Extracellular presence/release of galectins from HTR-8/SVneo extravillous trophoblast cells*

Danica ĆUJIĆ1,*, Maja KOSANOVIĆ2,*, Milica JOVANOVIĆ KRIVOKUĆA1,*, Ljiljana VIĆOVAC1, Miroslava JANKOVIĆ2

1Department of Biology of Reproduction, Institute for the Application of Nuclear Energy (INEP), University of Belgrade, Belgrade, Serbia
2Department of Immunochemistry and Glycobiology, Institute for the Application of Nuclear Energy (INEP), University of Belgrade, Belgrade, Serbia

Received: 06.04.2017 • Accepted/Published Online: 21.08.2017 • Final Version: 10.11.2017

Abstract: Galectins (gals) are β-galactoside binding lectins, involved in many processes at the fetomaternal interface where they can exert their roles both in and out of cells. The aim of this work was to explore the extracellular presence/release of HTR-8/SVneo extravillous trophoblast cell line galectins. To that end, conditioned medium (CM) from HTR-8/SVneo cell culture was fractionated into a high molecular mass fraction (HMF) and low molecular mass fraction (LMF) using 100 kDa cut-off concentrators. In addition, extracellular vesicles (EVs) were isolated from CM by ultracentrifugation. Size and shape of the EVs were analyzed by transmission electron microscopy and their galectin mRNA content was determined by real-time PCR. The presence of galectins in fractions of HTR-8/SVneo CM and EVs was detected by western blot. All three galectins expressed by HTR-8/SVneo cells (gal-1, gal-3, and gal-8) were detected in the CM, HMF, and EVs, while only gal-1 was found in the LMF. In addition, EVs contained all three galectin mRNAs. These results reveal that free, complexed, and EV-associated forms of galectins were released from extravillous trophoblast cells, suggesting their potential to exert extracellular functions both in their immediate vicinity at the fetomaternal interface and distant locations.

Key words: Galectin, trophoblast, HTR-8/SVneo, extracellular vesicles

1. Introduction
Galectins (gals) are β-galactoside binding lectins, involved in many processes important for cell functioning and survival (Perillo et al., 1998). Galectins manifest their roles both inside and outside of cells and are also present in the extracellular matrix (Compagno et al., 2014). Most galectins described in humans are present at the fetomaternal interface (Than et al., 2012). Three members of the galectin family are found in extravillous trophoblast (EVT): gal-1, gal-3 (Maquoi et al., 1997; Vićovac et al., 1998), and gal-8 (Kolundzic et al., 2011a). In EVT, gals may play multiple roles, possibly in cell adhesion and migration, as shown for gal-1 in EVT in vitro (Kolundzic et al., 2011b). Gal-1 has been also associated with different pregnancy disorders such as spontaneous abortion (Ramhorst et al., 2012), preeclampsia (Than et al., 2008), and gestational diabetes mellitus (Blois et al., 2014).

Extracellular vesicles (EVs) are nano-sized (30–150 nm) membrane-enclosed vesicles released from all cells. They are formed within multivesicular bodies and released upon their fusion with plasma membrane (exosomes) or by budding from the plasma membrane (microvesicles) (Thery et al., 2002). EVs have a pivotal role in intercellular communication, both between neighboring and distant cells (Compagno et al., 2014). EVs have been associated with most (patho)physiological processes studied so far (Vlassov et al., 2012; Record, 2014). EVs carry different molecular messengers: proteins, RNA (mRNA and microRNA), lipids, and small molecules (Valadi et al., 2007; Baig et al., 2014).

Gal-1, gal-3, and gal-8 have been detected in conditioned medium (CM) of trophoblast cell lines (Yang et al., 2011; Ćujić et al., 2013; Kolundzic et al., 2015). As gals lack a signal sequence, questions pertinent to the route of gal secretion in trophoblasts remain unresolved (Hughes, 1999). Among several mechanisms suggested, the involvement of EVs was proposed, since these lectins have been found in EVs from different sources (Klibi et al., 2009; Barres et al., 2010; Welton et al., 2010; Block et al., 2011).

Since gals have been proposed to have important functions in early pregnancy, we aimed to study released forms of gals using the HTR-8/SVneo EVT cell line as an important model in trophoblast research, with special
focus on their presence in EVs at both the protein and mRNA levels.

In order to separate different forms of released galectins, CM was fractionated into a high molecular mass fraction (HMF) and a low molecular mass fraction (LMF) using a 100 kDa cut-off. EVs were isolated from unfractionated CM and the presence of gal-1, gal-3, and gal-8 was detected in all three sample types.

2. Materials and methods

2.1. Cell culture

The HTR-8/SVneo EVT cell line was kindly provided by Dr Charles H Graham (Queen's University, Ontario, Canada). HTR-8/SVneo cells were cultured in RPMI 1640 medium (Lonza Group Ltd., Basel, Switzerland), supplemented with 5% (v/v) fetal bovine serum (Sigma-Aldrich, St Louis, MO, USA) at 37 °C and 5% CO₂. For EV isolation and gals analysis in CM, 4 × 10⁶ cells were seeded in T175 flasks and cultured for 96 h; that is close to confluence (approximately 80%). After washing twice with 0.05 M PBS (pH 7.2), cells were grown in serum-free medium for another 24 h prior to collection of CM, which was used for both fractionation and EV isolation.

2.2. Fractionation of conditioned medium

HTR-8/SVneo cell CM was separated into two fractions by ultrafiltration using 100 kDa cut-off concentrators (Millipore, Billerica, MA, USA). The 100 kDa concentrator retentate was designated as the HMF, while the respective ultrafiltrate was additionally concentrated using a 10 kDa cut-off concentrator, and the retentate was marked as the LMF. Unfractionated CM as well as the HMF and LMF were examined for the presence of gal-1, gal-3, and gal-8, as well as CD63 as an EV marker.

2.3. Isolation of EVs

EVs were isolated by differential centrifugation. Cells and cell debris were removed from CM by successive centrifugations at 300 × g for 15 min, 3000 × g for 20 min, and 17,000 × g for 25 min. The last supernatant was filtered through a 0.22 µm filter and ultracentrifuged at 100,000 × g for 2 h at 20 °C (Optima L-90K ultracentrifuge, Ti 50.2 rotor; Beckman Coulter, Brea, CA, USA; k factor = 157.7.). The pellet was resuspended in PBS and washed with six volumes of the same buffer. Total protein content in EV suspension was determined using the BCA Protein Quantification Kit (Abcam, Cambridge, UK). The EV suspension was stored at −80 °C until use.

2.4. Transmission electron microscopy (TEM)

EVs (10 µL) were applied to Formvar-coated copper grids by floating the grids on droplets of sample for 10 min at room temperature (RT). After excess fluid removal, grids were incubated for 10 min on droplets of 2.5% glutaraldehyde at RT to fix the adsorbed EVs. Glutaraldehyde was removed and the grids were allowed to air dry. Electron micrographs were obtained using a Philips CM12 electron microscope (Philips, Eindhoven, the Netherlands).

2.5. SDS-PAGE and western blot

Unfractionated CM, as well as HMF and LMF fractions and EV samples (8 µg of proteins per lane), were separated on 10% SDS-PAGE gel under reducing conditions on a Mini-PROTEAN 3 Cell (Bio-Rad Laboratories, Hercules, CA, USA). Subsequently, separated proteins were transferred to nitrocellulose membrane by semidy transfer (Trans-Blot SD Semi-Dry Transfer Cell, Bio-Rad Laboratories). After blocking in 1% casein-PBS (w/v) for 1 h at room temperature, membranes were probed with mouse anti-CD63 antibody (20 µg/mL; clone TS63, Abcam) or goat anti-gal-1, -3, and -8 antibodies (0.5 µg/mL; R&D Systems, Minneapolis, MN, USA) overnight at 4 °C with shaking. Membranes were further incubated with biotinylated goat antimouse IgG antibody (for CD63 detection) or biotinylated rabbit antigoat IgG antibody (for gal detection) (Vector Laboratories, Burlingame, CA, USA), followed by incubation with avidin-biotin peroxidase complex (ABC Elite Vectastain kit, Vector Laboratories). The reaction was visualized using Pierce ECL western blot substrate (Thermo Fisher Scientific, Rockford, IL, USA). Controls omitting the primary antibody were performed.

As a positive control for CD63 western blot, urinary EVs were used, isolated as described previously (Kosanovic and Jankovic, 2014). Previous studies have found these EVs to be CD63-positive (http://microvesicles.org/gene_summary?gene_id=967).

2.6. RNA isolation from EVs and gals specific mRNA analysis

Following ultracentrifugation at 100,000 × g, the EV pellet was resuspended in PBS and treated with RNase (Life Technologies, Carlsbad, CA, USA) for 30 min at 37 °C in order to remove any non-EV RNA. The reaction was stopped by adding 1 U/µL RNase inhibitor (SUPERase R Nase Inhibitor, Life Technologies). Treated EVs were washed once in PBS at 100,000 × g and the supernatant was carefully removed. EVs were restored in TRIzol (Ambion, Foster City, CA, USA) and stored at −80 °C. Total mRNA was extracted according to the manufacturer’s instruction. In order to minimize loss and maximize RNA detection, the whole amount of isolated mRNA was transcribed into cDNA, using oligo dT primers (Applied Biosystems, Foster City, CA, USA).

Analysis of gene expression was performed by real-time PCR. The reaction mixture contained cDNA, 5 µL of SYBR Green PCR Master Mix (Applied Biosystems), and a specific primer in a final concentration of 0.5 µM. The reaction was performed in a 7500 Real Time PCR System (Applied Biosystems). The sequences of gene-specific primers are listed in the Table. The specificity
of the amplification process was confirmed by melting curve analysis and data analysis was performed with 7500 Software v2.3.

3. Results
Unfractionated CM from the HTR-8/SVneo cell line contained gal-1, gal-3, and gal-8, as revealed by western blot analysis and evidenced as bands of the appropriate molecular mass (Figures 1A–1C). Upon separation into the HMF and LMF using 100 kDa as the cut-off, all examined gals were found within the HMF (Figures 1A–1C), suggesting their corelease in association with other molecules, i.e. in complexes of >100 kDa. Only gal-1 was detected in the LMF (Figure 1A). Under the given experimental conditions, EVs were not detectable in unfractionated CM or in HMF (before isolation), as evidenced by the absence of staining for EV marker CD63 (Figure 1D). This finding indicates that the gals detected in the unfractionated CM and HMF were of non-EV origin.

Size and shape of EVs derived from HTR-8/SVneo cells were determined by TEM. Isolated EVs were spherical and ranged from 25 to 60 nm in diameter (Figure 2A). The presence of EVs was additionally verified by detection of the vesicular marker CD63 using western blot (Figure 2B). All three studied gals were detected in isolated EVs by western blot as bands at their respective molecular masses: 14 kDa for gal-1, 29 kDa for gal-3, and 38 kDa for gal-8 (Figure 2B).

Real-time PCR analysis of EV mRNA revealed that, in addition to proteins, mRNA for all three studied gals was present in the EV cargo (Figure 3).

4. Discussion
The data presented show that all three analyzed gals were released from HTR-8/SVneo cells, as assessed by western blot of different fractions of CM. Our group previously demonstrated that gal-1 is secreted by HTR-8/SVneo cells (Čujić et al., 2013) and the presence of all three galectins in CM of the choriocarcinoma cell line JAr (Kolundzic et al., 2015). Secreted gal-3 was detected in CM from another choriocarcinoma cell line, BeWo (Yang et al., 2014). Our detection of gal-3 and gal-8 in CM of HTR-8/SVneo cells is the first report on the release of these gals from a normal EVT-derived cell line. Among gals studied here, only gal-1 was found in LMF, suggesting its presence in free/uncomplexed form in CM. This finding indicates the availability of gal-1 for binding to ligands present on both secreting and neighboring cells, as well as components of extracellular matrix.

### Table. Primer sequences used for real-time PCR analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>gal-1 (LGALS1)</td>
<td>F: 5'-TGCAACAGCAAGGACGGC-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CACCTCTGCAACACTTCCA-3'</td>
</tr>
<tr>
<td>gal-3 (LGALS3)</td>
<td>F: 5'-CAGAATTGCTTTAGATTCCAA-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-TTATCCAGCTTTGTATTGCAA-3'</td>
</tr>
<tr>
<td>gal-8 (LGALS8)</td>
<td>F: 5'-CTTAGGCTGCCATTCGCT-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-AAGCTTTTGGCATTGCA-3'</td>
</tr>
</tbody>
</table>

![Figure 1](image-url) Western blot analysis of galectins present in HTR-8/SVneo conditioned medium. Conditioned medium (CM) of HTR-8/SVneo cells was separated in a high molecular mass fraction (HMF) and low molecular mass fraction (LMF) using 100 kDa cut-off concentrator. The presence of galectin-1 (A: gal-1), galectin-3 (B: gal-3), and galectin-8 (C: gal-8) was detected by western blot in the CM, HMF, and LMF. EVs could not be detected in either CM, HMF, or LMF, as shown by the lack of CD63 reactivity (D). Numbers and arrowheads indicate positions of molecular mass standards. Representatives of three experimental replicates are shown.
On the other hand, all three gals were found in the HMF, indicative of their presence in molecular complexes. In a Chinese hamster ovary cell model, gal-1 was secreted in complex with other proteins (Seelenmeyer et al., 2005). In the present study, gals found in HMF were not associated with EVs, which were not detectable, as confirmed by the lack of the CD63 EV marker in CM fractions.

Hence, EVs were isolated and separately analyzed for the presence of galectins. All three studied gals were detected in EVs derived from HTR-8/SVneo cells at both the mRNA and protein levels. Notably, the galectin profile of EVs was the same as that of parental cells (Kolundzic et al., 2011b). Generally, gals are found in EVs from different cell types. The present results for gal-1 are in accordance with previous reports for HTR-8/SVneo cells (Salomon et al., 2014) and another trophoblast cell line, SWAN71 (Atay et al., 2011). The findings presented here are novel for gal-3 and gal-8, since no literature data regarding their presence in EVs of trophoblast origin have been reported so far. However, both gal-3 and gal-8 were found in EVs from different cell types or body fluids (Ogawa et al., 2008; Wang et al., 2012; Webber et al., 2014; Hurwitz et al., 2016).

Additionally, EVs from HTR-8/SVneo cells contained all three gal mRNAs. The galectin mRNA profile was comparable to that of parental HTR-8/SVneo cells (Kolundzic et al., 2011b). Valadi et al. (2007) showed that EVs may contain both mRNA and microRNA, which can be delivered to other cells and be functional in this new location. To our knowledge this is the first report on the presence of galectin mRNAs in trophoblast-derived EVs. However, mRNAs for all three gals were found in glioblastoma (Skog et al., 2008) and colorectal cancer cells (Hurwitz et al., 2016). The presence of gal mRNAs in EVs could indicate EVT cells’ potential to contribute to gal synthesis in target cells with increased need for these lectins.

These findings, along with the literature data, raise a question regarding the functional relevance of gals associated with EVs in human trophoblast. It is widely accepted that both gals (Than et al., 2012; Blois and Barrientos, 2014) and EVs (Redman and Sargent, 2008; Mincheva-Nilsson and Baranov, 2014) serve as important mediators in early pregnancy events. It is of interest to note that EVT cell invasion is increased by both gal-1 (Kolundzic et al., 2011b) and EVs from cytotrophoblast cells grown in a hypoxic environment (Salomon et al., 2013). Both gal-1 (Blois et al., 2007; Tirado-Gonzalez et al., 2013) and
placental EVs (Stenqvist et al., 2013; Mincheva-Nilsson and Baranov, 2014; Tannetta et al., 2014) are involved in immunoregulation during pregnancy. As for gal-3 and gal-8, their role in trophoblasts is still unresolved.

Taken together, these results reveal that the gals released from EVT cells are free, complexed, and EV-associated, suggesting a possibility of their multiple functions in cellular communication at the fetomaternal interface. Presence of gals, as important players in pregnancy-related processes, in trophoblast-derived EVs may indicate their role not only in immediate surroundings but also in interactions with distant targets, and merits further investigation.

Acknowledgment
This work was supported by the Ministry of Education, Science, and Technological Development of the Republic of Serbia, projects #173010 and #173004.

References


