Hyaluronan-CD44 Interaction with Neural Wiskott-Aldrich Syndrome Protein (N-WASP) Promotes Actin Polymerization and ErbB2 Activation Leading to β-Catenin Nuclear Translocation, Transcriptional Up-regulation, and Cell Migration in Ovarian Tumor Cells

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In this study we have investigated the interaction of hyaluronan (HA) and CD44 with the neuronal Wiskott-Aldrich syndrome protein (N-WASP) in regulating actin polymerization and ErbB2/β-catenin signaling in human ovarian tumor cells (SK-OV-3.ip1 cells). Biochemical and immunological analyses indicate that N-WASP is expressed in SK-OV-3.ip1 cells and that the binding of HA stimulates N-WASP association with CD44 and Arp2/Arp3 leading to filamentous actin formation and ovarian tumor cell migration. In addition, HA binding promotes CD44-N-WASP association with ErbB2 and activates ErbB2 kinase activity that in turn increases phosphorylation of the cytoskeletal protein, β-catenin. Subsequently, phosphorylated β-catenin is transported into the nucleus leading to β-catenin-mediated TCF/LEF transcriptional co-activation. Because HA-induced β-catenin phosphorylation, nuclear translocation, and TCF/LEF transcriptional activation is effectively blocked by the ErbB2 inhibitor, AG825, we conclude that HA/CD44-N-WASP-associated ErbB2 activation is required for β-catenin-mediated signaling events. Transfection of SK-OV-3.ip1 cells with N-WASP-VCA (verpolin homology, cofilin homology, and acidic domain) fragment cDNA not only blocks HA/CD44-induced N-WASP-Arp2/3 complex formation but also inhibits actin polymerization/F-actin assembly and tumor cell migration. Overexpression of the N-WASP-VCA domain also significantly reduces HA-induced ErbB2 recruitment to CD44, diminishes β-catenin phosphorylation/nuclear translocation, and abrogates TCF/LEF-specific transcriptional co-activation by β-catenin. Taken together, our findings strongly suggest that N-WASP plays a pivotal role in regulating HA-mediated CD44-ErbB2 interaction, β-catenin signaling, and actin cytoskeleton functions that are required for tumor-specific behaviors and ovarian cancer progression.

Ovarian carcinoma is the most lethal tumor of the female genital tract and continues to be a major cause of mortality in female cancer patients. A common mechanism for the spread of ovarian cancer is the shedding of cells from the primary tumor into the peritoneal cavity (1). This event is then followed by the formation of secondary tumor masses attached to the bowel and omental surface, both of which are covered by a single layer of mesothelial cells (2). Extracellular matrix components, such as hyaluronan (HA), 2 are present in large amounts in the mesothelial lining of the peritoneum (3, 4). HA is often bound to CD44, which is a ubiquitous, abundant, and functionally important surface receptor that displays HA-binding site(s) (5). Both CD44 and HA are overexpressed at sites of tumor attachment and are involved in tumor cell-specific functions (3, 6–9). Thus, it has been postulated that CD44 interaction with HA may be one of the important requirements for the spread of ovarian cancer.

CD44 denotes a family of cell-surface glycoproteins that are expressed in a variety of human solid neoplasms, particularly those of gynecologic origin (e.g. ovarian cancers) (6, 7). One of the distinct features of CD44 is the enormous heterogeneity in the molecular masses of this family of proteins. It is known that CD44 is encoded by a single gene that contains 19 exons (10), and out of the 19 exons, 12 exons can be alternatively spliced (10). Most often, the alternative splicing occurs between exons 5 and 15 leading to an insertion in tandem of one or more variant exons (v1–v10, involving exons 6–14 in human cells) within the membrane proximal region of the extracellular domain (10). The binding of HA to CD44 isoforms triggers oncogenic signals (9) and direct “cross-talk” between two different tyrosine kinase-linked (ErbB2 (p185HER2)/EGFR tyrosine kinases (11, 12) and c-Src kinase (13)) signaling pathways (cell growth versus cell migration, respectively). Most importantly, the cytoplasmic domain of CD44 isoforms selects its unique downstream effectors (e.g. cytoskeletal proteins, 2)

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2 The abbreviations used are: HA, hyaluronan; N-WASP, neuronal Wiskott-Aldrich syndrome protein; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorter; F-actin, filamentous actin; WASP, Wiskott-Aldrich syndrome protein; siRNA, small interfering RNA; TCF/LEF, T-cell factor/lymphocyte enhancer factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; WH1, WASP-homology 1.
ankyrin (14–16)/ERM (17) or various oncopgenic signaling molecules, Tiam1 (18), Vav2 (19), RhogeF/RhoA-activated ROK (20, 21), Cdc42-IQGAP1 (22), and PKN (23)) and coordinates intracellular signaling pathways (e.g. Rho/Ras signaling (12, 19) and receptor-linked (11, 12, 19, 24)/nonreceptor-linked tyrosine kinase/serine-threonine pathways (13, 23)) to generate a concomitant onset of multiple cellular functions (e.g. cytoskeleton activation, tumor cell adhesion, growth, migration, and invasion) leading to tumor progression.

The Wiskott-Aldrich syndrome protein (WASP) family of proteins includes two types of hematopoietic WASP proteins, the ubiquitously expressed neural WASP (N-WASP) (25, 26) and three WASP family verprolin-homology proteins (WAVEs) (27, 28). These proteins integrate upstream signaling events with changes in the actin cytoskeleton (29, 30). WASP family proteins such as N-WASP contain a number of functional domains and motifs known to interact with both the cytoskeleton and various signaling complexes. These domains include a verprolin homology, a coflin homology, and an acidic domain (VCA) at the C terminus; a proline-rich region in the center of the molecule, a GTPase binding domain/CRIB motif, a WASP-homology 1 (WH1) domain, and a basic region at the N terminus (25, 26, 28). Some of these motifs are involved in N-WASP interaction with specific proteins, such as Cdc42 (32), WASP-interacting protein (33), Src (34), Nck (35), Grb2/Ash (34), phospholipase Cγ (34), and phosphatidylinositol 3-kinase (34). In particular, the verprolin homology (V) binds to actin directly, whereas the coflin homology-acidic (CA) region interacts with an actin-related protein (Arp) 2/3 complex (a 7-component protein complex), initiating the nucleation reaction of actin filaments in vitro and inducing actin polymerization/filamentous actin (F-actin) formation in vivo (26–28, 36). Thus, these findings suggest that N-WASP not only serves as a scaffolding protein (mediating multiprotein Arp2/3 complex aggregation) but also directly participates in F-actin assembly and cytoskeleton reorganization. The VCA domain also interacts with the middle region of the N-WASP, generating an autoinhibitory configuration and preventing Arp2/3 association. Subsequently, this VCA-mediated autoinhibitory process blocks actin polymerization (37, 38). However, a number of signaling regulators (e.g. Cdc42, phosphatidylinositol bisphosphate (39), Grb2/Ash (40), Nck (41), WISH (42), and Src family tyrosine kinases (43)) can promote the ability of Arp2/3 to nucleate actin filaments by releasing the autoinhibitory conformation of inactive N-WASP. Thus, the C-terminal VCA sequence of N-WASP is considered as one of the important regulatory domains of N-WASP in regulating F-actin formation and cytoskeleton function required for a variety of cellular functions, including filopodia/lamellipodia formation (27, 37) and vesicle movement (44–47).

The HER2 oncogene (also called ErbB2 or neu) encodes a 185-kDa membrane protein (p185HER2) that contains a single transmembrane spanning region and a tyrosine kinase-associated cytoplasmic domain (48, 49). Overexpression or amplification of HER2 oncogenes appears to correlate with poor survival rates of many known cancers, including ovarian cancer (50, 51). However, the cellular and molecular mechanisms by which ErbB2 enhances the growth and survival of ovarian cancer cells are not completely understood. Previously, we have determined that CD44 and ErbB2 are physically linked to each other via interchain disulfide bonds (11) or signaling linker molecules (19) in human ovarian tumor cells (SK-OV-3.ipl cell line). The binding of HA to a CD44-associated ErbB2 complex stimulates ErbB2 kinase activity and promotes Ras-mediated stimulation of a downstream kinase cascade, which includes the Raf-1/MEK/MAPK(ERK) pathway leading to tumor cell growth and migration (11, 19).

The cytoskeletal protein β-catenin is a multifunctional protein known to play a key role in cell-cell adhesion and the Wnt signaling pathway (52–55). In normal cells, β-catenin is associated with E-cadherin and mediates cell-cell adhesion (52–55). To maintain homeostasis, the β-catenin/E-cadherin association is regulated in part by serine/tyrosine phosphorylation on specific residues. Serine phosphorylation in the N terminus of β-catenin targets it for proteosomal degradation, whereas tyrosine phosphorylation in the C terminus of β-catenin influences its interaction with E-cadherin (52–55). In the Wnt signaling pathway, glycogen synthase kinase-3β (GSK-3β, a kinase that normally phosphorylates excess β-catenin and causes its ubiquitination and degradation) is inactivated when soluble Wnt protein binds to the frizzled receptor (52–55). β-Catenin then accumulates in the cytoplasm and translocates to the nucleus where it can interact with members of the TCF/LEF-enhancing factor family as well as other transcription factors (56, 57).

β-Catenin also contributes to oncogenesis of some tumors (52, 55). In cancer cells, an uncomplexed phosphorylated form of β-catenin also accumulates in the cytoplasm prior to translocation into the nucleus where it binds to the TCF/LEF family of transcription factors and activates transcription of downstream genes such as cyclin D1 and c-myc (58, 75). β-Catenin has been shown to be linked to ErbB2 tyrosine kinase or other receptor tyrosine kinases (59–63). In particular, β-catenin phosphorylation by ErbB2 tyrosine kinase destabilizes the E-cadherin-β-catenin complexes leading to a decrease of cell adhesion and a shifting of β-catenin into the oncogenic pathway (59–64). Until the present time, the mechanism by which HA/CD44 activation of ErbB2 modulates β-catenin-mediated signal transduction and oncogenesis in ovarian tumor cells has not been addressed.

In this study, we have discovered a new mechanistic role for HA/CD44-mediated N-WASP signaling and ErbB2 activation that is centered on F-actin formation and β-catenin phosphorylation/nuclear translocation. These events then promote cell migration and transcriptional up-regulation in ovarian tumor cells.

MATERIALS AND METHODS

Cell Culture—The SK-OV-3.ipl cell line was established from ascites that developed in a nu/nu mouse given an intraperitoneal injection of SK-OV-3 human ovarian carcinoma cell line (obtained from the American Type Culture Collection) as described previously (11, 13, 19, 22). Cells were grown in Dulbecco’s modified Eagle’s medium/F-12 medium.
supplemented with 10% fetal bovine serum. Cells were routinely serum-starved (and therefore deprived of serum HA) before adding HA.

**Antibodies and Reagents**—Monoclonal anti-CD44 antibody (clone 020; isotype IgG2a; obtained from CMB-TECH, Inc., San Francisco) recognizes a determinant of the HA binding region common to CD44 and its principal variant isoforms (11–24). This rat anti-CD44 was routinely used for HA-related blocking experiments and immunoprecipitation. In SK-OV-3.ipl cells, the CD44 standard form (CD44s) is the predominant CD44 isoform precipitated by this anti-CD44 antibody. Immuno-reagents such as mouse anti-N-WASP antibody, goat anti-Arp2 antibody, rabbit anti-Arp3 antibody, and mouse anti-β-catenin antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Both rabbit anti-ErbB2 antibody and rabbit anti-phospho-ErbB2 antibody were obtained from Upstate (Charlottesville, VA). Mouse anti-phosphotyrosine antibody and AG825 were purchased from Cell Signaling Technology (Beverly, MA) and Calbiochem, respectively. FITC-labeled phalloidin and Topro-3 were obtained from Molecular Probes. Healon HA polymers (~500,000 dalton polymers) purchased from Pharmacia & Upjohn Co. (Kalamazoo, MI) were prepared by gel filtration column chromatography using a Sephacryl S1000 column. The purity of the HA polymers used in our experiments was further verified by anion exchange high performance liquid chromatography followed by protein and endotoxin analyses using BCA protein assay kit (Pierce) and an in vitro Limulus amebocyte lysate assay (Cambrex BioScience Walkersville Inc., Walkersville, MD), respectively. No protein or endotoxin contamination was detected in this HA preparation.

**DNA Constructs**—A construct for expression of the C-terminal part of rat N-WASP containing the verprolin homology, cofilin, and acidic domains (VCA, amino acids 391–501, N-WASP-VCA) in mammalian cells (kindly provided by Britta Qualmann and Regis B. Kelly, University of California, San Francisco) was generated by PCR with primer 5′-CGCTCGAGGGTGACCATCAAGTTCCAG-3′ and 5′-CGGAATTCA-GTCTTCCACTCATC-3′ using rat N-WASPcDNA as a template. The PCR product was cloned into the Xhol-EcoRI sites of a derivative of the pEGFP-C1 vector (Clontech), in which green fluorescent protein was replaced by the hemagglutinin tag.

**Cell Transfection**—To establish a transient expression system, SK-OV-3.ipl cells were transfected with various plasmid DNAs (e.g. hemagglutinin-tagged N-WASP-VCA or vector alone) using Lipofectamine 2000 methods (Invitrogen). Briefly, cells were plated at a density of 2 × 10^6 cells per 100-mm dish and transfected with 25 μg/dish plasmid cDNA using Lipofectamine 2000. Transfected cells were grown in the culture medium for at least 24–48 h. Various transfectants were then analyzed for their protein expression and functional properties as described above.

**RNA Oligonucleotides**—The siRNA sequence targeting human β-catenin (from mRNA sequence, GenBank™ accession number AJ251595) corresponds to the coding region relative to the first nucleotide of the start codon. Target sequences were selected using the software developed by Ambion Inc., UK. As recommended by Ambion, β-catenin-specific targeted regions were selected beginning 50–100 nucleotides downstream from the start codon. Sequences close to 50% G/C content were chosen. Specifically, the β-catenin target sequence (5′-AGCGUAUUAGUGAGAC-3′) and scrambled sequences (5′-AGUGCGGAAGUAUCCG-3′) were used. β-Catenin-specific target sequences were then aligned to the human genome data base in a BLAST search to eliminate sequences with significant homology to other genes. Sense and antisense oligonucleotides were provided by the Biomolecular Research Unit, University of California, San Francisco. For construction of the siRNA, a transcription-based kit from Ambion was used (Silencer™ siRNA construction kit). SK-OV-3.ipl cells were then transfected with siRNA using siPORT Lipid as transfection reagent (Silencer™ siRNA transfection kit; Ambion, TX) according to the protocol provided by Ambion. Cells were incubated with 50 pmol of β-catenin siRNA or 50 pmol of siRNA containing scrambled sequences or no siRNA for at least 48 h before biochemical experiments and/or functional assays were conducted as described below.

**Preparations of Cell Lysate, Cytosolic and Nucleus Fractions**—SK-OV-3.ipl cells (untransfected or transfected with N-WASP-VCAcDNA or vector alone) were serum-starved for 24 h followed by incubation with 50 μg/ml HA (or pretreated with anti-CD44 antibody or 25 μM AG825 for 1 h followed by HA treatment or no HA addition) for various time intervals (e.g. 0.5, or 30 min) at 37 °C. Cells were then lysed in a lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM MgCl2, 0.5% Nonidet P-40, 0.2 mM Na2VO4, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 5 μg/ml aprotinin). The lysate was then homogenized by 30 strokes in a tight fitting Dounce homogenizer. Both cytoplasmic and nuclear fractions were prepared using the extraction kit from Active Motif (Carlsbad, CA) with some modifications. Briefly, the cell homogenate was centrifuged at 1,500 × g for 5 min to sediment the nuclei. The supernatant was then recentrifuged at 15,000 × g for 5 min, and the resulting supernatants form “the cytosolic fraction.” The nuclear pellet was washed three times and resuspended in the same buffer containing 0.5 M NaCl to extract nuclear proteins. The extracted material was centrifuged at 15,000 × g for 10 min, and the resulting supernatant was termed “the nuclear fraction” (65).

**Immunoblotting and Immunoprecipitation Techniques**—The cell lysate or the cytosolic fraction isolated from SK-OV-3.ipl cells (untransfected or transfected with N-WASP-VCAdNA or vector alone in the presence of 50 μg/ml HA (or pretreated with anti-CD44 antibody or 25 μM AG825 for 1 h followed by HA treatment or no HA addition) for various time intervals (e.g. 0, 5, or 30 min) at 37 °C) was immunoblotted using various immunoreagents (e.g. mouse anti-N-WASP antibody (5 μg/ml) or rabbit anti-ErbB2 (5 μg/ml) or rabbit anti-phospho-ErbB2 (5 μg/ml)).

In addition, immunoprecipitation was conducted after homogenization of the cell lysate using rat anti-CD44 antibody followed by goat anti-rat IgG beads. Subsequently, the immunoprecipitated materials were solubilized in SDS sample buffer, electrophoresed, and blotted onto the nitrocellulose. After blocking nonspecific sites with 3% bovine serum albumin, the nitrocellulose filter was incubated with various antibodies (e.g.
mouse anti-N-WASP antibody (5 μg/ml), goat anti-Arp2 antibody (5 μg/ml), rabbit anti-Arp3 antibody (5 μg/ml), rabbit anti-ErbB2 antibody (5 μg/ml), or rat anti-CD44 antibody (5 μg/ml), respectively) for 1 h at room temperature. The supernatant fraction following anti-CD44-mediated immunoprecipitation was also used for anti-N-WASP-mediated immunoprecipitation plus immunoblotting with anti-Arp2/anti-Arp3/anti-ErbB2 or anti-ErbB2-mediated immunoprecipitation plus anti-ErbB2-mediated immunoblotting.

In some experiments, the cytosolic fraction isolated from SK-OV-3.ipl cells ((untransfected or transfected with N-WASP-VCAcDNA or vector alone) in the presence of 50 μg/ml HA (or pretreated with anti-CD44 antibody or 25 μM AG825 for 1 h followed by HA treatment or no HA addition) for various time intervals (e.g., 0, 5, or 30 min) at 37 °C) was immunoprecipitated with mouse anti-β-catenin antibody-conjugated beads followed by immunoblotting with mouse anti-phosphotyrosine antibody or mouse anti-β-catenin antibody. The cytosolic fraction of cells (transfected with N-WASP-VCAcDNA or vector alone) was also subjected to immunoprecipitation using mouse anti-N-WASP antibody-conjugated beads followed by immunoblotting with goat anti-Arp2 antibody or rabbit anti-Arp3 antibody. These samples were then incubated with horseradish peroxidase-conjugated secondary antibodies (e.g. rabbit anti-goat IgG, goat anti-rabbit IgG, or goat anti-mouse IgG (1:10,000 dilution)) at room temperature for 1 h. The blots were then developed using ECL reagent (Amer sham Biosciences) according to the manufacturer’s protocols.

In some cases, the nuclear fractions of SK-OV-3.ipl cells (untransfected, transfected with N-WASP-VCAcDNA, or vector alone and incubated with 50 μg/ml HA (or no HA) for various time intervals (e.g. 0, 5, or 30 min) at 37 °C) were processed for immunoprecipitation using mouse anti-β-catenin antibody-conjugated beads followed by immunoblotting with mouse anti-phosphotyrosine antibody or mouse anti-β-catenin antibody, respectively. In some experiments, anti-β-catenin-mediated immunoblot of cell lysates (isolated from β-catenin siRNA-scrambled sequence-treated cells or β-catenin siRNA-treated cells) was also carried out as described above.

Pyrene-Actin Assay—To measure actin polymerization in a cell-free system, pyrene-actin assay was performed as described previously (66). Specifically, 100 nm N-WASP (or recombinant VCA) isolated from SK-OV-3.ipl cells was incubated with 60 nm Arp2/3 complex, 2 μM G-actin, and 0.2 μM pyrenyl-actin in a buffer containing 10 mM HEPES (pH 7.9), 100 mM KCl, 2 mM MgCl₂, 0.2 mM CaCl₂, and 5 mM EGTA. Actin polymerization was then monitored with a fluorescence microscope.

FACS Quantitation of F-actin—For measuring total F-actin levels, FACS analysis was used to quantitate FITC-phalloidin staining in fixed cell populations (67). SK-OV-3.ipl cells ((untransfected or transfected with N-WASP-VCAcDNA or vector alone) were grown in 6-cm plates in the presence of 50 μg/ml HA (or pretreated with anti-CD44 antibody or 20 μg/ml cytochalasin D) for 1 h followed by HA treatment or no HA treatment) for various time intervals (e.g., 0, 5, or 30 min) at 37 °C. First, cells were trypsinized, washed sequentially with Dulbecco’s modified Eagle’s medium, 1% horse serum/PBS, and then resuspended in 0.5 ml of PBS. Cells were then fixed by the addition of 0.5 ml of 8% paraformaldehyde in PBS for 10 min at room temperature, washed in PBS, permeabilized in 1 ml of 0.3% Triton X-100 in PBS. Subsequently, permeabilized cells were labeled with 33 nM FITC-phalloidin to measure F-actin content in 5% fetal calf serum in PBS for 45 min at 37 °C. Finally, FITC-phalloidin-labeled cells were analyzed using a FACSCalibur™. Mean F-actin content was evaluated and expressed as a percentage of that of untransfected cells (with no HA treatment) or vector-transfected cells (with no HA treatment) from the same experiment.

Immunofluorescence Staining—SK-OV-3.ipl cells (transfected with N-WASP-VCAcDNA or vector alone) were incubated with HA (50 μg/ml) at 37 °C for various time intervals (e.g., 0, 10, 30, or 60 min) (or pretreated with various agents (e.g., anti-CD44 antibody or 25 μM AG825 or 20 μg/ml cytochalasin D) followed by adding HA (50 μg/ml) or no HA). These cells were then fixed with 2% paraformaldehyde. Subsequently, these cells were rendered permeable by ethanol treatment followed by incubating with FITC-conjugated anti-β-catenin antibody followed by a monoclonal cyanine nucleic acid staining, Topro-3 (a marker for nucleus) or stained with Texas Red-conjugated phalloidin alone (to locate F-actin). To detect nonspecific antibody binding, Topro-3-labeled cells were incubated with FITC-conjugated normal IgG, respectively. No labeling was observed in such control samples. These fluorescence-labeled samples were then examined with a confocal laser scanning microscope.

Luciferase Reporter Assays—Transactivation assays were conducted with SK-OV-3.ipl cells (untreated or pretreated with anti-CD44 antibody, 25 μM AG825, or 20 μg/ml cytochalasin D or transfected with N-WASP-VCAcDNA (or vector alone) or treated with 50 pmol of scrambled siRNA or 50 pmol of β-catenin siRNA). Following 24 h of HA (50 μg/ml) treatment (or no HA treatment), these cells (or transfectants) grown in 35-mm diameter dishes were transfected with 1.0 μg of a plasmid containing a multimeric TCF/LEF-1 consensus-binding sequence driving the luciferase reporter gene (pTop-flash) or a mutant inactive form (pFop-flash) (kindly provided by Robert Nissen, University of California and Veterans Affairs Medical Center, San Francisco). pTop-flash, but not pFop-flash, is responsive to co-activation of TCF/LEF by β-catenin (68). Therefore, relative luciferase units were expressed as the amount of pTopflash-derived luciferase activity divided by the amount from control pFop-flash. A plasmid encoding β-galactosidase (1.0 μg) was also co-transfected to enable normalization for transfection efficiency. After 24 h, expression of the reporter (luciferase) and the control (β-galactosidase) genes was determined using enzyme assay systems from Promega as per the manufacturer’s instructions.

Tumor Cell Migration Assays—Twenty four transwell units were used for monitoring in vitro cell migration as described previously (12, 13, 18—24). Specifically, the 5-μm porosity polycarbonate filters (CoStar Corp., Cambridge, MA) were used for the cell migration assay. SK-OV-3.ipl cells (untransfected, transfected with N-WASP-VCAcDNA, or vector alone in the presence or absence of rat anti-CD44 antibody (5 μg/ml), 20 μg/ml cytochalasin D, or 50 pmol of scrambled siRNA or 50 pmol of β-catenin siRNA) were placed in the upper chamber of the transwell unit. The medium containing 50 μg/ml HA or no
HA was placed in the lower chamber of the transwell unit. After 18 h of incubation at 37 °C in a humidified 95% air, 5% CO₂ atmosphere, cells on the upper side of the filter were removed by wiping with a cotton swab. Cell migration processes were determined by measuring the cells that migrated to the lower side of the polycarbonate filters by standard cell number counting methods. The CD44-specific cell migration was determined by subtracting nonspecific cell migration (i.e. cells migrate to the lower chamber in the presence of anti-CD44 antibody treatment). Each assay was performed in triplicate and repeated at least five times. The number of ovarian tumor cell migration in untreated SK-OV-3.ipl cells (control) or in SK-OV-3.ipl cells treated with no HA (or vector-transfected cells treated with no HA) is designated as 100%. All data were analyzed statistically by Student’s t test, and statistical significance was set at p < 0.01.

RESULTS

HA/CD44-activated N-WASP Signaling and F-actin Formation in Ovarian Tumor Cells

The basic cellular and molecular processes underlying ovarian tumor cell invasion and metastasis are poorly understood at the present time. It is quite clear, however, that transmembrane interactions between receptor(s) and actin cytoskeleton are involved in tumor cell motility, invasion of surrounding tissue, and metastasis (69, 70). The details of these interactions in ovarian tumor cell movement and infiltration of surrounding tissue remain largely unknown.

A number of studies have been aimed at identifying the specific molecules expressed by tumor cells that correlate with actin-based tumor motility and metastatic behavior. One possible candidate in this area is N-WASP, which has been shown to play an important role in signal transduction and F-actin formation (27–47). Using a specific anti-N-WASP-mediated immunoblot technique, we have found that significant amounts of N-WASP (molecular mass ~55 kDa) are expressed in SK-OV-3.ipl cells (Fig. 1A, lane 2). We believe that the N-WASP protein detected by anti-N-WASP-mediated immunoblot is specific because no protein is detected in those cells incubated with preimmune rabbit IgG (Fig. 1A, lane 1).

In addition, we have addressed the question of whether there is a physical linkage between CD44 and N-WASP in ovarian tumor cells (SK-OV-3.ipl cell line). To this end we first carried out anti-CD44-mediated immunoprecipitation followed by anti-N-WASP immunoblot (Fig. 1B–(I), panel a) or anti-CD44 immunoblot (Fig. 1B–(I), panel d), respectively, using cells treated with HA (or no HA). Our results indicate that HA treatment causes the recruitment of a significant amount of N-WASP (Fig. 1B–(I), panel a, lane 2) into the CD44 complex (Fig. 1B–(I), panel d, lane 2). In contrast, a low level of N-WASP (Fig. 1B–(I), panel a, lane 1) is present in the anti-CD44-immunoprecipitated materials (reblotted with anti-CD44) in cells treated with no HA (Fig. 1B–(I), panel d, lane 1) or in cells pretreated with anti-CD44 antibody followed by HA treatment (Fig. 1, B–(I), panel a, lane 3, and panel d, lane 3). These findings clearly establish that CD44 and N-WASP are closely associated with each other, and there is a significant increase in the complex following HA treatment of the ovarian tumor cells.

In response to external signals, the N-WASP often interacts with Arp2/3 complex and G-actin to stimulate actin polymerization in cells (26–28, 36). In this study we have demonstrated that HA induces recruitment of Arp2 (Fig. 1B–(I), panel b, lane 2) and Arp3 (Fig. 1B–(I), panel c, lane 2) into CD44-N-WASP complexes (Fig. 1, B–(I), panel a, lane 3, and panel d, lane 2). In contrast, only a small amount of Arp2/Arp3 complexes is detected in the CD44-N-WASP complexes isolated from cells treated with no HA (Fig. 1, B–(I), panel b, lane 1, and panel c, lane 1) or from cells pretreated with anti-CD44 antibody followed by HA treatment (Fig. 1, B–(I), panel b, lane 3, and panel c, lane 3). These observations suggest that the association between CD44-N-WASP complex (isolated from ovarian tumor cells) and Arp2/3 is HA-dependent and CD44-specific. Using the supernatant fraction following anti-CD44 immunoprecipitation, we have found that N-WASP (immunoprecipitated with anti-N-WASP antibody) contains neither Arp2 nor Arp3 (analyzed by anti-Arp2 or anti-Arp3-mediated immunoblotting) in SK-OV-3.ipl cells treated with HA (Fig. 1, B–(II), panel a, lane 2, and panel b, lane 2). Apparently, N-WASP only binds Arp2 and Arp3 when in complex with CD44 following HA stimulation.

Using a cell-free system and pyrene-labeled actin (a fluorescent derivative of actin known to display higher fluorescence intensity when actin is assembled into filaments), we have observed that N-WASP (isolated from SK-OV-3.ipl cells) markedly activates Arp2/3 complex-induced actin polymerization (Fig. 1C, line a). In contrast, the level of Arp2/3-mediated actin polymerization is relatively low in these samples treated with N-WASP alone (or Arp2/3 alone or the reaction buffer only) (Fig. 1C, lines b and c). These findings suggest that N-WASP is involved in Arp2/3-mediated actin assembly. In addition, our flow cytometry analyses of FITC-phalloidin-labeled SK-OV-3.ipl cells (an assay that selectively detect the expression of polymerized F-actin) indicate that HA-mediated actin polymerization is enhanced by ~295% in SK-OV-3.ipl cells (Table 1). In contrast, F-actin formation is greatly reduced in cells pretreated with anti-CD44 antibody followed by HA treatment (Table 1) or with no HA treatment (Table 1). Treatment of cells with cytochalasin D (an inhibitor known to impair F-actin polymerization) also blocks HA-mediated F-actin formation (Table 1). These findings suggest that HA-CD44 interaction promotes de novo actin polymerization in SK-OV-3.ipl cells.

Furthermore, using in vitro migration assays, we have observed that SK-OV-3.ipl cells undergo active cell migration (enhanced by ~285–290%) during HA treatment (Table 2). However, pretreatment of SK-OV-3.ipl cells with certain reagents, such as anti-CD44 antibody (Table 2, part A) and cytochalasin D (an inhibitor known to impair F-actin polymerization) (Table 2, part B), causes a significant inhibition of HA/CD44-mediated tumor cell migration (Table 2). Together, these findings indicate that HA/CD44-induced F-actin formation is required for ovarian tumor cell migration.
Previous studies have shown that CD44 and ErbB2 are both structurally and functionally linked in ovarian tumor cells (11, 19). To examine the possible relationship between the CD44-N-WASP complex and ErbB2 in SK-OV-3.ipl cells, we have analyzed the anti-CD44-mediated immunoprecipitates from cell lysates by immunoblotting with anti-ErbB2 (Fig. 2A, panel b, lane 1) or anti-N-WASP (Fig. 2A, panel c, lane 1) antibody, respectively. Our results demonstrate that ErbB2 (Fig. 2A, panel b, lane 1) is complexed with both CD44 (Fig. 2A, panel a, lane 1) and N-WASP (Fig. 2A, panel c, lane 1). Furthermore, we have observed that HA treatment of SK-OV-3.ipl cells stimulates a significant increase in the amount of ErbB2 (Fig. 2A, panel b, lane 2) and N-WASP (Fig. 2A, panel a, lane 2), recruited into the CD44-associated (Fig. 2A, panel c, lane 2) signaling complex. Using the supernatant fraction following anti-CD44 immunoprecipitation from cells (untreated (lane 1), treated with HA (50 μg/ml) for 5 min (lane 2), pretreated with anti-CD44 antibody (10 μg/ml) for 1 h followed by HA (50 μg/ml) incubation for 5 min (lane 3)) were immunoprecipitated (IP) with anti-CD44 antibody followed by immunoblotting with anti-N-WASP antibody (panel a), anti-Arp2 antibody (panel b), anti-Arp3 antibody (panel c), or reblotting with anti-CD44 antibody (panel d) as a loading control. B-I, the supernatant fraction following anti-CD44 immunoprecipitation using cells (untreated (lane 1), treated with HA (50 μg/ml) for 5 min (lane 2), or pretreated with anti-CD44 antibody (10 μg/ml) for 1 h followed by HA (50 μg/ml) incubation for 5 min (lane 3), as described in B-II) were immunoprecipitated with anti-N-WASP antibody followed by immunoblotting with anti-Arp2 antibody (panel a), anti-Arp3 antibody (panel b), or reblotting with anti-N-WASP antibody (panel c) as a loading control. C, assay for actin polymerization using 100 nM N-WASP (isolated from SK-OV-3.ipl cells) and 60 nM Arp2/3 complex (line a), 100 nM N-WASP alone (line b), or 60 nM Arp2/3 complex alone (line c). Pyrene fluorescence intensity was versus time (in minutes) after initiating polymerization. G-actin was added at a final concentration of 2 μM (9% pyrene-labeled) after a 5-min preincubation as described under “Materials and Methods.”

**FIGURE 1. Characterization of N-WASP and CD44-N-WASP complex in SK-OV-3.ipl cells.**

A, detection of N-WASP in SK-OV-3.ipl cells using preimmune serum (lane 1) and anti-N-WASP-mediated immunoblot (IB) (lane 2). B, analysis of CD44-N-WASP and Arp2/3 complex in SK-OV-3.ipl cells. B-I, SK-OV-3.ipl cells (untreated (lane 1), treated with HA (50 μg/ml) for 5 min (lane 2), pretreated with anti-CD44 antibody (10 μg/ml) for 1 h followed by HA (50 μg/ml) incubation for 5 min (lane 3)) were immunoprecipitated (IP) with anti-CD44 antibody followed by immunoblotting with anti-N-WASP antibody (panel a), anti-Arp2 antibody (panel b), anti-Arp3 antibody (panel c), or reblotting with anti-CD44 antibody (panel d) as a loading control. B-II, the supernatant fraction following anti-CD44 immunoprecipitation using cells (untreated (lane 1), treated with HA (50 μg/ml) for 5 min (lane 2), or pretreated with anti-CD44 antibody (10 μg/ml) for 1 h followed by HA (50 μg/ml) incubation for 5 min (lane 3), as described in B-I) were immunoprecipitated with anti-N-WASP antibody followed by immunoblotting with anti-Arp2 antibody (panel a), anti-Arp3 antibody (panel b), or reblotting with anti-N-WASP antibody (panel c) as a loading control. C, assay for actin polymerization using 100 nM N-WASP (isolated from SK-OV-3.ipl cells) and 60 nM Arp2/3 complex (line a), 100 nM N-WASP alone (line b), or 60 nM Arp2/3 complex alone (line c). Pyrene fluorescence intensity was versus time (in minutes) after initiating polymerization. G-actin was added at a final concentration of 2 μM (9% pyrene-labeled) after a 5-min preincubation as described under “Materials and Methods.”
**TABLE 1**
Measurement of total F-actin formation in SK-OV-3.ipl cells

| Treatments | F-actin formation (relative fluorescence unit) (% of control)* |
|------------|-------------------------------------------------------------|
| A. Effects of anti-CD44 antibody on HA-dependent F-actin formation | |
| No treatment (control) | 100 |
| HA treatment | 295 |
| Anti-CD44 IgG + HA treatment | 98 |
| B. Effects of cytochalasin D on HA-dependent F-actin formation | |
| No treatment (control) | 100 |
| HA treatment | 285 |
| Cytochalasin D + HA treatment | 28 |

* The amount of F-actin formation in SK-OV-3.ipl cells (treated with 50 µg/ml HA or pretreated with 20 µg/ml cytochalasin D or rat anti-CD44 IgG followed by adding 50 µg/ml HA or no HA or transfected with N-WASP-VCA cDNA or vector alone) was determined by FACS analysis as described under "Materials and Methods." Each assay was set up in triplicate and repeated at least three times. The F-actin content measured from SK-OV-3.ipl cells without any treatment (parts A and B) or SK-OV-3.ipl cells transfected with vector alone without any treatment (part C) is designated as 100%. The values expressed in this table represent an average of triplicate determinations of 3–5 experiments with an S.D. of less than ±5%. All data were analyzed statistically using the Student’s t test, and statistical significance was set at p < 0.01.

**TABLE 2**
Analyses of ovarian tumor cell migration

| Treatments | Ovarian tumor cell migration (% of control)* |
|------------|---------------------------------------------|
| A. Effects of anti-CD44 antibody on HA-dependent tumor cell migration | |
| No treatment (control) | 100 |
| HA treatment | 289 |
| Anti-CD44 IgG + HA treatment | 95 |
| B. Effects of cytochalasin D on HA-dependent tumor cell migration | |
| No drug treatment (control) | 100 |
| HA treatment | 286 |
| Cytochalasin D + HA treatment | 25 |
| C. Effect of N-WASP-VCA overexpression (by transfecting SK-OV-3.ipl cells with N-WASP-VCA cDNA or vector alone) on HA-dependent F-actin formation | |
| Vector-transfected cells (control) | 100% |
| N-WASP-VCA cDNA-transfected cells | 285% |
| D. Effect of β-catenin siRNA on HA-dependent tumor cell migration | |
| β-catenin siRNA-scrambled sequence treatment (control) | 100% |
| β-catenin-siRNA-treated cells | 292% |

* SK-OV-3.ipl cells (treated with 50 µg/ml HA, pretreated with 20 µg/ml cytochalasin D or rat anti-CD44 IgG, or 50 pmol of β-catenin siRNA-scrambled sequences or 50 pmol of β-catenin siRNA followed by adding 50 µg/ml HA or no HA or transfected with N-WASP-VCA cDNA or vector-alone) were placed in the upper chamber of the transwell unit. Cell migration processes were determined by measuring the cells that migrated to the lower side of the polycarbonate filters (containing 50 µg/ml HA or no HA) as described under "Materials and Methods." The CD44-specific cell migration in cells without any treatment (parts A and B) or cells transfected with vector alone without any treatment (part C) or cells treated with β-catenin siRNA-scrambled sequences (part D) is designated as 100%. The values expressed in this table represent an average of triplicate determinations of 3–5 experiments with an S.D. of less than ±5%. All data were analyzed statistically using the Student’s t test, and statistical significance was set at p < 0.01.

SK-OV-3.ipl cells stimulates ErbB2 tyrosine kinase activity (Fig. 3A, panels a and b, lanes 1 and 2). Pretreatment of cells with anti-CD44 antibody (Fig. 3A, panels a and b, lane 3) or an ErbB2 inhibitor, AG825 (Fig. 3A, panels a and b, lane 4), readily inhibits HA-mediated ErbB2 kinase activity. These results suggest that HA-mediated ErbB2 activation in SK-OV-3.ipl cells is CD44-dependent and ErbB2 tyrosine kinase-sensitive.

The cytoskeletal protein, β-catenin, is known to serve as a substrate for ErbB2 tyrosine kinase (59–63). Here we have found that a 5-min HA treatment stimulates β-catenin tyrosine phosphorylation in the cytosol (Fig. 3B(I), panels a and b, lanes 1 and 2). Phosphorylated β-catenin then becomes translocated from the cytosol into the nuclear fraction (Fig. 3B(II), panels a and b, lane 2) after a 30-min HA treatment of SK-OV-3.ipl cells. When cells were pretreated with anti-CD44 or AG825 (an ErbB2 inhibitor), both HA-mediated β-catenin phosphorylation (Fig. 3B(I), panels a and b, lanes 3 and 4) and nuclear translocation (Fig. 3B(II), panels a and b, lanes 3 and 4) are greatly reduced. Therefore, it is likely that β-catenin phosphorylation and nuclear translocation are closely linked to HA/CD44-N-WASP-associated ErbB2 signaling.

There is a compelling evidence that transcriptional co-activation by β-catenin occurs through the binding to TCF/LEF transcription factors (56, 57). Consequently, we examined the potential impact of HA/CD44-ErbB2 activation on β-catenin signaling (β-catenin tyrosine phosphorylation and nuclear translocation)-mediated co-activation of TCF/LEF transcription using luciferase reporter assays. Specifically, we utilized firefly luciferase reporter plasmids containing either pTop-flash (a wild-type containing TCF/LEF-binding sites for β-catenin) or pOp-flash (a mutant lacking TCF/LEF-binding sites for β-catenin), which are transiently transfected into SK-OV-3.ipl cells. With this technique HA/CD44-ErbB2 signaling-related and β-catenin-mediated TCF/LEF-transcriptional co-activation can be measured by the ratio of pTop-flash to pOp-flash luciferase units. Our results indicate that the level of β-catenin-mediated TCF/LEF-transcriptional co-activation is low in cells treated with no HA (Fig. 4A, bar a) or pretreated with anti-CD44 followed by HA treatment (Fig. 4A, bar b). However, TCF/LEF transcriptional co-activation by β-catenin is greatly enhanced in SK-OV-3.ipl cells treated with HA (Fig. 4A, bar c). The level of β-catenin-mediated TCF/LEF transcriptional co-activation is greatly reduced if these cells were pretreated with AG825 followed by no HA or with HA addition (Fig. 4A, bars d and e). These findings clearly indicate that TCF/LEF-transcriptional co-activation by β-catenin requires HA/CD44-activated ErbB2 tyrosine kinase in SK-OV-3.ipl cells.

Although cytochalasin D treatment significantly blocks HA-mediated F-actin formation (Table 1), it does not cause a noticeable inhibition of HA-mediated phosphorylation of β-catenin (Fig. 3B(I), panels a and b, lane 5). Thus, F-actin assembly appears not to be involved in the initiation of HA/CD44 interaction (so-called “inside-out signaling”) in SK-OV-3.ipl cells. The fact that cytochalasin D treatment significantly abrogates HA/CD44-mediated β-catenin nuclear translocation (Fig. 3B(II), panels a and b, lane 5, and Fig. 3B(iii), panels a–c) and β-catenin-TCF/LEF transcriptional co-activation (Fig. 4A, bars f and g) suggests that the F-actin cytoskeleton (regulated...
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by N-WASP and Arp2/Arp3) is actively participating in HA/CD44-mediated “outside-in” signaling cascades in ovarian tumor cells.

Effects of N-WASP-VCA Overexpression on HA/CD44-N-WASP Signaling and ErbB2 Activation in SK-OV-3.ipl Cells

N-WASP Signaling—The VCA domain of N-WASP is an important region for Arp2/3 binding and actin polymerization (26–28, 36). Overexpression of the VCA domain of N-WASP in cells (by transfecting cells with N-WASP-VCA cDNA) has been shown to inhibit N-WASP-Arp2/3 signaling and impair actin cytoskeleton-mediated cellular functions (47). In this study we have used a VCA fragment construct that was cloned into a hemagglutinin-tagged expression vector (Fig. 5A) to examine the possible involvement of N-WASP-VCA in HA/CD44-mediated signaling. Using a cell-free system, we have confirmed that the hemagglutinin-tagged recombinant VCA fusion protein of N-WASP can promote a significant up-regulation of Arp2/3-induced actin polymerization (Fig. 5B, lane a) as compared with these samples treated with VCA alone (or Arp2/3 alone or reaction buffer alone) (Fig. 5B, lines b–d). Moreover, we have demonstrated that HA is capable of promoting the recruitment of endogenous Arp2 (Fig. 5C, panel a, lanes 1 and 2) together with Arp3 (Fig. 5C, panel b, lanes 1 and 2) into a complex with N-WASP (Fig. 5C, panel c, lanes 1 and 2) in vector-transfected cells. In contrast, transfection of SK-OV-3.ipl cells with N-WASP-VCA cDNA causes a significant reduction in HA-mediated endogenous Arp2/3 (Fig. 5C, panels a and b, lanes 3 and 4) association with N-WASP (Fig. 5C, panel c, lanes 3 and 4). These findings indicate that overexpression of the VCA domain acts as a potent competitive inhibitor for endogenous Arp2/3 binding to N-WASP in SK-OV-3.ipl cells.

Furthermore, we have examined both F-actin formation and tumor cell migration in vector-transfected or N-WASP-VCA cDNA-transfected cells treated with HA or without HA (Table 1, part C, and Table 2, part C). Our results indicate that HA promotes F-actin accumulation and tumor cell migration in vector-transfected cells (Table 1, part C, and Table 2, part C). In contrast, a low level of F-actin formation and tumor cell migration was observed in vector-transfected cells treated with no HA (Table 1, part C, and Table 2, part C). Overexpression of the N-WASP-VCA domain by transfecting SK-OV-3.ipl cells with N-WASP-VCA cDNA reduces F-actin formation (Table 1, part C) and inhibits tumor cell migration (Table 2, part C). All results support the notion that N-WASP (in particular, the VCA domain) is involved in HA/CD44-mediated actin cytoskeleton assembly and ovarian tumor cell migration.

Staining of SK-OV-3.ipl cells with fluorescent phalloidin reveals that the assembly of both cortical actin fibrils and cell body actin fibrils occurs in vector-transfected cells following HA treatment (Fig. 6, B and C). In the majority of those cells treated with HA for 10 min, the actin filaments were present in numerous stress fibers and in a thick layer immediately beneath the plasma membrane (Fig. 6B). After 60 min of HA treatment, radiating actin filaments start to disassemble and become reorganized/aggregated at the cell margins as well as the membranous projections (Fig. 6C). It is possible that the disassembly of cell body actin fibrils and reorganization/thickening of cortical fibril during 60 min of HA treatment is involved in membrane motility and cell migration. In contrast, we have found that a small amount of F-actin fragments was randomly distributed in the cytosol and located around the cell periphery in vector-transfected cells treated with no HA (Fig. 6A) or pretreated with anti-CD44 antibody...
plus HA (Fig. 6, B, inset i, and C, inset i). These findings suggest that HA-induced F-actin formation and reorganization are CD44-dependent. Furthermore, we have noted that stress fibers were no longer apparent, and the total amount of actin was greatly reduced, and the small amount of remaining actin was primarily located at the cell periphery in N-WASP-VCA cDNA-transfected cells either treated with (Fig. 6, E and F) or untreated with HA (Fig. 6D). These observations support the notion that disruption of the CD44/N-WASP interaction affects the assembly and reorganization of both cortical actin filaments and cell body actin fibers. Consequently, F-actin-based cytoskeletal function appears to be impaired during HA-mediated CD44 signaling.

ErbB2 Activation and β-Catenin Function—To assess the effects of the N-WASP-VCA domain in regulating HA/CD44-mediated ErbB2 and β-catenin signaling, we have transfected SK-OV-3.ipl cells with N-WASP-VCA cDNA. Our results show that these transfectants exhibit a marked inhibition of HA-mediated TCF/LEF transcriptional co-activation in SK-OV-3.ipl cells. These findings suggest that the N-WASP containing the VCA domain functions as a strong dominant-negative mutant for blocking N-WASP and ErbB2 accumulation into HA-induced CD44 signaling complexes.
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**A**

**FIGURE 5. Analyses of N-WASP-VCA interaction with Arp2/3 complex.** A, illustration of the full-length N-WASP (a) and VCA fragments (b) used in this study. A number of functional domains from N-WASP are indicated as follows: WH1, WASP homology domain; B, basic region; CRIB, cdc42/raf interactive binding region; Pro-rich, proline-rich region; Grb2/Ash, Nck, and WISH binding region; V, verproline-homology domain, actin binding region; C, cofilin-homology domain; A, acidic region, the CA region interacts with the Arp2/3 complex; and hemagglutinin-tagged N-WASP-VCAcDNA construct (b). B, assay for actin polymerization using 100 nM recombinant VCA and 60 nM Arp2/3 complex (line a), 100 nM VCA alone (line b), 60 nM Arp2/3 complex alone (line c), or reaction buffer alone (line d). Pyrene fluorescence intensity versus time (in minutes) after initiating polymerization. G-actin was added at a final concentration of 2 µM (9% pyrene-labeled) after a 5-min preincubation as described under "Materials and Methods." C, analyses of N-WASP and Arp2/3 complex in N-WASP-VCAcDNA-transfected/vector-transfected cells. Vector-transfected cells (untreated (lane 1) or treated with HA (50 µg/ml) for 5 min (lane 2)), N-WASP-VCAcDNA-transfected cells (untreated (lane 3) or treated with HA (50 µg/ml) for 5 min (lane 4)) were immunoprecipitated with anti-N-WASP antibody followed by immunoblotting with anti-Arp2 antibody (panel a) or anti-Arp3 antibody (panel b) or reblotting with anti-N-WASP antibody (panel c) (as a loading control).

Furthermore, we have examined signaling events (e.g. ErbB2 tyrosine kinase (Fig. 7B) and β-catenin phosphorylation/nuclear translocation (Fig. 7, C and D)) in vector-transfected or N-WASP-VCAcDNA-transfected cells treated with HA or without HA. Our results indicate that ErbB2 kinase activation (Fig. 7B, panels a and b, lanes 1 and 2) and β-catenin phospho-

**DISCUSSION**

Ovarian cancer has the highest mortality rate among all gynecological malignancies. HA, a glycosaminoglycan, is present as a loose pericellular layer that coats the mesothelium...
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required for both protection and lubrication within this body cavity (3, 4). Overexpression of HA has been found to be associated with ovarian tumor progression (3, 4). HA binds to specific tumor cell-surface receptors such as CD44, which is present in at least 94% of ovarian tumor cells (6, 7). HA-CD44 binding stimulates oncogenic signaling and ovarian tumor cell-specific properties (3, 6–9). Thus, it has been suggested that the interaction between the HA pericellular coat of mesothelial cells and CD44 on the surface of tumor cells is one of the important steps in the peritoneal spread of ovarian cancer.

The metastatic phenotype of ovarian tumor cells, characterized by tumor cell activation and migration, appears to be closely linked to cytoskeletal function. Previous studies have shown that HA-mediated CD44 cytoskeleton binding requires ankyrin (14–16) and ERM (17). Recent reports indicate that HA-mediated CD44 interaction with certain signaling activators (e.g. RhoGEFs (20, 21), Rho kinase (ROK) (20, 21), Vav2/Tiam1-Rac1 (18, 19), Cdc42-IQGAP1 (22), transforming growth factor-β receptors (24), EGFR (12), and ErbB2 (11, 19)) plays a pivotal role in the activation of N-WASP by interact-ting with Grb2/Ash (34, 40), Nck (35, 41), and WISH (42). The VCA domain contains a conserved tryptophan residue that is essential for triggering actin nucleation and polymerization by binding to the Arp2/3 complexes (25, 26, 28). Consequently, N-WASP-mediated actin assembly can be regulated by many different mechanisms and is apparently involved in the formation of membrane projections, vesicular trafficking, cell migration, and gene regulation (27, 37, 44–47). In this study we have focused on the role of N-WASP (in particular, the VCA domain) in regulating HA/CD44-mediated actin assembly and ErbB2-regulated β-catenin signaling as well as specific ovarian tumor cell behaviors.

Our initial results demonstrate that N-WASP is expressed in ovarian tumor cells (SK-OV-3.ipl cells) (Fig. 1). We have also presented evidence for an HA-induced physical interaction between N-WASP and the transmembrane glycoprotein, CD44, in SK-OV-3.ipl cells (Fig. 1). Treatment of cells with HA also promotes an association of the CD44-N-WASP complex with the actin-related protein (Arp) 2/3 complex (25, 26, 28). Both the basic region and the GBD/CRIB motifs contribute to N-WASP activation through phos-photidylinositol 4,5-bisphosphate/Cdc42 binding (32, 39). The proline-rich domain regulates the activation of N-WASP by interact-ing with Grb2/Ash (34, 40), Nck (35, 41), and WISH (42). The VCA domain contains a conserved tryptophan residue that is essential for triggering actin nucleation and polymerization by binding to the Arp2/3 complexes (25, 26, 28). Con-

that correlate with metastatic behavior, a prime candidate (named N-WASP), has been identified. N-WASP contains a number of functional domains known to interact with various signaling regulators and actin cytoskeleton (25, 26, 28). These include the WH1 domain, IQ region, a highly basic region, the GBD/CRIB motifs, a proline-rich region, and the VCA domain (25–36). Specifically, the WH1 domain binds to WASP-interacting protein family proteins that are important for N-WASP localization (25, 26, 28). Both the basic region and the GBD/CRIB motif contribute to N-WASP activation through phos-photidylinositol 4,5-bisphosphate/Cdc42 binding (32, 39). The proline-rich domain regulates the activation of N-WASP by interact-ing with Grb2/Ash (34, 40), Nck (35, 41), and WISH (42). The VCA domain contains a conserved tryptophan residue that is essential for triggering actin nucleation and polymerization by binding to the Arp2/3 complexes (25, 26, 28). Con-

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FIGURE 7. Analyses of CD44-N-WASP/ErbB2 complex and HA-mediated signaling events in N-WASP-VCAcDNA-transfected/vector-transfected cells. A, vector-transfected cells (untreated (lane 1) or treated with HA (50 μg/ml) for 5 min (lane 2)) or N-WASP-VCAcDNA-transfected cells (untreated (lane 3) or treated with HA (50 μg/ml) for 5 min (lane 4)) were immunoprecipitated (IP) with anti-CD44 antibody followed by immunoblotting with various immunoreagents (e.g. anti-N-WASP antibody (panel a) or anti-ErbB2 antibody (panel b) or replotting with anti-CD44 antibody (panel c) (as a loading control). B, Nonidet P-40-solubilized cell lysates obtained from vector-transfected cells (untreated (lane 1) or treated with HA (50 μg/ml) for 5 min (lane 2)) or from N-WASP-VCAcDNA-transfected cells (untreated (lane 3) or treated with HA (50 μg/ml) for 5 min (lane 4)) were immunoblotted with anti-phospho-ErbB2 antibody (panel a) or anti-ErbB2 antibody (panel b). C, (I), the cytoplasmic fraction isolated from vector-transfected cells (untreated (lane 1) or treated with HA (50 μg/ml) for 5 min (lane 2)) or from N-WASP-VCAcDNA-transfected cells (untreated (lane 3) or treated with HA (50 μg/ml) for 5 min (lane 4)) were immunoprecipitated with anti-β-catenin antibody followed by immunoblotting with anti-phosphotyrosine antibody (panel a) or anti-β-catenin antibody (as a loading control) (panel b). C, (II), the nuclear fraction isolated from vector-transfected cells (untreated (lane 1) or treated with HA (50 μg/ml) for 30 min (lane 2)) or from N-WASP-VCAcDNA-transfected cells (untreated (lane 3) or treated with HA (50 μg/ml) for 30 min (lane 4)) were immunoprecipitated with anti-β-catenin antibody followed by immunoblotting with anti-phosphotyrosine antibody (panel a) or anti-β-catenin antibody (as a loading control) (panel b).

the recruitment of a range of proteins resulting in the activation of certain intracellular signaling pathways. ErbB2 is implicated in important biological events such as proliferation, migration, and differentiation and has been shown to be overexpressed in a number of human cancers, including ovarian cancer. This overexpression often correlates with a more aggressive disease and a poor prognosis for the patient (50, 51).

Hyaluronan has been shown to constitutively regulate ErbB2 activity and to influence ErbB2 interaction with phosphatidylinositol 3-kinase/AKT signaling in tumor cells (71). Previously, we have determined that CD44 and p185<sup>HER2</sup> are physically linked to each other via interchain disulfide bonds in human ovarian tumor cells (SK-OV-3.3p cell line) (11). Most importantly, HA binding to a CD44-associated p185<sup>HER2</sup> complex activates the p185<sup>HER2</sup> tyrosine kinase activity and promotes ovarian carcinoma cell growth (2). We believe that direct cross-talk between the two surface molecules, CD44 and the p185<sup>HER2</sup>, may be one of the most important signaling events in human ovarian carcinoma development. HA-mediated CD44 association with p185<sup>HER2</sup> signaling complexes is also mediated by molecular scaffolds and adaptors such as Vav2 (a Rac-specific GEF) and Grb2 (19) in ovarian tumor cells. Specifically, endogenous Vav2 and Grb2 are associated with CD44 and p185<sup>HER2</sup> in a signaling complex, and HA treatment induces recruitment of both Vav2 and Grb2 into CD44v3-p185<sup>HER2</sup>-containing multimolecular complexes leading to the co-activation of Rac1 and Ras signaling and ovarian tumor cell growth and migration (19).

In this study we have demonstrated that the CD44-N-WASP complex interacts with ErbB2 (Fig. 5). HA treatment of SK-OV-3.3p cells causes a significant increase in the amount of ErbB2 recruited into the CD44-N-WASP complex and stimulates ErbB2 tyrosine kinase activity (Fig. 3) leading to β-catenin phosphorylation (Fig. 3) and nuclear translocation (Fig. 3). The cell-cell adhesion regulator, β-catenin, has been found to be a critical downstream mediator of Wnt signaling (52, 53). In the normal ovary β-catenin degradation through the ubiquitin pathway is facilitated by GSK-3β-mediated serine/threonine phosphorylation in connection with APC (the tumor suppressor gene product adenomatous polyposis coli) and Axin (72).

Overexpression of β-catenin has been shown to be closely associated with ovarian cancer progression (52, 59–64, 73, 74). Misregulated Wnt signaling in tumor cells often causes β-catenin accumulation in the cytoplasm and nuclear translocation (74). β-Catenin also binds to the transcription factor TCF/LEF in the nucleus. This is followed by transcriptional activation of target genes such as c-myc, E-cadherin, and cyclin D1 (75). A number of studies also indicate that ErbB2 kinase is capable of inducing tyrosine phosphorylation of β-catenin, which can shift β-catenin from a normal cell-cell adhesion state into the oncopgenic pathways (59 – 64, 76). Our results indicate that HA-mediated and CD44/N-WASP-associated ErbB2 kinase can induce β-catenin phosphorylation and nuclear translocation (Fig. 3 and Fig. 8) resulting in β-catenin-mediated TCF/LEF-specific transcription co-activation (Fig. 4) in ovarian tumor cells. The fact that ErbB2 kinase inhibitor (AG825) can effectively block HA-mediated β-catenin phosphorylation, nuclear translocation, and TCF/LEF-transcriptional co-activation (Figs. 3, 4, and 7) strongly suggests that HA interaction with CD44-N-WASP complex-associated ErbB2 kinase plays a critical role in β-catenin signaling and oncogetic events during SK-OV-3.3p cell activation. In human hepatocellular carcinoma, the extracellular signal-regulated kinase (ERK) has been shown to be involved in the inactivation of GSK-3β and the up-regulation of β-catenin (77). A previous study showed that the binding of HA to CD44 promotes the association of ERK
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**TABLE 3**

| Cells                                      | Relative luciferase activity units (% of control) |
|--------------------------------------------|--------------------------------------------------|
| Vector-transfected cells (control)         | 100                                              |
| N-WASP-VCADNA-transfected cells           | 92                                               |
| N-WASP-VCADNA-transfected cells           | 95                                               |

Both vector-transfected or N-WASP-VCADNA-transfected SK-OV-3.ipl cells were transfected with either pTop-fl ash or pFop-fl ash as described under “Materials and Methods.” After transfection, cells (no HA treatment or 24-h HA treatment) were lysed, and luciferase activities were determined by luminometry. Results are expressed as percentage (%) of control (untreated vector-transfected cells). Data expressed as relative luciferase units (pTop-fl ash units divided by mutant pFop-fl ash units) are the mean of 3–5 separate experiments with an S.D. of less than ±5%.

FIGURE 8. Immunofluorescence staining of β-catenin in SK-OV-3.ipl transfectants. SK-OV-3.ipl cells transfected with vector alone or N-WASP-VCADNA (in the presence or absence of HA) were fixed by 2% paraformaldehyde. Subsequently, cells were rendered permeable by ethanol treatment and stained with β-catenin and To pro-3 (a nuclear marker) as described under “Materials and Methods.” A, panels a–c, FITC-labeled anti-β-catenin (green color) (panel a), To pro-3 (red color) (panel b), and an overlay image (panel c) of panels a and b in vector-transfected cells treated with no HA, B, inset i, panels a–c, FITC-labeled anti-β-catenin (green color) (a), To pro-3 (red color) (b), and an overlay image (panel c) of panels a and b in vector-transfected cells treated with HA for 30 min. B, inset ii, panels a–c, FITC-labeled anti-β-catenin (green color) (inset i), To pro-3 (red color) (inset ii), and an overlay image (panel c) of panels a and b in vector-transfected cells pretreated with 25 μM A 8 G25 followed by 30 min of HA treatment. B inset iii, panels a–c, FITC-labeled anti-β-catenin (green color) (inset i), To pro-3 (red color) (inset ii) and an overlay image (panel c) of panels a and b in vector-transfected cells pretreated with 20 μg/ml cytochalasin D followed by 30 min of HA treatment. C, panels a–c, FITC-labeled anti-β-catenin (green color) (panel a), To pro-3 (red color) (panel b), and an overlay image (panel c) of panels a and b in N-WASP-VCADNA-transfected cells treated with no HA. D, panels a–c, FITC-labeled anti-β-catenin (green color) (panel a), To pro-3 (red color) (panel b), and an overlay image (panel c) of panels a and b in N-WASP-VCADNA-transfected cells treated with HA for 30 min.

The VCA domain of N-WASP is known to interact with Arp2/3 complexes and to promote actin polymerization (26–28, 36). In this study we have confirmed that the VCA domain of N-WASP is responsible for the Arp2/3-mediated actin polymerization in a cell-free system (Fig. 5). To further assess the role of the N-WASP-VCA domain in regulating CD44- and ErbB2-mediated cytoskeleton function and signaling events in human ovarian tumor cells (SK-OV-3.ipl cells), we have transfected SK-OV-3.ipl cells with N-WASP-VCADNA (Fig. 5) or vector alone. Our results indicate that transfection of SK-OV-3.ipl cells with N-WASP-VCADNA effectively blocks HA-induced endogenous Arp2/3 association with N-WASP (Fig. 5). These findings further support our conclusion that N-WASP-VCA domain acts as a potent competitive inhibitor that is capable of interfering with endogenous Arp2/3 interaction with N-WASP. Subsequently, HA-mediated actin polymerization (Table 1) and tumor cell migration (Table 2) in these transfectants are inhibited. These observations strongly indicate the importance of the VCA domain of N-WASP in regulating HA/CD44-mediated F-actin formation and ovarian tumor cell migration.

Moreover, we have observed that HA not only stimulates the recruitment of N-WASP and ErbB2 into a CD44 complex (Fig. 7) but also promotes ErbB2 signaling (e.g. ErbB2 kinase activation, β-catenin phosphorylation, nuclear translocation, and TCF/LEF-specific transcriptional co-activation) in vector-transfected cells (Fig. 7). In contrast, the failure of endogenous Arp2/3 to become associated with N-WASP in N-WASP-VCADNA-transfected cells (Fig. 5) almost completely abolishes HA-mediated N-WASP association with membrane proteins such as CD44 and ErbB2 leading to an
impairment of ErbB2-associated signaling events (e.g. ErbB2 kinase activity, β-catenin phosphorylation, nuclear translocation, and TCF/LEF-specific transcriptional co-activation) (Fig. 7 and Fig. 8; Table 3). Thus, these results provide strong evidence that N-WASP (via the VCA domain) not only serves as an activator for Arp2/3-induced actin assembly but also provides a novel linker function to recruit ErbB2 into a CD44 signaling complex resulting in HA-induced ErbB2-β-catenin signaling required for tumor-specific behaviors (e.g. tumor cell migration and transcriptional activation). Most importantly, we believe that successful identifications of specific linker molecule(s) between CD44 and tyrosine kinase receptors (e.g. ErbB2) could lead to the identification of potential new drug targets. For example, signaling perturbation procedures designed to overexpress a dominant-negative mutant protein, such as the VCA domain of N-WASP in ovarian tumor cells, could prevent the HA/CD44-induced activation of multiple signaling pathways from being initiated in the first place.

Although the ovarian carcinomas cells are frequently detected in an HA-enriched environment, the biological effects or activities generated by the HA polysaccharides may vary significantly depending on the HA sizes and concentrations. Apparently, both HA synthase isozymes (e.g. HA synthase 1 (HAS1), HA synthase 2 (HAS2), and HA synthase 3 (HAS3)) and hyaluronidases (e.g. hyaluronidase 1 (Hyal-1), hyaluronidase 2 (Hyal-2), and PH20) are involved in regulating HA sizes and concentrations required for outside-in signaling events (e.g. HA-CD44 binding, ErbB2-β-catenin signaling, and cytoskeleton organization). Therefore, it is possible that the enhanced ErbB2 signaling and β-catenin phosphorylation are triggered by locally accumulated HA at the tumor attachment site(s) when the required sizes and concentrations of HA become available. The question regarding how various HAS molecules and hyaluronidases are regulated in the extracellular matrix components during ovarian cancer progression will be addressed in our future studies. Preliminary data indicate that large size HA (>1–2 × 10^6 daltons) has a minimal signaling activation capability. In contrast, HA fragments ranging from 500,000 to 100,000 daltons induce a strong stimulation of cellular signaling. The cellular and molecular mechanisms involved in these selective signaling responses induced by large HA fragments versus small HA fragments await further investigation in our laboratory.

Previously, HA-mediated CD44 interaction with cytoskeleton proteins (e.g. ankyrin and ERM) and various signaling molecules (e.g. Tiam1, Vav2, RhoGEF/RhoA-activated ROK, Cdc42-IQGAP1, and PKN) have been reported (18–23). However, the regulatory mechanism involved in HA/CD44-mediated ErbB2 activation in ovarian tumor cells has not been well understood. In this study we have obtained new evidence indicating that N-WASP participates in a multimolecular complex formation containing CD44 and ErbB2. Most importantly, N-WASP regulates HA/CD44-mediated ErbB2 activation and β-catenin signaling (e.g. β-catenin phosphorylation/nuclear translocation and TCF/LEF transcriptional co-activation) needed for transcriptional activation and ovarian tumor cell growth. N-WASP is also involved in HA/CD44-mediated F-actin assembly required for cytoskeleton-dependent tumor-specific behavior such as tumor cell migration. These findings strongly suggest that N-WASP not only mediates multiprotein complex assembly but also participates in cytoskeleton activation and CD44-ErbB2 signaling cross-talk processes.

As summarized in Fig. 9, we would like to propose that upon binding of HA, CD44 is tightly coupled with N-WASP and ErbB2 in a complex (step 1) that induces the formation of the N-WASP-Arp2/3 complex (step 2a) and ErbB2 tyrosine kinase-mediated β-catenin phosphorylation (step 2b). These N-WASP-Arp2/3 complexes then stimulate actin polymerization (step 3a) and cytoskeleton activation resulting in tumor cell migration. At the same time, β-catenin phosphorylation (step 3b) because of HA-mediated CD44-N-WASP-ErbB2 tyrosine kinase activation (together with cytoskeletal function) promotes β-catenin nuclear translocation (step 4) and TCF/LEF-specific transcriptional up-regulation as well as tumor cell migration. Taken together, these results indicate that CD44 interaction with N-WASP and ErbB2 plays a pivotal role in stimulating HA-dependent actin polymerization and β-catenin signaling leading to the concomitant stimulation of cell migration and transcriptional activation required for ovarian cancer progression.
are known to be involved in transcription of specific target genes such as cyclin D1 and c-myc (58, 75). Cyclin D1 is a major regulator of the progression of cells into the proliferative stage of the cell cycle (78). Overexpression of cyclin D1 is associated with poor survival in epithelial ovarian cancers (79, 80). Proto-oncogene c-myc codes for several phosphoproteins that regulate cell cycle and cell proliferation (81). Amplification of c-myc also plays a critical role in the development of epithelial neoplasms (54, 79). Consequently, β-catenin-mediated TCF/LEF transcriptional co-activation of these target genes (e.g., cyclin D1 and c-myc) is a critical component in the tumorigenesis pathway by dysregulating cell cycle progression and cell growth. Taken together, these results clearly indicate that CD44 interaction with N-WASP and ErbB2 plays a pivotal role in stimulating HA-dependent actin polymerization and β-catenin signaling leading to the concomitant stimulation of cell migration and transcriptional activation required for ovarian cancer progression.

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REFERENCES

1. Hoskins, W. J. (1993) Cancer 71, 1534–1540
2. Fox, H. (1990) in Clinical Gynecological Cancer (Shepherd, J., and Monaghan, J., eds) pp. 188–217. Blackwell Scientific Publishers, Oxford
3. Laurent, T. C., and Fraser, J. R. E. (1992) J. Biol. Chem. 267, 2397–2404
4. Jones, L. M. H., Gardner, J. B., Catterall, J. B., and Turner, G. A. (1995) Clin. Exp. Metastasis 13, 373–380
5. Underhill, C. (1992) J. Cell Sci. 103, 293–298
6. Lesley, J., Hyman, R., English, N., Catterall, J. B., and Turner, G. A. (1997) Glycoconj. J. 14, 611–622
7. Naor, D., Sionov, R. V., and Ish-Shalom, D. (1997) Adv. Cancer Res. 71, 241–319
8. Toole, B. P., Wight, T., and Tammi, M. (2002) J. Biol. Chem. 277, 4593–4596
9. Turley, E. A., Noble, P. W., and Bourguignon, L. Y. W. (2002) J. Biol. Chem. 277, 4589–4592
10. Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D’Amico, M., Pestell, R. G., Saper, M. A., and Rosen, M. K. (1998) Nature 391, 93–96
11. Kim, A. S., Kakalis, L. T., Abdul-Manman, N., Liu, G. A., and Rosen, M. K. (2000) Nature 404, 151–158
12. Higgs, H. N., and Pollard, T. D. (2000) J. Cell Biol. 150, 1311–1320
13. Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D’Amico, M., Pestell, R. G., Saper, M. A., and Rosen, M. K. (1998) Nature 391, 93–96
14. Higgs, H. N., and Pollard, T. D. (2000) J. Cell Biol. 150, 1311–1320
15. Carlier, M. F., Nioche, P., Broutin-l’Hermitte, L., Boujemaa, R., Le Clainche, C., Egile, C., Garbay, C., Ducruix, A., Sansonetti, P., and Pantaloni, D. (2000) J. Biol. Chem. 275, 21946–21952
16. Carbognin, P., Pollard, T. D., and Shtutman, M. (2000) J. Cell Biol. 150, 1311–1320
17. Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D’Amico, M., Pestell, R. G., Saper, M. A., and Rosen, M. K. (1998) Nature 391, 93–96
18. Higgs, H. N., and Pollard, T. D. (2000) J. Cell Biol. 150, 1311–1320
19. Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D’Amico, M., Pestell, R. G., Saper, M. A., and Rosen, M. K. (1998) Nature 391, 93–96
20. Higgs, H. N., and Pollard, T. D. (2000) J. Cell Biol. 150, 1311–1320
21. Carlier, M. F., Nioche, P., Broutin-l’Hermitte, L., Boujemaa, R., Le Clainche, C., Egile, C., Garbay, C., Ducruix, A., Sansonetti, P., and Pantaloni, D. (2000) J. Biol. Chem. 275, 21946–21952
22. Carbognin, P., Pollard, T. D., and Shtutman, M. (2000) J. Cell Biol. 150, 1311–1320
23. Snapper, S. B., Rosen, F. S., Mizoguchi, E., Cohen, P., Khan, W., Liu, C., Hagermann, T. L., Kwan, S., Ferrini, R., Davidson, L., Bhan, A. K., and Alt, F. (1998) Immunity 9, 81–91
24. Snapper, S. B., Rosen, F. S., Mizoguchi, E., Cohen, P., Khan, W., Liu, C., Hagermann, T. L., Kwan, S., Ferrini, R., Davidson, L., Bhan, A. K., and Alt, F. (1998) Immunity 9, 81–91
25. Jiang, X., Shehahedini, A., Och, L. G. A., Butler, J., Somani, A., McGavin, M., Kozieradzki, I., los Santos, A. O., Nagy, A., Grinstein, S., Perninger, J. M., and Sinimotivich, K. A. (1999) J. Exp. Med. 190, 1329–1341
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59. Hazan, R. B., and Borton, L. (1998) J. Biol. Chem. 273, 9078–9084
60. Shibata, T., Ochiai, A., Kanai, Y., Akimoto, S., Gotoh, M., Yasui, N., Machinami, R., and Hirohashi, S. (1996) Oncogene 13, 883–889
61. Daniel, J. M., and Reynolds, A. B. (1997) BioEssays 19, 883–891
62. Ozawa, M., and Kemler, R. (1998) J. Biol. Chem. 273, 6166–6170
63. Christofori, G., and Semb, H. (1999) Trends Biochem. Sci. 24, 73–76
64. Muller, T., Choidas, A., Reichmann, E., and Ullrich, A. (1999) J. Biol. Chem. 274, 10173–10183
65. Lin, S. Y. A., Makino, K., Xia, W., Martin, A., Wen, Y., Yin, K., Bourguignon, L. Y. W., and Hung, M. C. (2001) Nat. Cell Biol. 3, 802–808
66. Rohatgi, R., Ma, L., Miki, H., Lopez, M., Kirchhausen, T., Takenawa, T., and Kirschner, M. W. (1999) Cell 97, 221–231
67. Howard, T. H., and Meyer, W. H. (1984) J. Cell Biol. 98, 1265–1271
68. van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loireiro, J., Ypma, A., Hursch, D., Jones, T., Beijsovec, A., Peifer, M., Martin, M., and Clevers, H. (1997) Cell 88, 789–799
69. Jiang, W. G., Puntis, M. C. A., and Hallett, M. B. (1994) Br. J. Surg. 8, 1576–1590
70. Lauffenburger, D. A., and Horwitz, A. F. (1996) Cell 84, 359–369
71. Ghatak, S., Misra, S., and Toole, B. P. (2004) J. Biol. Chem. 280, 8875–8883
72. Vainio, S., Heikkila, M., Kispert, A., Chin, N., and McMahon, A. P. (1999) Nature 397, 405–409
73. Boerboom, D., Paquet, M., Hsieh, M., Jinsong, L., Jamin, S. P., Behringer, R. R., Sirois, J., Taketo, M. M., and Richard, J. S. (2005) Cancer Res. 65, 9206–9215
74. Saegusa, M., Hamano, M., Kuwata, T., Yoshida, T., Hashimura, M., Akino, F., Watanabe, J., Kuramoto, H., and Okayasu, I. (2003) Cancer Sci. 94, 103–111
75. Roose, J., and Clevers, H. (1999) Biochim. Biophys. Acta 1424, M23–M37
76. Piedra, J., Martinez, D., Castano, J., Miravet, S., Dunach, M., and de Herrelos, A. G. (2001) J. Biol. Chem. 276, 20436–20443
77. Ding, Q., Xia, W., Liu, J. C., Yang, J. Y., Lee, D. F., Xia, J., Bartholomeusz, G., Li, Y., Pan, Y., Li, Z., Bargou, R. C., Qin, J., Lai, C. C., Tsai, F. J., Tsai, C. H., and Hung, M. C. (2005) Mol. Cell 19, 159–170
78. Sherr, C. J. (1996) Science 274, 1672–1677
79. Chen, C. H., Shen, J., Lee, W. J., and Chow, S. N. (2005) Int. J. Gynecol. Cancer 15, 878–883
80. Barbieri, F., Lorenzi, P., Ragni, N., Schettini, G., Bruzzo, C., and Pedulla, F. (2004) Oncology 66, 310–315
81. Pelengaris, S., Khan, M., and Evan, G. (2002) Nat. Rev. Cancer 2, 764–776