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Circulating Microvesicles Contain Critical Elements of the RISC Complex

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Abstract

Circulating microvesicles (cMV) are small, membrane-bound structures that are shed from cells and are thought to be involved in intracellular communication. It has been discovered that cMVs contain microRNAs (miRs), which are short RNA molecules known to regulate gene expression. In cells, miRs must be bound to an Argonaute (Ago) protein as part of the RNA-Induced Silencing Complex (RISC) in order to regulate mRNA translation. The protein GW182 is a functional partner of Ago, and is another important component of some types of RISC complexes. We investigate here whether miRs present in cMV are bound to Ago protein as a RISC complex, and whether GW182 is associated with Ago and cMV from human plasma and cultured cells.

First, we investigated whether RISC is present on the outside or inside of cMV. In order to determine this, we performed an immunoprecipitation of the proteins Argonaute 2 (Ago2) and CD81 (a cMV-specific marker) from purified cMV from cells in culture. Then, copy numbers for let-7a and miR-16 were determined from anti-Ago2 and anti-CD81 precipitates under both native and lysed conditions. We hypothesize that if Ago2 is bound on the outside of the cMV, then an immunoprecipitation with Ago2 will capture as many miRs as an immunoprecipitation with CD81. Our data demonstrates that under non-lysed conditions, the vast majority of these two miRs were found in the CD81 positive population, with minimal amounts in the Ago2 positive population. However, upon lysis the proportions reversed, and most of the miR was associated with Ago2. These results show that these miRs are loaded into Ago2 on the inside of exosomes. Therefore it is likely that following endocytosis of exosomes by recipient cells, these Ago2-miR complexes will be immediately functional and able to inhibit translation of the complementary mRNA in the absence of any RISC-loading requirements.

Next, we investigated the relationship of GW182 with Ago2 and cMV in human plasma. Antibodies directed toward Ago2 and GW182 were used to immunoprecipitate the proteins from plasma. Western blot analysis revealed that GW182 and Argonaute co-precipitate, suggesting that these two proteins retain their functional relationship in plasma. RNA was then isolated from the immunoprecipitates for miR detection and copy-number analysis. The GW182-associated miR profile from human plasma contained individual miRs whose abundance either equaled or surpassed that of their matched Ago2 immunoprecipitated miRs. This implies that GW182 maintains an association with the family of Argonaute proteins and a subset of cMV in human plasma.

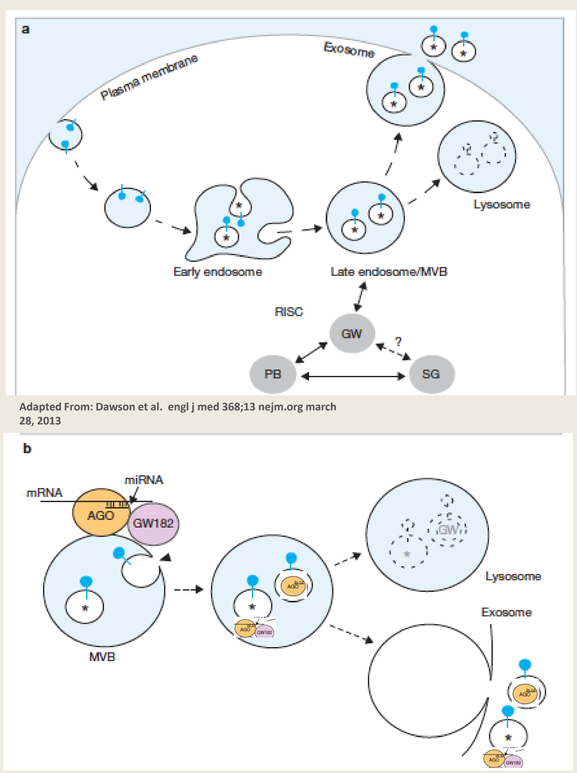


Figure 1. The RISC complex is formed in the MVB where exosomes are formed

Methods

Whole Exosomes vs. Lysed Exosomes Immunoprecipitations (IPs)

Exosomes were prepared from Vcap, LNcap and 22rv1 cell lines by ultracentrifugation. Exosomes were measured by BCA and equal total protein amounts were added for both IPs. Magnabind beads with pre-conjugated α-mouse IgG antibody incubated for 1 hour with either α-Ago2 monoclonal antibody (Abcam), α-CD81 monoclonal antibody (BD Biosciences), or α-BrdU monoclonal antibody (Invitrogen) and mouse normal IgG (Santa Cruz) as negative control. Unbound antibodies were washed with PBS + 1% BSA. Whole exosomes or the corresponding exosome lysates were added to the beads and incubated for 1 hour at RT.

Whole Exosome IP

Beads were washed with mild buffer: PBS pH 7.4 + 1% BSA.

Lysed Exosome IP

Prior to IP reaction, exosomes were lysed by lysis buffer: 20 mM HEPES pH 7.9, 10 mM NaCl, 1 mM $MgCl_2$, 0.5 M sucrose, 0.2 mM EDTA, 0.5 mM DTT, 0.35% Triton X100 (v/v) and protease inhibitor tablet (1 tablet/50 ml lysis buffer, Roche).

After incubation with antibody-bound beads, samples were washed with stringent buffer: Tris-HCl pH 7.5, 1% NP-40, 1% BSA, 1 mM EDTA and 300 mM NaCl.

Ago2 Plasma IP

The exosome lysates IP protocol was followed, but neat plasma was used in lieu of lysates.

RNA was extracted from all IP methods by TrizolLS (Invitrogen). TaqMan® miR analysis was performed according to the manufacturer (Applied Biosystems).

Results

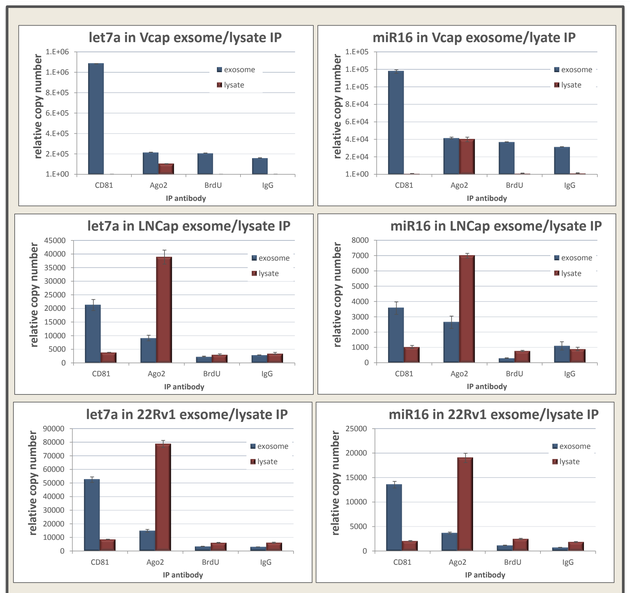


Figure 2. microRNA found inside exosomes, and Ago2 found inside exosomes are microRNA-loaded. Exosomes from 3 cell lines were tested by whole exosome IP and exosome lysates IP with α-CD81 (exosome surface marker), α-Ago2, α-BrdU and mouse normal IgG. α-CD81 IP with whole exosomes had greater miR recovery compared to α-Ago2 IP in all three cell lines; miR recovery using α-Ago2 antibody is similar to the negative control, indicating the miRs detected were from inside the exosomes. α-CD81 IP with lysed exosomes showed less miR recovery, while α-Ago2 IP showed much higher miR recovery compared to α-CD81, α-BrdU and mouse normal IgG IPs. α-CD81 IP miR recovery is similar to the negative control IP using BrdU antibody and mouse normal IgG, showing CD81 is an exosome surface mark and it is not a miR-interacting protein, suggesting the Ago2 inside the exosome is miR-loaded.

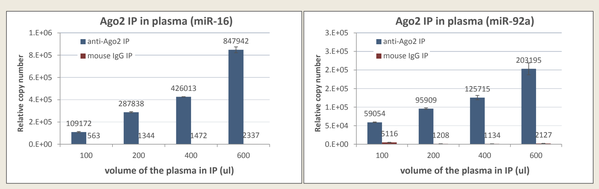


Figure 3. Ago2 is present in neat plasma and is miR-loaded. Ago2 was immunoprecipitated from various volumes of neat. Mouse normal IgG was used as a negative control. Detection of miRs16 and 92a were dependent upon plasma volume input, mouse IgG miR recovery is far less than Ago2 IP, suggesting Ago2 exists naturally in plasma and is miR-loaded.

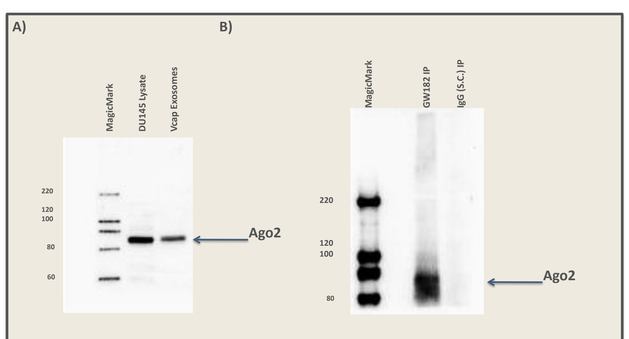


Figure 4. Ago2 co-precipitates with GW182 in human plasma. A) Western analysis for Ago2 in Du145 lysate and purified VCaP exosomes. B) IP of GW182 from human plasma reveals co-immunoprecipitation of Ago2 by Western blot.

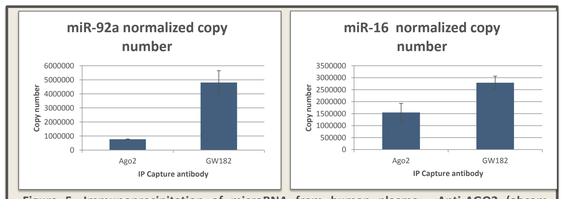


Figure 5. Immunoprecipitation of microRNA from human plasma. Anti-AGO2 (abcam, ab57113, lot GR29117-1), GW182 (Bethyl Labs, A302-330A) and IgG (Santa Cruz sc-2025) were conjugated to Magnabind protein G beads (Thermo Scientific _Cat. # 21349). Conjugated beads were incubated with human plasma. RNA was isolated and screened for select microRNAs (miR-16 and miR-92a) using ABI Taqman detection kits (ABI_391 and ABI_431), respectively. RNA was quantified against synthetic standards and normalized to IgG control.

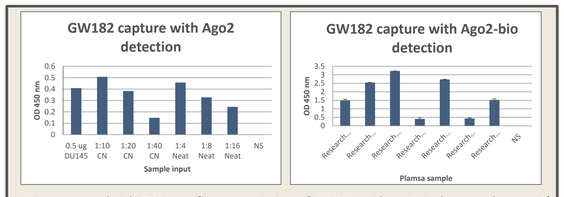
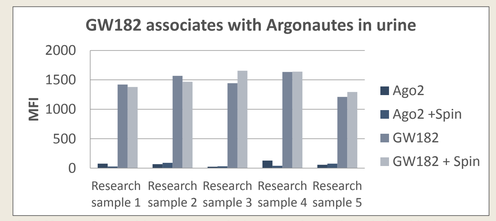


Figure 6. Sandwich ELISA confirms association of GW182 with Ago2 in human plasma. A) Titration of sample input using purified microvesicles and raw plasma by plate-based ELISA using anti-GW182 as a capture (GW182 (Bethyl Labs, A302-330A) and biotinylated anti-Ago2 (abcam, ab57113, lot GR29117-1) as a detector. Signal normalized to no sample control. B) A survey of seven research samples reveals variable levels of GW182:Ago2 binding in human plasma. Signal normalized to no sample control.



GW182 associates with Argonautes in human urine. The relationship between human GW182 and the Argonaute family of proteins was investigated in urine using Luminex. GW182 capture followed by Pan Argonaute detection was tested across five research samples. Conditions included raw vs cell + hard spun urine.

The presence of Argonaute 2 was confirmed in purified VCaP exosomes by Western blot. Precipitation of GW182 from human plasma revealed an association with Ago2 by Western analysis. RNA was isolated from samples following IP from human plasma using either anti-Ago2 or anti-GW182. The copy number of known circulating miRNAs was comparable across the IPs.

A plate-based ELISA was developed to evaluate the relationship of GW182 and Argonaute proteins in biological fluids. A signal that titrated with input was observed when GW182 was used as capture followed by Ago2 detection in either raw plasma or concentrated cMV from plasma. Additional research sample were surveyed using the plate ELISA strategy. The levels of GW182:Ago2 positive particles varied dramatically across the sample set. Lastly, an association of GW182 and the Argonaute family of proteins was confirmed across five urine samples using Luminex technology.

Conclusions

GW182 and Ago2 IP revealed a strong IP of circulating RNA. Both miR-16 and miR-92a were enriched in Ago2 and GW182 IPs. This is the first observation of the use of GW182 for the purpose of surveying miRNAs from human plasma and urine. The potential source(s) of miRNA from human plasma and urine include microvesicles/exosomes and/or circulating Ago2-bound ribonucleoprotein complexes (RNP). This evidence suggests that GW182 may be a useful tool in the identification of a unique subpopulation of biomarkers in biological fluids.