The Spy1/RINGO Family Represents a Novel Mechanism Regulating Mammary Growth and Tumorigenesis

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Abstract
Spy1A is a unique cell cycle activator known to mediate cell cycle progression and override the DNA damage response. This study focused on determining the role of this protein on postnatal mammary gland morphogenesis and neoplasia. Herein, we show that Spy1A levels are tightly regulated during mammary gland development and that ectopic expression stimulates precocious development and results in disrupted morphology of the gland. This follows the same trend as the oncogene c-Myc, and we show that Spy1A expression is regulated downstream of c-Myc signaling. Importantly, we show that overexpression of Spy1A accelerates tumorigenesis in vivo. Collectively, this work is the first report that the Spy1/RINGO family of proteins may play an essential role in regulating both normal and abnormal growth processes in the breast. [Cancer Res 2008;68(10):3591–600]

Introduction
Systematic movement through the cell division cycle is an intricately controlled process that lays at the heart of all cell fate decisions. Progression through this cycle relies on the production and destruction of cyclin proteins, which function by binding to their catalytic partners, the cyclin-dependent kinases (CDK). These regulatory cyclin/CDK complexes are subject to negative regulation when bound to the CDK inhibitors (CKI). Spy1, or Speedy, is an atypical cyclin known to play essential roles in cell proliferation and oocyte maturation. First isolated in Xenopus, Xenopus-Spy1 (X-Spy1) was shown to induce rapid meiotic maturation and premature activation of CDKs (1, 2). There are multiple isoforms of Spy1 that are now classified into a larger family of proteins, known as the Spy1/RINGO family. The originally characterized Spy1A isoform, herein called Spy1A1, is expressed in multiple human tissues and immortalized cell lines (3, 4). Previous data in multiple immortalized cell systems has shown that overexpression of Spy1A promotes a shortening of G1-S phase of the cell cycle, activation of CDK2, degradation of the CKI p27Kip1, and ultimately enhanced cell proliferation (3–5). Importantly, small interfering RNA (siRNA) knockdown of Spy1A prevents these events, thereby demonstrating the essentiality of Spy1A in cell growth mechanisms (4). Furthermore, Spy1A has been shown to override the DNA damage response, functioning to inhibit DNA damage-induced apoptosis (6). Hence, Spy1A plays a key role in regulating both cell growth and death processes.

The mammary gland provides an excellent model, wherein to study the in vivo function of Spy1A since the majority of proliferation and patterning required to develop the organ occurs postnatally (7). During puberty, hormones and growth factors stimulate rapid proliferation and extensive lateral branching of mammary gland epithelia (8). Early in pregnancy, the quiescent gland responds to hormones of pregnancy with an initial burst of epithelial cell proliferation (9). During pregnancy, signaling factors and hormones begin an early wave of mammary epithelial differentiation, which, after parturition, results in terminal differentiation to establish lactation (10). After weaning, withdrawal of growth-regulating hormones results in a rapid involution of the gland through the induction of apoptosis (11). Importantly, improper regulation of these signaling pathways can lead to mammary epithelial hyperplasia and ultimately to mammary gland tumorigenesis (12).

All breast cancers, whether familial or sporadic, depend on the accumulation of multiple genetic lesions for their malignant progression. Cytogenetic studies have indicated specific chromosomal regions that are subject to loss or gain of genetic material, including 8q24, the genomic locus for the proto-oncogene c-Myc (13). c-Myc has been shown to be overexpressed in ~70% of human breast cancers, wherein 16% of this is accounted for by an amplification of the c-Myc gene (14). c-Myc overexpression can occur in part due to mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase activation, which have been shown to facilitate c-Myc protein stabilization and translation, respectively (15, 16). Herein, we show a novel relationship between c-Myc and Spy1A. Interestingly, recent serial analysis of gene expression analysis and other microarray studies have indicated that Spy1A is elevated in various aggressive breast cancers (17–19).

Here, we show that Spy1A protein and mRNA levels are tightly regulated during the course of mammary gland development and that this regulation lay downstream of c-Myc and MAPK signaling pathways. We show that ectopic expression of Spy1A expression in the mammary gland leads to accelerated development and abnormal morphogenesis in vivo. Importantly, this work shows for the first time that Spy1A may play a role in the initiation and/or progression of tumorigenesis in vivo. Hence, the Spy1/RINGO family of cell cycle proteins represents a novel regulatory mechanism involved in both normal and abnormal mammary gland development; further study of this pathway may reveal novel treatment strategies for breast cancer.

Materials and Methods

Cell culture. Phoenix cells (purchased from American Type Culture Collection) and Rosa26-MycERT2 (Myc-ER) mouse embryonic fibroblast (MEF) cells were maintained in DMEM (Sigma) containing 10% (v/v) fetal...
bovine serum (Sigma), HC11, BALB/c mouse mammary epithelial cell line (provided by Dr. C. Shermanko, University of Calgary) were maintained in RPMI 1640 (Sigma) containing 10% (v/v) FCS (Sigma) and supplemented with 5 μg/mL insulin (Sigma) and 10 ng/mL epidermal growth factor (EGF; Invitrogen). All cell lines were maintained in medium containing 2 mM/L L-glutamine (Sigma), penicillin (Invitrogen), and streptomycin (Invitrogen) and were maintained in a 5% CO2 environment. During the differentiation time course, HC11 cells were grown to confluence and maintained for 2 d in growth media to confer competent cells capable of responding to lactogenic hormones. EGF-containing media was then removed; the cells were rinsed twice with PBS and then incubated in differentiation media containing RPMI 1640, 1% FCS, 2 mM/L L-glutamine, 5 μg/mL insulin, 1 μM dexamethasone (Sigma), 5 μg/mL prolactin (Sigma), penicillin, and streptomycin for the remainder of the time course; differentiation media was changed every 2 d. To assess differentiation, β-casein luciferase was transfected into HC11 cells and activity was monitored using the Luciferase Assay System (Promega) and Wallac Victor Reader (Perkin-Elmer).

Plasmids and antibodies. The β-casein-luciferase construct (provided by Dr. M. David, University of California-San Diego) contains residues –344 to –1 of the β-casein promoter in front of a promoterless luciferase gene, pSVOL β5Luc, which was constructed using the pLucDSS vector (20, 21). The flag-Spy1A-pLXSN construct was previously described (5). The siRNA against Myc and control siRNA were purchased (Santa Cruz). Human Spy1 antibody was generated as described previously (4). Other antibodies used and their distributors are as follows: Myc (9E10; Santa Cruz), actin (MAB1501R; Chemicon), proliferating cell nuclear antigen (PCNA; 90-1149; Zymed), IgG (22–24). Subconfluent phoenix cells were treated with 25 μM/L chloroquine (Sigma) 5 min before transfection and then transfected separately with flag-Spy1A-pLXSN and pLXSN using the BBS/calcium phosphate precipitation method (25). Infectious flag-Spy1A-pLXSN and pLXSN viruses were collected 48 h posttransfection. HC11 cells were incubated with retrovirus for 24 h in the presence of 5 μg/mL polybrene (Sigma). HC11 growth media was supplemented with the selection antibiotic, 400 μg/mL G418 (Sigma). G418 resistant clones were selected and maintained in HC11 cell growth media supplemented with 400 μg/mL G418. Individual colonies or mixed populations of cells were selected, and lysates were analyzed on 10% polyacrylamide-SDS gels and transferred to polyvinylidene difluoride PLUS (PVDF-PLUS) transfer membrane. Expression of flag-Spy1A was monitored by immunoblotting using both flag and endogenous Spy1 antibodies; Actin was used as a loading control. Colonies 3 and 6 (C3 and C6) were selected, as well as two individually infected mixed populations (M1 and M2), all of which had significantly elevated Spy1A expression. Integration was also monitored by PCR using genomic DNA and primers spanning the flag-tag and an internal Spy1A fragment. Transient transfections of HC11 cells were carried out using cells at 60% confluence with the ESCORT II transfection reagent following the protocol, as suggested by the manufacturer (Sigma).

Immunoblotting. Total protein was isolated from cell cultures by harvesting cells and lysing in 0.1% NP40 lysis buffer [5 mL 10% NP40, 10 mL 1mol/L Tris (pH 7.5), 5 mL 0.5 mol/L EDTA, 10 mL 0.5 mol/L NaCl up to 500 mL reverse osmosis water] containing protease inhibitors (10 μL/L phenylmethylsulfonyl fluoride, 3 μL/L aprotinin, and 10 μL/L leupeptin) for 30 min on ice. Total protein was extracted from frozen mammary gland tissue as previously reported for whole cell extract preparation from mammary gland (26). Protein concentration was determined by Bradford reagent (Sigma). Protein samples (20–30 μg) was subjected to electrophoresis on denaturing SDS–10% polyacrylamide gels and transferred to PVDF-PLUS transfer membranes (Osmontics, Inc.) for 3 h at 30 V using a wet transfer method. Blots were blocked for 2 h in TBS–TWEEN 20 (TBST) containing 3% nonfat dry milk (blocker) at room temperature; primary antibodies were reconstituted in blocker and incubated over night at 4°C, and secondary antibodies were used at a 1:10,000 dilution in blocker for 1 h at room temperature. Blots were washed thrice with TBST after incubation with both the primary and secondary antibodies. Washes were 20 min each after the primary antibody and 10 min each after the secondary antibody. Chemiluminescent peroxidase substrate-3 (Sigma) was used for detection, and images were quantified on an Alpha Immotech HD2 ( Fisher) using AlphaEase FC software.

Reverse transcription–PCR reactions. Total RNA was extracted using RNeasy Plus Mini Kit (Qagen). RNA quality and quantity were analyzed using RNA 6000 Nano Assay and 2100 Bioanalyzer (Agilent). DNase treatment of each RNA sample was performed before reverse transcription–PCR (RT-PCR) using RQ1 RNase-free DNase kit (Promega). For end point RT-PCR, reverse transcription was carried out using Superscript II Reverse Transcriptase (Invitrogen). Taq PCR (New England Biolab) was used for the PCR reaction using following primers, all designed to span an exon-exon junction:

PCR cycles were optimized to ensure that end-point measures were on a log-phase scale; in all plots shown, 25 amplification cycles were used for quantitative real-time PCR reactions, high-capacity cDNA reverse transcription kit, and Taqman gene expression assays for β-casein (Mm00839664_m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Mm99999915_g1). Taqman gene expression master mix was used according to the manufacturer's instruction (ABI). All PCR products were separated on 8% PAGE, quantitative real-time PCR (Q-RT-PCR) replicates were pooled, and end-point RNA was run to ensure proper amplicon size. Gels were visualized on an Alpha Immotech HD2 Gel Documentation System after ethidium bromide staining using AlphaEaseFC software.

Mammary gland development assays. Mammary glands were collected from C57BL/6 female mice during a developmental time course. C57BL/6 mice were maintained following the American Association of Laboratory Animal Care guidelines at University of Tennessee (protocol #1422). Fat pad transplant assays were conducted using BALB/c mice, which are syngeneic for the HC11 cell line. BALB/c mice were maintained following the Canadian Council on Animal Care guidelines at University of Windsor (protocol #06-19). Stable Spy1A-HC11 cells and Cntl-HC11 cells (5 × 10^5) were injected into the cleared fat pad of four pair mammary glands of 22-d-old mice and allowed to grow for 8 to 16 wk. Successful clearing was monitored by incorporating a control mouse with no injected cells into each set of experiments. Tumor incidence was monitored weekly after 4 wk by palpation of the gland. Glands were either fixed for whole mount analysis, paraffin embedded for immunocytochemistry, or flash frozen for use in Western blots or genomic or mRNA analysis. Whole mount carmine alum staining of mammary gland was performed, as previously described (27), and were visualized on a Leica MZFLII dissecting scope; pictures were taken using Northern Eclipse software. Expression was monitored using PCR of genomic DNA (isolated using PureLink Genomic DNA purification kit, Invitrogen) and primers for human Spy1 spanning an exon-exon junction Spy1A forward primer (A091) CCATGCGCTTTAGGAAAAAAC and reverse primer (A073) TGGCCATAA CCTCTCCACAAAAC or primers for pLXSN, forward primer (A061) CCCCTGACACTCCGTGCCCC and reverse primer (A065) GAGCTCTGGGACA ATTCCCCACAC. Immunohistochemistry was carried out using 10% formalin fixation followed by paraffin embedding, sectioning (6-μm sections), and staining with Mayer's H&E β-phoxine (Sigma), as previously described (28). Staining with specific antibodies was carried out as per manufacturers recommendations.

Results

Utilizing a mouse model of mammary gland development, tissue was collected at various time points during development and prepared for protein and mRNA analysis. Immunostaining shows that Spy1A is primarily localized to the epithelial cells and that expression is absent or weak in the myoepithelial cells, as well as
in adipose tissue (Fig. 1A). Expression patterns seen by immunostaining are mimicked by Western blot analysis, where Spy1A protein levels are high in virgin glands, elevated in early pregnancy, and dramatically reduced at peak lactation, coinciding with terminal differentiation. Interestingly, at 4 days postweaning, a period of intense apoptosis, Spy1A expression is up-regulated again (Fig. 1B). Figure 1C shows that Spy1A mRNA is also elevated during early pregnancy and is depleted at 15 days pregnancy, a time when the gland begins to undergo the early stages of secretory differentiation, as indicated by β-casein expression. Collectively, these data show that Spy1A protein is tightly regulated through the course of mammary gland development.

Using the mammary epithelial cell line HC11 as a model system, we have determined that Spy1A protein (Fig. 2A) and mRNA levels (Fig. 2B) are high in proliferating mammary cells and are drastically depleted at 3 days postdifferentiation, a time period that is coincident with the onset of β-casein expression (Fig. 2B, bottom and C). Cells grown to confluence, to infer competency to respond to differentiation stimuli, do not show any significant reduction in Spy1A expression over loading controls. Hence, Spy1A expression is tightly regulated at the protein and mRNA level during development in this model system.

To investigate the possible signaling pathways mediating controlled expression of Spy1A during development, we treated proliferating HC11 cells with a variety of different hormones or inhibitors and studied the direct effects on Spy1A protein expression. Figure 3A shows that treatment with inducers of differentiation, dexamethasone, and prolactin, do not directly down-regulate Spy1A protein levels compared with vehicle (DMSO) alone (top, lanes 1–3). However, treatment with the MAPK inhibitor...
Spy1A protein levels (top, lane 5), whereas levels of Spy1A were not altered by the control compound SB202474 (top, lane 4). These data suggest that Spy1A expression is dependent on active MAPK signaling.

Expression of c-Myc is tightly regulated in the mammary gland and reflects a very similar pattern of expression as that observed with Spy1A (29, 30). Hence, we wished to determine whether Spy1A could be a downstream target for c-Myc signaling. To this end, we used a Rosa26-MycER<sup>T2</sup> MEF inducible cell system which expresses a mutant murine form of the estrogen receptor (ER<sup>T2</sup>) that is no longer capable of binding to estrogen (E2) but instead binds to 4-hydroxytamoxifen (TAM; refs. 31, 32). TAM treatment removes the steric hindrance of the ER<sup>T2</sup> receptor and allows for activation of the fusion protein, c-Myc (32). Because MEFs do not express endogenous estrogen receptor, all the effects of TAM addition can be attributed to the action of c-Myc. Using MEF cells lacking the Myc-ER<sup>T2</sup> fusion as a control (Myc-ER<sup>T2</sup>−/−) or the Myc-ER<sup>T2</sup> MEF cell line (Myc-ER<sup>T2</sup>+), we addressed whether c-Myc has a direct effect on Spy1A expression. Figure 3B shows that the Myc-ER<sup>T2</sup> cells turn on Spy1 protein after 15 minutes of TAM treatment (top). This figure further shows that Spy1A protein levels are elevated significantly by 45 minutes after the induction of c-Myc (middle). Next, we wanted to determine whether this induction was occurring at the mRNA level. As seen in Fig. 3B (bottom), Spy1A mRNA levels accumulate significantly at 1-hour posttreatment with TAM. To determine whether Spy1A induction by c-Myc requires de novo protein synthesis, mRNA levels of Spy1A were monitored through a time course of TAM treatment in the presence of the protein synthesis inhibitor cyclohexamide. Even in the absence of de novo protein synthesis, Spy1A mRNA levels were elevated after treatment with TAM (Fig. 3C, top, lanes 2–5). This accumulation was not a result of the cyclohexamide treatment alone (lanes 6–8). These experiments show that c-Myc, an established regulator of mammary gland proliferation, is capable of activating Spy1A mRNA and protein expression. Whether c-Myc is functioning directly as a transcription factor for Spy1A remains to be determined. We then used the HC11 cell system in the presence of c-Myc siRNA (siMyc) or a scrambled siRNA sequence (siCntl) to determine the necessity for c-Myc on Spy1A expression in proliferating mammary cells (Fig. 3D). Densitometry shows that depletion of c-Myc protein by 36% with siRNA results in a 28% decrease in Spy1 protein levels. This exciting correlation between Spy1A and the oncogene c-Myc emphasizes the importance of understanding how Spy1A is regulated during mammary gland development since it may provide future relevance to the understanding of breast cancer.

Mammary gland epithelial cells bear several histologic features that are essential for proper cellular function; they retain a polarized morphology, have specialized cell-cell contacts, and are attached to an underlying basement membrane. These characteristics contribute toward the function and development of the mammary gland. Disruption of the epithelial cell architecture is a precipitating factor in the pathogenesis of epithelial tumors (33). The HC11 cell line is a nontransformed, clonal mammary epithelial cell line that can be used in the study of mammary cell morphogenesis. During differentiation, these cells secrete laminin, interact appropriately with the extracellular matrix, and form spherical structures which mimic the mammospheres formed by primary mouse mammary epithelial cells (Fig. 4A; Cntl-HC11; ref. 34). To determine the effects of Spy1A regulation on cell morphology, several plates of HC11 cells were infected with either flag-tagged Spy1A or an empty vector control using different batches of retrovirus. Stable cell lines were generated from two individual colonies, as well as two mixed populations of cells, for both flag-Spy1A (Spy1A-HC11) and empty vector control (Cntl-HC11). Spy1A-HC11 cells exhibited enhanced proliferation as measured by trypan blue analysis and BrdUrd incorporation, as previously described for many other cell types (data not shown; refs. 4–6). Interestingly, when cultured appropriately, the Spy1A-HC11 cells, unlike the Cntl-HC11 counterparts, are not capable of forming acini-like spheroids during development (Fig. 4A; Spy1A-HC11). Even during confluency, Spy1A-HC11 cells have a propensity to generate disorganized piles of cells rather than...
remaining as an organized blanket of cells, such as the Cntl-HC11 line (Fig. 4A, top). This shows that proper regulation of Spy1A levels plays an important role in maintaining mammary cell morphology.

To investigate the effect of Spy1A overexpression on differentiation events in vitro, Spy1A-HC11 and Cntl-HC11 were subject to a normal differentiation time course and mRNA was collected from cells throughout the phases of growth, confluency, and differentiation and analyzed using Q-RT-PCR. Results were quantified as the relative expression of β-casein to GAPDH and graphed as log relative quantification (Fig. 4B). Figure 4B shows that in Cntl-HC11 cells β-casein expression at day 1 postdifferentiation (D1) and accumulates gradually until day 4 differentiation (D4) with levels maintained in day 5 differentiation (D5). However, when Spy1A levels are maintained via a foreign promoter, β-casein levels are increased over 1,000-fold by D1 and accumulate only slightly more by D5; with final levels of β-casein expression for the Spy1A-HC11

Figure 3. Spy1A is a downstream target of MAPK and c-Myc signaling. A, proliferating HC11 cells were treated with dexamethasone (DEX), prolactin, vehicle control (DMSO), 1 μmol/L inhibitor control (SB203580), and 1 μmol/L MAPK inhibitor (PD98059). Lysates were run on 10% SDS page followed by immunoblotting with Spy1A antibody (top). Equal protein was loaded in each lane, as determined by Bradford protein assay and confirmed by immunoblotting the same blot with actin antibody (bottom). B, top, Myc-ERT2-control cells (left) and Myc-ERT2+ cells (right) were treated with 100 nmol/L TAM and total protein extracts collected at different time points (0, 15, 30, 45, 60, 90, and 240 min). Lysates were analyzed by 10% SDS-PAGE followed by immunoblotting with c-Myc antibody (top), Spy1A antibody (middle). Equal protein was loaded in each lane as determined by Bradford protein assay and confirmed by immunoblotting the same blot with actin antibody (bottom). B, bottom, Myc-ERT2+ cells were cultured and treated with 100 nmol/L TAM and total mRNA collected at various time points (0, 1, and 4 h). RT-PCR was performed using random hexamer primers to generate first-strand cDNA followed by PCR using Spy1A and GAPDH-specific PCR primers. Equal RNA was used in each RT-PCR reaction, as determined by RNA Nano assay and confirmed by equal GAPDH levels in all reactions. C, Myc-ERT2+ cells were cultured and treated with 50 μg/mL cyclohexamide 30 min before TAM treatment. Total mRNA was collected at various time points (0, 1, 2, 3, and 4 h) and analyzed by RT-PCR using random hexamer primers to generate first-strand cDNA followed by PCR using Spy1A-specific PCR primers. Equal RNA was used in each RT-PCR reaction as determined by RNA Nano assay and confirmed by equal GAPDH levels in all reactions. D, proliferating HC11 cells were treated with siRNA against c-Myc (siMyc) or control siRNA (scrambled sequence; siCntl) or c-Myc was transiently overexpressed (MycPCDNA3). Lysates were collected and analyzed via Western blot analysis. Immunoblots were probed with c-Myc (top) and Spy1A (middle) antibody. Equal loading was confirmed by Bradford protein assay and immunoblotting the same blot for actin antibody (bottom). Densitometry was performed using the AlphaInnotech, AlphaEaseFC Software.
Figure 4. Abundance of the Spy1A transcript disrupts cellular morphogenesis and accelerates terminal differentiation. Stable flag-tagged Spy1A over expressing HC11 cells (Spy1A-HC11) and control cell line (Cntl-HC11) were generated using retroviral gene transfer technology. Four separate colonies were isolated for experiments; this figure is depicting results gathered from colony M1; data were consistent for all colonies. A, effects of Spy1A overexpression on the ability of HC11 cells to acquire a differentiated morphology were determined by culturing Cntl-HC11 and Spy1A-HC11 stable cell lines to confluence and adding differentiation media. Top, bright field images of confluent Cntl-HC11 or Spy1A-HC11 cells; bottom, cells after differentiation (10× objective, middle; 63× objective, bottom). Square brackets indicate acini-like formations in Cntl-HC11 and disrupted aggregates in Spy1A-HC11 cells. B, total mRNA was collected from Spy1A-HC11 (dark columns) or Cntl-HC11 cells (light gray columns) during proliferation day 1, proliferation day 2, confluency (C), and postdifferentiation over a period of 6 d (D1-D5). RNA quality and quantity were determined by RNA Nano assay, and samples were analyzed by Q-RT-PCR using Taqman assay. β-Casein levels were normalized with corresponding GAPDH levels and are graphically depicted. The X axis reflects development time course, and the Y axis indicates log₁₀ of the relative β-casein expression. Error bars, SE over three replicate wells.
cells being just over 100-fold elevated compared with Cntl-HC11 cells. Collectively, these data show that, at a cellular level, Spy1A overexpression grossly alters the rate of mammary cell differentiation, resulting in abnormal morphogenesis.

To investigate the effects of Spy1A overexpression in vivo, we took advantage of the cleared fat pad transplant technique using HC11 syngeneic mice (BALB/c). Here, the prepubertal inguinal mammary glands are cleared from the terminal end buds, and the remaining fat pad is used as an injection site. The injected cells use the cleared mammary fat pad as an environment to reestablish the mature gland. It has been previously shown that the HC11 cells are a useful model system with which to study tumorigenesis in vivo using orthotopic transplant techniques (34–36). Carmine alum staining of whole mounts from four virgin transplanted mice show that fat pads injected with Spy1A-HC11 cells filled the mammary fat pad at a faster rate and exhibit increased ductal branching at only 10 weeks postsurgery (Fig. 5A, top right) compared with Cntl-HC11–transplanted fat pads (Fig. 5A, top).

Figure 5. Spy1A induces precocious lobular development in the virgin mammary gland in vivo. Spy1A-HC11 and Cntl-HC11 stable cells (colonies M1, M2, C3 and C6) were injected into the cleared fat pads of the fourth inguinal mammary glands of virgin, 22-d-old BALB/c mice. Whole mounts of several transplanted glands were analyzed using carmine alum staining at different time points during puberty. A, top, at 10 wk postsurgery, Spy1A-injected glands fill the cleared mammary fat pads completely and show increased ductal side branching (right) compared with Cntl-injected glands (middle). A saline-injected gland was used as a control for clearing (left). Data depict results from colony C3. Bottom, graph represents total numbers of side branches calculated from a control (Cntl)-injected gland (gray columns), as well as two glands injected with number 3 and number 6 colonies of Spy1A (dark columns). Error bars, SE over three fields of view from two separate samples from each colony. B, PCR analysis of genomic DNA collected from control (pLXSN) and Spy1-overexpressing glands from each colony (Spy1A-pLXSN: Mixed pop#1, Mixed pop#2, Colony#3, Colony#6). Top, pLXSN-Spy1 amplification using primers spanning the vector and tag of Spy1, as well as an internal primer at an exon-exon boundary (130-bp amplicon); bottom, the pLXSN empty vector using primers flanking either side of the multicloning site of the vector (117-bp amplicon). C, top, serial sections from 10 wk Cntl (left) and Spy1A (right) transplanted mice were taken and stained for PCNA and hematoxylin. Data depict gland using colony#3. Bottom, PCNA-positive cells were counted over three fields of view (>200 cells per view) for both Spy1A (dark columns) and Cntl glands (gray columns). Results are graphically depicted as percentage of cells stained. Error bars, SE over three fields of view from three separate samples from both colonies 3 and 6.
A saline-injected gland is used as a control for the clearing procedure (Fig. 5A, top left). Quantification of the number of side branches per mammary gland at 10 weeks shows that Spy1A-HC11 injected glands had 34% to 39% more side branching than pair-matched Cntl-HC11–injected glands (Fig. 5A, bottom). Integration of the empty pLXSN control or the individual Spy1A-pLXSN colonies were screened for genomic DNA purifications and PCR analysis using primers for Spy1-pLXSN (Fig. 5B, top) or the pLXSN backbone (Fig. 5B, bottom). Enhanced proliferation of the Spy1A-overexpressing glands is further confirmed by PCNA staining of sections at 10 weeks posttransplantation (Fig. 5C, top). At 10 weeks, there was a 60% increase in PCNA staining of the Spy1A-HC11 glands over Cntl-HC11 glands (n = 4; Fig. 5C, bottom). Hence, Spy1A expression enhanced proliferation and development of the mammary gland in vivo.

To determine whether the aberrant morphology and precocious growth characteristics for Spy1A both in vitro and in vivo could stimulate tumorigenesis in vivo, we further studied the HC11-transplanted glands over time. It is notable that the HC11 cells grow continuously in culture and have two mutant p53 alleles and therefore may be more sensitive to initiation of tumorigenesis; therefore, we used this model strictly to assess the role of Spy1A on the rate of early onset of tumorigenesis. By 13 weeks posttransplantation, large tumors were visibly detected in 83% of the mice.
that had been transplanted with Spy1A-HC11 cells from four different stable cell populations. No visible tumors occurred on the control contralateral gland of the animal that had been transplanted withCntl-HC11 cells (Fig. 6A). When excised, all but one of the fat pads injected with Spy1A-HC11 cells were larger in size, had numerous large solid mammary tumors, and had invaded into the stomach cavity and fifth mammary gland (Fig. 6B, middle). All but one of the Cntl-HC11–injected fat pads were smaller with no visible outgrowths (Fig. 6B, right). One control gland was larger and seemed infected but did not have palpable or visible outgrowths.

This is the first direct evidence that Spy1A is capable of promoting tumor formation. The 83% incidence seen in this experiment is particularly surprising because the incidence of mammary tumors in virgin, young BALB/c mouse strain is very low (37). Whole mounts of early stage tumor glands extracted immediately after detection of a palpable tumor revealed extensive hyperplasia resulting in severe abnormalities in the morphology of the ductal tree (data not shown). H&E staining of six Spy1A glands at 16 weeks posttransplant show the presence of primarily two histologically distinct tumor phenotypes. Four of the tumors had larger pleomorphic nuclei with sparse basophilic cytoplasm and vesicular chromatin patterns and grew in ill-defined glandular patterns or in diffuse sheets (Fig. 6C). The remainder of the tumors primarily consisted of uniform cells with abundant eosinophilic cytoplasm with small ovoid nucleus and dense chromatin. PCNA staining of tumor sections showed a 30% increase in PCNA-positive cells in the Spy1A-transplanted tissue compared with Cntl-transplanted glands (Fig. 6D). Spy1A-derived tumors harbor a large number of keratin 8/18–positive cells, indicative of the luminal epithelial cell lineage, and there were very few anti–smooth muscle actin–positive cells found in the tumor tissue, indicating a low number of myoepithelial cells (data not shown). Together these findings show that Spy1A is directly implicated in the initiation and/or the progression of carcinogenesis. Importantly, this is the first evidence that the Spy1/RINGO family of cell cycle regulators plays a role in both normal and abnormal mammary gland development, potentially via a MAPK/c-Myc–mediated pathway.

Discussion

There are five Spy1/RINGO family members found in mammals; each differ in their expression profile, yet all contain a Spy1/RINGO box and bind and activate CDKs (reviewed in ref. 38). The only isoform identified to date in the mammary gland, and the focus of this study, is Spy1A. In both the developing mouse gland and the HC11 cell system, we have shown that Spy1A protein and mRNA levels are naturally elevated during proliferative stages of mammary gland development and are rapidly down-regulated at the onset of lactogenesis. Our data show that Spy1A protein levels are not altered by treatment with prolactin or dexamethasone alone; however, Spy1A is down-regulated after MAPK inhibition in proliferating HC11 cells. This shows that MAPK may play an essential role in maintaining high levels of Spy1A during proliferation of the gland. EGF is one of the primary components of HC11 cell growth media, and this component is removed to allow for differentiation (39, 40). It is well established that EGF activates different signaling pathways, such as the MAPK pathway (41). In vivo another strong candidate for MAPK activation is the estrogen signaling pathway (42). Previous data have implicated MAPK as a downstream mediator of X-Spy1; MAPK is required to support X-Spy1–induced oocyte maturation, and injection of X-Spy1 into oocytes results in the activation of the MAPK pathway (2). It is plausible that the involvement of MAPK upstream of Spy1A, as our data suggest, is dependent on the nature of the stimulus and/or is a cell type–dependent mechanism. It may also reflect the presence of a positive feedback loop between Spy1 and the MAPK pathway; details with regard to the involvement of MAPK on Spy1 regulation and mechanism of action require further investigation.

Mammary fat pad experiments show that Spy1A overexpression increases the rate of ductal morphogenesis, the presence of ductal side branching, and terminal end bud formation in the virgin mammary gland. This precocious development phenotype resembles data seen in p27 hemizygous knockout mice (43). Spy1A is known to play a direct role in enhancing the degradation of p27 (5, 38). It is interesting to note that, in glands null of p27 (p27/−/−) or fat pads reconstituted with p27/−/− epithelium present with an inverse phenotype to the hemizygous glands and to the Spy1A overexpressing glands, there is reduced ductal branching and hypoplasia (43). The fact that this phenotype was not seen in the Spy1A-overexpressing glands shows that Spy1A does not interfere with critical events for p27 that may happen earlier in the cell cycle. Whether down-regulation of p27 regulates Spy1-mediated tumorigenesis remains to be determined. Surprisingly, Spy1A ectopic overexpression also leads to premature alveolar development both in vitro and in vivo. This seemingly converse role in promoting both proliferation and differentiation is not unique. It has recently been shown that the consequences of aberrant c-Myc activation in the mammary gland are dependent upon the developmental stage of the gland at the time of c-Myc exposure. If activated during a specific 72-h window during midpregnancy, c-Myc also promotes precocious differentiation, which leads to premature involution of the gland (44). Spy1A follows the same expression pattern as c-Myc during normal mammary development and is expressed downstream of c-Myc, whether Spy1A functions to regulate c-Myc effects in the breast or function similarly during pregnancy in vivo remains to be determined.

Importantly, Spy1A overexpression in vitro and in vivo leads to abnormal morphogenesis and eventually into rapid, invasive tumorigenesis. It is known that lactogenesis represents full-term pregnancy and confers significant protection against breast cancer when it occurs in the young animal or human (45). Whether dysregulation in the timing of lactogenesis by Spy1A and/or whether known downstream targets of Spy1A, CDK2, and p27Cip1 are implicated in Spy1A-mediated tumorigenesis remains to be determined. Collectively, these data provide exciting results, demonstrating for the first time that Spy1A plays a role in mammary development and tumorigenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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