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Comparative analysis of galectin-1, galectin-3, and galectin-8 signatures in normal and choriocarcinoma trophoblast cell lines

Milica Jovanović Krivokuća[1], Janko Legner[1], Danica Ćujić[1], Aleksandra Vilotić[1], Ivana Grubiša[2], Ljiljana Vićovac[1], and Žanka Bojić-Trbojević[1,*]

[1]*Department for the Biology of Reproduction, Institute for the Application of Nuclear Energy, INEP, University of Belgrade*, Belgrade, Serbia

[2]*Department of Human Genetics, Zvezdara University Medical Center, University of Belgrade*, Belgrade, Serbia

***Corresponding author**: zana@inep.co.rs

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Abstract: Galectins are found in the placenta during both normal and pathological conditions. Specifically, galectins -1, -3, and -8 have been identified in extravillous trophoblast cells, with galectins -1, and -3 playing notable roles in trophoblast cell function. Altered expression profiles of galectins are linked to various cancers. This study explores the expression and localization of galectins -1, -3, and -8 in a normal extravillous HTR-8/SVneo cell line and two choriocarcinoma cell lines, JAR and JEG-3. Expression levels of these galectins at both mRNA and protein levels were evaluated using PCR, qPCR, and Western blotting, while their cellular localization and distribution were assessed through immunocytochemistry, subcellular fractionation, and Western blotting. Galectins -1, -3, and -8 were expressed at lower levels in JAR and JEG-3 cells compared to HTR-8/SVneo cells. Additionally, different splice variants of galectin-8 were detected across all cell lines. Galectin-1 was predominantly localized at the plasma membrane. Notable differences in galectin-3 localization and distribution were observed between HTR-8/SVneo cells and choriocarcinoma cells. These findings highlight variations in the expression, staining patterns, and subcellular distribution of galectins -1, -3, and -8, suggesting their potential involvement in the pathology of choriocarcinoma.

Keywords: galectin-1, galectin-3, galectin-8, extravillous trophoblast, choriocarcinoma cells

INTRODUCTION

Pregnancy success depends on a dynamic crosstalk between various molecules [1]. Galectins, as glycan-binding proteins, are recognized as modulators of pregnancy-associated processes, such as implantation, angiogenesis, feto-maternal immunotolerance, and placentation [2-4]. Galectins are involved in many biological functions, manifesting their roles inside and outside cells. Members of the galectin family are reported at the feto-maternal interface and at different stages of gestation [5-7]. As in other tissues, expression-profiling studies described the presence of galectins -1 to -3 and galectin -7 to -10 in various placental cell types such as syncytiotrophoblast, cytotrophoblast, and extravillous trophoblast cells [5,8-10], while galectins -13, -14, and -16 were found exclusively in the placenta [6,9]. Our previous studies showed that galectins -1, -3, and -8 are strongly expressed in extravillous trophoblast cells of the first-trimester placenta along the invasion pathway, indicating their involvement in cell-cell and cell-matrix interactions of trophoblast during placentation [5,11,12]. Moreover, galectins -1 and -3 are identified as important molecules involved in the invasion of normal human trophoblasts *in vitro* [11,13], partially through interaction with glycans, whereas galectin-8 in trophoblast cell functioning requires elucidation.

An increasing amount of data provides evidence of dysregulated galectin -1 and -3 expression in connection with pregnancy-related disorders, including early/recurrent fetal loss, preeclampsia, and gestational trophoblastic disease [14-17]. Gestational choriocarcinoma is a highly malignant tumor characterized by abnormal trophoblastic hyperplasia, the absence of chorionic villi, hemorrhage, and necrosis, and it is

composed exclusively of cytotrophoblasts and syncytiotrophoblasts. This type of carcinoma has a high metastatic potential and a pronounced vascular invasion [18]. Choriocarcinoma cells frequently invade the uterus and nearby organs and often spread to distant sites, particularly the lungs, brain, liver, spleen, kidneys, and bowels [18,19]. Molecular mechanisms leading to the pathogenesis of gestational diseases remain largely unknown.

Given the proposed involvement of galectins -1, -3, and -8 in tumorigenesis and metastasis [20-22], we investigated their profiles in normal extravillous trophoblast and choriocarcinoma cell lines under identical experimental conditions.

MATERIALS AND METHODS

Reagents and antibodies

DMEM/F12 medium and fetal bovine serum (FBS) were from Sigma-Aldrich (USA). RPMI 1640 medium was from Gibco (Thermo Scientific, USA); the antibiotic/antimycotic solution was obtained from Capricorn Scientific (Germany). Primers, SYBR® Green PCR MasterMix, RevertAid reverse transcriptase, dNTPs, TRI Reagent Solution, and Oligo(dT) 12-18 primers were from Thermo Scientific (USA). Goat anti-galectin-1 (AF1152), goat anti-galectin-3 (AF1154), and goat-anti-galectin-8 (AF1305) were from R&D (UK); mouse anti-galectin-8 (sc-377133) was from Santa Cruz Biotechnology (USA) and rabbit anti-galectin-1 from Dr. Kadoya (Maebashi Institute of Technology, Japan). Rabbit anti-GAPDH (2118S) and anti-mouse IgG HRP-linked (7076S) were purchased from Cell Signaling Technology (USA). Rabbit anti-β-actin (A5060) was from Sigma-Aldrich (USA). Anti-goat IgG antibody AlexaFluor 488 (A11001) was from Molecular Probes (Thermo Scientific, USA). Biotinylated goat anti-rabbit IgG (BA-1000), biotinylated horse anti-goat IgG (BA-5000), avidin-biotinylated peroxidase complex (ABC), and VECTASHIELD mounting medium with DAPI were from Vector Laboratories (USA).

Cell lines

The normal extravillous trophoblast cell line HTR-8/SVneo was kindly provided by Dr. Charles H. Graham (Queen's University, Kingston, Canada). Choriocarcinoma cell line JAR was from ATCC (USA) and JEG-3 was from ECACC (UK). HTR-8/SVneo and JAR cells were cultured in RPMI 1640, while JEG-3 were cultured in DMEM/F12; both were supplemented with 10% fetal bovine serum (FBS) in a 1% antibiotic/antimycotic mixture. HTR-8/SVneo and JAR cell lines were authenticated by the European Collection of Authenticated Cell Cultures (ECACC) using short tandem repeat analysis. All cell lines tested negative for *Mycoplasma* contamination. For immunocytochemical analysis, the cells were seeded (3×10^5) in 3 mL of respective media into a 65-mm Petri dish, cultured for 48 h on glass cover slips, fixed with ice-cold acetone-methanol (1:1), and kept frozen until staining.

For sodium dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE), HTR-8/SVneo, JAR, and JEG-3 cells were grown in complete media for 24 h, washed with 0.05 M phosphate-buffered saline (PBS), pH 7.2, lysed in sample buffer for SDS-PAGE containing protease inhibitor cocktail, boiled for 5 min, and centrifuged at 17,000×*g* for 10 min at 4 °C. The supernatant was used for electrophoresis.

PCR and quantitative real-time PCR (qPCR)

Total RNA was isolated from HTR-8/SVneo, JAR, and JEG-3 cells after 24 h in culture using TRI Reagent Solution. First-strand cDNA was synthesized from 2 µg (PCR) or 1 µg (qPCR) of total RNA, using 0.5 µg of Oligo(dT) 12-18 primers, 250 µM of each dNTP, and 200 U of RevertAid reverse transcriptase.

PCR was performed using a T100 Thermal Cycler (Bio-Rad Laboratories, USA). Each gene was amplified in a 25-µL reaction mixture containing 1 µL (*LGALS1, GAPDH*) or 2 µL (*LGALS3, LGALS8*) cDNA, 200 µM of each dNTP, 0.625 U of AmpliTaq Gold DNA (Applied Biosystems, USA), and 0.2 µM (*LGALS1, LGALS8, GAPDH*) or 0.3 µM (*LGALS3*) of each primer. The reactions were run at 95°C for 10 min followed by 40 cycles at 95°C for 15 s, at 56°C for 30 s, and at 72°C for 1 min with the final extension step at 72°C for 5 min. PCR products were analyzed electrophoretically on a 1% agarose gel that contained SYBR® Safe DNA gel stain (Thermo Scientific, USA), and visualized by UV light using ChemiDoc MP Imaging System (Bio-Rad Laboratories, USA).

The reaction mixture for qPCR contained 1 μL of cDNA, 5 μL 2×SYBR® Green PCR MasterMix, and specific forward and reverse primers in a final concentration of 0.5 μM. Reactions were run at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C, and for 1 min at 60°C. Melting curve analysis was performed to verify amplification specificity. qPCR was performed using the 7500 Real-Time PCR System (Applied Biosystems, USA). Relative gene expression levels of *LGALS1, LGALS3,* and *LGALS8* were normalized to *GAPDH*. Calculations were made by the comparative $2^{-\Delta Ct}$ method. The sequences of specific primers, as described in a previous study, are given in Supplementary Table S1 [23].

Immunocytochemistry

HTR-8/SVneo, JAR, and JEG-3 cells cultured on glass coverslips were incubated with anti-galectin-1 (1 μg/mL, AF1152), anti-galectin-3 (1 μg/mL, AF1154), and anti-galectin-8 (1 μg/mL, AF1305) antibodies. Staining was visualized with anti-goat AlexaFluor 488. Slides were mounted with VECTASHIELD mounting medium containing DAPI and examined using a Carl Zeiss Axio Imager microscope with an AxioCam HR Camera (Carl Zeiss, Germany).

Subcellular fractionation

Subcellular fractionation of HTR-8/SVneo, JAR, and JEG-3 cells cultured in complete respective media for 24 h was carried out using the Subcellular Protein Fractionation Kit for Cultured Cells (Thermo Scientific, USA), according to the manufacturer's instructions. The protein concentration of the cytosolic, membrane, nuclear soluble, and nuclear chromatin fractions was determined using the BCA Protein Assay kit (Thermo Scientific, USA); equal amounts of protein of each fraction were loaded for SDS-PAGE.

SDS-PAGE and Western blotting

Galectin -1, -3, and -8 protein levels of HTR-8/SVneo, JAR, and JEG-3 whole-cell lysates and subcellular fractions were estimated by Western blotting. Following electrophoresis under reducing and denaturing conditions on 10% (galectin -3 and -8) or 12.5% (galectin-1) polyacrylamide gels, the samples were transferred to membranes and incubated with rabbit anti-galectin-1 (0.9 μg/mL), goat anti-galectin-3 (1 μg/mL), and goat anti-galectin-8 (1 μg/mL) (subcellular fractions) or mouse anti-galectin-8 (0.4 μg/mL, whole cell lysate) overnight at 4°C with constant shaking. Staining for GAPDH (anti-GAPDH 1:12000) was used as the loading control for galectins -1 and -3, while β-actin (anti-β-actin 1:6000) was used as the loading control for galectin-8. Proteins were detected with Pierce ECL Western Blotting Substrate (Pierce Biotechnology, USA). The obtained signals were scanned and analyzed by the ImageMaster TotalLab v2.01 program (Amersham Biosciences, Inc., USA).

Statistical analysis

The data obtained were analyzed with GraphPad Prism Demo software (GraphPad Software, Inc., USA) using one-way analysis of variance (ANOVA) with Tukey's post-hoc test (α=0.05). All experiments were performed at least three times. Values were considered significantly different when $P<0.05$.

RESULTS

Comparative analysis of galectin-1, -3, and -8 expression

The expression of galectins -1, -3, and -8 at mRNA and protein levels was investigated in established trophoblast cell lines, normal extravillous trophoblast HTR-8/SVneo, and choriocarcinoma JAR and JEG-3 cell lines. Under the experimental conditions used herein, all examined trophoblast cells consistently expressed *LGALS1*, *LGALS3*, and *LGALS8* (Fig. 1A, B, C, D). However, trophoblast cell lines differed in the relative abundance for each of the three galectins examined. PCR detected single transcripts of 408 bp for LGALS1 and 753 bp for LGALS3 in all examined cell lines, with the highest expression levels observed in HTR-8/SVneo cells compared to choriocarcinoma cells. (Fig. 1A). For *LGALS8*, two transcripts of 957 bp and 1083 bp were observed in all examined cell lines. Primer blast prediction of PCR products indicated the presence of 2 transcripts of 957 bp (*LGALS8* transcript variants 2 and 3), and 2 transcripts of 1083 bp (*LGALS8* transcript variants 1 and 4). However, it

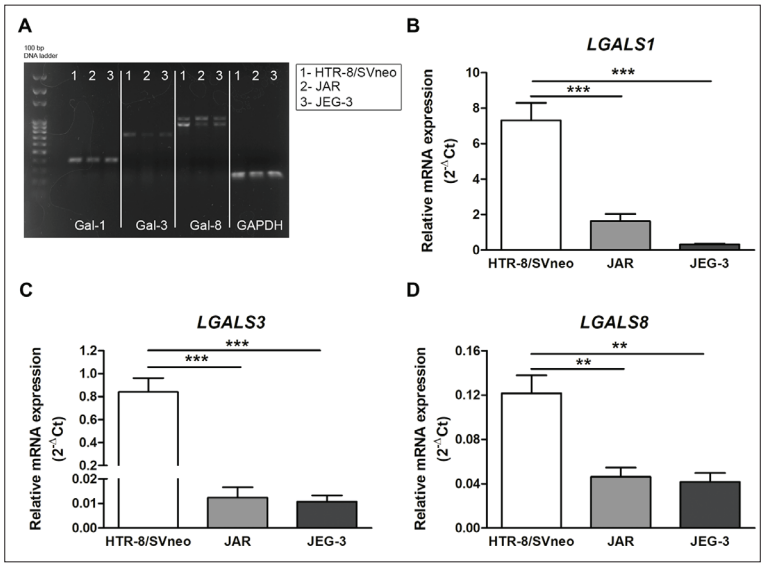

Fig. 1. Expression profiles of *LGALS1*, *LGALS3*, and *LGALS8* in HTR-8/SVneo, JAR, and JEG-3 trophoblast-derived cell lines. Agarose gel electrophoresis following PCR with fulllength primers (**A**). Expression of *LGALS1* (**B**), *LGALS3* (**C**), and *LGALS8* (**D**) as determined by qPCR in HTR-8/SVneo, JAR, and JEG-3 cells. Statistical analysis was performed using one-way ANOVA with Tukey's post-hoc test. Data are expressed as the mean+SEM from three independent experiments, **P<0.01, ***P<0.001; n=6.

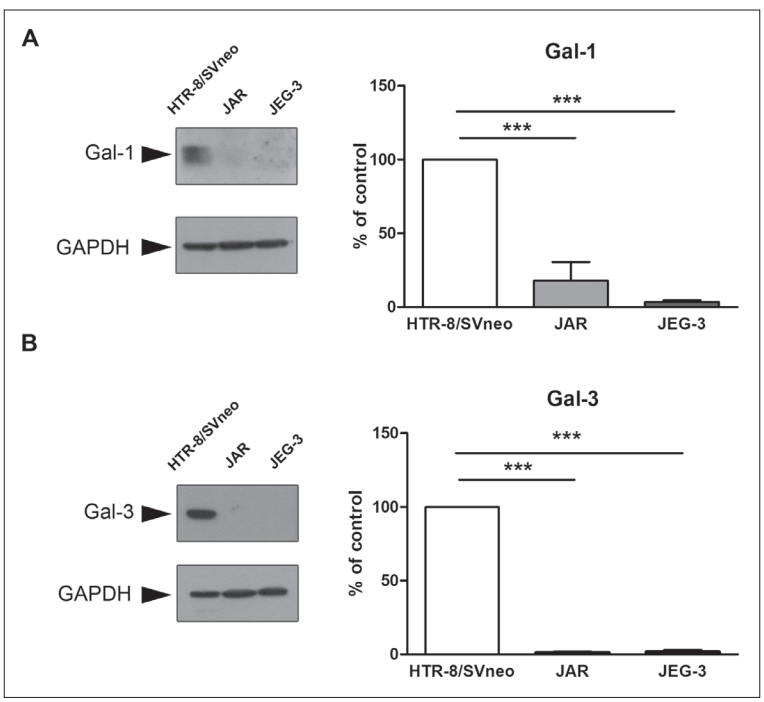

Fig. 2. Galectin-1 and -3 protein levels in extravillous HTR-8/SVneo, JAR, and JEG-3 choriocarcinoma whole cell lysates. Charts show relative expression of galectins compared to HTR-8/SVneo cells, assessed by densitometric analysis following Western blotting (**A**, **B**). Representative Western blots are shown. GAPDH served as a loading control. Statistical analysis was performed using one-way ANOVA with Tukey's post-hoc test. Data are expressed as the mean+SEM, ***P<0.001; n=4 for Gal-1 and n=5 for Gal-3.

was not possible to determine whether one or both transcripts were present for each PCR product length. Interestingly, in HTR-8/SVneo cells, the 957 bp transcript was more abundant, whereas both choriocarcinoma cell lines exhibited higher levels of the 1083 bp transcripts (Fig. 1A).

The results obtained by qPCR analysis confirmed that HTR-8/SVneo cells contained higher levels of *LGALS1*, *LGALS3*, and *LGALS8* than choriocarcinoma cell lines. The relative levels of galectins -1, -3, and -8 mRNA were significantly higher in HTR-8/SVneo cells (7.32 arbitrary units (AU), P<0.001, 0.84 AU, P<0.001, 0.12 AU, P<0.01, respectively) compared to JAR (1.62 AU, 0.01 AU, 0.05 AU, respectively) and JEG-3 cells (0.32 AU, 0.01 AU, 0.04 AU, respectively; Fig. 1 B, C, D).

The expression of galectins -1, -3, and -8 at the protein level was examined by immunoblotting (Figs. 2 and 3). In HTR-8/SVneo, JAR, and JEG-3 lysates, bands corresponding to galectin-1 at 14 kDa, galectin-3 at 30 kDa, and two galectin-8 bands at 34 and 40 kDa were detected. The data regarding the relative content of each galectin corroborated the qPCR data. The level of galectin-1 protein expression in HTR-8/SVneo cells was significantly higher compared to the expression in JAR and JEG-3 cells (18% and 3.6% of the HTR-8/SVneo galectin-1 content, respectively; P<0.001, Fig. 2A). Differences in galectin-3 expression between normal trophoblast and choriocarcinoma cell lines were even more pronounced; relative galectin-3 protein levels in JAR and JEG-3 cells were 1.3% and 1.8% of the HTR-8/SVneo galectin-3 content, respectively (P<0.001, Fig. 2B).

Galectin-8 in all cell lines had two bands, a shorter ~34 kDa form (Gal-8S) and a longer ~40 kDa form (Gal-8L) (Fig. 3A). In line with the PCR results, a significantly higher level of the ~34 kDa isoform in HTR-8/SVneo cells was observed, while levels in JAR and JEG-3 were 35.9% and 19.9% of the HTR-8/SVneo

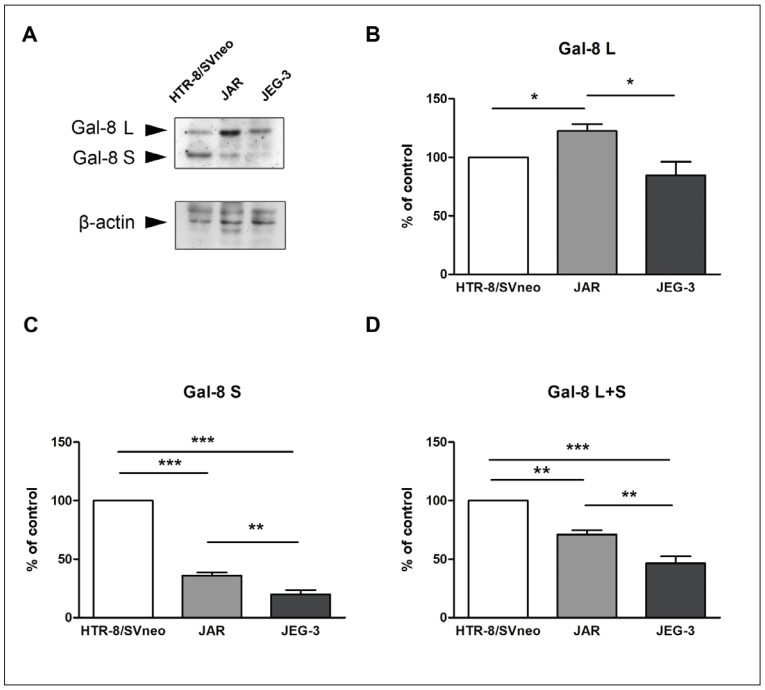

Fig. 3. Galectin-8 expression in extravillous HTR-8/SVneo and choriocarcinoma JAR and JEG-3 whole cell lysates. Galectin-8 expression (**A**, **D**), Gal-8 L (**B**), and Gal-8 S (**C**) represent the relative expression of the lectin compared to HTR-8/SVneo cells, assessed by densitometric analysis. β-actin served as the loading control; representative Western blots are shown in **A**. Statistical analysis was performed using one-way ANOVA with Tukey's post-hoc test. Data are expressed as the mean+SEM, *P < 0.05; **P < 0.01; ***P < 0.001; n=4.

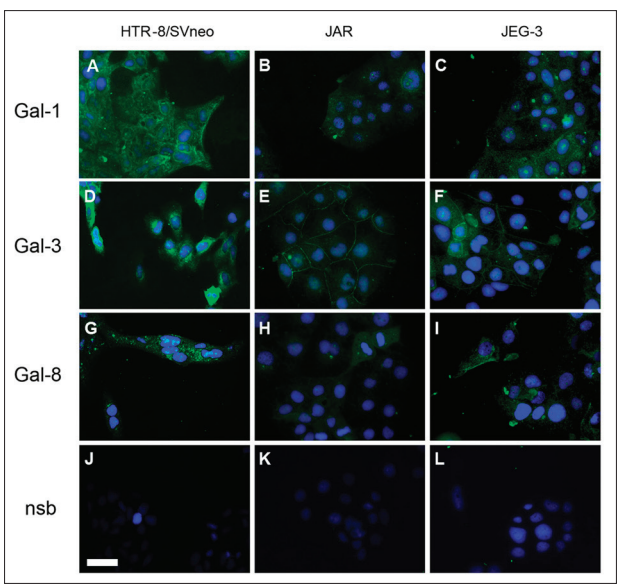

Fig. 4. Immunolocalization of investigated galectins in HTR-8/SVneo, JAR, and JEG-3 cells. Representative images of immunofluorescence staining are shown for galectin-1 (**A**, **B**, **C**), galectin-3 (**D**, **E**, **F**), and galectin-8 (**G**, **H**, **I**). Non-specific binding is shown for all studied cell lines (**J**, **K**, **L**). Nuclei were stained with DAPI. Scale bar represents 10 μm.

level (HTR-8/SVneo *vs* JAR P<0.001; HTR-8/SVneo vs. JEG-3 P<0.001; JAR *vs* JEG-3 P<0.01; Fig. 3C). Gal-8L was significantly higher in JAR (122.5%, P<0.05; Fig. 3B) compared to HTR-8/SVneo and JEG-3 cells (85%, P<0.05; Fig. 3B). However, when the galectin-8 protein levels were compared, significantly lower expression was detected in JAR and JEG-3 cells compared to HTR-8/SVneo cells (71%, P<0.01 and 46.4%, P<0.001, respectively; Fig. 3D). In some experiments, a band of ~50 kDa appeared in investigated cell lysates (not shown). Collectively, the data point to lower galectin -1, -3, and -8 expression in choriocarcinoma JAR and JEG-3 cells than in normal extravillous trophoblast HTR-8/SVneo cells.

Cellular localization and distribution of galectins -1, -3, and -8

Immunocytochemical analysis of galectin -1, -3, and -8 expression in the studied trophoblast cell lines demonstrated differences in staining patterns and intensity (Fig. 4). Strong membrane and intracellular galectin-1 expression in HTR-8/SVneo cells was detected (Fig. 4A). A similar but less intense staining pattern for galectin-1 was observed in JEG-3 cells (Fig. 4C) while in JAR only weak and diffuse cytoplasmic staining was detected (Fig. 4B). Galectin-3 was present at the plasma membrane, and in the cytoplasm and nuclei of HTR-8/SVneo cells (Fig. 4D). In contrast to this finding, galectin-3 was localized at the JAR and JEG-3 cell membrane (Fig. 4E and F). Intracellular and nuclear staining of galectin-3 was either weak or absent in both choriocarcinoma cells. The galectin-8 staining pattern observed for all trophoblast cell lines varied in intensity but was almost exclusively intracellular (Fig. 4G-I).

Analysis of subcellular distribution showed that a band of 14 kDa corresponding to galectin-1 is present in all investigated HTR-8/SVneo and JAR subcellular fractions, with the most intense band in cytoplasmic and membrane fractions observed in both cell lines (Fig. 5A). A faint band of 14 kDa was detected in

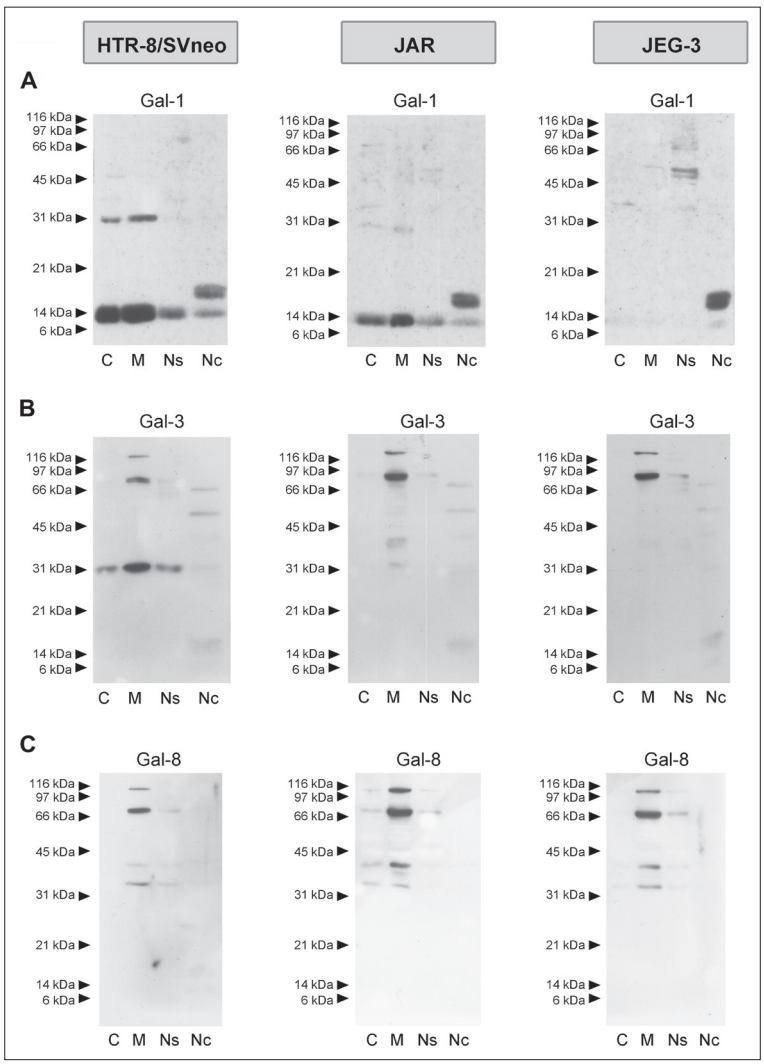

Fig. 5. Localization of galectins -1, -3, and -8 in HTR-8/SVneo, JAR, and JEG-3 cellular compartments. Subcellular fractions prepared from trophoblast-derived cell lines were analyzed by Western blotting using antibodies for galectin-1 (**A**), galectin-3 (**B**), and galectin-8 (**C**). The abbreviations for subcellular fractions are C – cytoplasmic, M – membrane, N – nuclear soluble, Nc – nuclear chromatin. Molecular masses are in kDa.

the cytoplasmic fraction of JEG-3 cells (Fig. 5A). In HTR-8/SVneo cytoplasmic and membrane fractions an additional ~30 kDa band was detected. The faint band at ~30 kDa was also present in JAR cytoplasmic and membrane fractions. Bands of 14 kDa and ~16 kDa were detected in the nuclear chromatin fraction of all three cell lines. Additionally, analysis of JEG-3 subcellular fractions showed galectin-1 bands in the nuclear soluble fraction ranging from 45-60 kDa (Fig. 5A). Galectin-3 was detected as a 30 kDa band in all HTR-8/SVneo subcellular fractions and the JAR membrane fraction (Fig. 5B). In the membrane fraction, two bands of ~80 kDa and >116 kDa were also present, while the band at ~40 kDa was detected in JAR and JEG-3 membrane fraction (Fig. 5B). In the nuclear soluble fraction of all examined cell lines, a faint band at ~100 kDa was present. The nuclear chromatin fraction of all investigated cell lines also contained 45-66 kDa bands (Fig. 5B). When subcellular fractions were analyzed for the presence of galectin-8, the ~34 kDa lectin was detected in the membrane fraction of all three cell lines (Fig. 5C). Additional bands from ~40 kDa to ~100 kDa were also present. In the nuclear soluble fraction of all cell lines, faint galectin-8 bands were also detected from ~40 kDa to ~100 kDa. Faint galectin-8 immunoreactive bands of ~34 kDa and from ~40 kDa to 100 kDa were also detected in the cytoplasmic fractions of JAR and JEG cells (Fig. 5C).

DISCUSSION

Galectins are involved in diverse physiological and pathological processes. In trophoblasts, galectin family members are recognized as contributing factors in various reproductive events, including blastocyst implantation, placental development, angiogenesis, and feto-maternal immune tolerance. Galectins are implicated in different malignancies, including choriocarcinoma. Although tumor and trophoblast cell invasions share similarities, such as epithelial-mesenchymal transition (EMT) and extracellular matrix (ECM) degradation, the key difference is that trophoblast invasion is tightly regulated in both time and space. It is important to elucidate whether these galectins could be involved in the pathophysiological processes of choriocarcinoma, starting from their expression and localization in the normal trophoblast and choriocarcinoma cells examined in this study.

Galectins -1, -3, and -8 have been previously documented in extravillous and choriocarcinoma cells [11,12,24]. In the present study, much higher mRNA and protein levels of examined galectins were detected in HTR-8/SVneo cells compared to JAR and JEG-3 cells. Differential expression of galectins -1, -3, and -8 has

been documented in various normal and cancer cell lines, highlighting the variability of galectin expression profiles across cell lines of different origins [25,26]. Comparative analysis of galectin-1 and galectin-3 in human embryonic kidney 293 cells (HEK-293), drug-sensitive human breast cancer MCF-7, and human colon carcinoma cell lines HCT-116 and HT-29 revealed the presence of transcribed galectins. However, translated proteins were not detected in HEK-293 and HT-29 cells [26]. The analysis of galectin-1 expression in cervical cancer cell lines revealed its elevated expression compared to non-cancerous cells [27]. The data presented here point to lower basal expression levels of galectins -1, -3, and -8 in choriocarcinoma cell lines compared to HTR-8/SVneo cells derived from normal extravillous trophoblasts. In contrast, an immunohistological study showed overexpression of galectins -1 and -3 in transformed trophoblast cells of choriocarcinoma compared to normal first-trimester trophoblasts [14]. These contradictory findings may be attributed not only to the specific tumor environment and methodologies used but also suggest that the role of the investigated galectins could vary depending on the cell type. Given the relatively low galectin-1, -3, and -8 mRNA and protein levels in choriocarcinoma cells compared to normal extravillous cells, the involvement of different regulatory pathways, specific gene expression, and glycosylation patterns in HTR-8/SVneo, and JAR and JEG-3 cells could be anticipated.

Comparative analysis of galectin-8 identified more than one *LGALS8* transcript and galectin-8 isoforms suggesting that alternative splicing occurred in the examined cell lines. Multiple galectin-8 isoforms have been shown in different cancer cell types [23,28]. Western blot analysis revealed different ratios of galectin-8 splice variants, showing a higher amount of the ~34 kDa variant and a lower abundance of the ~40 kDa form in HTR-8/SVneo cells than in JAR and JEG-3 cell lines. The same analysis also detected differences in galectin-8 protein in JAR and JEG-3 cells in contrast to similar levels of *LGALS8* transcripts in both cell lines, suggesting the presence of a fine control of lectin translation. Using a polyclonal anti-galectin-8 antibody we detected one galectin-8 isoform in HTR-8/SVneo cells and multiple isoforms in JAR cells [12,24]. The monoclonal anti-galectin-8 antibody used herein detected at least two galectin-8 isoforms in every examined cell line. Variations in galectin-8 isoform expression were observed in osteoclasts and the SK-MES-1 human lung squamous cell line [28,29]. To date, the function of the detected isoforms has only been demonstrated in osteoclasts, showing a predominant role in bone resorption of the short galectin-8 isoform [29]. Differences in the prevalence of short and long variants of galectin-8 in HTR-8/SVneo and choriocarcinoma cell lines indicate an altered expression pattern of this protein in choriocarcinomas. Future studies should investigate the levels and biological role of the detected isoforms and their significance for trophoblast function and choriocarcinoma.

The cellular localization of galectins -1, -3, and -8 in placental tissue, as well as in primary human trophoblast cells, and extravillous and choriocarcinoma cell lines, has been previously described [5,8,11,12,24]. The present study was designed to systematically analyze the expression of selected galectins in cells cultured under consistent conditions. The approach using cell fractionation and immunocytochemistry allowed the detection of fine variations, pointing to cell type-specific differences in distribution and potential to interact with binding partners within cell compartments, which merits further investigation. For instance, there was an apparent difference in galectin-3 cellular distribution in HTR-8/SVneo and choriocarcinoma cell lines. However, no correlation between immunocytochemistry and Western analysis of cellular fractions can be made. Namely, the cell fractionation protocol produces fractions of complex composition; thus, the cell membrane compartment contains not only the plasma membrane but also mitochondrial and endoplasmic reticulum/Golgi membranes, which can influence the results. In addition, the procedure involves chemical treatment that could affect epitope availability to antibody binding in immunostaining techniques.

We examined the differences in staining patterns and galectin presence across cellular compartments in normal versus choriocarcinoma cells. As expected, galectin-1 showed the most prominent plasma membrane and/or ECM association, especially in HTR-8/SVneo and JEG-3 cells, supporting its relevance for trophoblast invasiveness. Several experiments in different cell types have also shown galectin-1 plasma membrane localization [26]. In normal and JAR choriocarcinoma cells, galectin-1 associated with the cytoplasmic and

membrane fractions was most abundant and consistent with the presence of both galectin-1 monomers and dimers. Our finding of galectin-1 in nuclear compartments of all examined trophoblast-derived cell lines agrees with the data on its role in gene transcription [30]. However, the exact nature of the ~16 kDa and 45-60 kDa bands remains to be determined. Galectin-3 localization and cellular distribution revealed substantial differences between HTR-8/SVneo and choriocarcinoma cells. In HTR-8/SVneo cells, galectin-3 was detected mainly in the cytoplasm and to a lesser extent the plasma membrane; in both choriocarcinoma cell lines, a strong membrane staining pattern was observed. Recently, we showed that galectin-3 acts as a pro-invasive molecule in primary trophoblast cell culture and in HTR-8/SVneo cells [13]. Although the role of galectin-3 in choriocarcinoma cells is not well-documented, the expression patterns observed in JAR and JEG-3 cells suggest that galectin-3 may be exported with specific ligands and/or bind to cell membrane ligands. However, additional research into the molecular mechanism of galectin-3 export and its cell surface receptors in choriocarcinoma cell lines is needed. These results align with the distribution of galectin-3 across different cell compartments, indicating that this protein is abundant in the membrane fraction of all investigated cell lines. Our findings further support the interaction of galectin-3 with trophoblast ligands, since together with the 30 kDa band, higher molecular mass bands were also detected. The presence of galectin-3 in nuclear compartments of trophoblast-derived cell lines supports its involvement in nuclear export and pre-mRNA splicing, as described in other cell types [31]. Our results show similar galectin-8 cellular staining patterns in all investigated cell lines. On the other hand, galectin-8 is primarily detected in the cell membrane compartment, not only as a ~34 kDa band but also in regions of higher molecular mass. This finding suggests that galectin-8 binds to previously unidentified biological ligands in trophoblasts. Based on published data for other cells [32,33], it could be expected that galectin-8 may act intra- and extracellularly in trophoblasts. These results show that the examined galectins exist either as free molecules or in high molecular weight complexes. Many proteins in trophoblasts, such as laminin, fibronectin, integrins, and mucin 1, have the potential to interact with the examined galectins. However, many potential interactors remain unidentified. In addition, it is necessary to determine whether and how different protein complexes participate in regulating the invasion of normal and transformed trophoblast cells and other trophoblast functions. Identifying new galectin-binding partners in complexes will be the focus of our future research.

The data provided here show that all three examined galectins are present in normal and transformed trophoblast cells and that they are expressed at different levels and localizations, which suggests their possible involvement in the pathology of choriocarcinoma. Given their association with many cancers, galectins may serve as targets for various therapeutic approaches. These include galectin-targeting compounds such as siRNAs, neutralizing antibodies, and both carbohydrate- and non-carbohydrate-based inhibitors [37,38].

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Data availability: Data underlying the reported findings have been provided as a raw dataset available here: https://www.serbiosoc.org.rs/NewUploads/Uploads/Jovanovic%20Krivokuca%20Raw%20Dataset.docx

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SUPPLEMENTARY MATERIAL

Supplementary Table S1. Primers used for PCR or qPCR

Gene	PCR	qPCR
LGALS1	F: ATGGCTTGTGGTCTGGTC R: TCAGTCAAAGGCCACACA	F: TGCAACAGCAAGGACGGC R: CACCTCTGCAACACTTCCA
LGALS3	F: ATGGCAGACAATTTTTCG R: TTATATCATGGTATATGAAGCAC	F: CAGAATTGCTTTAGATTTCCAA R: TTATCCAGCTTTGTATTGCAA
LGALS8	F: AGAATGATGTTGTCCTTAAAC R: CTACCAGCTCCTTACTTCC	F: CTTAGGCTGCCATTCGCT R: AAGCTTTTGGCATTTGCA
GAPDH	F: GAAGGTGAAGGTCGGAGT R: GAAGATGGTGATGGGATTTC	F: GAAGGTGAAGGTCGGAGT R: GAAGATGGTGATGGGATTTC