

A Direct Mechanistic Link between Growth Control and a Tumor Cell Immune Function: Increased Interleukin-8 Secretion Accounts for Elimination of Oct-1 Antisense Transformants from *Scid* Mice

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Abstract. *Background:* Tumorigenesis involves the aberrant function of proteins that regulate growth control, including Oct-1. Oct-1 is a DNA binding transcription factor that activates genes that encode proteins required for S-phase and cell growth. For example, Oct-1 activates the histone H2B promoter and the promoters for the snRNPs. Oct-1 also represses certain promoters, including promoters of immune function genes, such as the IL-8 and the HLA-DRA genes. *Materials, Methods and Results:* Oct-1 antisense transformants were determined to have reduced growth rates and other characteristics of growth control. Also, Oct-1 antisense transformants endured for a shorter time in scid mice, being attributable to the increased expression of IL-8 by the Oct-1 antisense transformants. *Conclusion:* These results may help resolve the conundrum of why growth control de-regulation alone is not enough for tumorigenicity. The results also support the conclusion that the molecular mechanisms of growth control de-regulation and tumor cell immune functions are directly linked.

Oct-1 is a ubiquitously expressed transcription factor that binds to an eight-nucleotide DNA sequence (ATTTGCAT). Oct-1 DNA binding activity is regulated by serine and threonine phosphorylation. The highly phosphorylated form of Oct-1 does not bind DNA. DNA binding occurs following a reduction in the level of phosphorylation, but it is not known

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which phosphorylation sites must be dephosphorylated for promoter binding to occur. Oct-1 is a member of a family of transcription factors containing a POU domain, a 155 to 162 amino acid region which consists of a bipartite DNA-binding domain, containing an N-terminal POU sub-domain and a C-terminal POUHD homeodomain (4, 14, 15).

Oct-1 has been implicated in oncogenesis because a truncated form of Oct-1 facilitates tumor formation in mice (12) and because the molecular functions of Oct-1 are consistent with rapid growth. Oct-1 activates the H2B and snRNP promoters (3, 6), representing proteins required for S-phase and rapid cell growth. Oct-1 also plays a role in DNA synthesis in adenoviral replication (2). Upon infection with an adenovirus, the binding activity of Oct-1 is stimulated. This most likely occurs due to a decrease in phosphorylation of Oct-1 resulting in an increase in the level of the active protein. Oct-1 DNA binding sites are found in the viral terminal repeat sequences which are important for the initiation of DNA replication.

While the function of Oct-1 is consistent with de-regulated growth control, it remains to be determined how Oct-1 facilitates tumor formation in animals. More recently, it has become apparent that tumor formation can be dependent on tumor cell immune functions. For example, the transformation of retinoblastoma protein (Rb) defective cells with an Rb-expression vector leads to an increase in interleukin-8 (IL-8) secretion and this IL-8 plays a role in eradicating Rb-transformed tumor cells from the mouse (17, 18). Rb transformation leads to increased Oct-1 phosphorylation, consistent with its role in reducing cell growth rates. Oct-1 binding to the IL-8 promoter inhibits IL-8 mRNA transcription (16), thus explaining, in part, the increased IL-8 expression attributable to the reconstitution of tumor cells with Rb. There are numerous other tumor cell immune functions that are attributable to de-regulated

growth control (1). However, only a few, if any cases represent a direct connection between the molecular mechanism of de-regulated growth control and the molecular mechanisms of tumor cell immune functions. To further elucidate such connections in general, and to further elucidate the connection between Rb expression and IL-8 expression, we established Oct-1 knockdown (antisense) transformants, which have a reduced transformed phenotype, according to *in vitro* parameters; express an increased level of IL-8 mRNA and protein; and dissipate rapidly when injected into scid mice. Furthermore, the duration of the Oct-1 knockdown tumor cells in mice can be extended by treating the mice with anti-IL-8 blocking antibody, establishing a direct mechanistic linkage between Oct-1 function and IL-8 function in tumor formation.

Materials and Methods

Construction of the pAS-Oct-1 plasmid harboring the anti-sense Oct-1 cDNA. The full-length 2.3 kb Oct-1 cDNA was excised from the pCG-Oct-1 vector (generous gift of Winship Herr) by BamHI/XbaI digestion. The Oct-1 cDNA fragment was gel purified (Qiagen). BamHI/XbaI digested pcDNA3 was ligated to the Oct-1 cDNA in a reaction containing 1.0 μ l of 10X ligase buffer, 1.0 μ l of T4 DNA ligase and 3.8 μ l of water. The ligation reaction was incubated at room temperature for two hours and used to transform bacteria.

Transformation of 5637 cells. The human bladder carcinoma cell line, 5637 (ATCC HTB9), was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U penicillin-streptomycin per milliliter, 3 mM L-glutamine and 1 mM sodium pyruvate. The cells were maintained at 37°C and 5% CO₂ in a humidified tissue culture incubator. One million 5637 cells were plated on 100-mm plates 24 hours prior to DNA transfection. One plate was transfected with pAS-Oct-1, the second plate was transfected with the pcDNA3 empty vector control, and the third plate was used for a mock transfection. The indicated amounts of plasmid DNA were transfected as per the manufacturer's instructions using the cationic lipid reagent TransIT-LT1 (PanVera, Madison, WI, USA) at a ratio of 2 μ l TransIT-LT1 per 1 mg of DNA transfected. The transfection was performed in 3 mL of complete media per plate. The cells were incubated for six hours. The serum-free media was replaced with 10 mL of supplemented RPMI 1640 media and left overnight. The plates were subsequently split into serial dilutions the following day. The Oct-1 antisense and control DNA cells were plated at four dilutions: 1:100, 1:1000, 1:10,000 and 1:100,000. The antibiotic Geneticin (G418) was added to each plate at a concentration of 600 μ g/mL. Individual colonies from the Oct-1 antisense and control DNA dilution plates were selected for expansion using sterile cloning rings. Twenty microliters of trypsin (2.5 mg/mL) was added to the rings to remove the cells from the tissue culture plate. Five antisense and two control colonies were expanded into 96-well tissue culture plates and subsequently split into larger volume flasks.

Oct-1 immunohistochemistry. Oct-1 antisense cells or control DNA cells were plated on a 25x75 mm tissue culture chamber slide containing 1.3x10⁵ cells in 2 ml of G418-containing media. The

slides were incubated overnight, after which the media and chamber were removed. The slides were fixed with 4% paraformaldehyde solution for 30 minutes and incubated with the anti-Oct-1 antibody (Santa Cruz).

Measurement of a growth curve for Oct-1 antisense and control transformants. Cells were plated at 100 cells per well in 24-well tissue culture plates. Each well was supplemented with 1 ml of media. Cell growth was counted on days four, eight, ten, twelve, and fifteen. Counts were obtained by removing the media, rinsing thoroughly twice with 1 ml of 1X PBS, and trypsinizing in 1 ml for 30 minutes. The cells were agitated and counted using a Z Series Coulter Counter.

Colony formation in low serum. Twenty-four hours after plating, the media containing 10% serum was removed, and the cells were rinsed once with 1X PBS. Three milliliters of media containing 0.2% serum was added to each well. The cells were incubated for 14 days to allow for colony formation, with one re-feeding of the 0.2% serum. After 14 days, the media was removed and each well stained with 2.5 ml of a crystal violet/ethanol solution for three minutes to stain and fix the cells. The wells were rinsed with water.

Scid mice tumor growth and antibody injections. The preparation of cells for injection into mice, antibody treatments of tumor-bearing mice, and measurements of tumor cell masses were all done exactly as previously described (18).

Results

Generation and characterization of Oct-1 antisense transformants. A series of stable Oct-1 antisense transformants were generated using the Oct-1 antisense expression vector indicated (Figure 1A) and a series of control transformants were generated using the empty vector. The Oct-1 antisense and control transformants were generated from 5637 cells, representing a bladder carcinoma. The 5637 cells are defective for the retinoblastoma tumor suppressor protein (Rb), and Rb-reconstitution of these cells increases Oct-1 phosphorylation, decreases Oct-1 DNA binding activity, and increases IL-8 secretion (17, 18).

The Oct-1 antisense transformants were assayed by Western analysis, which indicated reduced Oct-1 expression (10). The transformants were also examined by immunohistochemistry (Figure 1B) which, like the Western analysis, indicated that the Oct-1 expression was reduced in the Oct-1 antisense transformants.

To determine whether Oct-1 knockdown affected the canonical *in vitro* parameters of malignant transformation, the growth rates of Oct-1 antisense and control transformants were compared and indicated that knockdown of Oct-1 reduced cell growth rates (Figure 2). Oct-1 knockdown also reduced colony forming ability in low serum (Figure 3). These data indicated that Oct-1 plays a role in maintaining the malignant, transformed phenotype, consistent with one other report indicating that a truncated Oct-1 stimulated tumor development in transgenic mice (12).

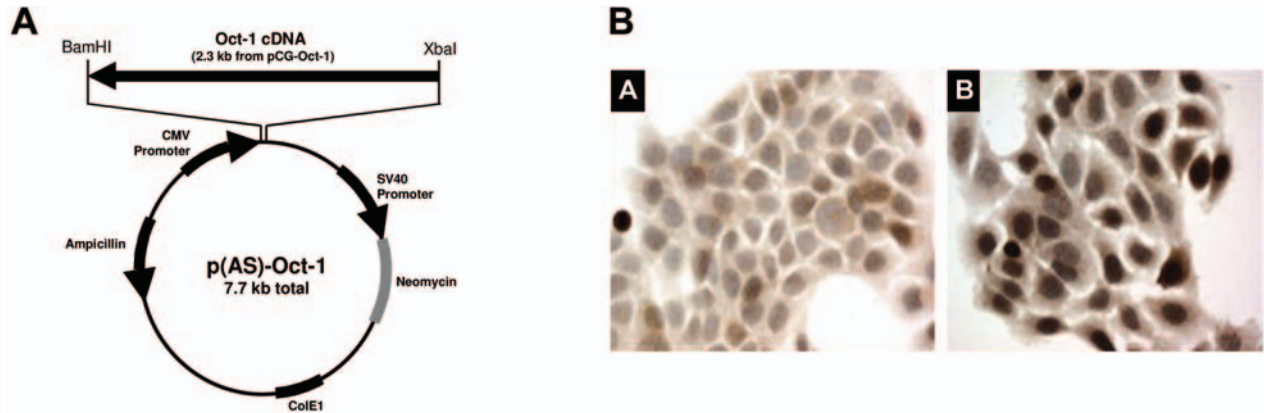


Figure 1. A) Map of the recombinant plasmid pAS-Oct-1 created from the ligation of pcDNA3 (5.4 kb) with full length, antisense Oct-1 cDNA (2.3 kb). B) Nuclear staining of Oct-1 antisense A1 (A) and control C1 (B) transformants with anti-Oct-1.

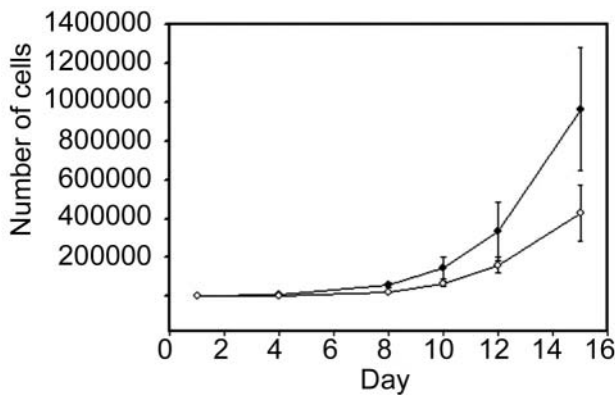
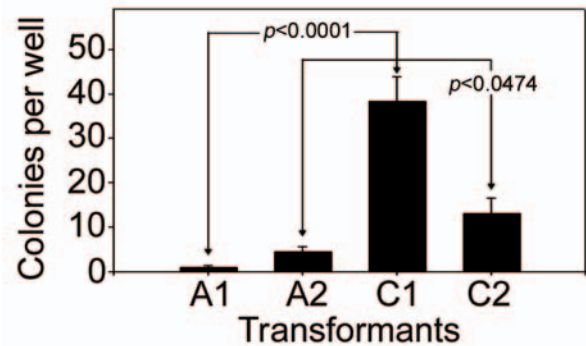


Figure 2. Growth rates for Oct-1 antisense transformant A1 (open circles) and control transformant C1 (closed circles). Similar data were obtained for a second set of Oct-1 antisense and control transformants. Combining these data for day fifteen resulted in a p-value of 0.0214.

Oct-1 knockdown leads to increased IL-8 secretion. Oct-1 has been shown previously to repress the IL-8 promoter in transient transfection experiments (7, 9, 17). To determine whether Oct-1 affects the levels of endogenous IL-8 mRNA, an RT-PCR analysis was performed on AS-1, which indicated that Oct-1 knockdown led to increased IL-8 mRNA (Figure 4), consistent with a role for Oct-1 in repressing the IL-8 promoter. Oct-1 knockdown cells also had an increased level of IL-8 secretion (Figure 5).

Oct-1 knockdown reduces the durability of tumor cells in scid mice. The Oct-1 antisense and control transformants were injected into scid mice exactly as described (18). The tumor size was measured every three days as previously described. The size of the tumor cell mass generated by the Oct-1 antisense transformants reduced more quickly than did the size of the tumor cell mass generated by injecting the



Colony growth in 0.2% serum

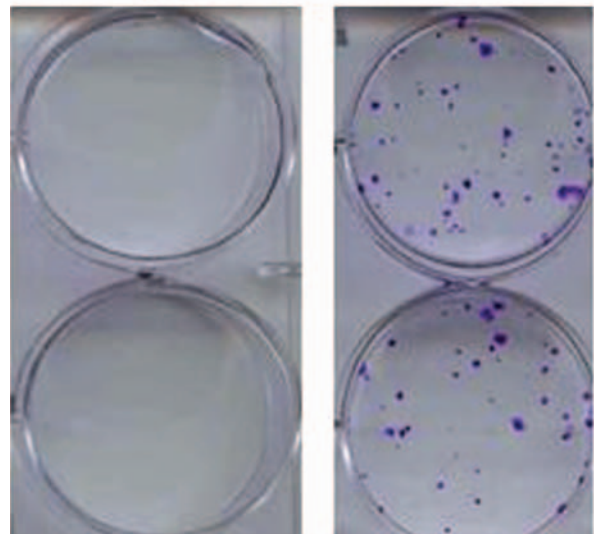


Figure 3. Crystal violet-stained colonies formed in 0.2% serum for two weeks and quantification and statistical analyses of the formation of the colonies by the Oct-1 antisense transformants (lower left panel) and the control transformants (lower right panel). Bar graphs represent independent Oct-1 antisense transformants A1, A2; and independent control transformants C1, C2.

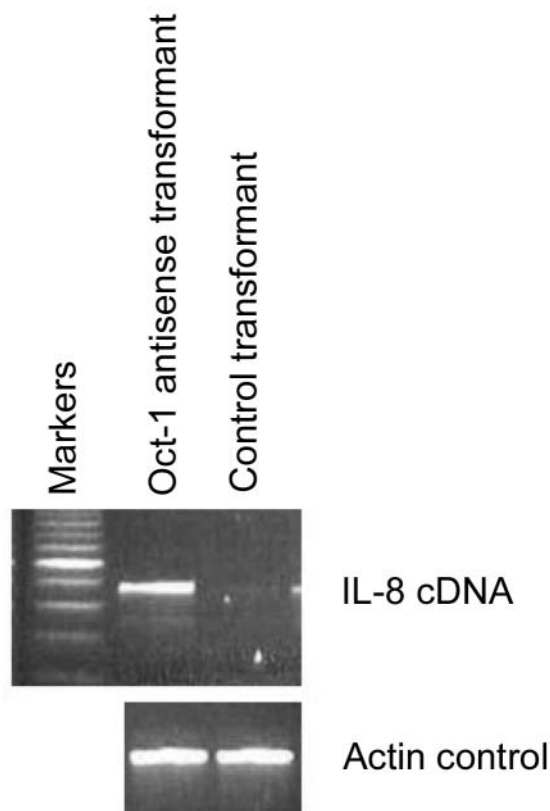


Figure 4. De-repression of Oct-1 mRNA expression in an Oct-1 antisense transformant. RNA was prepared from an Oct-1 antisense transformant and a control transformant and assayed by RT-PCR using standard techniques and using the following primers: agcttctaggacaagac (forward, first exon), ggataccacagagaatga (reverse, fourth exon). The RT-PCR product was detected by gel electrophoresis.

control transformants (Figure 6). The tumor size data were analyzed using both the ANOVA and the Wilcoxon Mann-Whitney tests for statistical significance, as indicated in detail in the figure legend.

Reduced duration of Oct-1 antisense transformants in scid mice is due to IL-8 secretion. We previously determined that Rb-transformants had a reduced durability in nude mice as a result of increased IL-8 secretion (18). When the mice receiving the Rb-transformants were treated with an anti-IL-8 antibody that neutralizes IL-8, the Rb-transformants survived significantly longer. Furthermore, this survival was accompanied by a sharp decrease in neutrophil infiltrates into the tumor mass, as in other reports where IL-8 mediated tumor cell destruction, for example ref. (5). To determine whether the reduced duration of the Oct-1 antisense cells in the scid mice was related to IL-8 secretion, the mice receiving Oct-1 antisense cells were treated with either anti-IL-8 or isotype control antibody, exactly as described (18). The

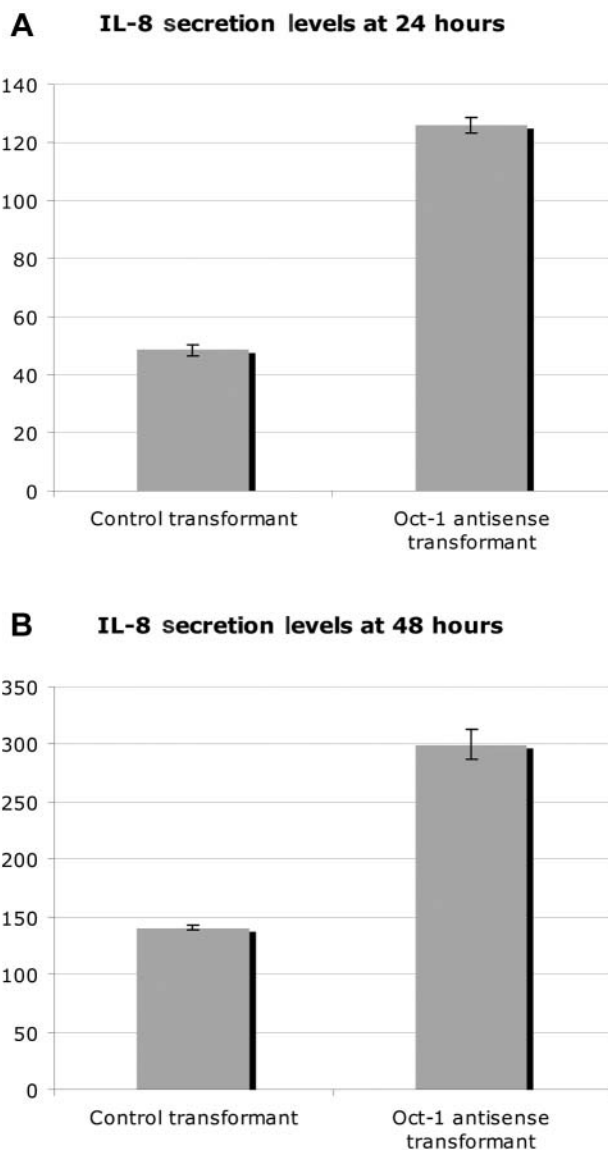


Figure 5. IL-8 secretion by Oct-1 antisense transformant cells and control transformant cells. Serum-free media was obtained after 24 or 48 hours (as indicated) and assayed by ELISA for IL-8 content (Cytokine Core Laboratory, Baltimore, MD, USA).

results indicated that IL-8 blockade, by treatment with the anti-IL-8 antibody, prolonged the survival of the Oct-1 antisense transformants in the scid mice (Figure 7).

Discussion

The molecular mechanisms of oncoprotein functions have mainly been studied because of their relationship to traditional parameters of growth control. However, cancer that kills a patient involves several aspects of tumor cell

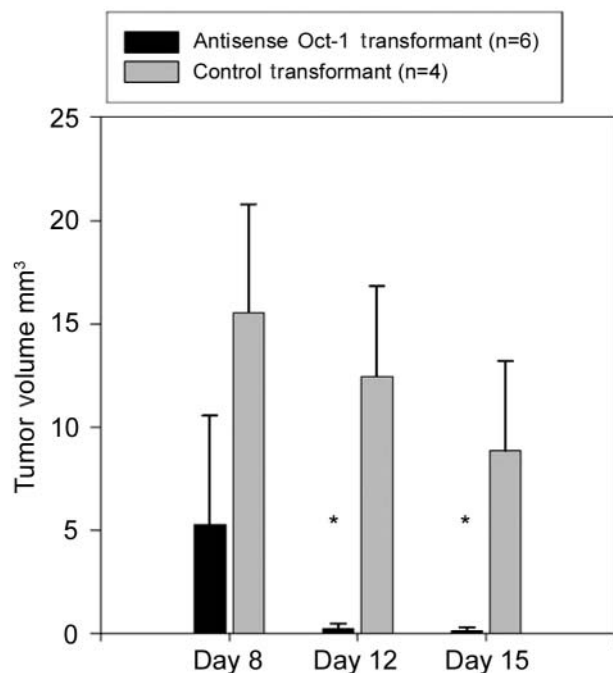


Figure 6. Duration of Oct-1 antisense transformant and control transformant tumors in scid mice. The indicated number of mice were inoculated with equal numbers of cells as previously described (18). Error bars represent standard error of the mean. The asterisks indicate statistical significance. A repeated ANOVA and non-parametric Wilcoxon Mann-Whitney rank-sum were used to determine statistically significant results in tumor volume changes ($p > 0.05$). P-values were 0.008 for day 12 for the ANOVA test and 0.005 for the Wilcoxon test; 0.035 for day 15 for the ANOVA test and 0.005 for the Wilcoxon test.

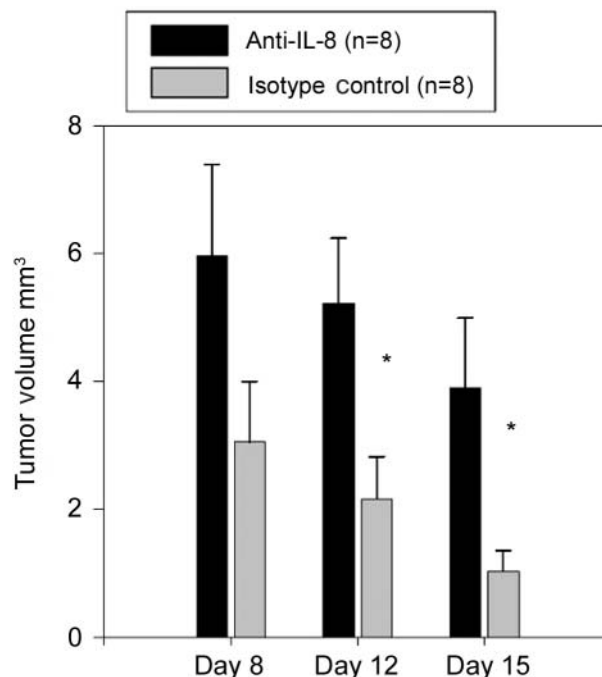


Figure 7. Duration of Oct-1 antisense transformant tumor in scid mice with and without anti-IL-8 treatment. The indicated numbers of mice were inoculated with equal numbers of cells and were injected with anti-IL-8 or isotype control antibody as previously described (18). Error bars represent standard error of the mean. The asterisks indicate statistical significance. A repeated ANOVA and non-parametric Wilcoxon Mann-Whitney rank-sum were used to determine statistically significant results in tumor volume changes ($p < 0.05$). P-values were 0.029 for day 12 for the ANOVA test and 0.034 for the Wilcoxon test; 0.034 for day 15 for the ANOVA test and 0.010 for the Wilcoxon test.

physiology beyond the regulation of cell division and growth, such as tissue invasion, angiogenesis and counteracting the antitumor immune response. An emerging issue is to what extent does an oncoprotein or tumor suppressor protein, as defined by parameters of growth control, also affect the other aspects of tumor cell physiology that facilitate cancer?

The above described experiments and data indicate: (i) that Oct-1 represses the endogenous IL-8 promoter, consistent with previously described experiments using a model system involving transient transfection and an IL-8 promoter reporter plasmid; and (ii) that the binding of Oct-1 to the IL-8 promoter mediates the repression of IL-8: (a) that is relieved by Rb expression and (b) that is partially responsible for the tumorigenicity of cells lacking the Rb tumor suppressor protein.

Oct-1 has been shown to function as an oncoprotein with regard to several parameters of growth control (Figures 2, 3 and ref. (12)), and several Oct-1 functions, namely the activation of promoters of genes important for cell division, are consistent with Oct-1's oncogenic properties.

Tumor cell IL-8 has previously been shown to recruit neutrophils to the site of tumor development and the presence of these neutrophils has been correlated with eradication of IL-8-secreting tumor cells (5, 18). Thus, the Oct-1 knockdown described here most probably leads to a more rapid eradication of the tumor cells because the knockdown of Oct-1 increases tumor cell secretion of IL-8, which in turn leads to neutrophil recruitment to the site of the tumor.

Oct-1 now joins a growing list of oncoproteins and tumor suppressor proteins defined by conventional parameters of growth control but shown to also direct functions related to other aspects of cancer development (1). For example, mutant ras (13) and STAT3 (8) have growth control functions and induce VEGF, important for tumor angiogenesis and for inhibiting dendritic cell function (11).

Acknowledgements

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