Manuscript Type: Article

Title: Mycoplasma exploits mammalian tunneling nanotubes for cell-to-cell dissemination

Author's name: Bong-Woo Kim[1,2], Jae-Seon Lee[3] & Young-Gyu Ko[1,4,*]

Affiliation: [1]Tunneling Nanotube Research Center, Korea University, Seoul 02841, [2]Skin Innovation R&D centre, HnB9 Co., Ltd. Cheongju 28161, [3]Department of Molecular Medicine, College of Medicine, Inha University, Incheon 22212, [4]Division of Life Sciences, Korea University, Seoul 02841, Republic of Korea.

Running Title: Mycoplasma propagation through tunneling nanotubes

Keywords: Tunneling nanotube, Mycoplasma, Fibroblast, Rac1

Corresponding Author's Information: Tel: +82-2-3290-3453, Fax: +82-2-927-9028; E-mail: ygko@korea.ac.kr

SUPPLEMENTARY MATERIALS AND METHODS

Cell culture, antibodies and reagents

The NIH3T3 (ATTC CRL-1658), CCD986-sk (ATCC CRL-1947), Chang Liver (ATCC CCL-13), and B16F10 (ATCC CRL-6475) cells were purchased from the American Type culture Collection (ATCC, Manassas, VA, USA) and cultured according to the provider's instructions. The Huh7 cells were obtained from Korean Cell Line Bank (Seoul, Korea). The anti-*Mycoplasma hyorhinis* (P70 surface protein) mouse antibodies were obtained from Kerafast (Boston, MA, USA). The anti-GFP, GAPDH, and Rac1 mouse antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). The anti-Rac1, anti-Cdc42, anti-RhoA, and anti-pan Ras mouse antibodies were obtained from Cell Biolabs, Inc (San Diego, CA, USA). DAPI (4′,6-diamidino-2-phenylindole), gentamicin, paclitaxel, cytochalasin B, and NSC23766 were obtained from Sigma Aldrich (St. Louis, MO, USA). Mycoplasma removal reagent (MRA) was obtained from MP Biomedicals (Santa Ana, CA, USA). CellMask™ was purchased from Thermo Fisher scientific (Waltham, MA, USA).

Plasmids, RNA interference, and transfection

The plasmids expressing pcDNA3-EGFP-Rac1-wt, pcDNA3-EGFP-Rac1-Q61L, and pcDNA3-EGFP-Rac1-T17N were obtained from Addgene (Cambridge, MA, USA). The pEGFP-C1 and ptdTomato vectors were obtained from Clontech (Mountain View, CA, USA). For the transient transfections, NIH3T3 and Chang liver cells were transfected with DNA (10 μg/1x10^7 cells) by a Neon® transfection system (Thermo Fisher scientific) following the manufacturer's instructions. For the siRNA experiments, NIH3T3 cells were plated in 100

mm dishes (SPL Life Science, Korea) at 1 x 10^6 cells and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Thermo Fisher scientific, HyClone™) supplemented with 10 % FBS (HyClone™) for 24 h. In addition, the cells were transfected with either control siRNA or Rac1 specific Stealth siRNA (Thermo Fisher scientific, Invitrogen) at a 400 pmol concentration using a Neon® transfection system.

Mycoplasma cultivation and invasion

Mycoplasma hyorhinis was obtained from ATCC (ATCC 17981). The mycoplasmas were grown at 37 °C in BBL™ Mycoplasma Broth Base (PPLO Broth Base, BD Biosciences, San Diego, USA)) containing BBL™ Mycoplasma enrichment (BD Biosciences). Prior to the infection, the mycoplasmas were harvested by centrifugation for 20 min at 12,000 rpm, washed and resuspended in DMEM. The number of mycoplasmas was determined by a MycoAlert™ Plus mycoplasma detection kit (Lonza, Basel, Switzerland). Briefly, serial dilutions of mycoplasma were centrifuged at 200 g for 5 min, and the supernatant was mixed with MycoAlert™ Plus reagents and substrates according to the provider's instructions. Mycoplasmas with a luminescence value of 1,000 were used for the invasion experiments, and this value represents the number of cells with approximately 10^7 CFUs (colony-forming units). The mycoplasma invasion experiment was carried out in 100-mm dishes containing DMEM and 10 % FBS for one week. For the internalization of mycoplasma, mycoplasma infected cells were washed three times with 1X PBS to remove the non-adherent mycoplasmas. Then, the cells were trypsinized and incubated with gentamicin (600 μg/ml) containing DMEM for 6 h at 37 °C in 5 % CO_2. After the gentamicin treatment, the cells were centrifuged at 1,000 g, re-suspended, diluted in DMEM medium and plated.

Fluorescence analysis, small GTPase activity, and immunoblotting

For the immunofluorescence staining, a monoclonal mouse anti-Mycoplasma hyorhinis P70 antibody was used. NIH3T3 cells were infected with mycoplasma as described above and fixed at room temperature for 10 min with 4 % paraformaldehyde in PBS. The cells were then washed with PBS, permeabilized by 0.2 % Triton X-100 in PBS, and incubated for 30 min with 2 % BSA, followed by incubation for 1 h at room temperature with an anti-P70 antibody. Then, the cells were incubated for 1 h with an anti-mouse Alexa 488- or Alexa 594-conjugated IgG antibody (Thermo Fisher scientific) and stained with DAPI (3 μM) for 10 min. The plasma membrane was stained with CellMask™ according to the manufacturer's instructions and observed by LSM700 confocal microscopy (Carl Zeiss). F-Actin of NIH3T3 cells is stained with 165 nM phalloidin-alexa fluor™ 488 (Thermo Fisher scientific) for 30 min after fixation with 4 % paraformaldehyde and permeabilization with 0.2 % Triton X-100.

The small GTPase activities were measured by a small GTPase activation assay kit from Cell Biolabs according to the provider's instructions. For the immunoblotting, the cells were lysed with RIPA buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na_2EDTA, 0.1 % SDS, 1 % NP-40, 1 % sodium deoxycholate, and 2.5 mM sodium pyrophosphate, pH 7.5), and the proteins were separated on SDS polyacrylamide gels and transferred to a nitrocellulose membrane. The antigens were analyzed by sequential treatment with specific primary antibodies and peroxidase conjugated secondary antibodies.

Co-culture system

In the mixed co-culture system, NIH3T3 cells (donor cells) expressing EGFP were treated with gentamicin (600 μg/ml) as described above, mixed with Chang liver cells (recipient

cells) expressing tdTomato at the ratio of 1:1, and plated on μ-Dish^{35mm} (ibidi, Biovalley, France) at a density that allows confluence to be reached after 24 h. After the 24-h incubation, the cells were washed with DMEM and incubated with DAPI to stain the mycoplasma for 10 min at 37 °C. The transfer of the mycoplasma through TNTs was analyzed in living cells by LSM700 confocal microscopy and qualified statistically. In the spatially separated co-culture system, the donor cells and recipient cells were also plated in a co-culture dish (209260, SPL life sciences) separately and incubated for 24 h.

FACS analysis for determination of mycoplasma-infected cells on co-culture system

For detection of mycoplasma-recipient cells on co-culture system, EGFP-expressing NIH3T3 cells and tdTomato-expressing Chang liver cells were dispersed with 0.25% Trypsin-EDTA and washed with 1X cold PBS after co-culture on 100 mm dish for 24 h. Subsequently, 2 x 10^6 cells per milliliter were analyzed and sorted on a fluorescence-activated cell sorting FACSAria (BD Biosciences) using FACSDiva software (BD Biosciences). EGFP and tdTomato were excited with standard 488-nm and 640-nm lasers, and green fluorescence was detected using 530/30 filters and red fluorescence using 585/42 filters. All analyses were repeated three times, and purified cells were lysed by RIPA buffer for the immunoblotting.

Statistical analysis

Statistical values are presented as the mean ± S.E. A two-tailed Student′s *t*-test was used to calculate the *P* values.

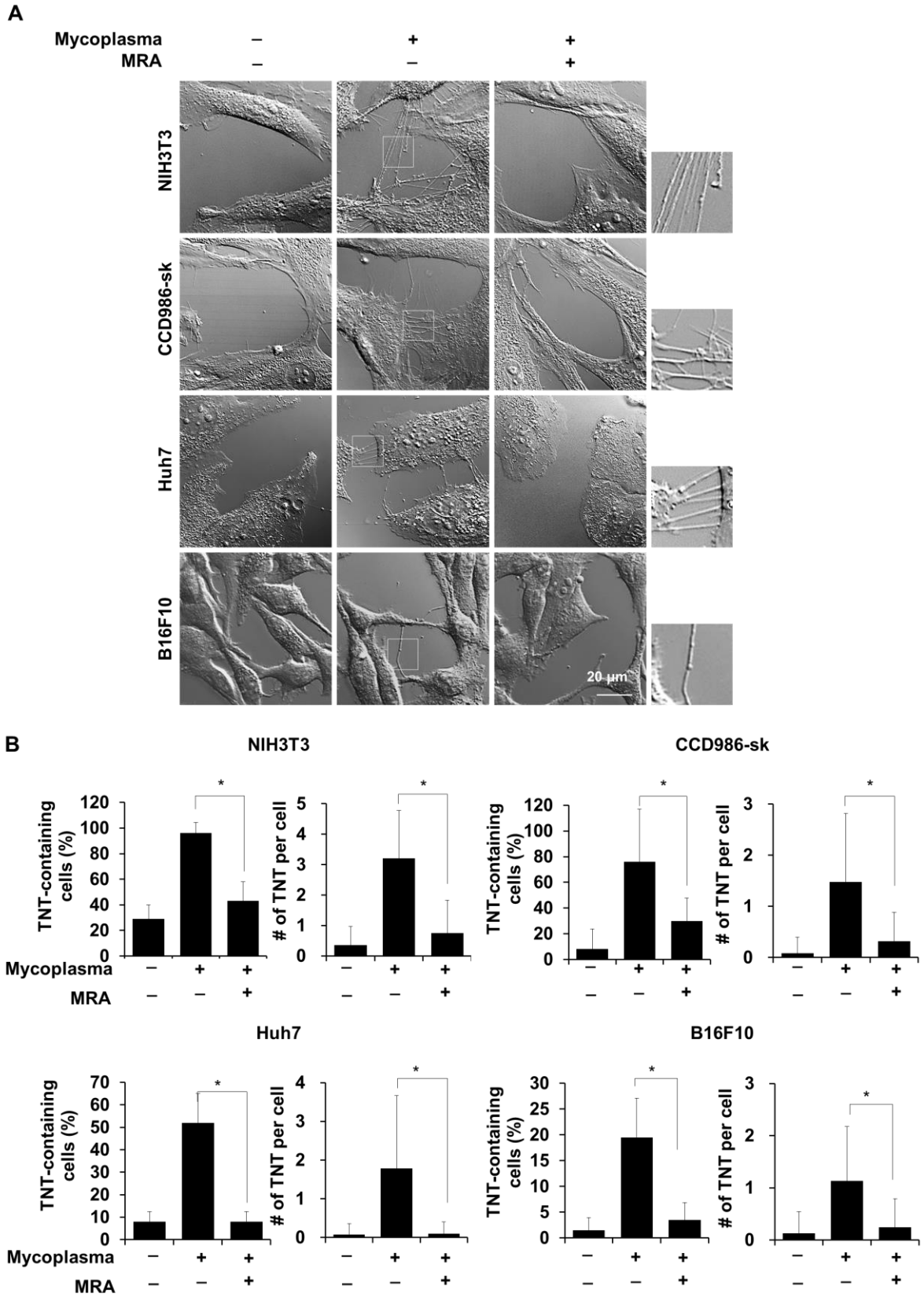
Supplementary Figure Legends

Supplementary Figure 1. *M. hyorhinis* infection induces TNT generation in various mammalian cells. (A) NIH3T3 murine fibroblasts, CCD986-sk human fibroblasts, Huh7 human hepatomas and B16F10 murine melanomas were infected with *M. hyorhinis* in the absence or presence of Mycoplasma Removal Agent (MRA, 0.1 μg/ml) for 7 days. The TNTs between the cells were observed by differential interference contrast (DIC) microscopy. Boxed regions are enlarged in the right panels. (B) The number of TNTs per cell and the percentage of TNT-containing cells among all cells were statistically determined and are represented as the mean ± s.d. $*p < 0.01$. One hundred cells were observed in ten different fields under each condition.

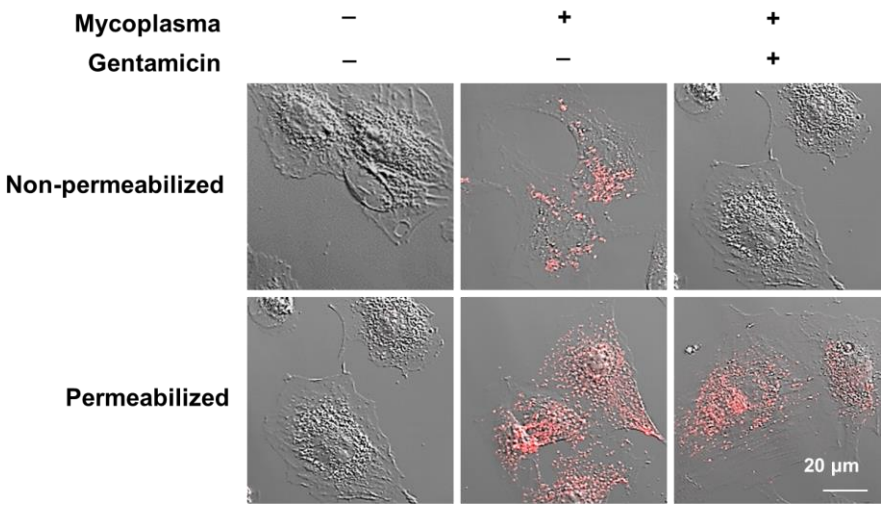
Supplementary Figure 2. Extracellular and intracellular mycoplasmas were observed in *M. hyorhinis*-infected NIH3T3 cells. Extracellular and intracellular mycoplasmas were observed in *M. hyorhinis*-infected NIH3T3 cells by P70 immunofluorescence with or without a permeabilization step. Intracellular mycoplasmas were observed after removing extracellular mycoplasma by treating with gentamicin (600 ng/ml) as described in Supplementary materials and methods.

Supplementary Figure 3. **TNTs possess intracellular *M. hyorhinis* in NIH3T3 cells.** (A) *M. hyorhinis*-infected NIH3T3 cells were stained with CellMast^{TM}, which is a specific tracker of the cell membrane, in the live state, fixed with 4 % paraformaldehyde (fixation) and permeabilized with 0.1 % Triton X-100 (permeabilization). Boxed regions are enlarged in

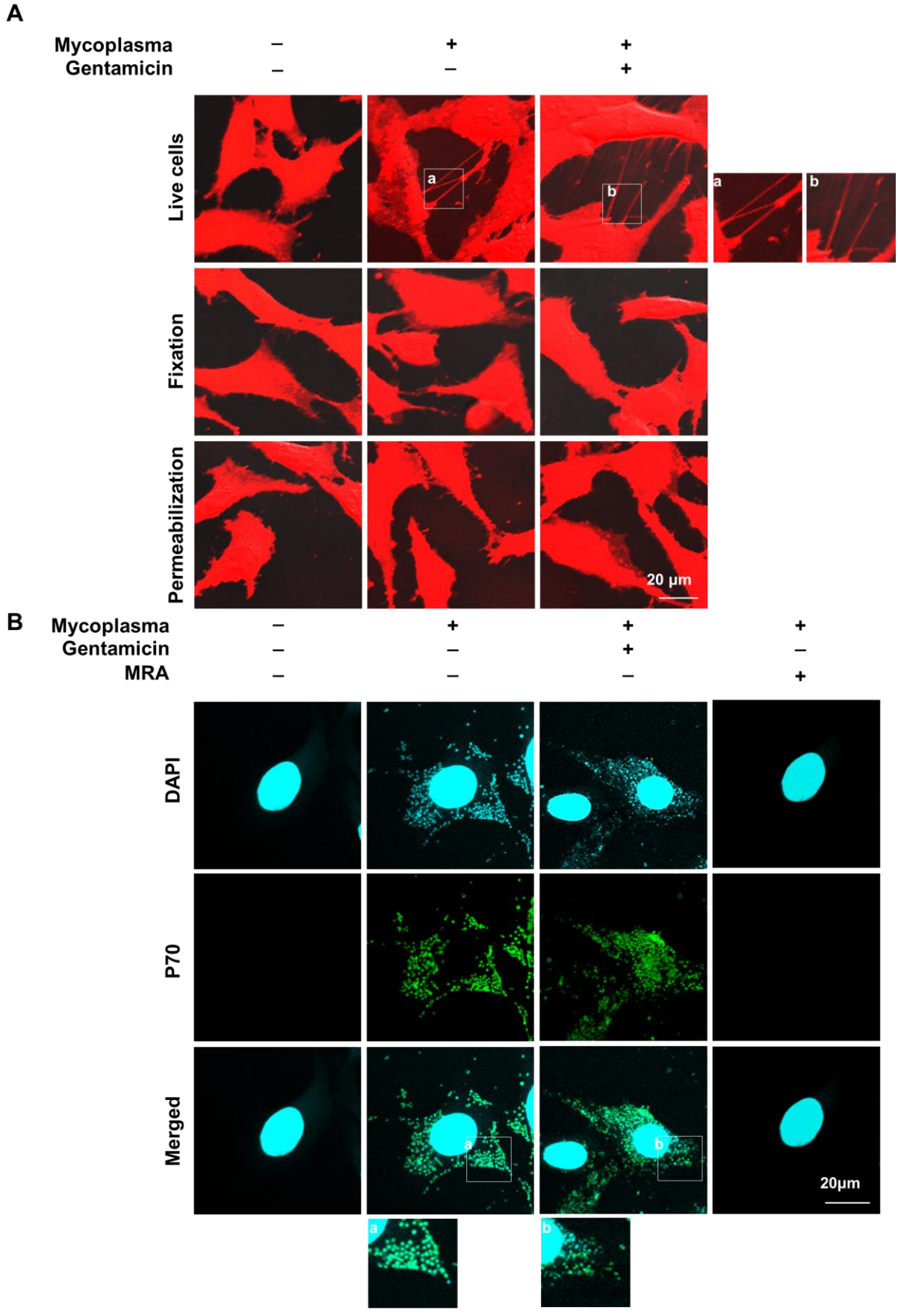
right panels. (B) *M. hyorhinis*-infected NIH3T3 cells were treated with gentamicin (600 ng/ml) or MRA for 16 h. Cells were fixed and permeabilized for P70 immunofluorescence and stained with DAPI (300 nM) for 10 min. Boxed regions are enlarged in the bottom panels.



Supplementary Fig. 1



Supplementary Fig. 2



Supplementary Fig. 3