行政院國家科學委員會補助專題研究計畫
期中進度報告

細胞凋亡調控基因(sFRP2)在犬乳腺腫瘤之細胞凋亡調控角色及相關調節因子的研究 (2/3)

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一、中文摘要

乳腺腫瘤對動物與人類皆是重要且常見的腫瘤，其產生的病因是複雜為多因子牽涉的結果。Secreted frizzled related proteins (sFRPs)是近年的報告發現與 Wnt-Frizzled 訊息傳遞傳導路徑的調節和細胞凋亡(apoptosis)的調節中扮演著雙重角色的蛋白質。我們實驗室最近發現 sFRP2 基因在人類與犬隻乳腺腫瘤中有大量的表現及活化，但是在正常犬乳腺組織中則沒有表現(前 NSC project, 已發表於期刊 Breast Cancer Research and Treatment)。我們為了有系統地進一步研究犬隻乳腺腫瘤中的 sFRP2 在功能上的角色與腫瘤分子生物學上的機制，擬定了下列的幾項研究策略。

這個計畫包括了六個主要的部分，需要 3 年的研究時間：第一年首先主要的工作在於建立並分析多種新犬隻乳腺腫瘤組織的初代培養(primary culture)細胞株並且純化、分析乳腺上皮細胞。在第一年我們已成功地建立並分析數個本地病例之犬乳腺腫瘤細胞株。這些細胞利用下列技術分析其特性，包括增殖速度(by MTT assay)、 反轉錄鏈聚合胺反應(RT-PCR)、原位雜交法(*in situ* hybridization)與免疫化學染色(immunohistochemistry)及西方墨點法偵測 sFRP2 的表現。結果發現 sFRP2 基因之 mRNA 及蛋白質在犬乳腺腫瘤細胞株有大量之表現，然而在犬正常乳腺細胞及其他非 MGT 細胞株則無表現，第一年的成果已發表刊登。在第二階段，犬 sFRP2 被轉殖入含有 GFP 基因與 CMV 啟動子的哺乳類細胞表現載體，藉由 lipofection 方式將 GFP-sFRP2 穩定地轉染入(transfect)犬隻乳腺腫瘤初代培養與商品化乳腺腫瘤的細胞株，在本年度之計畫中進行更進一步的 sFRP2 調控細胞凋亡的功能分析。

在本年度之研究成果及進度方面，我們已非常辛苦地分析確認 sFRP2 基因確具有抗細胞凋亡之功能，且分析複雜的調控細胞凋亡之相關訊息傳遞途徑為 fibronectin-integrin signal transduction pathway，非過去傳統所知之 Wnt signaling，此重要發現為此新基因族之首篇調控細胞凋亡功能訊息傳遞途徑之新發現，此階段之研究成果也獲期刊 Journal of Biological Chemistry (impact factor:7.0)接受刊登。本階段之研究結果，預期將提供重要之學術資訊，以了解 sFRP 基因族在犬乳腺腫瘤細胞之調控情形。此外，此計劃也為未來下一階段進一步研究 sFRP 基因族不同成員之各種功能，及了解犬乳腺腫瘤複雜之病因，提供進一步研究分析之基礎。

雖然本階段的努力有獲得重大之進展及新發現，然而調控細胞凋亡功能之訊息傳遞途徑為十分複雜的研究領域，我們還須持續努力，以解開更多的謎團。

關鍵詞:分泌性細胞凋亡基因,分泌性 frizzled 蛋白基因，細胞凋亡，訊息傳遞，乳腺腫瘤

二、計畫英文摘要 (Abstract)

Mammary neoplasms are important and common tumors in both animals and humans and the etiology is complex. The secreted frizzled related proteins (sFRPs) are newly identified proteins and implicated to have dual roles of modulation of Wnt-Frizzled signal transduction pathway and regulation of apoptosis. We have recently found that sFRP2 was expressed abundantly in human and canine mammary gland tumors (MGT) tissues but was undetectable in normal canine mammary gland. To systematically

investigate the functional roles and molecular mechanisms of sFRP2 in canine MGT, several strategies are to be carried out as described below. The project is comprised of six major parts for a period of 3 years: In the 1st year, new primary cell cultures from native canine MGT tissues has been established and purified for mammary epithelial cells. We have successfully established and analyzed more native primary MGT cell lines from surgically excised MGT specimens. The cells are characterized for their cell origins, proliferation rate (by MTT assay), expression of sFRP2 by RT-PCR, *in situ* hybridization, and immunohistochemistry, and Western blotting. Expression experiments revealed the sFRP2 was abundantly expressed in canine MGT cell lines, but not expressed in normal canine MG cells nor other commercial non-MGT cell lines (previous NSC project, published in the Breast Cancer Research and Treatment). Canine sFRP2 is cloned into a mammalian expression vector with GFP reporter gene and CMV promoter. The GFP-sFRP2 is stably transfected into primary canine MGT and commercial MGT cell lines by lipofection for further analysis from the next stage of the project.

To elucidate the role of SFRP2 in the tumorigenesis of MGTs, apoptosis regulation mediated by SFRP2 was investigated by overexpression of SFRP2 in MGT and MCF7 cells. 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. To analyze the pathways through which SFRP2 transduces anti-apoptosis signals, co-immunoprecipitation and cell adhesion assays were carried out. SFRP2 was found secreted from cells and associated with the fibronectin-integrin protein complex and could promote cell adhesion. Moreover, by using heparin to block the SFRP2-fibronectin interaction or anti-integrin $\alpha 5\beta 1$ antibody to interrupt the fibronectin-integrin connection, the anti-apoptosis activity of SFRP2 was decreased. Taken together, these results suggest that SFRP2 exert its anti-apoptotic function in mammary cancer cells through association with the fibronectin-integrin signal transduction pathway, not the Wnt signaling as previous thought. The important data has been published and accepted by the Journal of Biological Chemistry (impact factor: 7.0).

The results of this stage of the project should offer important scientific basis and information to understand the roles of sFRP gene family in canine neoplastic cells. It also provides a basis for further analysis of functions of different members of the sFRP gene family and elucidation of the complex etiology and signaling pathways of mammary tumors.

Keywords: secreted apoptosis related protein secreted frizzled related protein, apoptosis, signal transduction, gene transfection, mammary neoplasia

二、緣由與目的

The frizzled and secreted frizzled related protein family is thought to modulate Wnt-Frizzled signal transduction pathway which plays an important role in normal development and oncogenesis, particularly in mammary neoplasia. More recently it has been reported that the sARP1 (also named sFRP2) possesses anti-apoptosis activity while sARP2 (also named sFRP1) induces pro-apoptosis in the breast tumor cells. In our previous NSC project (NSC 91-2313-B-002-404), sFRP2 was found to be expressed abundantly in 31 different canine MGT tissues, but not expressed in normal MG tissues. This striking finding stimulated our interest in further investigation of the gene family. The roles of the gene family in tumor tissues remain to be determined.

Canine mammary gland tumor (MGT) is the canine counterpart of human breast cancer that shares significant similarities in several aspects. MGT is the most common tumor type in female dogs comprising of 52% of all neoplasms in the bitches. However, the etiology of MGT is mostly unknown and surprisingly very few advanced molecular studies regarding MGTs have been done to date. We would like to understand whether the dual roles of secreted frizzled related genes-mediated apoptosis and Wnt-signaling pathway play a part in the pathogenesis of canine MGT.

The purposes of the study at this stage include 1) to determine the apoptosis regulatory functions of sFRP2 in mammary cancer cell lines

2) to analyze and investigate the complex signaling pathways of sFRP2-mediated apoptosis regulatory functions by a variety of experiments.

三、結果

Please note that the following is the "summary" of all experimental results, not detailed data due to space limit of this report.

1. Overexpression of SFRP2 results in decrease of sensitivity to UV-induced apoptosis

Previously, SFRP2 has been reported to possess anti-apoptosis activity. To explore the molecular mechanisms underlying the anti-apoptosis effect of SFRP2 in mammary tissues, 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 and purified in our lab. After being selected by G418, the stable clones of MPG/pCMV-cSFRP2-Flag and MCF7/pCMV-cSFRP2-Flag were collected and the expression of SFRP2 was analyzed by Western blotting. **Figure 1a** showed that MPG cells have basal expression of SFRP2, and increasing amount of SFRP2-Flag protein expression was observed in both MPG and MCF7 cells. To further examine the functional activity of SFRP2, the stable cell lines described in **Figure 1a** were exposed to UV, and subjected to DNA fragmentation analysis. **Figure 1b** showed that overexpression of SFRP2 in MPG and MCF7 cells could inhibit the DNA fragmentation caused by UV (lanes 3 and 6). The caspase 3 activity in MPG cells was also decreased in the presence of SFRP2 (**Figure 1c**). Taken together, these results demonstrated that canine SFRP2 has anti-apoptosis activity in mammary cancer cells.

2. Interaction of SFRP2 with FN

The immunohistochemical staining results of canine MGT tissues showed that SFRP2 existed in connective tissues (**Figure 2a**). Furthermore**,** the presence of SFRP2 protein can be detected in the media from the stably expressed SFRP2 cells (**Figure 2b**). Thus, consistent with previous reports, SFRP2 is indeed a secreted protein. To assess the possibility of an interaction between SFRP2 and ECM, the immunoprecipitated SFRP2 complexes from culture media were subjected to Western blotting using anti-FN antibody. **Figure 2c** showed that the SFRP2 antibody could co-immunoprecipitate FN from MPG and MCF7 cell culture media. FN has been reported to interact with integrin receptor (Pytela *et al.*, 1985; Akiyama and Yamada, 1985). To dissect the signaling pathways of SFRP2, we further examine whether integrin receptor is present in the SFRP2 protein complexes. Expression of integrin $\alpha5\beta1$ in both the MPG and MCF7 cells was confirmed (**Figure 2d**), and co-immunoprecipitation experiment using the anti-SFRP2 antibody revealed that FN as well as integrin $\alpha5\beta1$ receptor associate with SFRP2 in MPG and MCF7 cells (**Figure 2e**). Taken together, theses results demonstrated that SFRP2 is a secreted protein and an interacting component of the FN-integrin receptor protein complexes.

3. The expression of SFRP2 could promote cell adhesion

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 (**Figure 3a**). It appeared that the presence of SFRP2 could enhance the formation of FN-integrin receptor complexes. Based on the knowledge that the interaction of FN and integrin receptor promotes the cell adhesion, we set out to compare the level of cell adhesion in the presence or absence of SFRP2. As shown in **Figure 3b**, overexpression of SFRP2 in MPG cells could promote cell adhesion. SFRP2 also could further promote cell adhesion in the presence of Mn^{2+}, an integrin $\alpha5\beta1$ receptor stimulator (Mould *et al.*, 1995). It is possible that, in addition to the integrin $\alpha5\beta1$-FN pathway, the SFRP2 may promote cell adhesion through additional pathways. **Figure 3c** showed that the stimulatory effect of SFRP2 was specific to FN. The similar results were obtained in MCF7 cells (**Figure 3d**). Thus, we can conclude that SFRP2 promotes cell adhesion through enhancing integrin $\alpha5\beta1$-FN binding.

4. The possible signaling pathway downstream of the SFRP2-FN interaction

To delineate the anti-apoptotic signaling pathways mediated by SFRP2, we attempted to examine the expression of some signaling factors. Since focal adhesion kinase (FAK) is a key mediator of integrin signaling, we evaluated the tyrosine phosphorylation status of FAK. When MPG cells harboring the SFRP2 expression vector were allowed to survive upon UV-irradiation, the tyrosine phosphorylation of FAK was enhanced (**Figure 4a**). Cleaved side products of FAK were observed under UV-induced condition, consistent with a previous report (Chan *et al.*, 1999). Two additional apoptotic effectors, β-catenin and JNK, were also analyzed**;** and **Figure 4b** showed that the expression of SFRP2 elevated the intracellular level of β-catenin regardless of UV treatment. JNK could be activated by UV-irradiation in the control cell line, whereas it was down-regulated in the SFRP2-overexpressing cells.

5. SFRP2-FN interaction is required to reduce cell susceptibility to UV-induced apoptosis

We have provided evidence that expression of SFRP2 could protect cell from UV-induced apoptosis and that SFRP2 interacts with FN. To examine whether SFRP2's interaction with FN is essential for its anti-apoptosis activity, we used inhibitors to interrupt the interaction between SFRP2 and FN. Heparin has been reported to be able to bind to either SFRP2 or FN and release FRP from the cell surface (Finch *et al.*, 1997). Immunoprecipitation assay using anti-SFRP2 antibody was done on lysates prepared from cells previously treated with heparin. As shown in **Figure 5a**, the level of co-immunoprecipitated FN was decreased in the presence of heparin. In addition, anti-integrin α5β1 antibody appeared to block the association of integrin with the SFRP2-FN complexes, as revealed by the co-immunoprecipitation experiment (**Figure 5b**). Moreover, result of the DNA fragmentation analysis indicated that the anti-apoptosis effect of SFRP2 was abrogated in the presence of heparin or anti-integrin α5β1 antibody (**Figure 5c**).

四、討論

Despite the well-established role of Wnt signaling in oncogenesis (Peifer and Polakis, 2000), the involvement of the SFRP gene family in cancer is still under investigation. In the past few years, several reports have described the different expression patterns of SFRPs in various cancers. For example, SFRP1 is down-regulated in breast cancer (Zhou *et al.*, 1998; Ugolini *et al.*, 1999; Wong *et al.*, 2002), but is overexpressed in uterine leimyoma (Fukuhara *et al.*, 2002). frpHE (SFRP4) is up-regulated in the stroma of endometrial and breast carcinomas (Abu-Jawdeh *et al.*, 1999) and SFRP1 and SFRP2 are produced by the majority of long-term and *ex vivo* malignant glioma cell lines (Roth *et al.*, 2000). Our previous study demonstrated that canine SFRP2 was expressed in MGTs, but absent in normal mammary glands. In this study, we showed that overexpression of canine SFRP2 in MCF7 and our cloned canine MGT cell line could decrease cell sensitivity to UV-induced apoptosis. This result is consistent with the observation that mouse homologue of SFRP2 could also enhance the cell viability of MCF7 cells in response to the presence of TNF or ceramide (Melkonyan *et al.*, 1997). Apoptosis is the physiological process by which unwanted or useless cells are eliminated during development and other normal biological processes. When apoptosis malfunctions, the results may be: cancer and autoimmune diseases when there is too little apoptosis occurs; or possibly stroke damage or the neurodegeneration of Alzheimer's disease when there is too much apoptosis. The anti-apoptotic activity of SFRP2 offers a clue that it may be involved in either the formation or progression of mammary tumors. Also consistent with this idea, investigating new drugs that potentially restore the apoptotic defense mechanism (such as heparin or anti-integrin α5β1 antibody mentioned in this study) will possess substantial therapeutic benefit for mammary tumors.

SFRP2 is a secreted glycoprotein. Functional characterization of this protein is our main research focus in this project. Interactions of cells with the extracellular environment regulate many basic cellular functions, including differentiation, migration, cell growth, and apoptosis (Aplin *et al.*, 1999; Ilic *et al.*, 1997; Sonoda *et al.*, 1999; Lee and Juliano, 2000). Most normal cells require attachment to ECM for survival. Cell adhesion to ECM is mainly mediated by integrins, a family of

cell surface receptors widely expressed on all tissues. The αβ integrin is heterodimeric transmembrane receptors with overlapping specificity toward ECM components (Damsky and Werb, 1992; Meredith *et al.*, 1993; Howlett *et al.*, 1995; Giancotti and Ruoslanhti, 1999). One of ECM components, FN, is particularly effective in providing survival signals for several cell types**;** and these survival signals were found to be transduced by FAK (Frisch *et al.*, 1996; Ilic *et al.*, 1998). The physical interaction of SFRP2 with FN-integrin protein complex was demonstrated by co-immunoprecipitation, and it was 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 anti-apoptotic 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. In an attempt to examine the possible underlying mechanisms of such anti-apoptotic function, we discovered that, under UV-stimulation, the overexpression of SFRP2 causes the activation of FAK, elevation of β-catanin protein, and inactivation JNK. According to the published observations, these cellular features are an indication that cells is in a survival mode (Sonoda *et al.*, 1999; Chen *et al.*, 2001; Harding *et al.*, 2001). 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.

SFRPs, Wnt and FN possess a highly basic region that confers strong affinity for heparin (Finch *et al.*, 1997; Üren *et al.*, 2000). If tissue proteoglycan content is decreased, either by genetic or biochemical manipulation, the Wnt signaling is impaired (Hacker *et al.*, 1997; Binari *et al.*, 1997). For instance, heparin or endogenous HSPG might promote SFRP1/Wg binding by stabilizing conformation of either SFRP1 or Wg that would increase their mutual affinity or by enhancing ligand or receptor oligomerization (Üren *et al.*, 2000). Heparinase treatment of endothelial cells inhibited endothelial cell proliferation and in vivo neovascularization (Sasisekharan *et al.*, 1994), emphasizing the role of heparin-like molecules in tumorigenesis.

Estrogens are known to stimulate the proliferation of hormone-dependent breast and ovarian cancers through their interaction with estrogen receptors (ERs) and the subsequent expression of a number of genes, some of them involved in the control of cell proliferation (Dickson *et al.*, 1986; Vignon *et al.*, 1986). In addition, several recent *in vitro* and *in vivo* studies have demonstrated that ECM proteins, including FN, may be important paracrine factors in mammary gland growth, morphogenesis, and lactation (Xie and Haslam, 1997; Woodward *et al.*, 2000). The progestin, R5020, significantly stimulates proliferation of primary mouse mammary epithelial cells cultured on FN but not on collagen I (Col I), laminin (LM), or tenascin (Xie and Haslam., 1997). In this study, our results link FN to mammary tumorigenesis as part of the SFRP2-regulated integrin-mediated anti-apoptotic signaling pathway. Taken together, the available data suggest that estrogen/ER and FN/SFRP2 both play critical roles in breast cancer development. However, some studies have demonstrated that the ER-positive breast cancer cells are in general poorly invasive *in vivo* (better prognosis) and *in vitro* as compared with ER-negative cancer cells (Price *et al.*, 1990). One hypothesis for this contrasting observation is that FN/SFRP2 is a key factor. In related studies, estradiol and fibulin-1 can inhibit human ovarian- and breast-cancer cell motility induced by FN and therefore have the potential to inhibit tumor invasion (Hayashido *et al.*, 1998). The expression levels of the ECM protein FN and the α5β1 integrin are enhanced by estrogen in normal mouse mammary gland (Woodward *et al.*, 2001) and by the Wnt/Frizzled-mediated β-catenin/Tcf transcription (Gradl *et al.*, 1999). In contrast to FN, the concentrations of Col I, Col IV, and LM do not exhibit major changes during mammary development (Woodward *et al.*, 2001). Therefore, ER-positive breast cancer patients have better prognosis due to the enhanced FN/SFRP2-mediated signal. Additionally, further studies can be undertaken to determine whether SFRP2 and FN might be ideal prognostic markers in breast cancers.

五、計畫成果自評

During the second year of this 3-year project, we have obtained important research data and progress. Firstly, we have established and characterized the apoptosis regulatory functions of sFRP2 in mammary cancer cell lines.

Secondly, the complex signaling pathways of sFRP2-mediated apoptosis regulation were investigated and elucidated by a number of complex experiments. It is an important and the first finding regarding signaling pathways of sFRP2-mediated apoptosis regulation in cancer cell lines.

The research results will be very helpful to elucidate the relation between apoptosis, the pathogenesis of mammary cancers, and sFRP gene family. Further studies will be required to understand the complex signaling pathways in sFRP2 and other apoptosis regulatory sFRP gene family in mammary cancer lines from the next stage.

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行政院國家科學委員會專題研究計畫
期中進度報告

實驗結果圖表部分

細胞凋亡調控基因(sFRP2)在犬乳腺腫瘤之細胞凋亡調控角色及相關調節因子的研究 (2/3)

計畫編號：NSC 92-2313-B-002-133

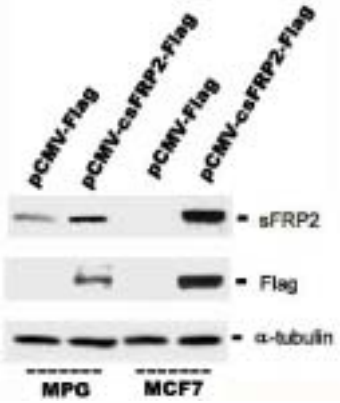
執行期限：92 年 8 月 1 日至 93 年 7 月 31 日

主持人：林中天　　國立台灣大學獸醫學系

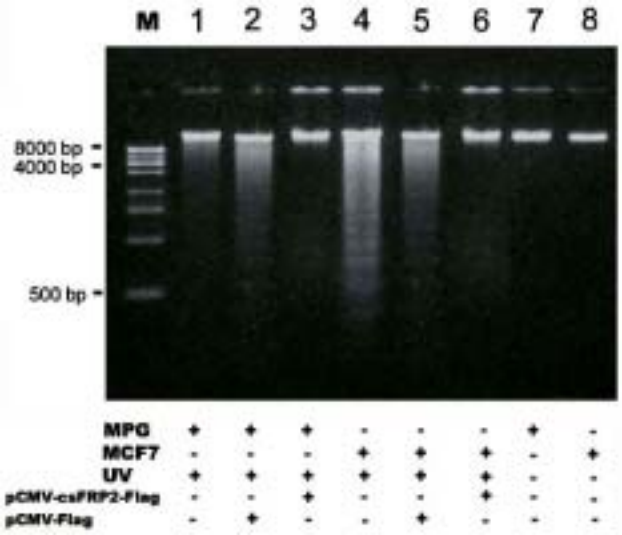
計畫參與人員: 李佳霖、林念儀、謝岱儒　等研究生

Figure 1

a


pCMV-Flag
pCMV-csFRP2-Flag
pCMV-Flag
pCMV-csFRP2-Flag
sFRP2
Flag
α-tubulin
MPG
MCF7

b


M 1 2 3 4 5 6 7 8
8000 bp
4000 bp
500 bp
MPG + + + - - - + -
MCF7 - - - + + + - +
UV + + + + + + - -
pCMV-csFRP2-Flag - - + - - + - -
pCMV-Flag - + - - + - - -

c

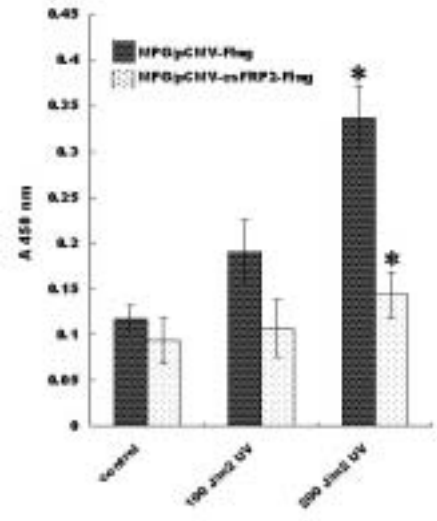
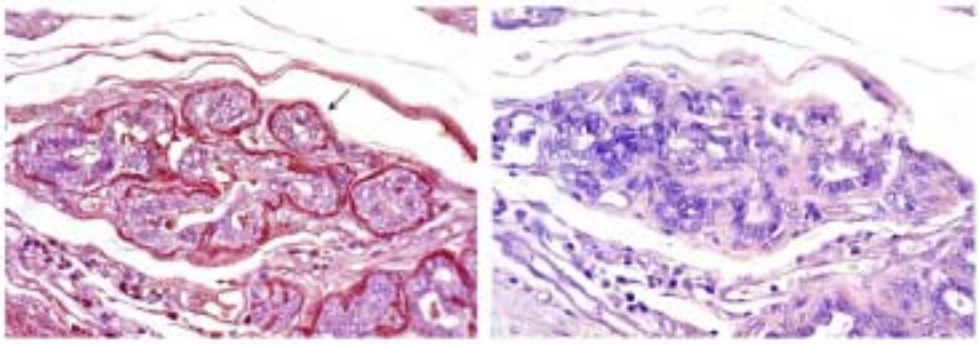
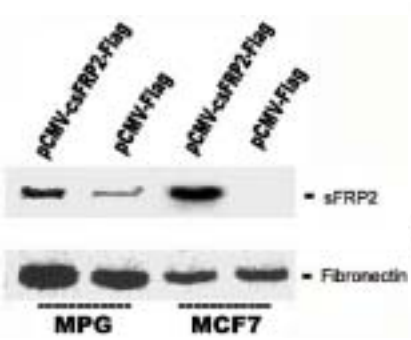

MPG/pCMV-Flag
MPG/pCMV-csFRP3-Flag
A 450 nm
control
100 J/m2 UV
200 J/m2 UV

Figure 2

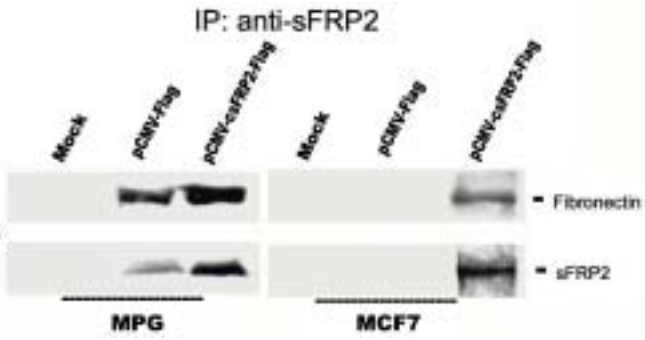
a



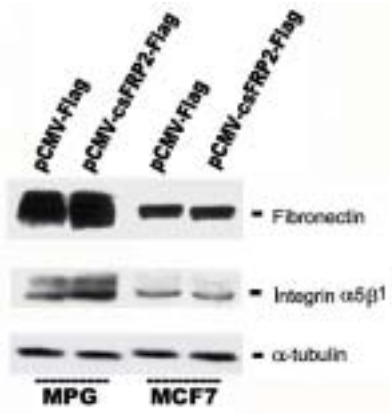
b



c



d



e

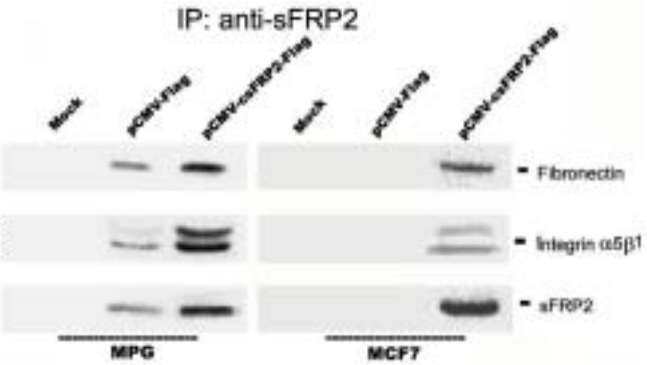


Figure 3

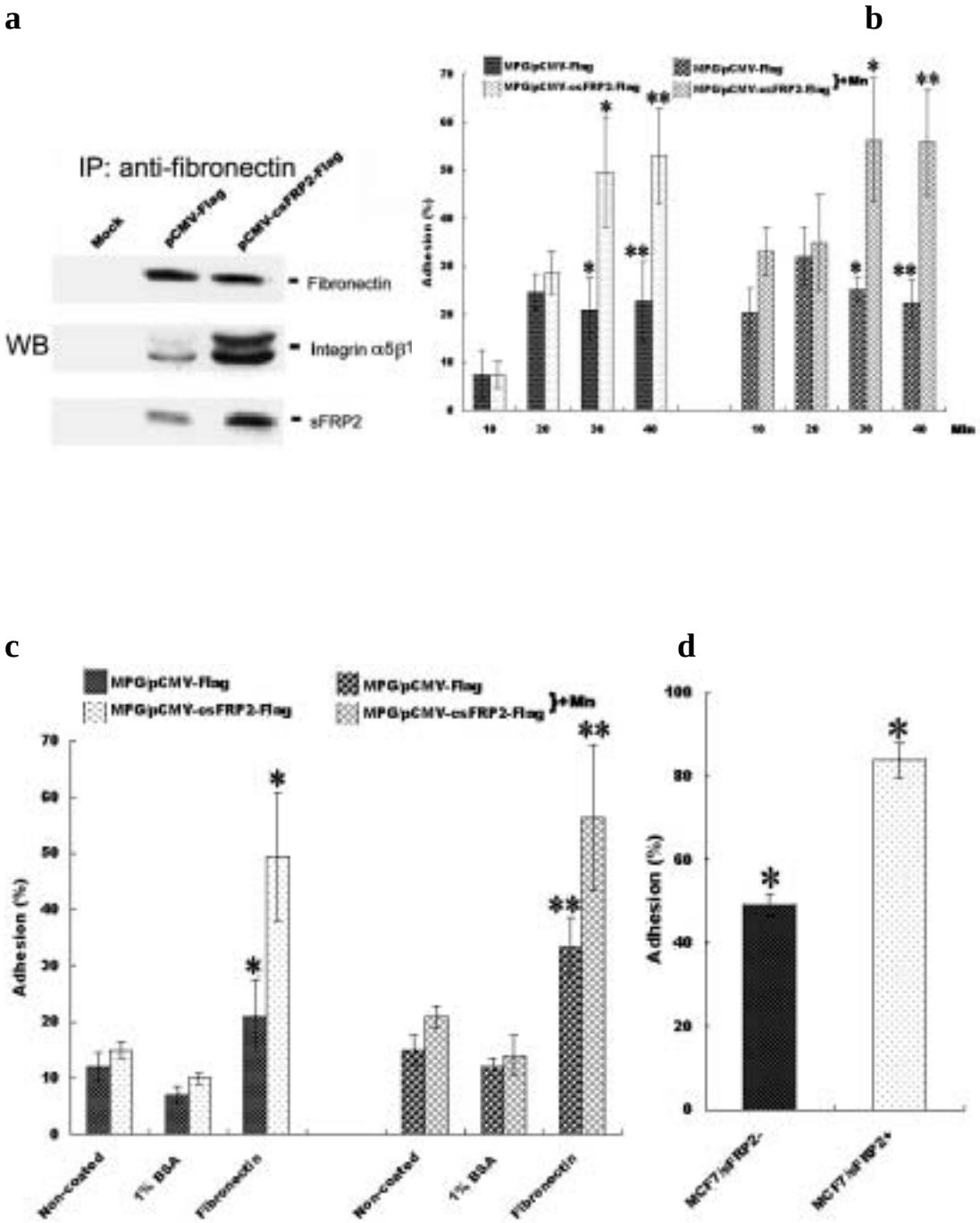
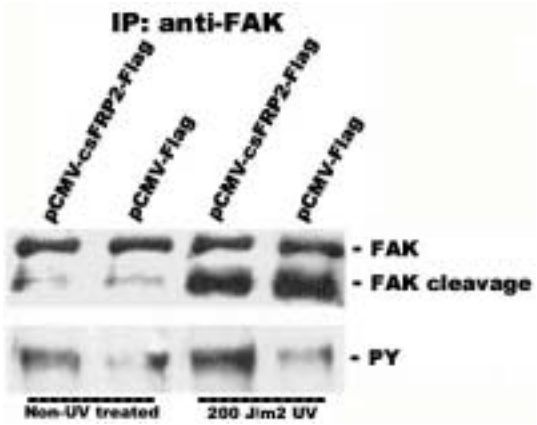
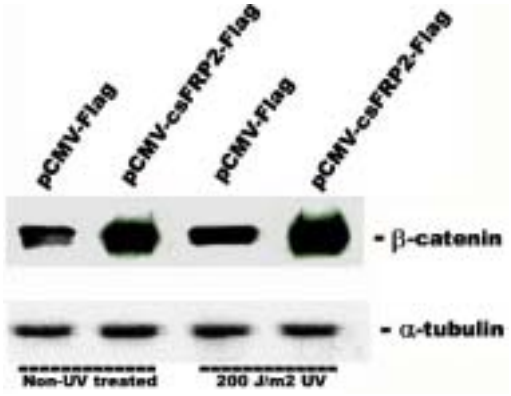

a
b
IP: anti-fibronectin
Mock
pCMV-Flag
pCMV-csFRP2-Flag
WB
Fibronectin
Integrin α5β1
sFRP2
MPG/pCMV-Flag
MPG/pCMV-csFRP2-Flag
+Mn
Adhesion (%)
Min
c
d
Non-coated
1% BSA
Fibronectin
MCF7/sFRP2-
MCF7/sFRP2+

Figure 4

a


IP: anti-FAK
pCMV-csFRP2-Flag
pCMV-Flag
pCMV-csFRP2-Flag
pCMV-Flag
- FAK
- FAK cleavage
- PY
Non-UV treated
200 J/m2 UV

b


pCMV-Flag
pCMV-csFRP2-Flag
pCMV-Flag
pCMV-csFRP2-Flag
- β-catenin
- α-tubulin
Non-UV treated
200 J/m2 UV

c

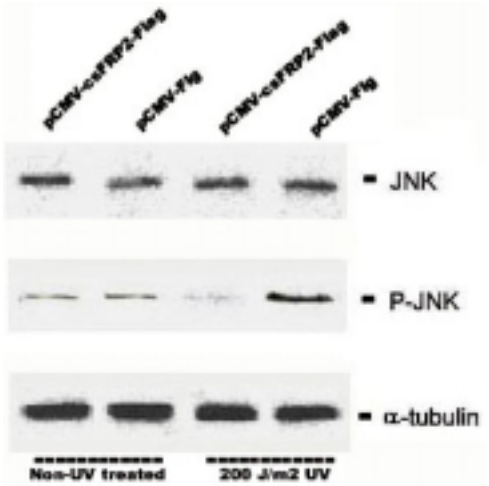
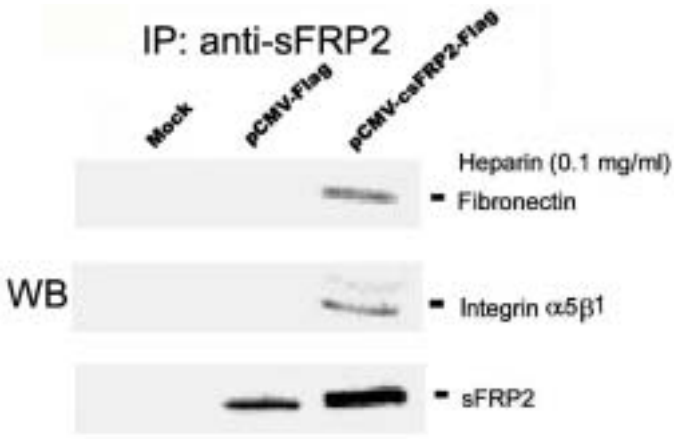
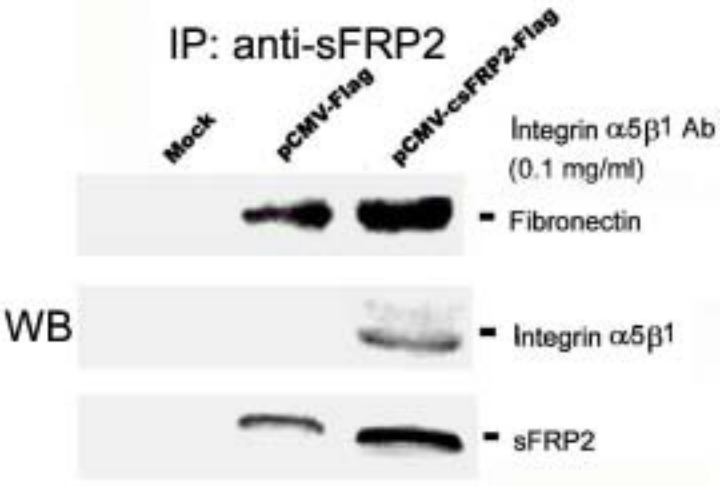

pCMV-csFRP2-Flag
pCMV-Flag
pCMV-csFRP2-Flag
pCMV-Flag
- JNK
- P-JNK
- α-tubulin
Non-UV treated
200 J/m2 UV

Figure 5

a


IP: anti-sFRP2
Mock
pCMV-Flag
pCMV-csFRP2-Flag
Heparin (0.1 mg/ml)
Fibronectin
WB
Integrin α5β1
sFRP2

b


IP: anti-sFRP2
Mock
pCMV-Flag
pCMV-csFRP2-Flag
Integrin α5β1 Ab
(0.1 mg/ml)
Fibronectin
WB
Integrin α5β1
sFRP2

c

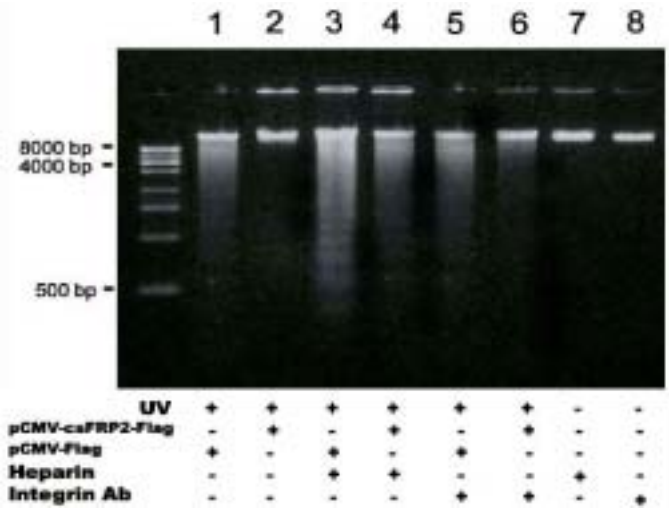

1 2 3 4 5 6 7 8
8000 bp
4000 bp
500 bp
UV + + + + + + - -
pCMV-csFRP2-Flag - + - + - + - -
pCMV-Flag + - + - + - - -
Heparin - - + + - - + -
Integrin Ab - - - - + + - +

FIGURE LEGENDS

Figure 1 Overexpression of SFRP2 reduces cell sensitivity to UV-induced apoptosis. **(a)** Expression of the endogenous or exogenous SFRP2. MPG and MCF7 cells were transfected with expression vectors for SFRP2 proteins (MPG/pCMV-cSFRP2-Flag, MCF7/pCMV-cSFRP2-Flag cells) or the vector backbone (MPG/pCMV-Flag, MCF7/pCMV-Flag cells) and selected by G418. Expression of the SFRP2 proteins was determined by Western blot analysis. **(b, c)** Apoptotic analysis of the MPG and MCF7 transfectants. After selection by G418, the cells were irradiated with UV at 200 J/m^2 in **b** or 100, 200 J/m^2 in **c**. 24 hours after irradiation, the cells were analyzed for DNA fragmentation by DNA laddering in **b** and Cell-Death Detection ELISA in **c**. Results are represent the means ± SE of three independent experiments. *: $P< 0.05$.

Figure 2 SFRP2 is secreted from the cells and associated with FN. (a) Expression of SFRP2 in tissue sections of canine MGTs (magnification x 400). Expression of the SFRP2 proteins were detected by immunohistochemical analysis; anti-SFRP2 antibody as indicated (left) and negative control (right). Note the positive signal around cells (arrow). **(b)** Expression of the SFRP2 and FN proteins in cultured medium were determined by Western blot analysis. Proteins were prepared from cultured medium of MPG/pCMV-cSFRP2-Flag, MPG/pCMV-Flag, MCF7/pCMV-cSFRP2-Flag, and MCF7/pCMV-Flag cells. **(c)** Immunoprecipitation of the SFRP2 proteins in cultured medium. Proteins were prepared as in **b**. SFRP2 was immunoprecipitated with anti-SFRP2 antibody as indicated. SFRP2 and FN were detected by Western blot analysis. **(d)** Expression of the SFRP2, integrin $\alpha5\beta1$ and FN proteins of whole cell extracts were determined by Western blot analysis. Whole cell extracts were prepared from the same cells as in **b**. **(e)** Immunoprecipitation of the SFRP2 proteins of whole cell extracts was done as in **c**.

Figure 3 SFRP2 promotes integrin-mediated cell adhesion by enhancing integrin $\alpha5\beta1$-FN binding

affinity. **(a)** Immunoprecipitation of the FN proteins. Whole cell extracts were prepared from MPG/pCMV-cSFRP2-Flag and MPG/pCMV-Flag cells. FN was immunoprecipitated with anti-FN antibody as indicated. SFRP2, integrin α5β1, and FN were detected by Western blot analysis. **(b, c)** Adhesion assay. The same cells as in **a** were incubated without (left) or with (right) 2 mM of $MnCl_2$ for 30 min. Cells were replated on FN and allowed to adhere for 10, 20, 30, or 40 min in **b**, or on non-coated, 1%BSA, FN for 30 min in **c**, respectively. The percentage of adhesion was calculated as described in Materials and Methods. Data from three separate experiments were shown as means ± SE. *, **: $P< 0.05$. **(d)** MCF7 cells were incubated with cultured medium of MCF7/pCMV-cSFRP2-Flag (SFRP2+) or MCF7/pCMV-Flag (SFRP2-) cells. The cells were replated on FN and allowed to adhere for 30 min as indicated. Steps were the same as in **b**. *: $P< 0.05$.

Figure 4 SFRP2 activates FAK and β-catenin but suppresses JNK activity. (a) Immunoprecipitation of the FAK proteins. MPG/pCMV-cSFRP2-Flag and MPG/pCMV-Flag cells were irradiated with or without UV at 200 J/m^2. After 24 h of incubation, the cells were lysed and FAK, FAK cleavage, and FAK tyrosine phophorylation were detected by immunoprecipitation with anti-FAK antibody, followed by western blot analysis using anti-FAK antibody and antibody to phosphotyrosine, as indicated. **(b, c)** Expression of the β-catenin in **b** and JNK, phospho-JNK in **c** proteins were determined by Western blot analysis. Whole cell extracts were prepared as in **a**. (α-tubulin was used as internal control).

Figure 5 SFRP2 requires ECM components to decrease susceptibility to UV-induced apoptosis. **(a, b)** Immunoprecipitation of the SFRP2 proteins. MPG/pCMV-cSFRP2-Flag and MPG/pCMV-Flag cells were incubated with 0.1 mg/mL Heparin in **a** or 0.1 mg/mL integrin α5β1 antibody in **b**. Experimental steps were the same as Figure 2-**e**. **(c)** Apoptotic analysis. The same cells as in **a** and **b** were irradiated with UV at 200 J/m^2. 24 hours after irradiation, the cells were analyzed for DNA fragmentation by DNA laddering experiment.