High-density lipoproteins induce miR-223-3p biogenesis and export from myeloid cells-role of scavenger receptor BI-mediated lipid transfer

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Supplementary Methods:

***Preparation of Reconstituted HDL*:**

Discoidal reconstituted HDL (rHDL) containing apoA-I and 1-palmitoyl-2-linoleoyl phosphatidylcholine (PLPC) (Avanti Polar Lipids, Alabaster, AL) (initial PLPC:apoA-I molar ratio 100:1, final PLPC:apoA-I molar ratio 80:1) were prepared by cholate dialysis method(1). Particle sizes were determined by electrophoresis on 3-40% non-denaturing polyacrylamide gradient gels with Coomassie staining to high molecular weight standards of known diameter(2).

***Messenger RNA quantification and Primers Specificity*:**

For mRNA quantification, total RNA was isolated from PMNs or HMDMs using a miRNeasy mini kit (Qiagen). Reverse transcription was performed using 0.1 µg of RNA. Real-time PCR was performed with MyiQ single colour real-time PCR detection system, using iQ SYBR Green Supermix (Bio-Rad) for relative mRNA quantification of human SR-BI (Primers: Forward: 5'-GCACACTCCTTGTTCCTGG -3' and Reverse 5'- GTCCAATGCCTGCGACAGAT -3'), human Dicer (Primers: Forward: 5'- ACAAACAGATGGAAGCAGAA -3' and Reverse 5'-CAAATCAAAACGAACCACCA -3') or human beta actin (Primers: Forward: 5'-AACTACCTTC AACTCCATCA -3' and Reverse 5'- TGTCCACCTT CCAGCAGAT -3'). All real-time PCR values were expressed as $2^{-(CT[Gene\ of\ Interest]-CT[beta\text{-}actin])}$.

Agarose gel electrophoresis was used to demonstrate the specificity and purity of the SR-BI primers. Human macrophages cDNA samples were amplified (25, 30 and 35 PCR cycles) and visualized on a 2% agarose gel. Amplicon size was confirmed at 105 bp.

***Superoxide Anion Generation*:**

Superoxide anion generation was measured with Superoxide Anion Assay Kit (CS1000, Sigma-Aldrich). Briefly, PMNs were incubated with either PBS or fMLP (1 μM) for 45 min at 37° C. Superoxide anion production oxidized luminol and the chemiluminescence was measured using the EnSpire multimode plate reader (PerkinElmer).

***Protein Kinase C Activity*:**

PMNs were incubated with PBS or fMLP (1 μM) for 45 min at 37°C. Cells were then centrifuged at 2,000 rpm for 5 min and the cell pellets were washed with ice-cold PBS. Cell pellets were re-suspended in cell lysis buffer (20 mmol/l Tris [pH 7.5], 0.5 mmol/l EDTA, 0.5 mmol/l EGTA, Triton X-100 [0.5%] and protease inhibitor [1:1000]) and incubated on ice for 10 min. Cell lysates were sonicated (3 x 20 seconds) and centrifuged at 13,000 rpm for 15 minutes. The supernatant was collected and the protein concentration was measured and normalised to 1 mg/ml for all samples. A PKC Kinase Activity Kit (ab139437, Abcam, Australia) was used to measure the activity of all PKC isoforms in each sample with active PKC as a positive control, according to manufacturer's instructions.

***Western Blotting*:**

Proteins were extracted from Hela, HEK, Jurkat, plasma and DGUC-HDL in protein extraction buffer (50 mM HEPES, 150 mM NaCl, 0.2 mM EDTA, 2 mM $MgCl_2$, 10% glycerol, 1% triton, protease inhibitors). 25 μg of protein was loaded onto a 4-12% NuPAGE Bis-Tris polyacrylamide gels (Life Technologies), then transferred to a nitrocellulose membrane and confirmed with Ponceau staining. Western blotting was performed using primary antibodies against apoA-I (1:8000; Meridian Life Science), Alix (1:500; Cell Signaling), HSP70 (1:200;

Santa Cruz), TSG101 (1:500; Abcam), and flotillin-1 (1:1000; BD Bioscience). Membranes were washed, incubated with goat anti-rabbit IR Dye 800nm or goat anti-mouse IR Dye 680nm (Licor), and imaged with the Odyssey system.

Supplementary Figures and Legends:

Figure S1

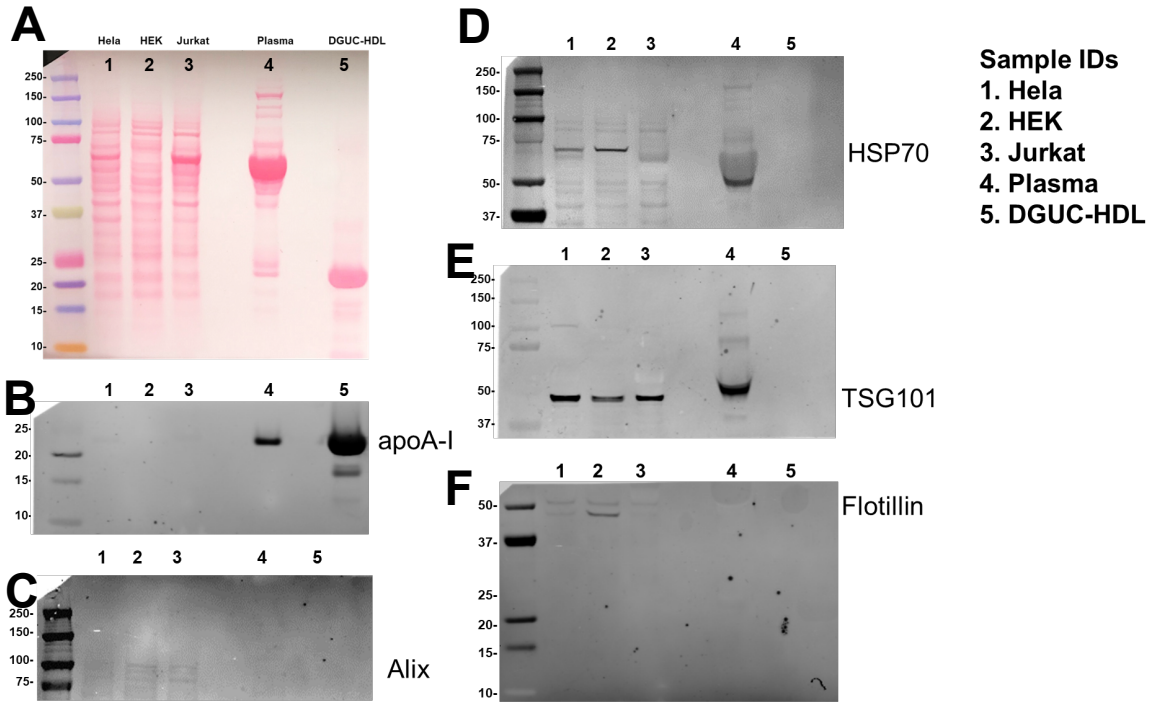

Figure S1. Immunoblotting of extracellular vesicles markers in HeLa cells, HEK cells, Jurkat cells, whole plasma and native HDL protein purified by DGUC (DGUC-HDL). **(A)** Ponceau staining of the nitrocellulose membrane confirming protein transfer. Immunoblotting for apoA-I **(B)**, Alix **(C)**, HSP70 **(D)**, TSG101 **(E)**, Flotilin-1 **(F)** in HeLa cells, HEK cells, Jurkat cells, whole plasma and DGUC-HDL.

Figure S2

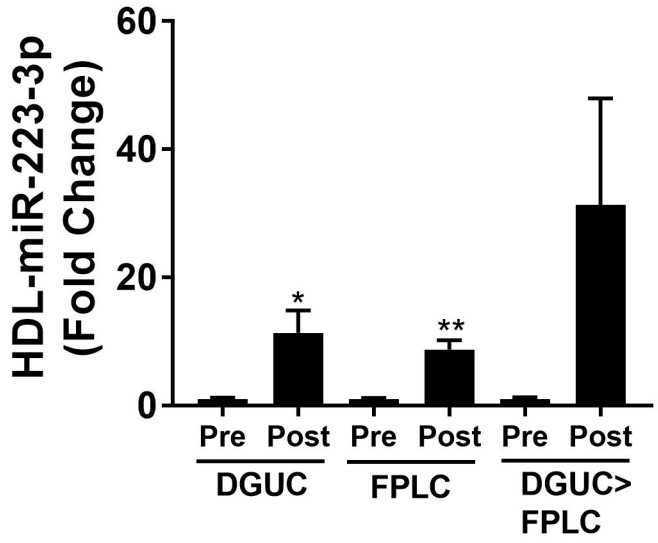

Figure S2. HDL-associated miR-223-3p levels prior and following 4 h incubation with PMNs (1 mg/ml;). HDL were isolated from plasma by either density-gradient ultracentrifugation (DGUC), fast-protein liquid chromatography (FPLC) or DGUC followed by FPLC (DGUC>FPLC).

Figure S3

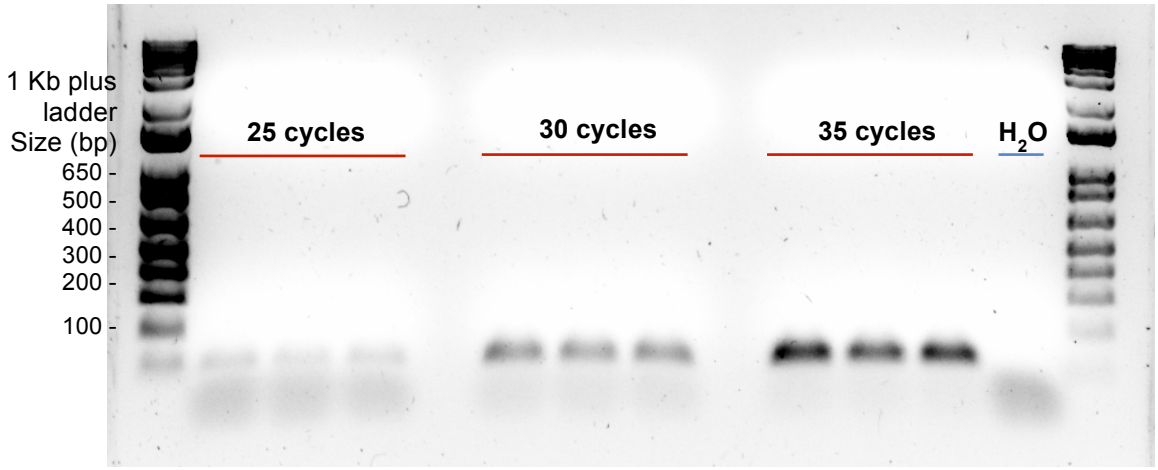

Figure S3. Specificity and purity of the SR-BI primers. Human macrophages cDNA amplicons were run on a 2% agarose gel. Amplicon size was confirmed at 105 bp.

References

1. Matz, C. E., and Jonas, A. (1982) Micellar complexes of human apolipoprotein A-I with phosphatidylcholines and cholesterol prepared from cholate-lipid dispersions. *The Journal of biological chemistry* **257**, 4535-4540
2. Rainwater, D. L., Andres, D. W., Ford, A. L., Lowe, F., Blanche, P. J., and Krauss, R. M. (1992) Production of polyacrylamide gradient gels for the electrophoretic resolution of lipoproteins. *Journal of lipid research* **33**, 1876-1881