RaIA and RaIB: Antagonistic Relatives in Cancer Cell Migration

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Abstract

The RaI family of small G proteins has been implicated in tumorigenesis, invasion, and metastasis. However, little emphasis has been placed on clarifying the individual roles of the two RaI proteins, RaIA and RaIB, in these processes in view of their high sequence homology. Here we analyze the separate contributions of RaIA and RaIB in regulating cell migration, a necessary component of the invasive phenotype, in two human cancer cell lines; UMUC-3, a bladder carcinoma line, and the prostate carcinoma line, DU145. Although inhibiting RaIA protein expression by ~80% with two different small interfering RNA duplexes had no effect on migration, inhibiting RaIB expression to the same extent with two different duplexes resulted in a marked reduction in migration. Inhibiting RaIB expression did trigger a significant loss of actin cytoskeleton fibers in UMUC-3 that was not seen with inhibition of RaIA expression. Interestingly, simultaneous inhibition of RaIA and RaIB expression had no effect on migration. However, dual inhibition of RaIA and RaIB expression in UMUC-3 did result in an almost total loss of actin fibers as well as a reduction in proliferation, particularly in reduced serum conditions. These results suggest that RaIA and RaIB have different roles in cell migration and that they may in fact act as antagonists with regard to this phenotype. As further verification of this hypothesis, we found that expression of constitutively active RaIA inhibited migration, whereas expression of constitutively active RaIB stimulated migration, consistent with this model. In summary, we present the first demonstration that despite their significant sequence homology, RaIA and RaIB have nonoverlapping and opposing functions in cancer cell migration but overlapping functions in cell growth. (Cancer Res 2005; 65(16): 7111-20)

Introduction

With >100 members in humans, the Ras superfamily of monomeric G proteins (1) contribute to the regulation of many, if not most, cellular functions; from gene expression (2), to cell division (3), membrane traffic (4), and cytoskeletal dynamics (5). In general, these functions are dependent upon the ability of these proteins to cycle between two conformations by binding and hydrolyzing GTP. These two conformations usually differ in both their affinity for other protein effectors and their cellular localization, hence allowing these G proteins to alter cellular pathways both temporally and spatially (6). The intrinsic GTP hydrolase (GTPase) activity of these proteins is further regulated in cells by three other classes of proteins: guanine nucleotide exchange factors (GEF; ref. 7), which promote the GTP-bound, active conformation; GTPase-activating proteins (GAP; ref. 8), which promote the GDP-bound, inactive conformation; and guanine nucleotide dissociation inhibitors (GDI; ref. 9), which block activation and also facilitate transfer of G proteins between membrane compartments. It is estimated that there are at least as many, if not more, GEFs, GAPs, and GDIs as there are monomeric G proteins, which contributes greatly to the complexity of understanding G protein function and regulation. Although there is no consensus, a common classification scheme splits the superfamily into six families—Ras, Rho, Arf, Rab, Ran, and Ral/Gem (6). Members of the Ras family, particularly H-Ras, K-Ras, and N-Ras, are known to contribute widely to human tumorigenesis through both activating mutations and overexpression (10, 11). Rho family members such as RhoA, RhoC, Rac1, and cdc42, are also involved in many human cancers, primarily by overexpression (12). Recent data also suggest that members of the Arf (13) and Rab (14) families might contribute to tumor cell invasion and metastasis. Overexpression of GEFs (15) and loss of expression and/or inactivation of GAPs (16) and GDIs (17) also occur in human cancer. It is therefore not surprising that Ras and Rho family members and their regulators are now targets for new strategies of therapeutic intervention in cancer (18, 19).

Within the Ras family of monomeric G proteins, RaIA and RaIB are highly similar proteins (85% amino acid identity) that participate in diverse cellular functions (20). Among the processes regulated by RaI proteins are endocytosis, exocytosis, actin cytoskeletal dynamics, and transcription (21). In some cases, RaI involvement in these processes has been shown to be mediated through effectors such as RaIBP1, Sec5, filamin, and phospholipase D1; in other cases, particular RaI-effector interactions responsible for downstream functions remain to be determined. Recent results have also indicated roles for RaI proteins in tumorigenesis and cancer progression (22, 23). For example, GEFs for RaI seem to be the most crucial determinants downstream of Ras in transforming human cells (24, 25). Also, using small interfering RNA (siRNA) duplexes specific for RaIA or RaIB, it was shown that RaIA and RaIB have separate, nonoverlapping roles in regulating anchorage-independent proliferation and survival, respectively, in several human cancer cell lines (26). We have previously shown that RaI is necessary for epidermal growth factor–stimulated migration in human bladder cancer cells (27). Here, we address the critical issue of whether RaIA and RaIB have similar or different contributions on cell migration and find that RaIA and RaIB play opposing roles in regulating this process, with RaIB stimulating and RaIA inhibiting motility. In contrast to migration, we describe overlapping roles for RaIA and RaIB in regulating cancer cell proliferation. These results suggest the existence of distinct migration and proliferation signaling networks downstream of RaI family proteins which have fundamental implications for the development of therapeutics.
Materials and Methods

Cells. UMUC-3 human bladder cancer cells were obtained from American Type Culture Collection (Rockville, MD) and cultured in MEM with 2 mmol/L L-glutamine and Earle's balanced salt solution adjusted to contain 1.5 g/L sodium bicarbonate, 1.0 mmol/L sodium pyruvate, and 10% fetal bovine serum (FBS). DU145 human prostate cancer cells were also obtained from American Type Culture Collection and cultured in MEM with 2 mmol/L L-glutamine and Earle's balanced salt solution adjusted to contain 1.5 g/L sodium bicarbonate, 1.0 mmol/L sodium pyruvate, 0.1 mmol/L nonessential amino acids, and 10% FBS. HeLa human cervical cancer cells were also obtained from the University of Virginia Tissue Culture Facility and were cultured in the same media as DU145 above.

Plasmids. pcDNA 3.1+/?RalB was obtained from the Guthrie cdna Resource Center (Sayre, PA, http://www.cdna.org/). RalA cDNA was amplified from bladder cancer cell RNA by RT-PCR using the following primers: forward, 5'-GTCCTGACATCACCTGGAATAGCCCGAGG-3'; reverse, 5'-TAGACTCGAGTTTAAATGATCGAGATCTTTG-3', and cloned into pcDNA 3.1+ using BamH I/Xho I digestion and ligation. Constitutively active mutants (G2SV) of RalA and RalB were made using a QuikChange mutagenesis kit (Stratagene, La Jolla, CA) with corresponding primers. Wild-type and activated mutants of RalA and RalB were NH2-terminally taged by cloning into pFLAG-CMV-4 (Sigma, St. Louis, MO) using Hind III/Xba I sites. Plasmids were transfected into cells in six-well plates using FuGENE (Roche, Indianapolis, IN) according to the manufacturer's instructions.

Small interfering RNA. siRNA duplexes were chemically synthesized, deprotected and annealed by Dharmacon (La Flame, CO), with the following target sequences: RalA 1, 5'-GACAGGUUUCUUGAAGA-3'; RalA II, 5'-CAGACCUGACAGCUUGUAAU-3'; RalB I, 5'-GGUGAUCUGUGCUCCGACGAC-3'; RalB II, 5'-AAGUGACAUAAUCAGGAA-3'; RalB I, RalA/B, 5'-GCAUAAACAGCUGCAAAG-3'; luciferase (GL2), 5'-CTGAC CGGGATACTTCCA-3'. RalA duplexes I and II and RalB duplex I are the same as described in Chien and White (26) (internal RalAG-tagged by cloning into pFLAG-CMV-4 (Sigma, St. Louis, MO) using Hind III/Xba I sites. Plasmids were transfected into cells in six-well plates using OligofectAMINE (Invitrogen, Carlsbad, CA) according to manufacturer's instructions.

Western blotting and Ral activation assays. Either 24 hours (DNA) or 72 hours (siRNA) posttransfection, cells were lysed in 50 mmol/L Tris-HCl (pH 7.5), 200 mmol/L NaCl, 1% Igepal CA-630 (Sigma), 10 mmol/L MgCl2, Lysates were cleared by centrifugation and protein amounts were quantitated by bichorronic acid assay (Pierce, Rockford, IL). Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with antibodies against RalA and RalB (PharMingen, San Diego, CA) or FLAG (Sigma). Because the RalB antibody became unavailable, experiments involving HeLa cells used a RalB antibody generously provided by Dr. Raj Bhullar. Blots were developed using Super Signal Femto Chemiluminescence (Pierce) and results were visualized and quantitated using an Alphalnnotech (San Leandro, CA) imaging system. Ral activation assays were done as previously described (27) using RalBP1 agarose to pull down activated RalA and RalB (Upstate, Charlotteville, VA).

Cell migration and plating efficiency assays. Either 24 hours (DNA) or 72 hours (siRNA) after transfection, cells were harvested, counted in a hemacytometer, and resuspended in serum-free media. Cells (20,000) were added in triplicate to the upper chambers of transwell filters (8.0 μm pores, Becton Dickinson, Franklin Lakes, NJ) in a 24-well tissue culture plate. The lower chambers contained media with 2% FBS. Plating control assays were done in triplicate in adjacent wells containing the same media but without transwell filters. After 6 hours (for UMUC-3) or 18 hours (for DU145 and HeLa), cells remaining on the upper surface of the filters were removed with cotton swabs and cells on the lower surface were fixed with 100% methanol, stained with crystal violet, and counted with the aid of a gridded coverslip. Cell numbers in plating control assay wells were estimated using CyQuant (Molecular Probes, Eugene, OR) according to manufacturer's instructions.

Actin cytoskeleton visualization. UMUC-3 cells were plated in six-well plates containing coverslips and transfected with either siRNA or FLAG-Ral expression constructs as described above. One day (DNA) or 3 days (siRNA) after transfections, cells were fixed with 4% paraformaldehyde for 15 minutes, stained with Hoechst 33342 (1:200) and Phalloloid-AlexaFluor 594 (1:200; both from Molecular Probes) for 20 minutes and allowed to air-dry overnight. Cells transfected with FLAG-Ral alleles were also visualized by FLAG immunofluorescence using M2 anti-FLAG mouse monoclonal antibody (1:7500; Sigma) and Oregon Green conjugated anti-mouse IgG (1:200; Molecular Probes).

Growth curves and cell cycle analysis. Twenty four hours after siRNA transfection as previously described, UMUC-3 cells were harvested, counted using a hemacytometer, and resuspended in media with either 2% or 10% FBS. One thousand cells/well were added to wells of 96-well plates and were then incubated at 37°C, 5% CO2 for 24 to 96 hours. After incubation, cell numbers were estimated using CyQuant. For evaluation of cell cycle and detection of sub-G0 populations, 120 hours after siRNA transfection and after 4 days in reduced (2%) serum, cells were permeabilized with 70% ethanol for 1 hour and stained with propidium iodide (0.1% sodium citrate, 0.1% Triton X-100, 50 μg/mL propidium iodide and 100 units/mL RNase) for 30 minutes. Flow cytometry was done on a FACScalibur system using excitation light at 488 nm and Cell Quest software (Becton Dickinson).

Results

Whereas previous results have indicated the importance of Ral proteins in cell motility (27, 28), these studies have assumed complete functional overlap between RalA and RalB and have been largely based upon the use of dominant-negative alleles of RalA or RalB. These dominant-negative Ral proteins are postulated to inhibit endogenous GEF function by preventing Ral binding to its GEFs (29). Because the specificities of the Ral GEFs for RalA versus RalB are not well understood, it must be assumed that any dominant-negative Ral allele likely inhibits both RalA and RalB activation. Therefore, to better understand the individual roles of RalA and RalB in bladder cancer cell migration, we undertook a siRNA approach. Chien and White (26) developed siRNA duplexes that specifically inhibited the expression of either RalA or RalB. They used these to show that inhibition of RalB expression triggers apoptosis and cell death in selected cancer cell lines, but that non–cancer cell lines did not undergo apoptosis in response to RalB knockdown. Surprisingly, they showed that simultaneous inhibition of RalA and RalB expression reversed the apoptotic and cell death effects of RalB knockdown alone.

We chose to focus initially on two highly motile human cancer cell lines which have a strong invasive and metastatic phenotype; UMUC-3, a bladder cancer line isolated from a high-grade, invasive transitional cell carcinoma, and DU145, a prostate cancer line isolated from a brain metastasis. Figure 1A and B shows that these two cell lines have similar levels of RalA and RalB, and that the activation levels of RalA under standard growth conditions are similar between these cell lines, RalB activation is somewhat lower in UMUC-3 than in DU145. The effectiveness and specificity of siRNA-mediated reduction in RalA and RalB expression in these two cell lines is shown in Fig. 1C and D. Two different duplexes for RalA reduced RalA expression at the protein level by ~80% in both cell lines, whereas having little effect on RalB expression. Similarly, two different duplexes targeting RalB reduced its expression by 80%, with little change in RalA expression. Similar results were obtained in HeLa cells, in which these siRNA duplexes had been previously shown to be effective (26), although RalB depletion in HeLa was less pronounced than in UMUC-3 or DU145 (Fig. 1D). A nonspecific control siRNA (GL2, which targets firefly luciferase; ref. 30) did not have any effect on either RalA or RalB expression. In all cases, the level of protein inhibition reached a maximum
between 48 and 72 hours after transfection and persisted until at least 120 hours posttransfection (data not shown).

Using the aforementioned duplexes, we proceeded to evaluate the effects of inhibiting RalA or RalB expression on cell migration. Relative to the control siRNA, inhibition of RalA expression with either RalA duplex I or II had no effect on motility toward 2% FBS (Fig. 2A). In contrast, inhibition of RalB expression with either RalB duplex I or II significantly reduced transwell migration (35-55% reduction) in both UMUC-3 and DU145 (Fig. 2C). However, neither RalA nor RalB depletion had any effect on HeLa motility in this assay. In all cases, there was no detectable motility in the absence of FBS (data not shown). Because of data showing that RalB siRNA can trigger cell death via apoptosis in cancer cells (26), we sought to determine whether the effect of RalB siRNA on motility was due to a specific effect on migration or a more general reduction in plating efficiency and/or viability. Plating control assays done in parallel to migration assays indicated that, in both the RalA and RalB siRNA–transfected cells, there was no reduction in plating efficiency relative to control siRNA–transfected cells (Fig. 2B and D). Nor were there any changes in gross cell morphology.
upon siRNA-mediated depletion of RalA or RalB (data not shown).
Taken together, these data indicate that the effect of RalB depletion on cell migration in UMUC-3 and DU145 is specific and not due to the production of either nonadherent or nonviable cells. Inhibition of motility by RalB, but not RalA, siRNA points toward RalB having a specific promigratory role in these cells.

Because apoptosis triggered by RalB siRNA in cancer cells can be blocked by simultaneous transfection of RalA and RalB siRNA (26), we sought to determine the effect on motility of both RalA and RalB depletion. A priori, three results seem possible. RalA depletion could have no effect on the ability of RalB depletion to inhibit motility, suggesting that RalA plays no role in motility. Alternatively, RalA depletion could further inhibit motility in the context of RalB depletion, which could be interpreted that RalB plays a dominant role in motility that RalA can partially fill in the absence of RalB. Thirdly, RalA depletion could reverse the RalB siRNA inhibition of motility, indicating a possible antagonistic relationship between RalA and RalB in regulating motility. In Fig. 3A, B, C, and D, cells transfected with RalA siRNA duplexes (*, P < 0.01; t test).

Figure 2. Effects of RalA and/or RalB depletion on transwell migration. Triplicate Boyden chambers were loaded with 20,000 siRNA-transfected UMUC-3, DU145, or HeLa cells in serum-free media and cells were allowed to migrate toward 2% serum for 6 hours (UMUC-3) or 18 hours (DU145 and HeLa). After removing nonmotile cells from the upper surface of the filter, cells on the lower surface were fixed, stained, and counted. Columns, means; bars, ± SD. Plating control wells did not contain transwell chambers and were quantitated using CyQUANT (Molecular Probes). Data shown are representative of three separate experiments. In each case, Western blotting of duplicate siRNA transfections indicated reduction in RalA or RalB expression of >75%. A and B, cells transfected with RalA siRNA duplexes. C and D, cells transfected with RalB siRNA duplexes.
siRNA-mediated depletion of both RalA and RalB is shown. RalA/B is a single siRNA duplex that, in UMUC-3, is able to reduce expression of both RalA and RalB. In DU145, however, this duplex is much less effective at reducing RalA expression. This is consistent with it being used previously as a RalB-specific siRNA (26), although it has only a single base mismatch with RalA mRNA. It has been shown that siRNA duplexes with single base mismatches are often inefficient at targeting mRNA destruction via DICER, but can still inhibit protein expression, presumably by interfering with translation (31). Interestingly, from our results, it seems that this is a cell type–specific phenomenon. Thus, to address this issue, we transfected both siRNA duplexes and achieved >80% protein reduction in this cell line (Fig. 3). When we looked at the effect of simultaneous RalA and RalB depletion on motility, we found that, in UMUC-3, normal motility was restored using either a single siRNA duplex targeting both RalA and RalB or individual duplexes for each. In DU145, only the two-duplex approach actually reduced RalA and RalB expression to levels similar to that found in single
Figure 4. Effects of constitutively active RalA and RalB on transwell migration. UMUC-3 cells were transfected with expression constructs (in pFLAG vector) for either wild-type (wt) or constitutively active (act) RalA and RalB. A, Ral activation assays showing that act-RalA and act-RalB have much higher activation levels than wt-RalA and wt-RalB. B, quantitation of results in (A) using AlphaInnotech software. C, 24 hours after transfection, triplicate Boyden chambers were loaded with 20,000 cells in serum-free media and allowed to migrate toward 2% serum for 6 hours, as in Fig. 2. Plating controls as described in Fig. 2. Columns, means; bars, ± SD (*, P < 0.01; **, P < 0.05; t test). Data shown are representative of three separate experiments. Inset, expression levels of wt- and act-RalA and RalB (FLAG) by Western blot.
structures were also visualized in these cells, as shown in Fig. 5 expressing constitutively active RalA and RalB, actin-based normal. Because motility was also altered in UMUC-3 cells where both RalA and RalB were depleted, stress fibers were almost organization and/or maintenance of these actin fibers. In cells depleted of RalB, suggesting that RalB has a role in the prominence of these stress fibers. In contrast, there were markedly prominent actin stress fibers running most of the length or width of the cell. These fibers were also numerous and prominent in Fig. 5A. Cells transfected with control siRNA (GL2) show many prominent actin stress fibers running most of the length or width of the cell. These fibers were also numerous and prominent in untransfected UMUC-3 cells (data not shown). In cells that had been depleted of RalB by siRNA, there was little change in the number or prominence of these stress fibers. In contrast, there were markedly fewer and less prominent stress fibers in cells that had been depleted of RalB, suggesting that RalB has a role in the organization and/or maintenance of these actin fibers. In cells where both RalA and RalB were depleted, stress fibers were almost nonexistent, and many of the cells were much more elongated than normal. Because motility was also altered in UMUC-3 cells expressing constitutively active RalA and RalB, actin-based structures were also visualized in these cells, as shown in Fig. 5B. The number and prominence of stress fibers in cells expressing act-RalA or act-RalB seemed unchanged compared with vector-transfected cells or untransfected neighbors.

Because Ral proteins have been shown to play important roles in regulating cancer cell proliferation (26, 33), we studied the effect of selective inhibition of RalA and/or RalB expression on cell proliferation by doing growth curves on UMUC-3 cells depleted of RalA and/or RalB by siRNA transfection. Reducing either RalA (Fig. 6A) or RalB expression (Fig. 6B) had no effect on UMUC-3 cells’ ability to grow in medium containing normal serum (10%) and only minor effects on their ability to grow under reduced serum conditions (2%). In contrast, simultaneous reduction of RalA and RalB expression with either one or two duplexes reduced proliferation in medium with 10% serum, and had an even more profound effect on growth in reduced serum conditions (Fig. 6C).

**Discussion**

In this report, we describe an antagonistic relationship between RalA and RalB in regulating motility of bladder and prostate cancer cells. Although Ral proteins have previously been shown to be important for the motility of skeletal myoblasts and bladder cancer cells (27, 28), this is the first indication that RalA and RalB have nonoverlapping functions in cell motility. That these nonoverlapping functions may actually be antagonistic in cell migration is surprising and novel. Interestingly, our results showing that RalB is promigratory, whereas RalA inhibits motility, stand somewhat in contrast to recent results showing that RalA activation is necessary for the formation of lamellipodia in moving COS-7 and Madin-Darby canine kidney cells (34). Whether this indicates different roles for RalA and RalB in normal versus cancer cells awaits further experimentation, although the lack of an effect on motility upon RalB depletion in HeLa cells indicates that RalB is not universally required for cancer cell motility. What also remains unclear is why migration is not stimulated in cells depleted of RalA.

So how might RalA and RalB antagonistically regulate cell motility? Of the known Ral effectors—RalBP1, Sec5, Exo84, Filamin, and phospholipase D1 (21)—none are known to bind selectively to RalA or RalB. In fact, RalBP1, Sec5, and Exo84 have been shown to bind to both RalA and RalB, at least in yeast two-hybrid studies (35, 36). Recently, it has been shown that RalA binds the exocyst complex more effectively than RalB, at least in Madin-Darby canine kidney cells, allowing RalA to target E-cadherin delivery to the basolateral membrane (37). They also described nonoverlapping localizations of RalA and RalB, with RalB, but not RalA, associated with recycling endosomes. This still leaves open the possibility of selective interaction of RalA and RalB with other known effectors in vivo, as well as undiscovered Ral effectors that might specifically interact with RalA or RalB. Alternatively, this antagonism may reflect the importance of intracellular protein localization in cell motility. Perhaps different cellular localizations of RalA and RalB, particularly in a moving cell, allow for different subsets of effector interactions that result in opposing effects on whole cell migration. Given that all the known Ral effectors have functions consistent with roles in motility (21), dissecting out the relative affinities of RalA and RalB for these effectors in cells, particularly moving cells (if possible), should be quite informative.

We have also shown that RalB depletion has a much more profound effect than RalA depletion on actin cytoskeletal organization in UMUC-3 cells, causing a dramatic reduction in stress fibers. However, this probably doesn’t explain why RalB depletion inhibits motility, because depletion of both RalA and RalB, which restores normal motility, causes an even greater loss of stress fibers, as well as a shift to a more elongated shape in many cells. The hypothesis that RalB depletion inhibits motility independent of its effect on the actin cytoskeleton is further supported by the observation that expression of act-RalA, which inhibits motility, and act-RalB, which stimulates motility, both have no visible effect on the actin cytoskeleton. Given the dramatic differences in actin cytoskeleton organization and morphology, and yet similar motility, between control siRNA-transfected
cells and cells depleted for both RalA and RalB, two alternatives present themselves. Either these differences in cell shape and actin fibers have no effect on motility, or cells with reduced levels of RalA and RalB have acquired a different mode of motility. Recent results indicate that cancer cells can shift between two modes of invasion; an elongated, lamellipodia-driven mode, dependent upon extracellular proteases, and a rounded mode dependent upon ROCK and ezrin (38). Further experiments may elucidate what roles, if any, RalA and RalB play in these two modes of tumor cell invasion.

We have also shown that the growth of UMUC-3 cells, particularly in reduced serum conditions, is inhibited by simultaneous depletion of RalA and RalB. This suggests that RalA and RalB may have an overlapping yet essential role in regulating cell proliferation, with the presence of at least one Ral protein necessary for optimal growth. These findings may help to explain
previous observations showing involvement of Ral in the growth of breast cancer cells (33) and in regulating cyclin D1 expression (39). This effect of RalA and RalB depletion on proliferation does not seem to be due to triggering apoptosis because cell cycle analysis of siRNA-transfected cells showed no increase in apoptotic cells (G1) 120 hours after transfection (data not shown). In light of the Ral effectors discussed above, it seems that the Ral effector(s) that regulate motility are different than the effector(s) that regulate proliferation. Taken together with the plating control data, this also indicates that, in contrast to at least some other cancer cell lines (26), UMUC-3 and DU145 do not undergo apoptosis resulting in cell death in response to reduction in RalB expression.

Whatever the mechanism, the antagonistic regulation of bladder cancer cell migration by RalA and RalB indicates that even highly similar proteins may have different and even opposing functions, highlighting the need for careful study using, wherever possible, specific tools to study specific protein function. In this case, such an approach has uncovered novel, opposing roles for RalA and RalB in cancer cell migration, which seem distinct from their overlapping roles in cell proliferation. Furthermore, these results highlight the potential pitfalls in designing pharmacologic compounds against a whole class of molecules without first understanding the biological function of each separately, even in cases where the molecules are highly homologous.

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Figure 6. Effects of RalA and/or RalB depletion on cell proliferation. UMUC-3 cells were transfected with siRNA duplexes targeting RalA and/or RalB. Twenty-four hours after transfection, cells were harvested, resuspended in medium containing either 10% or 2% FBS, reseeded in 96-well plates, and allowed to grow an additional 24 to 96 hours. Cell numbers were estimated by CyQuant fluorescence. In each case, Western blotting of duplicate siRNA transfections indicated reduction in RalA and/or RalB expression of >75%.

A, cells transfected with RalA siRNA duplexes. B, cells transfected with RalB siRNA duplexes. C, cells transfected with siRNA duplexes for both RalA and RalB.
References

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