Galectin-3 deficiency in pregnancy increases the risk of fetal growth restriction (FGR) via placental insufficiency

Nancy Freitag1,2, Irene Tirado-Gonzalez1, Gabriela Barrientos3, Katie L. Powell4, Philipp Boehm-Sturm5,6, Stefan P. Koch5,6, Kurt Hecher7, Anne C. Staff8,9, Petra C. Arck7, Anke Diemert7 and Sandra M. Blois1,7

Abstract
Fetal growth restriction (FGR) is the most common pregnancy complication in developed countries. Pregnancies affected by FGR, frequently concur with complications and high risk of neonatal morbidity and mortality. To date, no approved treatment is available for pregnant women affected with FGR. The objective of this study was to investigate the contribution of galectin-3 (gal-3), a β-galactoside binding protein involved in pregnancy, placental function and fetal growth. We demonstrated that lack of gal-3 during mouse pregnancy leads to placental dysfunction and drives FGR in the absence of a maternal preeclampsia syndrome. Analysis of gal-3 deficient dams revealed placental inflammation and malperfusion, as well as uterine natural killer cell infiltration with aberrant activation. Our results also show that FGR is associated with a failure to increase maternal circulating gal-3 levels during the second and third trimester in human pregnancies. Placentas from human pregnancies affected by FGR displayed lower gal-3 expression, which correlated with placental dysfunction. These data highlight the importance of gal-3 in the promotion of proper placental function, as its absence leads to placental disease and subsequent FGR.

Introduction
During the establishment of a mammalian pregnancy, the placenta plays a critical role in controlling fetal-maternal resource allocation and mediating fetal programming of future disease. Placental function depends on a healthy decidual environment, and inadequate endometrial receptivity or inappropriate implantation increase the incidence of anomalous placentation. Defects in placentation often result in fetal growth restriction (FGR), one of the most complex pregnancy complications without currently available treatment aside delivery, most often preterm1. Affecting up to 8% of pregnancies worldwide, FGR is also associated with significant perinatal complications, such as increased risk of stillbirth, neonatal long-term morbidity, obesity, type 2 diabetes, and coronary disease later in life1–4. When not attributable to structural or genetic defects of the fetus, FGR is primarily caused by placental insufficiency5,6, but the biological processes that promote placental insufficiency and the subsequent progression to FGR are currently poorly understood. To date, no treatment options are available1.

Galectins, a family of soluble glycan-binding proteins are increasingly recognized as powerful modulators of pregnancy-associated processes including proper placent development. Among the galectin family members, the chimera-type galectin-3 (gal-3, encoded by the Lgals3 gene) is highly expressed at the fetal-maternal interface7–10. Gal-3 knock-down in mouse endometrium results in
substantially less implanted embryos, and dysregulation of gal-3 is associated with several obstetrical complications resulting from placental dysfunction. These include preeclampsia (PE), hemolysis elevated liver enzymes and low platelets (HELLP) syndrome, small-for-gestational age, and gestational trophoblastic disease. In the placental compartment, lack of gal-3 compromised placental vascularization and perfusion resulting in placental insufficiency and the subsequent development of asymmetric FGR in mice. Using the prospective birth cohort PRINCE (Prenatal Determinants of Children’s Health), we showed that development of FGR is accompanied by an altered kinetics of circulating maternal gal-3 levels during gestation. We also found that placental gal-3 expression is downregulated in human pregnancies complicated with FGR, demonstrating that gal-3 also plays a role in human FGR pathology. Finally, using reciprocal matings, we showed that gal-3 within the maternal compartment is required for proper placental development and fetal growth. Our findings identify gal-3 as a key component of the molecular program of decidu/placental development and offspring health, as well as a potential target for future strategies aimed at minimizing adverse outcomes in pregnancies at high risk for FGR.

Results
Gal-3 deficiency in pregnant mice leads to FGR
To evaluate the contribution of gal-3 during gestation, we analysed pregnancy outcome and fetal growth in gal-3 deficient mice. Although gestational length was similar between gal-3 wild type (Lgals3+/+) and deficient (Lgals3−/−) dams, gal-3 deficient dams displayed an increased frequency of fetal demise when compared to their wild type counterparts (Fig. 1a), which is in agreement with observations from other group. Fetuses carried by Lgals3−/− dams showed a weight reduction of 20–40%, compared with Lgals3+/+ fetuses during pregnancy.
pregnancy, assessed on embryonic day (E13 and E17 (Fig. 1b). The reduced fetal weight was accompanied by a delay in fetal development, as the majority of Lgals3−/− fetuses only reached the Theiler stage (TS)21 on E13, whereas Lgals3+/− offspring reached the TS22, which is appropriate for the gestational age. The immaturity of gal-3 deficient fetuses was still present on E17, evident as fewer skin wrinkles, smaller whiskers, and eyes visible through the eyelids corresponding to TS25 (Fig. 1b). In addition, we confirmed that Lgals3−/− fetuses suffered from asymmetric FGR, as denoted by an increased brain-to-liver weight ratio (Fig. 1d). The reduction of fetal weight carried over into the postnatal period, as Lgals3−/− offspring also showed a significantly reduced body weight until P14 (Fig. 1c). Upon weaning at 3 weeks of age, both Lgals3−/− and Lgals3+/− offspring displayed similar body weights (Fig. 1c).

Lack of gal-3 during murine gestation is linked to features of placental insufficiency

Since asymmetric fetal growth primarily arises from placental pathologies15,16, we next investigated whether the lack of gal-3 causes placental insufficiency. We observed reduced placental weight on E13 and E17 in Lgals3−/− mice (Fig. 1e). Furthermore, in contrast to wild type dams, the placental disc in E10 and E13 Lgals3−/− placentas displayed reduced trophoblast layers with a correspondingly enlarged maternal decidua (Fig. 1e). Improper placent al development in Lgals3−/− mice was accompanied by a significantly increased abundance of uterine NK (uNK) cells in the decidua. Here a higher frequency of uNK cells contained cell-free, perforin-reactive granules (Fig. 1f). As shown in Fig. 1g, gal-3 deficiency was associated with a significant reduction in P4 levels. In line with this finding, mRNA expression of inflammatory cytokines (e.g. INF-γ, TNF-α, and IL-17) was significantly increased in Lgals3−/− decidual tissue (Fig. 1h).

Placental gal-3 deficiency may also affect trophoblast differentiation during placentaetion. To address this possibility, we assessed the expression of trophoblast cell markers between E10 and E13. Our analysis revealed altered expression of the trophoblast Junb, Gab1, and Gcm1 genes, a phenomenon that likely contributed to the failure of placental labyrinth differentiation in Lgals3−/− placentas (Fig. 2a). To further examine the placental labyrinth structure, we performed morphological and functional magnetic resonance imaging (MRI) during E13. As illustrated in Fig. 2b, placental volume was reduced in Lgals3−/− compared to Lgals3+/− implantations. After injection of an albumin-bound contrast agent, initial contrast enhancement was significantly higher for Lgals3−/− mice but contrast material dynamics described by rate of enhancement were similar (Fig. 2c). Interestingly, the variability of both parameters was much higher in Lgals3−/− animals. Finally, we used Isolectin B4 staining to further characterize the Lgals3−/− placental labyrinth zone. As expected, gal-3 deficiency resulted in reduced placental labyrinth total vessel length and vessel area (Fig. 2d).

Reduced maternal levels of gal-3 are linked to human FGR

We next sought to assess the translational relevance of our findings in mice and determined gal-3 levels in human pregnancies using biological samples from two independent patient cohorts. In a subgroup of women participating in the prospective pregnancy cohort PRINCE carried out in Germany (clinical data shown in Supplementary information), we observed that serum gal-3 levels continuously increased in normally progressing pregnancies (n = 33) (range 3.02–16.70 ng/ml, Fig. 3a). Conversely, in pregnant women affected by FGR (n = 32), levels of serum gal-3 were significantly lower as early as the first trimester and levels stalled throughout gestation (range 1.00–9.79 ng/ml). In order to assess the interaction between serum gal-3 levels in human pregnancies and placental function, we analysed its correlation with uterine artery perfusion (Ut PI). As suspected, gal-3 peripheral levels were found to correlate inversely with Ut PI values (r = −0.5065, P < 0.05) in pregnancies complicated with FGR. In addition, we used placental biopsies taken at birth from uneventful (n = 27) and FGR pregnancies with placental pathology (n = 19) in an independent cohort recruited in Australia (clinical data shown in Supplementary information18). Similar to our observations in mice, FGR placentas expressed decreased protein levels of gal-3 in general as revealed by western blot (Fig. 3b), or when specifically assessed within the extravillous CK7+ trophoblasts (Fig. 3c, Supplementary Fig. 1).

Since placental insufficiency is not exclusive to FGR, we wondered whether gal-3 dysregulation also occurs in pregnancies complicated by preeclampsia. Placental expression and circulating levels of gal-3 in preeclamptic women (n = 36) from the Oslo study were not different from those of uneventful pregnancies (n = 23) (Supplementary Fig. 2a–d; clinical data shown in Supplementary information). Supporting this, gal-3 deficient mice did not develop preeclampsia-like features (increased blood pressure, new-onset proteinuria, production of Angiotensin II receptor type 1 autoantibody (AT1AA) or circulating anti-angiogenic factors (Supplementary Fig. 2e–h). In addition, no differences were found in E10 and E13 decidual spiral artery wall thickness between WT and KO dams (Supplementary Fig. 2i). We conclude that gal-3 deficiency is involved in the placental-fetal axis and confined to the fetal phenotype downstream of placental pathology without significantly affecting maternal vascular health in pregnancy.
Maternally-derived gal-3 drives proper placental function and fetal growth

We next investigated whether maternal gal-3 plays a role in placental and fetal development. As shown in Fig. 3d, absence of maternal gal-3 increased fetal demise. Most notably, we observed that the body weight of Lgals3<sup>−/−</sup> fetuses from Lgals3<sup>−/−</sup> dams (maternal KO, mKO) was significantly reduced compared to Lgals3<sup>+/−</sup> fetuses carried by Lgals3<sup>++/−</sup> dams (maternal KO, pKO). Also, an immature Theiler stage (TS21), characterized by a backwards pinna and unseparated fingers (Fig. 3e), was detected in fetuses from Lgals3<sup>−/−</sup> dams. In addition, Lgals3<sup>−/−</sup> implantations carried by Lgals3<sup>−/−</sup> dams displayed a decreased fetal/placental weight ratio, indicative of placental insufficiency (Fig. 3f), along with a reduction of PAS<sup>+</sup> glycogen cells in the junctional zone (Fig. 3g, Supplementary Fig. 3a). We also identified an increased uNK cell abundance in the decidua of Lgals3<sup>−/−</sup> dams, compared to the reciprocal matings (Fig. 3g, Supplementary Fig. 3b). These findings highlight that
maternally-derived gal-3 maintains placental function. If maternal gal-3 expression is low, the incidence of developing FGR is increased.

Discussion

FGR remains one of the “great obstetrical syndromes”, and is a leading contributor worldwide to perinatal mortality and morbidity, as well as playing an important role in mediating non-communicable diseases in adult life, including cardiovascular disease, diabetes, metabolic syndrome, and obesity. Although significant advances have been made on its understanding, there is still no definitive cure or treatment for pregnancies complicated by FGR. Thus, the current knowledge gap emphasizes the need for a better understanding of how fetal development is normally achieved and how it is dysregulated in FGR.

Since normal placental development and functional integrity are essential for normal fetal growth, the identification of alterations during critical windows of placental development could help to optimize antepartum monitoring and timely delivery of FGR infants. Our study unveils that a dysregulation of gal-3, especially at the maternal compartment, is a significant factor involved in abnormal placentation and development of FGR. This phenotype appears to be mirrored in human FGR patients, suggesting that gal-3 can assert similar effects in the human placenta.

Galectin-3 was initially reported to be a factor related to endometrial receptivity due to its increased expression in the endometrium and trophoblast during embryo...
implantation. Subsequent studies have revealed that endometrial gal-3 is involved in embryo implantation and dysregulation of placental gal-3 is associated with adverse pregnancy outcomes. The present study demonstrates that gal-3 is critical for orchestrating healthy fetal-maternal interface interactions and its dysregulation results in asymmetric FGR. Affecting approximately 70% of growth-restricted fetuses, the asymmetric type of FGR occurs when brain growth and maturity is maintained while the visceral organs (especially the liver) have relatively reduced weights, and represents a later timing of embryonic damage that results from maternal vascular factors and/or placental insufficiency. The fact that gal-3 deficiency-induced asymmetric growth restriction followed by relatively rapid catch-up growth is of significant importance since human babies with these characteristics, particularly those with accelerated postnatal weight gain after in utero growth restriction, have a greatly enhanced risk of developing type 2 diabetes, obesity and cardiovascular disease.

Studies in gal-3 deficient dams enabled us to identify this chimera lectin as a key player in regulating decidualization and maternal immune adaptation during early gestation. The absence of gal-3 per se induced decidual enlargement, inflammatory cytokine gene expression, and NK cell infiltration with aberrant activation. Supporting our observations, these features were also found in a different mouse FGR model established by independent researchers. Gal-3 is known to regulate immune responses, acting as a pro-inflammatory signal with diverse innate immune cell targets to promote their activation, degranulation, and cytokine production. In this context, recent in vitro studies have shown that endogenous expression of gal-3 in human NK cells can be stimulated by activating cytokines (IL-2, IL-15) and correlates functionally with their degree of cytotoxic degranulation. While similar functions remain to be examined in the context of pregnancy, the identification of an endogenous gal-3 ligand localizing specifically to mouse uNK perforin granules implies a potential role of this lectin in controlling the activation status of this particular lymphocyte subset, which has been ascribed important roles in the modulation of decidual development, trophoblast invasion, and maternal vascular remodeling during early pregnancy.

Gal-3 deficiency impacts placental function by means of intrinsic effects on trophoblast biology. Specifically, we showed that gal-3 deficiency during gestation alters trophoblast differentiation, vascularization of the labyrinth zone and subsequently causes changes in placental perfusion. While the structural organization of the fetal-placental interface differs between human and mouse (villous versus labyrinthine placenta), the syncytiotrophoblast separates fetal and maternal blood in both species and serves analogous functions. Therefore, it is not surprising that gal-3 deficiency compromised the labyrinth layer during mouse placentation. Since inflow and clearance of the contrast agent strongly depend on perfusion, we speculate on more heterogeneous maternal circulation in gal-3 deficient compared to control placentas. Indeed, gal-3 deficiency caused reduced total vessel length and vessel area in the placental labyrinth, suggesting that absence of this lectin during gestation alters vascularization of the labyrinth and subsequently results in placental malperfusion. On the other hand, maternal deficiency of gal-3 also provoked alterations in placental structure, particularly in glycogen cells of the junctional zone, emphasizing the importance of an interplay between fetal and maternal sources of gal-3 expression for normal placental development. Alterations in placental energy storage (glycogen cells) are a typical feature of FGR models and likely to contribute to the detrimental maternal effect on fetal growth. A reduced placental glycogen reserve will definitely impact on placental well-being and fetal growth as the main source of energy for the placenta comes from glucose and also, the glycogen stores may be destined to the fetal demands. In addition, glycogen trophoblasts may serve other functions likely to impact fetal growth, as these cells invade the decidua congregating near spiral arteries to support their remodeling and also have an endocrine role, being the source of several mediators including retinoic acid, prolactin-like hormones and particularly IGF-2, a crucial modulator of placental and fetal growth.

Finally, our results showing dysregulated gal-3 expression in human pregnancies affected by impaired fetal growth are consistent with the role played by this lectin in pregnancy orchestration as demonstrated in our mouse studies. Particularly, the fact that besides sharing a common placental histopathology, FGR and PE placentas differed in their pattern of regulation of gal-3 expression is of great importance. Our clinical and experimental findings demonstrated that gal-3 loss of function is not sufficient to provoke a maternal PE-like syndrome in mice, and women with both early and late-onset PE showed no alterations in gal-3 expression. Because gal-1 and gal-3 are the most prominent galectins associated with critical processes in early gestation, we hypothesize that unique properties exerted by gal-3 in supporting placental development, fetal growth, and pregnancy cannot be substituted by gal-1. Our study is clinically relevant as it provides important insights into the role of gal-3 during gestation, highlighting its requirement for proper placental development and function and fetal growth. It also reinforces the concept that unique functional properties in support of healthy pregnancy are specific to each of the different members of the placental galectin network.
**Materials and methods**

**Mice**

C57BL/6 Lgals3+/+ and Lgals3−/− mice were purchased from Jackson Laboratories. The presence of a vaginal plug after mating was denoted as embryonic day (E) 0. Timed pregnant Lgals3+/+ and Lgals3−/− mice were evaluated at E7, E10, E13, E17, P4, and P21 (n = 7–9 mice per group) and approved by Charité and Berlin authority for Animal Use in Research and Education. A second experiment used Lgals3+/+ or Lgals3−/− females mated with Lgals3−/− or Lgals3+/+ males respectively to create the “paternal” (pKO) or “maternal” (mKO) gal-3 deficiency. On E13, after euthanizing the mice, the percentage of failed implantations was calculated (non-viable implantations/total implantation ×100) and whole implantation sites were frozen for histological sectioning and isolation of total protein or total RNA according to our published procedures. Embryos were fixed in Bouin’s solution and subsequently cleared in 70% v/v ethanol for body weight measurement and Theiler stage analysis. Placenta efficiency was calculated as “fetal/placental weight ratio” and used to measure nutrient transfer capacity from the placenta to the fetus.

**Human samples**

Three patient cohorts were used in this study. The prospective human low-Risk pregnancy cohort PRINCE (Prenatal Identification of Children’s Health, Ethics Committee: PV3694) is based at the university medical center in Hamburg, Germany. Since 2012 a total number of 721 women have been included during early pregnancy and examined a total of three times during the course of the pregnancy (14th, 24th, and 36th week of pregnancy). In addition to collecting demographic details and psychosocial factors, these prenatal study visits involved extensive ultrasound examinations of fetal growth and placental function, nutritional behavior, medication intake, and vaccinations during pregnancy. Blood was drawn at each of the three-time points.

For analyses of gal-3 expression in pregnancy affected only by FGR, patient samples from the research bio-bank collection at the Kolling Institute, Sydney, Australia were used. Use was approved by the Northern Sydney Local Health District Human research Ethics Committee, (St Leonards, NSW, Australia) and was assigned the site-specific assessment number 0912-348 M and the Australian national ethics application form number HREC/09/HARBR/165 as described. For analyses of gal-3 expression in pregnancy affected by preeclampsia, patient samples from the Oslo Pregnancy Biobank research collection at Oslo University Hospital, Oslo, approved by the Regional Committee of Medical Research Ethics in Eastern Norway, were used as described. Circulating soluble fms-like tyrosine kinase-1 (sFlt-1) and placenta growth factor (PIGF) levels were determined by commercial ELISA from R&D Systems following the manufacturer’s recommendations. The clinical characteristics of the human cohorts are summarized in Supplementary information.

**Histological analysis**

Serial paraffin-embedded uterine sections from Lgals3+/+ and Lgals3−/− mice at E13 were cut into 4 µm-thick sections and stained with hematoxylin-eosin (H&E), PAS, Masson-Goldner’s trichrome, Dolichos biflorus agglutinin (DBA) lectin/PAS and Isolectin B4 as previously described. Whole implantation sites were stained and digitally scanned by a high-resolution bright field and fluorescence slide scanner (Pannoramic MIDI BF/FL, 3DHISTECH Ltd.), and staining was evaluated on virtual slides using Pannoramic Viewer 1.15.4 (3DHISTECH Ltd.) by two examiners blinded to the pregnancy outcome.

**Magnetic resonance imaging**

MRI measurements for in vivo phenotype of Lgals-3-deficient mice at E13 were performed in a dedicated 7 T small animal scanner (Biomspec, Bruker BioSpin, Ettlingen, Germany). The imaging protocol was adapted from a previous study by Plaks and coworkers. Full details on the MRI protocol is provided in the Supplementary information.

**Galectin-3 ELISA**

Gal-3 concentrations in the serum of pregnant patients were determined by ELISA as described previously. Briefly, immunolon 2 ELISA plates (Dynatech Laboratories, USA) were covered with polyclonal antihuman gal-3 antibody (1 µg/ml; AF1154, R&D Systems, USA) and washed with washing buffer (0.5% Tween-20 in PBS). Plates were blocked with 3% BSA in PBS. Individual wells were incubated with serial dilutions of gal-3 (1154-GA, R&D Systems, USA) or serum samples (diluted 1/10) for 2 h at RT. Wells were washed and incubated with biotinylated polyclonal antihuman gal-3 antibody (0.25 µg/ml in PBS 0.1% BSA; BAF1154, R&D Systems, USA). Plates were washed six times and incubated with horseradish peroxidase (HRP)-conjugated streptavidin (Calbiochem, USA). After eight additional washes, a colorimetric reaction was developed with the 3,3′,5,5′-tetramethyl benzidine (TMB) substrate (Pierce Biotechnology, USA). The reaction was stopped by adding one volume of 4 N H2SO4. Absorbance at 450 nm was recorded. Each reported value is the mean of triplicate assays.

**Gal-3 western blotting**

Protein was extracted from frozen placental tissue and 10 µg of protein lysate was separated by SDS-PAGE as described previously. Immunoblotting was performed
for gal-3 (1:1000, Santa Cruz Biotechnology SC-20157) or β-actin (loading control) and protein expression was detected using the appropriate HRP-conjugated secondary antibodies (1:3000, Bio-Rad).

**Galecin-3 staining in human samples**

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded placental tissues from both normal and FGR pregnancies as previously described19. Slides were incubated with gal-3 (0.5 µg/ml; Santa Cruz Biotechnology sc-32790), cytokeratin 7 (0.09 µg/ml; Abcam ab68459) or isotype controls at equivalent concentrations, visualized using NovaRed peroxidase HRP substrate kit (Vector Laboratories) and staining quantitated using ImageJ.

**Statistical analysis**

Data are expressed as mean ± SEM. Mouse and human data were analysed using the non-parametric Mann–Whitney U-test. GraphPad Prism 8.0 (GraphPad Software, Inc.) was used to analyse the data and a P value <0.05 was considered as statistically significant.

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