Involvement of Cancer Biomarker C7orf24 in the Growth of Human Osteosarcoma

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Abstract. *Background: Up-regulation of the expression of the gene C7orf24, encoding γ-glutamyl cyclotransferase, is a common event in cancers derived from various tissues, but its involvement in osteosarcomas (OS) has not yet been demonstrated. Materials and Methods: The expression of C7orf24 was analyzed in human OS cell lines and primary tumor samples. The biological effects of C7orf24 on growth, motility, and invasion in the OS cell lines were investigated using siRNA for C7orf24. Genes related to the function of C7orf24 were sought by genome-wide gene expression profiling. Results: The level of C7orf24 expression was much higher in the OS cell lines and OS primary tumors than in normal osteoblasts. Down-regulation of C7orf24 expression inhibited the growth of the cell lines in association with enhancement of cell-clustering. Treatment with C7orf24-siRNA inhibited cell motility and invasion. Gene ontology suggested the function of C7orf24 to be related to cell adhesion and protein transport. Conclusion: C7orf24 is also involved in the growth of OS, and is a potential biomarker for this type of tumor.*

Osteosarcoma (OS) is a primary bone malignancy generally affecting the young, with 60% of cases occurring before the age of 25 years and peak incidence at 15 years (1). Current standard treatment for OS involves neoadjuvant (preoperative) chemotherapy, definitive surgery on the primary tumor, and adjuvant (postoperative) chemotherapy, and the survival rate has improved significantly and reached more than 70% at 5 years (2-5). Further improvement, however, may be difficult without developing novel approaches such as molecular target therapy. A number of studies have been performed to identify molecules involved in the malignant phenotype of OS cells (6). The expression level of Ezrin, an adaptor protein linked to the cell membrane, correlated with the metastatic activity of OS (7), which led to clinical trails of the mammalian target of rapamycin (mTOR) inhibitor for OS patients (6). Inhibition of growth factors such as insulin-like growth factor (IGF), either by an antibody or by inhibitor for IGF receptors, prevented the growth of OS (8, 9). Although the functional involvement is not known, the expression level of the chemokine (C-X-C motif) receptor, CXCR4, correlated with the incidence of metastasis (10). Recent genome-wide gene expression analyses identified the receptor tyrosine kinase-like orphan receptor 2 (*ROR2*) gene as being up-regulated in OS tumors, and that the signal through a putative ligand, WNT5B, to ROR2 was involved in the growth of OS (11). Array-based analyses identified some miRNAs related to the resistance of chemotherapy such as miR-140 (12), and miR-92a, miR-99b, miR-132, miR-193a-5p and miR-422a (13). It remains to be resolved how these multiple factors affect the overall phenotype of OS, and whether any other molecules are also involved.

We have identified chromosome 7 open reading frame 24 (C7orf24) as an up-regulated protein of unknown function in bladder cancer (14), which was independently identified as

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a 21-kDa cytochrome *c*-releasing factor in the cytosolic fraction of human leukemia U937 cells after treatment with geranylgeraniol (15). Geranylgeraniol has potent apoptosis-inducing activity in various tumor cell lines, implicating the C7orf24 protein in apoptotic pathways of cancer cells (15). *In silico* analyses utilizing a panel of gene expression profiles of cancer cells also identified *C7orf24* as a gene up-regulated in many types of cancer (16, 17). Here we investigated *C7orf24* expression in human OS, and its association with cell motility and invasion.

Materials and Methods

Tissue specimens and cell lines. Tumor tissues were obtained at either biopsy or resection surgery and kept at –80˚C. Informed consent was obtained from each patient, and tumor samples were approved for analysis by the Ethics Committee of the Faculty of Medicine, Kyoto University. The human OS cell lines Saos2, HuO, HOS, MG63, U2OS, and G292 were obtained from the ATCC (Rockville, MD, USA) or the Japanese Cancer Research Resources Bank (Tokyo, Japan). OS690 cells were established in our laboratory from a tumor of a 10-year-old girl with an osteoblastic OS in the femur. Normal human osteoblasts (NHOst) were obtained from TaKaRa (TaKaRa Bio, Shiga, Japan). These cells were cultured in DMEM containing 10% fetal bovine serum (FBS) supplemented with 100 units/ml of penicillin and 100 g/ml of streptomycin.

Reverse transcription (RT-PCR). All RT reactions were performed using 1 μg of total RNA with the Superscript III first-strand system (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. PCR was performed for the *C7orf24* and β-actin (*ACTB*) genes using standard procedures. PCR products were loaded on 1% agarose gel and visualized by ethidium bromide staining. A pair of intron-spanning primers specific for human C7orf24 cDNA (GenBank accession number NM_024051; sense primer, 5'-ACAAGTCAAACTTGGCATGGAG-3'; antisense primer, 5'-TCTTGATACTCC AGCGGCAAAC-3') was used to amplify the 296 bp product.

RT-quantitative PCR (RT-qPCR). The relative amount of *C7orf24* mRNA was assessed by TaqMan real-time PCR with the ABI PRISM 7700 sequence detection system (Life Technologies). A 75-bp fragment from +293 (exon 2) to +408 (exon 3) of the *C7orf24* cDNA was amplified using specific primers (sense, 5'-TCCCAAGGCAAAACAAGTCAA-3'; antisense, 5'-TTAACCC CTTCTTGCTC ATCCA-3') and labeled with a TaqMan probe (5'-FAM-CACCATTTTTCAGAG TCCTG-GCGATGA-3'-TAMRA). NHOsts were used as the internal control, and all of the reactions were run in duplicate. The ratio of *C7orf24* of OS cell lines and sample/NHOst in each sample was calculated, and the expression level of *C7orf24* was demonstrated as a relative value using the *C7orf24/18S* ratio as a standard.

Western blotting. Whole-cell lysate in SDS sample buffer was prepared from each cell line. Aliquots of the extracts were electrophoresed in 15% polyacrylamide gels. Subsequently, proteins were transferred onto Immobilon-P Transfer Membranes (Millipore, Billerica, MA, USA). After blocking with 5% skim milk, membranes were probed with an anti-human C7orf24 monoclonal antibody, 6.1E (14), at a 1:40,000 dilution overnight. After 1 h of incubation at room temperature with secondary antibody (horseradish peroxidase-conjugated rabbit IgG against mouse Ig; Dako, Kyoto, Japan) at 1:20,000, immunoreactive bands were detected with ECL Western Blotting Buffer Detection Reagents (GE Healthcare, Biosciences, Piscataway, NJ, USA). The densitometric analysis was conducted using ImageJ (http://rsb.info.nih.gov/ij/) and values were normalized with those of NHOst.

siRNA synthesis and transfection. The following target sequences were used to generate siRNA (Qiagen, Chatworth, CA, USA): sequence no. 1 (057), 5'-CUUUGCCUACGGCAGCAAC-3' (nucleotides 184-202); sequence no. 2 (498), 5'-UGACUAUACAGGAAAGGUC- GA-3' (nucleotides 625-643); sequence no. 3 (570), 5'-CAUAACA GAAUAUAU- CUAA-3' (nucleotides 697-715); GL3 (firefly luciferase), 5'-CUUACGCUGAGU-ACUUCUUCGA-3'. The synthetic siRNA duplexes were transfected to cells using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. For validation of the knockdown effect, 5×10^5 cells in a 60 mm dish were transfected with siRNAs. The medium was changed 24 h after the transfection, and the cells were harvested at 48 h.

Water-soluble tetrazolium salts (WST)-1 assay. The antiproliferative activities of siRNAs were measured by WST-1 assay (Dojindo, Kumamoto, Japan). Appropriate numbers of cells were seeded into 96-well plates 24 h after the transfection of siRNAs. The WST-1 assay was carried out 48 h after seeding, and repeated every 24 h until the 144 h mark. Colorimetric measurements at 450 nm were made in a microplate reader (Thermo Labsystems, Waltham, MA, USA).

Cytochemistry. Cells were seeded in 8-well chamber slides 24 h after the transfection of siRNAs (10 nM). After a 48-h culture, slides were washed with phosphate-buffered saline (PBS) and fixed with 4% para-formaldehyde in PBS. They were then incubated with rhodamine-phalloidin conjugate (10 U/ml) (Life Techonologies) at room temperature for 30 min. After washing with PBS, slides were counterstained with 4,6-diamidino-2-phenylindole, and viewed under fluorescence microscopy.

Matrigel invasion assay. Cell motility and invasion were assayed using BioCoat Matrigel Invasion Chambers (BD Biosciences, Franklin Lakes, NJ, USA). Twenty-four hours after the transfection of *C7orf24*- or *GL3*-siRNA, cells (2.5×10^4) suspended in serum-free DMEM were placed in the upper chamber of 8 μm Control Cell Culture Inserts (BD Biosciences), and DMEM plus 5% FBS was placed in the lower chamber as a source of chemoattractant. Cells were allowed to migrate through a porous, uncoated membrane for 24 h at 37˚C. The cells remaining in the upper chamber were then removed with a cotton-tip applicator. The cells on the lower surface were fixed with methanol and stained with 1% toluidine blue. The number of migrating cells was determined by counting in five randomly chosen fields under a magnification of ×100. For invasion assays, cells (2.5×104/well) in serum-free DMEM were seeded in the upper chamber coated with Matrigel. DMEM plus 5% FBS was placed in the lower chamber. Incubation was carried out for 24 h at 37˚C. The membrane was processed as described for the motility assay. Cell invasiveness was calculated by dividing the number of cells invading through the Matrigel membrane by the number invading the control insert. Experiments were performed three times in triplicate.

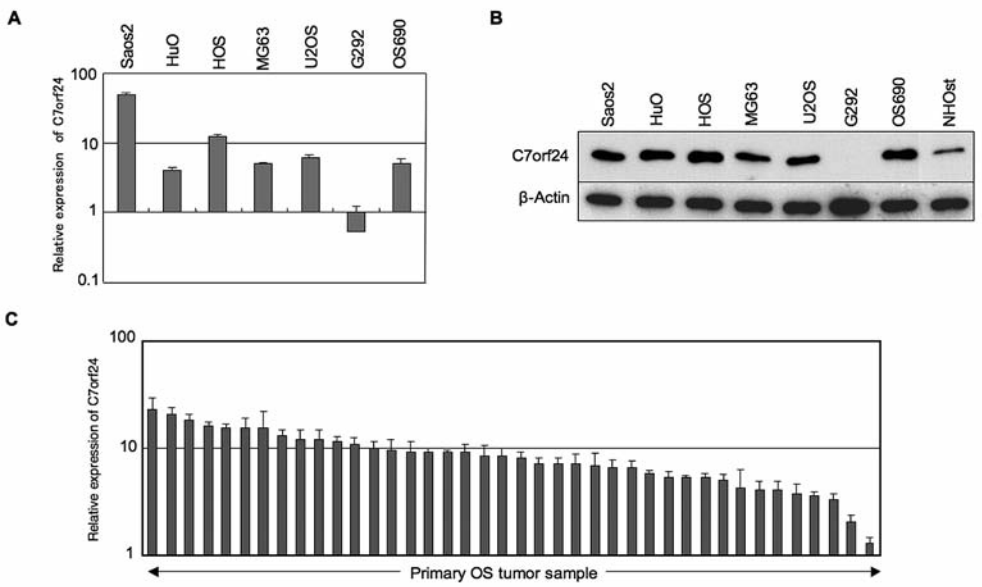

Figure 1. *Expression of C7orf24 in OS cell lines and primary tumors. mRNA expression of the C7orf24 gene in OS cell lines (A) and primary tumors (C). Expression levels of the C7orf24 gene in OS cell lines are indicated relative to that in NHOst. B: Expression of C7orf24 protein in OS cell lines and NHOst.*

Gene expression on transfection with siRNA. RNA was extracted from HOS cells 36 h or 72 h after the transfection of either *C7orf24*- or *GL3*-siRNA (10 nM), and processed for the microarray analyses using a GeneChip Human Genome U133 Plus 2.0 Array containing 54,675 probes (Affymetrix, Santa Clara, CA, USA).

Statistical analysis. Statistical analyses were performed using StatView software (Abacus Concepts Inc., Piscataway, NJ, USA). For comparisons of two individual data points, a two-sided Student's *t*-test was applied to assess statistical significance. An ANOVA with post hoc testing was used for comparisons of more than three data points.

Results

Expression of C7orf24 mRNA in human OS cell lines and primary tumors. Expression of the *C7orf24* mRNA was investigated by RT-qPCR in seven OS cell lines (Saos2, HuO, HOS, MG63, U2OS, G292, and OS690), as well as in normal human osteoblasts (NHOst). Relative to the value in NHOst, the level of *C7orf24* gene expression in the OS cell lines, except for G292 was increased by 3- to 55-fold (Figure 1A). The up-regulation of *C7orf24* expression was further confirmed at the protein level. The amount of C7orf24 protein was higher in OS cell lines, except G292, than in NHOst (Figure 1B), which corresponded to the results of the mRNA analyses (Figure 1A). The expression of *C7orf24* mRNA in tumor tissues was also analyzed by RT-qPCR using 40 primary OS samples. Relative to the expression in NHOst, the level of *C7orf24* was 2- to 24-fold higher in OS tumors (Figure 1C).

Knockdown of the expression of C7orf24 by siRNA. To knockdown the expression of *C7orf24*, three siRNA targeting *C7orf24* were designed and transfected into HOS by lipofection. An siRNA targeting the firefly luciferase gene was used as a control (*GL3*-siRNA). The efficacy of transfection was more than 70% based on the number of positive cells transfected with the fluorescence-labeled gene (data not shown). The expression of *C7orf24* was relatively unchanged in cells transfected with *GL3*-siRNA, but significantly down-regulated in cells transfected with 498- and 570-siRNA at 48 h, and the siRNA remained effective until 96 h after transfection (Figure 2A). Transfection of 498-siRNA also reduced the expression of the *C7orf24* gene in MG63, Saos2, and OS690 (Figure 2B) and also in U2OS, HuO, and G292 (data not shown). Based on these results, 498-siRNA was used in subsequent experiments as *C7orf24*-siRNA.

Down-regulation of C7orf24 expression inhibited the growth of OS cell lines. Either *C7orf24*-siRNA or *GL3*-siRNA was transfected into seven OS cell lines, as well as NHOst, and growth profiles were examined by WST-1 assay. No significant change in growth was observed in any cell line transfected with *GL3*-siRNA (data not shown). The growth profile of NHOst transfected with *C7orf24*-siRNA showed no change even at the highest concentration. G292 cells, which expressed *C7orf24* at the lowest level among the OS cell lines, showed no significant change either. In MG63 cells, the growth-

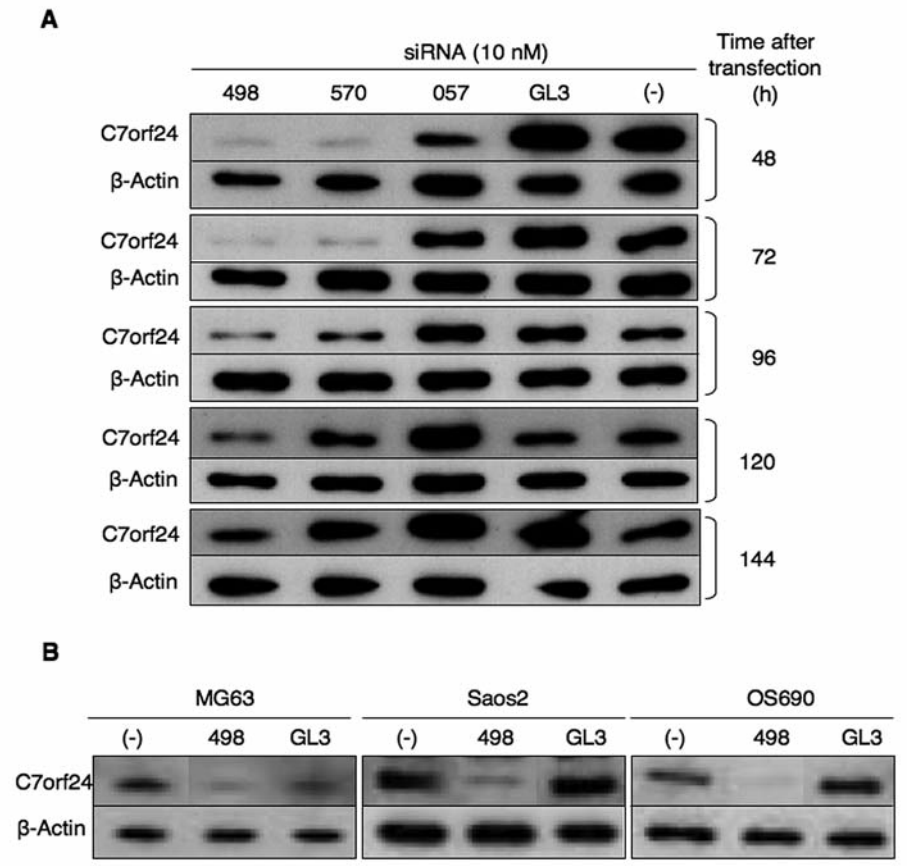

Figure 2. *Knockdown of the expression of C7orf24 by siRNA. A: mRNA expression of the C7orf24 gene in HOS cells after the transfection of siRNAs. RNAs were extracted at the indicated time points after transfection of siRNAs targeting C7orf24 (498, 570 or 057) or GL3. B: mRNA expression of the C7orf24 gene in OS cell lines after the transfection of siRNAs. RNAs were extracted from MG63, Saos2, and OS690 cells 48 h after the transfection of siRNAs targeting C7orf24 (498) or GL3.*

inhibitory effect of *C7orf24*-siRNA was observed only after 144 h at the highest concentration. In contrast, a time- and dose-dependent reduction in growth was observed in the other five OS cell lines (Figure 3).

Down-regulation of C7orf24 expression induced clustering in OS cell lines. Parental HOS cells retained their original spindle shape and proliferated with less cell-to-cell contact until confluent (Figure 4A, left), which was also observed in HOS cells transfected with *GL3*-siRNA (Figure 4A, middle). In contrast, HOS cells transfected with *C7orf24*-siRNA were polygonal to cuboidal in shape and tended to cluster via cell-to-cell attachments (Figure 4A, right). This change was more clearly observed when actin fibers were stained (Figure 4B). The morphological change gradually reversed with time (Figure 4C), which seemed to correspond to the loss of inhibitory effect of siRNA (Figure 1A). Similar but less significant changes were observed in U2OS, SaOS2 and OS690 cells, the growth of which was inhibited even at lower concentrations of *C7orf24*-siRNA (Figure 3). In contrast, no obvious morphological changes were observed in MG63 and G292 cells, in which the growth inhibitory effect of C7orf24 was not remarkable (Figure 3).

Down-regulation of C7orf24 expression reduced the motility and invasiveness of OS cell lines. The morphological changes induced by *C7orf24*-siRNA suggested that *C7orf24* down-regulation affects cell motility. The migration and invasion by HOS cells transfected with either *C7orf24*- or *GL3*-siRNA were evaluated using the matrigel invasion chambers. The number of cells passing through the control membrane was counted as an index of cell motility. The introduction of *C7orf24*-siRNA significantly reduced the number of cells passing through the control membrane (Figure 5A). Cell invasion was also assayed using a matrigel-coated membrane. As well as cell motility, cell invasion was reduced by the introduction of *C7orf24*-siRNA (Figure 5B).

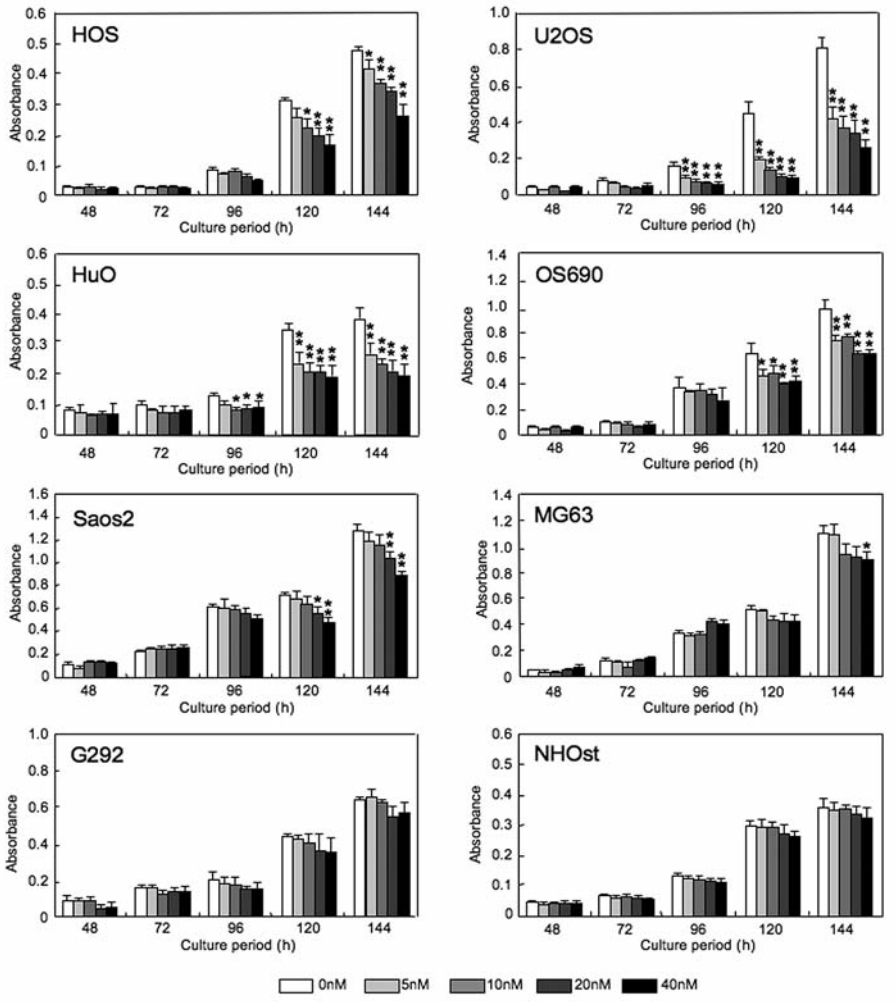

Figure 3. *Growth profiles of OS cell lines treated with C7orf24-siRNA. C7orf24-siRNA in the indicated concentration was transfected into OS, as well as NHOst, cell lines. Cell numbers were evaluated by the WST1 assay 48 h after transfection and every 24 h thereafter. *p<0.05; **p<0.01.*

Genes up- or down-regulated by the knockdown of C7orf24. To elucidate the functional relevance of *C7orf24*, the gene expression profiles of HOS cells transfected with *C7orf24*-siRNA and *GL3*-siRNA were compared, and genes up- or down-regulated by the knockdown of *C7orf24* were identified. RNAs were isolated at two time points (36 and 72 h after transfection of each siRNA), and used for the Affymetrix gene chip. The criteria for up- and down-regulated genes were an expression level higher by more than two-fold and lower by less than half in *C7orf24*-siRNA-treated cells than in *GL3*-siRNA-treated cells at both time points, respectively. One hundred and ninety-seven genes were identified as being up-regulated, and the ontological analyses revealed that the biological function of the genes with the highest *p*-value was cell adhesion followed by system development (Table I). Two hundred and seventy-seven genes were identified as being down-regulated, and the biological function of the genes with the highest *p*-value was intracellular protein transport followed by protein localization (Table I).

Discussion

We performed a proteomic analysis of bladder cancer using narrow range pH two-dimensional gel electrophoresis (2DE) to find new proteins that can be used for cancer diagnosis or treatment (14). Fifteen spots were identified as proteins up-regulated in cancerous tissue, including C7orf24. Functional analyses using expression vectors and siRNA revealed that C7orf24 was involved in the growth of cancer cells (14). Xu *et al*. identified *C7orf24* as 1 out of 46 genes that form a common cancer signature in a study that directly merged

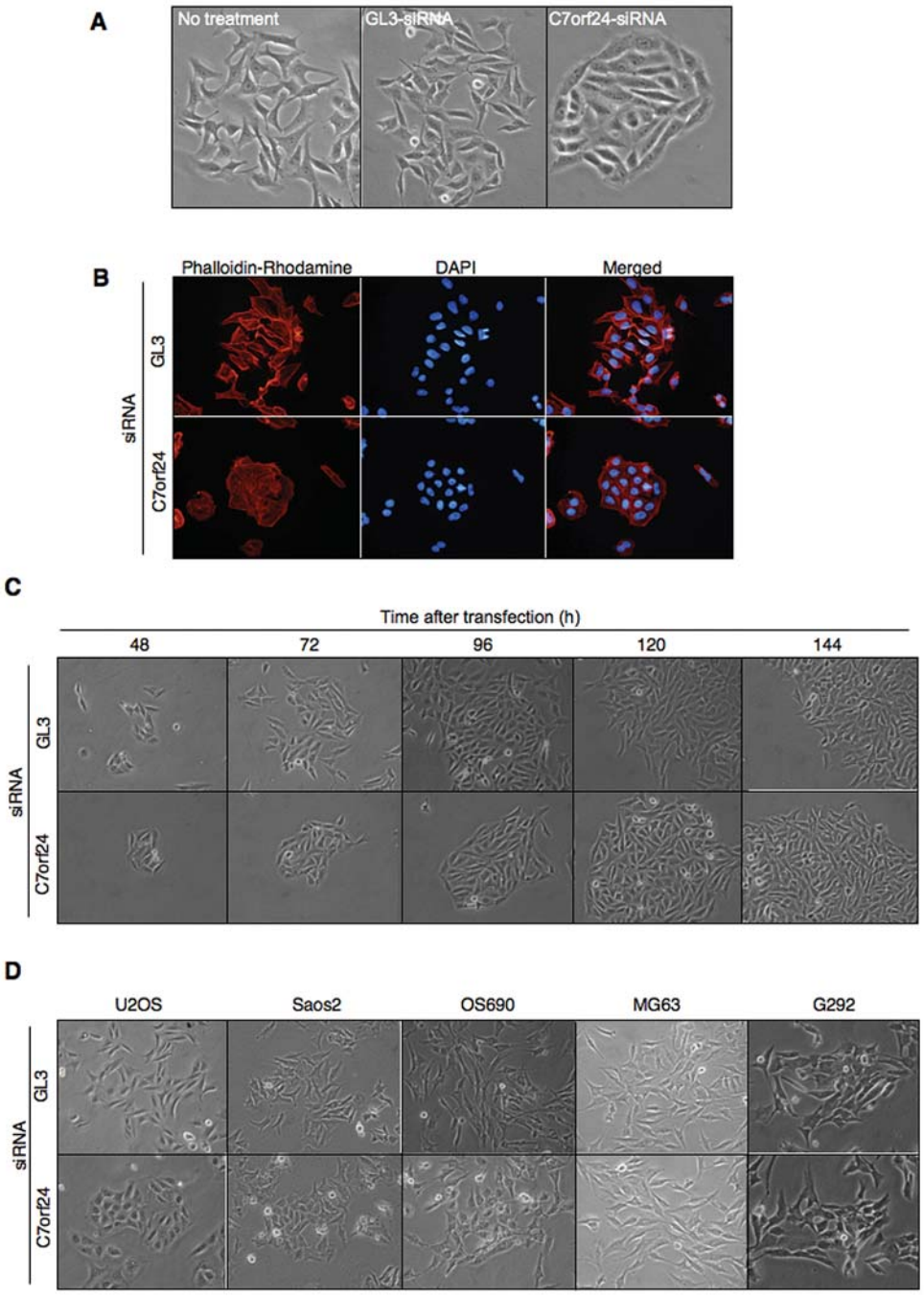

Figure 4. *Morphological changes of OS cells treated with C7orf24-siRNA. A: Phase-contrast micrographs of HOS 72 h after the transfection of siRNAs (10 nM). B: Fluorescent micrographs of HOS 72 h after the transfection of siRNAs (10 nM). C: Phase-contrast micrographs of HOS cells treated with siRNSs (10 nM) for the period indicated. D: Phase-contrast micrographs of OS cell lines 72 h after the transfection of siRNAs (10 nM).*

cancer/normal whole-genome microarray data sets to form an integrated training data set with 799 samples from 21 tissue types, not including bone sarcomas (17). Here we demonstrated that *C7orf24* expression is also up-regulated in bone sarcomas.

Oakley *et al*. tried to purify human γ-glutamyl cyclotransferase (GGCT) (18), an enzyme in the γ-glutamyl cycle that catalyzes the formation of 5-oxoproline from γ-glutamyl dipeptides and potentially plays a role in glutathione homeostasis (19, 20). They found that the gene

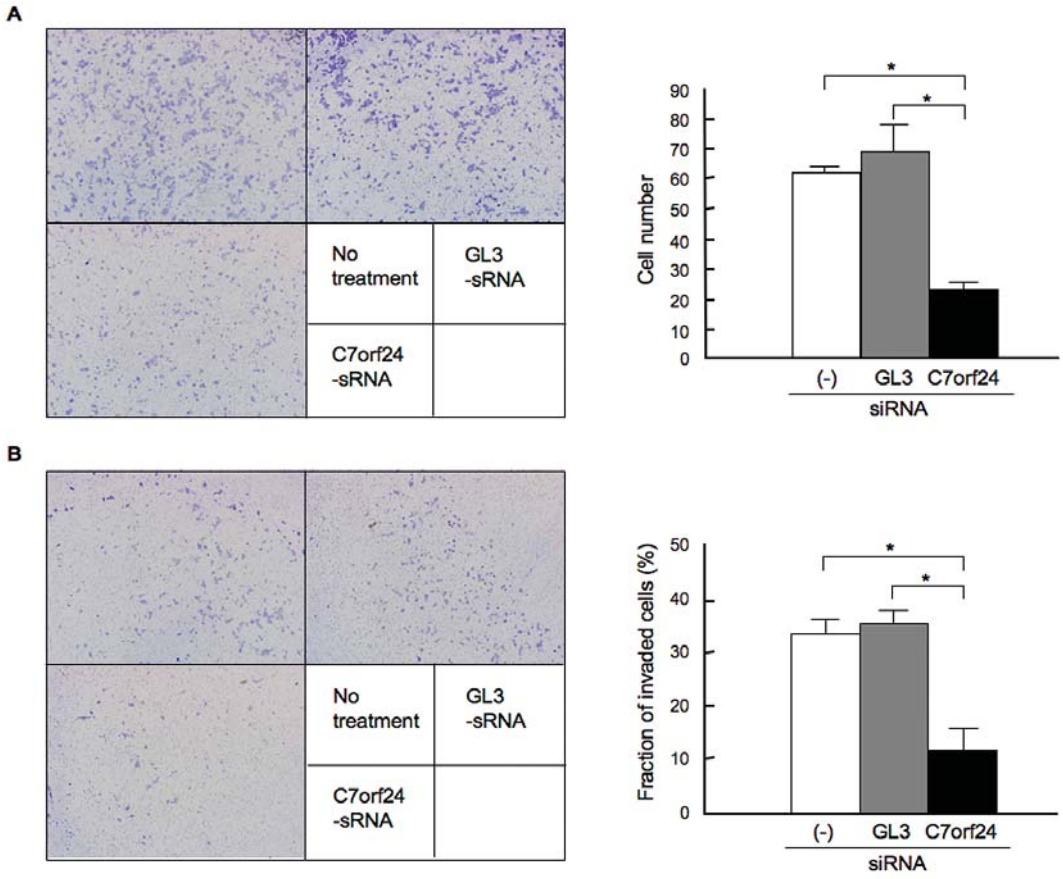

Figure 5. *Motility and invasiveness of HOS cells treated with siRNAs. A: Motility of HOS cells. Cells that passed through the pore of the control membrane were stained (left panel) and the positively stained cells were counted (right panel). B: Invasiveness of HOS cells. Cells that passed through the matrigel were stained (left panel) and the positive by stained cells were counted. The fraction of invading cells was determined as described in Materials and Methods and is indicated in the right panel.*

encoding GGCT is *C7orf24* (18), although the functional relevance to the growth of cancer cells is not known. Recently, Gromov *et al*. identified C7orf24 as an up-regulated protein by 2DE proteomic analyses in 123 samples of breast cancer (21). They validated the up-regulation of C7orf24 expression using a larger number of samples (2,197 samples) and found that approximately one fourth of tumors expressed C7orf24. Interestingly, the prognosis was poorer for patients with C7orf24-positive tumors than those with C7orf24-negative tumors. They also analyzed other types of cancer, including cervical, lung and colon cancer, and found that a significant proportion expressed C7orf24 (58%, 38%, and 72%, respectively). In addition, they established a method to monitor the level of C7orf24 in serum, and proposed C7orf24 as a general cancer biomarker.

In this study, we demonstrated that the knock-down of *C7orf24* expression inhibited the growth of OS cells, as we previously observed for bladder cancer cell lines. The molecular mechanism responsible for this inhibition is not yet known. Although the knockdown effect of *C7orf24*-siRNA showed no significant differences, the growth-inhibitory effect differed among cell lines. On treatment with *C7orf24*-siRNA, significant morphological changes were observed in HOS and, to a lesser degree, in other OS cell lines. Because the extent of the morphological change seemed to correspond to the degree of growth reduction in each cell line, these two phenotypes may be related to each other. In the case of HOS, the knockdown of *C7orf24* reduced cell motility and invasion, which also may relate to morphological changes. Based on these biological consequences, it is rational that gene ontology identified a set of genes related to cell adhesion as being up-regulated using *C7orf24*-siRNA. It is also intriguing that a set of genes relating to protein transport and localization was identified as being down-regulated using *C7orf24*-siRNA. Although we have no clear explanation of how these molecules contribute to the phenotype observed in this study, the current findings will be useful for understanding the role of C7orf24 in cancer.

Table I. *Genes up- or down-regulated by C7orf24-siRNA.*

Entrez gene number	Description	Ontological term	
		Cell adhesion	System development
Up-regulated genes			
780	Discoidin domain receptor family, member 1	+	
999	Cadherin 1, type 1, E cadherin (epithelial)	+	
1525	Coxsackie virus and adenovirus receptor	+	
1952	Cadherin, EGF LAG seven pass G type receptor 2 (flamingo homolog, Drosophila)	+	
3693	Integrin, beta 5	+	
3728	Junction plakoglobin	+	
4240	Milk fat globule EGF factor 8 protein	+	
4753	NEL-like 2 (chicken)	+	
4973	Oxidised low density lipoprotein (lectin-like) receptor 1	+	
10100	Tetraspanin 2	+	
10516	Fbulin 5	+	
50509	Collagen, type V, alpha 3	+	
130271	Pleckstrin homology domain containing, family H (with MyTH4 domain) member 2	+	
3730	Kallmann syndrome 1 sequence	+	+
3897	L1 cell adhesion molecule	+	+
7057	Thrombospondin 1	+	+
5376	Peripheral myelin protein 22		+
8522	Growth arrest specific 7		+
9241	Noggin		+
9723	Sema domain, immunoglobulin domain, short basic domain, secreted, (semaphorin) 3E		+
11075	Stathmin like 2		+
50861	Stathmin like 3		+
84612	Par 6 partitioning defective 6 homolog beta (*C. elegans*)		+

Entrez gene number	Description	Ontological term	
		Intracellular protein transport	Protein localization
Down-regulated genes			
5192	Peroxisome biogenesis factor 10	+	+
5824	Peroxisomal biogenesis factor 19	+	+
6747	Signal sequence receptor, gamma (translocon-associated protein gamma)	+	+
9590	A kinase (PRKA) anchor protein (gravin) 12	+	+
10254	Signal transducing adaptor molecule (SH3 domain and ITAM motif) 2	+	+
10802	SEC24 related gene family, member A (*S. cerevisiae*)	+	+
26985	Adaptor-related protein complex 3, mu 1 subunit	+	+
79716	Aminopeptidase like 1	+	+
89781	Hermansky Pudlak syndrome 4	+	+
130340	Adaptor-related protein complex 1, sigma 3 subunit	+	+
8934	RAB7, member RAS oncogene family like 1		+
51715	RAB23, member RAS oncogene family		+
54832	Vacuolar protein sorting 13 homolog C (*S. cerevisiae*)		+

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