integrin-mediated antiapoptotic signaling pathway. Moreover our studies highlight the importance that SFRP2, in addition to the Wnt/Frizzled pathway, can potentially modulate other signaling pathway(s). The detailed mechanism underlying the SFRP2-mediated signaling will be a subject of future research.

Extracellular complex polysaccharides, belonging to the family of heparin/heparan sulfate glycosaminoglycans, have recently received increasing attention because of their important roles in several biological processes at the cell-tissue-organ interface (41). The antitumor effect of heparin has previously been reported. The use of heparins to treat venous thrombosis in cancer patients in large clinical trials has led to the observations that this treatment prolongs cancer patient survival

(42, 43). Moreover these clinical findings have been substantiated in various animal models, which have demonstrated that heparin inhibits tumor invasion and metastasis (44). Therefore, heparin is a multifaceted drug that binds to several factors and therefore impinges on various biological processes. However, the exact mechanism through which heparin mediates the *in vivo* tumor blockade is largely unknown. Interestingly SFRPs (45), Wnt (46), and FN (47) proteins possess highly basic regions that confer strong affinity for heparin. Consistently we discovered in our study that, in the presence of soluble heparin, the association of SFRP2 with cell surface and ECM was weakened. Furthermore, upon heparin-mediated disruption of the SFRP2-FN-integrin interaction, mammary cancer cells became more prone to UV-induced apoptosis. Therefore, experimental evidence provided by our work links heparin, as well as SFRP2, to the development of breast cancer and provides potential leads for future studies.

Some studies have demonstrated that the estrogen receptor-

positive breast cancer cells are in general poorly invasive *in vivo* (better prognosis) and *in vitro* as compared with estrogen receptor-negative cancer cells (48). The exact mechanism has yet to be established. However, studies have shown that estradiol and fibulin-1 can inhibit FN-induced human ovarian and breast cancer cell motility and can therefore block tumor invasion (49). One hypothesis for the contrasting tumor invasive properties is thus the potential involvement of FN/SFRP2. The expression levels of the ECM protein FN and the $\alpha_5\beta_1$ integrin are enhanced by estrogen in normal mouse mammary gland (50) and by the Wnt/Frizzled-mediated β -catenin/Tcf transcription (51). On the other hand, the concentrations of collagen I, collagen IV, and laminin do not exhibit major changes during mammary development (49). Therefore, estrogen receptor-positive breast cancer patients have a better prognosis due to the consequently elevated FN/SFRP2-mediated signal. In addition, future studies can be undertaken to determine whether SFRP2 and FN might be ideal prognostic markers in breast cancers.

In this report, we have provided several lines of evidence demonstrating the antiapoptotic function of SFRP2 that are consistent with its unique, tumor-specific expression and presumed role in tumorigenesis. Acting in an autocrine manner, SFRP2 can prevent mammary cancer cells from undergoing apoptosis. Furthermore SFRP2 is secreted from mammary cancer cells and concentrated locally via association with ECM. Paracrine SFRP2 induces tumorous transformation of and inhibits apoptosis in the neighboring normal cells. Thus, like Wnt, SFRP2 may act as a morphogen or oncogene in mammary tissues and alter the biological behavior of target cells in a concentration-dependent manner. Our report is the first extensive study on the interplay between SFRP2 and ECM in mammary tumors. It has also contributed valuable information to understanding the role of SFRP2 in the development of mammary tumor.

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REFERENCES

- Steller, H. (1995) *Science* **267**, 1445–1448
- Thompson, C. B. (1995) *Science* **267**, 1456–1462
- Pawlak, G., and Helfman, D. M. (2001) *Curr. Opin. Genet. Dev.* **11**, 41–47
- Medina, D. (1996) *J. Mammary Gland Biol. Neoplasia* **1**, 5–19
- Carson, D. A., and Ribeiro, J. M. (1993) *Lancet* **341**, 1251–1254
- Kumar, R., Vadlamudi, R. K., and Adam, L. (2000) *Endocr. Relat. Cancer* **7**, 257–269
- Chen, S., Guttridge, D. C., You, Z., Zhang, Z., Fribley, A., Mayo, M. W., Kitajewski, J., and Wang, C. Y. (2001) *J. Cell Biol.* **152**, 87–96
- Huguet, E. L., McMahon, J. A., McMahon, A. P., Bicknell, R., and Harris, A. L. (1994) *Cancer Res.* **54**, 2615–2621
- Dale, T. C., Weber-Hall, S. J., Smith, K., Huguet, E. L., Jayatilake, H., Gusterson, B. A., Shuttleworth, G., O'Hare, M., and Harris, A. L. (1996) *Cancer Res.* **56**, 4320–4323
- Jones, S. E., and Jomary, C. (2002) *Bioessays* **24**, 811–820
- Finch, P. W., He, X., Kelley, M. J., Uren, A., Schaudies, R. P., Popescu, N. C., Rudikoff, S., Aaronson, S. A., Varmus, H. E., and Rubin, J. S. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 6770–6775
- Melkonyan, H. S., Chang, W. C., Shapiro, J. P., Mahadevappa, M., Fitzpatrick, P. A., Kiefer, M. C., Tomei, L. D., and Urmansky, S. R. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 13636–13641
- Leyns, L., Bouwmeester, T., Kim, S. H., Piccolo, S., and De Robertis, E. M. (1997) *Cell* **88**, 747–756
- Wang, S., Krinks, M., Lin, K., Luyten, F. P., and Moos, M., Jr. (1997) *Cell* **88**, 757–766
- Lee, J.-L., Chang, C.-J., Wu, S.-Y., Sargan, D. R., and Lin, C.-T. (2004) *Breast Cancer Res. Treat.*, in press
- Miller, J. R., Hocking, A. M., Brown, J. D., and Moon, R. T. (1999) *Oncogene* **18**, 7860–7872
- Kim, A. S., Anderson, S. A., Rubenstein, J. L. R., Lowenstein, D. H., and Pleasure, S. J. (2001) *J. Neurosci.* **21**, RC132(1–5)
- Das, S. K., Tan, J., Raja, S., Halder, J., Paria, B. C., and Dey, S. K. (2000) *J. Biol. Chem.* **275**, 28834–28842
- Jones, S. E., Jomary, C., Grist, J., Stewart, H. J., and Neal, M. J. (2000) *Investig. Ophthalmol. Vis. Sci.* **41**, 1297–1301
- Roth, W., Wild-Bode, C., Platten, M., Grimmel, C., Melkonyan, H. S., Dichgans, J., and Weller, M. (2000) *Oncogene* **19**, 4210–4220
- Bafico, A., Gazit, A., Pramila, T., Finch, P. W., Yaniv, A., and Aaronson, S. A. (1999) *J. Biol. Chem.* **274**, 16180–16187
- Lee, J. L., Chang, C. J., Chueh, L. L., and Lin, C. T. (2003) *In Vitro Cell Dev. Biol. Anim.* **35**, 221–227
- Lin, C. T., and Sargan, D. R. (1997) *Nucleic Acids Res.*, **25**, 4427–4428
- Lin, C. T., and Sargan, D. R. (2001) *Biochem. Biophys. Res. Commun.* **282**, 394–403
- Xu, Y., and Yu, Q. (2001) *J. Biol. Chem.* **276**, 34990–34998
- Chang, C. J., Chen, Y. L., and Lee, S. C. (1998) *Mol. Cell. Biol.* **18**, 5880–5887
- Schlegler, K., Heck, R., Niketeghad, F., Schirmacher, P., Radaeva, S., Oesch, F., Dienes, H. P., Bannasch, P., and Steinberg, P. (1997) *Exp. Cell Res.* **236**, 418–426
- Chan, P. C., Lai, J. F., Cheng, C. H., Tang, M. J., Chiu, C. C., and Chen, H. C. (1999) *J. Biol. Chem.* **274**, 26901–26906
- Ruest, L. B., Marcotte, R., and Wang, E. (2002) *J. Biol. Chem.* **277**, 5418–5425
- Mould, A. P., Akiyama, S. K., and Humphries, M. J. (1995) *J. Biol. Chem.* **270**, 26270–26277
- Pytela, R., Pierschbacher, M. D., and Ruoslahti, E. (1985) *Cell* **40**, 191–198
- Akiyama, S. K., and Yamada, K. M. (1985) *J. Biol. Chem.* **260**, 4492–4500
- Frisch, S. M., and Francis, H. (1994) *J. Cell Biol.* **124**, 619–626
- Peifer, M., and Polakis, P. (2000) *Science* **287**, 1606–1609
- Wong, S. C. C., Lo, S. F. E., Lee, K. C., Yam, J. W. P., Chan, J. K. C., and Hsiao, W. L. W. (2002) *J. Pathol.* **196**, 145–153
- Fukuhara, K., Kariya, M., Kita, M., Shime, H., Kanamori, T., Kosaka, C., Orii, A., Fujita, J., and Fujii, S. (2002) *J. Clin. Endocr.* **87**, 1729–1736
- Abu-Jawdeh, G., Comella, N., Tomita, Y., Brown, L. F., Tognazzi, K., Sokol, S. Y., and Kocher, O. (1999) *Lab. Invest.* **79**, 439–447
- Woodward, T. L., Xie, J., Fendrick, J. L., and Haslam, S. Z. (2000) *Endocrinology* **141**, 3578–3586
- Weaver, V. M., Petersen, O. W., Wang, F., Larabell, C. A., Briand, P., Damsky, C., and Bissell, M. J. (1997) *J. Cell Biol.* **137**, 231–245
- Danen, E. H. J., and Yamada, K. M. (2001) *J. Cell. Phys.* **189**, 1–13
- Perrimon, N., and Bernfield, M. (2000) *Nature* **404**, 725–728
- Kakkar, A. K., and Williamson, R. C. (1999) *BMJ* **318**, 1571–1572
- Cosgrove, R. H., Zacharski, L. R., Racine, E., and Andersen, J. C. (2002) *Semin. Thromb. Hemost.* **28**, 79–88
- Smorenburg, S. M., and Van Noorden, C. J. (2001) *Pharmacol. Rev.* **53**, 93–105
- Üren, A., Reichsman, F., Anest, V., Taylor, W. G., Muraiso, K., Bottaro, D. P., Cumberledge, S., and Rubin, J. S. (2000) *J. Biol. Chem.* **275**, 4374–4382
- Reichsman, F., Smith, L., and Cumberledge, S. (1996) *J. Cell Biol.* **135**, 819–827
- Ruoslahti, E., Hayman, E. G., Engvall, E., Cothran, W. C., and Butler, W. T. (1981) *J. Cell Biol.* **256**, 7277–7281
- Price, J. E., Polyzos, A., Zhang, R. D., and Daniels, L. M. (1990) *Cancer Res.* **50**, 717–721
- Hayashido, Y., Lucas, A., Rougeot, C., Godyna, S., Argraves, W. S., and Rochefort, H. (1998) *Int. J. Cancer* **75**, 654–658
- Woodward, T. L., Mienaltowski, A. S., Modi, R. R., Bennett, J. M., and Haslam, S. Z. (2001) *Endocrinology* **142**, 3214–3222
- Gradi, D., Kuhl, M., and Wedlich, D. (1999) *Mol. Cell. Biol.* **19**, 5576–5587