Supplementary Information

MicroRNAs bind to Toll-like receptors to induce prometastatic inflammatory response

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METHODS

Cell culture, transfection and treatment. All cell lines were purchased from American Type Culture Collection unless indicated otherwise. Human embryonic kidney-derived HEK-293 cells were maintained using standard conditions and were grown in Dulbecco's modified Eagle Medium (DMEM) (Gibco), supplemented with 10% (vol/vol) fetal bovine serum (FBS). Human HEKBlue-TLR7 and TLR8 293 cells (Invivogen) (indicated as TLR7-HEK-293 or TLR8-HEK-293, respectively) were cultured in DMEM supplemented with 10% FBS, normocin (50μg/ml), blasticidin (10 μg/ml), zeocin (100 μg/ml) (Invivogen).

Human A-549, SK-MES and murine LLC cells were maintained in RPMI 1640, supplemented with 10% FBS (vol/vol). RAW 264.7 cells were instead maintained in DMEM supplemented with 20% FBS (vol/vol). All cells were transfected using Lipofectamine LTX and Plus Reagent (Invitrogen) following the manufacturer's instructions.

For all experiments, cells were treated with 15 μg of HPLC-purified synthetic miRNAs (Integrated DNA Technologies) complexed with Dotap Liposomal Transfection Reagent (Roche) as previously described (1).

For the experiment with Bafilomycin A, TLR8-HEK-293 cells were seeded in a 24 well-plate at a density of 200,000 cells/well. The day after cells were pre-incubated with Bafilomycin A (Sigma) 10 nM for 30 min and then treated with exosomes purified from LLC cells for 24h in the presence or absence of Bafilomycin A 10 nM. Cell supernatants were finally collected, harvested and QUANTI-Blue Assay was performed.

Exosome purification. Serum free-conditioned media were collected after cell treatment at the indicated time points from all the mentioned cell lines. Media were then harvested at 14,000 g for 15 min to eliminate cell debris. Exosomes were precipitated by using Exosomes Precipitation Solution (ExoQuick, System Bioscience) following the manufacturer's instructions.

NanoString nCounter assays. Both cells and exosomes purified from 300 µl of SK-MES and A-549 conditioned media were processed in hextuplicate for RNA extraction, using Trizol reagent (Invitrogen) and following the manufacturer's instructions. The RNA concentration and quality were estimated by Nanodrop assay (Nanodrop Spectrophotometer 2000), and 100 ng were used as input for nCounter miRNA sample preparation reactions. All sample preparation was performed according to manufacturer's instructions (NanoString Technologies). Preparation of small RNA samples involves the ligation of a specific DNA tag onto the 3' end of each mature miRNA. These tags are designed to normalize the melting temperatures of the miRNAs as well as to provide a unique identification for each miRNA species in the sample. The tagging is accomplished in a multiplexed ligation reaction using reverse-complementary bridge oligonucleotides to direct the ligation of each miRNA to its designated tag. Following the ligation reaction, excess tags and bridges are removed, and the resulting material is hybridized with a panel of miRNA:tag-specific nCounter capture and barcoded reporter probes. Hybridization reactions were performed according to manufacturer's instructions with 5 µl of the 5-fold diluted sample preparation reaction. All hybridization reactions were incubated at 64°C for a minimum of 18h. Hybridized probes were purified using the nCounter Prep Station (NanoString Technologies) following the manufacturer's instructions to remove excess

capture and reporter probes and to immobilize transcript-specific ternary complexes on a streptavidin-coated cartridge. Data collection was carried out on the nCounter Digital Analyzer (NanoString Technologies) following the manufacturer's instructions to count individual fluorescent barcodes and quantify target RNA molecules present in each sample. For each assay, a high-density scan (600 fields of view) was performed. For the analysis, only the miRNAs that in both cell lines had a number of code counts ≥ 50 (twice as much as the average negative controls of 25 code counts) were further considered. The threshold for **Fig. 1*A*** was chosen in order to eliminate the background noise and make sure that the levels of identified miRNAs represented real exosome enrichment. The average plus a multiple of the standard deviation of all negative control probe counts in a lane could be defined as background for that lane. Therefore, and also as recommended in the NanoString Expression Data Analysis Guide, we operated as follows:

1. For each lane, we calculated the average of all the negative control counts.
2. For each lane, we calculated the standard deviation of all the negative control counts.
3. We added a multiple of the standard deviation to the average. Specifically, we multiplied the standard deviation by two before adding to the average.
4. This is the background threshold.
5. In order to restrict the analysis to those miRNAs that are really over-expressed in the exosomes, we used even a more stringent threshold, by further doubling the background threshold (as calculated in 4). This calculation gave us a threshold value of 51.04, which was rounded to 50.

Quantitative Real-Time PCR. Quantitative Real-Time PCR analysis for miRNAs was performed in triplicate with the TaqMan MicroRNA assays kit (Applied Biosystems),

according to the instructions of the manufacturer. RNU44 (human samples) or snoRNA234 (murine samples) was used to normalize the quantitative Real-Time PCR on RNAs extracted from the cells. Total RNA concentration was used to normalize quantitative Real-Time PCR performed on miRNAs purified from exosomes.

Immunofluorescence. HEK-293 cells were seeded 24h before transfection onto 60 mm plates and allowed to grow to 50% confluence. They were then transfected with 1 μg of GFP-TLR8 encoding plasmid (Origene). After 48h cells were treated with the indicated mature miRNA oligos conjugated to Cy-5 fluorophore as described above, washed 4 times with PBS and finally incubated for 15 min with Lysotracker blue DND-22 (Invitrogen) diluted 1:25,000 in PBS.

For the Immunofluorescence experiment with physiological exosomes HEK-293 cells were transfected with 1 μg of GFP-CD9 encoding plasmid (Origene). After 24h cells were detached and co-cultured with RAW 264.7 cells, previously seeded onto a 40 mm coverslip at a density of 700,000 cells/ml and stained with blue cell tracker (Invitrogen). After 30 of incubation, for allowing HEK-293 cells to seed, the co-culture was finally stained with Lysotracker red DND-99 (Invitrogen) diluted 1:25,000 in PBS and coverslips were analyzed with confocal microscope.

Co-Immunoprecipitation experiments. TLR8-HEK-293 cells were treated with the indicated miRNAs and incubated at 37ºC for 20 min. They were then extensively washed with 1.5 ml of ice-cold PBS, collected and lysis performed through a 5 min incubation with 150 μl of Polysome lysis buffer on ice, as previously described (2). Lysates were finally frozen in dry ice for 1 h, and then harvested at 14,000 g for 15 min. 100 μl of each lysate was added to 50 μl of A/G protein (Santa Cruz), which was previously pre-

incubated with 25 µl of NT2 buffer 5% BSA for 1h and then with 100 µl of anti-TLR8 antibody in rotation at 4ºC overnight. Lysates were further incubated with beads (extensively washed with NT2 buffer, according to the manufacturer's protocol) (Mylteni) in a final volume of 850 µl of NT2 buffer supplemented with 1 µl of 1M DTT and 34 µl of 0.5M EDTA. Immunoprecipitation was performed for 5h at 4ºC in rotation. Beads were washed 4x with NT2 buffer, and was incubated with 100 µl of NT2 buffer and Proteinase K (Qiagen) at 55 ºC for 30 min, then RNA was extracted with Trizol and processed for real-time analysis, as previously reported.

The miRNA immunoprecipitation for TLR8 was performed as follows. TLR8-GFP-HEK-293 cells were treated with 5'-biotinylated mature miR-16, 29 or RNA40 (15 µg/mL final concentration) at 37ºC for 20 min. Cells were fixed and lysed as previously described[2], and co-immunoprecipitation was performed by using streptavidin-conjugated magnetic beads (Miltenyi), according to the manufacturer's instructions. TLR8-GFP protein was detected with anti-GFP antibody.

Western blotting. Cells were washed in PBS, scraped and collected in a 15 ml tube, then harvested at 1,500 g for 10 min. The pellet was resuspended in NP-40 Cell Lysis Buffer (Invitrogen) supplemented with protease inhibitors (Roche) for 30 min at 4º C. The suspension was finally harvested at 14,000 g for 10 min and the supernatant, containing solubilized proteins, collected. Proteins were quantified using Bio-Rad Protein Assay (Bio-Rad), following the manufacturer's instructions, and 30 µg of each sample were loaded on a Criterion Tris-HCl 4-20% pre-cast gel (Bio-Rad), transferred onto PVDF membranes and probed with anti-GFP (Novus Biologicals) and anti-TLR8 (Santa Cruz) antibodies. Isotype-matched, horseradish-peroxidase-conjugated secondary antibodies

(GE Healthcare) were used, followed by chemi-luminescence detection (Denville Scientific, Inc.). Also, for immunoblotting the following antibodies were used: anti-CD9, and anti-CD63 (Santa Cruz), and anti-phospho p65 (Cell Signaling).

LNA-ISH Our protocol for protein/microRNA as well as protein/protein co-expression analysis has been previously published (3). In brief, *in situ* hybridization for the microRNA is done using the 5' digoxigenin tagged LNA probe (Exiqon). After the in situ hybridization, we used the Benchmark LT automated system from Ventana Medical Systems according to the manufacturer's specifications to do the immunohistochemistry for CD9 (1:200, antigen retrieval for 30 min), *TLR7* (1:250, antigen retrieval for 30 min), and IL-6 antibodies (1:300, antigen retrieval for 30 min). The data is then analyzed with the Nuance system (Cambridge Research Institutes) which separates the colorimetric based signal for the different chromogens, converts these to fluorescence-based signals, then mixes them using a computer-based analysis system.

Animals. Wild type B6 mice (WT), B6 *TLR7*$^{-/-}$ mice and nude mice were purchased from Jackson laboratories. Seven WT B6 mice and seven *TLR7*$^{-/-}$ B6 mice matched for age and sex (males, 7 week-old) were injected with 1.8 x 10^6 LLC cells in the tail vein, and followed for survival. At the moment of death, or after sacrificing the surviving mice at 36 days from injection, necropsy was performed and multiplicities of lung metastases were photographed and counted.

The *in vivo* experiment with LLC cells transfected with anti miRNAs was conducted in six male, 7-week old B6 mice/group (total *n*=18). LNA anti scrambled=mice injected with LLC cells transfected with LNA anti scrambled used as control; LNA anti miR-16=mice injected with LLC cells transfected with LNA anti miR-16; LNA anti miR-

21/29a=mice injected with LLC cells transfected with LNA anti miR-21 and LNA anti miR-29a. Mice were injected with 1.8 x 10^6 LLC cells in the tail vein, and sacrificed after 15 days. Necropsy was performed and lung multiplicities were photographed and counted.

The *in vivo* rescue experiment with LLC cells was conducted in 15 male, 7-week old B6 mice. Mice were injected with 1.8 x 10^6 LLC cells in the tail vein in 300 μl of volume (T_0). After 4 days (T_4) we have started intra-peritoneal injections of 10 mice with GW4869 (1.25 mg/kg/day), an inhibitor of exosome secretion also able to reduce the content of miRNAs in secreted exosomes, and 5 mice with Dimethylsulphoxide (DMSO, solvent of GW4869) as a control, daily for 5 consecutive days. After 1 week from the first injection of GW4869 (T_{11}) we have performed intra-tail vein injection of exosomes purified from 1 ml/mouse of wt LLC supernatant in half (*n=5*) of the mice which previously were treated with GW4869. A second injection of LLC derived exosomes was performed in these same mice after 3 days from the previous one (T_{14}). All mice were sacrificed at T_{18}, necropsy performed and lung multiplicities counted.

LLC cells transfected with the above mentioned anti miRNAs were also injected subcutaneously into the left flanks of 9 nude mice (8 x 10^6 cells/mouse, 3 mice per condition) and tumor growth was monitored for the following 3 weeks. Tumor size was assessed once a week using a digital caliper. Tumor volumes were determined by measuring the length (l) and the width (w) of the tumor and calculating the volume ($V= lw^2/2$).

All procedures used in this study complied with federal guidelines and institutional policies by the Ohio State University animal care and use committee.

Isolation of primary human and murine cells. Total splenocytes derived from WT and *TLR7*$^{-/-}$ age and sex-matched mice were prepared by harvesting the spleen and preparing a single cell suspension. Red blood cells were eliminated by osmotic lysis using red blood cell lysis buffer (e-bioscience). Total splenocytes were seeded in a 96-well plate (1 × 10^6 cells in 200 μl of medium per well) and then stimulated with synthetic miRNAs or with purified exosomes for 18 h. Cells were stained with PE-conjugated anti-CD69 (BioLegend) antibody and analyzed using FACS Calibur flow cytometer (Becton Dickinson).

Macrophages derived from the peritoneal cavity were isolated from WT and *TLR7*$^{-/-}$ age and sex matched mice as previously described (4).

PBMC were isolated from heparinized blood of healthy donors by Ficoll-Paque (Pharmacia) centrifugation (500g) following manufacturer's instructions and immediately plated for the stimulation. 300,000 cells (human PBMCs or murine peritoneal macrophages) were stimulated with synthetic miRNAs for 24h; then ELISA assays for TNF-α and IL-6 were performed on the conditioned media using Multi-Analyte ELISArray Kits (SABioscience) following the manufacturer's instructions.

Growth curve, cell viability and cell cycle. For the determination of the growth curve cells were seeded at a density of 10,000 cells/ml in a 48 well plate. After 24, 48 and 72h cells were collected and counted with a haemocytometer (Beckman Coulter).

Cell viability was examined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTS)-Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega) following the manufacturer's instructions. Metabolically active cells were detected by

adding 20 μl of MTS to each well. After 1 h of incubation, the plate was analyzed in a Multilabel Counter (Bio-Rad Laboratories).

Cell cycle analysis was performed by propidium iodide staining (50 μg/ml in PBS) of cells fixed in methanol followed by flow cytometry analysis (FACScan Becton-Dickinson).

Cell migration and invasion. Cell migration and invasion through a 3D-extracellular matrix was assessed by using transwell migration chamber (Corning).

Briefly, for migration, transwells were saturated 1 hr at room temperature with PBS 1% BSA. LNA-transfected cells were seeded in the transwell upper chamber and the incubated at 37ºC for 6 h or 24 h. The lower chamber was filled with serum free medium or serum supplemented with 10% FBS, as indicated. After 24 or 6 h, filters were washed, fixed, and stained with Coomassie Brilliant Blue (Sigma-Aldrich Corp.). Cells that had invaded to the lower surface of the filter were counted under the microscope.

For invasion experiments, transwells were coated overnight at 4°C with 80 μg/ml MatrigelTM (Becton Dickinson) and then saturated 2 h at room temperature with PBS 1% BSA. LNA-transfected cells were then processed as described above and allowed to invade the matrix up to 24 h.

NF-κB activity assay. NF-κB activity was assessed by QUANTI-Blue Assay (Invivogen). HEK-Blue-TLR7 and TLR8 293 cells allow verification of the activation of NF-κB after the stimulation of the respective TLRs. Both these cell lines were obtained by co-transfection of the TLR7 and 8 genes and an optimized secreted embryonic alkaline phosphatase reporter gene (*SEAP*) under the control of the *IFN-α* minimal promoter fused to five NF-κB and AP-1-binding sites into HEK-293 cells. Stimulation

with a TLR7 or 8 ligand activates NF-κB and AP-1, which induces the production of SEAP. Levels of SEAP can be easily determined with QUANTI-Blue, a detection medium that turns purple/blue in the presence of alkaline phosphatase.

Supernatants derived from treated TLR7-HEK-293 and TLR8-HEK-293 cells were harvested at 14,000 g for 15 min to eliminate debris; 20 µl were then transferred into a 96-well plate and added to 180 µl of QUANTI-Blue solution (Invivogen). The plate was then incubated at 37ºC for 1-3 h and SEAP levels were determined by reading the plate with a spectrophotometer at 620-655 nm (SpectraMax, Molecular Devices).

Statistical analysis of data Statistical data are presented as mean ± standard deviation (s.d.), unless otherwise specified. Significance was calculated by Student's *t-test* or by Anova test with Bonferroni correction. Kaplan-Meier survival curves were calculated with the Log-rank (Mantel-Cox) method, by using the SPSS statistics software (IBM).

For the LNA-ISH analysis, mean and standard deviation were calculated by using InStat software and significance was determined by Student's *t-test* via InStat.

SUPPLEMENTARY FIGURE LEGENDS

Fig. S1. Distribution of CD9, CD63 and miR-29a. (*A*) Immunoblotting for CD9 and CD63 showing that the purified fractions from the supernatants of A-549 and SK-MES are enriched in CD9 and CD63 proteins, two well known markers of exosomes. (*B*) Statistical analysis performed on mouse tumor samples processed for *in situ* hybridization for miR-29a. Cancer cells were considered positive for miR-29a when their blue signal was at least 5 fold greater than the background stain as measured by the Nuance system. miR-29 positive cancer cells were counted in multiple 200X fields for the different slides tested, then mean ± s.d. were determined by using InStat software. **, $P< 0.005$.

Fig. S2. In human lung cancer CD9 is mainly produced at the tumor interface. (*A*) ISH for exosomal marker CD9 performed on human lung cancer samples. The arrow depicts cells highly enriched in CD9, stained in red, at the tumor interface. In blue is haematoxylin counterstain. (*B*) Higher magnification of tumor interface in human lung cancer sample as seen in panel *A*. CD9 is mainly located in cells that display the oval/folded nuclei and ample cytoplasm features typical of macrophages.

Fig. S3. At the tumor interface miR-29a is co-expressed with macrophage marker F-11, with *TLR7* receptor but not with cancer-associated epithelial marker cytokeratin. (*A*) LNA-ISH performed on mouse tumor showing that miR-29a and macrophage marker F-11 are co-expressed only at the level of tumor interface but not in the adjacent normal

lung. The Nuance- converted image depicts miR-29a as fluorescent blue and F-11 as fluorescent green, while fluorescent yellow indicates their co-expression. (*B*) Mouse tumors were stained for miR-29a LNA-ISH and the cancer-associated epithelial marker cytokeratin AE1/3. Cells positive for miR-29a (fluorescent blue) co-localize with cytokeratin only in the tumor (fluorescent yellow), but not in the tumor interface, where there are cells miR-29a positive and cytokeratin negative. (*C*) LNA-ISH of miR-29a and *TLR7* on a mouse tumor. The Nuance-converted image shows miR-29a as fluorescent blue and *TLR7* as fluorescent green. Their co-expression is indicated by fluorescent yellow signal.

Fig. S4. miRNA sequence analysis. (*A*) BLAST analysis showing the similarity between the sequences of mature miR-147 and 574-5p with that of RNA33. RNA33 corresponds to a GU-rich oligonucleotide which has been demonstrated to induce the secretion of cytokines in human and murine immune cells via human TLR8 and murine *TLR7*, respectively. The exact matching bases are highlighted in red. (*B*) List of miRNA sequences that were taken in consideration for the study of point mutations. miR-21 sequence was mutated in G18 and U20 (in red), while miR-29a was mutated in U20 and U21: these bases were substituted with the bases occupying the same position in the miR-16 sequence. Also a sequence with the combination of the two considered point mutations was generated.

Fig. S5. Co-expression of miR-29a and IL-6 in human macrophages. LNA-ISH (Nuance-converted image) for miR-29a (depicted as fluorescence blue, upper-left panel) and IL-6

(fluorescent red, upper-right panel) performed on a human lung cancer sample. The merged image (lower-left panel) displays in fluorescent yellow macrophages that co-express both miR-29a and IL-6. The lower-right panel is the corresponding RGB image: its comparison with the merged image confirms that only macrophages that express miR-29a at the tumor interface also co-express IL-6 (see arrow). Conversely, macrophages that do not express miR-29a do not express IL-6 either.

Fig. S6. NF-κB is required for miR-21 and 29a dependent (and TLR mediated) secretion of TNF-α. (*A*) Immunoblotting for phospho p65 in RAW 264.7 cells incubated with Dotap formulations of miR-16, 21 and 29a or treated with LPS (as a positive control). Vinculin was used as a normalizer to show equal protein loading. (*B*) ELISA assay for TNF-α performed on conditioned medium of RAW 264.7 cells transfected with a plasmid encoding a dominant negative form of IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha, indicated as "IκBαDN"), a dominant negative form of Iκκ2 (inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta, indicated as "Iκκ2DN") or the corresponding empty vector (indicated as "EV") as a control. The assay was performed 48h after cells were treated with Dotap formulations of the indicated miRNAs. The experiment was conducted in triplicate and data are presented as average ± s.d. *, $P<0.05$. (*C*) Study of the structural features in the sequence of miR-21 and 29a. TLR8-HEK-293 cells were incubated with Dotap formulations of the indicated mature miRNAs (cfr. Fig. S4 (*B*)) for 24 h, and QUANTI-Blue Assay was performed. Dotap alone was used as a negative control, and ssRNA40 as

positive control for NF-κB activation. The experiment was conducted in quintuplicate and presented as average ± s.d. **, $P< 0.0001$.

Fig. S7. Functional studies performed with miRNA-containing exosomes. (*A*) Quantitative Real-Time PCR for miR-16, 21, 27b, and 29a in the RNA extracted from exosomes in the supernatant of LLC and A-549 cells. The experiments were conducted in triplicate and presented as average ± s.d. **, $P<0.0001$. (*B, C*) ELISA assay for TNF-α (*B*) and IL-6 (*C*) performed on conditioned medium of peritoneal macrophages isolated from WT (n=3) B6 mice and incubated with LLC supernatant containing exosomes (Conditioned Medium) or with LLC supernatant after removal of exosomes by ultra-centrifugation (Ultra-centrifuged Medium). (*D*) CD69 activation detected by cytofluorimetry performed on spleen cells isolated from WT (n=3) B6 mice and treated as in *B* and *C*. Data are presented as average ± s.d. **, $P<0.005$. (*E*) NF-κB activation in TLR8-HEK-293 cells treated with LLC-released exosomes. TLR8-HEK-293 cells were not pre-treated ("Exosomes" group), or pre-treated with Bafilomycin A ("Exosomes + Bafilo" group). As a positive control, TLR8-HEK-293 cells were treated with artificial exosomes containing the TLR8 activator ssRNA40 ("ssRNA40" group). The experiments were conducted nine times, and presented as average ± s.d.. **, $P< 0.0005$ ("Exosomes" vs "Exosomes+Bafilo"). (*F*) LLC cells were transfected with LNA anti scrambled (control), or LNA anti miRNAs for miR-16 (LNA anti miR-16) and for both miR-21 and 29a (LNA anti miR-21/29a). After 48h cells were collected and RNA extracted from both LLC cells and exosomes purified from their supernatants. The level of all three miRNAs was detected by quantitative Real-Time PCR both in LLC cells (left panels, labeled as

"LLC cells"), and in the exosomes purified from their supernatants (right panels, labeled as "LLC Exosomes"). The experiments were conducted in triplicate and presented as average ± s.d. **, $P<0.001$. (*G*) Lung multiplicities in B6 mice injected with wild type LLC cells followed by injections of Dimethylsulphoxide (DMSO) ($n=5$, group "LLC wt-ctrl"), with wild type LLC cells followed by injections of the miRNA and exosome release inhibitor GW4869 ($n=5$, group "LLC wt-GW4869"), or with wild type LLC cells followed by injections of GW4869 and two intra-tail vein rescue injections of LLC-derived exosomes ($n=5$, group "LLC wt-GW4869 rescue"). Data are presented as mean ± s.d.. *, $P<0.05$; **, $P<0.005$.

Fig. S8. Only miR-29a expressing cancer cells are able to form lung tumor multiplicities. *In situ* hybridization for miR-29a (left panel), and for anti miR-29a (right panel) in a lung multiplicity developed by a mouse injected with LLC cells transfected with LNA anti miR-21/29a. Blue is the probe staining and pink is the counterstain. This image is representative of findings in all studied multiplicities from mice injected with LNA anti miR-21/29a.

Fig. S9. Effects of miR-16, 21 and 29a silencing on LLC cell biology. (*A*) Growth curve of LLC cells determined 24 h, 48 h and 72 h after transfection with LNA anti scrambled (control), LNA anti miR-16 or LNA anti miR-21 and 29a in combination (LNA anti miR-21/29a). (*B*) MTS assay on LLC transfected cells at the indicated time points. LLC viability is represented as the OD value obtained by reading the plate at 490 nm. (*C*) Cell cycle assay of LLC cells collected 48h after transfection and performed by

cytofluorimetry. (*D*) Invasiveness assay performed on transfected LLC. The percentage of cell invasion for each treatment was normalized with respect to the control (LNA anti scrambled). (*E*) Migration assay of transfected LLC cells assessed by incubating the cells in the transwell with 10% FBS - supplemented medium for 6 h or with serum-free medium for 24 h. (*F*) Tumor growth in xenograft nude mice ($n=9$) injected subcutaneously with LLC cells transfected with LNA anti scrambled (control), LNA anti miR-16 (LNA anti miR-16) or LNA anti miR-21 and miR-29a (LNA anti miR-21/29a). Data are normalized with respect to the control. The experiments were conducted in triplicate unless otherwise specified and presented as average ± s.d. **, $P<0.005$.

SUPPLEMENTARY REFERENCES

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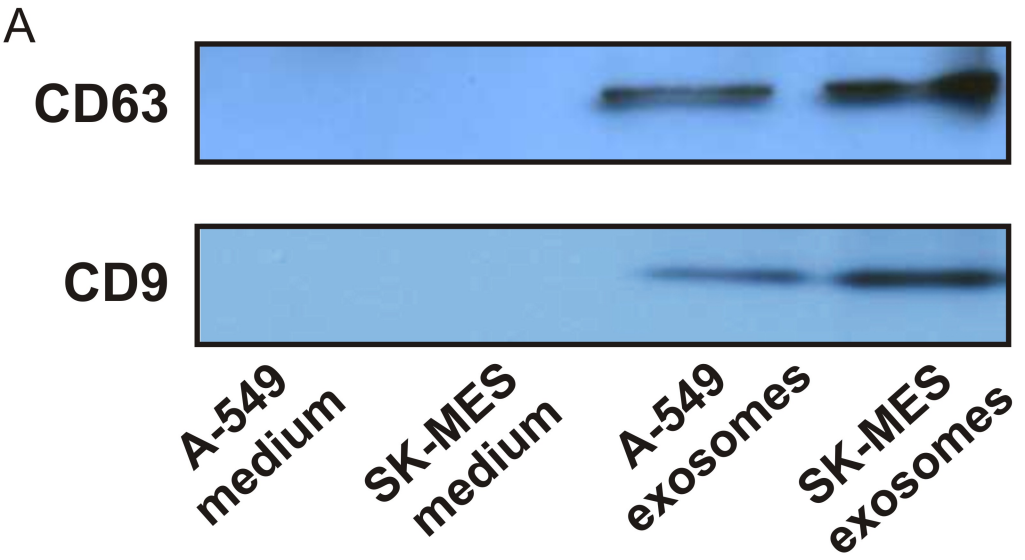
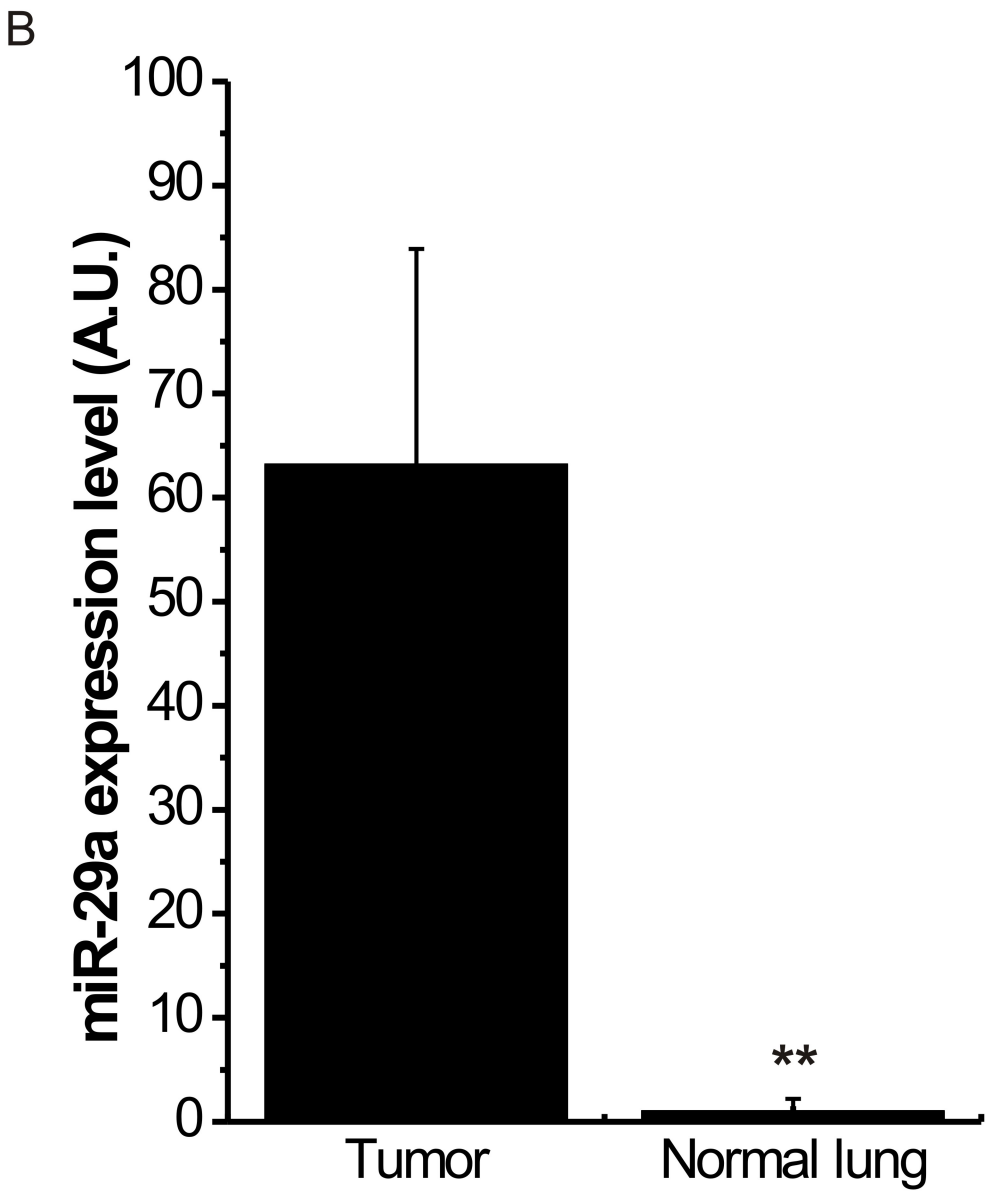
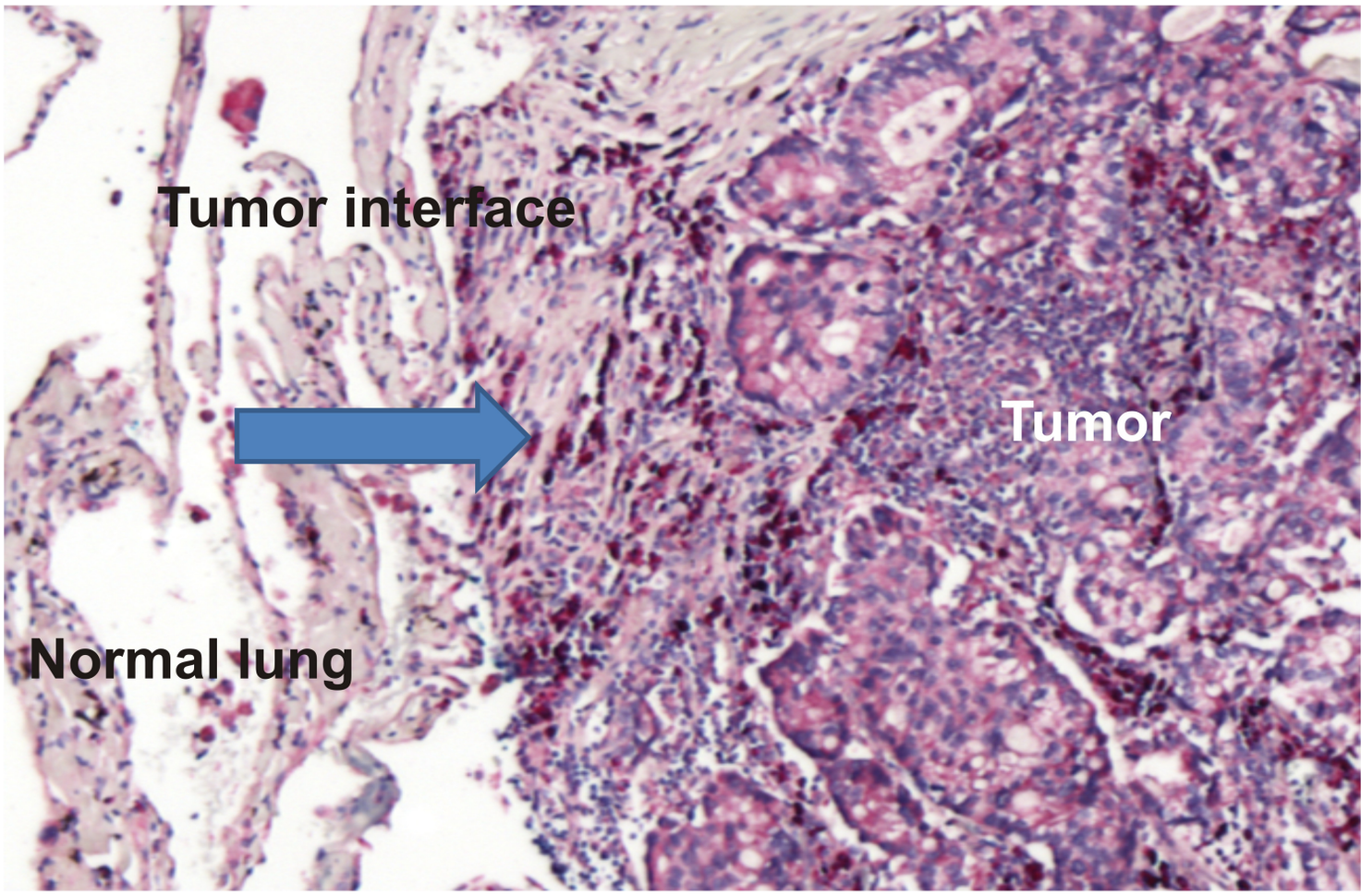

A
CD63
CD9
A-549 medium
SK-MES medium
A-549 exosomes
SK-MES exosomes

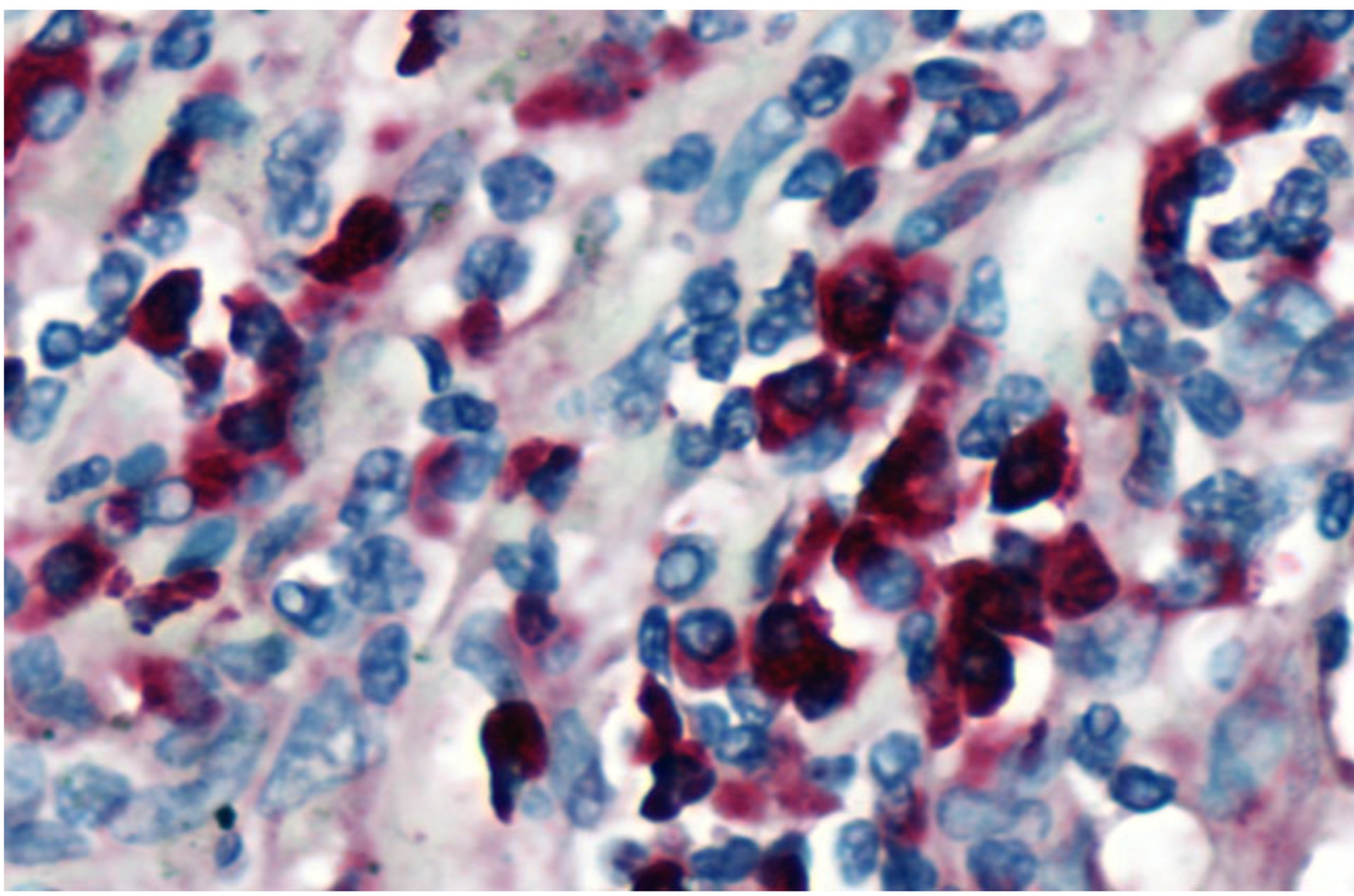

B
miR-29a expression level (A.U.)
100
90
80
70
60
50
40
30
20
10
0
**
Tumor
Normal lung

A



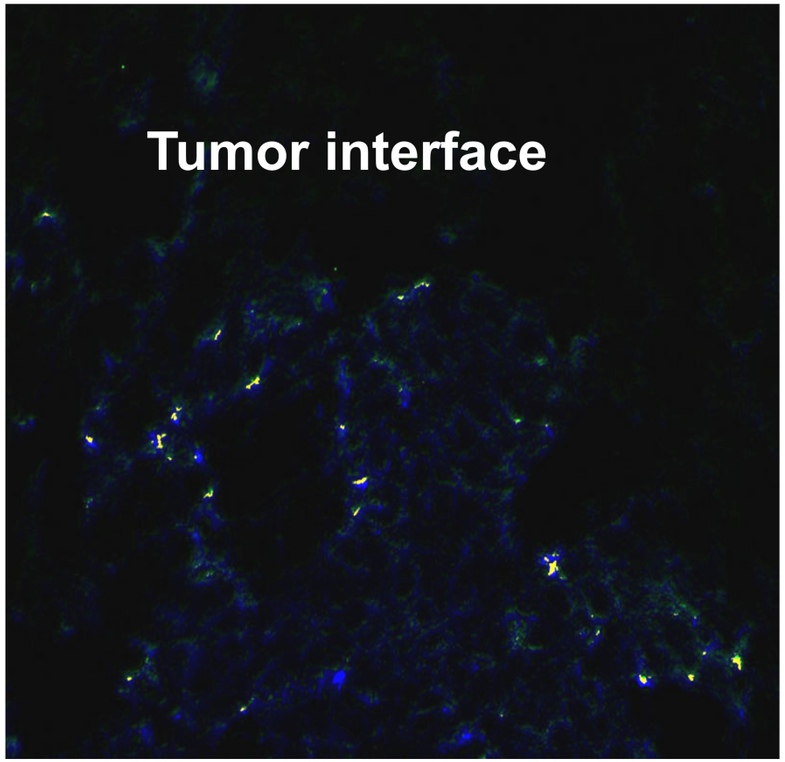
CD9 (red)

B



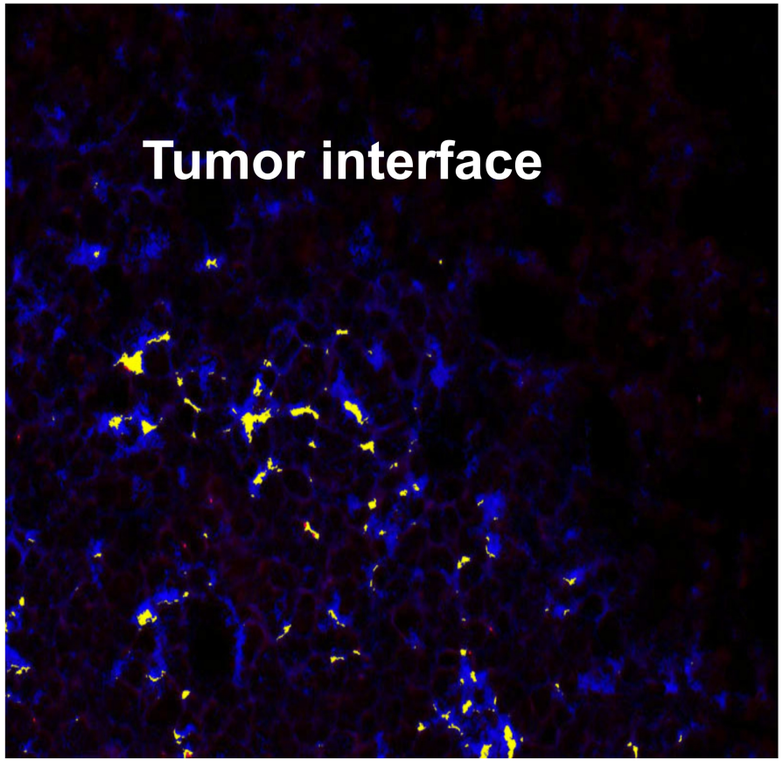
CD9 (red) distribution in macrophages
higher magnification

A



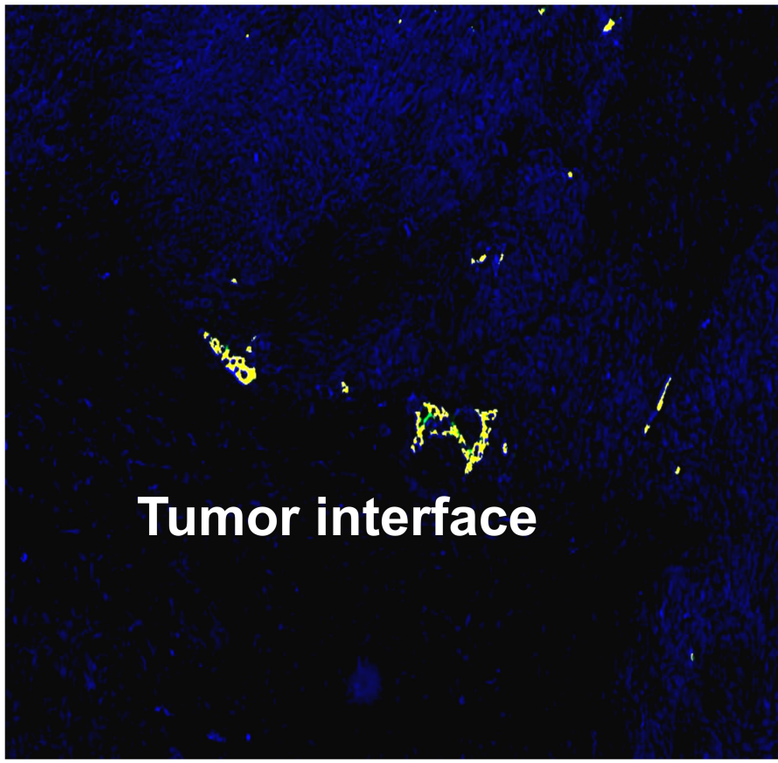
Nuance-converted
miR-29a (blue) and F-11 (green)

B



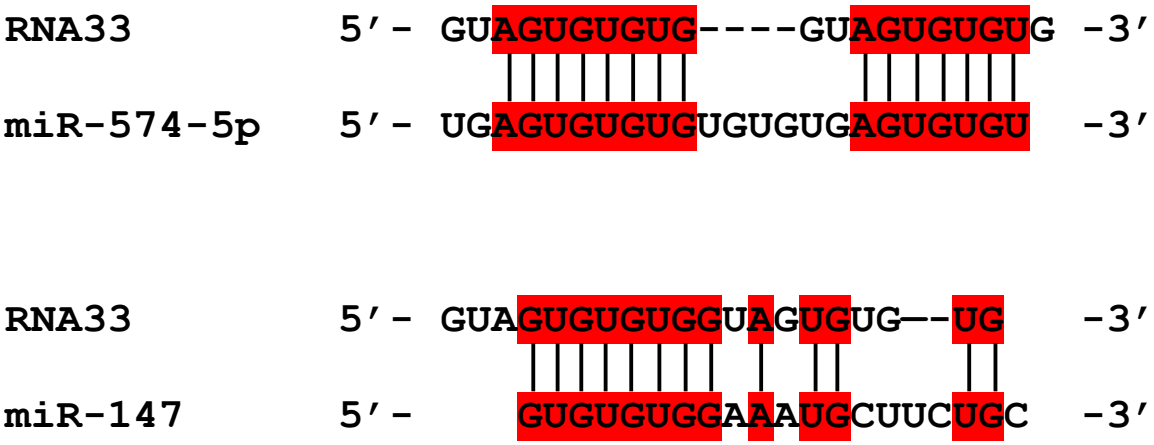
Nuance-converted
Cytokeratin (red) and miR-29a (blue)

C



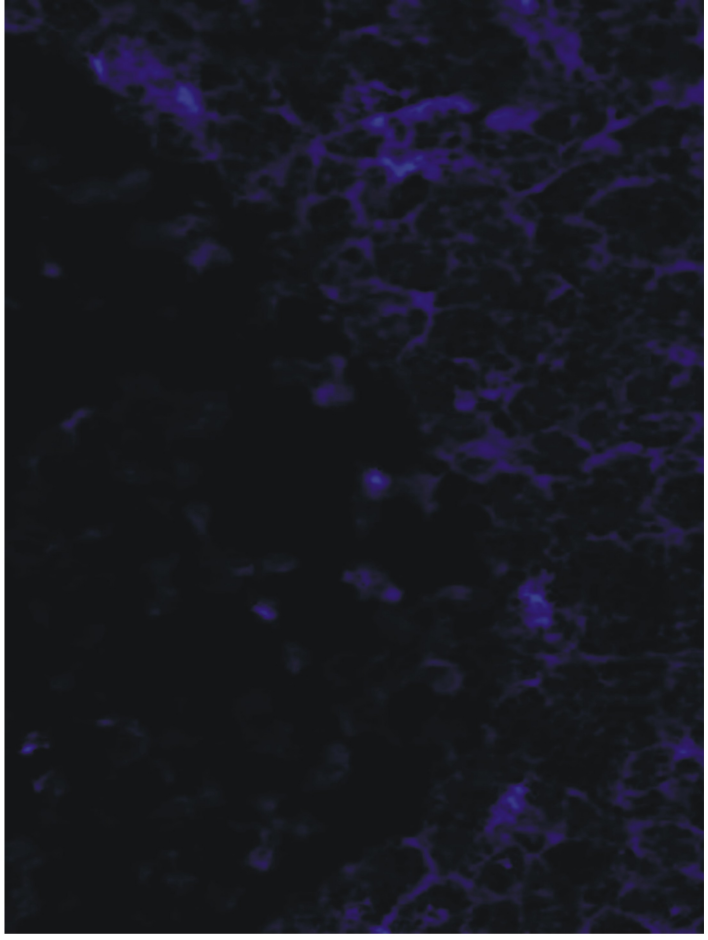
Nuance-converted
TLR7 (green) and miR-29a (blue)

A

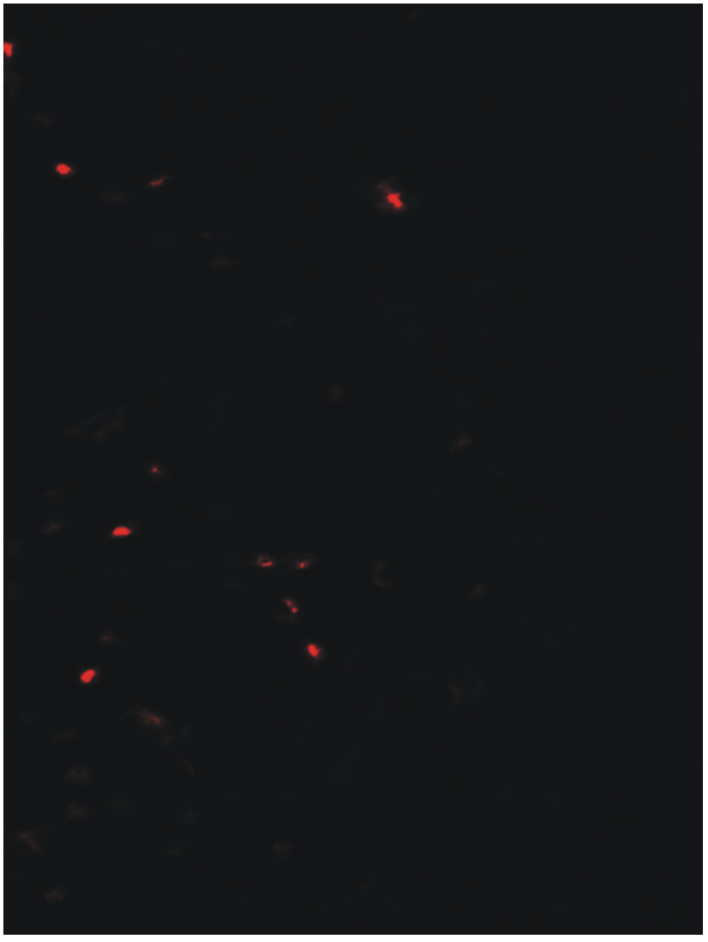


B

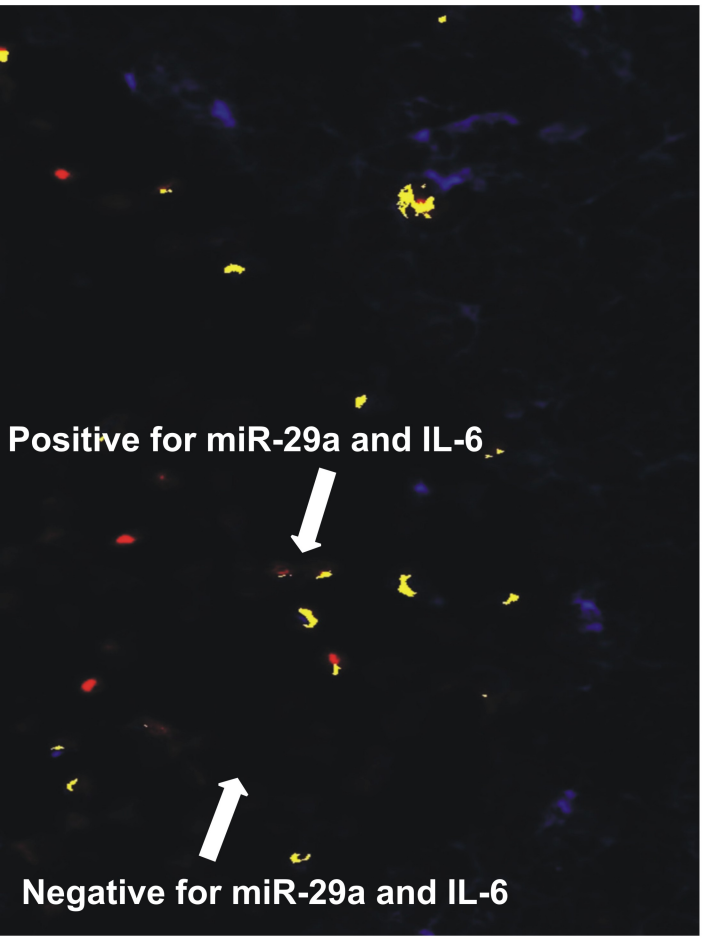
miRNAs	Sequences
miR-16	UAGCAGCACGUAAAUAUUGGCG
miR-21	UAGCUUAUCAGACUGAUGUUGA
miR-21 G18 modified	UAGCUUAUCAGACUGAUUUUGA
miR-21 U20 modified	UAGCUUAUCAGACUGAUGUGGA
miR-21 G18+U20 modified	UAGCUUAUCAGACUGAUUUGGA
miR-29a	UAGCACCAUCUGAAAUCGGUUA
miR-29a U20 modified	UAGCACCAUCUGAAAUCGGGUA
miR-29a U21 modified	UAGCACCAUCUGAAAUCGGUCA
miR-29a U20+U21 modified	UAGCACCAUCUGAAAUCGGGCA



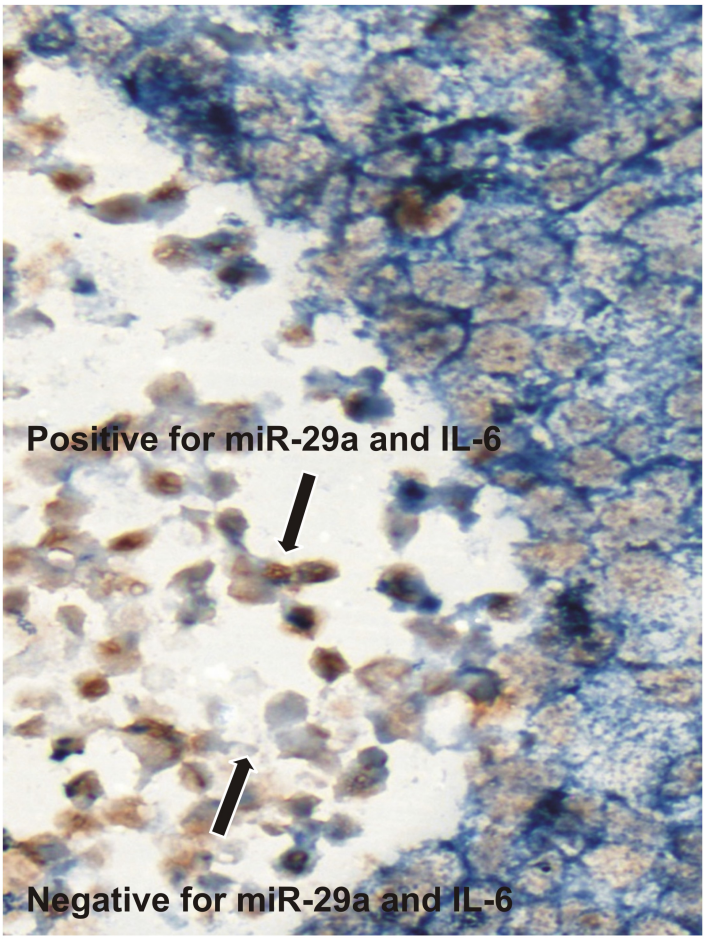
miR-29a (blue)



IL-6 (red)

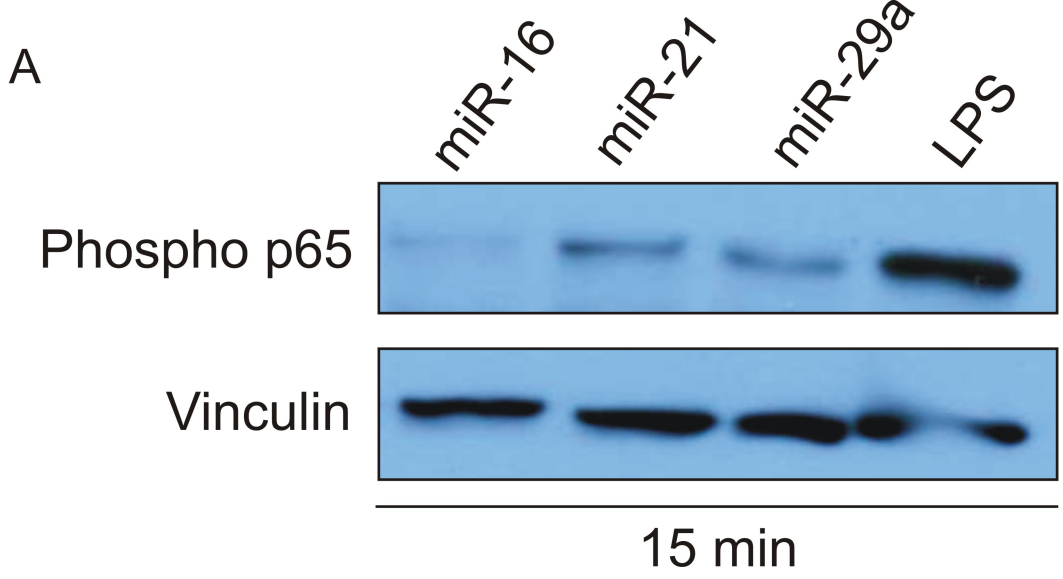


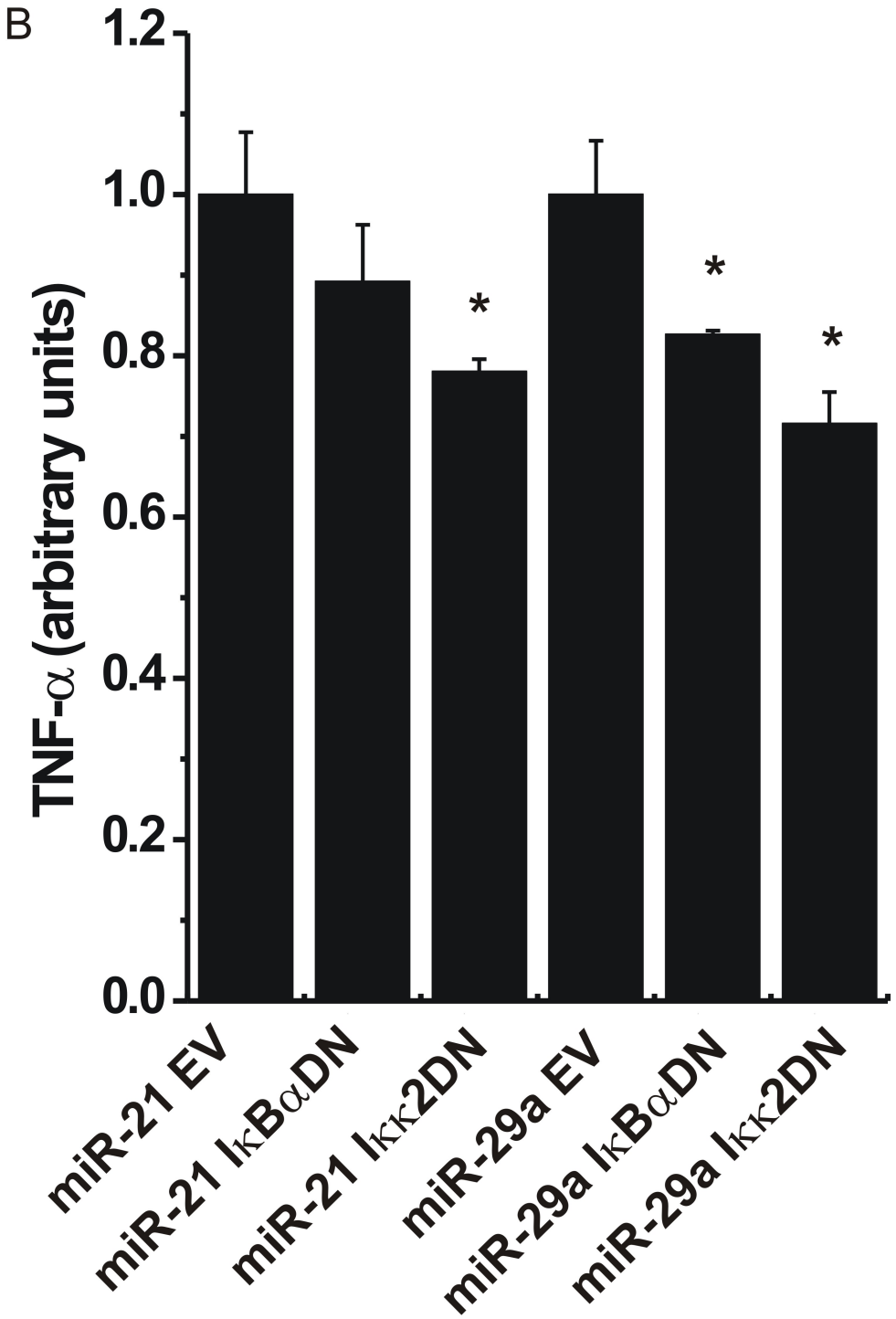
Merge

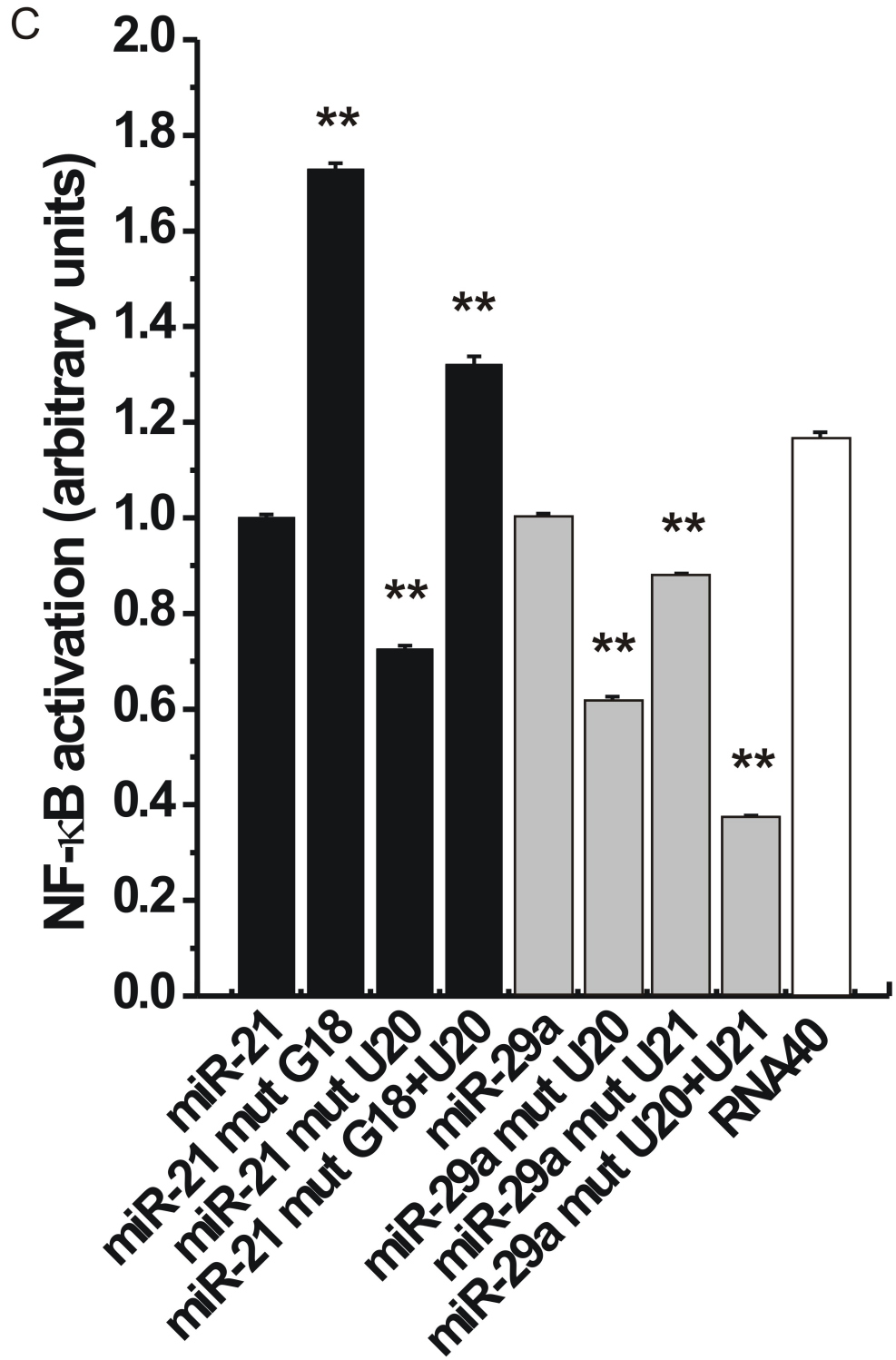


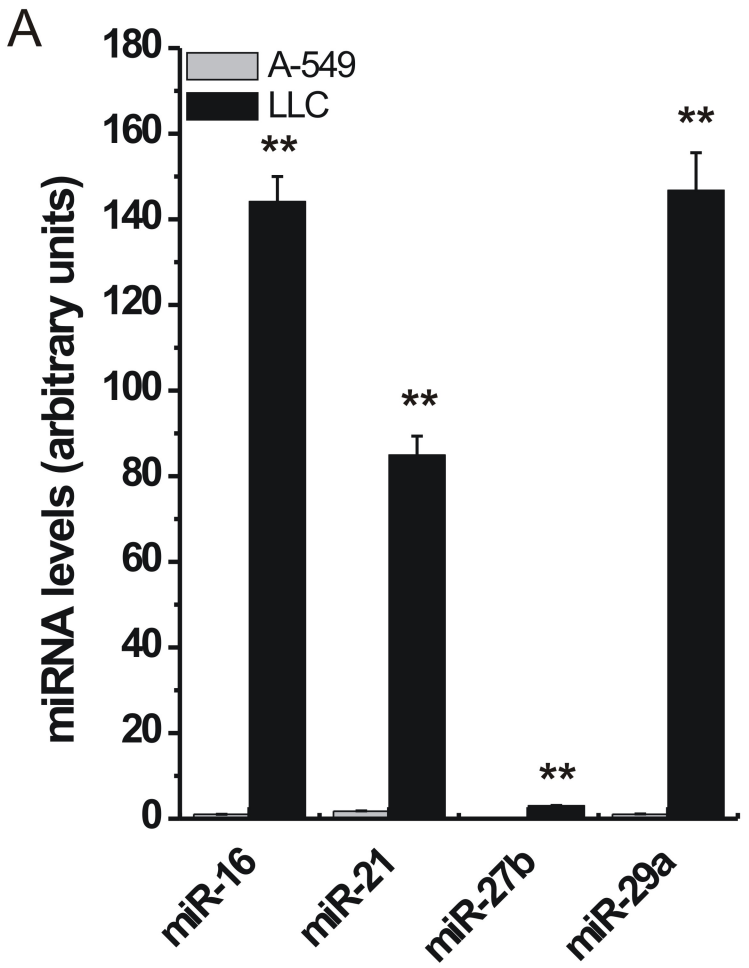
RGB
IL-6 (brown) and miR-29a (blue)

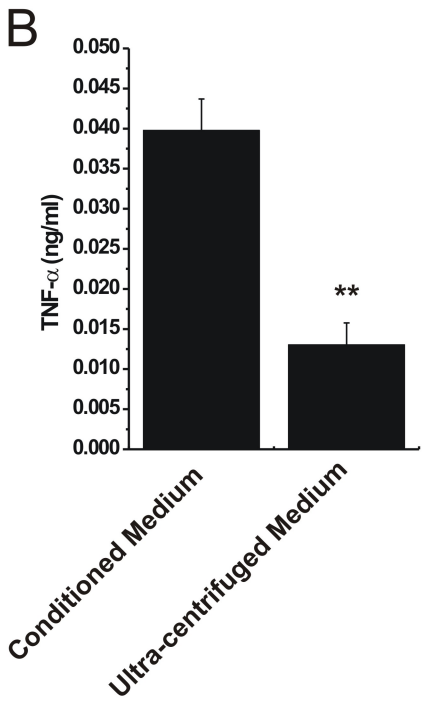
SI5

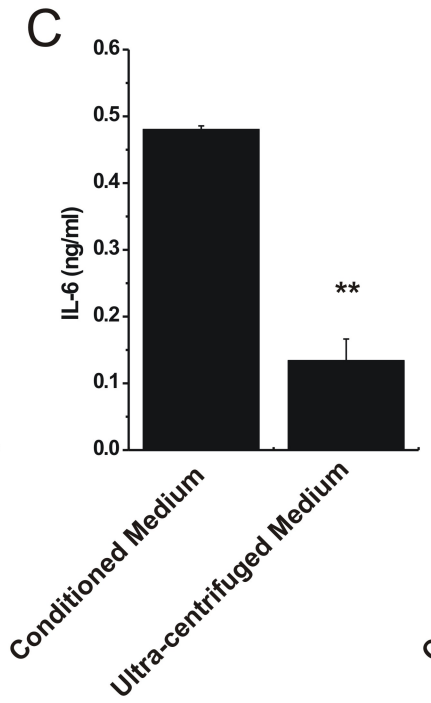

A
miR-16
miR-21
miR-29a
LPS
Phospho p65
Vinculin
15 min

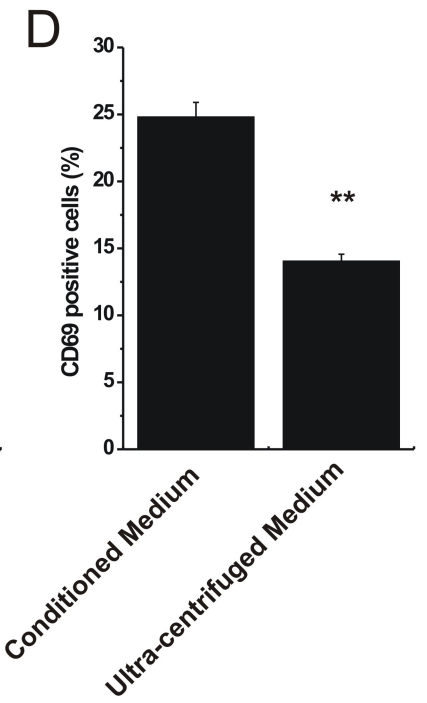

B
TNF-α (arbitrary units)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
miR-21 EV
miR-21 IκBαDN
miR-21 IκκβDN
miR-29a EV
miR-29a IκBαDN
miR-29a IκκβDN

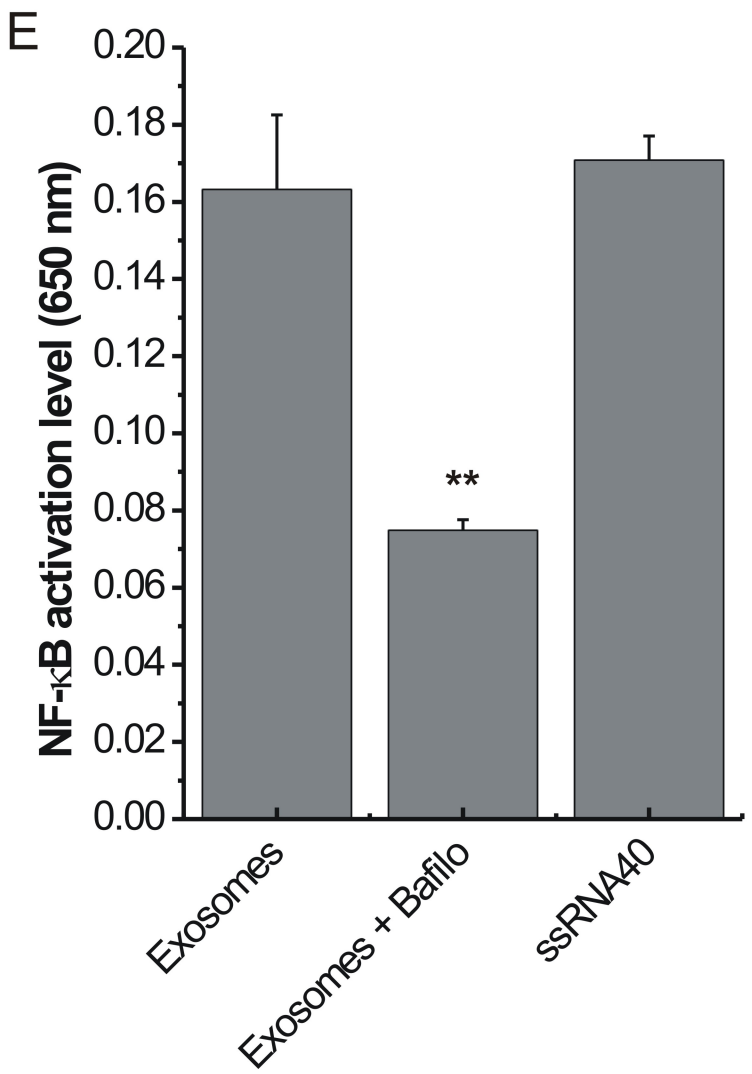

C
NF-κB activation (arbitrary units)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
miR-21
miR-21 mut G18
miR-21 mut U20
miR-21 mut G18+U20
miR-29a
miR-29a mut U20
miR-29a mut U21
miR-29a mut U20+U21
RNA40

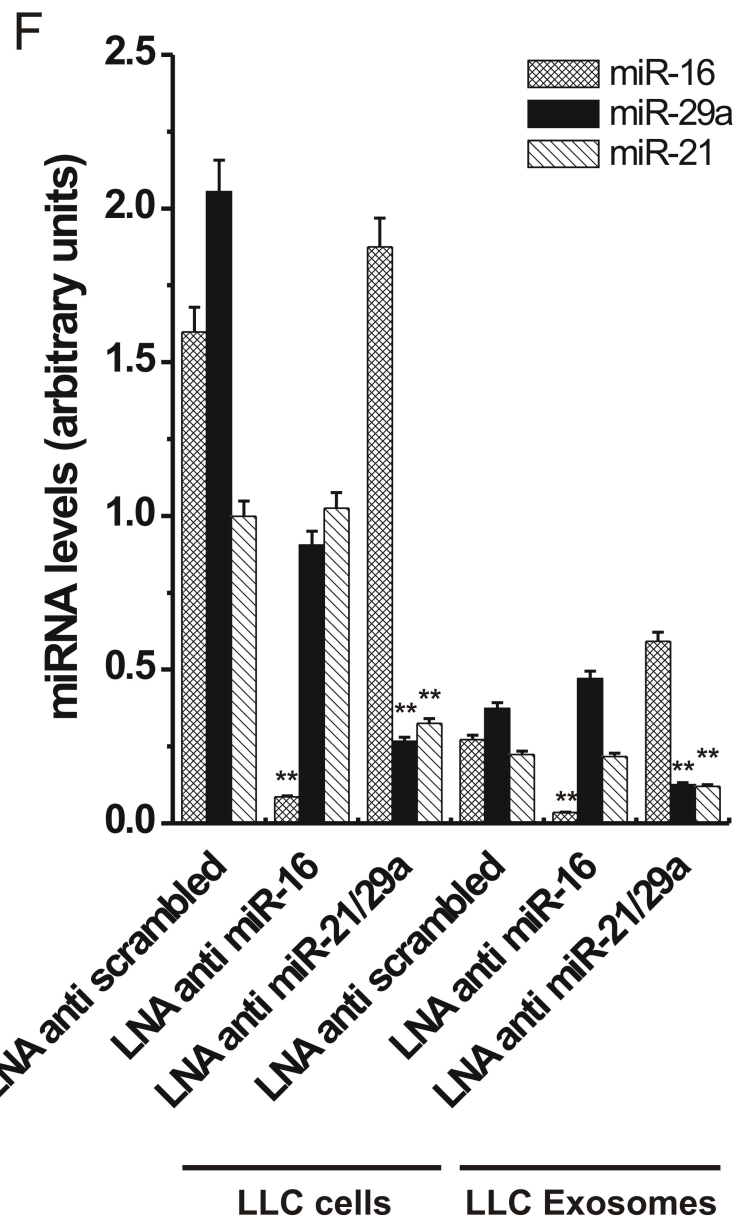

A
A-549
LLC
miRNA levels (arbitrary units)
miR-16
miR-21
miR-27b
miR-29a

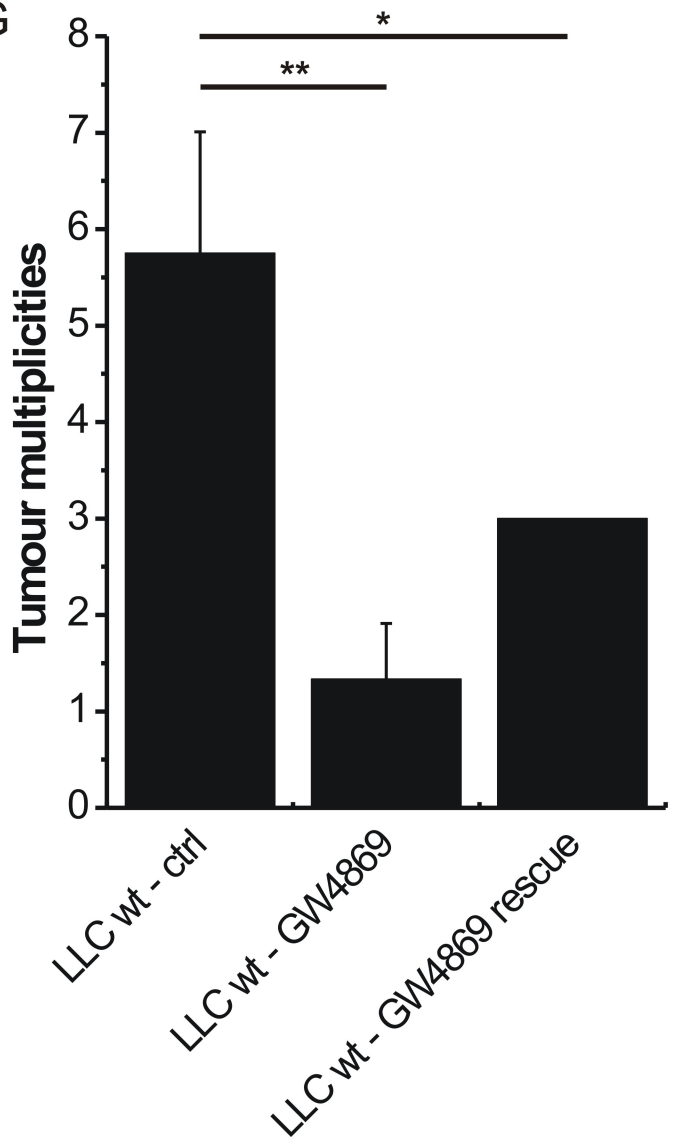

B
TNF-α (ng/ml)
Conditioned Medium
Ultra-centrifuged Medium

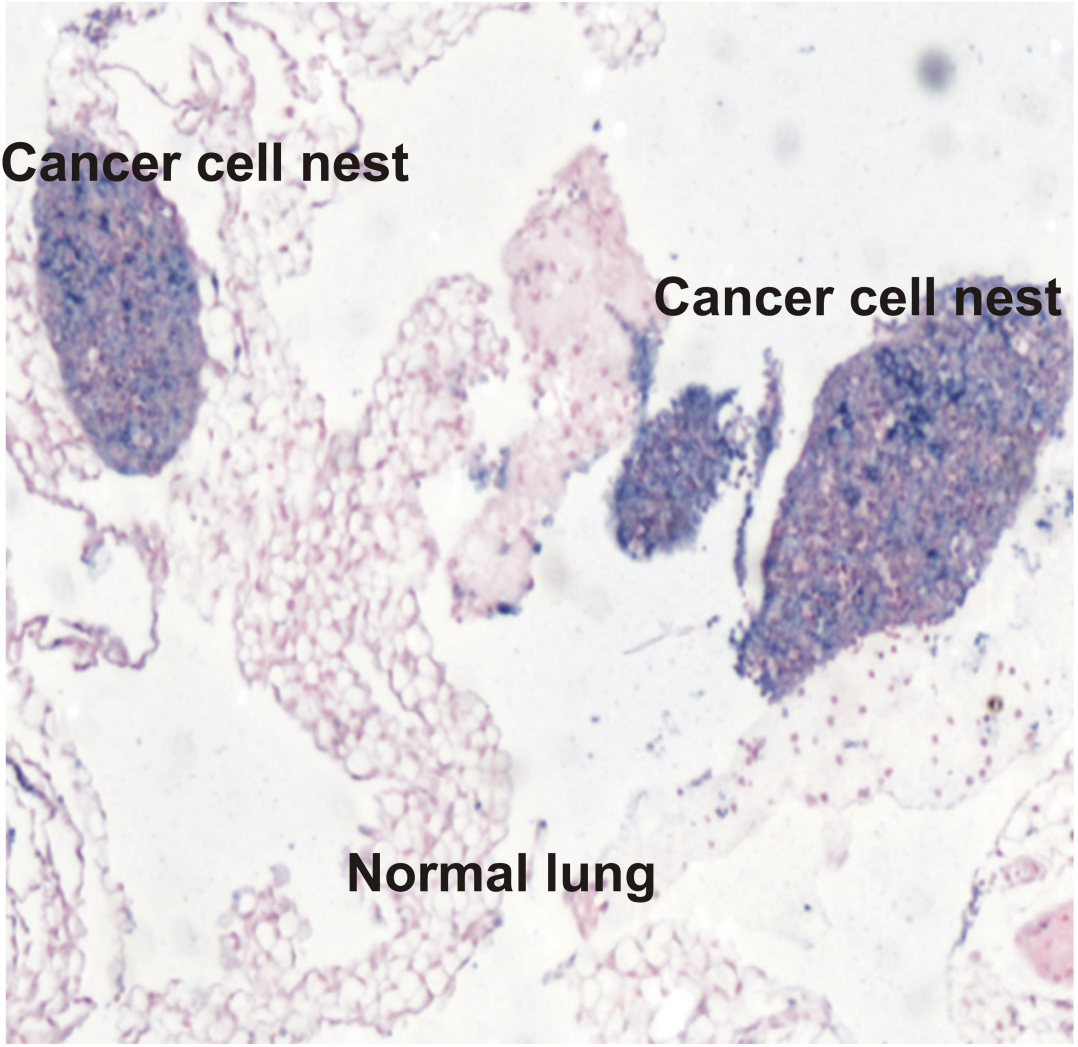

C
IL-6 (ng/ml)
Conditioned Medium
Ultra-centrifuged Medium


D
CD69 positive cells (%)
Conditioned Medium
Ultra-centrifuged Medium

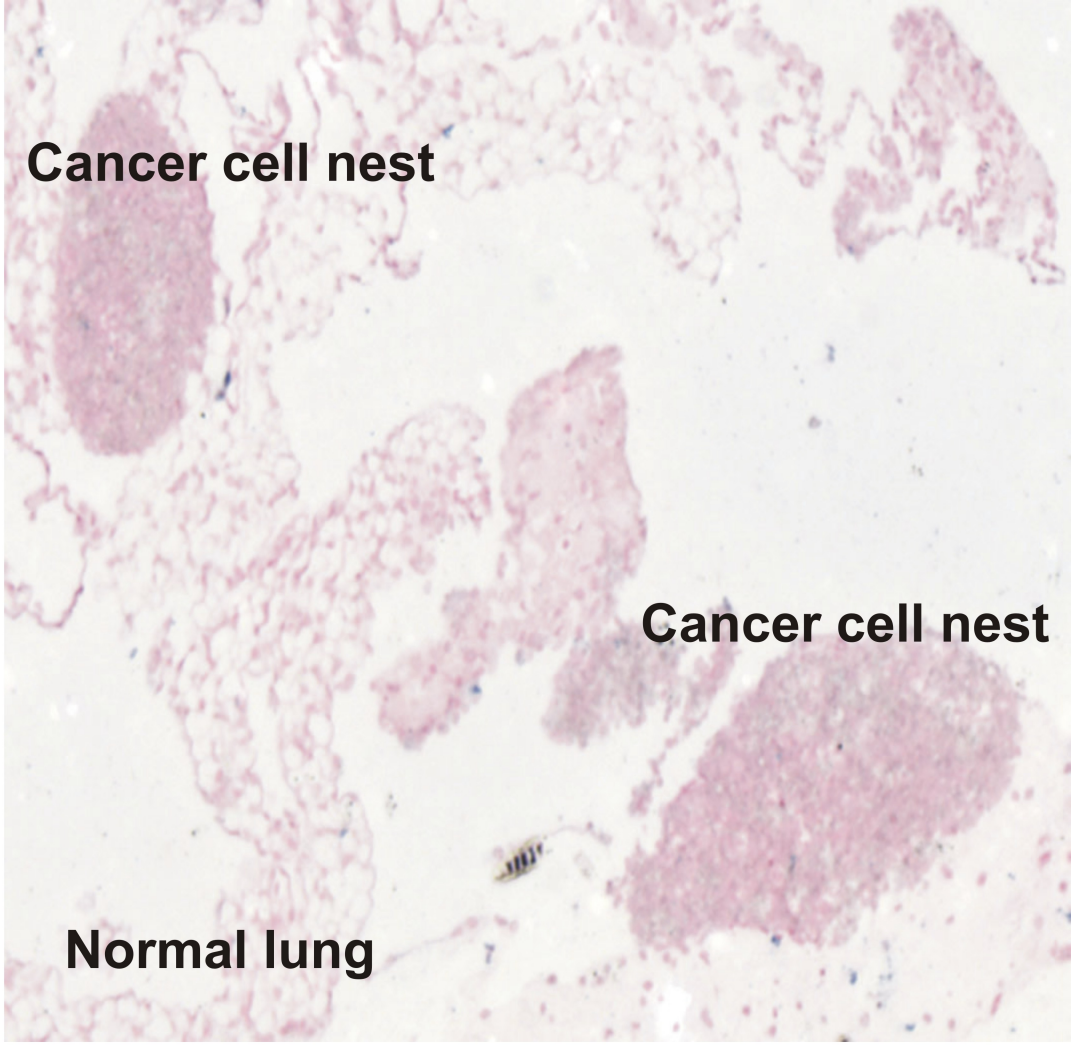

E
NF-κB activation level (650 nm)
Exosomes
Exosomes + Bafilo
ssRNA40


F
miR-16
miR-29a
miR-21
miRNA levels (arbitrary units)
LNA anti scrambled
LNA anti miR-16
LNA anti miR-21/29a
LNA anti scrambled
LNA anti miR-16
LNA anti miR-21/29a
LLC cells
LLC Exosomes

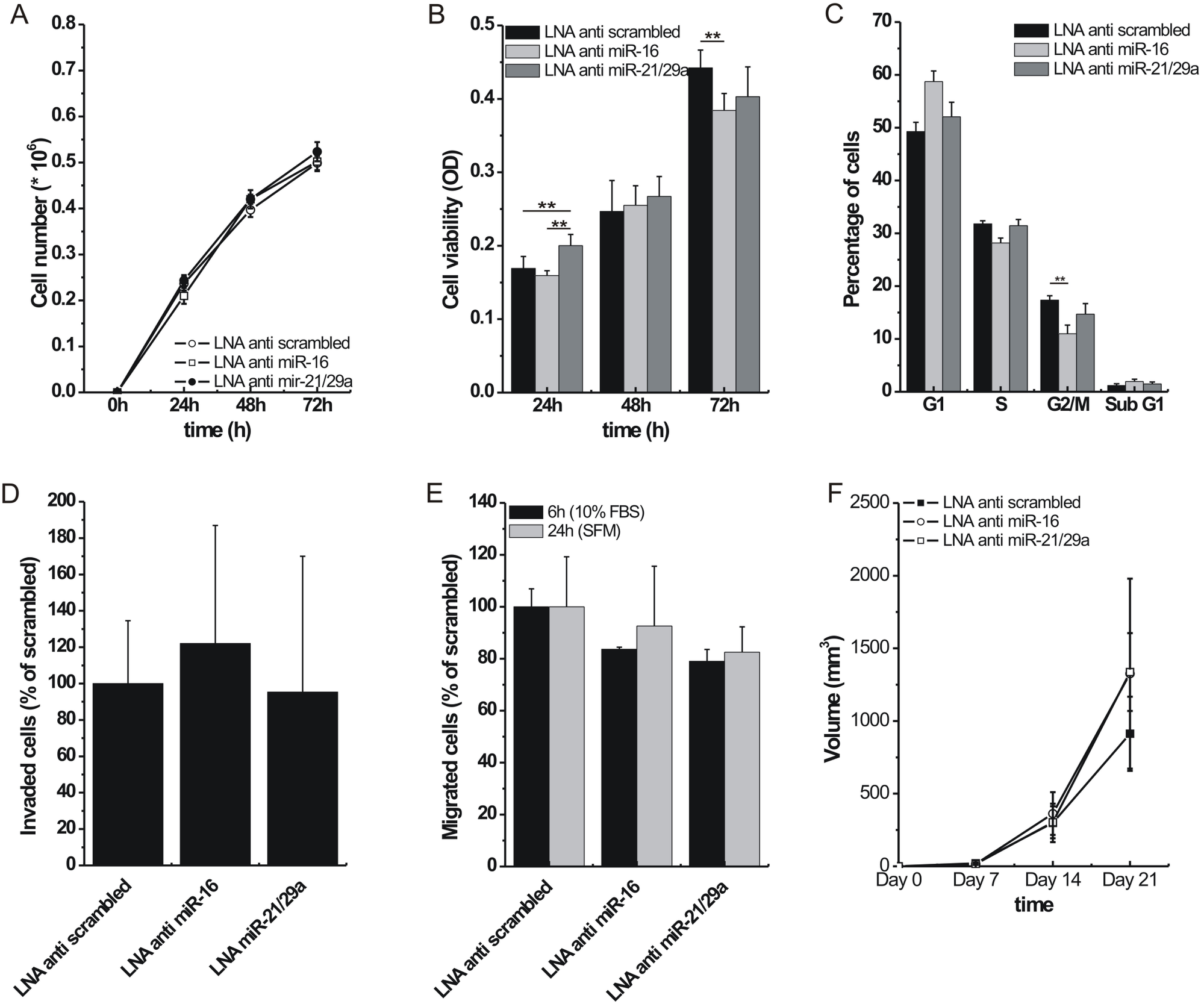

G
Tumour multiplicities
LLC wt - ctrl
LLC wt - GW4869
LLC wt - GW4869 rescue



Probe to miR-29a



Probe to anti miR-29a


A
Cell number (* 10^6)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0h
24h
48h
72h
time (h)
LNA anti scrambled
LNA anti miR-16
LNA anti mir-21/29a
B
Cell viability (OD)
0.0
0.1
0.2
0.3
0.4
0.5
24h
48h
72h
time (h)
**
**
**
LNA anti scrambled
LNA anti miR-16
LNA anti miR-21/29a
C
Percentage of cells
0
10
20
30
40
50
60
70
G1
S
G2/M
Sub G1
**
LNA anti scrambled
LNA anti miR-16
LNA anti miR-21/29a
D
Invaded cells (% of scrambled)
0
20
40
60
80
100
120
140
160
180
200
LNA anti scrambled
LNA anti miR-16
LNA miR-21/29a
E
Migrated cells (% of scrambled)
0
20
40
60
80
100
120
140
6h (10% FBS)
24h (SFM)
LNA anti scrambled
LNA anti miR-16
LNA anti miR-21/29a
F
Volume (mm^3)
0
500
1000
1500
2000
2500
Day 0
Day 7
Day 14
Day 21
time
LNA anti scrambled
LNA anti miR-16
LNA anti miR-21/29a