

Inhibition of Cell Growth by Nuclear Receptor COUP-TFI: Possible Involvement of Decorin in Growth Inhibition

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Abstract

The transcription factor, Chicken Ovalbumin Upstream Promoter-Transcription Factor I (COUP-TFI) is a member of the steroid/thyroid hormone receptor superfamily and plays important roles especially in development and differentiation. To investigate the effects of COUP-TFI on cell growth, we transfected this factor stably into Ha-ras-transformed NIH3T3 (ras-NIH3T3) cells. Ectopic expression of COUP-TFI resulted in reduction of both proliferation rates and anchorage-independent growth. Flow cytometric analysis showed that progression through mid-S to G2/M phase was delayed in COUP-TFI transfectants. cDNA microarray analysis was performed to elucidate candidate genes whose expression levels were changed in response to COUP-TFI introduction. mRNA levels of decorin and cyclin G were elevated in COUP-TFI transfectants. Luciferase reporter assay revealed that the upstream promoter regions between -252 and -205 bp and between -204 and +42 bp of the decorin gene were responsible for COUP-TFI. These suggest that decorin transactivated by COUP-TFI is one of the molecules which account for the growth-inhibitory effects of COUP-TFI.

Keywords: Steroid/thyroid receptor superfamily; Stable transfection; Anchorage-independent growth; Flow cytometry; Cell cycle; cDNA microarray; Decorin

Introduction

Chicken Ovalbumin Upstream Promoter-Transcription Factors (COUP-TFs) [1] are the member of the steroid/thyroid hormone-activated nuclear receptor superfamily. Since no ligand has yet been identified, COUP-TFs are regarded as orphan receptors. Two distinct genes for COUP-TFs, COUP-TFI/Ear3 [1,2] and COUP-TFII/ARP-1 [3], have been identified, and their sequence homology was 98 and 96% in the DNA-binding domain and in the putative ligand-binding domain, respectively [4]. Although COUP-TFI was originally shown to stimulate transcription of the ovalbumin gene [5], a number of studies have demonstrated that COUP-TFI antagonistically represses transactivation by other nuclear receptors [6-10], mainly through competitive occupancy of a target binding sequence. However, it has been recently reported that COUP-TFs can function as transactivators [11-13] as well as an auxiliary cofactor for other transcription factors [14,15].

Targeted disruption of the COUP-TFI gene resulted in defects in neurogenesis, axon guidance, and arborization [16], whereas targeted deletion of COUP-TFII resulted in embryonic lethality with defects in angiogenesis, vascular remodeling, and fetal heart development [17]. Therefore, COUP-TFs are essential for early development and cell differentiation [18,19], and could be associated with cell proliferation.

Recent studies have reported that COUP-TFI was required for growth inhibition and apoptosis in response to retinoic acid in some cancer cell lines [20], and that ectopic expression of COUP-TFII in

breast cancer cell lines caused growth arrest associated with modulation in expression of cell-cycle regulatory proteins such as p21Cip1 and cyclin D1 [21]. On the other hand, COUP-TFI stimulates proliferation [22] and migration [23] of breast cancer cells by activating estrogen signaling or EGF signaling.

In the present study, we stably introduced COUP-TFI into Ha-ras-transformed NIH3T3 cells and examined its effect on cell growth. Ectopic expression of COUP-TFI led to decrease in cell proliferation rates, reduction of anchorage-independent growth, and delay of progression through mid-S to G2/M phase. Results of cDNA microarray analysis and reporter assay suggested that decorin is one of the transcriptional targets of COUP-TFI and mediates the growth-inhibitory effects of COUP-TFI.

Materials and Methods

Cells and culture

A cell strain, Ha-ras-transformed NIH3T3 (ras-NIH3T3, clone F25) was kindly supplied by T. Sekiya [24] and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% calf serum.

Stable transfection

Transfection of plasmids into ras-NIH3T3 cells and selection of stable transfectants were done essentially as described [25]. Cells were plated at a density of 2×10^5 cells in 35 mm dishes. Twenty-four hours later, the cells were co-transfected with the plasmid expressing human COUP-TFI cDNA [7] under the control of the elongation factor 1 α promoter of pEF-BOS [26], in addition to neo gene-bearing plasmid pcDNA3 (Thermo Fisher Scientific, Waltham, MA) at a ratio of 10:1.

Control cells were co-transfected with pEF-BOS and pcDNA3. For transfection, FuGENE6 reagent (Roche Diagnostics, Mannheim, Germany) was used according to manufacturer's protocol. The next day, the cells were replated onto 100 mm dishes in growth medium containing 400 µg/ml G418 (Thermo Fisher Scientific). After 3 weeks, the G418-resistant colonies were isolated.

Analysis of cell proliferation rates and cell cycle

Cells (2×10^4) were seeded in 35 mm plates in triplicate. After the culture for various periods, cells were trypsinized and counted using Coulter Counter ZM (Beckman Coulter, Fullerton, CA). For cell cycle analysis, cells were collected by centrifugation and washed three times with Phosphate-Buffered Saline (PBS). The cells were then resuspended in 0.9 ml of staining solution (0.1% Triton X100, 100 µg/ml of RNase A, 40 µg/ml of propidium iodide and 4 mM sodium citrate), and incubated at room temperature for 20 min in the dark. Following addition of 0.1 ml of 1.5 M NaCl, samples were analyzed with FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA) as described previously [27]. Twenty thousand events of individual samples were collected for each analysis.

Semi-synchronized cell cycle analysis

To synchronize cell cycle at least partially, cells were incubated in DMEM containing 0.2% calf serum for two days, and then released from serum starvation by addition of calf serum to 5%. After the culture for various periods, cell cycle distribution was measured by the flow cytometry using FACScan.

Anchorage-independent growth

Anchorage-independent growth was tested by colony-forming assay using soft agar medium essentially as described [28]. The top layer in a 60 mm dish contained 600 cells in 2 ml of 0.4% Sea Plaque Agarose (BioWhittaker Molecular Applications, Rockland, ME) in DMEM supplemented with 5% calf serum, while the bottom layer consisted of 3 ml of 0.6% agarose in DMEM supplemented with 5% calf serum. Cells were incubated at 37°C in 5% CO₂ for 3 weeks, and colonies with diameters larger than 0.1 mm were scored under a phase-contrast microscope.

Northern blot analysis

Total cellular RNA was prepared by the acid-guanidine thiocyanate-phenol/chloroform extraction method [29]. The RNA was subjected to denaturing agarose gel electrophoresis and transferred to a nylon membrane. Hybridization with digoxigenin-labeled antisense RNA probes followed by chemiluminescent detection on X-ray films was done according to manufacturer's protocol (Roche Diagnostics) as described previously [30].

Western blot analysis

Cells were lysed in the protein lysis buffer (0.5% Nonidet P-40, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethanesulfonyl fluoride, 50 µM leupeptin, 50 µM antipain, 50 µM pepstatin A and 50 µM N-acetyl-leucyl-leucyl-norleucinal), and protein concentrations were determined using the Bradford method [31]. Equal amounts of sample lysates were separated by SDS-polyacrylamide gel electrophoresis and electrically transferred onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat

dry milk in a buffer consisting of 20 mM Tris-HCl (pH 7.6), 137 mM NaCl and 0.1% Tween 20, and incubated with specific primary antibodies against COUP-TFI (a gift from MJ Tsai) [4]. After incubation with horseradish peroxidase-conjugated secondary antibody, immunoreactivity was detected with the ECL kit (GE Healthcare, Buckinghamshire, England) as described previously [32].

cDNA microarray analysis

A microarray chip harboring 2,304 cDNA clones derived from the mouse embryo was prepared essentially as described [33]. Total RNA was prepared from COUP-TFI-transfected cells, and from parental cells. Poly(A)⁺ RNA was isolated, using oligo(dT)-paramagnetic beads Dynabeads Oligo(dT)25 (Dyna, Oslo, Norway). Two µg of poly(A)⁺ RNA and 4.5 µg of oligo(dT) in 15 µl of solution were heat-denatured at 70°C for 10 min, and chilled on ice immediately. The solution was made up to 30 µl of the mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 0.5 mM each of dATP, dCTP, and dGTP, 0.2 mM dTTP, 0.1 mM Cy3- or Cy5-dUTP, and 400 units of reverse transcriptase SuperScript II (Thermo Fisher Scientific). After the incubation at 42°C for 1 h, template mRNAs were degraded at 65°C for 10 min in the presence of 1.5 µl of 1 N NaOH/20 mM EDTA, and then neutralized with 270 µl of 10 mM Tris-HCl (pH 8.0)/1 mM EDTA and 1.5 µl of 1 N HCl. The Cy3- or Cy5-labeled cDNA was purified with Centricon-30 microconcentrators (Millipore, Bedford, MA). Hybridization was performed in a solution containing 2 µg/µl of yeast RNA, 2 µg/µl of poly(A), 3.4×SSC (1×SSC consists of 0.15 M NaCl/15 mM sodium citrate) and 0.3% SDS at 65°C overnight under humidified condition. After the hybridization, the arrays were washed twice for 5 min with 2×SSC/0.1% SDS at room temperature, twice for 5 min with 0.2 x SSC/0.1% SDS at 40°C, and finally rinsed with 0.2×SSC. The fluorescent images were scanned with a laser-scanning device (ScanArray 4000, GSI Lumonics, Bedford, MA).

Luciferase reporter assay

Genomic DNA was isolated from T.Tn human esophageal carcinoma cell line [34] and used as a template. The promoter regions of -1301 to -205, -252 to +42, and -204 to +42 of the decorin gene were amplified by PCR and inserted into the XhoI-HindIII site of the firefly luciferase reporter plasmid, pGL3-Basic (Promega, Madison, WI), to construct DecoLuc:-1338/-205, DecoLuc:-252/+42 and DecoLuc:-204/+42. By using them as templates, DecoLuc:-252/-205, DecoLuc:-252/-231, DecoLuc:-231/-205 were constructed by reverse PCR method using KOD-Plus-Mutagenesis kit (Toyobo, Osaka, Japan) according to the manufacturer's instruction. Base sequences of these constructs were confirmed by sequencing analysis.

The cells (1×10^5 cells/well) seeded on 24-well plates were transfected with COUP-TFI expression plasmid [7] or control empty vector pEF-BOS (0.1 µg) together with firefly luciferase reporter plasmid (0.4 µg) and Renilla luciferase reporter plasmid CMV-Rluc [25] by using LipofectAMINE-Plus (Thermo Fisher Scientific). Two days after the transfection, firefly and Renilla luciferase activities were measured with a Dual Luciferase Assay System (Promega) and a luminescencer (Atto, Tokyo, Japan). Firefly luciferase activities were normalized with respect to parallel Renilla luciferase activities, to correct for differences in transfection efficiency.

Results

Isolation of stable transfectants of COUP-TFI

To examine the biological activity of COUP-TFI, Ha-ras-transformed NIH3T3 (ras-NIH3T3) cells were stably transfected with COUP-TFI-expressing plasmids. Among G418-selected clones, COUP-TFI-transfected clones (FCO-3, 7 and 10) showed higher expression of COUP-TFI at both mRNA and protein levels than the parental cells and a vector-transfected clone (Figure 1a and b).

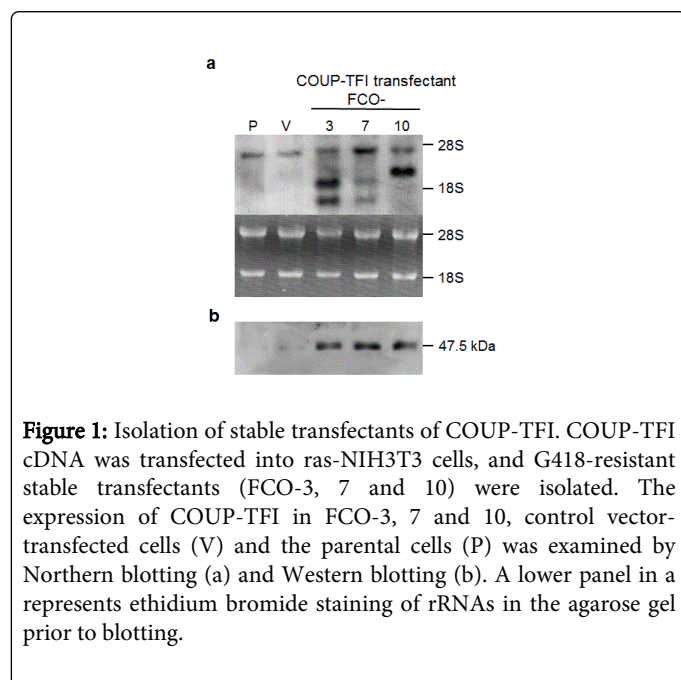


Figure 1: Isolation of stable transfectants of COUP-TFI. COUP-TFI cDNA was transfected into ras-NIH3T3 cells, and G418-resistant stable transfectants (FCO-3, 7 and 10) were isolated. The expression of COUP-TFI in FCO-3, 7 and 10, control vector-transfected cells (V) and the parental cells (P) was examined by Northern blotting (a) and Western blotting (b). A lower panel in a represents ethidium bromide staining of rRNAs in the agarose gel prior to blotting.

Reduced proliferation rates and delay of cell cycle progression through mid-S to G2 phase in COUP-TFI transfected cells

When the proliferation rates were examined by direct counting of cell numbers, all of COUP-TFI-transfected cells grew much more slowly than the parental and vector-transfected control cells (Figure 2). The apparent inhibitory effect of COUP-TFI on cell proliferation prompted us to investigate changes in cell cycle distribution by the flow cytometric analysis.

As shown in Figure 3a, a different cell cycle distribution was observed between COUP-TFI-transfected cells and control cells: COUP-TFI transfectants with the exception of one clone (FCO-7) showed accumulation in late S to G2/M phase of the cell cycle. A further analysis of cell cycle distribution with semi-synchronized cells at various times after release from serum starvation was performed (Figure 3b). Serum starvation for two days led to an increase in G0/G1 population and a concomitant decrease in S and G2/M populations in parental ras-NIH3T3 cells. A small but apparent portion of hypodiploid apoptotic cells was also detected in the parental cells. On the other hand, a substantial amount of the G2/M population was still detectable in FCO-3 cells.

Although the parental cells entered S phase around 12 h after serum stimulation, an apparent delay was observed in progression of FCO-3

cells from S phase to the sequential phase, suggesting that COUP-TFI repressed mid-S to G2/M progression.

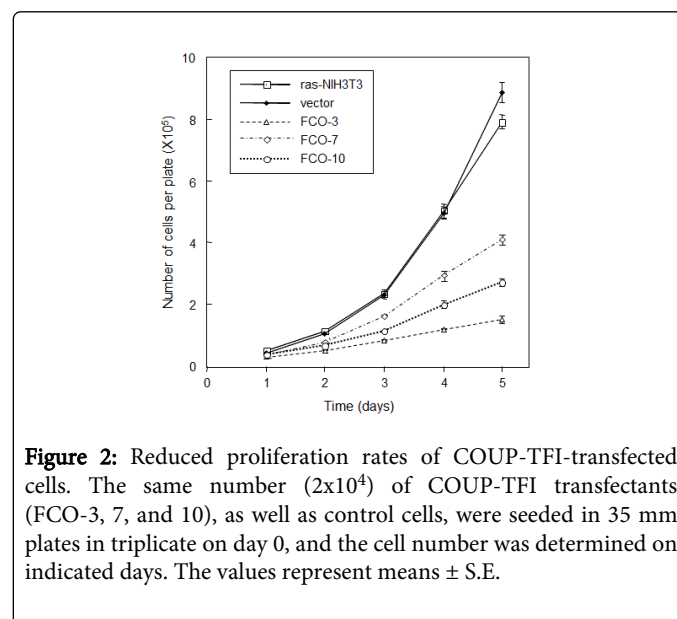


Figure 2: Reduced proliferation rates of COUP-TFI-transfected cells. The same number (2×10^4) of COUP-TFI transfectants (FCO-3, 7, and 10), as well as control cells, were seeded in 35 mm plates in triplicate on day 0, and the cell number was determined on indicated days. The values represent means \pm S.E.

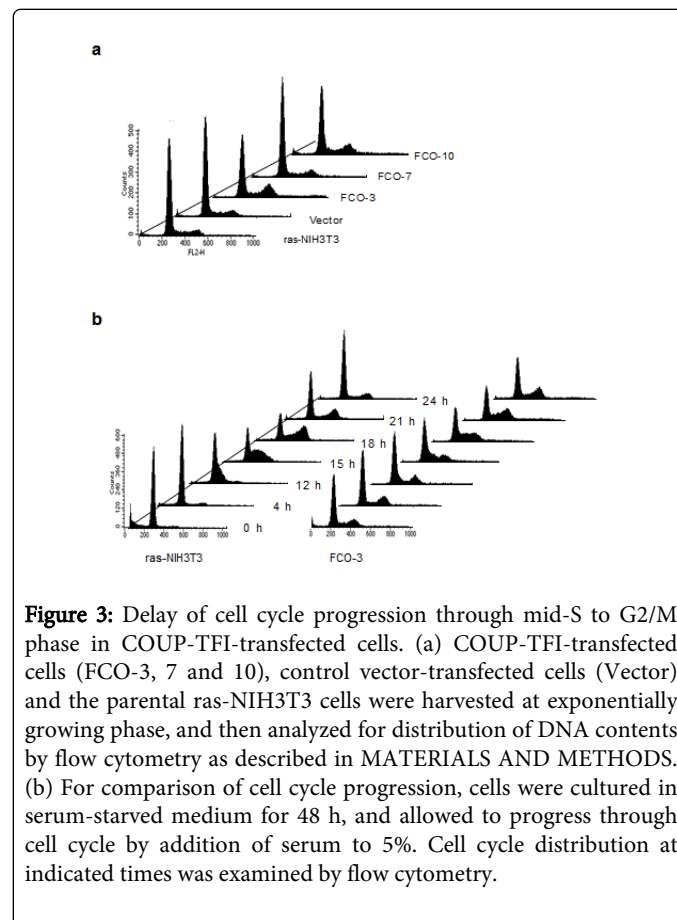


Figure 3: Delay of cell cycle progression through mid-S to G2/M phase in COUP-TFI-transfected cells. (a) COUP-TFI-transfected cells (FCO-3, 7 and 10), control vector-transfected cells (Vector) and the parental ras-NIH3T3 cells were harvested at exponentially growing phase, and then analyzed for distribution of DNA contents by flow cytometry as described in MATERIALS AND METHODS. (b) For comparison of cell cycle progression, cells were cultured in serum-starved medium for 48 h, and allowed to progress through cell cycle by addition of serum to 5%. Cell cycle distribution at indicated times was examined by flow cytometry.

The extent of prolongation in mid-S to G2/M progression correlated well with that of decreases in cell proliferation rates (Figure 2). Taken together, we concluded that a delay of progression through

mid-S to G2/M phase is responsible for growth inhibition in COUP-TFI-transfected cells.

Reduced anchorage-independent growth in COUP-TFI transfectants

Colony-forming ability in soft agar medium is well established as a useful mean to assess the transformed state of a given cell population, and was examined on COUP-TFI transfectants.

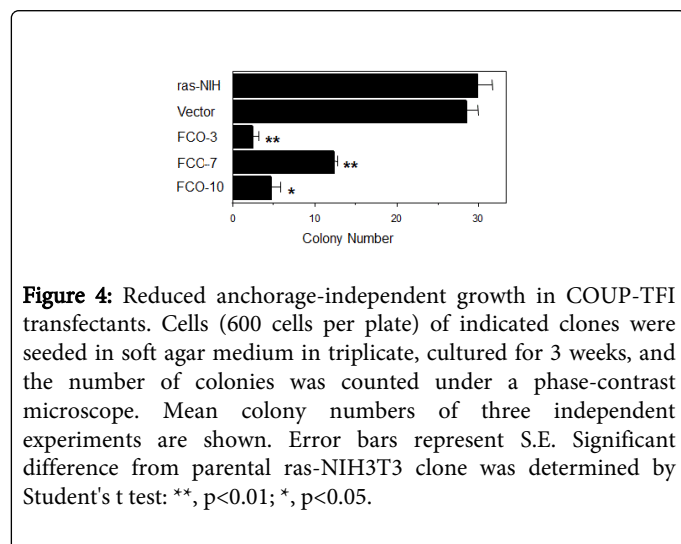


Figure 4: Reduced anchorage-independent growth in COUP-TFI transfectants. Cells (600 cells per plate) of indicated clones were seeded in soft agar medium in triplicate, cultured for 3 weeks, and the number of colonies was counted under a phase-contrast microscope. Mean colony numbers of three independent experiments are shown. Error bars represent S.E. Significant difference from parental ras-NIH3T3 clone was determined by Student's t test: **, $p < 0.01$; *, $p < 0.05$.

As shown in Figure 4, the number of colonies formed in soft agar decreased significantly in COUP-TFI transfectants compared with parental cells and a vector-transfected clone, suggesting that COUP-TFI may have a potential to suppress a transformed phenotype of tumor cells.

cDNA microarray and northern blot analysis

cDNA microarray analysis was performed to elucidate candidates for genes that are targets of COUP-TFI, and that may account for the growth-inhibitory effects. Total RNAs prepared from three COUP-TFI-transfected clones, FCO-3, 7 and 10, were each mixed before poly(A)+ RNA isolation, in order to avoid detecting non-specific alterations in mRNA levels fluctuating from one clone to another. For the control, a mixture of total RNAs prepared from parental cells harvested at two different times was used. Possible difference of labeling efficiency of Cy3 and Cy5 was controlled by interconverting the two dyes in cDNA synthesis. Genes whose expressions were altered by transfection of COUP-TFI are listed in Table 1.

Levels of mRNAs for six genes were significantly elevated in COUP-TFI-transfected cells. Among these genes, the decorin and cyclin G genes were especially noteworthy because they are involved in the control of cell proliferation [35,36]. Contrary to our expectations, none was listed as the gene down-regulated by introduction of COUP-TFI, which often works as a transcriptional repressor in a reporter transfection assay [6-10].

Elevated mRNA expression levels of decorin and cyclin G in COUP-TFI-transfectants

To confirm changes in decorin and cyclin G mRNA levels, we performed Northern blot analysis (Figure 5).

Decorin mRNA levels were elevated in all three clones of COUP-TFI transfectants as compared to those of parental cells and a control vector-transfected clone. Cyclin G mRNA levels were elevated in at least two of COUP-TFI transfectants. Interestingly, the extents of elevation in decorin and cyclin G mRNA levels in COUP-TFI transfectants (Figure 5) seem to correlate roughly with degrees of cell growth inhibition (Figure 2), changes in cell cycle distribution (Figure 3a) and suppression of anchorage-independent growth (Figure 4).

Activation of decorin promoter by COUP-TFI

To examine whether decorin is a target of COUP-TFI, luciferase reporter assay was performed using reporter plasmids containing the promoter region of the decorin gene. The regions between -252 and +42 and between -204 and +42 were activated approximately 30-fold by COUP-TFI as compared to the empty vector (Figure 6).

Among several constructs, the region between -252 and -205 was most activated by COUP-TFI, although the ratio of activation by COUP-TFI versus activation by control vector was also 30 fold. Further dividing this region into two portions indicated COUP-TFI-responsive domains are located in both regions between -252 and -231 and between -231 and -205. Activation of the region between -252 and +42 was lower than that between -252 and -205, suggesting the presence of cis-acting suppressive element(s) in the region between -204 and +42. Thus, multiple COUP-TFI-responsive elements may locate in the promoter region of the decorin gene.

Discussion

In this study, we examined the effects of COUP-TFI on cell growth, and observed that COUP-TFI transfection resulted in reduction of proliferation rates and anchorage-independent growth. This growth-inhibitory activity of COUP-TFI is well consistent with the recent report that COUP-TFI is down-regulated in bladder transitional cell carcinoma [37]. Cell cycle progression of COUP-TFI-transfected cells was mainly blocked around G2/M phase and the transition from mid-S to G2/M phase was also delayed. A previous study using breast cancer cell lines has demonstrated that COUP-TFII inhibits cell growth and cell cycle progression by modulating the expression of p21Cip1, cyclin D1, and the activity of cdk2 [21]. In the present study, we could not detect any elevation of the p21Cip1 level in COUP-TFI transfectants (data not shown). In addition, cDNA microarray analysis and succeeding Northern blot analysis revealed elevation of mRNA levels for decorin and cyclin G in COUP-TFI transfectants.

Cyclin G was initially identified as a target of the c-src family kinase pathway in rat fibroblasts [38]. It has attracted attention because the cyclin G gene is one of the target genes of p53 tumor suppressor protein: two distinct high-affinity p53-binding sites were found in the promoter region of cyclin G, and its expression is induced after DNA damage via p53 [39,40]. In addition, it was reported that cyclin G is involved in G2/M checkpoint control [36]. Therefore, cyclin G might cause G2/M arrest through its direct induction by p53. However, in other studies, ectopic overexpression of cyclin G in human RKO colon carcinoma cells accelerated cell growth [41], and cyclin G1 was essential for the growth of human osteosarcoma cells [42]. This contradiction may be attributable to difference in cell types and/or in the expression levels of cyclin G (ectopic enforced expression etc.).

We also identified decorin as a candidate for the cell growth inhibitor under the control of COUP-TFI. Decorin is a member of small leucine-rich proteoglycan gene family [43]. It has been reported

that decorin inhibits cell proliferation by neutralizing transforming growth factor β (TGF β) activity [35,44] and/or by inducing p21Cip1 through epidermal growth factor receptor (EGFR) activation [45-47]. The latter mechanism may not account for the growth inhibition in the present study because EGFR is not expressed in ras-NIH3T3 cells (data not shown) and also because p21 was not induced in COUP-TFI transfectants (data not shown). On the other hand, it is plausible that suppression of TGF β activity by decorin is the cause of growth inhibition in response to COUP-TFI introduction, because TGF β stimulates the proliferation of NIH3T3 cells [48]. It is noteworthy that decorin-overexpressing COUP-TFI transfectants showed apparent reduction of anchorage-independent growth (Figure 4). This

observation is concordant with the notion that decorin plays a role in suppression of some transformed phenotypes of tumor cells. Indeed, it has been demonstrated that de novo decorin gene expression suppressed the malignant phenotype in human colon cancer cells [49]. We also isolated stable decorin transfectants, which showed similar cell cycle distribution to that of COUP-TFI transfectants (Supplemental Figure S1).

The accumulation at mid-S to G2/M phase was more prominent than COUP-TFI-transfectants, possibly due to the enforced overexpression of decorin.

Accession Number	Name	Ratio (Cy3/Cy5)	Ratio (Cy4/Cy3)
X53929	MMPG II	2.85	3.64
AF111103	MMHC332F16 Mouse major histocompatibility complex region containing the Q region of class I	2.73	2.01
X13460	MMP68 Mouse mRNA for p68 protein of the lipocortin family	2.37	2.12
L07095	MUSMTHYPA	2.16	2.61
Z37110	MMCYCLGMR	1.82	1.92
J05287	MUSLAMP2 Mouse lysosomal membrane glycoprotein (LAMP-2) mRNA	1.65	1.68

Table 1: Results of cDNA microarray analysis for genes whose mRNA levels are changed in COUP-TFI transfectants. Ratios represent fold over-expression for microarray data based on ratio of fluorescence for COUP-TFI-transfected cells compared to parental cells. Cy3- and Cy5-labeling for the two mRNA samples were interconverted to control difference of incorporation efficiency for the two dyes.

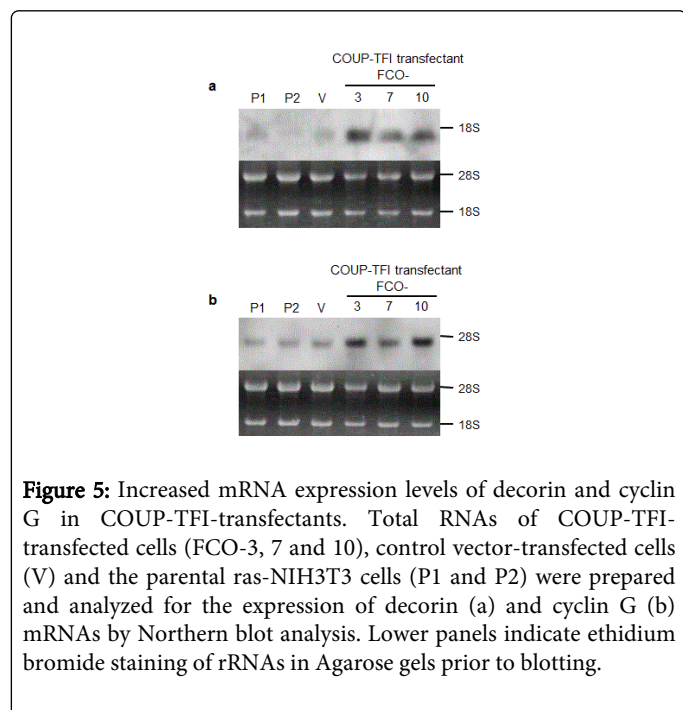


Figure 5: Increased mRNA expression levels of decorin and cyclin G in COUP-TFI-transfectants. Total RNAs of COUP-TFI-transfected cells (FCO-3, 7 and 10), control vector-transfected cells (V) and the parental ras-NIH3T3 cells (P1 and P2) were prepared and analyzed for the expression of decorin (a) and cyclin G (b) mRNAs by Northern blot analysis. Lower panels indicate ethidium bromide staining of rRNAs in Agarose gels prior to blotting.

COUP-TFs belongs to the superfamily of the steroid/thyroid hormone-activated nuclear receptors [1], which binds to direct repeat of the hexanucleotide 5'-AGGTCA-3' motif (DR1 motif) [50,51]. Our present study showed that the regions between -252 and -205 bp and between -204 and +42 in the decorin promoter are responsive to COUP-TFI (Figure 6). The 30-fold transactivation of the reporter gene

suggests the direct activation of the decorin promoter by COUP-TFI. However, this region contains no similar DR1 consensus sequence. Therefore, we cannot rule out the possibility that the decorin promoter was indirectly activated by COUP-TFI via some other transcription factor(s). We also failed to find any DR1-like motif in the promoter region of the cyclin G. However, the potent growth-inhibition and the specific mid-S to G2/M arrest of cell cycle progression by both decorin and cyclin G suggest that these two molecules have a key role in the growth-inhibitory activity of COUP-TFI.

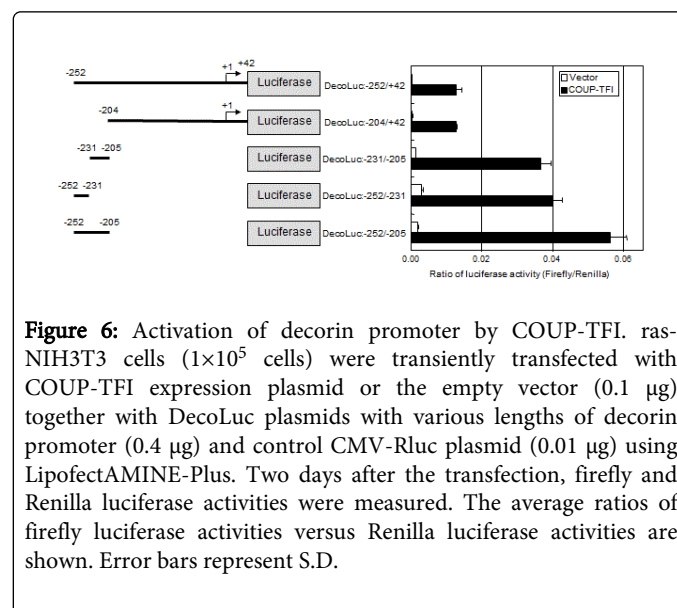
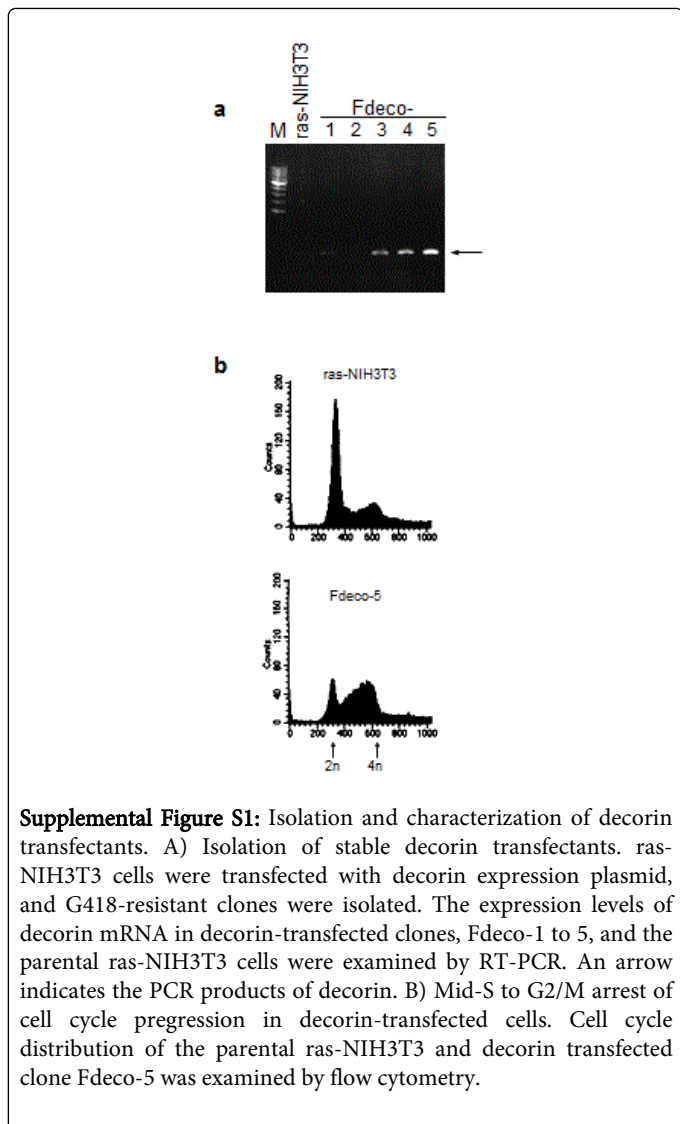


Figure 6: Activation of decorin promoter by COUP-TFI. ras-NIH3T3 cells (1×10^5 cells) were transiently transfected with COUP-TFI expression plasmid or the empty vector (0.1 μ g) together with DecoLuc plasmids with various lengths of decorin promoter (0.4 μ g) and control CMV-Rluc plasmid (0.01 μ g) using LipofectAMINE-Plus. Two days after the transfection, firefly and Renilla luciferase activities were measured. The average ratios of firefly luciferase activities versus Renilla luciferase activities are shown. Error bars represent S.D.



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Competing interests: The authors declare that they have no competing interests.

References

1. Wang LH, Tsai SY, Cook RG, Beattie WG, Tsai MJ, et al. (1989) COUP transcription factor is a member of the steroid receptor superfamily. *Nature* 340: 163-166.
2. Miyajima N, Kadowaki Y, Fukushige S, Shimizu S, Semba K, et al. (1988) Identification of two novel members of erbA superfamily by molecular

- cloning: the gene products of the two are highly related to each other. *Nucleic Acids Res* 16: 11057-11074.
3. Ladias JA, Karathanasis SK (1991) Regulation of the apolipoprotein AI gene by ARP-1, a novel member of the steroid receptor superfamily. *Science* 251: 561-565.
4. Tsai SY, Tsai MJ (1997) Chick ovalbumin upstream promoter-transcription factors (COUP-TFs): coming of age. *Endocr Rev* 18: 229-240.
5. Pastorcic M, Wang H, Elbrecht A, Tsai SY, Tsai MJ, et al. (1986) Control of transcription initiation in vitro requires binding of a transcription factor to the distal promoter of the ovalbumin gene. *Mol Cell Biol* 6: 2784-2791.
6. Mietus-Snyder M, Sladek FM, Ginsburg GS, Kuo CF, Ladias JA, et al. (1992) Antagonism between apolipoprotein AI regulatory protein 1, Ear3/COUP-TF, and hepatocyte nuclear factor 4 modulates apolipoprotein CIII gene expression in liver and intestinal cells. *Mol Cell Biol* 12: 1708-1718.
7. Kimura A, Nishiyori A, Murakami T, Tsukamoto T, Hata S, et al. (1993) Chicken ovalbumin upstream promoter-transcription factor (COUP-TF) represses transcription from the promoter of the gene for ornithine transcarbamylase in a manner antagonistic to hepatocyte nuclear factor-4 (HNF-4). *J Biol Chem* 268: 11125-11133.
8. Nakshatri H, Chambon P (1994) The directly repeated RG(G/T)TCA motifs of the rat and mouse cellular retinol-binding protein II genes are promiscuous binding sites for RAR, RXR, HNF-4, and ARP-1 homo- and heterodimers. *J Biol Chem* 269: 890-902.
9. Tran P, Zhang XK, Salbert G, Hermann T, Lehmann JM, et al. (1992) COUP orphan receptors are negative regulators of retinoic acid response pathways. *Mol Cell Biol* 12: 4666-4676.
10. Cooney AJ, Tsai SY, O'Malley BW, Tsai MJ (1992) Chicken ovalbumin upstream promoter transcription factor (COUP-TF) dimers bind to different GGTC A response elements, allowing COUP-TF to repress hormonal induction of the vitamin D3, thyroid hormone, and retinoic acid receptors. *Mol Cell Biol* 12: 4153-4163.
11. Lu XP, Salbert G, Pfahl M (1994) An evolutionary conserved COUP-TF binding element in a neural-specific gene and COUP-TF expression patterns support a major role for COUP-TF in neural development. *Mol Endocrinol* 8: 1774-1788.
12. Power SC, Cereghini S (1996) Positive regulation of the vHNF1 promoter by the orphan receptors COUP-TF1/Ear3 and COUP-TFII/Arp1. *Mol Cell Biol* 16: 778-791.
13. Lazennec G, Kern L, Valotaire Y, Salbert G (1997) The nuclear orphan receptors COUP-TF and ARP-1 positively regulate the trout estrogen receptor gene through enhancing autoregulation. *Mol Cell Biol* 17: 5053-5066.
14. Hall RK, Sladek FM, Granner DK (1995) The orphan receptors COUP-TF and HNF-4 serve as accessory factors required for induction of phosphoenolpyruvate carboxykinase gene transcription by glucocorticoids. *Proc Natl Acad Sci U S A* 92: 412-416.
15. Ktistaki E, Talianidis I (1997) Chicken ovalbumin upstream promoter transcription factors act as auxiliary cofactors for hepatocyte nuclear factor 4 and enhance hepatic gene expression. *Mol Cell Biol* 17: 2790-2797.
16. Qiu Y, Pereira FA, DeMayo FJ, Lydon JP, Tsai SY, et al. (1997) Null mutation of mCOUP-TFI results in defects in morphogenesis of the glossopharyngeal ganglion, axonal projection, and arborization. *Genes Dev* 11: 1925-1937.
17. Pereira FA, Qiu Y, Zhou G, Tsai MJ, Tsai SY (1999) The orphan nuclear receptor COUP-TFII is required for angiogenesis and heart development. *Genes Dev* 13: 1037-1049.
18. Alfano C, Magrinelli E, Harb K, Studer M (2014) The nuclear receptors COUP-TF: a long-lasting experience in forebrain assembly. *Cell Mol Life Sci* 71: 43-62.
19. Park JI, Tsai SY, Tsai MJ (2003) Molecular mechanism of chicken ovalbumin upstream promoter-transcription factor (COUP-TF) actions. *Keio J Med* 52: 174-181.

20. Lin B, Chen GQ, Xiao D, Kolluri SK, Cao X, et al. (2000) Orphan receptor COUP-TF is required for induction of retinoic acid receptor beta, growth inhibition, and apoptosis by retinoic acid in cancer cells. *Mol Cell Biol* 20: 957-970.
21. Nakshatri H, Mendonca MS, Bhat-Nakshatri P, Patel NM, Goulet RJ Jr, et al. (2000) The orphan receptor COUP-TFII regulates G2/M progression of breast cancer cells by modulating the expression/activity of p21(WAF1/CIP1), cyclin D1, and cdk2. *Biochem Biophys Res Commun* 270: 1144-1153.
22. Le Dily F, Métivier R, Guéguen MM, Le Péron C, Flouriot G, et al. (2008) COUP-TFI modulates estrogen signaling and influences proliferation, survival and migration of breast cancer cells. *Breast Cancer Res Treat* 110: 69-83.
23. Boudot A, Kerdivel G, Lecomte S, Flouriot G, Desille M, et al. (2014) COUP-TFI modifies CXCL12 and CXCR4 expression by activating EGF signaling and stimulates breast cancer cell migration. *BMC Cancer* 14: 407.
24. Sekiya T, Fushimi M, Hori H, Hirohashi S, Nishimura S, et al. (1984) Molecular cloning and the total nucleotide sequence of the human c-Ha-ras-1 gene activated in a melanoma from a Japanese patient. *Proc Natl Acad Sci U S A* 81: 4771-4775.
25. Liu TL, Shimada H, Ochiai T, Shiratori T, Lin SE, et al. (2006) Enhancement of chemosensitivity toward peplomycin by calpastatin-stabilized NF-kappaB p65 in esophageal carcinoma cells: possible involvement of Fas/Fas-L synergism. *Apoptosis* 11: 1025-1037.
26. Mizushima S, Nagata S (1990) pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res* 18: 5322.
27. Sakaida T, Iwadate Y, Yamaji S, Uehara M, Noda A, et al. (2003) Acquisition of drug resistance by constitutive suppression of NF-kappaB in ras-transformed NIH3T3 mouse fibroblasts. *Int J Oncol* 23: 1071-1077.
28. Hiwasa T, Sawada T, Sakiyama S (1996) Synergistic induction of anchorage-independent growth of NIH3T3 mouse fibroblasts by cysteine proteinase inhibitors and a tumor promoter. *J Biol Chem* 271: 9181-9184.
29. Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156-159.
30. Sakao E, Ishihara A, Horikawa K, Akiyama M, Arai M, et al. (2003) Two-peaked synchronization in day/night expression rhythms of the fibrinogen gene cluster in the mouse liver. *J Biol Chem* 278: 30450-30457.
31. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248-254.
32. Hiwasa T, Arase Y, Kikuno K, Hasegawa R, Sugaya S, et al. (2000) Increase in ultraviolet sensitivity by overexpression of calpastatin in ultraviolet-resistant Uvr-1 cells derived from ultraviolet-sensitive human Rsa cells. *Cell Death Diff* 7: 531-537.
33. Yoshikawa T, Nagasugi Y, Azuma T, Kato M, Sugano S, et al. (2000) Isolation of novel mouse genes differentially expressed in brain using cDNA microarray. *Biochem Biophys Res Commun* 275: 532-537.
34. Takahashi K, Kanazawa H, Chan H, Hosono T, Takahara M, Sato K (1990) A case of esophageal carcinoma metastatic to the mandible and characterization of two cell lines (T. T and T. Tn) established from the oral tumor. *Jpn J Oral Maxillofac Surg* 36: 307-316.
35. Yamaguchi Y, Ruoslahti E (1988) Expression of human proteoglycan in Chinese hamster ovary cells inhibits cell proliferation. *Nature* 336: 244-246.
36. Jensen MR, Factor VM, Thorgeirsson SS (1998) Regulation of cyclin G1 during murine hepatic regeneration following Dipin-induced DNA damage. *Hepatology* 28: 537-546.
37. Ham WS, Lee JH, Yu HS, Choi YD (2008) Expression of chicken ovalbumin upstream promoter-transcription factor I (COUP-TFI) in bladder transitional cell carcinoma. *Urology* 72: 921-926.
38. Tamura K, Kanaoka Y, Jinno S, Nagata A, Ogiso Y, et al. (1993) Cyclin G: a new mammalian cyclin with homology to fission yeast Cig1. *Oncogene* 8: 2113-2118.
39. Okamoto K, Beach D (1994) Cyclin G is a transcriptional target of the p53 tumor suppressor protein. *EMBO J* 13: 4816-4822.
40. Zauberman A, Lupo A, Oren M (1995) Identification of p53 target genes through immune selection of genomic DNA: the cyclin G gene contains two distinct p53 binding sites. *Oncogene* 10: 2361-2366.
41. Smith ML, Kontny HU, Bortnick R, Fornace AJ Jr (1997) The p53-regulated cyclin G gene promotes cell growth: p53 downstream effectors cyclin G and Gadd45 exert different effects on cisplatin chemosensitivity. *Exp Cell Res* 230: 61-68.
42. Skotzko M, Wu L, Anderson WF, Gordon EM, Hall FL (1995) Retroviral vector-mediated gene transfer of antisense cyclin G1 (CYCG1) inhibits proliferation of human osteogenic sarcoma cells. *Cancer Res* 55: 5493-5498.
43. Fisher LW, Termine JD, Young MF (1989) Deduced protein sequence of bone small proteoglycan I (biglycan) shows homology with proteoglycan II (decorin) and several nonconnective tissue proteins in a variety of species. *J Biol Chem* 264: 4571-4576.
44. Yamaguchi Y, Mann DM, Ruoslahti E (1990) Negative regulation of transforming growth factor-beta by the proteoglycan decorin. *Nature* 346: 281-284.
45. Moscatello DK, Santra M, Mann DM, McQuillan DJ, Wong AJ, et al. (1998) Decorin suppresses tumor cell growth by activating the epidermal growth factor receptor. *J Clin Invest* 101: 406-412.
46. Patel S, Santra M, McQuillan DJ, Iozzo RV, Thomas AP (1998) Decorin activates the epidermal growth factor receptor and elevates cytosolic Ca2+ in A431 carcinoma cells. *J Biol Chem* 273: 3121-3124.
47. Iozzo RV, Moscatello DK, McQuillan DJ, Eichstetter I (1999) Decorin is a biological ligand for the epidermal growth factor receptor. *J Biol Chem* 274: 4489-4492.
48. Benzakour O, Merzak A, Dooghe Y, Pironin M, Lawrence D, et al. (1992) Transforming growth factor beta stimulates mitogenically mouse NIH3T3 fibroblasts and those cells transformed by the EJ-H ras oncogene. *Growth Factors* 6: 265-275.
49. Santra M, Skorski T, Calabretta B, Lattime EC, Iozzo RV (1995) De novo decorin gene expression suppresses the malignant phenotype in human colon cancer cells. *Proc Natl Acad Sci U S A* 92: 7016-7020.
50. Umesono K, Murakami KK, Thompson CC, Evans RM (1991) Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D3 receptors. *Cell* 65: 1255-1266.
51. Näär AM, Boutin JM, Lipkin SM, Yu VC, Holloway JM, et al. (1991) The orientation and spacing of core DNA-binding motifs dictate selective transcriptional responses to three nuclear receptors. *Cell* 65: 1267-1279.

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