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Msx1 Gene Overexpression Induces G1 Phase Cell Arrest in Human Ovarian Cancer Cell Line OVCAR3

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Recent evidence suggested an involvement of homeobox genes in tumorigenesis. Here we investigated whether one of homeobox-containing genes, Msx1, might be involved in the regulation of cell proliferation and cell cycle using Msx1 overexpressing human ovarian cancer cell line, OVCAR3. Overexpression of Msx1 in OVCAR3 cells inhibited cell proliferation by markedly increasing the length of the G1 phase of the cell cycle over control cells. Consistent with this result, dramatic suppression of cyclins D1, D3, E, cyclin-dependent kinase 4, c-Jun, and Rb was observed. Elevated expression of genes involved in the growth arrest and apoptosis (GADD153 and apoptotic cystein protease MCH4) and suppression of proliferation sociated protein gene (PAG) in Msx1-overexpressing cells by cDNA expression array analysis provide further evidence for a potential repressor function of Msx1 in cell cycle progression. © 2001 Academic Press

Key Words: **homeobox gene; Msx1 overexpression; growth arrest; human ovarian cancer cell; cell cycle; cDNA expression array.**

Homeobox (Hox) genes are a family of genes containing a common nucleotide Ôequence of 180 bp, which was first discovered in genes regulating *Drosophila* development. This motif codes for a 60-amino acid homeodomain that forms a helix-turn-helix structure and is in¦olved in DNA Ôequence-specific recognition. Homeodomain-containing proteins function as transcriptional regulator that control cellular(a)-27.9(proliferation)]TJT*[(and)-348.3(differentiation)-348.3(during)-348 ment. Thus, alteration of homeobox genes leads to the abnormal phenotype and cell growth (1, 2). Recent evidence suggested that homeobox genes were involved in malignant process (3). Especially, Hox gene expression was altered in various neoplasia including solid tumors (4, 5). Moreover various homeobox genes, HB24, Hoxb8, and HoxA1, exhibited a transforming potential in several murine cell lines. Namely, these homeobox genes are silent in normal cells but become active in transformed cells. (6, 7).

The Msx genes, *Drosophila msh*-like genes, has been identified in various species, including mouse, frog, chick, zebrafish, and human (8, 9). The mouse Msx1, previously known as Hox-7/7.1, was mapped on chromosome 5 and shared 94% similarity with human MSX1, especially 100% identity within the homeodomain (9). High levels of Msx1 gene expression were observed in regions of epithelial-mesenchymal interaction, such as the developing limb bud, heart, neural tube, and molar teeth (10). Previous studies demonstrated that altered expression of Msx1 is associated with a number of morphological differences and targeted gene disruption of Msx1 led to severe developmental abnormality (10). Msx1 deleted mice died at early developmental stage due to their defects of craninofacial structures (11). A point mutation and chromosomal deletion in Msx1 gene resulted in selective tooth agenesis and Wolf-Hirshhorn syndrome, respectively, in humans (12, 13). However, the function of Msx1 both in cell proliferation and cell cycle progression as well as the underlying molecular mechanisms by which Msx1 regulates the target gene expression are less well understood because its downstream target genes are unknown.

Ovarian carcinoma is the most lethal tumor of the female genital tract and has no diagnostic marker and effective treatment so far. Our previous studies demonstrated that the mRNA level of one of homeobox genes, Msx1, was decreased in human cervical tumors compared with that in normal cervix (14) suggesting a potential suppressor role of Msx1 in cervical tumors. As an initial step to explore the critical role of Msx1 during ovarian tumorigenesis, first, we have estab-

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lished stable human ovarian OVCAR3 cell lines, and, second, we investigated its role in the control of cell proliferation and cell cycle progression. Our results demonstrate, for the first time, that overexpression of Msx1 gene leads to the suppression of the cell proliferation as well as the induction of G1 cell cycle arrest in human ovarian cancer cells. Our results lead to the speculation that Msx1 is one of important repressors involved in cell proliferation and cell cycle progression in human ovarian cancer cells.

MATERIALS AND METHODS

Cell culture and transfection. Human ovary carcinoma cell line, OVCAR3, was maintained in RPMI 1640 supplemented with 10% FBS (fetal bovine serum), penicillin/streptomycin (100 units/ml), Fungizon (Life Technologies Inc.), and anti-PPLO (Life Technologies Inc.) at 37°C in a humidified 5% CO_2 incubator. For stable transfections, OVCAR3 cells were plated 24 h before transfection at 1×10^5 cells onto 60-mm dish. The cells were transfected with 5 μg of pCB6[1]/Msx1(1–297) cDNA using DOTAP transfection reagent according to the supplier's protocol (Boehringer Mannheim, Germany). pCB6[1] vector without Msx1 cDNA insert was used as a control vector. Forty-eight hours after transfection, transfected cells were split and maintained in the presence of 200 μg/ml of G418 in RPMI 1640 supplemented with 10% FBS (Gibco BRL). G418-resistant clones were selected and analyzed for the expression of Msx1 by Northern blot and Western blot analysis (15).

Growth curve and thymidine uptake. For determination of cell numbers, cells were seeded in a 6-well plate at a density of 1×10^4 cells/well and grown in RPMI 1640 containing 10% FBS. Viable cells were manually counted after trypan blue staining using a hemocytometer. Cell counting was performed for 7 days. Relative rates of DNA synthesis were assessed by determining [^{3}H] thymidine incorporation into trichloroacetic acid-precipitable material. Cells grown in 5 replicate wells in 24-well plate (1×10^4 cells/well) were pulsed for 4 h with [^{3}H] thymidine (5 μCi/ml), and the amount of [^{3}H] thymidine incorporated to the culture was measured by using Liquid Scintillation counter (Wallac Pharmacia).

Flow cytometric analysis. Synchronization of cells was performed by a method described previously (16). In brief, cells were seeded at 1×10^4 cells/well (24-well plate) one day before treatment. After aspirating the medium, the cells were washed with PBS thoroughly and then were exposed to RPMI 1640 containing 0.1% BSA for 48 h. After 48 h, the serum-deficient medium was replaced by a medium containing 10% FBS. Six hours later, hydroxyurea stock solution was added to each dish to reach a final concentration of 1.5 mM for 14 h. The media was replaced with medium containing 10% FBS, and then, cells were collected every 4 h and analyzed for DNA content by flow cytometry. Cells were trypsinized, fixed with 70% ethanol, and treated with 1U of DNase-free RNase (Sigma). The DNA content was measured by staining with propidium iodide (0.05 mg/ml). The stained cells were analyzed by FACS Vantage (Becton Dickinson, San Jose, CA). The percentage of cells in each phase of the cell cycle was calculated using the ModFit LT program.

Northern blot analysis. Total RNAs from cell lines were isolated by acidic guanidinum phenol-chloroform extraction method (17). Northern membrane with 10 μg of total RNA was hybridized for 24 h at 65°C with [α-^{32}P]dCTP-labeled probe prepared by Rediprime cDNA synthesis kit (Amersham Life Science). After hybridization, the membrane was washed under standard conditions and autoradiographed.

Western blot analysis. Whole cell extracts were prepared from subconfluent control and Msx1-overexpressing cell lines in the log phase of growth, respectively. After lysing in a buffer containing 1% SDS, 10 mM Tris–HCl (pH 7.0), 1 mM phenylmethylsulfonyl fluoride, and 10% glycerol, cell extracts were centrifuged at 10,000*g* for 30 min. Total protein concentration of the lysates was determined with a BIO-RAD protein assay kit. Total 20 μg of cell lysates were separated on 8 or 12% denaturing SDS–PAGE and transferred electrophoretically to an ECL membrane (Amersham). The blotted membrane was then blocked with 5% nonfat dry milk in 1× TBS for 1 h at room temperature (RT). Antibodies against cyclins A, B1, D1, D3, E, Rb, c-Myc, and CDK2 were purchased from Pharmingen (San Diego, CA) and antibodies against CDK4, *c*-Jun, *c*-Fos, and CDC25A were from Santa Cruz Biotechnology (Santa Cruz, CA). The unbounded antibody was washed with 0.1% TBS/Tween 20. Blots were incubated with HRP-conjugated secondary antibody (Zymed) at dilutions of 1:5000 to 1:10,000 for 1 h at room temperature. The protein bands were visualized using the ECL chemiluminescence kit (Amersham International PLC).

cDNA synthesis and hybridization. The ^{32}P-labled cDNA were synthesized with total RNA from the control and Msx1 overexpressing stable cells in the presence of [α-^{32}P]dCTP. Briefly, total RNAs (20 μg each) were denatured at 75°C for 10 min and cDNAs were synthesized by incubation at 37°C for 1 h in a master mix containing 3 μl of dNTP (500 μM, without dCTP), 5 μl of [α-^{32}P]dCTP (3000 Ci/mmol; Amersham Life Science, Cleveland, OH), 4 μl of 0.1 M $MgCl_2$, and 2 μl of SuperScript II reverse transcriptase (200 U/l, Gibco BRL) in 10× RT buffer (Gibco BRL). The reaction was terminated by heating for 5 min at 75°C, and applied to the spin column (Chroma Spin 200; Clontech Laboratories Inc., Palo Alto, CA) for purification of the sample. Membranes were prehybridized at 68°C for at least 2 h prior to probe addition. The labeled cDNAs were denatured by boiling for 5 min and then hybridized to Atlas human cDNA array blots (Clontech, Palo Alto, CA) in hybridization solution. Hybridization was performed at 68°C in a roller bottle overnight. After the first two washes with 2× SSC and 0.1% SDS at 68°C for 20 min, the membranes were subjected to a stringent wash with 0.1× SSC, 0.5% SDS, and 0.1 mM EDTA at 68°C. Membranes were then exposed to X-ray film (Hyperfilm, Amersham) for 1 or 3 days at −70°C.

RESULTS

Overexpression of Msx1 Decreases the Growth Rate by Increasing the Length of the G1 Phase of the Cell Cycle

We have established a number of human ovarian cancer OVCAR3 cell lines overexpressing Msx1. Stable transfectants were examined for Msx1 expression via Northern blot and Western blot analysis (Fig. 1A). To characterize the role of Msx1 on the cell growth and the cell cycle, first, growth rates of the Msx1-overexpressing cells were compared to those of control cells. As shown in Fig. 1B, dramatic differences were observed in the Msx1-overexpressing cell line when the growth rates of these two cell lines were compared. The control cells exhibited the immediate onset of cell proliferation that maintained throughout the entire 6-day time course. In contrast, the Msx1-overexpressing cells showed a very slow growth rate. When grown in complete medium with 10% FBS, the Msx1-overexpressing cells displayed 2-fold increase in doubling time over controls (24 vs 48 h). Second, we measured the relative rate of DNA synthesis by [^{3}H] thymidine incorporation in these cell lines (Fig. 1C). A

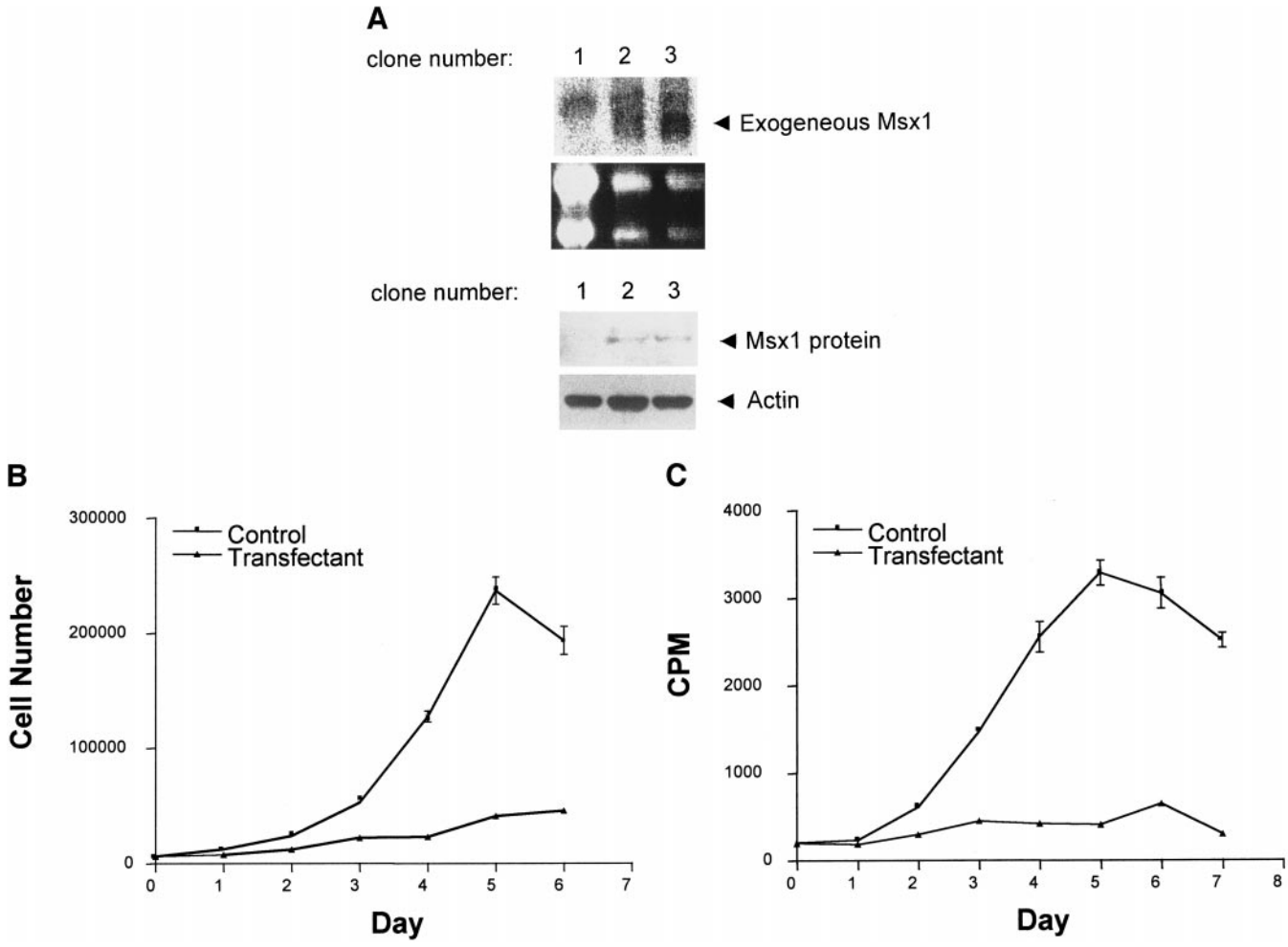

FIG. 1. Comparison of cell proliferation and Thymidine incorporation for control and Msx1 transfectant cells. (A) Northern blot (upper) and Western blot (lower) analysis of Msx1 transfectant and control cells. Clones were transfected with the pCB6[1]/Msx1(1–297) cDNA. EtBr staining was used as a loading control in Northern blot analysis. Western blot analysis for transfectant, anti-Myc (Invitrogen) monoclonal antibody was used. As described in reference, pCB6[1]/Msx1(1–297) plasmid contain sequences encoding a heterologous epitope from the c-Myc protein which is recognized by an anti-Myc monoclonal antibody. (B) Growth curve of control and Msx1-overexpressing cells. Cells were seeded at 1 3 10^4 cell/well of 24-well plate in six replicate wells in RPMI 1640 with 10% FBS and antibiotics. Every 24 h, viable cell counts were made using hemocytometer. (C) Comparison of cell proliferation between control and Msx1-transfectants. Control and Msx1 transfectants (1 3 10^4/well) were incubated in 24-well plates. 5 *m*Ci of [methyl-^{3}H] thymidine was added to each well for 4 h and tritium incorporation was measured by using a Backman LS 6000 SC counter.

dramatic decrease in DNA synthesis rate was also observed in the Msx1-overexpressing cells compared with control cells. The highest level of DNA synthesis was observed at the fifth day in the control cells in consistent with the data obtained from the growth rate studies. Third, we investigate the effect of Msx1 overexpression on cell cycle progression by flow cytometric analysis with synchronously growing cell cultures (Fig. 2A). Overexpression of Msx1 decreased the growth rate by increasing the length of the G1 phase compared with control (Fig. 2B). These results demonstrated that overexpression of Msx1 in human ovarian cancer cell line resulted in the growth inhibition due to the lengthened G1 phase of the cell cycle.

Overexpression of Msx1 Is Associated with Decreased Expression of the Cyclins D1, D3, E, CDK4, Rb, and c-Jun Proteins

To understand the mode of cell cycle arrest in Msx1-overexpressing cells, total cellular extracts were analyzed for the expression of the cell cycle regulators by Western blot assays. We found that the Msx1-overexpressing cells expressed relatively low levels of the cyclins D1, D3, and E, CDK4, Rb, and *c*-Jun proteins. These results are consistent with previous findings showing the association of these genes with G1 phase cell cycle arrest (18). Cyclins D1 and D3, and CDK4 levels were decreased by 5-fold in Msx1-

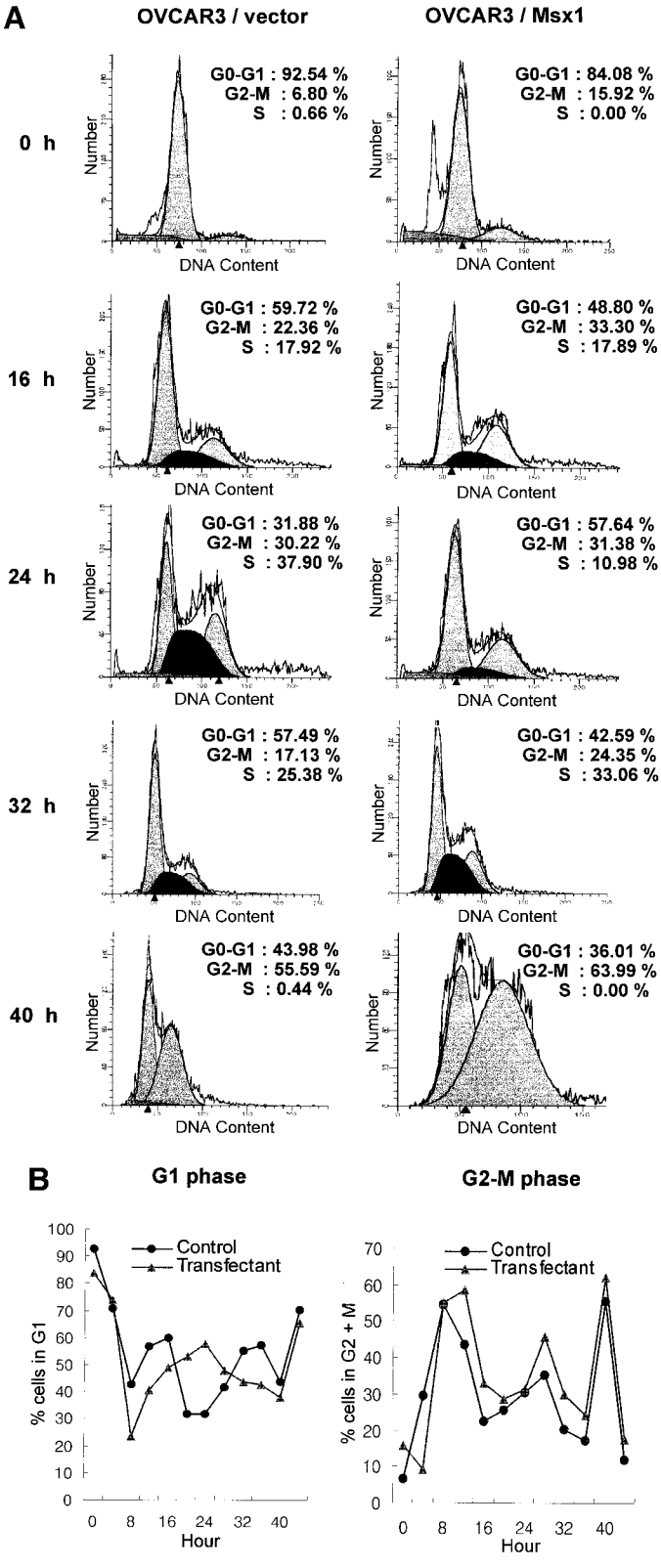

FIG. 2. Cell cycle analysis by flow cytometry of the effect of Msx1 on ovarian cancer cell line OVCAR3. (A) Distribution of the cells in the cell cycle. Cells were synchronized and replaced by a medium containing 10% FBS. Every 4 h, cells were collected, fixed, and stained with propidium iodide. DNA contents were analyzed by flow cytometry. The percentage of cells within the G1, S, and G2-M phase of the cell cycle was determined as described under Materials and Methods. (B) Graphic representation of the percentage of cells in either G1 or G2-M phase at every 4 h for 48 h. Note that Msx1-overexpressing cells progress through the G1 phase at slower rate than control cells.

overexpressing cells compared to those in control cells. There was no change or only a slight decrease (, 1.5-fold) in the levels of the following proteins; cyclins A and B1, CDK2, CDC25A, *c*-Fos, and *c*-Myc. Other regulatory proteins such as p16 and p27 were not also changed in these cells (data not shown). The most salient feature was the expression pattern of cyclin E protein. We found more than 20-fold decrease in the level of cyclin E protein in Msx1-overexpressing cells compared to that of controls. Most of the Rb proteins were hyperphosphorylated in the control cells (note the more slowly migrating phosphorylated Rb band in Fig. 3). The Msx1-overexpressing cells also displayed a marked decrease (9-fold) in the level of the *c*-Jun protein in Msx1-overexpressing cells when compared to the control cells. In contrast, protein levels of *c*-Myc and *c*-Fos showed no change. Taken together, overexpression of Msx1 gene resulted in the increase in the cell cycle regulators involved in the G1 arrest of the cell cycle.

Complementary DNA Expression Array Reveals the Differential Expression of Growth Arrest and Cell Proliferation Associated Genes in Msx1 Overexpressing Cells

To identify target genes for Msx1, total RNAs were isolated from control and Msx1 overexpressing OVCAR3 cells and used to examine the expression patterns of 588 known genes using the Atlas cDNA Expression Array. Each gene is represented by two parallel dots in order to differentiate specific hybridization signal from non-specific background signal (Fig. 4). The hybridization results were summarized in Table 1. Genes with expression levels that were altered more than . 2-fold between two cell lines were included (Table 1). Nine genes were differentially expressed in our analyses. Genes that showed increased expression in Msx1-overexpressing cells are growth arrest and DNA-damage-inducible protein GADD153, 40S ribosomal protein S19, apoptotic cysteine protease MCH4, and Guanine Nucleotide-binding protein G-S (alpha subunit). Among them, the GADD 153 gene expression pattern was the most prominent in that there was no signal at all in control cells even after 3 days of exposure to X-ray film. On the other hand, expression of PAG gene implicated in cell proliferation was suppressed in Msx1 overexpressing cells.

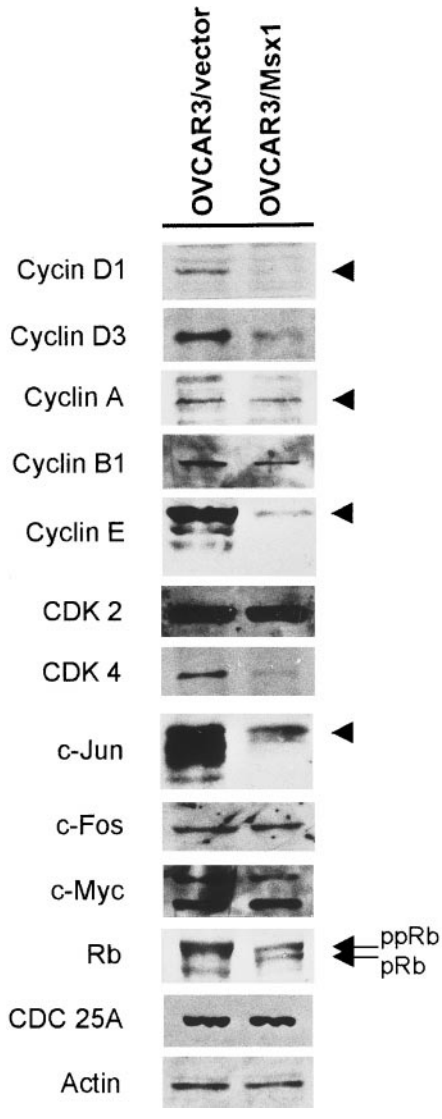

FIG. 3. Expression of cyclins, Cdks, and Cdk inhibitors in control and Msx1-overexpressing cells. Subconfluent control and Msx1-overexpressing cell lines in the log phase of growth were used to examine the expression of the proteins shown in figures. Total 20 *m*g of whole cell lysates were run on 8 or 12% SDS–polyacrylamide gel and were subjected to Western blot analysis with antibody to the specified protein. The arrow indicates the respective protein with the following molecular weight: cyclin D1, 36 kDa; cyclin D3, 34 kDa; cyclin A, 60 kDa; cyclin B1, 62 kDa; cyclin E, 50 kDa; CDC2, 34kDa; CDC4, 100kDa; *c*-Jun, 39 kDa; c-Fos, 62 kDa; c-Myc, 62 kD; Rb, 116 kDa; CDC25A, 70kDa; Actin, 43 kDa.

DISCUSSION

In this study, we have investigated the role of Msx1 in cell proliferation and cell cycle progression using human ovarian cancer cell lines. Overexpression of Msx1 gene inhibited cell proliferation by increasing the cell doubling time. The growth rate of OVCAR3 ovarian stable cell clones showed a strong correlation to the level of Msx1 expression (data not shown). The same results were obtained with other ovarian cancer cell lines, PA-1, and SKOV3 (data not shown).

Overexpression of some homeobox genes was shown to increase in cell growth and tumorigenicity either due to their interaction with deregulated target genes or due to deregulation of its function. Some murine homeobox genes were able to transform murine cells both *in vivo* and *in vitro* (6). In humans, some divergent homeobox genes are involved in chromosomal translocations or their expression associ-

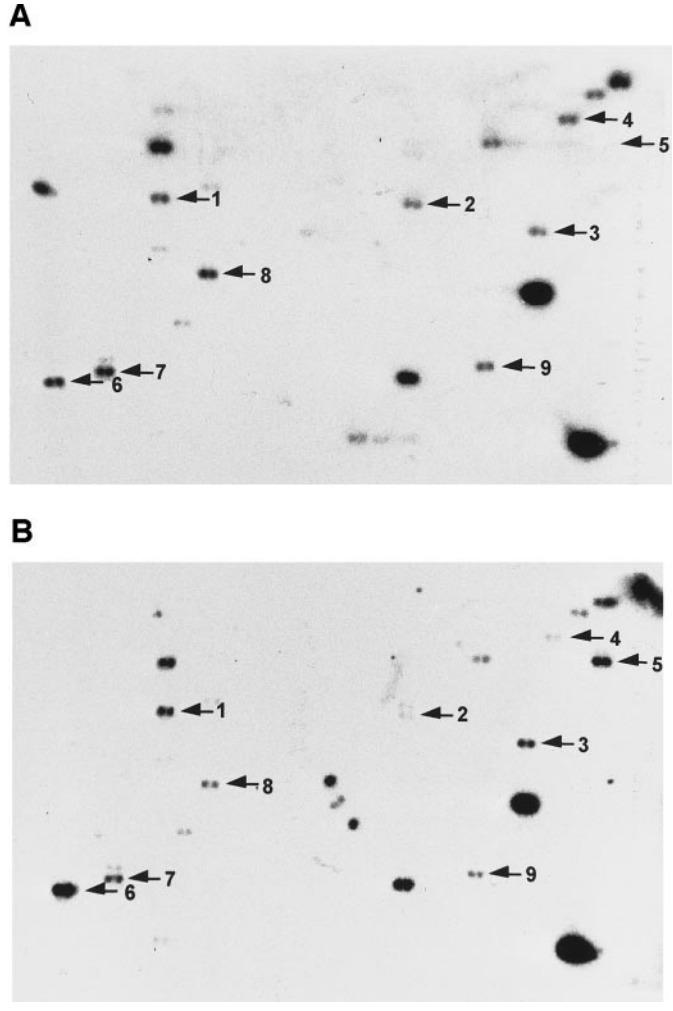

FIG. 4. Gene expression profiles of control and Msx1-overexpressing cell lines. Cells were cultured to a confluence of 60–70%, then total RNAs were isolated and used for gene expression analyses in a cDNA array assay. ^{32}P-labled cDNA was synthesized by RT of total RNAs isolated from control (A) and Msx1-overexpressing cell lines (B) and hybridized to the Atlas cDNA array blot. The blots were exposed to X-ray films for 3 days and autoradiographed. Examples of differentially expressed genes are indicated: 1, 40S ribosomal protein S19; 2, heat shock 27-kDa; 3, apoptotic cystein protease MCH4; 4, HDLC1 (cytoplasmic dynein light chain1); 5, growth arrest and DNA-damage-inducible protein GADD153; 6, guanine nucleotide-binding protein G-S (alpha subunit); 7, 60S ribosomal protein L6 (DNA-binding protein TAX); 8, proliferation-associated protein PAG; 9, Rantes protein T-cell specific.

TABLE 1

Summary of Differentially Expressed Genes in Control and Msx1-Overexpressing Cell Lines

Position[a]	Name of gene	Case[b]	
		Control	Transfectant
1	40S Ribosomal protein S19		,
2	Heat shock 27-kDa protein 1		.
3	Apoptotic cystein protease MCH4		,
4	HDLC1 (cytoplasmic dynein light chain 1)		.
5	Growth arrest and DNA-Damage-Inducible protein GADD153		,
6	Guanine Nucleotide-binding protein G-S (alpha subunit)		,
7	60S Ribosomal protein L6 (DNA-binding protein TAX)		.
8	Proliferation-Associated protein PAG		.
9	Rantes protine T-cell specific		.

[a] A gene list is available at Clontech's world wide web site (http://www.clontech.com/clontech/APR97UPD/Atlasist.html).
[b] . , , twofold or more increased/decreased.

ated with different leukemias (19, 20). Previous reports demonstrated that major differences in HOX gene expression were observed between renal carcinomas and normal human kidney. For example, the HOXB5 and HOXB9 gene expressions were turned off whereas HOXC11gene was turned on in primary kidney tumors (reviewed in Ref. 3).

We presented evidences that overexpression of Msx1 induced the growth arrest in the G1 phase of the cell cycle in human ovarian cancer line OVCAR3. Although the precise biological function of Msx1 is still unclear at present, our findings strongly suggest a potential role of the Msx1 as a repressor in the cell cycle regulation. We found a significant increase in cell doubling time and the blocking at G1 phase of the cell cycle in Msx1-overexpressing cells. As shown above, overexpression of Msx1 decreased the proliferation rate of cells by increasing the length of the G1 phase without a reciprocal increase in the S and G2-M phase. Growth inhibition by Msx1 implied a potential role for Msx1 in cell cycle regulation. Our Western blot analysis of the cell cycle regulators is consistent with the previous reports showing the dramatic decrease in the expression of the cyclins D1, D3, E, CDK4, and *c*-Jun upon induction of G1 phase arrest. During passage through G1 phase, cyclin D1-associated kinase progressively phosphorylates pRB to activate E2F activity. This induces cyclin E expression that ensures a sustained phosphorylation and inactivation of pRB during the remaining period of G1 phase through a positive autoregulatory loop between pRB phosphorylation and cyclin E expression (21, 22). Previous studies in cancer cell lines and tumor specimens demonstrated that overexpression of cyclin D1 was necessary for malignancy and cyclin E might be directly involved in the process of tumor formation (23–25). Both *c-jun* and *jun*-B are 'immediate early genes' that are associated with cell proliferation and transformation in various cell types including ovarian cancer cell lines (26, 27). Expression pattern of *c-jun* and *jun*-B in renal cell cancer tissues and cell lines, and normal kidneys suggested that *c-jun* might have a role in inducing malignant transformation (28). Increased *c-jun* expression was shown to be associated with the invasive stage in colorectal tumor formation as well as with a higher response rate to chemotherapy and also with an accelerated acquisition of drug resistance in ovarian cancer (29). In breast cancer cells, the promoter of cyclin was shown to be activated by c-Jun (30). Therefore, blocking of G1 phase progression in Msx1-overexpressing cells might be due to the decreased expression of cyclins D1 and D3, and CDK4, which might subsequently induce an inactivation of pRB followed by decrease in cyclin E expression.

Using a cDNA expression array, we compared the gene expression patterns of control and Msx1-overexpressing cell lines. Human cDNA expression array is based on reverse Northern blot hybridization and simultaneously analyzed the expression pattern of 588 cellular genes that were immobilized in duplicate onto a nylon membrane (14). These 588 genes belong to six functional genes, including (a) oncogenes, tumor suppressor genes, and cell cycle regulators; (b) stress repressor genes, ion channels and transport genes, and intracellular signal transduction modulators and effectors; (c) apoptosis-related genes, and genes involved in DNA synthesis, DNA repair, and DNA recombination; (d) transcriptional factors and general DNA-binding proteins; (e) receptors, cell-surface antigens and cell adhesion; (f) cell-cell communication.

Genes showing differential expression patterns between Msx1-overexpressing cells and control cells belong to the group of genes implicated in growth arrest and proliferation. Among the genes involved in growth arrest and DNA damage inducing genes, expression of the GADD153 gene was markedly elevated in Msx1-overexpressing cells. The GADD153 gene was reported to induce the growth arrest by blocking the cells from

progression from G1 to S phase and to be involved in apoptosis pathway in a p53-independent fashion (31, 32). In contrast, the proliferation-associated protein, PAG was decreased in Msx1-overexpressing cells. Although the biological significance of these findings needs further study, our results suggest that altered expression of these genes may be related to the growth arrest of Msx1-overexpressing cells. The targets of Msx1 protein are not known. But it has been postulated that they include genes encoding extracellular matrix proteins, adhesion molecules, and growth factors, families of genes important for development as well as tumorigenesis and metastasis (33). Our findings suggest that cell cycle regulator genes are potential targets of Msx1. Further investigation will be necessary to identify the Msx1-downstream target genes and its regulatory mechanisms.

In conclusion, our study demonstrated that overexpression of the homeobox gene, Msx1, suppressed cell growth and cell cycle progression in human ovarian cancer cell line, OVCAR3, by regulating the expression of key cell cycle regulators. To our knowledge, this is the first report demonstrating that Msx1 might be a repressor in cell proliferation and cell cycle progression in human ovarian cancer cells.

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