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Changes in Apoptosis-related Gene Expression Induced by Repression of *FGFR1* by RNA Interference in Embryonic Fibroblasts and Cancerous Cells from Chicken

Sang In Lee, Bo Ram Lee, Young Sun Hwang, Deivendran Rengaraj and Jae Yong Han*

WCU Biomodulation Major, Department of Agricultural Biotechnology, Seoul National University, 599 Gwanak-ro, Gwanak-gu, Seoul 151-921, Korea

ABSTRACT

Fibroblast growth factor receptor 1 (*FGFR1*) plays roles in angiogenesis, wound healing, and embryonic development via the regulation of cell proliferation, differentiation, and survival. It is well known that ectopic expression of *FGFR1* is associated with cancer development. To characterize the function of *FGFR1* in the normal and cancer cell lines DF-1 and DT40, respectively, we performed *FGFR1* knockdown by RNA interference. In the DT40 cells, *FGFR1* knockdown induced upregulation of *FGFR2* and *FGFR3* expression, downregulation of pro-apoptosis-related genes, and upregulation of anti-apoptosis-related genes. However, in DF-1 cells, *FGFR1* knockdown induced upregulation of pro-apoptosis-related genes and downregulation of anti-apoptosis-related genes. Our data suggest that repression of *FGFR1* induced upregulation of other FGF receptors and anti-apoptosis-related genes in cancer cells and pro-apoptosis-related genes in normal cells.

(**Key words :** RNA interference, *FGFR1*, Apoptosis, DT40, DF1)

INTRODUCTION

Fibroblast growth factors (FGFs) were discovered as mitogens for cultured fibroblasts (Gospodarowicz, 1974). A total of 22 FGFs have been identified in various organisms (Ornitz & Itoh, 2001). FGFs induce cellular responses by binding to a family of FGF receptors (*FGFRs*), which are receptor tyrosine kinases (Jaye et al, 1992). *FGFRs* are involved in numerous biological processes during embryo development and homeostasis. *FGFR1* in particular is expressed during the development of the brain in the chick, mouse, and frog (Amaya et al, 1991; Yamaguchi et al, 1994). Also, *FGFR1* promotes proliferation and survival via activation of the mitogen-activated protein kinase pathway in different cell lines (Tomlinson et al, 2009).

The DT40 cell line, a B-cell lymphoma line, was permanently transformed with an avian leukosis virus to cause immunoglobulin gene conversion (Baba et al, 1985; Buerstedde et al, 1990). The DT40 cell line exhibits a high frequency of recombination and thus is often used as a model system for functional gene studies (Hudson et al, 2002; Johnson et al, 2009). The DF-1 cell line, a line of immortalized chicken embryonic fibroblasts, is susceptible to transformation by numerous oncogenes and efficiently replicates avian retroviruses (Himly et al, 1998). Small interfering RNA (siRNA) complementary to a target mRNA sequence in a cell can be used to downregulate target gene expression, making the RNA interference (RNAi) technique applicable in functional genomic studies. The RNAi technique has been used to study gene function related to somitogenesis and retinal development in early stages of chicken embryos *in ovo* (Harpavat & Cepko, 2006).

To investigate the function of *FGFR1* in different cell types, we designed a short hairpin RNA (shRNA) for *FGFR1* knockdown and compared gene expression patterns between normal DF-1 cells and DT40 cancer cells. Our results indicate that *FGFR1* silencing by RNA interference induced the upregulation of anti-apoptosis-related genes and other *FGFRs* in cancer cells, whereas pro-apoptosis-related genes were upregulated in normal fibroblasts.

* Corresponding author : Jae Yong Han, Ph.D., Seoul National University 599 Gwanak-ro, Gwanak-gu, Seoul 151-921, Korea. Tel: +82-2-880-4810, Fax: +82-2-874-4811, E-mail: jaehan@snu.ac.kr

MATERIALS AND METHODS

1. Design of target-specific shRNA and RNA interference vector construction

Based on the full-length *FGFR1* sequence, FGFR1-specific siRNAs were designed using siRNA Target Finder (http://www.ambion.com/techlib/misc/siRNA/finder.html). Candidate siRNA sequences were analyzed using the pSilencer™ Expression Vector Insert Design Tool for shRNA (Ambion, Austin, TX; http://www.ambion.com/techlib/misc/psilencer_converter.html), to generate the loop sequence (TTCAAGAGA) required for making a hairpin structure. The designed shRNAs were cloned into the BamHI and HindIII sites in pSilencer expression vector (Ambion).

2. Cell culture and transfection

The procedures for animal management, reproduction, and embryo manipulation followed standard operating protocols used in our laboratory. Cell culture and transfection were performed according to our previous report (Lee et al, 2010). The chicken cell lines DT40 and DF-1 were purchased from ATCC (Global Bioresource Center). DT40 and DF-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM, High glucose; Gibco, Grand Island, NY) containing 10% fetal bovine serum (Hyclone, Logan, UT), and 1% antibiotic-antimycotic solution (Gibco) at 37℃ in 5% CO_2 at 90% humidity. For transfection, DT40 or DF-1 cells were resuspended at 1×10^6 cells per 100 μl of Nucleofection-V solution (Lonza, Basel, Switzerland). DNA was added to the cell solution, and nucleofection was performed using the A-033 program (Lonza). Then, 500 μl of medium were added to each cell solution, and the cells were transferred to a 35-mm culture dish and cultured for 48 h, with daily changes of the medium.

3. Quantitative real-time PCR

RNA extraction, cDNA synthesis, and quantitative real-time PCR analysis were performed according to our previous reports (Lee et al, 2007; Lee et al, 2009). To estimate the expression and effect of gene silencing, total RNA was extracted from the cultured cells. Total RNA (1 μg) from each sample was used to create single-stranded cDNA, using a Superscript III first-strand synthesis system (Invitrogen). Sequence-specific primers were designed using the Primer3 program (http://frodo.wi.mit.edu/). Real-time PCR was performed using an iCycler iQ real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) and SYBR Green1 (Sigma, St. Louis, MO). Wells without cDNA were included as negative controls. Each test sample was run in triplicate. The PCR conditions were 94℃ for 3 min, followed by 40 cycles of 94℃ for 30 s, 59 to 61℃ for 30s, and 72℃ for 30 s, using a melting curve program (temperature increase from 55 to 95℃ at a rate of 0.5℃/10 s) and continuous fluorescence monitoring. The results are reported as the relative expression after normalization of the transcript to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an endogenous control, with the nonspecific control as a calibrator, using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

4. Statistical analysis

Student's t-test and analysis of variance (ANOVA) were performed to compare the effect of *FGFR1* knockdown in both cell lines. SAS software (SAS Institute, Cary, NC) was used for the calculations. A P-value <0.05 was considered to indicate statistical significance.

RESULTS

1. Repression of *FGFR1* expression by RNA interference in DT40 and DF-1 cells

To obtain *FGFR1*-specific shRNA, we designed two shRNA sequences that obeyed Thuthel's rule (http://www.ambion.com/techlib/misc/siRNA/finder.html). The target sequences were located at 401 and 2,561 bp, respectively, in the *FGFR1* mRNA sequence (Table 1). To confirm that the two shRNAs repressed the expression of *FGFR1* mRNA, expression vectors containing the shRNAs were introduced into DT40 and DF-1 cells by nucleofection, a modified electroporation technique. Nonspecific shRNA containing no complementary sequence in the chicken genome was used as a control.

The *FGFR1* mRNA expression levels were analyzed 48 h after nucleofection. The shRNA_401 vector produced 56±9% ($P<0.0001$) repression of *FGFR1* mRNA expression in DT40 cells (Fig. 1A) and 54±3% ($P<0.0001$) repression in DF-1 cells (Fig. 1B) compared with expression in the controls. The

Table 1. List of synthesized candidate shRNAs for *FGFR1* specific knockdown

Candidate shRNA	Target Sequence	Direction	Designed shRNA Sequence (5'-3')	Location*
shRNA_FGFR1_401	AACGTCTCAGACGCACTCCCT	Forward	GATCCGCGTCTCAGACGCACTCCCTTTCAAGAGAAGGGAGTGCGTCTGAGACGTTTTTTGGAAA	401
		Reverse	AGCTTTTCCAAAAAACGTCTCAGACGCACTCCCTTCTCTTGAAAGGGAGTGCGTCTGAGACGCG	
shRNA_FGF1_2561	AACTGCCAAAGCTTTCGGCT	Forward	GATCCGCTGCCCAAAGCTTTCGGCTTTCAAGAGAAGCCGAAAGCTTTGGGCAGTTTTTTGGAAA	2561
		Reverse	AGCTTTTCCAAAAAACTGCCCAAAGCTTTCGGCTTCTCTTGAAAGCCGAAAGCTTTGGGCAGCG	
Nonspecific shRNA[#]	GTCAGGCTATCGCGTATCG	Forward	GATCCGTCAGGCTATCGCGTATCGTTCAAGAGACGATACGCGATAGCCTGACTTTTTTGGAAA	N/A
		Reverse	AGCTTTTCCAAAAAAGTCAGGCTATCGCGTATCGTCTCTTGAACGATACGCGATAGCCTGACG	

[#] Nonspecific shRNA has no complementary sequence in the chicken genome and was used as a gene silencing control.
* Location refers to the first nucleotide of the target sequence in FGFR1 mRNA sequence.

introduction of pMax_GFP vector (Lonza), used to determine the transfection efficiency of nucleofection, revealed transfection efficiencies of 63±6% (P<0.01) and 60±6% (P<0.01) in DT40 and DF-1 cells, respectively (data not shown). We used the shRNA_401 vector for further experiments and analysis in both cell lines.

2. Effect of *FGFR1* repression on the expression pattern of *FGFR*s in DT40 and DF-1 cells

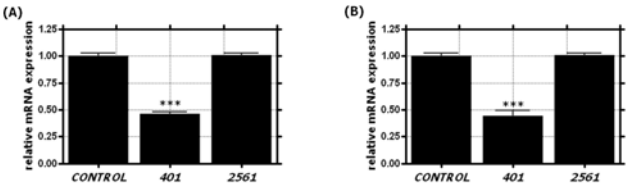

Fig. 1. Repression of *FGFR1* expression by target-specific shRNA in DT40 and DF-1 cell lines. Selected shRNA expression constructs were introduced into DT40 (A) and DF-1 (B) cells by nucleofection. Nonspecific shRNA, with no complementary sequence in the chicken genome, was used as the control. 401, shRNA_*FGFR1*_401 expression construct; 2561, shRNA_*FGFR1*_2561 expression construct; DT40, B-cell lymphoma cell line; DF-1, immortalized chicken embryonic fibroblast cell line. Quantitative real-time PCR analysis was conducted to measure relative expression normalized to *GAPDH* expression. All reactions were performed in triplicate. ***P < 0.001 (one-way ANOVA) compared with nonspecific shRNA. Error bars indicate the SE of triplicate analyses.

To examine the effect of repressed *FGFR1* on the expression pattern of *FGFRs*, we performed quantitative real-time PCR analysis in the DT40 and DF-1 cell lines. In the absence of shRNA_401, both cell lines showed stronger

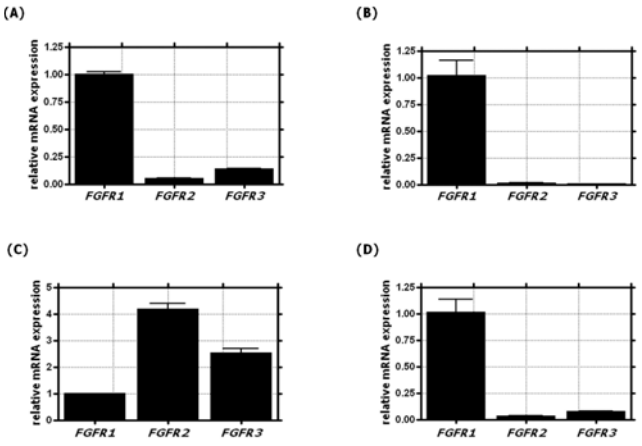

Fig. 2. Expression patterns of *FGFR*s in DT40 and DF-1 cell lines. (A) Relative mRNA expression of *FGFR*s in DT40 (B-cell lymphoma) cells, determined by quantitative real-time PCR. GAPDH was used as an endogenous control. (B) Relative mRNA expression of *FGFR*s in DF-1 (fibroblast) cells, determined by quantitative real-time PCR. (C) Changes in expression of *FGFR*s at 48 h after repression of *FGFR1* expression in DT40 cells. (D) Changes in expression of *FGFR*s at 48 h after repression of *FGFR1* expression in DF-1 cell. All reactions were performed in triplicate and normalized to *GAPDH* expression. Error bars indicate the SE of triplicate analyses.

Table 2. List of candidate genes by *FGFR1* knockdown and primer sequences for quantitative RT-PCR

Symbol	Description	Gene bank No.	Relative fold change using real_time quantitative PCR				Primer	
			DT40		DF1			
			CONTROL	FGFR1 KNOCKDOWN	CONTROL	FGFR1 KNOCKDOWN	Forward (5'−>3')	Reverse (5'−>3')
FADD	Fas (TNFRSF6)-associated via death domain	XM_421073	1.13±0.17	0.58±0.15**	1.01±0.20	2.96±0.12***	GGTCCAACCACC CTGCTGAA	CGCAGGTGACAGAGC ATTGG
TRADD	TNFRSF1A-associated via death domain	XM_414067	1.06±0.17	0.53±0.11*	1.00±0.04	1.32±0.11*	GAGAAAAGCCTG ACCGCCTG	GTGACCTGTGGGGAA AGGGA
CASP8	Caspase 8, apoptosis-related cysteine peptidase	NM_204592	1.17±0.31	0.73±0.08**	1.05±0.13	2.24±0.16**	TCTCAGCCTGGA GCACGTCA	AACAGGTCCCCCACC TCGAT
BID	BH3 interacting domain death agonist	NM_204552	1.07±0.23	1.11±0.10	0.97±0.10	2.03±0.13***	CTTTGCTTTCCTG GCGGAGT	CCACTTCGATTCCCAT CGGT
BCL2	B-cell CLL/lymphoma 2	NM_205339	1.07±0.13	1.79±0.05*	0.95±0.08	0.79±0.06	CTTTATCCTCCTG CCCCTCG	TTCTTCCGCTTCGTCA GCAA
BCL2L1	BCL2-like 1 or Bcl-x	NM_001025304	1.09±0.22	1.24±0.19	1.00±0.04	0.91±0.13	TTCAGCGACCTC ACCTCCCA	GCCCCCAGTTCACAC CATCA
CYCS	Cytochrome c, somatic	NM_418723	1.10±0.16	0.46±0.08**	1.04±0.21	2.19±0.17**	CCAGAAATGTTC CCAGTGCCA	GAGAAGCCCTCAGCT TGTCCTG
CASP3	Caspase 3, apoptosis-related cysteine peptidase	NM_204725	1.08±0.30	0.15±0.12***	1.03±0.07	1.88±0.17**	TTCAGGCACGGA TGCAGATG	CGCCATGGCTTAGCA ACACA
CASP9	Caspase 9, apoptosis-related cysteine peptidase	NM_424580	1.09±0.10	0.62±0.11*	1.09±0.24	1.46±0.08*	TGACCTGGCTGA CATGCTGG	ATGGACAAGCGTTCC GCAGT

* p<0.05 (t-test): significant difference compared to nonspecific shRNA as a control.
** p<0.005 (t-test): significant difference compared to nonspecific shRNA as a control.
*** p<0.001 (t-test): significant difference compared to nonspecific shRNA as a control.

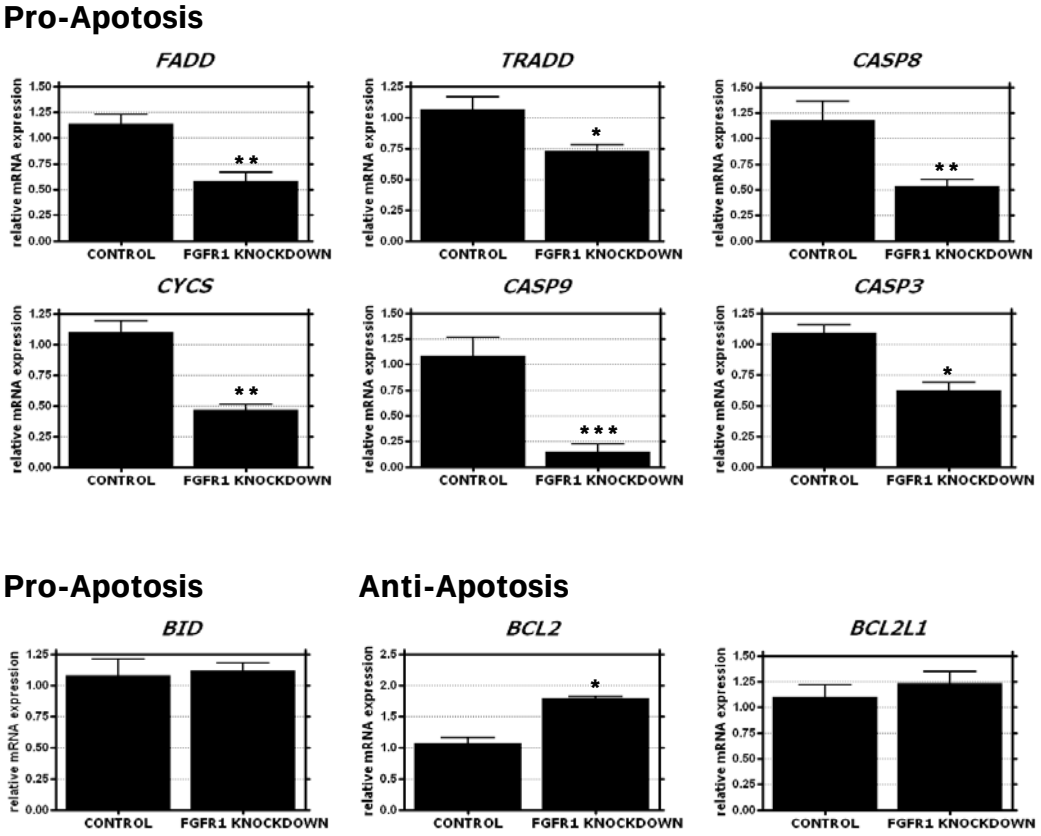

Fig. 3. Effect of *FGFR1* repression on apoptosis-related gene expression in DT40 cells. Changes in expression of apoptosis-related genes were determined by quantitative real-time PCR at 48 h after *FGFR1* repression. Each value was divided by the value of the nonspecific control. All reactions were performed in triplicate and normalized to GAPDH mRNA expression. *** P<0.0005, ** P<0.005, and * P<0.05 (t-test) compared with nonspecific shRNA. Error bars indicate the SE of triplicate analyses.

expression of *FGFR1* compared with *FGFR2* and *FGFR3* (Fig. 2A and 2B). In the presence of shRNA_401, *FGFR1* knockdown altered the expression pattern of *FGFRs* in DT40 cells; the expression of *FGFR2* and *FGFR3* was highly upregulated compared with *FGFR1* expression (Fig. 2C). In contrast, *FGFR1* knockdown did not alter the expression of *FGFRs* in DF-1 cells (Fig. 2D).

3. Effect of *FGFR1* repression on the expression of apoptosis-related genes in DT40 cells

The expression of apoptosis-related genes in control and *FGFR1*-knockdown DT40 cells was analyzed by quantitative real-time PCR (Fig. 3 and Table 2). The expression of two signal molecules that act up-stream in the apoptosis signal pathway, FADD [Fas (TNFRSF6)-associated via death domain] and *TRADD* (TNFRSF1A-associated via death domain), was downregulated in *FGFR1*-knockdown cells compared with expression in control cells. The downregulation of FADD and TRADD expression was associated with downregulated expression of *CASP8*, *CYCS*, *CASP9*, and *CASP3*, but not *BID*, in response to *FGFR1* repression. Concomitantly, expression of the anti-apoptosis signal gene *BCL2* was upregulated in *FGFR1*-knockdown cells, although *BCL2L1* expression was unchanged.

4. Effect of *FGFR1* repression on the expression of apoptosis-related genes in DF-1 cells

To investigate the effect of *FGFR1* repression on the expression of apoptosis-related genes in DF-1 cells, we performed quantitative real-time PCR (Fig. 4 and Table 2). In

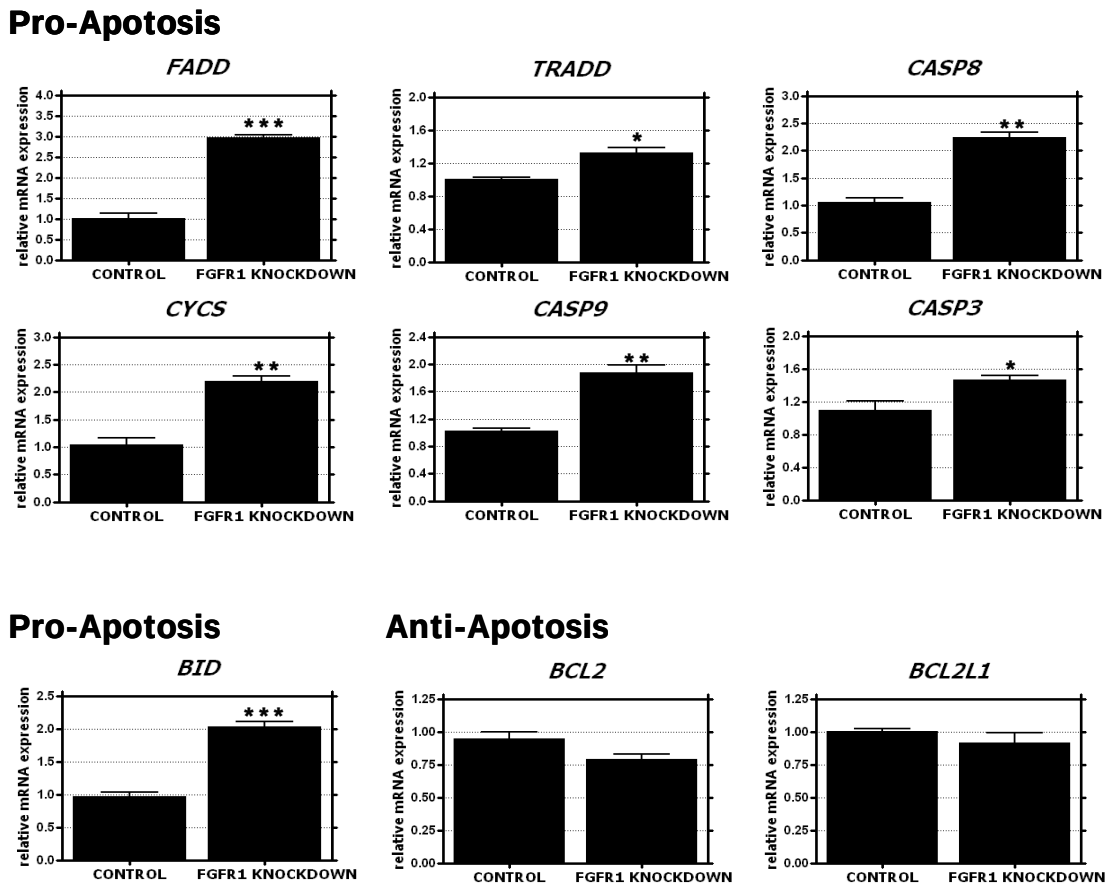

Fig. 4. Effect of *FGFR1* repression on apoptosis-related gene expression in DF-1 cells. line by quantitative real-time PCR. Changes in expression of apoptosis-related genes were determined by quantitative real-time PCR at 48 h after *FGFR1* repression. Each value was divided by the value of the nonspecific control. All reactions were performed in triplicate and normalized to *GAPDH* mRNA expression. *** P<0.0005, ** P<0.005, and * P<0.05 (t-test) compared with nonspecific shRNA. Error bars indicate the SE of triplicate analyses.

contrast to the results in DT40 cells, the expression of *FADD* and *TRADD*, and subsequently the expression of *CASP8*, *CYCS*, *CASP9*, *CASP3*, and *BID* were upregulated by the knockdown of *FGFR1* expression in DF-1 cells. The expression of the anti-apoptosis signal genes *BCL2* and *BCL2L1* was not changed by *FGFR1* repression in DF-1 cells.

DISCUSSION

Fibroblast growth factors (FGFs) function as mitogens in cultured cells such as somatic fibroblasts, germ cells, and blastodermal cells, and play regulatory roles during angiogenesis, wound healing, and various stages of embryonic development (Jung et al, 2005; Lee et al, 2010; Park & Han, 2000). The binding and activation of four *FGFRs* (*FGFR1* to -4) as well as binding to heparin or heparin sulfate proteoglycans mediate the actions of FGFs. The *FGFRs* have an extracellular ligand-binding site composed of three extracellular immunoglobulin-type domains (D1, D2, and D3), and FGFs interact with the D2 and D3 domains. Numerous functional studies have shown that abnormal regulation of FGFs and their receptors is associated with cancer. In particular, overexpression of *FGFR1* has been observed in breast cancers (Adnane et al, 1991; Penaultllorca et al, 1995) and the upregulation of FGFs and *FGFR1* has also been reported in other cancers (Yamaguchi et al, 1994; Yayon et al, 1997).

In chicken, *FGFR1* is expressed during mesoderm induction and is ubiquitously expressed during brain development. The present study shows that both DT40 and DF-1 cell lines express *FGFR1*, as well as low levels of *FGFR2* and *FGFR3*. The repression of *FGFR1* expression induced the upregulation of *FGFR2* and *FGFR3* in DT40, but not DF-1 cells, suggesting that the function of *FGFR1* may be different in each cell type. The DT40 cell line is a B-cell lymphoma cell line, whereas the DF-1 cell line is an immortalized fibroblast cell line. In normal fibroblasts, *FGFR1* knockdown may activate apoptosis signaling by inducing the upregulation of pro-apoptosis-related genes such as *FADD*, *TRADD*, *CASP8*, *BID*, *CYCS*, *CASP9*, and *CASP3*. In cancer cells, *FGFR1* knockdown induced the upregulation of *FGFR2* and *FGFR3*, downregulation of pro-apoptosis-related genes, and upregulation of the anti-apoptosis-related gene *BCL2*. This result imply that cancer cells and normal cells exhibit different mechanisms of cell survival. Jang (2005) reported that the reciprocal relationship between *FGFR1* and *FGFR3* in colorectal tissueplays a critical role in the progression of the carcinomas to malignancy; when *FGFR1* expression was repressed by *FGFR1* siRNA, *FGFR3* expression was effectively elevated (Jang, 2005).

These previous reports and the present results suggest that when cancer cell survival is threatened, as by the repression of *FGFR1*, the expression of anti-apoptosis-related genes as well as *FGFR2* and *FGFR3* may be induced; this response is in contrast to the induction of pro-apoptosis-related signals upon *FGFR1* repression in normal fibroblasts.

We demonstrated differential expression patterns of FGFRs in cancer cells versus normal fibroblasts, using RNA interference. In normal fibroblasts, *FGFR1* repression induced apoptosis through the upregulation of pro-apoptosis-related genes. However, when *FGFR1* was repressed in cancer cells, the expression of *FGFR2*, *FGFR3*, and anti-apoptosis-related genes was upregulated, thereby maintaining cancer cell survival. Further investigations of the phenotypic changes induced by *FGFR1* knockdown in other cancer cell types will provide new insight into cancer development and *FGFR1* functions.

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