Histol Histopathol (2016) 31: 1095-1111

http://www.hh.um.es

Histology and Histopathology

From Cell Biology to Tissue Engineering

Comparative analyses on expression of galectins1-4, 7-10 and 12 in first trimester placenta, decidua and isolated trophoblast cells *in vitro*

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Summary. Introduction: Galectins are members of the mammalian β-galactoside-binding proteins, which recognize Galβ1-4GlcNAc sequences of several cell surface oligosaccharides. Plenty of galectins are already described in human tissue, especially in placenta. Here, gal-1-4, 7-10 and gal-12 were investigated systematically in trophoblast and decidua cells of first trimester placentas.

Material and methods: Within this study, 15 first trimester placentas after induced abortion (7th-14th week of gestation) were examined with immunohistology and immunofluorescence based on a scoring system. Moreover, isolated and cultivated trophoblast cells from the first trimester were analyzed and evaluated for expression of gal-1-4, gal-7-10 and gal-12 at mRNA and protein level with real-time RT-Polymerase chain Reaction/PCR (Taq-Man). Double immunofluorescence with trophoblast specific markers identified galectin expressing cells at the feto-maternal interface.

Results: We could detect immunohistochemical staining of galectins 1-4, 7-10 and 12 in first trimester placenta: all examined galectins were found in the cytotrophoblast (CTB) and syncytiotrophoblast (SCT). Gal-1, -2, -3, -4, -7, -8, -9, -10 and -12 were identified in extravillous trophoblast cells (EVT) in immunohistology and immunoflourescence. The expression of gal-1, -9, -10, and gal-12 increased after 96h incubation *in vitro* without stimulation at mRNA level, while gal-2, -3, -4, -7 and -8 were decreased.

Discussion and conclusion: This study describes a systematic analysis of the expression of gal-1-4, gal-7-10 and gal-12 in first trimester placentas and isolated trophoblast cells. Expression levels at mRNA level and the change within 96h cultivation *in vitro* indicate a possible influence on syncytium building of trophoblast cell on expression of galectins. Therefore, an interaction of galectins *in vitro* in syncytium building is possible.

Key words: Galectins, Placenta, Tophoblast, Decidua, First trimester

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DOI: 10.14670/HH-11-739

Introduction

Galectins are galactose-binding proteins of the family of lectins and exhibit a characteristic amino acid sequence as well as features of cytoplasm proteins (Barondes et al., 1994; Kasai and Hirabayashi, 1996; Jeschke et al., 2013). Consisting of a highly conserved amino acid sequence motif in the globular galectin-type Carbohydrate Recognition Domains (CRD), they have β-galactoside binding affinity (Barondes et al., 1994; Ahmad et al., 2004). Galectins recognize galβ1-4GlcNAc sequences of cell surface oligosaccharides and bind to glycoconjugates on the plasma membrane and in the extracellular matrix (Barondes et al., 1988; Brinck et al., 1996; Jeschke et al., 2013). A common function of the galectins may be the crosslinking between galactose-containing structures found at cell surfaces and in the

extracellular matrix, or to crosslink different or the same type of cells (Bourne et al., 1994; Rubinstein et al., 2004). The eight residues that form the galactoside-binding site are conserved in most mammalian galectins (Arata et al., 2001a,b; Hirabayashi et al., 2002).

Most galectins have multiple sugar-binding sites due to the presence of two galectin-type CRDs in a single polypeptide or as a result of dimerization (Ozeki, 1997; Arata et al., 2001a,b). Therefore, galectins are classified into three types on the basis of their structural architecture: proto, chimera and tandem-repeat types (Hirabayashi et al., 2002; Jeschke et al., 2013).

Galectins are diversely expressed in human tissue. Different cells within an organism usually contain a different composition of galectins, but almost all cells have at least one galectin (Cooper et al., 1999; Wang et al., 2004). Gal-5, -6 and -15 so far have not been found in human tissues (Gray et al., 2005). Gal-1, -3, -7, -10, -11 and -14 are expressed in cytoplasm, nucleus and extracellular space of different tissues (Wang et al., 2004). Gal-4, -8, -9 were found in cytoplasm and extracellular space and gal-12 and -13 in cytoplasm and nuclei (Wang et al., 2004). However, most of the galectins are located intracellularly (cytoplasm and nuclei).

Galectins are expressed in the vast majority of immune cells and were found to be up-regulated in activated B- and T-cells, inflammatory macrophages and decidual natural killer (NK) cells (Blois et al., 2007; Than et al., 2009; Cedeno-Laurent et al., 2012). Therefore an immunomodulating function of galectins is assumed in neoplasia, but also in implantation in pregnancy (Rabinovich et al., 2000; Vasta, 2012). Many galectins are known to have an influence on different types of human T-cells, which may play a role in maternal-fetal immune tolerance (Aluvihare et al., 2004; Kubach et al., 2007; Paclik et al., 2008; Than et al., 2009). The mRNA-expression for gal-13, -14 and -16 were found to be placenta specific (Than et al., 2009).

Galectins have highly conserved structures and therefore they are considered to be closely connected to the evolution of placenta in eutherian mammals, including primates (Than et al., 2012).

With regard to placenta, several galectins are described in different stages of gestation. In the first trimester, placenta gal-1, a proto-type galectin, was found to be expressed mostly in the cytotrophoblast (CTB), while the SCT is not immunoreactive against gal-1 (Ramhorst et al., 2012).

Decreased levels of gal-1 may elucidate disturbed trophoblast differentiation and angiogenesis during early placentation leading to early pregnancy loss of fetus in mice (Blois et al., 2007; Freitag et al., 2013). But also in human pregnancy, a down regulation of gal-1 is described in patients suffering from early onset preeclampsia (PE) (Freitag et al., 2013). Within modulating HLA-G expression on trophoblast cells, circulating gal-1 levels are suggested as a predictive factor for pregnancy success in early human gestation (Tirado-Gonzalez et al., 2013).

Gal-1 binds to Thomsen-Friedenreich-(TF) antigen, which is a state-specific oncofetal carbohydrate antigen. The complex could modulate cell proliferation, trophoblast invasion and maintenance of pregnancy (Jeschke et al., 2006). Gal-1 and gal-3 may influence the invasion of blastocyst by an immunomodulatory process, but also on the development of the trophoblast (Maquoi et al., 1997; Jeschke et al., 2007). Gal-3 is localized in the human villous trophoblast (HVT) and extravillous trophoblast (EVT) (Vicovac et al., 1998; Tirado-Gonzalez et al., 2013). In gestational trophoblastic diseases (GTD), immunoreacivity of both gal-1 and gal-3 was increased in comparison to controls of 1st trimester placentas (Bozic et al., 2004). Moreover in preeclamptic and HELLP placentas of the third trimester, gal-1 and gal-3 expression was significantly up-regulated in the EVT (Jeschke et al., 2007).

Gal-9 is described in early pregnancy placenta. In endometrium an increase in mid and late secretory phase is known. Therefore, gal-9 is considered a marker of endometrial receptivity and could play a role during implantation (Popovici et al., 2005; Shimizu et al., 2008). Galectin-7 was recently found in the serum, endometrium and endometrial epithelial cells in first trimester placenta and its expression level is altered in pregnancies with preeclampsia or history of miscarriage (Menkhorst et al., 2014a,b).

Although there has been some investigation about gal-1, gal-3 as well as a little about gal-9 at the feto-maternal interface in the first trimester, more research on the other human galectins is necessary. Before approaching the functional aspects of further galectins in placental tissue in early pregnancy, expression of these lectins in first trimester has to be investigated systematically under the same test conditions. Therefore we analyzed first trimester placentas (7th-14th week of gestation) for expression of gal-1-4, gal-7-10 and gal-12.

Materials and methods

Tissue samples

A total of 15 placentas were obtained from legal terminations of normal pregnancies because of socio–economic reasons, each from the 7th to 14th week of gestation (first trimester of gestation). For further demographical and clinical characteristics see Tables 1, 2. The pregnancies were confirmed as healthy by an independent pathologist. The tissue was obtained after uterine curettage without hormonal pre-treatment.

Patients were screened for anatomic, chromosomal (parental and fetal) and endocrine disorders. Exclusion criteria were thrombophilia, autoimmune diseases, hydatidiform mole placentas, possible prostaglandin or progestin treatment before operation or chromosomal aberrations.

The study was approved by the ethical committee of the University of Munich and informed consent was

obtained from each patient in written form. Samples and clinical information were anonymized and encoded for statistical workup.

Tissue was obtained from three central parts of each placenta with about 1cm side length in every case. Each block had to contain both decidua and trophoblast proven by macroscopic inspection. One part was frozen in liquid nitrogen directly after termination of pregnancy and stored at -80°C for double immunoflourescence with cryosections. The other part of placental tissue was fixed immediately in 4% buffered formalin for 20-24 hours and embedded in paraffin for use in immunohistology (Jeschke et al., 2007). The third part was directly processed for cell culture (see below) and isolated cells were stored at -80°C.

Immunohistochemistry

Paraffin-embedded slides were dewaxed in xylol and washed in ethanol 100%. For inhibition of the endogen peroxidases, tissue samples were put to methanol with 3% H_2O_2 and rehydrated in a descending series of alcohol. For gal-2, gal-4, gal-7, gal-8, gal-9, gal-10 and gal-12 staining samples were heated in pressure cooker using sodium citrate buffer (pH 6.0), containing 0.1 M citric acid and 0.1 M sodium citrate in distilled water and after cooling washed in Aqua dest. and PBS.

For gal-1 and gal-3 no heating was required and slides were incubated with horse serum (20 min; Vector Laboratories, Burlingame, CA, USA). For gal-4 and gal-9 investigation slides were incubated for 5 min with Power Block (Bio Genex, 1:10 diluted in H_2O dest) to reduce non-specific staining in background. For gal-2, gal-7, gal-8, gal-10 and gal-12 blocking solution (Reagent 1, Zytochem-Plus HRP-Polymer-Kit (mouse/rabbit)) was used for incubation for 20 min.

The antibodies against these galectins were incubated. For dilution and incubation time see Table 3. Afterwards, second antibodies/complexes of detection kits were used following the manufacturer's protocols (Table 3).

Immunostaining was visualized with substrate and the chromogen-3,3'-diaminobenzidine (DAB; Dako, Glostrup, Denmark) for 30 s-2 min. Counterstaining was performed with Mayer's acid Hemalaun for 5 min with blueing in tap water. Slides were dehydrated in an ascending series of alcohol. After treatment with xylol, slides were cover-slipped with Consul-Mount™ medium. (ThermoSherton, Pittsburgh, USA)

As positive control in immunohistochemical staining an appropriate tissue was used, as listed in Table 3 with each antibody. Positive cells showed a brownish color and the negative control, as well as unstained cells, appeared blue (Mylonas et al., 2006).

Negative controls were used with the same control tissues and negative antibodies (Fig. 2). For isotype-control for gal-3 (mouse antibody) a mouse negative control antibody was used (Lot HK 1180313, BioGenex, The Hague, Netherlands).

The slides were analyzed under microscope by two independent observers. For the light microscopy analysis, a semi quantitative IRS score was used, which is the multiplication of optical staining intensity (grades: 0=no, 1=weak, 2=moderate and 3=strong staining) and the percentage range of positive stained cells (0=no staining; 1≤10% of the cells; 2=11–50% of the cells; 3=51-80% of the cells and 4≥81% of the cells were stained for the antibody, respectively). The score is calculated as followed: IRS=grade (0-3) x percentage (0-4). Therefore the minimum is 0 and the maximum is 12. Two independent observers scored the slides.

Negative controls were performed with the same tissue and negative control antibodies according to the same protocol. Images of negative controls are shown in Fig. 2.

Double Immunofluorescence staining

Cryosections of the tissue samples of first trimester placentas were examined for the characterization of galectin-expressing cells in decidua. All samples were fixed in acetone for 5 min. Antibodies used for the experiments are listed in Tables 3, 4. As CK7 stains in glandular structures of decidua, (Froehlich et al., 2012; Orazizadeh et al., 2013), HLA-G was chosen as a marker for extravillous trophoblast cells (AbDSerotec, Oxford, UK).

First, slides were blocked with Ultra V Block and were incubated with the different galectin antibodies and

Table 1. Demographic and clinical features of females taking part in the study.

characteristic	normal pregnancy n=15
maternal age [years]	33.0±6.7 (26-40)
gestational age [weeks]	9.1±1.6 (7-14)
gravidity	3.2±1.3 (1-6)
parity	1.6±0.7 (0-3)

Values are given as mean ± SD; the range is given in parentheses.

Table 2. Number of slides used for immunohistochemical staining for each week of gestational age

gestational age	number of slides in 1st trimester
7th week	3
8th week	5
9th week	1
10th week	3
11th week	1
12th week	1
13th week	0
14th week	1
	n=15

HLA-G (diluted 1:50 in Dako diluting medium, Dako) (Tables 3, 4) for 60 min at room temperature (gal 3, gal-9) or overnight at 4°C (rest).

After washing steps with PBS, Cy2- and Cy3-labeled antibodies were applied as fluorescent secondary antibodies. For gal-1, gal-2, gal-3, gal-4, gal-7, gal-8, gal-9, gal-10, gal-12, Cy3-labeled secondary Antibodies were used appearing red and for HLA-G Cy2- labelled Antibodies appearing green were applied and incubated for 30min (Table 4). After washing and drying, the slides were finally embedded in mounting buffer containing 4',6-diamino-2-phenylindole (DAPI) for blue staining of the nucleus (Jeppesen et al., 1989). Sections were examined with a Zeiss (Jena, Germany) Axiophot photomicroscope. Digital images were obtained with a digital camera system (Axiocam; Zeiss CF20DXC; KAPPA Messtechnik, Gleichen, Germany) and digitally stored.

Trophoblast cell purification and cell culture of villous cytotrophoblast cells

CTB cells were isolated and analysed for galectin expression from human first trimester placentas directly after abortion and after 96h in cell culture without any stimulation. For the 96h cell culture, cells from the placentas were cultured at 37°C in DMEM medium with 10% inactivated FCS and with antibiotics and antimycotics (Penicillin, Streptavidin and Amphothericin).

CTBs were prepared as published previously (Jeschke et al., 2002): the placental tissue was minced and transferred to HBSS-HEPES buffer containing trypsin (100 mg/ml) and DNase I (first step 0.05 mg/ml,

Table 3. Antibodies used in study for immunohistochemistry.

Anti-gene	Type	Species and isotype	Concentration/ dilution	Source of Ab	Incubation time/temp.	Blocking solution	Detection system	Positive control tissue
Gal-1	K8508	goat	100 µg/ml 1:3000 in 1:100 diluted Power Block	R&D Systems, Minneapolis, USA	Overnight/4°C	Horse serum Vectastain Elite Kit (Linaris, Dossenheim, Germany)	Vectastain Elite Kit (Linaris,)	Colon, Breast-Ca
Gal-2	H-45	rabbit	1:500 in Dako diluting medium	santacruz, Dallas, USA	1 h/room-temperature	Reagent 1, Zytochem-Plus HRP-Polymer-Kit (20 min)	Zytochem-Plus HRP-Polymer-Kit (Mouse/Rabbit) (Zytomed, Berlin Germany)	Colon
Gal-3	9C4	mouse IgG1	4,6 mg/ml/ 1:1000 in PBS	Novocastra	Overnight/4°C	Horse serum Vectastain Elite Kit (Linaris)	Vectastain Elite Kit (Linaris)	Colon
Gal-4	T 20	goat	200 µg/ml 1:200 in Dako diluting medium	santacruz	1 h/room-temperature	Power Block (Bio Genex, 1:10 diluted in H_2O dest); 5 min	HRP-Polymer Kit (Goat) (Biocare Medical, Concord, USA)	Colon
Gal-7	H 60	rabbit	200 µg/ml 1:150	santacruz sc-28253	1 h/room-temperature	Reagent 1, Zytochem-Plus HRP-Polymer-Kit (20 min)	Zytochem-Plus HRP-Polymer-Kit (Mouse/ Rabbit) (Zytomed)	Vagina, Cervix, Colon
Gal-8	H-80	rabbit	200 µg/ml 1:100 in Dako diluting medium	santacruz	1 h/room-temperature	Reagent 1, Zytochem-Plus HRP-Polymer-Kit (20 min)	Zytochem-Plus HRP-Polymer-Kit (Mouse/ Rabbit) (Zytomed)	Tonsil, Breast-Ca, small intestine
Gal-9	C-20	goat	200 µg/ml 1:50 in Dako diluting medium	santacruz	1 h/room-temperature	Power Block (Bio Genex, 1:10 diluted in H_2O dest); 5 min	HRP-Polymer Kit (Goat) (Biocare Medical)	Tonsilla, Colon
Gal-10	H-40	rabbit	200 µg/m 1:100 in Dako diluting medium	santacruz	1 h/room-temperature	Reagent 1, Zytochem-Plus HRP-Polymer-Kit (20 min)	Zytochem-Plus HRP-Polymer-Kit (Mouse/ Rabbit) (Zytomed)	Term Placenta, Sigma
Gal-12	H-166	rabbit	200 µg/ml 1:100 in Dako diluting medium	santacruz	1 h/room-temperature	Reagent 1, Zytochem-Plus HRP-Polymer-Kit (20 min)	Zytochem-Plus HRP-Polymer-Kit (Mouse/ Rabbit) (Zytomed)	Term Placenta, Vagina

Table 4. Supplement antibodies used in immunofluorescence.

Primary antibody		Blocking solution	Secondary antibody
HLA-G green	Mouse-IgG1 Clon MEM-6/9 (AbDSerotec) dilution 1:50 in Dako	Ultra V Block (15 min in RT)	Cy2-labeled Goat-Anti-Mouse IgG (Dianova) diluted 1:100 in Dako →green
Gal-2, 7, 8, 10, 12 red	See Table 3 all rabbits		Cy3-labeled Goat-Anti-Rabbit IgG (Dianova) diluted 1:500 in Dako →red
Gal-3 red	Rabbit polyclonal (Chemicon) 1:200 in Dako		
Gal-1, 4, 9 red	See Table 3		Cy3-labeled Donkey-Anti-Goat IgG (Dianova) diluted 1:500 in Dako →red

other steps 0.1 mg/ml). The cell suspensions obtained by three digestion steps were separated from tissue fragments. The first step of trophoblast cell purification was performed on a Percoll gradient (GE Healthcare, Chalfont St Giles, GB). After centrifugation, CTBs were aspired. Using antibodies against CD45 and CD9 (Dianova, Hamburg, Germany) and a magnetic cell sorting column (MiltenyiBiotec GmbH, Bergisch-Gladbach, Germany) the CTBs were immunopurified to eliminate contaminating leukocytes (CD45), EVT cells, fibroblasts and other mesenchymal components (CD9) (Jeschke et al., 2005).

Trophoblast identity of the isolated cells was determined morphologically by light microscopy and by

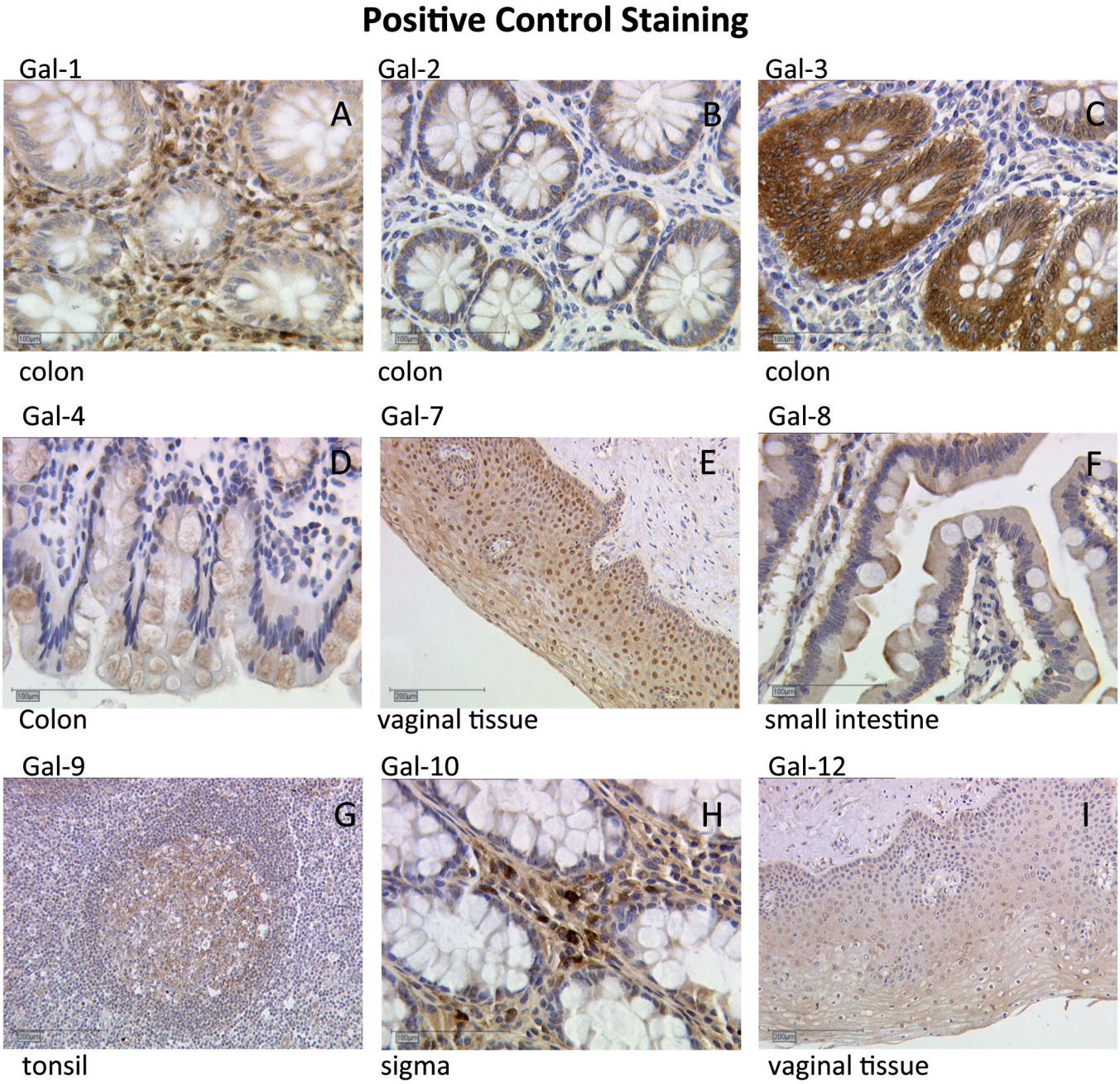

Fig. 1. Antibodies chosen for this study could be shown in control staining pictures (gal-1: **A**, gal-2: **B,** gal-3: **C,** gal-4: **D**) of human colon tissue. Gal-7 and gal-12 expression was verified in human vaginal tissue by the respective antibody used for this study **(E and I)**. Small intestine tissue was used to confirm gal-8 staining **(F).** Gal-9 expression was verified in human tonsil tissue by the antibody used for this study **(G).** We confirmed expression of gal-10 with the antibody used in sigma tissue **(H)**. All magnification are 25x lens, 1**(G)**: Scale bar 100 μm.

immunostaining with a monoclonal mouse anti-cytokeratin 7 antibody (ProgenBiotechnik, Heidelberg, Germany) (Jeschke et al., 2002) and HLA-G-staining (AbDSerotec, Oxford, UK). Isolated cells contained less than 1% digested fragments of the SCT.

Isolation of RNA for TaqMan RT-PCR

Total RNA was prepared using the acid NucleoSpin RNAII Kit (Macherey-Nagel, Nr. 740955.50) according to manufacturer's protocol:

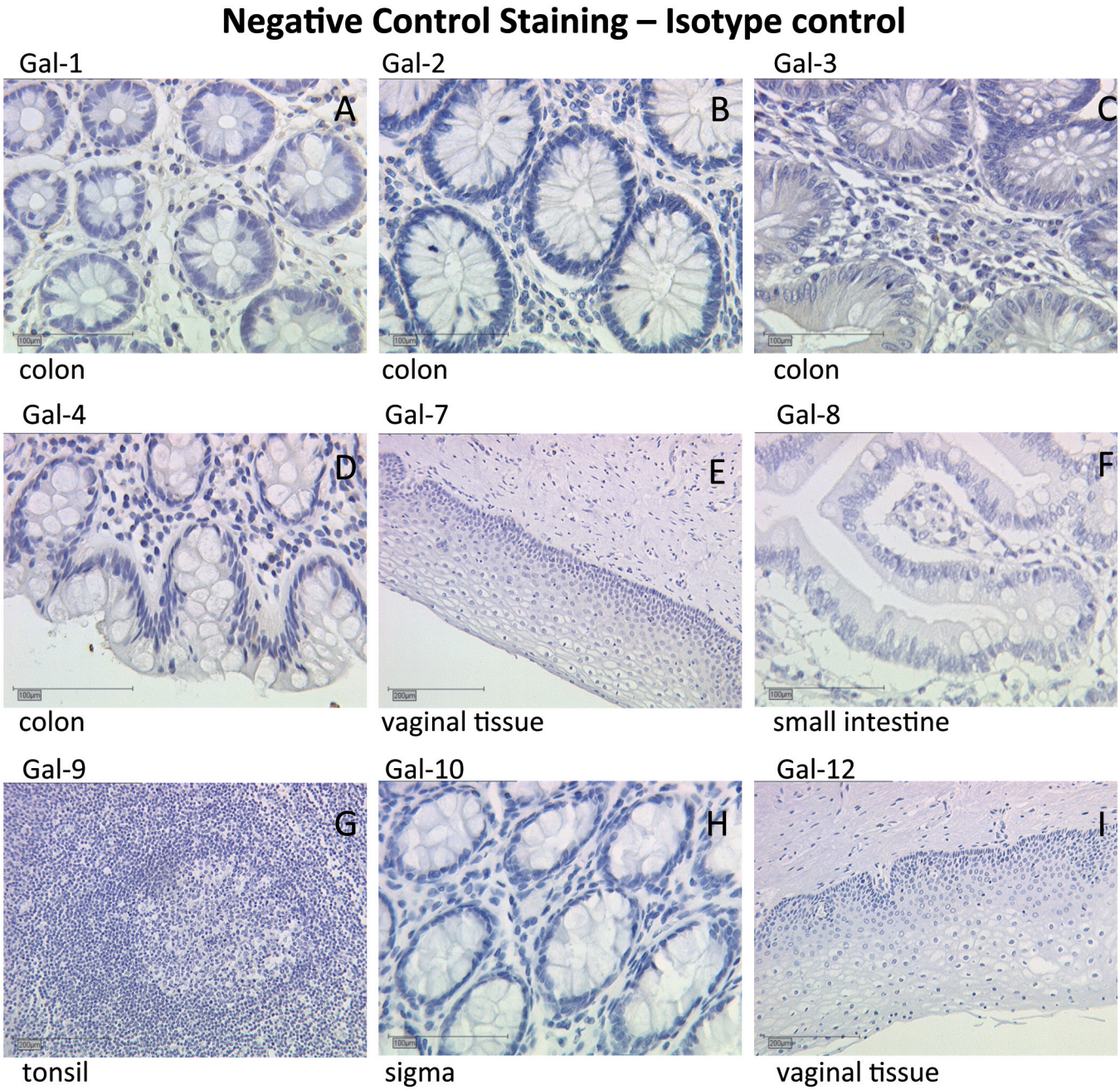

Fig. 2. Replacement of antibodies chosen for this study by appropriate isotype control antibodies showed no staining in negative control pictures (gal-1: **A**, gal-2: **B,** gal-3: **C**, gal-4: **D**) of human colon tissue. There was no gal-7 and gal-12 expression in human vaginal tissue after replacement of the respective antibody used for this study by isotype controls **(E and I)**. Small intestine tissue showed no expression of gal-8 **(F)** after replacement of the primary antibody with isotype control antibodies. Specificity of gal-9 expression was verified in human tonsil tissue by replacement of the antibody used for this study with isotype control antibodies **(G)**. We confirmed specificity of gal-10 by replacing the antibody used in sigma tissue with isotype control antibodies **(H)**. Scale bar 100 μm.

The CTBs from first trimester placentas and after 96h in cell culture were spinned for homogenisation. Cell-lysis was induced with RA1 and β–mercaptoethanol (3,5μl). With NucleoSpin-filter, the lysat was filtrated. For RNA binding NucleoSpin RNAII (Machery Nagel) was used. Membrane Desalting Buffer was added and once more centrifuged. With the adding of rDNAse and Reaction Buffer for rDNA (1:10) DNA is digested. The mixture was washed three times with RA2 or RA3 Buffer and eluted with RNAse free water.

cDNA-Synthesizing/ Reverse transcription

Reverse transcription for cDNA-Synthesizing was performed with TaqMan_EZ RT-PCR Kit (PE, Applied Biosystems). All RT-PCR reactions were performed with a final volume of 20 µl consisting of 10 µl2xRT-Mastermix and 10 µl mRNA. The reaction conditions were 10 min at 25°C, 120 min at 37°C, 5 min at 85°C and 4°C on hold and were performed with Eppendorf Mastercycler gradient (Eppendorf, Hamburg, Germany) (Jeschke et al., 2005)

Real-time-PCR

A volume of 20 μl containing 1 μl TaqMan® Gene Expression Assay 20x concentrated (Applied Biosystems, Weiterstadt, Germany), 10 μl TaqMan® Fast

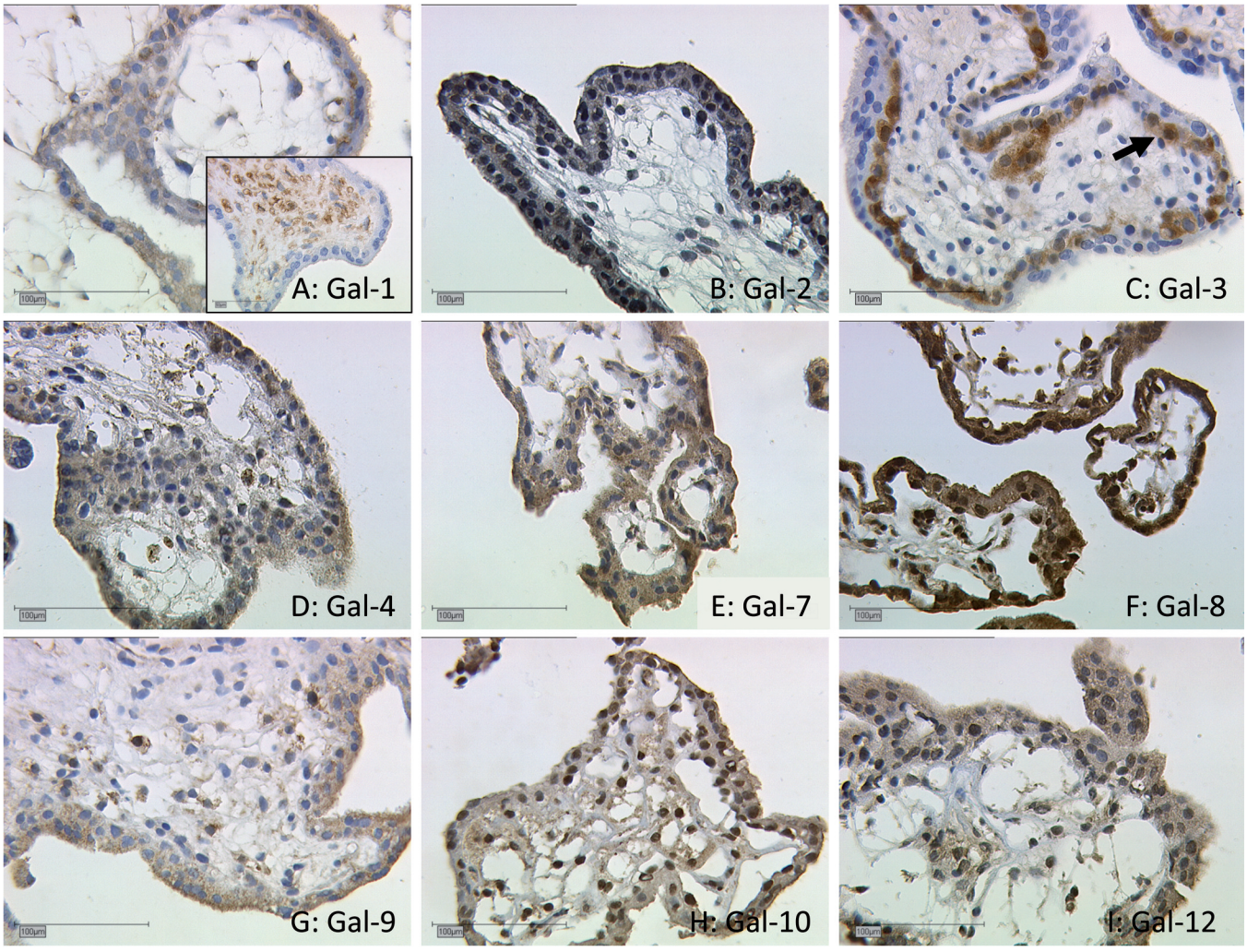

Fig. 3. Immunohistochemical staining of galectins in first trimester placentas in the villous trophoblast displays different staining patterns and strengths: For gal-1, the syncytiotrophoblast was staining weak, while staining is visible in the trophoblast and extracellular matrix **(A)**. Gal-2 showed a strong staining, especially in the CTB **(B)**. Gal-3 stained very weakly in the syncytiotrophoblast, but with moderate staining in the CTB cells (arrow) **(C)**. Gal-4 was moderately expressed in CTB **(D)**. Galectin-7 was expressed in SCT **(E)**. Gal-8 **(F)** with a similar strong staining pattern to gal-10 with mean IRS of 11 **(H)**, while gal-9 is expressed more weakly with the mean IRS of 4 **(G)**. Gal-12 is moderately expressed with mean IRS of 8 **(I)**. Scale bars: 100 μm; small image, 50 μm.

Universal PCR Master Mix 2x (Applied Biosystems), 2 μl cDNA template and 7 μl H_2O (DEPC treated DI water, Sigma, Taufkirchen, Germany) was used.

For the expression of LGALS7, a special primer was custom designed by Applied Biosystems (Primer sequence see Table 5). It contains a MGB probe, a forward and a reverse primer in a concentration of 10pmol/µl each.

1 μl of each named primer (Table 5; Applied Biosystems, Darmstadt, Germany), 10 μl TaqMan® Fast Universal PCR Master Mix 2x (Applied Biosystems), 2 μl cDNA template and 5 μl H_2O (DEPC treated DI water, Sigma, Taufkirchen, Germany) were used.

The mix was applied on an optical fast 96-well-plate (Applied Biosystems), covered by optical caps (Applied Biosystems) and put to ABI PRISM 7500 Fast (Applied

Table 5. Galectin primer used for PCR.

Marker	Purchase-No	gene
Galectin-1	Hs00169327_m1	LGALS1
Galectin-2	Hs00197810_m1	LGALS2
Galectin-3	Hs00173587_m1	LGALS3
Galectin-4	Hs00196223_m1	LGALS4
Galectin-7	Hs00170104_m1	LGALS7
Galectin-7	Custom made *	LGALS7
Galectin-8	Hs00374634_m1	LGALS8
Galectin-9	Hs00371321_m1	LGALS9
Galectin-10 (Charcot-Leyden crystal protein)	Hs00171342_m1	CLC
Galectin-12	Hs00263821_m1	LGALS12
Glycerinaldehyd-3-phosphate-dehydrogenase	Hs99999905_m1	GAPDH

* primer sequences for LGALS7: •Forward primer: 5‘ CTT GGT CTG GGT GGT TTC TGA 3‘ •reverse primer: 5’ CCC CGC ACA GCA GGT TTA 3’ •MGB probe: 5’ TCC TCT TCC AGG TTC CA 3’

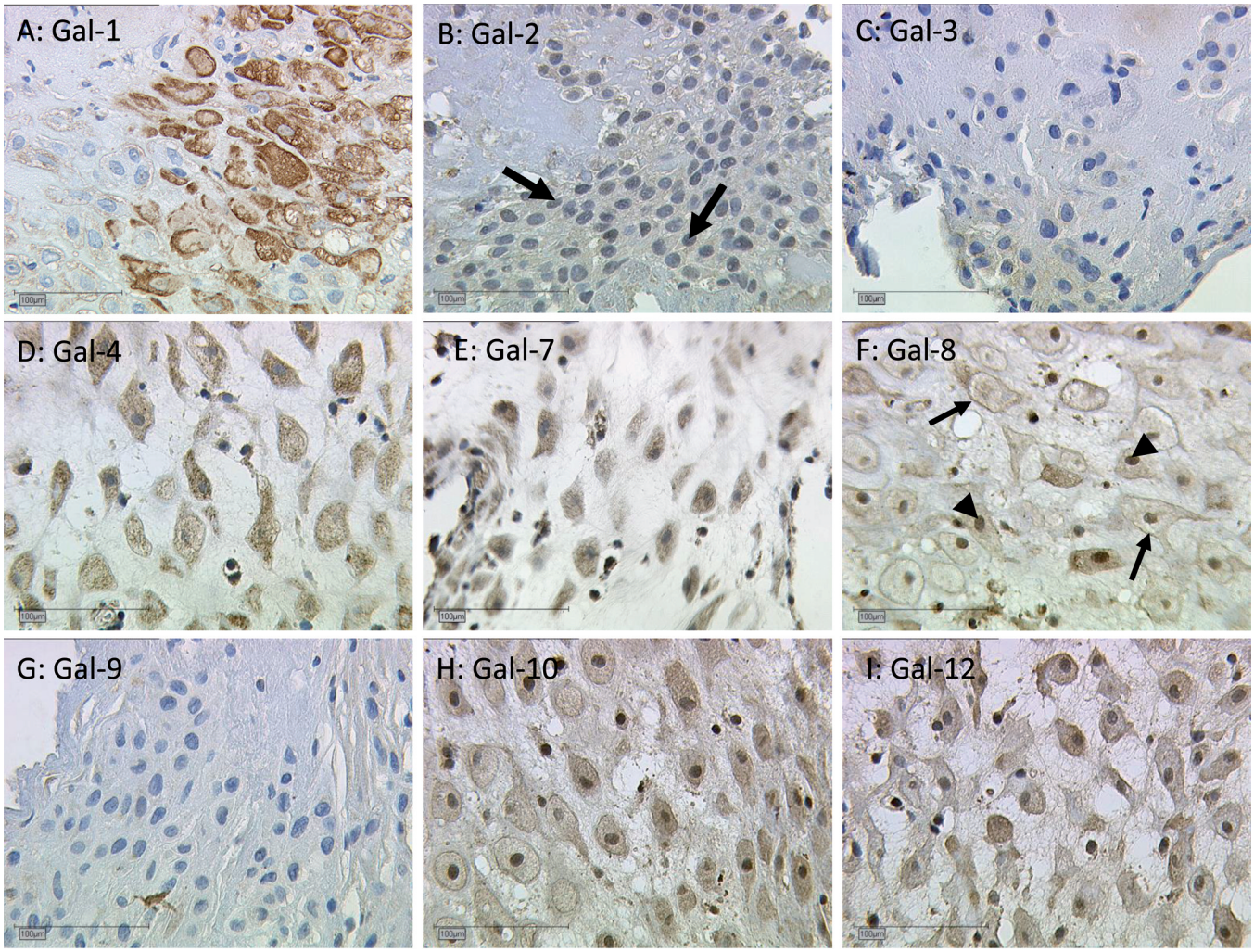

Fig. 4. Immunohistochemical staining of galectins in the decidua of first trimester placentas. Gal-1 staining was very strong in decidua **(A).** Gal-2 was staining moderately in the cytoplasm and in nuclei (arrow) **(B)**, while gal-3 staining was weak **(C)**. Gal-4 is moderately expressed **(D)**. Moreover, there was staining for gal-7 in the decidua **(E)**. Gal-8 was expressed strongly in some areas, even in nuclei (arrow head, **F**) and bound to membrane (small arrow, **F**). Gal-9 stained very weakly **(G)**. Gal-10 stained moderately, but also in nuclei **(H)**. Gal-12 stained moderately in decidual cells **(I)**. Scale bars: 100 μm.

Biosystems) to perform the PCR assays. Enzyme activation was performed for 20 s at 95°C. Real-time PCR ran for 40 cycles with denature temperature of 95°C for 3 s and annealing/extend temperature of 60°C for 30s in alternation.

For the statistics, the ΔCt results were used. Ct is defined as the first fluorescent signal reaching statistical significance. This is defined by the crossing of fluorescent signal over the auto-threshold of the taqman-machine. ΔCt values were calculated by normalizing to an endogenous control, for which glycerinaldehyd phosphate dehydrogenase (GAPDH) (Applied Biosystems) as housekeeping gene was used. For statistical and graphical analysis and relative quantification $2^{-\Delta\Delta Ct}$ was used (Fig. 7).

Control slides for gal staining

Gal-1, -2, -3 and -4 are all found in colon tissue (Hittelet et al., 2003; Barrow et al., 2011). Suitability of the antibodies chosen for this study could be shown in control staining pictures (gal-1: Fig. 1A, gal-2: Fig. 1B, gal-3: Fig. 1C, gal-4: Fig. 1D). Gal-7 and gal-12 are expressed in vaginal tissue (gal-7: Fig. 1E, gal-12: Fig. 1I). Gal-8 expression was found in small intestine tissue (Bidon et al., 2001). Gal-8 expression is shown in Fig. 1F. Gal-9 is known to be expressed in human tonsils (Suk et al., 1999). We could verify an expression of gal-9 in this organ by the antibody used for this study (Fig. 1G). The expression of gal-10 was described for normal sigma tissue (De Re et al., 2009). We verified expression

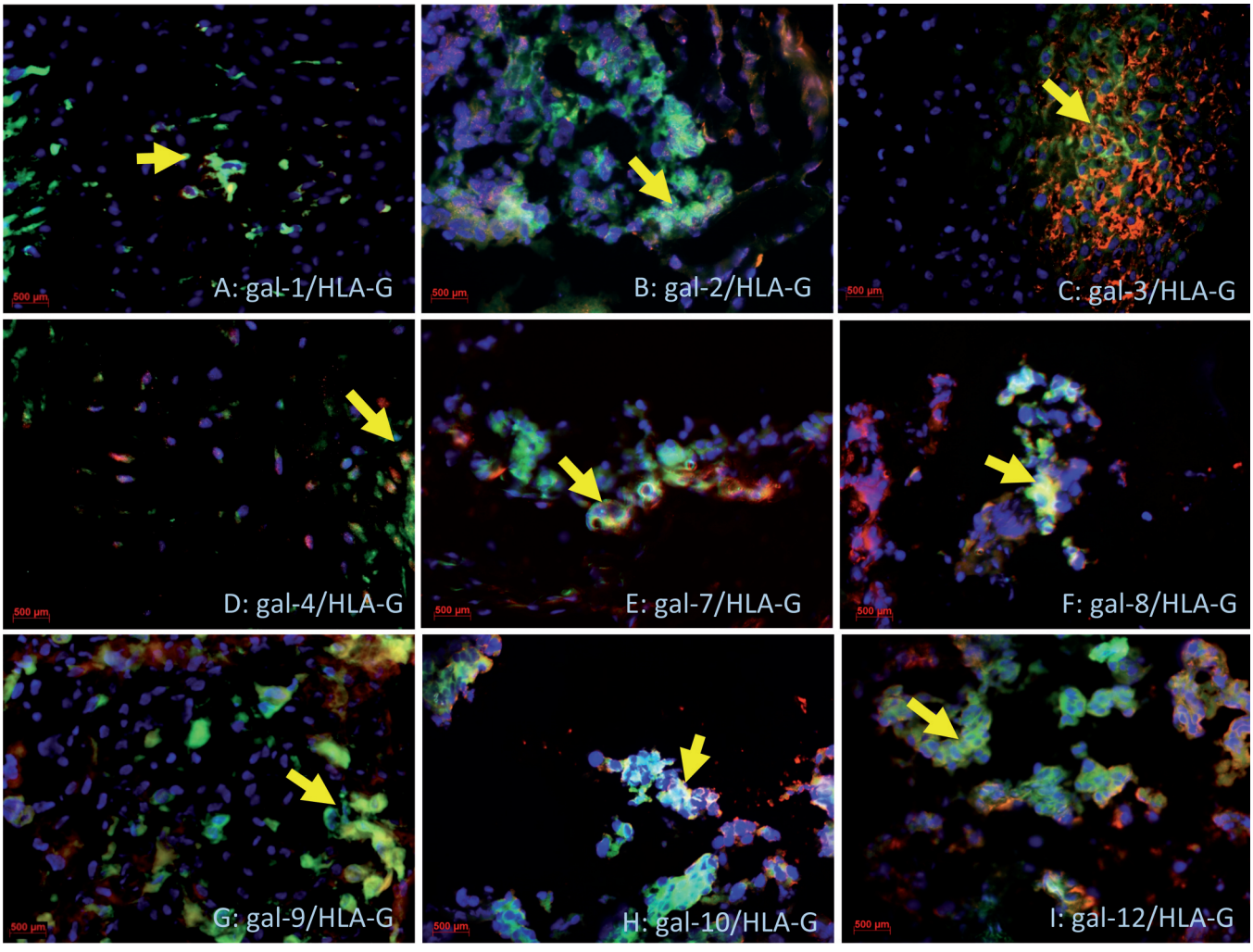

Fig. 5. Coexpression of galectins (red) and trophoblast markers (HLA-G in green) are shown in decidua (arrows and yellowish staining): gal-1 **(A)**, gal-2 **(B)**, gal-3 **(C)**, gal-4 **(D)**, gal-7 **(E)**, gal-8 **(F)**, gal-9 (**G)**, gal-10 **(H)**, and gal-12 **(I)**. Scale bar: 500 *µ*m.

of gal-10 with the antibody used in sigma tissue (Fig. 1H).

Replacement of the primary antibody with mouse, goat or rabbit IgG, respectively served as negative controls (Fig. 2).

Statistical analysis

Statistical analysis and Boxplots were performed using SPSS 21 (SPSS Inc., IBM, Chicago, USA) and Excel (Microsoft office 365). For analysis of clinical data, mean ranks were performed.

Results

Galectin expression in first trimester Placenta-Evaluation of staining results

The tissues from legal termination of normal healthy pregnancies (n=15) (Tables 1, 2) were examined in paraffin-immunohistochemical treated slides and in cryosections for immunoflourescence. Immunohistochemistry results were standardized with the semiquantitative IRS score, which was first developed for immunohistological diagnostics in breast-cancer (Remmele et al., 1987). We analyzed the different cell populations of placenta, decidua and villous trophoblast (HVT), each separately with IRS score according to different microscopic tissue structure. In general, mean IRS values are higher in SCT/HVT than in decidua, with the exception of gal-1 and gal-3.

Cytotrophoblast and syncytiotrophoblast

The expression of the different galectins in the subpopulations of human villous trophoblast (HVT), Cytotrophoblast (CTB) and Syncytiotrophoblast (SCT), respectively, was tested by immunohistochemistry. In general, we found very diverse staining strength and patterns between the different galectins, as depicted in Fig. 3. Overall staining for gal-1 in the SCT was very weak in first trimester samples, but underlying CTB and fetal macrophages (Hofbauer cells) were positive for gal-1 (mean IRS=2.8±0.2; Fig. 3A). Gal-2 showed a strong staining with mean IRS=8.9±3.5 (Fig. 3B), while staining was especially located at the border to maternal blood of the SCT and in the CTB. Moreover, some nuclei showed positive staining for gal-2 antibody in decidua (Fig. 4B). Gal-3 stained weakly in the SCT with mean IRS=0.8±1.4 (Fig. 3C), but the CTB cell layer showed a clear moderate staining. Gal-4 was expressed with a mean IRS=7.1±2.9 (Fig. 3D), with a stronger signal in the SCT than in the CTB. With a mean IRS=9.5±4.2 (Fig. 3E) gal-7 was expressed in the syncytium. Gal-8 as well as gal-10 showed strong expression levels both in SCT and CTB with mean IRS values for gal-8 with IRS IRS=11.4±3.3 (Fig. 3F) and for gal-10 with IRS =11.1±3.6 (Fig. 1H), respectively. Gal-9 was expressed by mean IRS=4.9±4.6 (Fig. 3G). Gal-9 staining was more prominent at the SCT layer and brush border (Fig. 3G), and Gal-12 was moderately expressed with a mean IRS of 8.3±3.5 (Fig. 3I). Moreover, cell nuclei stained strongly for gal-12

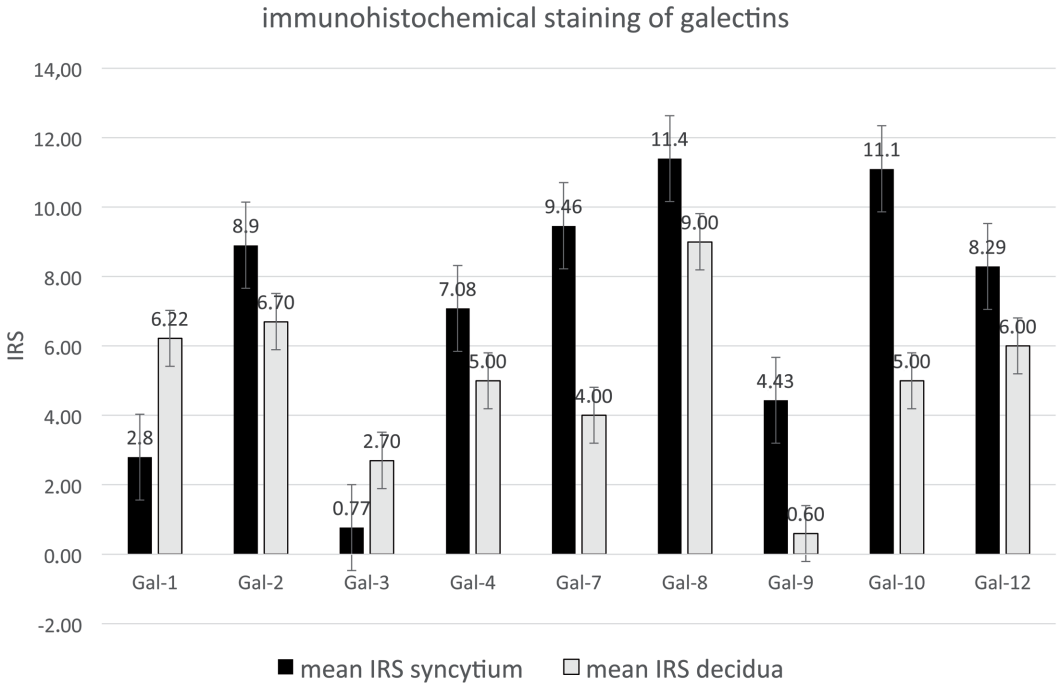

Fig. 6. Statistical overview over immunohistochemical staining of galectins in first trimester placenta. The mean IRS score is shown (product of optical staining intensity (0-3) and rating of percentage of positive stained cells (0-4)), differentiated for examination of decidua and syncytiotrophoblast.

antibody.

Evaluation and identification of galectin expressing cells at the feto-maternal interface

Maternal decidual stroma cells and invading fetal EVT cells can be found in decidua. The varying staining pattern and strength of different galectins in decidua is illustrated in Fig. 4. Staining of gal-1 in decidua (Fig. 4A) was very intense. Here, the cells showed a moderate expression with an IRS=6.2±0.4. Gal-2 was expressed with a mean IRS=6,7±4. Staining was also found in nuclei of the decidual cells (Fig. 4B). Gal-3 showed a statistically stronger staining in the decidua than in syncytium, however only to limited extent (IRS=2.7±3.7) (Figs. 3, 4C, 6). Gal-4 is expressed with a mean IRS=5.0±2.1, mainly in the cytoplasm of stained cells (Fig. 4D). These cells were identified in immunofluorescence as EVT (Fig. 5D). Gal-7 was mostly expressed in the cytoplasm of the decidua with a mean IRS=4.0±1.4 (Fig. 4E). Gal-8 was expressed with IRS=9.0±5.1 and showed strong staining in the nuclei and membrane border (Fig. 4F). Gal-9 antibody stained very weakly in decidua with mean IRS=0.6±1.0 (Fig. 4G). Gal-10 staining was moderate with IRS=5.0±2.3 in the cytoplasm, but more pronounced in the nuclei (Fig. 4H). With IRS=6.0±2.2, gal-12 also stained moderately in cytoplasm and additionally we observed some staining in the nuclei (Fig. 4I).

With double immunofluorescence staining, different cell populations (EVT and decidual stroma cells) of decidua were identified by double positive or single positive staining patterns. We used CK7 or HLA-G as markers for extravillous trophoblast cells depending on staining quality. Therefore, cells co-expressing CK7 or HLA-G and galectin proved to be EVT cells (Ristich et al., 2005).

In first trimester placentas, we found gal-1 coexpressed with HLA-G (Fig. 5A), as well as gal-2 (Fig. 5B). Gal-3 and gal-4 were expressed in HLA-G stained cells (Fig. 5C,D). Not all of the HLA-G stained cells expressed these galectins, especially in the case of gal-4/HLA-G (Fig. 5D). Gal-7 was coexpressed with HLA-G mostly in the cytoplasm and only in some cells (Fig. 5E). Gal-8 was coexpressed with HLA-G, too (Fig 5F), but gal-9 was only expressed in a few cells with HLA-G (Fig. 5G). Gal-10 staining can be shown in nearly every HLA-G expressing cell (Fig. 5H). Gal-12 is also co-expressed with HLA-G in nearly all cells of the visual field (Fig. 5I). In summary, all of our tested galectins were co-expressed with HLA-G, therefore leading to the conclusion that they are produced in extravillous trophoblast cells.

A synopsis of all investigated galectins in immunohistochemistry with mean IRS scores in decidua and SCT is shown in Fig. 6.

Galectin mRNA expression in freshly isolated cytotrophoblast cells and after onset of spontaneous fusion in vitro

We determined galectin mRNA expression with a real-time PCR on freshly isolated CTB cells directly after legal termination of pregnancy and isolation of trophoblast cells from the placenta. It is known that CTB cells spontaneously start to fuse *in vitro* (Fischer et al.,

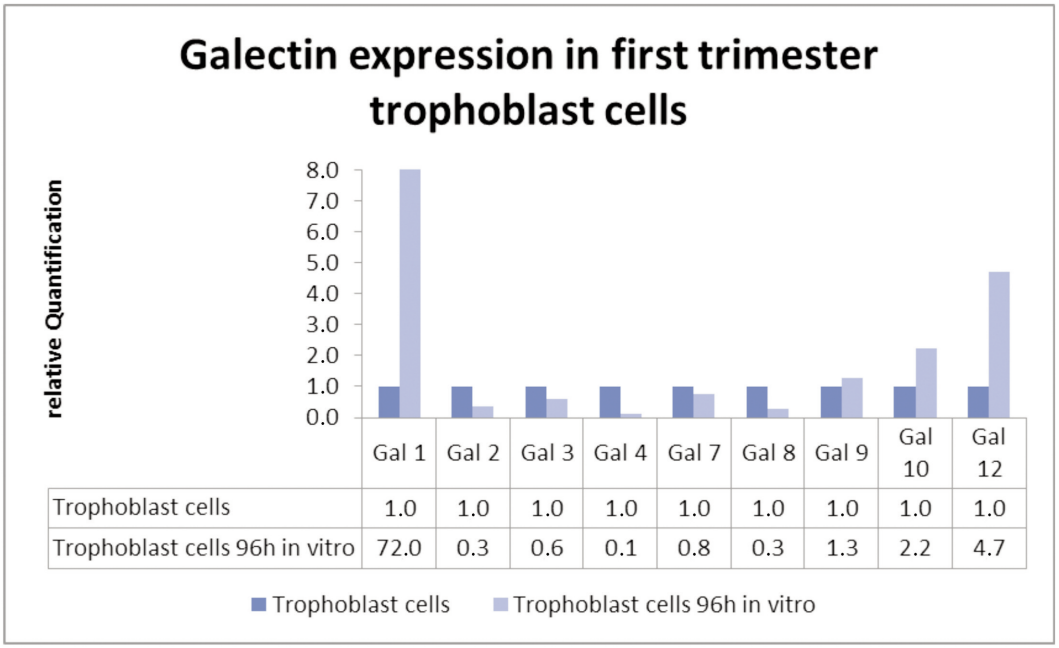

Fig. 7. Expression of mRNA of galectins in first trimester trophoblast cells. Blue columns show galectin expression in freshly isolated trophoblast cells. Light blue columns show galectin expression 96h after *in vitro* growth of trophoblast cells with onset of syncytium formation. Relative Quantification was shown with $2^{-\Delta Ct}$.

2010). Therefore, galectin mRNA expression with a real-time PCR was measured after 96h cultivation without additional stimulation. After that time, trophoblast cells in culture already started formation of a syncytium (Knofler and Pollheimer, 2013). So *in vitro* syncytium building and therefore galectin mRNA expression levels can be observed and correlated to the unfused state. The equation $2^{-\Delta\Delta Ct}$ shows the inverse number of cycles for a significant raising of the RNA-parts in comparison to an endogenous control. The expression of gal-1, 9, 10, and gal-12 increased after 96h incubation *in vitro* at mRNA level, while the rest of the investigated galectin mRNA decreased. Stronger differences can be seen in expression of gal-1. A summary of the results is presented in Fig. 7.

Discussion

The functions of galectins in term placenta have been studied especially with regard to gal-1 and gal-3 (Vicovac et al., 1998; Blois et al., 2007; Jeschke et al., 2013). Gal-1 is known as a regulator of cell apoptosis, cell differentiation and hormone synthesis. It is described as a pivotal regulator of the fetomaternal tolerance and might have potential therapeutic implications in threatened pregnancies, especially in trophoblast differentiation, invasion and in early pregnancy loss (Jeschke et al., 2006, 2010; Blois et al., 2007). Diminished levels of circulating gal-1 correlate positively with subsequent miscarriage and make gal-1 considerable as a predictive marker for spontaneous abortion (Tirado-Gonzalez et al., 2013). We have now investigated galectin expression in placental tissue in the first trimester.

For gal-1, in the first trimester placenta a weak immunohistochemical staining in SCT, but stronger staining in CTB cells (Fig. 3A) and extracellular matrix of the columns was found (insert Fig. 3A). In previous studies, expression of gal-1 in first trimester placenta was located to the CTB of middle and distal cell columns differentiating toward fully invasive trophoblast, while the SCT was not immunoreactive against gal-1, which supports our findings (Vicovac et al., 1998; Tirado-Gonzalez et al., 2013). Gal-1 was described in the EVT in previous studies (Ramhorst et al., 2012). In addition, expression of gal-1 in the EVT was very strong in our study. As it was shown recently, gal-1 is co-expressed with HLA-G and modulates and up-regulates its expression (Tirado-Gonzalez et al., 2013). This interaction is described for circulating gal-1, but not for expression of gal-1 in the placenta so far. The effect of high gal-1 levels on the expression of HLA-G, which we used as a EVT-marker in immunofluorescence remains unclear.

In PCR, gal-1 expression in CTB cells increased after 96h incubation *in vitro*, matching recent knowledge on increasing gal-1 expression in trophoblast cells during first trimester e.g. for syncytium-building (Fischer et al., 2010; Tirado-Gonzalez et al., 2013). In term placenta, gal-1 was found in higher expression for cases of severe preeclampsia than in normal placentas (Than et al., 2008). However, in preeclampsia a decreased fraction of peripheral blood T and NK cells expressing gal-1 was found (Molvarec et al., 2011). This is further evidence for the central role of gal-1 in immunotolerating processes in early pregnancy and led to the proposal that gal-1 bound to circulating peripheral blood lymphocytes might be involved in the establishment of a successful pregnancy (Molvarec et al., 2011). However, especially for first trimester, expression data in the placenta for development of preeclampsia or other gestational diseases are lacking. As in regular pregnancies gal-1 levels increase to a maximum at the second trimester. In the case of gestational diabetes mellitus (GDM), gal-1 was not increasing in this stage of pregnancy (Blois et al., 2014).

Gal-2 is described to bind on beta1-integrins on T-cells (or a closely associated glycoprotein), which can result in apoptosis of activated T cells or show cell specific responses on neutrophils (Ozaki et al., 2004; Sturm et al., 2004; Stowell et al., 2007). In placental tissue of the first trimester investigations are lacking. We found expression of gal-2 in first trimester placenta SCT with strong staining at the brush border to the intervillous space. However, in PCR gal-2 mRNA expression decreased during 96h cultivation in cell culture. EVT cells were also positive for gal-2, including nuclei, but with lower levels in comparison to the trophoblast. With immunoflourescence, we could prove co-expression with HLA-G, a trophoblast marker, but no nuclear staining. Thus, it will take further investigations about signal pathways in the nucleus and in cytoplasm. The strong immunohistochemical staining in both extravillous and villous trophoblast may be evidence for the immunomodulating role of gal-2 on immune cells, also in feto-maternal context in placenta (Stowell et al., 2007). So far gal-2 expression is obviously decreased in third trimester EVT cells in cases of PE on protein and mRNA level. Further implications for gal-2 research could be the involvement in spiral artery transformation in PE (Hutter et al., 2015).

Gal-3 is formed by carbohydrate recognition and collagen like domains. It interacts mostly with cell and extracellular matrix by modulating adhesive effects. Lack of gal-3 in cells results in poor interaction with extracellular matrices, especially in epithelial tissue (Jeschke et al., 2013). It is required for implantation in endometrium of mice (Yang et al., 2012). Additionally to these wide findings, we have observed weak gal-3 expression in healthy human first trimester placentas (7^{th}-14^{th} week) in immunohistochemistry. Staining appeared in both EVT and CTB/SCT. In PCR, mRNA expression levels in trophoblast cells decreased to a small extent after 96h cultivation (Fig. 7). In decidua, gal-3 was co-expressed with HLA-G as trophoblast marker, thus indicating gal-3 expression in the EVT. An inverse correlation between abundance of gal-3 expression in the human placenta and trophoblast

invasiveness has been further described for the course of gestation: reduced trophoblast invasion in pathologic pregnancies complicated with preeclampsia and HELLP syndrome may lead to compensatory elevated levels of gal-3 in EVT (Bozic et al., 2004; Jeschke et al., 2007). Moreover, gal-3 along with gal-1 is described as a factor for angiogenesis in early pregnancy and preeclampsia (Blois et al., 2015).

Important ligands for gal-4 on the cell surface are suggested to be sulphated glycosphingolipids (SB1a) and carcino embryonic antigen (CEA) (Ideo et al., 2005). So far, gal-4 is described in a serial analysis of gal-family expression in human placenta (Than et al., 2009). In a rat placenta trophoblast model, expression of gal-4 has been shown to be distributed in decidual and fetal tissue. Further results suggest down-regulated gal-4 expression during trophoblast differentiation (Arikawa et al., 2012). In our immunohistochemical study in healthy human first trimester placentas, gal-4 stained moderately in decidua and villous trophoblast. After differentiation of decidual staining, we detected gal-4 in the extravillous trophoblast in co-expression with HLA-G. Expression in trophoblast cells could also be shown in PCR. On mRNA level, gal-4 expression decreased in a small range after 96h cultivation. Similar findings were published by Arikawa et al. (2012). As we assume a spontaneous fusion of trophoblast cells, this little decrease of gal-4 might play a role in syncytium building and invasion of trophoblast into maternal tissue (Arikawa et al., 2012).

Recently, gal-7 was described in endometrium of first trimester placenta with immunohistochemistry, especially in SCT in the placenta and in EVTs in the cell column (Menkhorst et al., 2014b). Gal-7 also plays a role in menstrual cycle in terms of endometrial epithelial wound repair *in vitro* (Evans et al., 2014). In our study of first trimester placentas after induced abortion, gal-7 was also found in the SCT, but also in the decidua with less staining. At mRNA level, expression of gal-7 could only be detected by a custom made designed primer. As immunohistochemistry showed a clear staining for gal-7 antibody and has an intact negative control, we assume that the commercial LGALS-7 primer detected a gene region, which might be spliced in CTB in this stage of pregnancy (Liu et al., 2002). After 96h of cultivation without any stimulation the expression decreased nearly imperceptibly. We assume that the adhesive potential of gal-7 might have an influence on syncytium-building and immune tolerance during invasion of the trophoblast and decidualization.

Gal-8 shows a broad specificity for leukocytes and induces cell adhesion based on its di- or multivalent structure (Nishi et al., 2003; Zick et al., 2004). It was observed with intense expression in colon tissue and different cancer tissues (Nagy et al., 2002). Furthermore, gal-8 has been reported in villous and extravillous CTB in immunohistochemistry and under cell culture conditions (Kolundzic et al., 2011). Therein, staining of gal-8 in SCT was less intense than in proximal CTB whereas in EVT and decidual stroma cells, gal-8 staining was moderate to strong (Kolundzic et al., 2011). In our study, we similarly detected strong gal-8 staining in first trimester placenta tissue samples. Moreover, co-expression with trophoblast marker HLA-G was shown in decidual cells, but not exclusively (Fig. 5E), since gal-8 is also expressed by maternal decidual cells. In PCR, gal-8 expression in CTB cells was a little decreased after 96h cultivation. As our results refer to intracellular expression, it can be expected that gal-8 has intra- and extracellular effects on trophoblast cells. So far influence on cell growth and cell adhesion depending on the extracellular context i.e. binding special integrins is known (Hadari et al., 2000; Kolundzic et al., 2011).

Gal-9 decreases the levels of pro-inflammatory cytokines like IL-17, IL-12 and IFNγ. Moreover, it especially affects T-cells by inducing differentiation of naive T-cells to Treg cells and inhibition of the development of Th17 cells (Imaizumi et al., 2002, 2003; Popovici et al., 2005; Seki et al., 2008). Furthermore, gal-9 is also described to be expressed in human endometrial epithelium called uterodomes (Shimizu et al., 2008) and in endometrium, especially in late-secretory phase and with highest levels in early decidua (Popovici et al., 2005). There, gal-9 is overexpressed in the window of implantation (Popovici et al., 2005; Shimizu et al., 2008). In contrast, we have found low expression in first trimester decidua from 7th week on (Figs. 4, 6). In immunoflourescence, a few cells were co-expressing trophoblast marker HLA-G and gal-9, therefore supporting former observations of gal-9 (Shimizu et al., 2008). This lower expression after the window of implantation can be seen as a lower necessity of an anti-inflammatory reaction in the decidua and the suspected immunomodulatory function of gal-9 appears not to be required. At mRNA level in real-time PCR LGALS-9 expression shows a slight increase after 96 h of cultivation (Fig. 7). Consequently, we can state that gal-9 is expressed in villous and extravillous trophoblast cells in first trimester placenta.

Gal-10, also known as eosinophil Charcot-Leyden crystal protein (CLC), is similar to the structure of gal-13/PP-13 (Than et al., 1999). It was described to be essential for the functional properties of CD25(+) Treg cells and for differentiation of neutrophils (Abedin et al., 2003; Kubach et al., 2007). Gal-10 has been described in colon with different diseases and eosinophilic airway diseases (Agesen et al., 2011; Chua et al., 2012; Kwon et al., 2012). There are only few data available on the expression of gal-10 in the placenta, but recently LGALS-10 as part of Chr19 cluster galectin genes was described to be induced in trophoblast differentiation (Than et al., 2009, 2014). In first trimester placenta, we found a very strong staining in the SCT and to a lesser extent, though still moderate, in the decidua. We could show a coexpression of gal-10 with HLA-G in the decidua, which proves gal-10 expression in EVT. In PCR, gal-10 expression increased slightly after 96 h of cultivation. As syncytium-building happens during this

time, it can be assumed that in this process the functions of gal-10 described above may have an influence. As gal-10 is interacting with known inhibitors of eosinophil lysophospholipase activity it may have an influence on inflammatory and immunological diseases, as shown in the case of eosinophilic airway diseases (Ackerman et al., 2002; Chua et al., 2012). Therefore an immunomodulatory role can be assumed for this galectin.

Gal-12 is known in peripheral blood leukocytes and adipocytes (Jeschke et al., 2013). It seems to be required for differentiation of adipocytes with regard to signal transduction and induction of adipogenic factors (Hotta et al., 2001). So far, no findings about a role of gal-12 in human reproduction tissues have been described. In our study, we showed expression of gal-12 in first trimester placenta: In immunohistochemistry, gal-12 was highly expressed in villous trophoblast cells and slightly weaker in decidua and EVT, respectively, because of co-expression of gal-12 and HLA-G. In real-time PCR, increased levels after 96h of cultivation could be revealed. This raises the question, whether gal-12 is expressed in trophoblast cells or if gal-12 may play a role in cell fusion and syncytium building, because of increasing levels in cultivation and higher staining levels in immunohistochemistry in SCT, which requires further investigation.

Conclusion

We could reveal immunohistochemical staining of galectins 1-4, -7, -8, -10 and -12 in first trimester placenta, but with different staining strengths and patterns and in different compartments. These findings were confirmed for the same pattern at mRNA level in HVT. Gal-1, 2, -3, -4, -7, -9, -8, -10 and -12 were identified to be expressed in extravillous trophoblast cells (EVT). Regarding the recently discovered immunomodulatory functions of galectins it appears highly important to further characterize regular galectin expression patterns for all stages of pregnancy. More thorough investigations on expression patterns of galectins in gestational diseases would allow the identification of their functions concerning these pathologies. Recurrent spontaneous abortions, gestational diabetes, preeclampsia and HELLP syndrome are possible fields of interest.

Acknowledgements. This study was funded and supported by the Medical Faculty of the University of Munich and the Department of Gynecology and Obstetrics, Ludwig Maximilians University of Munich.

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Accepted February 22, 2016