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RNA interference-mediated URG4 gene silencing diminishes cyclin D1 mRNA expression in HepG2 cells

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ABSTRACT. Up-regulated gene 4 (URG4), stimulated by HBxAg, is a novel gene located on chromosome 7 (7p13). The full-length URG4 clone is 3.607 kb and encodes a polypeptide of 922 amino acids, with a molecular weight of 104 kDa (GeneID: 55665). It promotes cell growth, growth factor-independent survival, and anchorage-independent growth in HepG2 cells, and it accelerates tumor formation in nude mice. Hence, URG4 may be a natural effector of HBxAg and a putative oncogene that contributes to multi-step hepatocarcinogenesis. Cyclin D1 is frequently over-expressed in hepatocellular carcinoma, exhibiting a number of malignant phenotypes. We found that down-regulation of URG4 through RNA interference-mediated silencing suppressed cell proliferation in HepG2 cells. Over-expression of URG4 up-regulated cyclin D1 mRNA expression, whereas RNA interference-mediated URG4 silencing

diminished cyclin D1 mRNA expression in HepG2 cells. The data suggest that URG4 may play an important role in the development of hepatocellular carcinoma by partially regulating the expression of cyclin D1 and has potential for use as a therapeutic target for hepatocellular carcinoma.

Key words: URG4; Cyclin D1; Carcinogenesis

INTRODUCTION

Multiple changes in gene expression leading to dysregulated control of cell proliferation may result in tumor formation (Ruddon, 2007). This uncontrolled growth of cells can metastasize and cause significant morbidity and mortality (Lahiry et al., 2010). Hence, altered gene expressions particularly in oncogenes and tumor suppressor genes play an important role in the development of carcinogenesis (Planchard et al., 2009). Hepatitis B virus (HBV) is a major etiologic agent associated with chronic liver diseases, such as hepatitis, cirrhosis, and hepatocellular carcinoma (HCC; Arbuthnot and Kew, 2001). The relative risk of HBV carriers developing HCC is in excess of 100, suggesting that the relationship between HBV and HCC is one of tightest between a virus and a human cancer (Feitelson and Lee, 2007). The molecular mechanisms underlying this high risk for tumor development are incompletely understood. HBV encodes a protein known as hepatitis B x antigen (HBxAg or HBx), which appears to participate in the development of HCC (Feitelson et al., 2009). Recently, upregulated gene 4 (URG4, GenBank GeneID: 55665), a novel gene stimulated by HBxAg, was identified by polymerase chain reaction (PCR) select cDNA subtraction, using HBxAg-positive and HBxAg-negative HepG2 cells (Satiroglu-Tufan et al., 2002). URG4 was located on chromosome 7 (7p13). URG4 was strongly expressed in hepatitis B-infected liver and in HCC cells, where it costained with HBxAg, and was weakly expressed in uninfected liver, suggesting that URG4 was an effector of HBxAg *in vivo*. Over-expression of URG4 in HepG2 (Satiroglu-Tufan et al., 2002) and GES-1 cells (Song et al., 2006) promoted cell growth and survival in tissue culture and soft agar, and accelerated tumor development in nude mice, suggesting that URG4 may be associated with the onset of tumorigenesis. Over-expressed URG4 in osteosarcoma tissues is well correlated with tumor recurrence and metastasis, as well as with the proliferative activity of osteosarcoma cells. Patients with high expression of URG4 had shorter survival time, suggesting that URG4 may be a valuable prognostic marker in osteosarcoma patients (Huang et al., 2009).

The history of RNA interference (RNAi) is relatively short; however, siRNA-mediated gene silencing has become a crucial tool in genetics and molecular biology research (He et al., 2009). Chemically synthesized siRNA reagents targeting particular genes of interest in the human genome can be used for practical delivery *in vitro*, and RNA interference data can reveal novel and different pathways about the function of the gene. In the present study, the effects of RNAi-mediated URG4 gene silencing were determined in HepG2 cells.

MATERIAL AND METHODS

Cell lines and tissue culture conditions

The human hepatoblastoma cell lines, HepG2 (ATCC, Manassas, VA, USA), HepG2-

pcDNA3, HepG2-pcDNA3-URG4, and HepG2-pcDNA3-HBx, were cultured on type-1 rat tail collagen (Serva)-coated tissue culture dishes or plates. Cells were grown in Earle's modified Eagle's medium (Biological Industries, Beit Haemek, Israel) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Biological Industries), 100 µM minimal essential medium non-essential amino acids (Biological Industries), 1 mM sodium pyruvate (Biological Industries), as well as standard concentrations of penicillin plus streptomycin (Biological Industries).

Plasmids

pcDNA3 (Invitrogen, San Diego, CA, USA) was used to clone full-length URG4 cDNA under the control of the immediate early cytomegalovirus (CMV) promoter and also used for the cloning of the HBx open reading frame, as described previously (Lian et al., 1999).

Construction of URG4-overexpressing HepG2 cells

To study the properties of URG4 at the cellular level, separate cultures of 1 x 10^6 HepG2 cells were stably transfected with 10 µg pcDNA3, pcDNA3-URG4, or pcDNA3-HBx using SuperFect transfection reagent (QIAGEN, Valencia, CA, USA) according to manufacturer instructions. Cells were selected with G418 (800 µg/mL) for 4 weeks. URG4 and HBx mRNA expression was confirmed by semiquantitative RT-PCR.

RNAi-mediated URG4 silencing in HepG2 cells

To study the effects of RNAi-mediated URG4 silencing, HepG2-pcDNA3 cells were seeded in 6-well plates in duplicate (25 x 10^4 cells/per well) or in 96-well plates in triplicate (1 x 10^4 cells/per well) and incubated at 37°C with 5% CO_2 overnight. Separate cultures of HepG2-pcDNA3 cells were then transfected with chemically synthesized siRNA reagents for URG4 (ON-TARGETplus SMARTpool human URG4 siRNA, Dharmacon, Lafayette, CO, USA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; ON-TARGETplus siCONTROL human GAPDH Control siRNA, Dharmacon) and negative control (ON-TARGETplus siCONTROL Non-targeting siRNA, Dharmacon) using Dharmafect I transfection reagent (Dharmacon) according to manufacturer instructions. HepG2-pcDNA3-Mock-transfected cells were also included in this study. At 48 h after the siRNA transfection, RNA was isolated and the expression level of URG4 mRNA was determined by semiquantitative RT-PCR.

RNA isolation and semiquantitative RT-PCR analysis

Forty-eight hours after siRNA transfection, total RNA was isolated with Tri-Reagent (Sigma, St. Louis, MO, USA) according to manufacturer instructions with minor modifications. The single-tube one-step RT-PCR was standardized using the one-step RT-PCR kit (Qiagen, USA). Briefly, one-step RT-PCR was carried out in a 25-µL reaction mixture containing 1 µg total RNA, 20 pmol each primer, 5 µL 5X buffer (12.5 mM $MgCl_2$) 1 µL dNTPs mix (containing 10 mM of each dNTP), and 1 µL of a mixure of Ominiscript and Sensiscripts reverse transcriptases and Hot Star Taq DNA polymerase. The primer sequences (URG4 primer 1, URG4

primer 2, GAPDH, and cyclin D1) used in this study and cycling conditions are summarized in Table 1. The RT-PCR products were analyzed by electrophoresis using 2% Molecular Screening Agarose gel (Roche Diagnostics, GmbH, Mannheim, Germany).

Table 1. Primers used for one-step RT-PCR.

Primer name	Sequence	Annealing temperature (°C)	Amplicon size (bp)
URG4 (1)	URG4 (1) F: 5'-CGGGAGATGGGACAGTTTTA-3' URG4 (1) R: 5'-CATGGTGTTGAGGAGTGTGG-3'	55	285
URG4 (2)	URG4 (2) F: 5'-CTTCATCCTGAGTCCCTACCG-3' URG4 (2) R: 5'-GCCGTTCTGCTGCATTCG-3'	55	472
Cyclin D1	Cyclin D1_F: 5'-AGCTCCTGTGCTGCGAAGTGGAAAC-3' Cyclin D1_R: 5'-AGTGTTCAATGAAATCGTGCGGGGT-3'	60	480
GAPDH	GAPDH F: 5'-CCCCACACACATGCACTTACC-3' GAPDH R: 5'-CCTAGTCCCAGGGCTTTGATT-3'	55	98

Cell proliferation assay

HepG2 cells stably transfected with pcDNA3 and pcDNA3-URG4 were seeded into 96-well plates in triplicate (1 x 10^4 cells/per well) and grown in complete medium. RNAi-mediated silencing has been done using chemically synthesized siRNA reagents for URG4 (ON-TARGETplus SMARTpool human URG4 siRNA), GAPDH (ON-TARGETplus siCONTROL human GAPDH Control siRNA), and negative control (ON-TARGETplus siCONTROL Non-targeting siRNA) in HepG2-pcDNA3 cells according to manufacturer instructions with minor modifications. HepG2-pcDNA3-Mock-transfected cells were also included in this experiment. Cell viability was determined daily for up to 5 days using the modified tetrazolium salt (XTT) assay, as described by the manufacturer of the Cell Proliferation Assay with XTT Reagent (Biological Industries).

Flow cytometry

To study the effects of URG4 and RNAi-mediated URG4 silencing on cell growth, cells were seeded in 6-well plates in duplicate (25 x 10^4 cells/per well) and incubated overnight in complete medium at 37°C with 5% CO_2. After the cells were synchronized in serum-free medium for 48 h, HepG2-pcDNA3 cells were transfected with chemically synthesized siRNA reagent for URG4 (ON-TARGETplus SMARTpool human URG4 siRNA), according to the protocol. For DNA content analysis, siRNA-transfected cells and control cells were incubated in 10% FBS-containing medium for 48 h. Cell pellets were fixed in 70% ethanol, stained with propidium iodine and analyzed by the Flow Cytometry Facility at Thomas Jefferson University, Kimmel Cancer Center, Philadelphia, PA, USA.

RESULTS

Construction of URG4-overexpressing HepG2 cells

To functionally characterize URG4, independent cultures of HepG2 cells were stably transfected with pcDNA3, pcDNA3-URG4 or pcDNA3-HBx, and each of the cell lines selected in G418. The levels of URG4 mRNA were then determined in each of the cell lines

by RT-PCR (Figure 1A and B). The first set of one-step multiplex RT-PCR analysis (Figure 1A) showed two bands of 285 bp (URG4 1) and 98 bp (GAPDH) in all cell lines. The levels of URG4 expression were significantly higher in HepG2-pcDNA3-URG4 cells (lane 3) and in HepG2-pcDNA3-HBx cells (lane 4) compared to the levels of URG4 expression in HepG2 (lane 1) and HepG2-pcDNA3 cells (lane 2). Again, a second set of one-step multiplex RT-PCR analysis (Figure 1B) showed two bands of 472 bp (URG4 2) and 98 bp (GAPDH) in all cell lines. The levels of URG4 expression were significantly higher in HepG2-pcDNA3-URG4 cells (lane 3) and in HepG2-pcDNA3-HBx cells (lane 4) compared to the levels of URG4 expression in HepG2 (lane 1) and HepG2-pcDNA3 cells (lane 2).

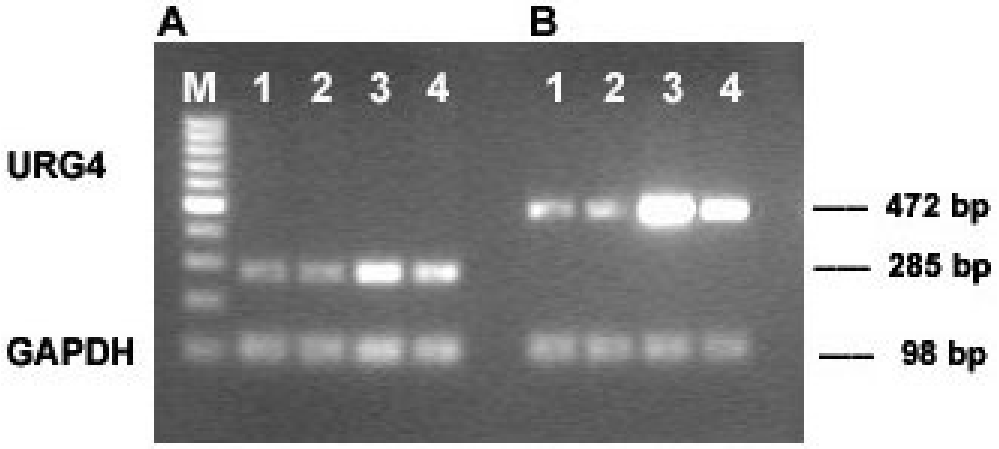

Figure 1. Expression of up-regulated gene 4 (URG4) mRNA in HepG2 cells stably transfected with pcDNA3, pcDNA3-URG4 or pcDNA3-HBx cells. One-step multiplex RT-PCR analysis was carried out on RNA isolated from HepG2 (*lane 1*), HepG2-pcDNA3 (*lane 2*), HepG2-pcDNA3-URG4 (*lane 3*) and HepG2-pcDNA3-HBx (*lane 4*) cells using primers. **A.** URG4 1 and GAPDH. **B.** URG4 2 and GAPDH. M = molecular marker. GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

RNAi-mediated URG4 gene silencing in HepG2 cells

In the present study, the effects of RNAi-mediated URG4 gene silencing were determined. To confirm the RNAi-mediated URG4 silencing in HepG2 cells, mRNA expression of URG4 was determined in HepG2-pcDNA3, HepG2-pcDNA3+URG4 siRNA, HepG2-pcDNA3-URG4, HepG2-pcDNA3+GAPDH siRNA, HepG2-pcDNA3+Non-targeting siRNA, and HepG2-pcDNA3+Mock cell lines. The first set of one-step multiplex RT-PCR analysis (Figure 2A) showed two bands of 472 bp (URG4 2) and 98 bp (GAPDH), and the second set of one-step multiplex RT-PCR analysis (Figure 2B) showed two bands of 285 bp (URG4 1) and 98 bp (GAPDH) in all cell lines. As a result of URG4 siRNA transfection, the expression level of URG4 mRNA was significantly reduced in HepG2-pcDNA3+URG4 siRNA cells (Figure 2A and B, lane 2), compared to the URG4 mRNA expression level in HepG2-pcDNA3 cells (Figure 2A and B, lane 1). The levels of URG4 expression were significantly higher in HepG2-pcDNA3-URG4 cells (Figure 2A and B, lane 3), and as a result of GAPDH siRNA transfection, the expression level of GAPDH mRNA was significantly

reduced in HepG2-pcDNA3+GAPDH siRNA cells (Figure 2A and B, lane 4). No difference in URG4 mRNA expression level was observed in HepG2-pcDNA3+GAPDH siRNA, HepG2-pcDNA3+Non-targeting siRNA, HepG2-pcDNA3+Mock cell lines (Figure 2A and B, lanes 4, 5 and 6, respectively).

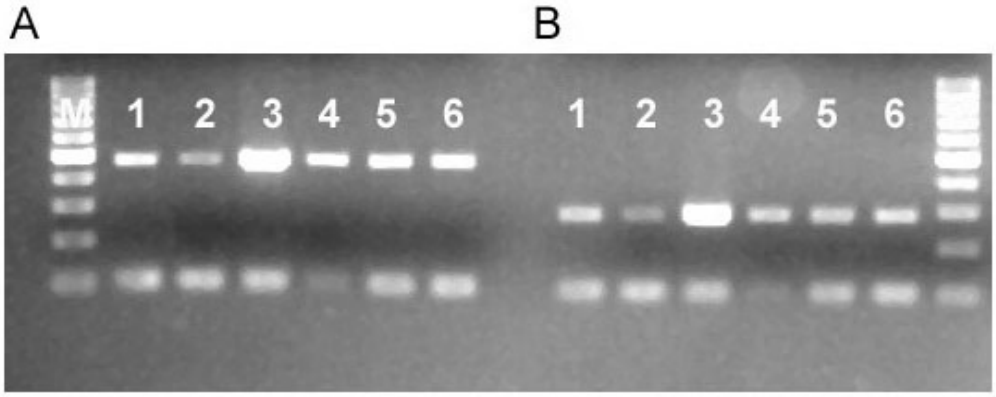

Figure 2. Expression of URG4 mRNA in RNAi-mediated URG4-silenced HepG2 cells. One-step multiplex RT-PCR analysis was carried out on RNA isolated from HepG2-pcDNA3 (*lane 1*), HepG2-pcDNA3+URG4 siRNA (*lane 2*), HepG2-pcDNA3-URG4 (*lane 3*), HepG2-pcDNA3+GAPDH siRNA (*lane 4*), HepG2-pcDNA3+Non-targeting siRNA (*lane 5*), and HepG2-pcDNA3+Mock (*lane 6*) cells using primers. **A.** URG4 2 and GAPDH. **B.** URG4 1 and GAPDH, 48 h after siRNA transfection. M = molecular marker.

RNAi-mediated URG4 silencing suppresses the growth of HepG2 cells

Previous publications of our group and others have revealed that URG4 over-expression stimulates cell growth in HepG2 and GES-1 cells (Satiroglu-Tufan et al., 2002; Song et al., 2006). Hence, experiments were designed to determine whether the inhibition of URG4 expression could suppress the growth of hepatocarcinoma cells. RNAi-mediated URG4 silencing was carried out according to the manufacturer protocol and cell viability was determined daily up to 5 days, using the XTT assay. When these cell lines were grown in tissue culture medium containing 10% FBS (Figure 3), HepG2-pcDNA3-URG4 cells grew faster than HepG2-pcDNA3 cells as expected. Statistically significant growth inhibition was observed in HepG2-pcDNA3+siURG4 cells compared to control cells (Figure 3), suggesting that the effects of these URG4 siRNA on proliferation and viability are likely caused by the repression of URG4 expression.

To confirm whether URG4 silencing suppress cell growth, these cell lines were also analyzed by flow cytometry. Forty-eight hours after siRNA transfection, 19.6% HepG2-pcDNA3 cells were in S phase (Figure 4A), whereas 12% HepG2-pcDNA3+siURG4 cells were in S phase (Figure 4B), and the percentage for HepG2-pcDNA3-URG4 cells in S phase was 27.8% (Figure 4C). Hence, these data suggest that URG4 silencing suppresses the entry of cells into S phase and cell growth in culture.

URG4 stimulates cyclin D1 mRNA expression in HepG2 cells

Since URG4 is a putative effector of HBx and promotes the entry of cells into S phase,

experiments were further designed to analyze cyclin D1 expression in URG4 over-expressing cell lines and cell lines with RNAi-mediated URG4 silencing. Over-expression of URG4 stimulated cyclin D1 mRNA expression (Figure 5, lane 3), whereas RNA interference-mediated URG4 silencing diminished cyclin D1 mRNA expression in HepG2 cells (Figure 5, lane 2).

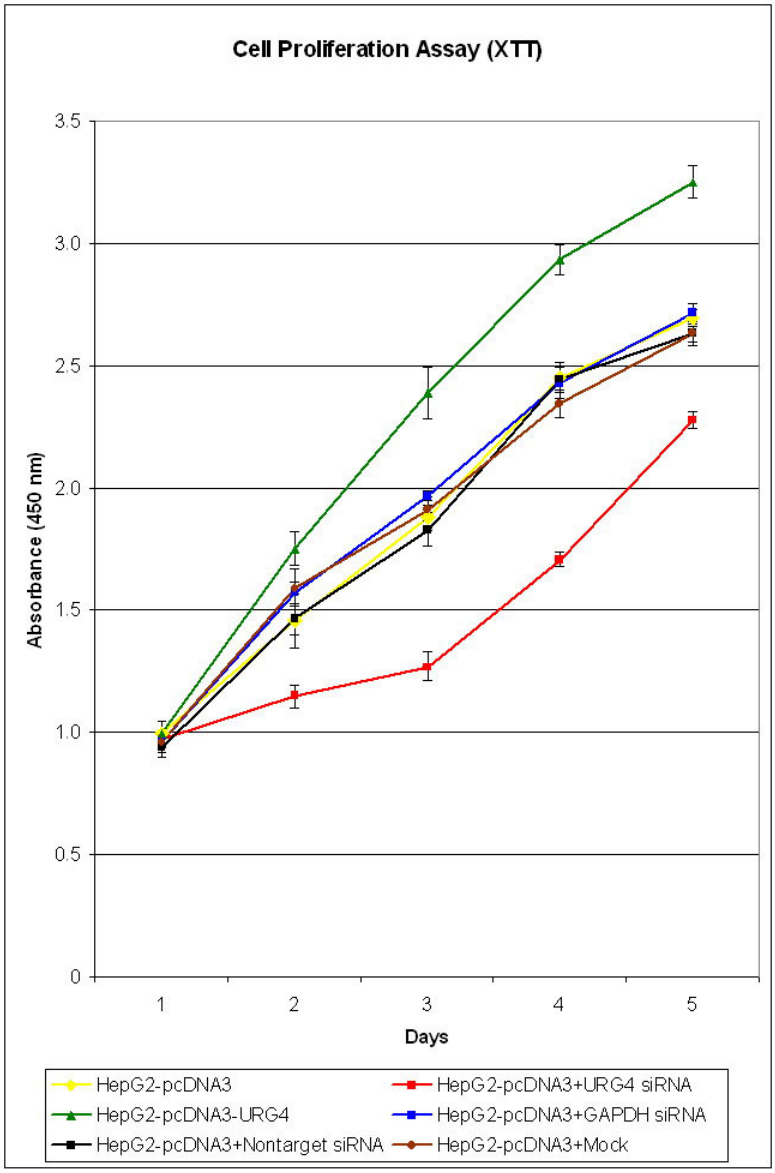

Figure 3. RNAi-mediated URG4 silencing suppresses the growth of HepG2 cells. Growth curves for HepG2-pcDNA3, HepG2-pcDNA3+URG4 siRNA, HepG2-pcDNA3-URG4, HepG2-pcDNA3+GAPDH siRNA, HepG2-pcDNA3+Non-targeting siRNA, and HepG2-pcDNA3+Mock cells in complete medium containing 10% FBS. The curves represent the average of two independent experiments, each done in triplicate. XTT = tetrazolium salt.

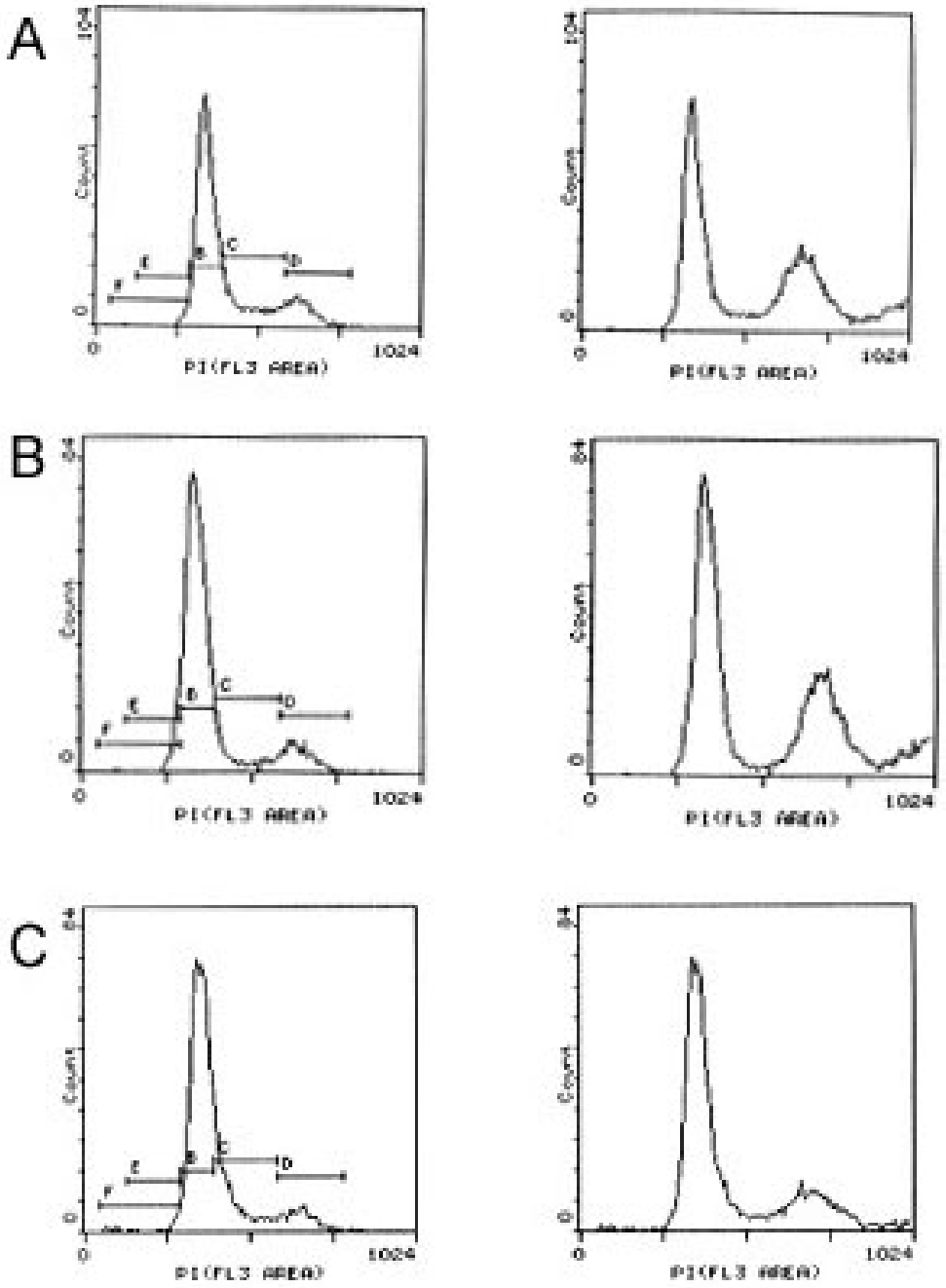

Figure 4. Flow cytometry analysis. Nineteen and six percent HepG2-pcDNA3 (A), 12% HepG2-pcDNA3+siURG4 (B) and 27.8% HepG2-pcDNA3-URG4 (C) cells were in S phase at 48 h after siRNA transfection. The results shown here illustrate one of the two independent analyses, each done in duplicate.

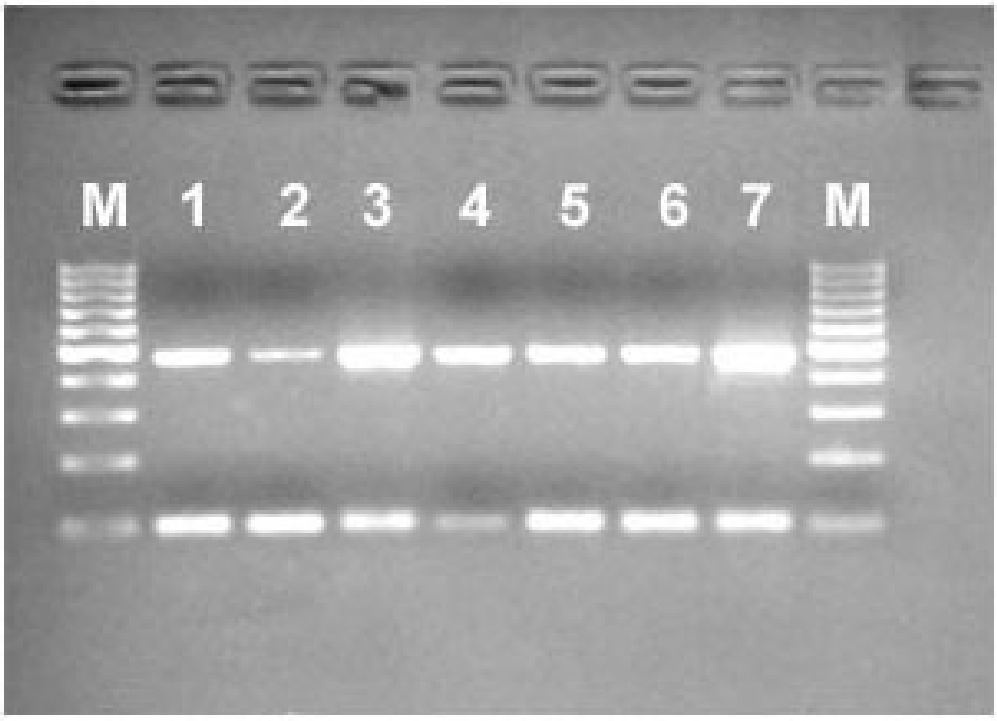

Figure 5. Expression of cyclin D1 mRNA in RNAi-mediated URG4-silenced HepG2 cells. One-step multiplex RT-PCR analysis was carried out on RNA isolated from HepG2-pcDNA3 (*lane 1*), HepG2-pcDNA3+URG4 siRNA (*lane 2*), HepG2-pcDNA3-URG4 (*lane 3*), HepG2-pcDNA3+GAPDH siRNA (*lane 4*), HepG2-pcDNA3+Non-targeting siRNA (*lane 5*), HepG2-pcDNA3+Mock (*lane 6*), and HepG2-pcDNA3-HBx (*lane 7*) cells using cyclin D1 and GAPDH primers, 48 h after siRNA transfection. M = molecular marker.

DISCUSSION

Identification and characterization of differentially expressed genes in cancer tissues provide important information to understand the possible molecular oncogenic mechanisms responsible for the development of carcinogenesis and metastasis. Recently, remarkable progress has been made in the techniques used to identify the differences in gene expression between cell populations. Previously, we have analyzed the differentially expressed genes in the presence of HBxAg in HepG2 cells, a hepatoblastoma cell line, and showed that HBx up-regulates the expression of a novel gene, URG4, which is differentially expressed in tumor compared to non-tumor liver specimens from HBV-infected HCC patients. Over-expression of URG4 in HepG2 and GES-1 cells promoted cell growth and survival in tissue culture and soft agar, and accelerated tumor development in nude mice, suggesting that URG4 may be associated with the onset of tumorigenesis (Satiroglu-Tufan et al., 2002; Song et al., 2006). Over-expression of URG4 in osteosarcoma tissues is well correlated with tumor recurrence and metastasis, as well as with the proliferative activity of osteosarcoma cells. Patients with high expression of URG4 had shorter survival time, suggesting that URG4 may be a valu-

able prognostic marker in osteosarcoma patients (Huang et al., 2009).

Advances in molecular technology are providing new tools to identify the molecular signatures of signaling pathways in the development of carcinogenesis. The ability to knock down gene function through the use of RNAi has become a crucial tool in functional genomics research (He et al., 2009). Chemically synthesized siRNA reagents targeting particular genes of interest in the human genome can be used for practical delivery *in vitro*, and RNA interference data can reveal novel and different pathways about the function of the gene. In the present study, the effects of RNAi-mediated URG4 gene silencing were examined. URG4-specific siRNA partially blocked the ability of URG4 to promote DNA synthesis and growth of HepG2 cells.

Cyclin D1 is a key factor in cell cycle that functions between G0/G1- and S-phase check points (Alao, 2007). It forms an active complex with cyclin-dependent kinase 4 and 6 (CDK4 and CDK6), and promotes cell cycle progression by phosphorylating and inactivating retinoblastoma protein (Weinberg, 1995; Lundberg and Weinberg, 1998). It is frequently over-expressed in HCC patients with enhanced malignant phenotypes and high mortality (Alao, 2007). Previous publications revealed that HBx up-regulates cyclin D1 promoter in Chang liver and HepG2 cells, which may have an important role in the HBx-mediated HCC development and progression (Park et al., 2006). Over-expression of URG4 stimulated cyclin D1 mRNA expression, and RNAi-mediated URG4 silencing also diminished cyclin D1 mRNA expression in HepG2 cells. These results suggest that cyclin D1 up-regulation contributes importantly to the mechanism of URG4-mediated hepatocellular growth. Our results give new insights into the complex phenomenon of HBV-induced hepatocellular carcinogenesis; however, there is much to be investigated to better understand the molecular mechanism(s) of action of novel URG4. Recently, the HUGO Gene Nomenclature Committee approved URG4's name as "upregulator of cell proliferation" (URGCP) (HGNC:30890 http://www.genenames.org/data/hgnc_data.php?hgncid=30890). Our ongoing studies include the detailed investigation of URG4's molecular function in oncogenic signaling pathways and cell cycle regulation.

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