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Merlin negative regulation by miR-146a promotes cell transformation

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ABSTRACT

Inactivation of the tumor suppressor Merlin, by deleterious mutations or by protein degradation via sustained growth factor receptor signaling-mediated mechanisms, results in cell transformation and tumor development. In addition to these mechanisms, here we show that, miRNA-dependent negative regulation of Merlin protein levels also promotes cell transformation. We provide experimental evidences showing that miR-146a negatively regulates Merlin protein levels through its interaction with an evolutionary conserved sequence in the 3′ untranslated region of the *NF2* mRNA. Merlin downregulation by miR-146a in A549 lung epithelial cells resulted in enhanced cell proliferation, migration and tissue invasion. Accordingly, stable miR-146a-transfectant cells formed tumors with metastatic capacity *in vivo*. Together our results uncover miRNAs as yet another negative mechanism controlling Merlin tumor suppressor functions.

1. Introduction

The tumor suppressor Merlin plays a key role in tissue homeostasis by controlling cell contact inhibition of growth [1]. Upon ligand binding, the epidermal growth factor receptor (EGFR) promotes Merlin inactivation through a conformational change imposed by p21-PAK kinase-mediated phosphorylation on Merlin S518 [2]; additionally, Merlin phosphorylation on T230 and S315 residues by AKT leads to Merlin degradation [3] thus favoring cell proliferation. In contrast, cell–cell adhesion or cell-extracellular matrix interaction through cadherins or CD44, respectively, result in Merlin reactivation, through p21-PAK inactivation and the activation of the phosphatase MYPT-1 that mediates Merlin S518 dephosphorylation [2]. Active Merlin then leads to the activation of the Hippo pathway that negatively regulates the transcription factor YAP and thus prevents the expression of the genes involved in cell proliferation and survival [4]. Cumulative evidence indicates that alteration in this fine tuned mechanism, either by gain of function resulting from constitutive active oncogenes or lost of function resulting from Merlin deleterious mutations, lead to cell transformation and tumor development [5].

In addition to the well-characterized mechanisms controlling the function of tumor suppressors, another level of regulation involves microRNAs (miRNAs). miRNAs have a central role in the fine tuning of distinct biological processes including, development, metabolism, cellular differentiation, cellular proliferation and programmed cell death [6]. In cellular transformation and cancer development, miRNAs with tumor suppressor function as well as oncogenic functions (oncomiRs) have been documented. OncomiRs negatively regulate tumor suppressors by blocking protein translation mechanisms through miRNA specific interaction with sequences located within the 3′ untranslated region (3′UTR) of their target mRNAs. Given that Merlin plays a crucial role in controlling cell number and tumor development and the plethora of distinct mechanisms negatively regulating Merlin function to allow cell proliferation under normal and pathological conditions, we speculated the existence of miRNAs targeting Merlin. A bioinformatic analysis revealed that the Merlin mRNA 3′UTR contains sequences that could mediate the interaction with specific miRNAs. Here we show that miR-146a, miR-25, miR-32, and miR-7 negatively regulate Merlin protein levels by targeting the Merlin 3′UTR. Accordingly, persistent Merlin downregulation by miR-146a expression in A549 lung epithelial cells blocked cell contact inhibition of proliferation and enhanced cell migration, tumor formation and metastasis. These phenotypes correlated with alterations in both Hippo

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and EGFR signaling pathways. Thus, our results indicate that targeting Merlin by miRNAs constitutes another mechanism controlling cell proliferation and tumor development.

2. Materials and methods

2.1. Mice

nu/nu mice were maintained in our animal facility with food and water *ad libitum*. Experiments were carried according to institutional guidelines and approved by the Bioethics Committee of the Instituto de Biotecnología, UNAM.

2.2. Cell lines

The human alveolar adenocarcinoma A549 cell line was obtained from the ATCC and cultured in DMEM-F12 (Gibco) medium with 5% fetal bovine serum (FBS), 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin at 37 °C and 5% CO_2. Human embryonic kidney 293 (HEK-293) cells were grown in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% FBS, 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin.

2.3. Antibodies

The anti-Myc, -Cyclin D, -Merlin, and -actin antibodies were from Santa Cruz Biotechnology. The anti-pAKT, -AKT, -pSTAT3, -pYAP, -YAP and anti-pEGFR antibodies were from Cell Signaling.

2.4. Plasmid constructs

The 3′UTR of the human *NF2* gene and the *pre-miR-146a* gene were amplified by PCR from human genomic DNA using the primers shown in Supplementary Table I.

The *NF2*-3′UTR fragment was cloned downstream the *Renilla* luciferase reporter gene, in the psiCHECKTM-2 vector (Promega). The different pre-miRNAs were cloned into pcDNA™3.1/myc-His A vector (Invitrogen). A mutant version of the *NF2*-3′UTR (Luc-mut *NF2*-3′UTR) was generated in which TT nucleotides of the seed sequence for miR-146a binding site (AGTTCTC) were substituted by AA using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies). miR-7, miR-145 and miR-881 expression vectors have been previously described [7].

2.5. Transfection and luciferase assays

2.5×10^5 HEK-293 or A549 cells were transfected with either 100 ng of empty psiCHECK-2 vector, 100 ng of the psi/Merlin construct or 100 ng of the psi/Merlin-Mut construct together with the indicated amount of empty pcDNA vector, pc/miR-146a, pc/miR-145 or pc/miR881 using Lipofectamine 2000 (Invitrogen). 48 h post-transfection, luciferase activity was determined using the Dual-Luciferase® Reporter Assay System Kit (Promega). Relative luciferase units (RLUs) were calculated as the ratio of (SAMPLEmiR-$X_{Renilla/firefly}$/SAMPLEpcDNA$_{Renilla/firefly}$)/(CONTROLmiR-$X_{Renilla/firefly}$/CONTROLpcDNA$_{Renilla/firefly}$) in which SAMPLE refers to *NF2* or Mut-*NF2* and CONTROL refers to psiCHECK-2. After RLUs were calculated for each miRNA all were normalized taking empty vector (pcDNA) data as 1. The antagomiRNA (anti-miR-146a) has been previously described [8], 10 nM was transfected into A549 cells using Lipofectamine 2000, after 4 h media was removed and cells were cultured for additional 24 h and subjected to the indicated biological assay.

2.6. Stable cell lines

A549 cells were transfected with 3 μg of linearized pcDNA vector or linearized pc/miR-146a using Lipofectamine 2000. Clones were obtained by Geneticin (Gibco) selection. Clones showing miR-146a overexpression and decreased Merlin protein levels as determined by RT-PCR and Western blot, respectively were selected for further experiments. At least, four independent clones showing normal (pcDNA) or reduced Merlin protein levels (miR-146a) were used for all biological assays. Moreover, clones co-transfected with miR-146a and Merlin expression vectors showing increased miR-146a levels and normal Merlin protein levels were selected for the rescue experiments.

2.7. Cell proliferation

2×10^4 cells were seeded in 24 well plates and cultured for the indicated times in supplemented medium, cells were harvested with trypsin, washed and counted. Where indicated, cells were allowed to reach confluence and new medium was added, cells were then cultured for the indicated periods of time.

2.8. Wound healing assay

Wound healing assays were performed as previously described [9]. When the first cells closed the wound, the area not healed in the other plates was measured using the TScratch software and reported relative to 100% healing. The wound healing process was recorded by using a Nikon TE300 inverted bioluminescence microscope.

2.9. Migration assays

5×10^4 A549 stable cells were seeded into Millicell Hanging Cell Culture Inserts (Millipore, pore size 8.0 μm) in DMEM-F12 medium supplemented with 0.5% FBS. In the lower chamber the bottom side of the inserts was immersed in DMEM-F12 medium with 20% FBS. Cells were allowed to migrate for 16 h at 37 °C and were stained with trypan blue and counted as described [7].

2.10. Invasion assays

These assays were performed essentially as the migration assays except that cells were cultured onto BioCoat-Matrigel Invasion Chambers (Corning).

2.11. In vivo tumor formation

A549 cells (3×10^6) clones stable transfected with empty pcDNA, miR-146a or miR-146a + Merlin expression vectors were injected subcutaneously to 2 months old male *nu/nu* mice. After one month, animals were sacrificed, the tumors were surgically excised and their mass was determined. RNA and protein extracts were prepared and stored at −70 °C until use.

2.12. Protein extracts

Cells or tumors were lysed in 100 μl of lysis buffer (20 mM Tris pH 7.4, 137 mM NaCl, 2 mM PPiNa, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 0.5 mM DTT, 25 mM β-glycerophosphate, 200 mM Na_3VO_4, 1 mM PMSF, 5 mg/ml leupeptin, 5 mg/ml aprotinin, 5 mg/ml antipapain) for 10 min at 4 °C. Lysates were centrifugated at 14,000 × *g* for 15 min at 4 °C and stored at −70 °C until use.