Abstract

Increasing evidence indicates that macrophages in tumor stroma can significantly modify the malignant phenotypes of tumors. Osteopontin (OPN) is frequently overexpressed in cancers with high metastatic capacity and, thus, has been considered as a potential therapeutic target. To find out whether macrophages can affect the outcome of OPN-knockdown tumor cells, we used RNA interference (RNAi) to stably silence the OPN expression in the highly invasive human hepatoma cell line SK-Hep-1. Silencing of OPN markedly decreased the motility and invasiveness of the SK-Hep-1 cells. Further studies using this cell model revealed that coculture with human macrophages or macrophage-conditioned medium largely restored the migration and invasion potential of OPN-knockdown tumor cells. Moreover, such macrophage-promoted motility can be effectively blocked either by the addition of OPN-neutralizing antibody to the cocultured medium or by silencing OPN expression in macrophages. These results indicate that macrophage-derived OPN can compensate for the decrease of OPN and thereby restore the metastatic potential of OPN-knockdown tumor cells. Further characterization of the underlying mechanisms disclosed that macrophage-derived OPN exerted its function independently of the actin cytoskeleton rearrangement or the activation of matrix metalloproteinase and Rho families. Our results suggest that there are fine-tuned complex interactions between cancer cells and stroma cells, which may modify the outcome of cancer therapy, and therefore should be considered for the rational design of anticancer strategy.

Introduction

Solid tumors comprise not only cancer cells but also many other nonmalignant stromal cells, which produce a unique microenvironment to modify the neoplastic properties of the tumor (1–4). Emerging evidence indicates that an abnormal tumor microenvironment contributes to tumor formation and progression, whereas normalizing of the stromal environment is able to slow or even to reverse tumor progression (5, 6). For example, recent studies have shown that the outcome of primary oncogenic events in epithelial cells can be significantly modified by the nature of the surrounding nonmalignant cells (7, 8), indicating that these stromal cells can serve as novel intervention targets.

The macrophage is a pivotal member within tumor microenvironment. Upon activation, macrophages can release a vast diversity of cytokines, proteolytic enzymes, growth factors, and inflammatory mediators that may directly influence the behavior of tumor cells (9–11). Although the activated macrophages may have antitumor activity under certain circumstances, increased macrophage density in tumor stroma has been shown to strongly correlate with poor prognosis in different types of solid tumors (11–13). Moreover, recent studies have revealed that macrophages can enhance the invasiveness of cancer cells both in vitro (11, 14, 15) and in vivo (16, 17). Despite the undoubted relevance of macrophages to the regulation of tumor progression, the underlying mechanisms by which macrophages modulate the metastatic capacity of cancer cells are still largely unknown.

Osteopontin (OPN) is a secreted glycoprotein that is widely expressed in various kinds of cells, including osteoclasts, osteoblasts, epithelial cells, endothelial cells, and activated immune cells such as T cells and macrophages (18–20). It is involved in normal tissue-remodeling processes as well as certain diseases such as tumorigenesis (20–22). OPN has been shown to enhance cell proliferation and promote migration and invasion by interacting with its receptors (22–25). Furthermore, overexpression of OPN has been detected in different types of tumor tissues, especially those with high metastatic capacity (26–31). Therefore, OPN has been considered as a potential therapeutic target in cancer therapy.

Considering the importance of stromal cells in promoting tumor progression, treatment only targeting the malignant cells would not be efficient to prevent metastasis. However, there has been an almost exclusive experimental focus on targeting the malignant cells, whereas the influence of the stromal cells on the outcome of treated tumor cells has thus far been largely ignored. To investigate the effect of macrophages on the outcome of tumor cells whose metastatic potential has been inhibited, we established a stable OPN-knockdown cell line that showed a marked decrease in motility and invasiveness. Further studies using this cell model revealed that macrophage-derived OPN can compensate for the decrease of OPN and thereby restore the metastatic potential of OPN-knockdown tumor cells. These findings provide important new insight into the significance of cancer-stroma cell interactions in influencing the outcome of cancer therapy, which should be helpful for the rational design of anticancer strategy.

Materials and Methods

Reagents. Antibodies and reagents were purchased as follows: Matrigel, OPN-neutralizing antibody (Ab; AF1433) and the isotype matched control (AB-108-C, R&D Systems); anti-Rac1 monoclonal Ab (mAb; Upstate Biotechnology); anti-RhoA mAb (26C4, Santa Cruz Biotechnology); Stealth small interfering RNA (siRNA), LipofectAMINE 2000, Opti-MEM, G418.
TRIzol, Alexa Fluor 532 phalloidin (Invitrogen); DMEM (HyClone); fetal bovine serum (FBS; PAA); and Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega). All other reagents were obtained from Sigma-Aldrich unless otherwise indicated in the text.

**Construction of siRNA expression plasmids.** The plasmid pSi-Vector (Fig. 1A), which contains a U6 RNA promoter and an enhanced green fluorescent protein (EGFP) expression cassette under the control of cytomegalovirus (CMV) promoter, was constructed as follows based on plasmid pcDNA3.0 (Invitrogen): EGFP was generated by PCR from pEGFP-C1 (Clontech) using the primers 5'-GATGGTGCATACAAAGC CCTTCTGACGTCCGACC and 5'-GATGGATCATCCCTGGTGACGACCC. The amplified EGFP fragment was then inserted into the KpnI and BamHI sites of pcDNA3.0. The human U6 promoter was cloned from human genome by PCR and inserted between the BamHI and EcoRI sites of pcDNA3.0. The siRNA sequences targeted human OPN transcript (GeneBank accession no. NM_000582) were designed using the software developed by Invitrogen, Inc. Two candidate sequences in human OPN gene, classified as OPN-1 (5'-CGCTCTAGATGATGTAATTGAG) and OPN-2 (5'-GCGAGGTGTGAAATGTGGCATACAA), were selected for RNA interference (RNAi). These sequences showed no homology with other known human genes. The antisense siRNA sequences, the loop sequence (5'-TCTCAAGACA) and the flanking EcoRI and XbaI sites were synthesized chemically (Fig. 1B). Two complementary oligonucleotides were then annealed and ligated into the linearized pSi-Vector. Each construct was sequenced to confirm the right sequence of insert. The construct containing Si-OPN-1 sequence was designated as pSi-OPN-1, and the construct with Si-OPN-2 sequence was designated as pSi-OPN-2. The plasmid with scrambled Si-OPN-NC sequence was named as pSi-OPN-NC.

**Establishment of stable OPN-knockdown tumor cells by vector-based RNAi system.** SK-Hep-1, a highly invasive human hepatoma cell line, was grown as monolayer in DMEM supplemented with 10% FBS and 1% nonadherent cells were washed out with warm Hanks solution, and the derived monocytes were cultured in DMEM containing 10% human AB serum. The medium was changed every 3 days, and the derived macrophages were used to 8 days after culture.

**Preparation of human monocye-derived macrophages.** The macrophages were prepared as follows: Human mononuclear cells were isolated from peripheral blood of healthy donors by Ficoll density gradient centrifugation at 450 × g for 30 min at room temperature. The mononuclear cells were washed thrice with PBS and plated at 5 × 10^5 per well in 24-well plates for 1 h in DMEM alone. Thereafter, the nonadherent cells were washed out with warm Hank’s solution, and the adherent monocytes were cultured in DMEM containing 10% human AB serum. The medium was changed every 3 days, and the derived macrophages were used to 5 days after culture.

**Transient transfection of human macrophages with OPN-siRNA.** The Stealth siRNA used is 25-bp duplex oligoribonucleotides with sequences corresponding to the sense and antisense strands of Si-OPN-1, Si-OPN-2, and Si-OPN-NC (Fig. 1B), respectively. Equal amounts of Si-OPN-1 and Si-OPN-2 were mixed and diluted in Opti-MEM to a final concentration of 100 or 400 nmol/L and transiently transfected into the macrophages using LipofectAMINE 2000 according to the manufacturer's protocol. As controls, the macrophages were transfected with 400 nmol/L of Si-OPN-NC.

**Analysis of OPN mRNA level by reverse transcription-PCR.** Total RNA was isolated using the TRIzol reagent. Aliquots of 2 μg total RNA was transcribed reversely using MMLV reverse transcriptase. The specific primers used to amplify the OPN gene and the housekeeping genes human porphobilinogen deaminase (HPBGD) and β-actin (Fig. 1C) were as follows: OPN, 5'-AGGCTTCTTGCAAGCAACG and 5'-GACCTATTGGAAGGAGTCTG; HPGD, 5'-TCTTGATACGCTGTCATGCA and 5'-GCAGATGGCTCCGATTG-GTG; β-actin, 5'-CCTTAATGTCACGCACGATTTC and 5'-GCAGCTCGG- TAGCTCTTTCGCA. HPGD or β-actin was amplified together with OPN in the same reaction, serving as internal control. PCR products were resolved on 1.5% agarose gel and visualized by ethidium bromide staining.

**Analysis of OPN protein level in condition media by Western blotting.** Conditioned media from tumor cells or macrophages were prepared as follows: ten hours after 100-mm dish or 48 h after macrophages were transfected with Stealth siRNA, the medium was removed, and each dish was washed once with warm PBS, followed by washing twice with serum-free Opti-MEM. The cells were then cultured in serum-free Opti-MEM for 2 h, refreshed with new serum-free Opti-MEM, and incubated for another 24 h. Following the incubation period, the conditioned medium from each dish was collected, centrifuged at 10,000 × g for 10 min to remove cell debris, and then concentrated by ultrafiltration in Centricon-30 mini-concentrators as per the manufacturer's protocol (Millipore). Each corresponding dish was trypsinized, and the number of cells was counted to allow appropriate correction in loading. The concentrated conditioned media were fractionated on 8% SDS-PAGE and then electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked with 5% bovine serum albumin,
and the OPN protein was detected using specific Ab (MAB14331, R&D Systems) and commercial enhanced chemiluminescence kit (Pierce).

**Assay for cell proliferation and foci formation.** For the analysis of cell proliferation, parental, mock, and OPN-knockdown tumor cells were seeded at 5 × 10³ per well in six-well plates (Corning Inc.) and then harvested at different time points. The number of living cells was counted with trypan blue exclusion.

For the assay of foci formation, a total of 400 cells were seeded onto a 100-mm dish in regular growth medium. Fourteen days later, colonies were counted under a light microscope. Migration assay was done by the same procedure, except that the membrane was not coated with Matrigel, and the membranes were removed. The migrated tumor cells on the lower surface of transwell polycarbonate membranes are visible in the background (original magnification, ×400).

**Analysis of actin cytoskeleton by confocal microscopy.** Cells were plated on poly-L-lysine–coated coverslips and cultured in DMEM with or without (for serum starvation) 10% FBS. After washing and fixation, the cells were stained with Alexa Fluor 532 phalloidin for filamentous actin and observed using confocal microscopy (Leica).

**Analysis of activated Rac1 and RhoA by affinity precipitation.** Activation of Rac1 in cells was determined as described previously (34). Plasmid encoding the p21-binding domain of human p21-activated kinase 1 as a fusion protein with glutathione S-transferase (GST-PBD) was a generous gift from Dr. G.M. Bokoch (Scrpps Research Institute, La Jolla, CA). Cells grown in 100-mm tissue culture plates were serum-starved for 48 h and lysed in ice-cold lysis buffer. The activated Rac1 in the cleared lysates were determined by pulldown assays using GST-PBD and immunoblotting. The specificity of this assay was confirmed by omitting GST-PBD as a negative control and by adding 100 μmol/L GTPγS as a positive control. Active RhoA was detected in a similar fashion as described for Rac1. Plasmid encoding the Rho-binding domain of Rhotekin (GST-RBD) was a generous gift from Dr. X.D. Ren (Scrpps Research Institute, La Jolla, CA; ref. 35).

**Statistical analysis.** Differences between experimental groups were analyzed for statistical significance using the Student’s *t* test. The *P* value <0.05 was considered statistically significant.

**Results**

Silencing of OPN decreases the motility and invasiveness of SK-Hep-1 cells. To investigate whether stromal cells can affect the outcome of tumor cells in which metastatic potential has been inhibited, we first set out to establish an OPN-knockdown model in SK-Hep-1, a highly invasive human hepatoma cell line with a high level of OPN (31). The vector-based RNAi system was employed to stably suppress the expression of OPN in SK-Hep-1 cells. The derived pSi-OPN-1– and pSi-OPN-2–stable transfectants were classified as OPN-knockdown tumor cells (OPN-KD), whereas the derived pSi-OPN-NC–stable transfectant was classified as mock
cells. In comparison with the parental SK-Hep-1 cells, both pSi-OPN-1 and pSi-OPN-2 transfectants showed a significant decrease of OPN in both mRNA and protein levels (Fig. 2A), indicating the successful knockdown of OPN in these derived clones. Furthermore, there was no difference in OPN expression level between the mock cells and the parental SK-Hep-1 cells (Fig. 2A).

Next, we evaluated whether down-regulation of OPN expression would alter the in vitro metastatic phenotype of SK-Hep-1 cells. The cell motility and invasiveness were analyzed by transwell chamber assay. As expected, both pSi-OPN-1 and pSi-OPN-2 transfectants revealed a pronounced reduction in both cell migration and invasiveness when compared with the mock and parental SK-Hep-1 cells (Fig. 2B–D and data not shown), whereas the mock and parental SK-Hep-1 cells showed similar potential of migration and invasion (Fig. 2B–D). These results indicate that OPN is closely related with the metastatic potential of SK-Hep-1 cells.

We further examined the effects of OPN silencing on the other malignant phenotypes of SK-Hep-1 cells, including the growth rate and foci formation. The results revealed that the capacity of both foci formation (Fig. 3A) and cell proliferation (Fig. 3B) was dramatically decreased in OPN-knockdown tumor cells, compared with that in the parental and mock cells. Furthermore, OPN-knockdown tumor cells grew as clusters, whereas the parental and mock cells scattered extensively in culture (Fig. 3C). Then, we investigated whether the reduced proliferation rate of the OPN-knockdown tumor cells was due to an increased apoptosis. The rate of apoptosis was analyzed by staining the nucleus with Giemsa and calculating the percentage of cells with condensed chromatin and/or fragmented nucleus. In comparison with the parental and mock cells, the OPN-knockdown tumor cells did not show increased rate of apoptosis (data not shown). Furthermore, the parental, mock, and OPN-knockdown tumor cells displayed similar apoptosis rate after their exposure to different compounds, including mitomycin, oxaliplatin, and curcumin (data not shown).

Coculture with macrophages increases the motility and invasiveness of OPN-knockdown tumor cells. To investigate the effect of macrophages on OPN-knockdown tumor cells, we used transwell chambers to assess the in vitro metastatic potential of the tumor cells in the presence or absence of macrophages. The tumor cells were plated in the upper chamber of the transwell, whereas the macrophages were preseeded in the lower chamber. The coculture was done for 10 h using 10% human AB serum as a chemoattractant. The results showed that the coculture with macrophages slightly enhanced the migration and invasion of the parental and mock cells (Fig. 2B–D). Surprisingly, in comparison with those noncocultured, the OPN-knockdown tumor cells cocultured with macrophages displayed as high as 14- and 8-fold increase in cell motility and invasiveness, respectively (Fig. 2B–D). Interestingly, the coculture with macrophages could restore the metastatic potential of OPN-knockdown tumor cell to the value of its wild-type counterparts (Fig. 2B–D). Furthermore, coculture with macrophage-conditioned medium also markedly promoted the migration/invasion of OPN-knockdown tumor cells, albeit not as pronounced as with macrophage itself (data not shown). These results clearly indicate that factors secreted by macrophages can significantly enhance the motility and invasiveness of OPN-knockdown tumor cells.

Macrophage-derived OPN plays a crucial role in macrophage-promoted migration. To characterize the mechanisms underlying macrophage-promoted migration in OPN-knockdown tumor cells, we first analyzed whether coculture with macrophages could alter the OPN expression level in tumor cells or macrophages. As reported earlier (18), we found that macrophages expressed high levels of OPN (Fig. 4A). However, the coculture did not alter the mRNA level of OPN in either tumor cells or macrophages (Fig. 4A).

Because OPN is a secreted glycoprotein, it may regulate the cellular responses in autocrine and paracrine manners. Therefore, a high level of OPN released by macrophages might be sufficient to compensate for OPN knockdown and thereby promote the motility of OPN-knockdown tumor cells, although coculture per se does not enhance the OPN expression in tumor cells. To test this hypothesis, a neutralizing Ab that can inhibit the OPN activity was added into the lower chamber of transwell preseeded with macrophages. An
isotype-matched normal goat immunoglobulin G (IgG) was used as negative control. After 1 h incubation, the upper chambers containing $3 \times 10^4$ cancer cells were inserted into the transwell, and the macrophtages/cancer cells were cocultured for an additional 10 h in the presence of Ab. As shown in Fig. 4B, OPN-neutralizing Ab could effectively blocked the coculture-stimulated motility of OPN-knockdown tumor cells in a dose-dependent manner, whereas the isotype-matched control Ab had no effect. These results suggest that macrophage-derived OPN is a prerequisite for coculture-stimulated migration in OPN-knockdown tumor cells.

To corroborate the above finding that macrophage-derived OPN promotes the motility of OPN-knockdown tumor cells, siRNA that targets the OPN gene was transduced into macrophages. We found that siRNA significantly reduced both the OPN mRNA level in macrophages and the amount of OPN protein in macrophage-conditioned media (Fig. 5A), indicating that macrophage-derived OPN can be effectively suppressed by RNAi. Next, we examined whether OPN knockdown in macrophages could prevent macrophage-promoted motility. Forty-eight hours after transfection, the siRNA-transfected macrophages were cocultured with OPN-knockdown tumor cells for 10 h in the transwell apparatus. As shown in Fig. 5B, 100 nmol/L of OPN-targeting siRNA could effectively inhibit the macrophage-promoted migration of OPN-knockdown tumor cells, whereas 400 nmol/L of siRNA conferred an even more dramatic effect. As a negative control, 400 nmol/L of scrambled siRNA displayed no effect at all. These results further confirm that macrophage-derived OPN plays a crucial role in macrophage-promoted migration of OPN-knockdown tumor cells.

Macrophage-derived OPN exerts its function independently of the actin cytoskeleton rearrangement or the activation of MMP and Rho families. It has been shown that OPN-promoted invasiveness is correlated with an increased expression and secretion of matrix metalloproteinases in tumor cells (33, 36, 37). To find out if such a mechanism is also involved in our model system, we first examined the gelatinolytic activity of the cultured media. Surprisingly, no discernible alterations in the mRNA level and the activities of matrix metalloproteinase (MMP)-2 and MMP-9 were observed in OPN-knockdown tumor cells, in comparison with the parental and mock cells (data not shown). Furthermore, neither the macrophages/cancer cells coculture nor the OPN-neutralizing Ab showed any effects on the activities of MMP-2 as well as MMP-9 (data not shown).

The rearrangement of actin cytoskeleton is essential for cell motility, and the Rac/Rho-GTPase family proteins are the key regulators for cytoskeleton rearrangement (38, 39). Therefore, we investigated whether actin cytoskeleton and Rac/Rho-GTPase activation were involved in macrophage-promoted motility. However, neither cytoskeletal structures nor the activity of RhoA and Rac1 displayed significant differences in the mock and OPN-knockdown tumor cells (data not shown). Moreover, cytoskeletal structures and Rho/Rac activity were rather similar in OPN-knockdown tumor cells cocultured with or without macrophage-conditioned media (data not shown).

These results suggest that macrophage-derived OPN exerts its function independently of the actin cytoskeleton rearrangement or the activation of MMP and Rho families in our model system.

**Discussion**

Metastasis is, in fact, what makes cancer so lethal. Effective prevention and therapy of metastasis will largely improve the survival of cancer patients. Accumulative evidence suggests that OPN overexpression is closely associated with metastatic phenotype of cancer cells (28–31). In the present study, we observed that OPN knockdown resulted in remarkable inhibition of invasiveness of human hepatoma cell line. Our result, together with the recent report that OPN silencing suppresses the metastasis of murine colon adenocarcinoma (37), strongly supports OPN as a potential therapeutic target for tumor metastasis. Interestingly, we found that the reduced metastatic potential in OPN-knockdown cells can be restored by coculturing with human macrophages, a pivotal member within the solid tumor microenvironment. These findings suggest that stromal cells are not the innocent bystanders in tumor metastasis, but rather the active regulators of the malignant phenotypes of cancer cells as well as the outcome of cancer therapy. Therefore, simultaneously targeting nonmalignant cells within tumor stroma should be considered whenever developing the anticancer strategy.

Previous studies have shown that autocrine OPN is associated with the migration and invasion of cancer cells (40–42). Our results indicate that OPN secreted by macrophages can also regulate the metastatic potential of tumor cells in a paracrine manner. This conclusion is based on the following observations. First, OPN was
highly expressed in macrophages, at a level much higher than that in SK-Hep-1 cells (Fig. 4A). Second, coculture with macrophages or conditioned medium from macrophages largely restored the motility of OPN-knockdown tumor cells without altering the OPN expression in these cells (Figs. 2 and 4A), indicating that the factors which promoted the motility should be derived from macrophages.

Third, both neutralization of OPN by Ab and reduction of macrophage-secreted OPN by siRNA could significantly inhibit the coculture-stimulated motility of OPN-knockdown tumor cells (Figs. 4B and 5B).

Interestingly, the motility of either cocultured (Fig. 4B) or noncocultured mock cells (data not shown) was not affected by the OPN-neutralizing Ab, even when high concentration (10 μg/mL) of Ab was used. These results indicate that the abundant autocrine OPN produced by the mock cell itself may bind to its receptors before it is effectively blocked by neutralizing Ab, and/or other factors are also involved in cell motility.

Besides OPN, macrophages also produce a vast array of mediators, such as cytokines, growth factors, and proteases, to exert their diverse actions on tumor progression and metastasis (9–11). In this context, we observed that either neutralization of OPN or reduction of macrophage-secreted OPN significantly reduced, but not completely blocked the coculture-stimulated motility of OPN-knockdown tumor cells (Figs. 4B and 5B), indicating that additional factors derived from macrophages are also involved in this process. Indeed, we found a mild increase in the motility of OPN-abundant mock cells after coculturing with macrophages, and this effect was not blocked by OPN-neutralizing Ab (Fig. 4B). We noted that the activity of MMP-9 secreted by macrophages was much higher than that from cancer cells (data not shown). Considering the important role of MMP-9 on tumor metastasis (43, 44), this enzyme could be one of the factors derived from macrophages that promote the cell motility, although to a lesser extent than OPN. In addition, it is known that macrophages can be activated or “educated” by cancer cells to release various mediators, which in turn aid the tumor progression. Such a cross-talk between cancer-stroma cells may also play a role in the present study because coculture with macrophages had a greater capacity to enhance the motility of OPN-knockdown tumor cells than macrophage-conditioned media did. Previous studies have shown that some cytokines, such as tumor necrosis factor α (TNFα) and interleukin 6 (IL6), are involved in macrophage-cancer cells interaction (11, 14, 15). In an attempt to clarify the involvement of cytokines, we analyzed the amount of TNFα, IL1β, IL6, IL10, and IL12 in the conditioned media by ELISA. However, no difference was observed among the parental, mock, and OPN-knockdown tumor cells or with and without macrophage coculture (data not shown).

The OPN-promoted motility of cancer cells observed in our study seems to be independent of the actin cytoskeleton rearrangement or the activation of MMP and Rho families. In other systems, addition or silencing of OPN has been shown to result in an altered activity of the MMP family members, especially MMP-2 and MMP-9 (33, 36, 37). However, we could not find any difference in MMP-2 or MMP-9 activity between the media derived from OPN-knockdown tumor cells and parental or mock cells. Neither coculture with macrophages nor the OPN-neutralizing Ab was used. These results indicate that the abundant autocrine OPN-neutralizing Ab, even when high concentration (10 g/mL) of Ab was used. The OPN-promoted motility of cancer cells observed in our study seems to be independent of the actin cytoskeleton rearrangement or the activation of MMP and Rho families. However, no difference was observed among the parental, mock, and OPN-knockdown tumor cells or with and without macrophage coculture (data not shown).

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Tumor progression and metastasis are now recognized as the product of an evolving cross-talk between different types of cells...
within the tumor microenvironment. Our findings that OPN derived from macrophages can effectively modulate the metastatic potential in paracrine manner may assist us to explore the complex cancer/stroma cell interactions that influence the process of cancer metastasis. The results presented also give important new insight into the significance of cancer-stroma cell interactions that influence the process of cancer within the tumor microenvironment. Our findings that OPN...
Human Macrophages Promote the Motility and Invasiveness of Osteopontin-Knockdown Tumor Cells

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