Long-range function of secreted small nucleolar RNAs that direct 2'-O-methylation

Jamie M. Rimer  
*Washington University School of Medicine in St. Louis*

Jiyeon Lee  
*Washington University School of Medicine in St. Louis*

Christopher L. Holley  
*Washington University School of Medicine in St. Louis*

Robert J. Crowder  
*Washington University School of Medicine in St. Louis*

Delphine L. Chen  
*Washington University School of Medicine in St. Louis*

See next page for additional authors

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Authors
Jamie M. Rimer, Jiyeon Lee, Christopher L. Holley, Robert J. Crowder, Delphine L. Chen, Phyllis I. Hanson, Daniel S. Ory, and Jean E. Schaffer
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Jamie M. Rimer‡, Jiyeon Lee†, Christopher L. Holley‡, Robert J. Crowder‡, Delphine L. Chen‡§, Phyllis I. Hanson§, Daniel S. Ory‡, and © Jean E. Schaffer†

From the †Department of Medicine, ‡Mallinckrodt Institute of Radiology, and §Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110

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Small nucleolar RNAs (snoRNAs) are noncoding RNAs that guide chemical modifications of structural RNAs. Whereas snoRNAs primarily localize in the nucleolus, where their canonical function is to target nascent ribosomal RNAs for 2′-O-methylation, recent studies provide evidence that snoRNAs traffic out of the nucleus. Furthermore, RNA-Seq data indicate that extracellular vesicles released from cells contain snoRNAs. However, it is not known whether snoRNA secretion is regulated or whether secreted snoRNAs are functional. Here, we show that inflammation stimulates secretion of Rpl13a snoRNAs U32a (SNORD32a), U33 (SNORD33), U34 (SNORD34), and U35a (SNORD35a) from cultured macrophages, in mice, and in human subjects. Secreted snoRNAs co-fractionate with extracellular vesicles and are taken up by recipient cells. In a murine parabiosis model, we demonstrate that snoRNAs travel through the circulation to function in distant tissues. These findings support a previously unappreciated link between inflammation and snoRNA secretion in mice and humans and uncover a potential role for secreted snoRNAs in cell–cell communication.

The nearly 400 snoRNAs encoded within mammalian genomes function in 2′-O-methylation, pseudouridylation, or processing of other cellular RNAs (1, 2). Typically, snoRNAs direct these modifications on rRNAs and small nuclear RNAs, with specificity in targeting achieved by base pairing between the snoRNA antisense elements and their targets. Most mammalian snoRNAs are encoded within introns of host genes and are released from intron lariats during splicing by debranching and exonucleolytic cleavage (3). A small number of snoRNAs are independently transcribed by RNA polymerase II, with an m7G cap structure that becomes hypermethylated and a short 3′ extension that is cleaved (4). The vast majority of cellular snoRNAs localize with their substrates in the nucleus. Intronic snoRNAs are produced within the nucleolus, complexed with proteins, and transported to the nucleoli, where they are well positioned to encounter nascent rRNA substrates (5). By contrast, independently transcribed snoRNAs traffic to the cytoplasm during their biogenesis and are efficiently retrieved to the nucleus (6, 7).

A number of studies suggest that snoRNA localization to the nucleus is dynamic and regulated. In the setting of serum starvation of fibroblasts, biogenesis of snoRNA U3 increases, and abundance of U3 in the cytoplasm increases (8). Our own work has demonstrated that the snoRNAs encoded in the Rpl13a locus, U32a (SNORD32a), U33 (SNORD33), U34 (SNORD34), and U35a (SNORD35a), are essential for the cell death response to metabolic stress and accumulate in the cytosol of fibroblasts and myoblasts in response to lipotoxic and oxidative stress (9). RNA-Seq studies from our laboratory provided evidence that box C/D snoRNAs as a class rapidly relocalize to the cytoplasm following treatment of cardiomyoblasts with doxorubicin, a known inducer of oxidative stress (10). This redistribution of snoRNAs depends on superoxide and NADPH oxidase activity and does not simply result from compromise of the nuclear compartment. We also recently found that nuclear export factor 3 (NXF3) regulates the trafficking of snoRNAs between the nucleus and cytosol (11). Importantly, regulated appearance of snoRNAs in the cytoplasm raises the possibility of novel functions for snoRNAs outside the nucleus.

Microarray and deep sequencing approaches have led to the discovery of small noncoding RNAs in the extracellular space. The most well studied of these species are miRNAs, which are found in plasma in small membrane-bound vesicles or exosomes and/or bound to lipoproteins (12, 13). The abundance of different miRNAs in plasma, serum, and urine tracks with a spectrum of neurological, cardiovascular, oncologic, metabolic, and hematological disorders, suggesting that miRNAs in the blood can serve as biomarkers for disease processes (14). The observations that miRNAs are transferred from donor to recipient cells in which they impact gene expression and biological functions indicate that miRNAs can play roles in cell–cell communication (13, 15, 16).
RNA-Seq has also revealed the presence of snoRNAs in the medium of cultured cells (17–19) and in human plasma exosomes (20, 21). However, it is unclear whether secretion of this class of noncoding RNAs is regulated, and in contrast to miRNAs, their potential to function in non-cell-autonomous roles is unknown. The present study was undertaken to further characterize the extracellular trafficking of the four intronic box C/D snoRNAs produced from the Rpl13a locus.

**Results**

**Rpl13a snoRNAs accumulate in the culture medium of lipopolysaccharide (LPS)-stimulated macrophages**

The Rpl13a snoRNAs U32a, U33, U34, and U35a accumulate outside the nucleus of fibroblasts and cardiomyoblasts in the setting of oxidative stress (9, 10, 22). To extend these findings to another cell type in which oxidative stress plays a central role in pathophysiologically important responses, we treated bone marrow–derived macrophages with LPS, a stimulus known to induce reactive oxygen species and inflammatory gene expression (23). Following exposure of macrophages to LPS, intracellular reactive oxygen species are increased, as evidenced by enhanced 2′,7′-dichlorodihydrofluorescein diacetate (DCF) staining (Fig. 1A) and expression of the well-characterized inflammatory response genes, cyclooxygenase 2 and tumor necrosis factor-α (COX2 and TNFα; Fig. 1B). Stimulation with LPS did not cause loss of cellular integrity, as assessed by LDH release (Fig. 1C), or apoptosis, as assessed by caspase 3/7 activity (Fig. 1D). Using snoRNA-specific stem loop primers for reverse transcription with qPCR amplification for detection, we found that the Rpl13a snoRNAs rapidly increased in the

![Image](https://example.com/image.png)
medium following 1 h of stimulation and were cleared over the subsequent 3 h (Fig. 1F). Increases in snoRNAs in the medium were not accompanied by increases in cytosolic or nuclear snoRNA abundance (Fig. 1, F and G). Absolute quantification of snoRNAs using standard curves for each species demonstrated that LPS-induced increases in snoRNAs in the medium are significant and specific, but small relative to nuclear and total cellular snoRNA abundance, pools that do not significantly change with treatment (Fig. 2, A–D). Control PCRs indicated that the target-specific stem-loop primed reverse transcription qPCR method detects mature snoRNAs, but not snoRNA sequences in DNA or pre-mRNA templates (Fig. 2E). These findings indicate that LPS induces macrophage snoRNA secretion. Rapid clearance of the snoRNAs from the medium suggests they may be degraded, and decreases in cytosolic snoRNAs at 4 h (significant for U33 and U34 only) may reflect the cumulative export over several hours. In addition to the Rpl13a snoRNAs, LPS also stimulates the secretion of SNORD82, SNORD92, and SNORA73b (Fig. 3), suggesting that LPS broadly stimulates secretion of box C/D and box H/ACA snoRNAs.

**Rpl13a snoRNAs are released with extracellular vesicles**

A growing body of literature provides evidence that RNAs are released from cells in membrane-bound structures, generally referred to as extracellular vesicles (EVs) (24). Release of Rpl13a snoRNAs from LPS-stimulated macrophages was unab-
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Secreted RNAs can impact the biology of target cells by engaging cell surface receptors or by uptake through endocytosis or macropinocytosis. To determine whether RNA from macrophage-derived EVs is incorporated into recipient cells, EVs were enriched from the medium of donor cells that had been metabolically labeled with ethynyl uridine and incubated for 45 min with unlabeled recipient cells. Following washing to remove surface-bound material, recipient cells were probed with an Alexa Fluor 594–linked click reagent. RNA from donor cell EVs was detected in the nucleus, where it co-localized with fibrillarin (Fig. 7A). The dye-only control indicates that any small amount of free dye that might remain after labeling and cleanup does not cause detectable nucleolar staining. In an alternate approach, RNA and lipids of isolated EVs were stained with Syto RNAselect and 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindodicarbocyanine perchlorate (DiD), respectively, and incubated with recipient cells. In the recipient cells, labeled EV RNA and lipids were transferred throughout the cytoplasm and nucleus and concentrated in nuclear structures suggestive of nucleoli (arrowheads), a pattern that was distinct from background autofluorescence in the dye-only control (Fig. 7B). DiD was also transferred to recipient cells and was distributed in cytosolic puncta. Together, these findings indicate that both RNA and lipid constituents of EVs are transferred.

Approaches in which RNA is followed by a metabolic label or dye cannot formally distinguish between uptake of exogenous EV components and transfer of free label or dye that has partitioned into the EVs. To specifically assess whether secreted snoRNAs are taken up into recipient cells, we isolated cells from mice with germ line knockout of all four Rpl13a snoRNAs (KO) (28). KO mice are viable and fertile. To determine whether RNA from macrophage-derived EVs is incorporated into recipient cells, we isolated cells from WT mice and from mice with germ line knockout of all four Rpl13a snoRNAs (KO) (28). KO mice are viable and fertile. To distinguish between co-fractionation and true association of Rpl13a snoRNAs with EVs, we separated EVs isolated by ultracentrifugation from potentially nonvesicular co-sedimenting components by floating the EVs into a continuous sucrose gradient. Both Rpl13a snoRNAs and EV membrane protein markers were enriched at a density between 1.14 and 1.20 g/cm³, the characteristic density for EVs (Fig. 6A). Rpl13a snoRNAs in these EVs were resistant to RNase treatment but degraded when treated with RNase in the presence of detergent (Fig. 6B), suggesting that the snoRNAs are protected by a membrane. Together, our findings support a model in which macrophages release EVs containing snoRNAs.
macrophages demonstrated significantly increased nuclear punctae, consistent with transfer of U33. Whereas the signal for this single snoRNA species is small compared with the signal for total labeled RNA (Fig. 7), the co-culture approach (Fig. 8) provides further evidence of snoRNA transfer under more physiological conditions.

LPS increases Rpl13a snoRNAs in the serum of murine model and human volunteers

Because LPS triggers EV release of snoRNAs from cultured macrophages, we asked whether this inflammatory stimulus precipitated secretion of snoRNAs in vivo by treating mice with LPS. Compared with PBS-treated mice, animals that received LPS demonstrated a rapid increase in EV-associated Rpl13a snoRNAs in the serum over 4 h, concomitant with an increase in hepatic inflammatory gene expression (Fig. 9, A and B). We extended these findings by quantifying serum snoRNAs in healthy human volunteers undergoing intrabronchial instillation of LPS for a study of lung inflammation (29). Although the dose of LPS was small and delivered locally in the lungs, an increase in C-reactive protein, white blood cell count, and percentage of polymorphonuclear leukocytes 6 h following the procedure indicated the presence of systemic inflammation (Fig. 9C). Compared with pretreatment conditions, levels of U32a, U33, and U35a in serum EVs were significantly increased, and levels of U34 trended up (Fig. 9D). Thus, in mice and in humans, as we previously observed in cultured macrophages, LPS induces secretion of snoRNAs, most likely in EVs.

Secreted snoRNAs direct new 2′-O-methylation in vivo

To determine whether secreted snoRNAs can function in distant tissues in vivo, we employed a parabiosis model in which WT mice expressing GFP in all tissues were surgically joined to Rpl13a snoRNA KO mice (Fig. 10A). PCR analysis of DNA from tail blood of the parabionts revealed the presence of both the WT and KO alleles in the circulation of each parabiont by 1 week following surgery (Fig. 10B), consistent with reports of rapid development of shared circulation in this model (30). To test for the function of the Rpl13a snoRNAs, we utilized a qPCR-based method for detection of 2′-O-methylation of RNA, the modification that these snoRNAs direct on their canonical rRNA targets (31, 32). In this assay, the covalent modifications of the template cause reverse transcriptase to stall at low deoxyribonucleotide concentrations, such that larger amounts of qPCR product indicate less modification (Fig. 11A). This approach revealed the expected increases in amplification across Rpl13a snoRNA–directed 2′-O-methylation sites on the 18S and 28S rRNAs in KO compared with WT macrophages (Fig. 11B).

We applied this method for analysis of Rpl13a snoRNA–directed rRNA modifications to tissues harvested 3 weeks following surgery from KO and WT parabionts. Because new rRNA transcription correlates with cell replication (33) and 2′-O-methylation occurs on nascent rRNAs (34), we chose for study the intestinal epithelium, where cells turn over every 2–3 days (35). Liver tissue served as a negative control, because...
hepatocytes divide approximately once in 150 days in the adult mouse (36), and therefore new rRNA synthesis and 2'-O-methylation are expected to occur at much slower rates. Compared with KO mice that had not undergone parabiosis, enterocytes of KO parabionts showed a significant decrease in amplification (relative quantification; RQ), indicating an increase in modification of all four Rpl13a snoRNA target sites (lower RQ) and phenocopying the WT parabiont and WT animal control (Fig. 12A). By contrast, in liver tissue, we observed no significant difference in modification (RQ) at target sites for U32a and U33 between KO controls and KO parabionts, despite a clear difference between KO controls and either WT controls or WT parabionts (Fig. 12B). Unexpectedly, there was no difference between WT and KO controls in apparent methylation in liver tissue at the U34 or U35a snoRNA target sites, raising the possibility that these target sites are unmodified in WT livers or that other snoRNAs could be up-regulated specifically in the KO livers to compensate for the loss of the Rpl13a snoRNAs. Importantly, GFP expression remained undetectable in the KO parabiont (Fig. 12, C and D), indicating the lack of transfer of cellular material. Taken together, our data provide evidence that Rpl13a snoRNAs travel through the circulation and are taken up by distant tissues in which they function to modify their established RNA targets.

Discussion

Herein, we demonstrate that cultured macrophages secrete Rpl13a snoRNAs in response to the inflammatory stimulus LPS, most likely as cargo inside of EVs. Furthermore, we show that LPS stimulates secretion of these snoRNAs in both mouse models and in human subjects. By leveraging a recently generated mouse model with selective loss of the Rpl13a snoRNAs, we show that these extracellular noncoding RNAs are taken up by cultured macrophages, in which they traffic to the nucleus. In parabiosis experiments, we demonstrate that these snoRNAs are not simply inert passengers in EVs, but are also capable of functioning in distant tissues. Together, our findings provide a new understanding of the biology of snoRNAs and the repertoire of EVs.

EVs secreted from macrophages and other cell types can carry not only membrane and protein components from their cells of origin, but also RNAs that can be detected by sensitive PCR or RNA-Seq–based methods from concentrated preparations of EVs. Microarray and RNA-Seq analyses have provided
Figure 6. Rpl13a snoRNAs are contained within secreted EVs. A, qRT-PCR for snoRNAs (relative to *C. elegans* miR39 spike-in) and Western blotting of fractions collected from a continuous sucrose gradient (0.25–2 M) after floating EVs enriched by differential centrifugation from overnight macrophage culture (1.6 × 10^7 cells). B, qRT-PCR of snoRNAs from EV-enriched fraction treated with RNase, Triton X-100, and RNase inhibitor (Inh) as indicated. Results are mean ± S.E. (error bars) for *n* = 3. *, *p* < 0.05 using ANOVA with Dunnett’s multiple-comparison test.

Figure 7. EV tRNA. A, WT cells cultured with EVs from ethynyl uridine–labeled WT cells (EU RNA), fixed and stained for labeled RNA with Alexa Fluor 594 azide and α-fibrillarin (FIB) antibody. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Dye control used EVs from cells not labeled with ethynyl uridine. Scale bar, 20 μm. B, representative images of WT cells cultured with DiD– and Syto RNAselect–labeled WT EVs and fixed and stained with 4′,6-diamidino-2-phenylindole. Scale bar, 20 μm. Images are representative of three independent experiments.
evidence that in addition to mRNAs, long noncoding RNAs, and microRNAs, a range of snoRNA species are present in EVs secreted from cultured cells and in EVs isolated from human blood (17–19, 21, 37). Our work extends these observations by showing that concentrated EVs transferred EV RNA and transferred U33 snoRNA, we were unable to consistently detect new 2′-O-methylation in cultured recipient cells treated with concentrated EVs or in co-cultured macrophages. We hypothesize that this is because snoRNAs direct 2′-O-methyl modifications of newly transcribed pre-rRNAs in the nucleolus, but not mature rRNAs (34). Transcription of new rRNA is linked to progression through the cell cycle, which is extremely slow in these nonreplicating cells.

By contrast, in the parabiosis model using WT and KO mice, we find that Rpl13a snoRNAs are transferred through the bloodstream and can function in distant tissues, in a setting that does not involve concentration of large quantities of biological fluids. Two features of this model probably facilitated detection of functional snoRNA transfer. First, although the mice were not treated with LPS, sterile inflammation secondary to the surgical procedure may have contributed to EV secretion. Second, our data indicate that shared circulation is established early. Because synthesis of new rRNAs is low in post-mitotic cells and tissues, chronic exposure to snoRNA-containing EVs over several weeks may have enabled detection of snoRNA
function in rapidly replicating enterocytes, which must synthesize and 2′-O-methylate new rRNAs. Although our studies demonstrate acquisition of new snoRNA-directed modifications, we were unable to consistently detect Rpl13a snoRNAs by qRT-PCR in KO parabiont tissues (not shown). This suggests that snoRNAs are short-lived in recipient tissues relative to the RNA marks they generate and/or that a relatively small number of transferred snoRNA molecules catalyze a substantially larger number of methylation events.

Macrophages play key roles in the response to infection or tissue damage through their activation, polarization, and communication with the extracellular environment. Although it has long been appreciated that the secreted protein cytokines are key mediators of communication between macrophages and distant tissues, evidence is emerging that macrophage-secreted EVs play important roles in pathophysiological responses to inflammation. In the setting of infections with the intracellular pathogens Mycobacteria and Listeria, macrophages secrete EVs that promote inflammatory signaling, cell migration, and protective T cell-mediated immune responses in vivo (38–40).

Macrophase-derived EVs have also been shown to play a critical role in the regenerative response of intestinal stem cells following radiation injury (41). In some instances, these functions have been associated with EV transfer of protein antigens (39) or signaling molecules (41). Thus, macrophase EVs have been implicated to serve as immunoregulators. Our data show that macrophase-derived EVs contain snoRNAs that are known to target rRNAs for 2′-O-methylation, suggesting that the functional repertoire of macrophase EVs may include broader processes for cellular homeostasis, such as protein expression or cellular growth.

Figure 10. Parabiosis model establishes shared circulation. A, parabiosis surgery was performed to join the circulations of WT (+/+) and KO (−/−) mice. The WT mice express GFP (*) in all tissues. Following parabiosis, the presence of GFP in blood stream of both parabionts indicates shared circulation. Analyses for snoRNA transfer to parenchymal tissues of KO parabiont were performed 3 weeks after surgery following perfusion to remove blood. B, representative agarose gel of genotyping PCR on DNA recovered from blood collected before and 1 week after surgery from three parabiosis pairs with WT and KO controls.

Future studies will be required to elucidate the spectrum of signals that regulate snoRNA secretion, the mechanisms for selection of snoRNAs as EV cargo, and whether specific sub-classes of EVs carry snoRNAs. The rapidity of release of EVs containing snoRNAs following treatment with ionomycin or LPS suggests that these stimuli promote release of preformed snoRNA-containing EVs. Although secreted snoRNAs that direct rRNA modifications in recipient cells may mediate cell–cell communication, the physiological role of snoRNA secretion remains to be determined. The finding of augmented mitochondrial proton leak in tissues of the Rpl13a-snoRNA KO mouse suggests that a noncanonical function of the snoRNAs is to promote tightly coupled respiration (28). Based on this, we hypothesize that EV-mediated snoRNA transfer could enhance metabolic efficiency in recipient cells, thereby improving cell survival in the face of stress and/or infection. Characterization of the physiological contexts that precipitate secretion of snoRNAs could reveal opportunities in which the Rpl13a snoRNAs or other snoRNAs might serve as biomarkers for detection or management of disease. Given that snoRNAs are sensitive to antisense knockdown, these noncoding RNAs have the potential to serve as targets for new therapeutic approaches.
Experimental procedures

Reagents

LPS, BFA, IO, CD, and staurosporine were from Sigma-Aldrich. α-Fibrillarin (ab4566), α-nucleophosmin (ab15440), α-flotillin 1 (ab50673), and α-calnexin (ab22595) antibodies were from Abcam (Cambridge, MA). α-Histone H3 (catalog no. 9717) antibody was from Cell Signaling (Danvers, MA). α-CD9 (catalog no. 564236) was from BD Biosciences.

Mice

FVB and FVB.Cg-Tg(CAG-EGFP)B5Nagy/J were obtained from the Jackson Laboratory (Bar Harbor, ME), and Rpl13a-snoless (KO) mice (28) were maintained in facilities under specific pathogen-free conditions at Washington University (St. Louis, MO). Bone marrow was isolated as described below. Blood samples were obtained from male and female mice 4 h following intraperitoneal injection of 10 mg/kg LPS. All experimental procedures were approved by the Washington University Animal Studies Committee and were conducted in accordance with the United States Department of Agriculture Animal Welfare Act and the Public Health Service Policy for the Humane Care and Use of Laboratory Animals.

Bone marrow–derived macrophages

Bone marrow was harvested from the tibia and femur of male and female mice. Cells were seeded and differentiated for 6 days in 10% CMG14.12 conditioned medium (42). CMG14.12 cells were a gift from the laboratory of Deborah Veis (Washington University). Cells were treated with 50 ng/ml LPS for the indicated times, 10 μg/ml BFA for 90 min, 0.5 μM IO for 15 min, or 2% CD for 60 min. Cell death was measured using the CytoTox-96 nonradioactive cytotoxicity assay (Promega, Madison, WI). LDH+ controls contained LDH from the kit (1:5,000 dilution of 1,600 units/ml solution), and the maximum signal was determined by measuring LDH activity in the medium of cells cultured under identical conditions and lysed by quickly freezing and thawing the plate. Apoptosis was quantified by a caspase 3/7 Glo assay (Promega, Madison, WI). ROS was
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detected by measuring fluorescence on a TECAN Infinite 200 PRO multimode reader (Tecan, Männedorf, Switzerland) after cells were loaded with 10 μM DCF (Thermo Fisher Scientific, Carlsbad, CA). Sequential detergent extraction was performed as described previously to generate nuclear and cytosolic fractions (10).

**EV enrichment, labeling, and transfer**

EVs were isolated from cell culture medium using Total ExoSome isolation reagent (Thermo Fisher Scientific) (43), or EVs were isolated by differential centrifugation (44). Serum EVs were isolated with ExoQuick (System Biosciences, Mountain View, CA) according to the manufacturer’s instructions (38). Culture medium used for EV labeling/transfer and floatation/RNase treatment experiments was depleted of EVs before use by centrifugation at 120,000 × g for 90 min. Conditioned medium was cleared by centrifugation for 10 min at 1,000 × g, followed by centrifugation for 20 min at 2,000 × g, and then concentrated using an Amicon Ultra-15 centrifugal filter unit (3,500 kDa cut-off) (forward) and TGG GAG TAG ACA AGG TAC AAC CC (reverse); and for Rplp0, they were ATC CCT GAC GCA CCG CCG TGA (forward) and TGC ATC TGC TTG GAG CCC ACG TT (reverse). The method for qRT-PCR for Rpl13a snoRNAs used target-specific stem loop RT primers, a target-specific forward PCR primer, and a universal reverse PCR primer (46). This method can detect as few as 10^3 copies of target RNAs, is able to distinguish between small RNA species that differ by only 1 nucleotide, is not affected by genomic contamination, distinguishes mature snoRNAs and miRNAs from their precursor small RNA species, and has been validated against RNA-Seq for snoRNAs (10, 47, 48). mRNA quantification was calculated using Rplp0 as an endogenous control. For quantification of EV RNA, Caenorhabditis elegans mir39 (500 fmol) or luciferase mRNA (0.3 fmol; Promega) was added to EV samples before RNA isolation and used as a reference control. Amplification of reference genes varied by <1 cycle across samples. A plate of 2 × 10^6 cells was used for cellular fractionation by sequential detergent extraction, and medium was pooled from three plates.

**Microscopy**

Freeze fracture/deep etch EM was performed according to published protocols with minor modifications (49). A 4-μl droplet of sample was placed on top of a 4 × 4-mm acid-cleaned and air-dried coverslip and then topped with a 3-mm round sapphire disc. Samples were immediately frozen by abrupt application of the sample against a liquid helium−cooled copper block with a Cryopress freezing machine. Frozen samples were transferred to a liquid nitrogen−cooled Balzers 400 vacuum evaporator, fractured at −100 °C by removal of the sapphire disc with the instrument’s nitrogen cooled knife. Samples were etched for 2 min at −100 °C and rotary replicated with ~2-nm platinum deposited from a 20° angle above the horizontal, followed by an immediate ~4-nm stabilization film of pure carbon deposited from an 85° angle. Replicas were floated onto a dish of concentrated hydrofluoric acid for coverslip removal and then transferred through several rinses of distilled H₂O with a loopful of Photoflo, picked up on Formvar-coated grids, and photographed on a JEOL 1400 microscope with attached AMT digital camera. Confocal images were taken on a Zeiss LSM 880 Airyscan confocal microscope with a 0.8-μm pinhole through a focal plane in the center of the nuclei and processed using Zen software (Zeiss, Oberkochen, Germany).

**RNase protection**

EVs enriched by ultracentrifugation were suspended in 250 μl of PBS and treated with 0.3% Triton X-100, 1 unit of RNase A, 40 units of RNase T1 (Ambion RNase mixture, Thermo Fisher Scientific), and 100 units of SUPERase-In RNase inhibitor (Thermo Fisher Scientific) for 30 min at 20 °C, and then RNase inhibitor was added to all samples. RNA was isolated using TRizol LS.

**Human subjects**

Blood samples were collected from human volunteers for a study of lung inflammation before and 6 h following endobronchial instillation of 4 ng/kg LPS (29). This study was approved by the Washington University Human Research Protection...
Office and complies with the Declaration of Helsinki principles. Informed consent was obtained from all subjects.

Parabiosis

The Washington University Mouse Cardiovascular Pheno-
typing Core performed parabiosis surgery using female WT
mice expressing GFP and Rpl13a snoRNA KO mice as
described (30). Tail vein blood was sampled serially from the
parabionts for PCR analysis of DNA to detect the WT and KO
Rpl13a loci with primers AGA AGG TTG CTG CTC AGG
AAG TAA ATGG (forward) and CCA GAC CTG CTT TCA
GAC TTT AGC CTG (reverse). Three weeks following surgery,
parabionts were perfused with PBS to clear blood from tissues
and euthanized. Enterocytes and liver were recovered and
flash-frozen in liquid nitrogen. For PCR analysis, tissues were
homogenized in TRIzol.

Primer extension qRT-PCR

Primer extension PCR to detect rRNA methylation was
adapted from Dong et al. (31). For each target, 20 ng of total
RNA was used in cDNA synthesis using the Super Script III kit
(Thermo Fisher Scientific) with 0.3 μl of SSIII enzyme per reac-
tion and dNTP at either the normal stock concentration (50
μM) or diluted 1:1000 (50 nM). The following primers were
used for each RT reaction: m18S RT (U32a, G1328), CAA CTA
AGA ACG GCC ATG CA; m18S RT (U33, U1326), AGA ACG
GCC ATG CAC C; m28S RT (U34, U2590), GAC TCT CTC
TAC CTA; and m28S RT (U35a, C4188), AAC CTG TCT
CAC GAC G. qPCR was done on a 7500 fast real-time PCR
system with SYBR Green real-time PCR master mix (Thermo
Fisher Scientific). PCR primers used were as follows: m18S F2
(U32a/33), GCC CGG CTT AAT TTG AC; m18S R2 (U32/33),
ATG CAC CAC CAC CCA CG; m28S F1 (U34), TCT
CGC TGG CCC TTG AAA AT; m28S R1 (U34), ACC TAC
ATT GTT CCA ACA TGC C; m28S F1 (U35a), CTT CGA TGT
CGG CTC TTC CT; m28S R1 (U35a), TCA CGA CGG TCT
AAA CCC AG. RT efficiency was calculated by normalizing the
Ct value for the low dNTP concentration to the high as an endog-
enous control (RQ). For each modification site within a specific
tissue, values are reported relative to the mean WT value.

Author contributions—J. M. R. and J. E. S. designed the research
with input from P. I. H. and D. S. O.; J. M. R. and R. J. C. performed
experiments; J. L. and C. L. H. contributed critical reagents and
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Long-range function of secreted snoRNAs


33. Long-range function of secreted snoRNAs


Long-range function of secreted small nucleolar RNAs that direct 2′-O-methylation
Jamie M. Rimer, Jiyeon Lee, Christopher L. Holley, Robert J. Crowder, Delphine L. Chen, Phyllis I. Hanson, Daniel S. Ory and Jean E. Schaffer

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