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Modeling NF2 with human arachnoidal and meningioma cell culture systems:

NF2 silencing reflects the benign character of tumor growth

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Abstract

Meningiomas, common tumors arising from arachnoidal cells of the meninges, may occur sporadically, or in association with the inherited disorder, neurofibromatosis 2 (NF2). Most sporadic meningiomas result from NF2 inactivation, resulting in loss of tumor suppressor merlin, implicated in regulating membrane-cytoskeletal organization. To investigate merlin function in an authentic target cell type for NF2 tumor formation, we established primary cultures from genetically-matched meningioma and normal arachnoidal tissues. Our studies revealed novel and distinct cell biological and biochemical properties unique to merlin-deficient meningioma cells compared to merlin-expressing arachnoidal and meningioma cells, and other NF2-deficient cell types. Merlin-deficient meningioma cells displayed cytoskeletal and cell contact defects, altered cell morphology and growth properties, most notably cell senescence, implicating the activation of senescence pathways in limiting benign meningioma growth. Merlin suppression by RNAi in arachnoidal cells replicated merlin-deficient meningioma features, thus establishing these cell systems as disease-relevant models for studying NF2 tumorigenesis.

Keywords

merlin; NF2; neurofibromatosis 2; meningioma; arachnoidal; senescence; actin cytoskeleton; α -catenin; contact inhibition; RNA interference

Introduction

Meningiomas arise from the arachnoid lining covering the brain and spinal cord and account for ~25% of all primary central nervous system neoplasms (Lantos et al. 1996; Perry et al. 2004). Although generally slow-growing and benign, these tumors cause significant morbidity

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by compressing adjacent cranial nerves or blood vessels. They may occur sporadically, or in the familial tumor disorder neurofibromatosis 2 (NF2), which also exhibits schwannomas (Gutmann et al. 1997; Evans et al. 2005;). About 60% of sporadic meningiomas result from biallelic inactivation of the *NF2* tumor suppressor gene and consequent inactivation of the merlin protein (Seizinger et al. 1987; Trofatter et al. 1993; Rouleau et al. 1993). Other genetic aberrations are implicated in the initiation of non-NF2 associated meningiomas and in the progression of benign meningiomas to higher-grade tumors (Collins 2004; Lamszus 2004; Perry et al. 2004).

Merlin is related to ezrin, radixin and moesin (ERM) of the band 4.1 superfamily of integral membrane-cytoskeletal linkers (Trofatter et al. 1993; Rouleau et al. 1993) and is believed to overlap functionally with these proteins, which are essential for regulating many cellular processes including cell shape, motility, membrane transport and signal transduction pathways (Gonzalez-Agosti et al. 1996; Pelton et al. 1998; Hall 1998; Tsukita and Yonemura 1999; Bretscher et al. 2002; McClatchey and Giovannini 2005). However, in spite of the wealth of information gained from mouse models (McClatchey and Cichowski 2001; Gutmann and Giovannini 2002; Kalamarides et al. 2002) and comparative ERM studies, merlin's growth regulatory mechanisms have not been fully elucidated. Actin-cytoskeletal disruption, cellular overproliferation and loss of cell contact inhibition have been observed in several different cell lines lacking merlin or expressing dominant-negative merlin (Lutchman and Rouleau 1995; McCartney and Fehon 1996; Pelton et al. 1998; Morrison et al. 2001; Johnson et al. 2002; Lallemand et al. 2003; Okada et al. 2005). Conversely, merlin overexpression results in inhibition of many signaling pathways (Gutmann et al. 1999; Lutchman and Rouleau 1995; Sun et al. 2002; Xiao et al. 2005). Reports of inconsistencies in merlin functions have often been attributed to species- or cell type-dependent differences. Importantly, only a small number of studies have been conducted in human target cell types that form tumors *in vivo* in response to merlin inactivation. Elegant studies performed in primary human schwannoma cells have been limited by the intrinsic difficulties in culturing sufficient pure Schwann cell populations from schwannoma and control tissue for comprehensive biochemical analysis (Pelton et al. 1998; Rosenbaum et al. 1998; Bashour et al. 2002; Kaempchen et al. 2003; Utermark et al. 2005; Nakai et al. 2006). Similarly, the scarcity of reliable human meningioma cell culture models has also hindered the direct examination of merlin's activity. No studies to date have analyzed *NF2*-deficient human tumor cells with relevant genetically-matched control cells.

Here, we report for the first time novel biochemical and cell biological properties of primary human merlin-deficient (merlin(-)) meningioma cells compared to their matched, non-neoplastic arachnoidal cell counterparts, with and without merlin suppression by RNAi. Our findings suggest that merlin mediates growth control by maintaining appropriate actin cytoskeletal organization and cell-cell communication, possibly by regulating α -catenin expression, but that merlin inactivation may also lead to premature activation of cell senescence programs that may restrict the growth of benign meningiomas *in vivo*.

Material and Methods

Human meningioma and arachnoidal cell culture

Thirty meningioma and six normal arachnoid specimens, including five sets of patient-matched samples, were received from surgical specimens, or from autopsies. Tumors were classified as sporadic or associated with NF2 based on diagnoses from referring physicians. These studies were approved by the Institutional Review Board (IRB) of Massachusetts General Hospital/Partners HealthCare and informed consent was signed by all living individuals donating tissue to this work. Tissues were harvested fresh: half quick-frozen for histological and/or mutational analysis and half cultured for the establishment of cell cultures as similarly described (Rutka et al. 1986; Ng and Wong 1993). Briefly, the tumors were rinsed in Hank's balanced salt

solution, minced into pieces smaller than 1 mm³ and disaggregated into single cells by exposure to 0.02% collagenous III (Worthington Biochemical Corp., Lakewood, NJ) in complete medium [Improved MEM with L-glutamine (Richter's Modified; Cellgro, Mediatech, Inc.), 15% fetal bovine serum, 100 u/ml penicillin and streptomycin, and insulin 4 mg/l (Gibco, Life Technologies, Inc.)] and incubated at 37°C for 20 minutes. The tissue was pelleted 5 min at 200 X g, resuspended in complete medium, and seeded evenly in 25-cm² flasks and incubated at 37°C in humidified atmosphere (5% CO₂). Normal arachnoidal tissues were cultured by an analogous method (Frank et al. 1983; Motohashi et al. 1994). Confluent cultures were split using 0.05% trypsin/EDTA, and medium was changed twice a week. Phase contrast images were visualized with an inverted Nikon Phase Contrast-2 microscope and photographed using a Hamamatsu CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan) and IPLab imaging software (Scanalytics, Inc., Fairfax, VA). Alternatively, images were photographed using a SPOT RT Slider CCD camera and SPOT imaging software (Diagnostics Instruments Inc., Sterling Heights, MI). Cell size is represented as the mean $\pm$ SD of the average area (μ m²) of at least 30 cells from three independent arachnoidal (AC006, AC007, AC016), merlin (-) meningioma (MN302, MN355, MN363) and merlin(+) meningioma (MN276, MN299, MN354) cell lines plated subconfluently at similar passages (p6 or p7). The cell area was determined by tracing the cell perimeter using SPOT imaging software.

Histopathology

Primary tumors were formalin-fixed, paraffin-embedded, and sections were stained with hematoxylin and eosin and evaluated histologically by a neuropathologist (A.S.R). Tumors were classified as transitional, fibroblastic or meningothelial histological subtypes and graded as meningioma, atypical meningioma and malignant meningioma as established by the World Health Organization (WHO) criteria (Kleihues et al. 2002).

Mutation analysis

Exon scanning using single-strand conformation polymorphism (SSCP) was carried out on five tumor/and or peripheral blood specimens from patients where matched meningioma and arachnoidal samples had been obtained. In brief, the 15 exons known to harbor pathogenic alterations of the *NF2* gene were PCR amplified from genomic DNA in the presence of [α -³³P]dATP as previously described (Jacoby et al. 1996; Heinrich et al. 2003). When aberrations were detected by SSCP, the specific exon was re-amplified without [α -³³P] and the PCR product was purified using a PCR purification kit (Qiagen). Sequencing was carried out bidirectionally using the Big Dye Terminator Sequencing Kit (Applied Biosystems, Foster City, CA) or by manual sequencing using a radiolabeled terminator cycle sequencing kit (USB, Cleveland, OH). Loss of heterozygosity (LOH) in the *NF2* region was determined at the markers *D22S1148*, *D22S1163* and *D22S193* (centromeric to *NF2*), *NF2TET* and *D22S929* (intragenic) and *D22S430* (telomeric to *NF2*). PCR amplification using [α -³³P]dATP and gel electrophoresis was carried out as described previously (Heinrich et al. 2003).

Antibodies

Merlin monoclonal antibody, 1C4, and affinity-eluted rabbit polyclonal antibodies C26 and MP4 were produced as previously described (Gonzalez-Agosti et al. 1996; Wiederhold et al. 2004). Merlin and ERM proteins ezrin and moesin were detected with rabbit polyclonal N21 (Wiederhold et al. 2004). Epithelial membrane antigen (EMA) and cytokeratin 18 monoclonal antibodies were purchased from DAKO Corporation (Carpinteria, CA). The β -catenin polyclonal antibody and α -catenin, cyclin D1 and paxillin monoclonal antibodies were purchased from BD Bioscience (San Jose, CA). The GAPDH monoclonal antibody was purchased from Chemicon International (Temecula, CA). Polyclonal Erk1/2 and phospho-Erk1/2(Thr²⁰²/Tyr²⁰⁴) were purchased from Cell Signaling Technologies (Beverly, MA).

Immunocytochemistry

Primary cells established from twenty-five meningioma and six normal arachnoidal samples were plated on glass or laminin/poly-D-lysine-coated coverslips (BD Bioscience) as indicated and fixed with 4% paraformaldehyde (PFA)/phosphate-buffered saline (PBS). Immunofluorescent staining of F-actin using Alexa Fluor 594-conjugated phalloidin (Molecular Probes, Inc, Eugene, OR), and of focal adhesions, using a paxillin monoclonal antibody and Alexa Fluor secondary, were described previously (James et al. 2004). Cells labeled with EMA and cytokeratin 18 were fixed and stained according to manufacturers recommendations (DAKO). Briefly, cells were fixed with sequential incubations of cold acetone and methanol at -20°C for 10 minutes each, followed by hydration in PBS. Cells were blocked and incubated with primary antibodies and secondary Alexa Fluor-conjugated antibodies as described above. Images were acquired using LSM 5 Pascal software coupled to a Zeiss LSM Pascal Vario 2 RGB confocal system.

Senescence-associated (SA) β -galactosidase staining assay

Arachnoidal and merlin(-) meningioma cells were fixed and stained for SA β -galactosidase activity using the Senescence Detection Kit (Calbiochem, EMD Biosciences, Inc., San Diego, CA) according to manufacturer's instructions. Images were visualized with a Nikon Inverted microscope, Eclipse TE2000-U, and photographed using a SPOT RT Slider CCD camera and SPOT imaging software (Diagnostics Instruments Inc., Sterling Heights, MI).

Lentivirus-mediated merlin expression

Merlin isoforms 1 or 2, or a mixture of both, were introduced into five different merlin(-) meningioma cell lines using the CSCW2 lentivirus delivery system (Sena-Esteves et al. 2004). The CSCW2 lentivirus vector encodes the gene of interest and EGFP under the control of a CMV promoter. Full-length human merlin isoforms 1 and 2 were PCR-amplified with *NheI* restriction enzyme sites engineered into the 5' and 3' ends, gel-purified and subcloned into the *NheI* linearized CSCW2 vector. Orientation was confirmed by restriction enzyme mapping and CSCW2 expression constructs were sequenced prior to packaging. Purified, viral particles were harvested, and inoculated into merlin(-) meningioma cells at multiplicity of infections (MOIs) ranging from one to 100. Transduction efficiency was obtained from the average of the ratio of EGFP positive cells to total cells in three random fields (of at least 20 cells) from three independent experiments. Merlin re-expression levels were determined by comparing the densitometric values of merlin immunoblots of total cell lysates of infected cells to merlin(+) meningioma cells (See *Immunoblot analysis* below).

RNA interference of arachnoidal cells

Two merlin RNAi oligonucleotides were synthesized and assayed for their effectiveness at downregulating merlin expression in cell culture systems. Merlin RNAi sequences, named meri-5 and meri-6, were designed based on optimal sequence predictive software obtained through OligonEngine Inc. (Seattle, WA). The 21-bp merlin RNAi target sequence for meri-5 was 5' gaggaagcaaccaagacgtt 3' (exon 1), and for meri-6, 5' gatactgacatgaagcggctt 3' (exon 14). A non-specific RNAi "scrambled" (scr) transcript, 5' cagtcgcgtttgcgactgg 3', served as a negative control. The sense and antisense RNAi oligonucleotides, with intervening hairpin sequences, were annealed, phosphorylated and subcloned into *AgeI* and *EcoRI* restriction sites of the lentivirus-based delivery cassette, pLKOpuo1, which provides a high rate of stable integration and puromycin selection for obtaining homogenous populations of infected cells (Stewart et al. 2003). The constructs were sequenced prior to packaging and resultant viral particles were used to inoculate arachnoidal cells at various MOIs. Five different arachnoidal cell lines were infected with control vector, scr and merlin RNAi viral particles at a MOI of 10 and were selected with puromycin at 0.4 μ g/ml if maintained in culture greater than three

days. Changes in merlin expression were determined by immunoblot analysis of total cell lysates. No toxicity was observed in response to viral infections at a MOI of 10.

Immunoblot analysis

Meningioma and arachnoidal cells were harvested by washing cell layers twice in cold PBS and lysing in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet p-40 (NP-40), 0.5% deoxycholate acid, 0.1% SDS, 2 mM EDTA) containing phosphatase inhibitors (2 mM sodium orthovanadate, 50 mM sodium fluoride and 50 nM calyculin A) and a 1X Complete protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN). Total cell lysates were incubated for 30 min on ice, clarified by centrifugation (13,000 X g) and resuspended in 1X sample buffer (33% glycerol, 6.7% SDS, 330 mM dithiothreitol). Proteins were resolved by SDS-PAGE, transferred electrophoretically to nitrocellulose membranes (Bio-Rad, Hercules, CA) and subjected to immunoblot analysis using appropriate antibodies, horseradish peroxidase (HRP)-conjugated secondary antibodies and the enhanced chemiluminescence (ECL) detection systems (Amersham Pharmacia Biotechnology). Expression levels were quantified on a GS-800 Calibrated Densitometer (BioRAD) and *p*-values were determined using Paired Student's *t*-test.

BrdU incorporation assay

The Cell Proliferation ELISA BrdU colorimetric method (Roche) was used according to manufacturer's instructions to quantify cell proliferation based on the measurement of BrdU incorporation during DNA synthesis. Arachnoidal cells were infected with control (scr) or merlin RNAi (meri-5) expressing viral particles at an MOI of 10. Three days prior to obtaining BrdU measurements, cells were counted and 100 μ l aliquots (1×10^4 cells/ml) were seeded in quadruplicate in 96-well tissue culture plates and incubated at 37°C. Twenty-four hours prior to obtaining measurements, cells were treated with a 10 μ M 5-bromo-2'-deoxyuridine (BrdU) solution (Roche Diagnostics). Sample absorbance at 370 nm (reference wavelength 492 nm) was measured in an ELISA plate reader (BioRAD).

Results

Characterization of matched meningioma and arachnoidal cells

We processed for primary cell culture meningioma specimens and adjacent normal arachnoid membrane (when available) from individuals with a sporadic tumor or with NF2. Meningeal origin was confirmed by indirect immunocytochemical staining of cells using a panel of antibodies to established cell biomarkers, including epithelial membrane antigen (EMA), vimentin and various cytokeratins. Figure 1A illustrates typical positive staining of arachnoidal cells with cytokeratin 18 and EMA.

As 80-90% of meningiomas are classified as WHO Grade 1, we conducted experiments on histologically benign meningioma samples unless otherwise specified. At least two different matched arachnoidal and merlin(-) meningioma cell pairs were analyzed, in all cases with consistent findings. As expected, mutation and immunoblot analyses performed on a subset of these samples showed that meningioma cells from NF2-associated tumors did not express merlin (Fig. 1B; Table 1). In contrast, N21, an antibody that detects ERM proteins ezrin and moesin in addition to merlin, demonstrated comparable moesin expression levels in matched normal and tumor cells, and variable ezrin expression, which did not correlate with merlin expression. Unmatched sporadic and NF2-associated meningioma cells also were characterized by immunoblot analyses to determine merlin expression status (Fig. 1C; Table 2).

Merlin-deficient meningioma cells are morphologically distinct from arachnoidal cells

Previous investigation of the origin of meningiomas from arachnoidal tissue focused on their significant histological and ultrastructural similarities (Yamashima 1996). In a detailed examination in cell culture, we have detected striking morphological and growth differences between arachnoidal cells and merlin(-) and merlin(+) meningioma cells, regardless of histological subtype. Merlin(-) meningioma cells at early cell passage (p0-p4) exhibited a more fibroblast-like cell morphology, possessing numerous long cellular processes and increased associated granularity compared to arachnoidal cells, which displayed more epithelial or crescent-shaped cytology, and lacked remarkable granularity and long cellular processes (Fig. 2A). Furthermore, merlin(-) meningioma cultures typically exhibited more divergent cell morphologies than arachnoidal cultures, consistent with previous studies reporting that mixed histological phenotypes frequently coexist in meningiomas due to the expression of both epithelial and mesenchymal characters (Beschet et al. 1999). Merlin(-) meningioma cells cultured for longer periods demonstrated a greater proportion of enlarged, flattened cells with increased granules and vacuoles at much earlier passages (~p6) than arachnoidal cultures, which did not display this phenotype until advanced stages of growth (~p18-p20) (Fig. 2B). The size (mean area $\pm$ SD) of arachnoidal, merlin(-) meningioma and merlin(+) meningioma cells was calculated at 1175 ± 169 , 5270 ± 810 and $1649 \pm 427 \mu\text{m}^2$, respectively, by quantitating the average area of at least 30 cells from three independent cell lines. Arachnoidal cultures exhibited relatively less heterogeneity in cell morphology and size with increased passaging. Merlin(+) meningioma cultures were more similar morphologically to arachnoidal cells than to merlin(-) meningioma cells, lacking extensive granularity, long cellular processes and the premature acquisition of enlarged, flattened cell phenotypes (Fig. 2B).

Merlin-deficient meningioma cells possess altered growth properties

Subconfluent, matched merlin(-) meningioma and arachnoidal cells from the same individual generally proliferated with similar apparent growth rates at early passages, although proliferative rates of meningioma cells from different tumors were variable. However, upon reaching confluence, arachnoidal cells formed a cell contact inhibited monolayer, whereas most merlin(-) meningioma cultures, or a subpopulation of cells therein, displayed loss of cell contact inhibition of growth (Fig. 3A). A small proportion of merlin(-) meningioma cultures failed to reach confluence and could be distinguished from arachnoidal or merlin(+) meningioma cells at similar passages by slowing growth and enlarged, flat-cell morphology. Moreover, with continuous subculturing, we consistently observed a marked decrease in the number of passages that all merlin(-) meningioma cells could be maintained compared to matched normal arachnoidal cells. Merlin(-) meningioma cell cultures failed to exhibit steady growth rates as early as passage six, and could not be maintained in culture for greater than ten passages, whereas arachnoidal cells proliferated steadily and could be maintained in culture ~20 passages. Because reduced growth rate, enlarged, flattened cell appearance and increased granular material and vacuoles are distinct features of senescent cells, we stained matched arachnoidal and merlin(-) meningioma cells for senescence-associated β -galactosidase (SA- β -Gal), a hallmark of senescent cells (Dimri et al. 1995). Strong positive staining for SA- β -Gal was observed in merlin(-) meningioma cells (p9) compared to arachnoidal cells (p10), which appeared negative, or weakly stained, suggesting that senescence programs are activated more rapidly in merlin(-) meningioma cells (Fig. 3B). Interestingly, merlin(+) meningioma cells possessed growth characteristics intermediate between merlin(-) meningioma and normal arachnoidal cells and typically could be maintained in culture approximately 15 passages (data not shown).

In addition to reduced proliferative potential and concomitant increase in cell size (Fig. 2B), compared to matched arachnoidal cells with extended time in culture, merlin(-) meningioma cultures also developed sparse regions of rapidly growing cellular foci resulting from apparent

loss of cell contact inhibition of growth (Fig. 3C). Although foci developed from minor subpopulations of cells in culture, interspersed among large, flat, slow growing cells, their formation was unique to merlin(-) meningioma cells as they never developed in arachnoidal or merlin(+) meningioma cell cultures. Development of foci was observed in 73.3% of benign merlin(-) meningioma cultures (n=15), forming between passages 2 and 10. Counting of foci in four merlin(-) meningioma cultures revealed development of 8.7 ± 3.6 foci per 10^6 cells versus complete absence of foci in comparable arachnoidal cultures. Coincident with the intermittent development of cellular foci, large gaps in merlin(-) meningioma cell layers and increased numbers of floating cells were observed during routine passaging, suggesting a reduced capacity to maintain adherence to the substratum (Fig. 3C). Similarly maintained arachnoidal cells seeded efficiently and did not demonstrate increased numbers of floating cells. We were unable to reproducibly quantify the rates of cell proliferation of matched arachnoidal and meningioma cell pairs due to the inability of merlin(-) meningioma cells to proliferate steadily and remain attached to cell matrices in culture. Subculturing merlin(-) meningioma cells obtained from cellular foci was unsuccessful because they did not adhere to fresh plate surfaces, or coated surfaces of laminin/poly-D-lysine or fibronectin.

Merlin-deficient meningioma cells exhibit disrupted intercellular junction sites

Apparent loss of cell contact inhibition of growth exhibited by merlin(-) meningioma cultures was characterized by the overlap of adjacent cells, even under subconfluent conditions, in contrast to arachnoidal cultures, whose cells maintained tight associations at cell-cell boundaries even after extensive culturing (Fig. 4A). To assess the potential role of merlin in regulating cell contact inhibition of growth, we examined the integrity of intercellular junction sites by indirect immunofluorescent staining of one of its core components, β -catenin. Cytoplasmic α - and β -catenin proteins structurally link transmembrane receptors (cadherins) of neighboring cells to the actin cytoskeleton. Previous studies showed that merlin localizes to cell-cell boundaries and coimmunoprecipitates β -catenin (McCartney and Fehon 1996; Maeda et al. 1999; Lallemand et al. 2003). We found disrupted β -catenin localization in merlin(-) meningioma cells at adherens junctions or desmosomes compared to matched arachnoidal cells (Fig. 4B). Merlin(-) meningioma cells possessed poorly defined sites of cell-cell contact, as β -catenin staining was less distinct and diffusely distributed throughout the cytoplasm. By contrast, arachnoidal cells exhibited well-defined, punctate β -catenin staining at cell-cell boundaries. To determine whether merlin(-) meningioma cells demonstrated altered expression of the intercellular junction proteins α - and β -catenin, we carried out immunoblot analysis on matched meningioma and arachnoidal cell lysates. Our findings indicated that α -catenin protein levels may be reduced in merlin(-) meningioma cells compared to arachnoidal cells (Fig. 4C).

Merlin-deficient meningioma cells demonstrate abnormal actin stress fibers and increased ruffling membranes

Merlin's reported roles as both a structural linker between membrane and cytoskeletal elements, as well as a negative regulator of RacGTPase signaling, suggested that careful examination of the actin cytoskeleton in meningioma and normal arachnoidal cells was warranted. Our immunocytochemical analyses of primary patient merlin-deficient meningioma cells revealed two distinct abnormal stress fiber phenotypes that differed remarkably from normal matched arachnoidal cells. Consequently, focal contacts that associate with actin microfilaments at their cytoplasmic aspects and play an important role in the regulation of actin organization (Zamir and Geiger 2001) were also altered. Unlike arachnoidal cells which demonstrated fine, organized actin stress fibers oriented parallel to the long axis of the cell, and well-defined, punctate focal contacts localized primarily at the cell periphery (Figs. 5A and 5B), merlin(-) meningioma cells exhibited short, dense, disorganized stress fiber bundles that were scattered throughout the cell, terminating at numerous focal contacts (Fig. 5A). Other merlin(-) meningioma cells, often within the same culture, displayed aberrant, web-

or mesh-like stress fibers, with poorly-defined focal contacts at the cell periphery (Fig. 5B). Although the number of cells expressing the latter phenotype varied considerably (20%-100% of cells) among individual merlin(-) meningioma cultures, this feature was never observed in arachnoidal or merlin(+) meningioma cultures. This abnormal web-like stress fiber phenotype was not a response to slowed cell division or increased cell size as it was observed at early passages (p2), in steadily proliferating merlin(-) meningioma cultures. In addition to the pronounced web-like phenotype, increased actin accumulation at ruffling edges and lamellipodia formation was also prominent in merlin(-) meningioma cells compared to matched normal arachnoidal cells plated on laminin-coated coverslips (Fig. 5B). Interestingly, merlin (+) meningioma cells lacked the striking cytoskeletal defects displayed by merlin(-) meningioma cells and exhibited a more organized actin cytoskeleton and distinct, peripheral focal contacts, phenotypes that more closely resembled those of arachnoidal cells (Figs. 5A and 5B). Taken together, these data suggest a role for merlin in maintaining the integrity of the actin cytoskeleton and/or focal adhesion formation in the target cells for meningioma formation.

Merlin overexpression reverses some but not all actin cytoskeletal defects

To determine whether defects in actin cytoskeletal organization characteristic of merlin(-) meningioma cells can be attributed specifically to merlin deficiency, we introduced full-length merlin isoforms into merlin(-) meningioma cells in an effort to rescue the aberrant cytoskeletal phenotypes. Using a lentiviral-based delivery system, we transduced merlin(-) meningioma cells with constructs encoding merlin isoforms 1 or 2, or a mixture of both, at various multiplicity of infections (MOIs). Exogenous merlin expression at a MOI of 2, individually (Figs. 6A and B), or together (data not shown), resulted in a reduction of lamellipodia formation, prominent membrane ruffling and aggregation of actin at the cell periphery, compared to vector-control infected EGFP positive cells, or uninfected merlin(-) meningioma cells within the same field. However, merlin re-expression did not rescue the aberrant mesh- or web-like stress fiber phenotype characteristic of merlin(-) meningioma cells (Figs. 5B), indicating the existence of some cytoskeletal abnormalities that are not reversed by merlin reintroduction. This suggests that even in benign tumors, additional genetic or epigenetic events in addition to merlin inactivation may have already occurred during tumor formation. These studies were valuable for distinguishing specific merlin-mediated actin cytoskeletal regulatory activities; however, this model has its drawbacks. At a MOI of 2, we obtained a 12% infection rate and yet western blot analysis suggested merlin isoform 1 expression at 2-fold higher than endogenous levels (Fig. 6A; data not shown). This indicates that in infected cells merlin isoform 1 re-expression per cell was approximately 16-fold higher than endogenous levels, far outside the physiological range. Although the expression of the re-introduced merlin isoform 2 was lower, at only 23% of isoform 1 levels (Fig. 6A), it was still approximately 4-fold higher than endogenous levels in infected cells (Fig. 6B). Thus, as the re-expression model was complicated by these high per cell merlin re-expression levels which might have additional consequences beyond rescue of merlin deficiency phenotypes, we limited further analyses with this system in favor of a RNA interference (RNAi) strategy to suppress merlin below physiological levels of merlin in arachnoidal cells.

Merlin suppression in arachnoidal cells results in actin cytoskeletal defects similar to merlin (-) meningioma cells

To determine whether the abnormal growth properties and cytoskeletal defects observed in merlin(-) meningioma cells are a direct and specific effect of merlin inactivation, we downregulated endogenous merlin expression in arachnoidal cells by RNA interference (RNAi) using stable incorporation and expression of short hairpin merlin RNAs (shRNAs) (Stewart et al. 2003). This resulted in a dramatic reduction in endogenous merlin expression in arachnoidal cells expressing different merlin RNAi transcripts, termed meri-5 and meri-6,

compared to cells expressing a control “scrambled” (scr), non-specific RNAi transcript (Fig. 7A). Treated arachnoidal cells showed no compensatory increase in ERM protein levels and no effect on CyclinD1, but did display a slight, reproducible elevation in activation of Erk1/2 (1.21-fold, $p=0.003$), key regulators of many cellular processes including cell growth, proliferation and differentiation (Fig. 7B). A significant reduction (2.21-fold; $p = 0.002$) of α -catenin expression was also observed in merlin knockdown arachnoidal cells (Fig. 7C). Merlin suppression also produced most but not all of the aberrant actin cytoskeletal phenotypes observed in merlin(-) meningioma cells. These included a marked increase in lamellipodia formation (Fig. 7C; arrowheads) and disruption of stress fibers which appeared as short, thick, disorganized bundles terminating at numerous focal contacts scattered throughout the cell. Greater than 90% of merlin RNAi-treated arachnoidal exhibited these characteristics, while virtually no cells from control RNAi arachnoidal cultures displayed these phenotypes. However, merlin knockdown in arachnoidal cells did not recapitulate the mesh- or web-like stress fiber phenotype that often characterized merlin(-) meningioma cells (Figs. 5B), consistent with the view that additional genetic changes may occur in merlin(-) meningiomas.

Merlin-suppressed arachnoidal cells exhibit loss of cell contact inhibition and induction of cell senescent phenotypes

Suppression of merlin expression in arachnoidal cells also resulted in striking morphological changes. Within one week, ~70% of treated cells showed an increased cell size compared to control cells, similar to that observed for merlin(-) meningioma cells (compare Figs. 8A and 2B). After ~21 days in culture, merlin RNAi-treated cultures also developed sparse multilayers of cells or refractive foci (Fig. 8A), a feature unique to merlin(-) meningioma cells (Fig. 3C). Foci did not develop in untreated, vector transfected or scr RNAi arachnoidal cultures (or in merlin(+) cultures), indicating that merlin functions to block the loss of cell contact inhibition of growth. Additionally, many floating cells were observed in merlin RNAi cultures compared to controls, suggesting that merlin loss may result in reduced cell adherence after protracted periods in culture (data not shown). Identical results were obtained with two different RNAi target sequences (data not shown), indicating that the RNAi-induced phenotypes did not result from off-target effects, but rather through merlin suppression. Thus, merlin suppression in arachnoidal cells reproduces the fundamental morphological and growth characteristics that are observed in merlin(-) meningioma cultures.

To assess the rate of proliferation in merlin knockdown arachnoidal cells, we performed BrdU incorporation assays. Arachnoidal cells expressing merlin RNAi transcripts exhibited rates of BrdU incorporation initially similar to scr RNAi-infected cells as demonstrated at three days after RNAi treatment (Fig. 8B). By seven days, merlin RNAi arachnoidal cells exhibited a 25% reduction in the mean rate of BrdU incorporation compared to scr RNAi arachnoidal cells. Thus, the DNA synthesis rates of merlin RNAi suppressed arachnoidal cells reflected early passage and later passage growth phases of merlin(-) meningiomas cells. Collectively, these data suggest that the initial loss of merlin in human NF2 target cells results in deregulation of growth control through mechanisms that do not result in dramatic increases in cell proliferation. Subsequently, however, changes attributed to merlin loss may trigger a senescence program that limits the rate of tumor growth.

Discussion

We describe a novel and powerful human cell culture system for investigation of merlin's function in cells directly relevant to tumor formation due to NF2 insufficiency. Comparisons of merlin(-) meningioma to patient-matched arachnoidal cells revealed distinct differences in actin cytoskeletal organization, cell contact inhibition, cell size, proliferative capacity and unique signaling events. Further, NF2 gene silencing by RNAi in arachnoidal cells replicated

most of the distinguishing features of merlin(-) meningioma cells. These data support and extend previous findings, and also reveal novel features that may be reflective of unique mechanisms involved in formation and growth of benign tumors in humans.

Our data show that merlin may function to suppress tumor initiation by mediating appropriate cytoskeletal-based signaling events and/or cell-cell communication, in agreement with previous studies in several other cell types (Pelton et al. 1998; Lallemand et al. 2003). Primary merlin(-) meningioma cells exhibited intercellular adhesion defects and constitutively manifested a variety of cytoskeletal abnormalities, regardless of culture or plating conditions. To verify a functional role for merlin in regulating cytoskeletal and cell-cell adhesion pathways, we showed that merlin RNAi silencing in arachnoidal cells replicated these phenotypes, including a significant reduction in α -catenin expression which has not previously been described. Reduced expression of intercellular junction proteins leading to the aberrant assembly of these structures is known to result in pronounced defects in actin cytoskeletal organization (Vasioukhin et al. 2000); however, it is unclear whether disruption of intercellular junctions is a cause or a consequence of aberrant actin cytoskeletal signaling, resulting in loss of cell contact inhibition. Our findings are indeed consistent with merlin in a role as a negative regulator of GTPase Rac1 (Pelton et al. 1998; Shaw et al. 2001; Okada et al. 2005), whose activation is reported to stimulate lamellipodia and ruffling membrane formation and loosening of cell-cell contacts (Hall 1998; Pujuguet et al. 2003). Interestingly, we also observed evidence of deregulated Rho-mediated events in response to merlin loss. As previously reported, activated Rac may also lead to Rho stimulation with the consequent formation of stress fibers that increase in number and thickness over a period of several hours, but subsequently become reduced to only a few fibers (Ridley et al. 1992; Machesky and Hall 1997). Moreover, direct Rho activation was shown to result in the assembly of disorganized actin filament bundles and production of focal contacts. Since abnormal stress fiber and focal contact phenotypes are prominently displayed by both merlin(-) meningioma and merlin-suppressed arachnoidal cells regardless of serum conditions, our data suggest that merlin loss may also lead to altered or persistent Rho activation, dependent on or independent of Rac activity.

The growth patterns of merlin-deficient meningeal cells exhibited similarities, as well as several remarkable differences compared to other cell models. Subconfluent, merlin(-) meningioma cells displayed proliferated at a rate comparable to control matched arachnoidal cells at early passages, in agreement with growth rates reported in a *Nf2* MEF model system, which demonstrated that merlin regulates cell growth only under specific culture conditions, such as increased cell density (Shaw et al. 1998; Lallemand et al. 2003; McClatchey and Giovannini 2005). However, with continuous subculturing, merlin(-) meningioma cells consistently exhibited a marked reduction in proliferative potential, as reflected by a decline in the number of cell passages that they could be cultured, and the acquisition of enlarged, flattened cells that stained positive for SA- β -Gal, distinct features of senescent cells (Dimri et al. 1995; Ben-Porath and Weinberg 2005). Merlin-suppressed arachnoidal cells likewise exhibited similar growth and phenotypic profiles. The slowed growth response of merlin RNAi cells compared to control arachnoidal cells indicates that diminished growth rates of merlin(-) meningioma cells is not due to suboptimal cell culture conditions unique to tumor cells. Another unique growth property of merlin-deficient meningeal cells was the development of rapidly growing cellular foci. Cellular foci, which emerged regularly in predominately senescent merlin-deficient cultures, may represent cells that have escaped cell senescence programs as a result of additional secondary or mutational events subsequent to merlin loss. Interestingly, we also observed that merlin(+) meningioma cells more closely resembled normal arachnoidal cells, lacking the striking cytoskeletal, morphological and growth abnormalities characterizing merlin(-) meningioma cells, further implicating merlin in specific regulatory functions.

Our findings suggest that merlin inactivation may trigger programs that accelerate the onset of cell senescence in human meningiomas. Diminished proliferative capacity in response to merlin inactivation is seemingly contradictory for loss of tumor suppressor function; however, our findings are consistent with *in vivo* data from earlier reports demonstrating the absence of signs of active growth (as assessed by the number of mitoses) as characteristic of benign meningiomas, a feature also used as a WHO diagnostic criterion for grade I staging (Hakin-Smith et al. 2001). Induction of senescence is thought to be an important contributor to tumor suppression in benign meningiomas, although the mechanism by which this occurs is unclear (Hakin-Smith et al. 2001; Boldrini et al. 2003). In other tumor types, recent studies have demonstrated the existence of senescent cells in premalignant, early stage tumors, but not in malignant forms (Chen et al. 2005; Collado et al. 2005). Activation of cell senescence in response to merlin inactivation has not previously been reported but suggests that somatic inactivation of senescence pathway genes in NF2 target cells may be involved in progression from benign to more advanced grade meningiomas. Further studies are necessary to define merlin's role in regulating senescence signaling pathways.

The observations that merlin(-) meningioma cells were frequently marked by a premature onset of cell senescence underscores the potential for mechanistic differences in distinct species and cell types. *NF2* loss in humans results in the formation of slow growing, benign schwannomas, meningiomas and ependymomas that most often do not progress to higher-grade tumors, whereas, heterozygous *Nf2*^{+/-} mice spontaneously develop various metastatic tumors, including fibrosarcomas, osteosarcomas and hepatocellular carcinomas (McClatchey et al. 1998). In a recent study of NF1, acute deficiency of neurofibromin induced by RNAi rapidly triggered senescence in primary human cells, whereas it resulted in immortalization in primary MEFs (Courtois-Cox et al., 2006), consistent with the phenotype described previously for *Nf2*^{-/-} MEFs (Lallemand et al., 2003). Although the molecular mechanisms that trigger this response are not completely understood, our studies provide additional support for the view that target cells that give rise to benign tumors may initiate signaling programs that ultimately promote the senescence response, thus limiting the development of these lesions in the absence of additional cooperating mutations. Activation of the senescence response *in vitro* may contribute to the reduced BrdU incorporation of merlin RNAi arachnoidal cells seen one week after infection, as well as the striking differences between NF2-associated benign tumors in humans and the malignant tumors associated with *Nf2* inactivation in mouse models. Although the proliferative activity of patient-matched normal human Schwann cells (NHSC) to human schwannoma cells, or to NHSC in response to acute merlin inactivation, has not been assessed, previous studies showed that human schwannoma cells demonstrate spread areas approximating five-to-seven-fold greater than unmatched NHSC (Pelton et al. 1998), in agreement with our observations for merlin(-) meningioma and merlin RNAi arachnoidal cells. Consistent with slowing growth rates in confluent arachnoidal cells three days after RNAi treatment, we detected only a slight elevation in activated Erk1/2 MAPKs and none for cyclin D1, key mitogenic signaling proteins (Fig. 7B). This contrasts with dramatic effects on these proteins seen in other merlin-deficiency models (Lallemand et al., 2003; Xiao et al., 2005).

In conclusion, our findings suggest that meningioma formation initiated by merlin inactivation results from deregulation of cell-cell communication and/or aberrant actin cytoskeletal signaling, without sustained increases in the rate of proliferation. Unexpectedly, we found that *NF2* loss may hasten the onset of cellular senescence, potentially limiting the growth rate of benign tumors and their progression. These studies establish novel *in vitro* systems as relevant NF2 models for the study of benign human tumor growth and could support pharmacological approaches to reverse the aberrant cytoskeletal and cell adhesion properties unique to merlin (-) meningioma cells, or alternatively to accelerate cellular senescence programs in an effort to limit the growth of NF2-associated meningiomas. "Scoreable" phenotypes such as aberrant stress fiber formation, focus development and large cell size make this model amenable to high

throughput drug testing using high-content image-based cell assays. Additionally, the finding that merlin(+) meningioma cells possess distinct properties from merlin(-) meningioma cells suggests that targeting alternative signaling pathways may be necessary to control their growth. Thus, the disease-relevant *in vitro* model systems described here, along with the discovery of unique phenotypes that specifically characterize human NF2 target cell types, provide the platform for the development of novel therapeutic strategies for treating merlin(-) meningiomas.

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Fig. 1. Characterization of arachnoidal and meningioma cells

(A) Arachnoid (AC006) cells were plated on glass coverslips and stained by indirect immunofluorescence for meningotheial cell markers, cytokeratin 18 and epithelial membrane antigen (EMA). Cells showed typical positive staining. Bar, 20 μ m. (B) Endogenous merlin and ERM expression in matched arachnoid (AC) and meningioma (MN) cell lysates (100 μ g) was determined by immunoblot analysis using antibodies MP4 to detect merlin, or N21 to detect ezrin, moesin and merlin. Matched pair AC007 and MN307 was not used in immunofluorescent cell staining studies due to the detection of low levels of merlin in sample MN307. (C) Immunoblot analysis of unmatched merlin-deficient and merlin-expressing meningioma cell lines are indicated by (-) and (+), respectively. *, cDNA analysis of tumor MN297 confirmed a stable merlin splice-product consistent with skipping of exon 15, as described elsewhere (Sainio et al. 2000). Constant moesin expression indicated equal loading of samples. Ezrin expression appeared variable among merlin(-) and (+) meningioma cells.

Fig. 2. Arachnoidal and meningioma cell morphology

(A) Phase-contrast (top panels) and confocal (bottom panels) images of patient-matched arachnoidal, AC006, and merlin(-) meningioma, MN302(-), cells at early passages, p3 and p2, respectively. Phase-contrast images were taken from live cells plated in tissue culture plates, and confocal images were taken from fixed cells plated on glass coverslips and stained immunocytochemically with Alexa Fluor-conjugated phalloidin. Arachnoidal cells possessed a more epithelial-like morphology whereas merlin(-) meningioma cells exhibited long cell processes and increased granules. Bar, 100 μ m. (B) Phase-contrast images of patient-matched arachnoidal, AC006 p7, and merlin(-) meningioma cells, MN302(-) p6, and unmatched merlin (+) meningioma cells, MN354(+) p6. Merlin(-) meningioma cultures are enlarged and

flattened, and characterized by increased granularity and vacuoles that are not prominent features of arachnoidal and merlin(+) meningioma cells at similar passages. Bar, 50 μm .

Fig. 3. Cell growth characteristics of merlin(-) meningioma and arachnoidal cells in culture
Phase-contrast images of matched pair AC006 and MN302 at various cell passages. (A) Arachnoidal AC006 p15 cells formed confluent cell monolayers, while matched merlin(-) meningioma MN302(-) p4 cells continued to grow after saturating. Bar, 100 μ m. *Inset*, 2X (B) MN302(-) p9 cell cultures, predominantly populated by enlarged, flattened cells, stained positively for the senescence-associated biomarker β -galactosidase (SA- β -Gal). In contrast, AC006 p10 cells were negative, or weakly positive for expression of SA- β -Gal. Bar, 100 μ m. (C) MN302(-) cells at p9 were unable to reach cell confluency, unlike matched arachnoidal cells that proliferated steadily and formed confluent monolayers after extensive cell passaging (p26). Merlin(-) meningioma cultures (MN302 p9) developed refractive foci of rapidly

growing cells demonstrating loss of cell contact inhibition of growth among slow-growing senescent-like cells. Bar, 100 μ m.

Fig. 4. Merlin(-) meningioma cells demonstrate defects in cell-cell contact and altered α - and β -catenin expression

(A) Confocal images of phalloidin-stained matched arachnoidal AC006 and merlin(-) meningioma MN302(-) cells. AC006 p27 cells form contacts with adjacent cells at their cell peripheries, unlike MN302(-) p5 cells, which do not adequately recognize cell-cell boundaries, resulting in the overlap of neighboring cells. Bar, 20 μ m. (B) Arachnoidal cells (AC006 p5) exhibited distinct, punctate β -catenin staining at sites of cell-cell contact while matched MN302(-) p6 meningioma cells demonstrated a reduced or disrupted β -catenin expression pattern at adherens junction sites, and diffuse, non-specific staining throughout the cytoplasm. Cells were plated in complete media on laminin/poly-D-lysine-coated coverslips, and fixed and stained

for β -catenin immunocytochemically. Bar, 20 μ m. Inset panels are 2X. (C) Matched arachnoidal and merlin(-) meningioma cells were harvested for total cell lysates and examined for expression of adherens junctions proteins by immunoblotting with α - and β -catenin antibodies. The data suggested that the α -catenin protein levels were reduced in merlin(-) meningioma cells compared to arachnoidal cells, whereas the beta-catenin expression levels were not significantly diminished. Merlin was detected using the C26 rabbit polyclonal antibody that often detects a higher cross-reactive molecular weight band. GAPDH expression was used as a loading control.

Fig. 5. Abnormal actin stress fibers, focal contacts and ruffling edges characterized merlin(-) meningioma cells

(A) Merlin(-) meningioma cells (MN302 p6) plated on glass coverslips in serum-reduced media (0.4% FBS) for 16hr demonstrated aberrant and disorganized actin stress fibers compared to matched arachnoidal cells (AC006 p12). MN302(-) cells exhibited dense, disorganized stress fiber bundles, abundant focal contacts scattered throughout the ventral cell surface at stress fiber termini and reduced focal complex formation at the cell periphery. Under similar culture conditions, AC006 p12 cells exhibited fine actin stress fibers, and focal complex and contact formation at the cell periphery. F-actin was stained with Alexa Fluor 594-conjugated phalloidin, and focal contacts were visualized by indirect immunofluorescent staining using a

paxillin antibody. (B) Merlin(-) meningioma cells, MN302(-) p3, plated on laminin/poly-D-lysine-coated coverslips in complete media demonstrated increased ruffling membranes and actin aggregation at ruffling membranes (arrows) compared to arachnoidal and merlin(+) meningioma cells. Merlin(-) meningioma cells also exhibited web- or mesh-like stress fiber formation (inset) and lacked well-formed focal contacts at the cell periphery. Under similar conditions, matched AC006 p3 cells demonstrated diminished actin accumulation at lamellipodia, reduced ruffling membranes, and fine parallel stress fibers (inset), in association with well-developed focal contacts at the cell periphery. Merlin(+) meningioma cells, MN276 (+) p11, exhibited long, fine, parallel stress fibers, diminished ruffling membranes and well-defined focal contacts at the cell periphery. MN276(+) cells appeared larger than early passage MN302(-) and AC006 cells, due to prolonged culturing, however, they did not exhibit actin cytoskeletal defects. Cells were fixed and stained for F-actin and paxillin as described in (A). Bar, 20 μ m. *Insets*, 2X.

Fig. 6. Merlin re-expression rescued abnormal membrane ruffling and actin aggregation in merlin(-) meningioma cells

(A) Immunoblot analysis of exogenous merlin isoform 1 and 2 expression in merlin(-) meningioma cells, MN302(-), infected at a MOI of 2 (30 μ g protein/lane). Merlin isoforms were introduced via by the lentiviral expression vector CSCW2, and expression levels were determined densitometrically by comparison with endogenous merlin from two independent merlin(+) meningioma cell lines (data not shown). Isoform 1 expression was calculated to be 2-fold higher than endogenous levels across the entire culture, although only 12% of cells were infected. (B) Immunofluorescent staining of MN258(-) cells infected with lentiviral vector control, merlin isoform 1 or merlin isoform 2 viral particles at an MOI of 2. Infection frequencies were determined by quantifying the ratio of EGFP expressing cells to total cells. F-actin was visualized with AlexaFluor 594-conjugated phalloidin. Cells infected with vector alone particles exhibited ruffling membranes and actin aggregation at the cell periphery (arrows), characteristic of merlin(-) meningioma cells. In contrast, merlin(-) meningioma cells expressing merlin isoform 1 or 2 (EGFP positive cells) did not exhibit peripheral actin aggregation at lamellipodia or membrane ruffles, in contrast to uninfected neighboring cells within the same field (arrows). Bar, 20 μ m.

Fig. 7. Merlin suppression by RNAi in arachnoidal cells demonstrated phenotypes similar to merlin (-) meningioma cells

Total protein lysates were harvested from confluent AC006 arachnoidal cells 72 hrs after infection of control mock (con), scr or merlin-specific (meri-5 and meri-6) RNAi viral supernatants. (A) Immunoblot analysis of merlin protein expression in control and RNAi infected arachnoidal cells demonstrated that merlin expression was reduced substantially in response to meri-5 and meri-6 RNAi infections compared to con or scr RNAi. No consistent change in ERM protein expression levels was detected upon merlin downregulation. GAPDH expression was used as a control for equal protein loading. (B) Expression and activation of Erk and cyclin D1 in response to merlin RNAi-mediated downregulation were examined by

immunoblot analysis. Merlin knockdown arachnoidal cells exhibited a slight, but reproducible increase of 1.21 ± 0.05 , $p=0.003$ (mean fold $\pm$ SD) in Erk1/2 activation compared to control scr RNAi cells. Data were derived from multiple experiments ($n = 3$) and values were normalized to GAPDH levels to adjust for total protein loading. Shown are representative blots from a single experiment. There was no significant difference for cyclin D1. (C) Merlin RNAi arachnoidal cells exhibited a statistically significant decrease of 2.21-fold (0.45 ± 0.13 , $p=0.002$) in α -catenin expression levels relative to scr RNAi treated cells. Catenin protein levels were normalized to GAPDH to adjust for total protein loading, and values for mock control infected cells were arbitrarily set to 1.0. Data represent mean expression levels from multiple experiments ($n = 4$). Shown is a representative immunoblot. (D) Merlin knockdown (meri-5) in arachnoidal cells resulted in increased lamellipodia formation (arrowheads) and disorganized, short, dense stress fiber bundles that terminated at numerous focal contacts scattered throughout the cell. In contrast, scr RNAi arachnoidal cells did not exhibit prominent lamellipodial protrusions and displayed a more organized and filamentous stress fiber phenotype compared to merlin RNAi arachnoidal cells. Scr RNAi arachnoidal cells demonstrated focal contacts at the cell periphery and a marked reduction in the number and thickness of stress fiber bundles. Fixed cells were stained for F-actin and paxillin as described in *Materials and Methods* (Bar, 20 μ m).

Fig. 8. Merlin-suppressed arachnoidal cells demonstrated cell growth patterns similar to merlin(-) meningioma cells

(A) Phase-contrast images of RNAi-mediated merlin knockdown (meri-5) arachnoidal cells (AC006) demonstrated increased cell size in a majority of cells (~70%) by seven days post-infection compared to vector only (control) infected cells. Merlin RNAi arachnoidal cells maintained in culture for 21 days post-infection exhibited loss of cell-contact inhibition of growth resulting in the formation of refractive foci. Foci did not form in vector only infected cells (data not shown). Bar, 100 μm . (B) In separate experiments, DNA synthesis rates based on BrdU incorporation were assessed for merlin and control scr RNAi arachnoidal cells on days three and seven after RNAi treatment. On the third day after RNAi treatment, merlin RNAi arachnoidal cells demonstrated very similar DNA synthesis rates compared to scr RNAi cells, showing a mean elevation of 3%. By day seven, merlin RNAi-suppressed arachnoidal cells demonstrated a 25% reduction in DNA synthesis rates compared to scr RNAi cells. Shown are mean values from three assays derived from RNAi-treated AC006 and AC007 cells.

Table 1

Meningioma and normal arachnoidal tissue pairs

Meningioma line	Arachnoidal line	Patient diagnosis	Meningioma merlin expression ^a	NF2 mutation ^b		
				Exon	Sequence	Predicted result LOH
MN302	AC006	sporadic	negative	10	c.947delT	FS yes
MN307	AC007	NF2	low levels	15	c.1599_1602delGCAT	FS ND
MN324	AC010	NF2	negative	UF	NA	yes
MN327	AC012	NF2	low levels	4	c.447+1G>C	SP ND
MN359	AC016	NF2	negative	12	c.1340+1insT	SP ND

^a detection of low levels of merlin due to approximately 20% stromal contamination.

^b Mutations in italics are constitutional changes detected in a non-tumor tissue. FS-frameshift, SP-splice-site, NA-not applicable, UF-no mutation found by exon scanning, LOH-loss of heterozygosity of NF2 region markers, ND-not determined.

Table 2
Merlin-expressing and merlin-deficient meningioma cells

Meningioma sample	Merlin expression ^b	WHO grade	Histological subtype ^c
MN34	(-)	benign	fibroblastic
MN256	(-)	benign	fibroblastic
MN258	(-)	benign	transitional
MN261	(-)	benign	fibroblastic
MN290	(-)	atypical	meningothelial
MN297	(-) ^a	atypical	ND
MN302	(-)	benign	transitional
MN304	(-)	benign	fibroblastic
MN307	(-)	benign	ND
MN310	(-)	benign	fibroblastic
MN320	(-)	atypical	ND
MN324	(-)	benign	ND
MN327	(-)	benign	transitional
MN336	(-)	benign	ND
MN350	(-)	benign	transitional
MN355	(-)	benign	ND
MN357	(-)	benign	fibroblastic
MN359	(-)	ND	ND
MN363	(-)	benign	ND
MN47	(+)	benign	transitional
MN276	(+)	benign	meningothelial
MN291	(+)	atypical	ND
MN298	(+)	malignant	ND
MN299	(+)	benign	meningothelial
MN318	(+)	atypical	transitional
MN319	(+)	atypical	fibroblastic
MN330	(+)	benign	transitional
MN332	(+)	benign	ND
MN349	(+)	benign	ND
MN354	(+)	benign	ND

^a molecular weight significantly lower than wild-type merlin, consistent with expression of a stable truncation product.

^b expression determined by immunoblotting.

^c ND-not determined.