Priority Report

The Metastasis-Associated Gene *CD24* Is Regulated by Ral GTPase and Is a Mediator of Cell Proliferation and Survival in Human Cancer

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Abstract

Ral GTPases are important mediators of transformation, tumorigenesis, and cancer progression. We recently identified the metastasis-associated protein CD24, a glycosyl phosphatidyl inositol–linked surface protein, as a downstream target of Ral signaling by profiling the expression of RalA/B–depleted bladder carcinoma cells. Because CD24 is highly expressed in bladder and many other tumor types, we sought to determine if this protein plays an essential role in maintaining the malignant phenotype. Here, we show that loss of CD24 function in cell lines derived from common tumor types is associated with decreased rates of cell proliferation, clonogenicity in soft agar, changes in the actin cytoskeleton, and induction of apoptosis. Given these phenotypes, we evaluated a human bladder cancer tissue microarray by immunohistochemistry for CD24 to determine if CD24 is a prognostic cancer biomarker. Multivariate analysis showed that increased CD24 expression correlated with shorter patient disease-free survival (*P* = 0.07). In conclusion, we show that CD24 is a novel and functionally relevant Ral-regulated target and a potentially important prognostic marker. We suggest that these insights may lead to future therapeutic approaches that seek to eliminate CD24 function in cancer cells. (Cancer Res 2006; 66(4): 1917-22)

Introduction

Recent reports reveal a compelling role for Ral GTPase signaling in carcinogenesis and tumor progression (reviewed in ref. 1). The Ral family of GTPases, comprising the 85% identical paralogues RalA and RalB, constitutes a distinct group of Ras-related small GTPases that are activated in a Ras-dependent manner via several RalGEFs and signal through several effectors (2). Activation of the Ral pathway is among the most basic requirements for transformation of human cells, and Ras-mediated transformation depends on activation of RalA (3). Extending our prior findings of Ral involvement in cancer cell motility (4), we recently reported antagonistic roles for these two GTPases in cancer cell motility and a cooperative role for both in cancer cell growth (5). An analysis of gene expression in Ral-depleted cells identified CD24 as a putative transcriptional target.[6] CD24 is a mucin-like cell surface protein consisting of a short, heavily glycosylated protein core linked to plasma membrane raft domains via glycosyl-phosphatidylinositol.

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Historically, CD24 was developed as a cluster of differentiation (CD) marker of hematopoietic lineages that has been found to be overexpressed in many common malignancies (6) and has been associated with the metastatic phenotype (7). Reported functions for CD24 include leukocyte signal transduction (8), regulation of B-cell apoptosis (9), and leukocyte adhesion (10). Despite the multitude of human tissue studies correlating CD24 expression with malignancy, to date, no report has definitively evaluated whether CD24 is important in maintaining this phenotype or merely a transformation-associated epiphenomenon. Here, we report that CD24 is central to maintaining cancer cell growth, anchorage-independent proliferation, and survival. We further evaluated CD24 protein expression in a bladder cancer tissue microarray and found that CD24 is an independent prognostic factor for disease-free survival in patients. Taken together, these results are the first to report that the prognostic marker CD24 is an important mediator of tumor growth and survival.

Materials and Methods

Cell culture, proliferation and cell cycle assays, and microscopy. All cell lines used are derived from human cancer and maintained as described (American Type Culture Collection, Manassas, VA). Cells were transfected with small interfering RNA (siRNA) for GL2, RalA, RalB, RalA/B, or CD24 as described (5), then quantified daily using Alamar Blue (Biosource, Camarillo, CA). Clonogenicity assays for anchorage-independent growth employed cells 24 hours after transfection and evaluated as described (11). For cell cycle analysis, cells were transfected with siRNA, harvested, fixed in ethanol, and stained with propidium iodide. Cells (10,000) were analyzed on a FACSCalibur cytometer using CellQuest Software (Becton Dickinson, San Diego, CA). Actin cytoskeletal visualization and microscopy were as described (5). The CD24 siRNA Smartpool, Smartpool Upgrade Duplexes, and final optimized CD24 duplex were obtained from Dharmacon (Lafayette, CO). The target sequence of the CD24 siRNA duplex is 5′-CAACTAATGCCACCACCAA-3′.

RNA isolation, real-time reverse transcription-PCR, and immunoblotting. RNA was isolated and quantitative reverse transcription-PCR (RT-PCR) done in the ABI Prism 7900HT Sequence Detection System from Applied Biosystems (Foster City, CA) as described (11). Primers used for CD24 were forward, 5′-CAATATTAAATCTGCTGGAGTTTCATG-3′ and reverse, 5′-TCCATATTTCTCAAGCCACATTCA-3′. Our immunoblotting and detection protocol was employed as described (5), probing for CD24 (antibody SWA11, a gift of Dr. Peter Altevogt, Tumor Immunology Programme, German Cancer Research Center, Heidelberg, Germany), tubulin (Calbiochem, La Jolla, CA), or RalA and RalB (BD PharMingen, San Diego, CA).

Human tissue microarray analysis and immunohistochemistry. Our oligonucleotide microarray analysis of 23 human bladder carcinomas and control tissues has been described previously (11). Gene expression data

[6] G. Oxford et al., in preparation.

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from the Lenberg et al. oligonucleotide microarray series of normal kidney and renal cell carcinomas (12) and the Chen et al. cDNA array study of normal liver and hepatocellular carcinoma (13) are publicly available. The human bladder cancer tissue microarray and our immunohistochemistry methods have been described (14). Anti-CD24 (monoclonal; clone 24CO2; 1:100 dilution; Lab Vision, Fremont, CA) was applied to tissue microarray sections for 1 hour at room temperature. CD24 expression scoring was 0, 1+ (<10% positive cells), 2+ (10-50% positive cells), and 3+ (>50% positive cells). This was carried out by one pathologist (H.F.F.) blinded to patient outcome.

Statistical methods. *In vitro* and human oligonucleotide microarray studies were analyzed using a two-tailed Student's t test. In microarray studies of CD24 down-regulation with Ral knockdown, we employed a one-tailed t test. In CD24 immunohistochemistry analyses, stage and grade categories with low frequencies were combined with the next higher category of stage or grade. Associations with stage and grade were tested with Pearson's χ^2 test. The log-rank test was used to compare disease-free survival distributions among levels of expression. Cox proportional hazards models were used to estimate the effect of CD24 on survival time, adjusting for stage and grade, treating CD24 levels as categorical. Plots and analysis were carried out with the SAS software (SAS Institute, Inc., Cary, NC).

Results and Discussion

Ral GTPases regulate CD24 expression. To identify genes regulated by Ral signaling, we did a transcriptional analysis, using high-density oligonucleotide microarrays, of Ral-depleted UM-UC-3 urothelial carcinoma cells 72 hours after siRNA transfection (Fig. 1*A*).[6] We observed decreased expression of CD24, minimally in RalA-depleted (80.1% of control; $P = 0.33$) and RalB-depleted (50.2% of control; $P = 0.13$) cells but substantially (17.9% of control; $P = 0.05$) following double knockdown of both RalA and RalB (RalA/B) compared with a GL2 luciferase control non-targeting duplex (Fig. 1*B*). We confirmed this regulation by real-time RT-PCR in an independent experiment in UM-UC-3 cells (Fig. 1*C*) and extended it to HeLa cells (Fig. 1*C*), a commonly studied cancer cell line. RalA/B knockdown led to decreased CD24 on the level of protein as well, as assayed by immunoblotting in both UM-UC-3 and HeLa (Fig. 1*D*). CD24 appears as a wide band of varying molecular weights (~30-60 kDa) due to the presence of multiple glycoforms, as reported before (6). Although the specific transcriptional mechanism of Ral regulation of CD24 remains unknown, Ral has been shown to signal to transcription through signal transducers and activators of transcription, Jun, nuclear factor-κB, AFX, and TCF pathways, (reviewed in ref. 15). Our data suggest that transcriptional targets of Ral signaling, such as CD24, should not be overlooked in the search for "drugable" or otherwise therapeutically viable targets for cancer treatment.

CD24 plays a role in cell proliferation *in vitro*. To develop siRNA for CD24, we initially used a pool of four siRNAs to evaluate effects of CD24 depletion. We then tested four individual duplexes and identified two nonoverlapping siRNAs that consistently reduced growth. We finally selected one of these duplexes exhibiting effective CD24 depletion for further experiments and verified that this duplex did not substantially induce protein kinase R, a surrogate for the induction of an IFN response (ref. 16; data not shown).

We observed substantial knockdown of CD24 protein in a panel of tumor cell lines from common epithelial human cancers, including UM-UC-3 urothelial carcinoma cells, DU145 prostate carcinoma cells, HeLa cervical adenocarcinoma cells, MCF-7 breast adenocarcinoma, and SAOS-2 osteosarcoma cells (Fig. 2*A-E, insets*). Depletion of CD24 by siRNA ranged from nearly complete in UM-UC-3, DU145, and HeLa to partial in the cells with the highest CD24 expression, MCF-7, and SAOS-2. Following siRNA-mediated depletion of CD24 in these five cell lines, we observed significant

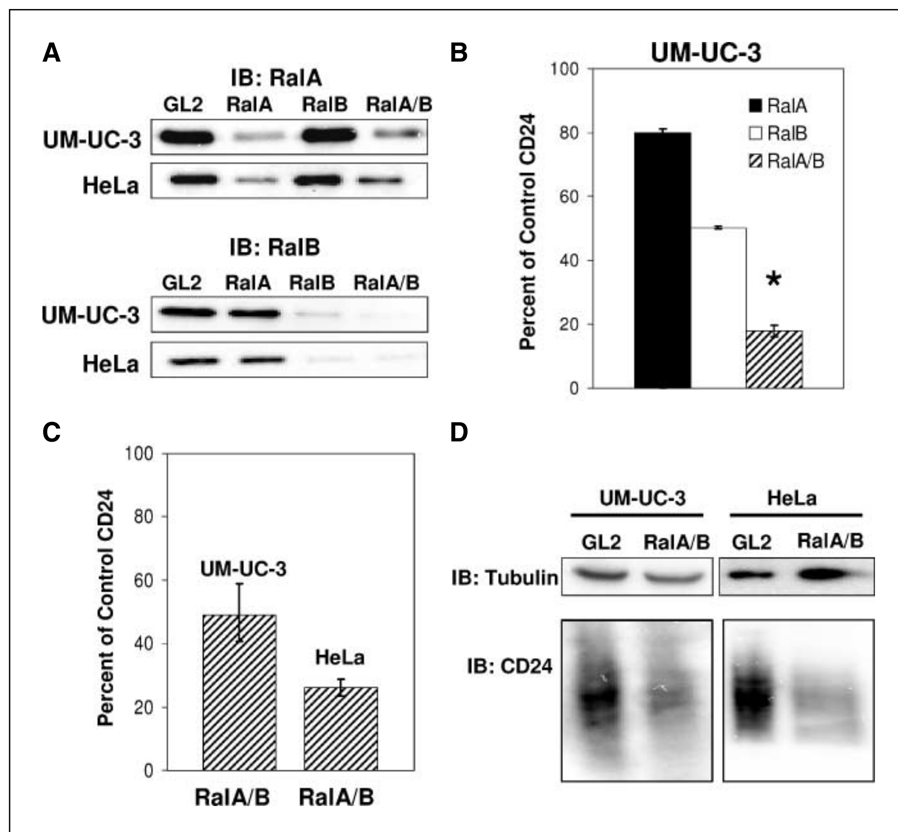

Figure 1. Ral regulates CD24. *A,* representative blots of Ral knockdown in UM-UC-3 and HeLa cells using control GL2, RalA, RalB, and the RalA/B duplex. Protein was quantitated using the bicinchoninic acid assay and equal amounts loaded. *Top,* blots probed for RalA; *bottom,* blots probed for RalB. *B,* oligonucleotide microarray analysis of CD24 mRNA expression in UM-UC-3 cells 72 hours after transfection with indicated siRNAs. *Columns,* % control CD24 expression in indicated Ral siRNA-treated sample; *bars,* SD. *C,* quantitative RT-PCR analysis of CD24 expression in UM-UC-3 and HeLa cells 72 hours after treatment with RalA/B or GL2 siRNA. *Columns,* % control CD24 expression; *bars,* SD. *D,* immunoblotting for CD24 in UM-UC-3 and HeLa cells 72 hours after treatment with RalA/B or GL2 siRNA. Blots were stripped and reprobed for tubulin as a loading control. RalA/B knockdown results in down-regulation of CD24. *, $P \leq 0.05$.

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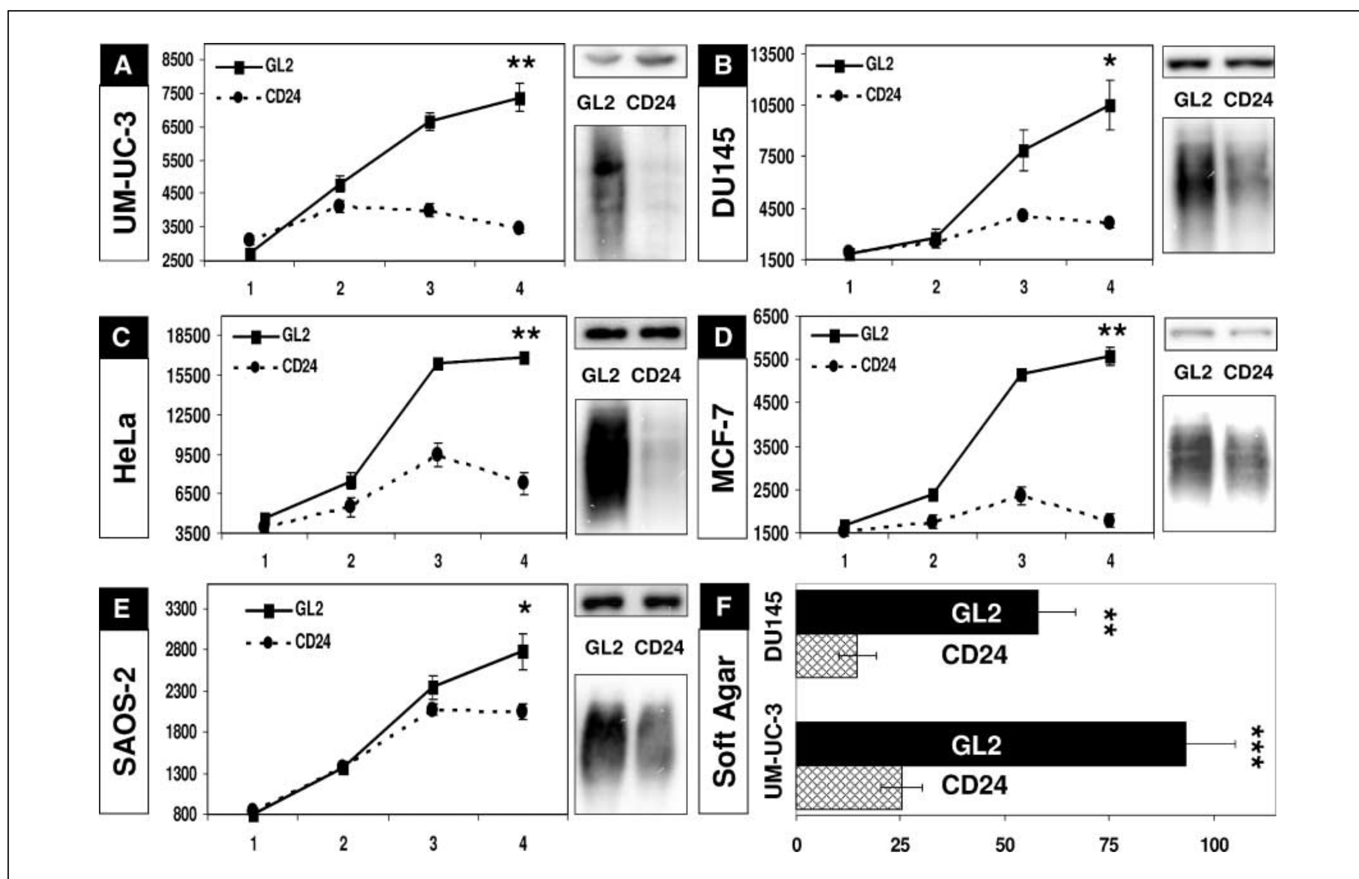

Figure 2. Growth curves and soft agar assays for CD24-depleted cells. After 4 days of growth, CD24 knockdown results in significantly less cells compared with GL2 nontargeting control in (*A*) UM-UC-3 (~53% less), (*B*) DU145 (~66% less), (*C*) HeLa (~57% less), (*D*) MCF-7 (~68% less), and (*E*) SAOS-2 (~26% less). *Points,* representative of multiple experiments using duplicate wells serially assayed using Alamar Blue, with fluorescence reported for each day; *bars,* SD. *Inset,* immunoblotting for CD24 72 hours after treatment with CD24 or GL2 siRNA in indicated cell lines. *Top,* anti-tubulin loading control; *bottom,* anti-CD24. *F,* in UM-UC-3 (15 days) and DU145 (25 days) cells, CD24 knockdown results in significantly reduced clonogenicity on soft agar compared with GL2 control. *Columns,* representative averages of counts of colonies in 1-cm diameter areas of triplicate wells in assays done at least twice; *bars,* SD. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

decreases in cell number. Specifically, over a 4-day period, the decreases in number compared with GL2 control ranged from 26% to 68% and in all cases were significant (Fig. 2*A-E*). This observation of reduced *in vitro* proliferation in five cell lines derived from diverse cancer types underscores an important and fundamental role for CD24 in cellular proliferation of cancers derived from both epithelial and mesenchymal origins.

CD24 knockdown modulates clonogenicity of cancer cells in soft agar. As anchorage-independent growth is one hallmark of tumorigenicity and metastatic competence, we used siRNA for CD24 to determine if UM-UC-3 and DU145 cells are dependent on CD24 expression for their clonogenicity in agar. In UM-UC-3, we observed a ~75% reduction ($P < 0.01$) in colony formation at 15 days, whereas in DU145, we observed a ~73% reduction ($P < 0.001$) in colony formation at 25 days compared with the same cells transfected with GL2 (Fig. 2*F*).

Knockdown of CD24 is associated with significant effects on the actin cytoskeleton and induction of apoptosis. We recently reported the observation that depletion of RalA/B in UM-UC-3 cells was associated with a significant decrease in stress fibers in adherent cells (5). Interestingly, in keeping with a role for CD24 as a Ral transcriptional target, depletion of CD24 was also associated with a significant change in cell morphology and the actin cytoskeleton. In phase contrast, we observed control-transfected cells growing to confluence (Fig. 3*A*) and displaying epithelial morphology identical to untreated cells at 72 hours (data not shown). At 48 hours (Fig. 3*B*) and 72 hours (Fig. 3*C*), CD24 siRNA-treated cells seemed more flat and rounded and exhibited unusual phase-dark radial protrusions. By 72 hours, CD24 siRNA-treated cells began to round up and lose adhesion to the substratum. Floating cells were evident in culture (data not shown). We stained for actin stress fibers in CD24 knockdown cells to ask whether CD24 depletion also resulted in modulation of the actin cytoskeleton. By fluorescence microscopy, at 72 hours, the GL2 control siRNA-treated cells displayed prominent stress fibers (Fig. 3*D*), as described before (5). However, in CD24 siRNA-treated cells at 48 hours (Fig. 3*E*) or 72 hours (Fig. 3*F*), cells showed increasingly disordered morphology, loss of stress fibers, rounding, and nuclear condensation evident in Hoechst 33342 nuclear staining at 72 hours, suggestive of apoptotic induction.

Having observed nuclear condensation microscopically, we did cell cycle analysis using propidium iodide staining and flow cytometry on UM-UC-3 and DU145 cells 96 hours after transfection to assess the proportion of cells undergoing apoptosis as a result of CD24 depletion and subsequent cytoskeletal rearrangements. Apoptosis, as defined by a sub-G_0-G_1 hypodiploid population in CD24 siRNA-treated samples of these cell lines (Fig. 3*G* and *H*), occurred in 12.2% and 13.2% of UM-UC-3 and DU145 cells,

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respectively, compared with GL2-treated controls (in both cell lines <1%). Taken together with the effects on agar growth, these results suggest that the effects of CD24 on the metastatic competence are likely mediated in part by its effects on survival.

CD24 protein expression in human cancer tissue is a prognostic factor for survival. CD24 overexpression has been reported in many tumor types (6). As we observed a striking *in vitro* phenotype following CD24 depletion in cell lines derived from breast and prostate cancer, in two common malignancies, where CD24 immunohistochemistry has been proven to be of prognostic value (6), we investigated a potential association of CD24 expression with bladder cancer.

Using previously generated microarray data (11), we found an ∼4.4 fold overexpression of CD24 in bladder cancer compared with normal mucosa ($P = 0.01$; Fig. 4*A*). We also analyzed other publicly available microarray data sets to ascertain if CD24 is a biomarker for tumor types not yet reported. Using the Lenberg et al. oligonucleotide microarray series of renal cell carcinomas and normal kidney tissue (12), we note that CD24 is ∼3.3-fold overexpressed in renal cell carcinoma ($P < 0.01$). In the Chen et al. cDNA microarray series of hepatocellular carcinomas and human liver tissue samples that are publicly available (13), we find that CD24 is overexpressed in hepatocellular carcinoma compared with normal liver ($P < 0.01$).

Based on these results, we carried out immunohistochemistry for CD24 on a tissue microarray of human bladder cancer patients (14). Although we found no statistically significant associations between clinical stage or grade with CD24 expression, adjustment for differences in stage and grade through multivariate analyses showed that patients with higher levels of CD24 (CD24, 3+) tended to have shorter disease-free survival times than those with lower levels (CD24, 1+), approaching significance even in our small cohort ($P = 0.07$). Figure 4*B* shows representative histologic sections for levels of CD24 staining, and Fig. 4*C* shows Kaplan-Meier estimation of disease-free survival time by CD24 expression. This observation that CD24 is an independent prognostic factor for bladder cancer disease-free survival is striking. It further extends the breadth of the prognostic use of CD24 immunohistochemistry to bladder cancer, in addition to breast, prostate, lung (6), and colon (17) cancers, which together comprise a vast majority of solid

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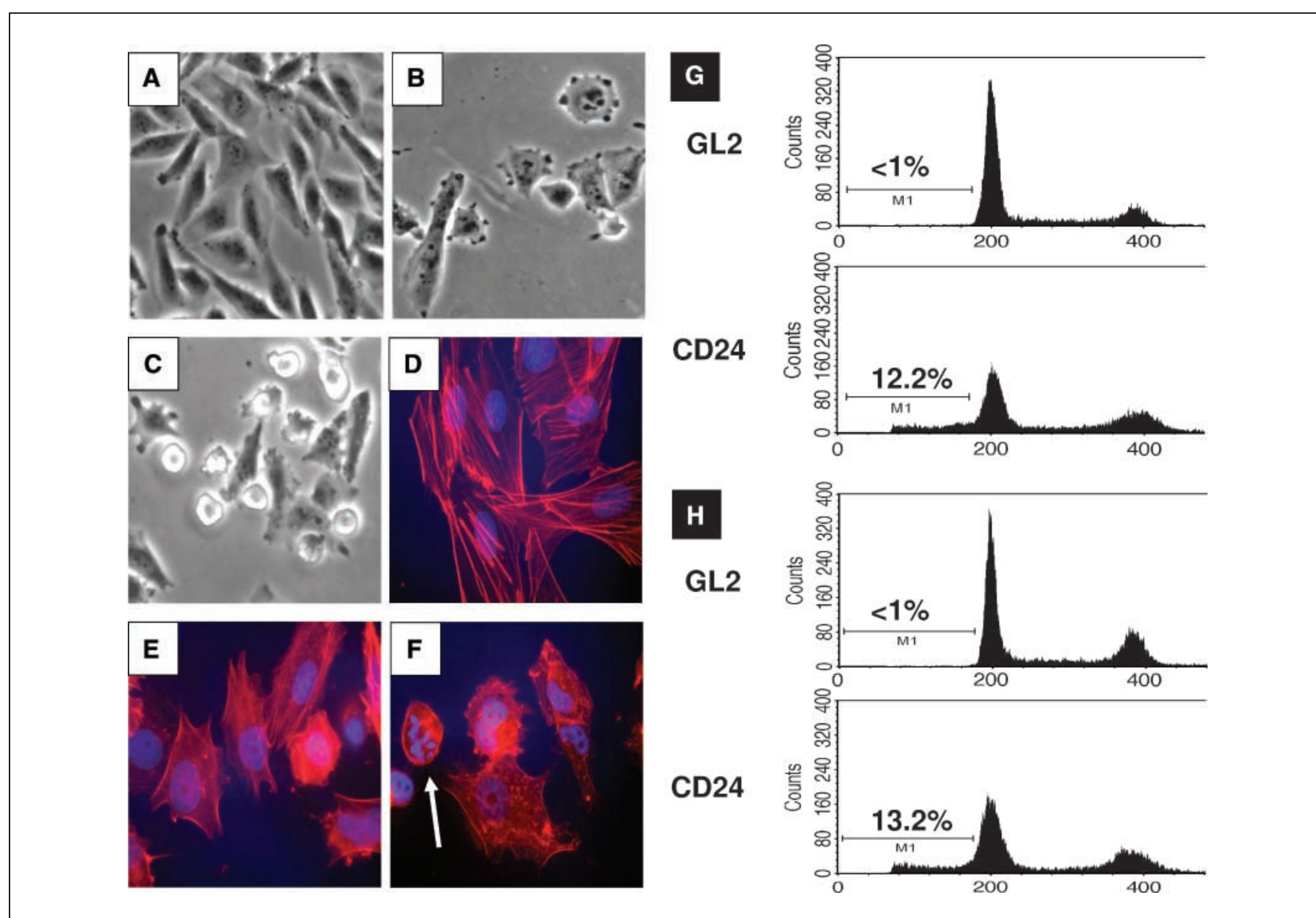

Figure 3. Microscopy and cell cycle analysis of CD24-depleted cells. *A,* phase-contrast microscopy of GL2 siRNA-treated UM-UC-3 cells reaching confluence at 72 hours after transfection. *B,* CD24 siRNA-treated UM-UC-3 cells at 48 hours are flattened and produce phase dark cytoplasmic protrusions. *C,* CD24 siRNA-treated cells at 72 hours rounded up and lost adhesion, and floating cells were apparent in culture. *D,* GL2 siRNA-treated UM-UC-3 cells at 72 hours exhibit latitudinal and longitudinal stress fibers on phalloidin staining, as previously reported. *E,* CD24 siRNA-treated cells at 48 hours seem to lose stress fiber organization of the actin cytoskeleton. *F,* CD24 siRNA-treated cells at 72 hours show loss of stress fibers, rounding, and an apoptotic figure visible on Hoechst 33342 nuclear staining (*arrow*). *G,* cell cycle analysis by propidium iodide staining for UM-UC-3 bladder cancer cells, 4 days after transfection with CD24 or GL2 siRNA, as indicated. Propidium iodide stains the DNA complement in cells, and DNA fragmentation, a hallmark of apoptosis, is represented by a shift in DNA content to the left of the main G_0-G_1 peak at 200, the hypodiploid range subtended by the M_1 markers indicated. *H,* similar analysis to that in (*G*) on DU145 prostate cancer cells.

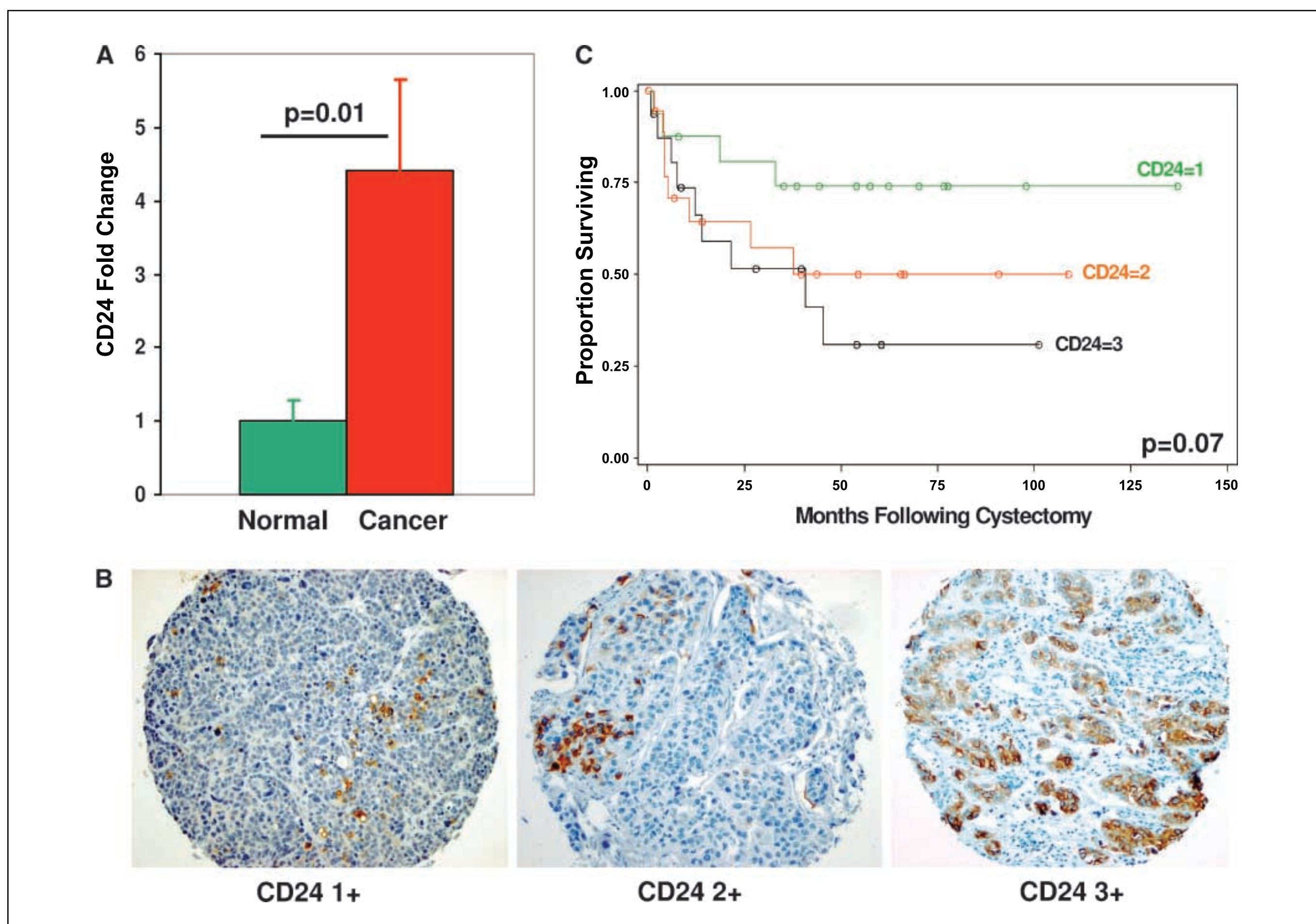

Figure 4. CD24 in human bladder cancer. *A,* Affymetrix analysis expression analysis of CD24 mRNA shows fold overexpression in bladder cancer compared with normal bladder mucosa. *Columns,* fold change; *bars,* SE. *B,* tissue microarray staining for CD24. Representative scoring of 1+, 2+, and 3+ levels. *C,* Kaplan-Meier estimation of disease-free survival as a function of CD24 immunohistochemical expression staining.

malignancies diagnosed in the United States (18). Furthermore, as most patients with bladder cancer die of metastatic disease, this supports the notion that CD24 is an important contributor to metastatic competence.

A model for CD24 function. To date, the functions ascribed to CD24 include induction of apoptosis (9), localization with lipid rafts (8, 9), and activation of tyrosine kinase signaling (8), assayed via inactivating approaches using extracellular antibody-mediated crosslinking. However, as study authors have noted (9), the physiologic correlate or specificity to CD24 function of these methods is unknown. Overexpression experiments are also limited, as invasive cancer cell lines may either not change their phenotypes due to limiting numbers of signaling intermediates or alter them in a potentially artifactual way. Here, we overcome these limitations by using a specific, reverse genetic approach to deplete CD24 expression and characterize effects in cell lines from a diverse set of cancer types.

Friederichs et al. recently reported that CD24 could function as a cell adhesion molecule in a murine tail vein model of metastasis (7). In this study, CD24, as a ligand for P-selectin, mediated pulmonary arrest of adenocarcinoma cells when stably cotransfected with an appropriate fucosyltransferase for sialylLe^x modification. A priori, this metastatic adhesive function alone could potentially explain the clinical prognostic associations of CD24. However, our findings of a CD24 role in proliferation and survival of cancer cells as well as the observations here and elsewhere (6) of overexpression of CD24 at many primary tumor sites support a role in transformation and tumorigenesis. Interestingly, CD24 has been shown to modulate integrin function, including LFA-1, or $\alpha_L\beta_2$ (19) and VLA-4, or $\alpha_4\beta_1$ (20), in leukocyte adhesion studies. Cell death in response to loss of integrin-mediated cell-matrix adhesion has been termed "anoikis," and recent reports find that "anoikis resistance" may be an essential property of metastasis-competent cells (reviewed in ref. 21). Given our actin cytoskeletal findings in CD24-depleted cells, we are currently investigating whether CD24 functions through regulating integrin-mediated survival signals in cancer cells. Taken together, these data support a dual-functional model for CD24 in cancer: both in proliferation and survival at the tumor origin and metastases as well as adhesive function during hematogenous dissemination of cancer cells. Clearly, *in vivo* systems employing cells stably expressing RNA interference for CD24 are necessary to address the relative contribution of CD24 to tumorigenesis versus metastasis.

In summary, our results suggest a novel and important role for CD24 in the regulation of proliferation and survival of cancer cells. We have shown that CD24 is regulated by Ral GTPases, which

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suggests that CD24, and potentially other transcriptional targets of Ral signaling, may mediate important functional aspects of this GTPase subfamily that is emerging as a central player in cancer. This report of a causal role for CD24 in growth and survival of many cancer cells of various histologic types, as well as the apparent generality of CD24 overexpression in different tumors, highlights the need to strongly consider this molecule as a therapeutic target.

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