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AT-rich interactive domain 1A protein expression in normal and pathological pregnancies complicated by preeclampsia

Sonia Fantone[1], Roberta Mazzucchelli[2], Stefano Raffaele Giannubilo[3], Andrea Ciavattini[3], Daniela Marzioni[1]*, Giovanni Tossetta[1,3]*

[1]Department of Experimental and Clinical Medicine, Università Politecnica delle Marche, Ancona, Italy

[2]Section of Pathological Anatomy, Department of Biomedical Sciences and Public Health, Università Politecnica delle Marche, United Hospitals, Ancona Italy

[3]Clinic of Obstetrics and Gynaecology, Department of Clinical Sciences, Università Politecnica delle Marche, Salesi Hospital, Azienda Ospedaliero Universitaria, Ancona, Italy

*These authors have contributed equally to this work

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Disclosure statement

No potential conflict of interest was reported by the author(s).

Ethic statement

The study was approved by the Ethics committee on investigations involving human samples (Università Politecnica delle Marche, Italy)

Consent statement

All patients gave their informed consent to participate to this study

Consent for publication statement

All authors have approved the final version of the article and gave their consent for publication

Abstract

AT-rich interactive domain 1A (ARID1A, as known as BAF250a) is a subunit of human Switch/sucrose nonfermentable chromatin remodeling complex with tumour suppressor function. Mutations of Arid1a have been reported in many human cancers and low expression of this protein has been correlated to a poor prognosis outcome in patients affected by some types of cancer. Although there are many studies regarding ARID1A functions in cancer, little is known about its role in regulating cell differentiation and normal tissues homeostasis. Here, we investigate ARID1A expression in normal placental tissues of first and third trimester of gestation and in pathological placental tissues of pregnancy complicated by preeclampsia (PE) and intrauterine growth restriction (IUGR) in order to evaluate a possible role of this protein in trophoblast differentiation. We found that ARID1A was specifically expressed in villous and extravillous cytotrophoblastic cells in normal placentas whereas syncytiotrophoblast was negative. Interestingly, ARID1A was expressed in both cytotrophoblastic cells and syncytiotrophoblast in placentas affected by PE and PE-IUGR. Moreover, ARID1A was also present in syncitial knots of pathological placentas. The present results indicate that ARID1A is a good marker of poor trophoblast differentiation in these pathologies because the significant high positive staining in syncytiotrophoblast nuclei may suggest a poor differentiation of this trophoblast layer due to the cytotrophoblast cells fusion with the syncytiotrophoblast overlaying before arresting their cell cycle.

Introduction

The placenta is an essential organ for normal *in utero* development in humans and mammals in general, playing different and essential functions during normal pregnancy (Costa 2016). Its multifaceted role has been demonstrated in different way by studying human pregnancy in normal and pathological conditions (Booker and Moroz 2019; Gurugubelli Krishna and Vishnu Bhat 2018; Phipps et al. 2019) and by using animal models, although the placenta varies from species to species in mammals (Carter and Enders 2016; Gundling and Wildman 2015; Knofler et al. 2019). The importance of placenta during pregnancy is highlighted when placental development is compromised, leading to the development of placental pathologies such as preeclampsia (PE) and intrauterine growth restriction (IUGR), two related but pathogenetically different placental disorders (Mayhew et al. 2007; Roberts and Post 2008). PE is a multisystem disorder that occurs in 5–7% of pregnancies (Rana et al. 2019). It is commonly diagnosed in the second half of pregnancy and it is clinically characterized by new onset of proteinuria and hypertension during pregnancy (Phipps et al. 2019; Rana et al. 2019). Although PE is diagnosed in the second half of pregnancy, placental impairment is due to a poor trophoblast invasion during the earliest stage of pregnancy (Fisher 2015; Ji et al. 2013; McMaster et al. 2004). Pregnancies complicated by PE have an increased risk of maternal and neonatal morbidity and mortality (Phipps et al. 2019; Rana et al. 2019). Moreover, PE is a leading cause of premature delivery and/or IUGR, term used to describe a foetus that has not

reached its potential growth in the uterus (Rasmussen and Irgens 2003; Sibai 2006). AT-rich interactive domain 1A (ARID1A, as known as BAF250a) is a protein of 250 kDa with tumour suppressor function and it is part of the Switch/Sucrose Non-fermentable (SWI/SNF) chromatin remodeling complex (Hargreaves and Crabtree 2011). This protein is widely expressed in the nuclei of many cells during cell cycle and cell differentiation (Flores-Alcantar et al. 2011; Nagl et al. 2007). Cancers where ARID1A expression was decreased or null have been associated with poor prognosis outcome (Wu and Roberts 2013). Although there are many studies regarding ARID1A expression in cancer, little is known about its role in regulating normal tissues homeostasis and cell differentiation. Interestingly, Hiramatsu et al, proved that ARID1A plays a pivotal role in regulating stem cell differentiation and self-renewal in normal intestinal tissues (Hiramatsu et al. 2019). Moreover, it has been proved that ARID1A protects hepatocytes by maintaining lipid and glucose metabolic homeostasis through PPARα and epigenetic regulation (Qu et al. 2019). In addition, Lei and colleagues showed ARID1A-mediated chromatin remodeling as pivotal epigenetic mechanism in cardiac progenitor cell differentiation (Lei et al. 2012). Since previous studies showed a pivotal role of ARID1A in regulating cellular homeostasis and differentiation, the aim of this study was to investigate ARID1A expression in normal and pathological placental tissues in order to evaluate a possible role of this protein in trophoblast differentiation of normal and pathological placentas.

Materials and methods

Tissue collection

The procedure for this study project complies with the last revision (2013) of the World Medical Association Declaration of Helsinki. All procedures were performed according to relevant national regulations and institutional guidelines. All patients gave their written informed consent and the permission of the Human Investigation Committee of Marche Region (IT) was granted (protocol number 2019.172; study ID 980; CERM number 172). In order to evaluate the expression of ARID1A in human placenta we analysed a total of 38 normal and pathological human placentas. Twenty normal placentas: 10 from first trimester (Obstetrics and Gynaecology of San Severino Hospital, MC, Italy), 10 from third trimester of gestation, 9 from gestations complicated by PE and 9 from gestation complicated by PE-IUGR (Department of Woman and Child Health, A. Gemelli Hospital, Università Cattolica Del Sacro Cuore Roma; Obstetrics and Gynecology, Department of Clinical Sciences, Polytechnic University of Marche, Ancona, Italy). First trimester samples were collected from clinically normal pregnancies interrupted by curettage (aspiration technique) for psycho-social and medical reasons that were unlikely to affect placental structure and function. Third trimester, PE and PE-IUGR gestation were terminated by caesarean sections. PE was defined as high blood pressure (systolic blood pressure ≥140 mm Hg and/or diastolic blood pressure ≥ 90 mm Hg on 2 occasions, at least 4 hours apart), developed after 20 weeks of gestation, with proteinuria (≥ 300 mg/24 hours or protein/creatinine ratio ≥ 0.3) (Roberts et al. 2013).

Intrauterine growth restriction was defined as estimated foetal weight by antenatal ultrasound below the 10th percentile for gestational age and gender, associated with abnormal umbilical and uterine artery (Gordijn et al. 2016) and confirmed by the new born birthweight. Patients with PE + IUGR presented with criteria aforementioned for both diseases. The gestational age estimation was based on the last menstrual period and validated by foetal ultrasound scanning before week 13 of gestation in the study groups (see table 1, Clinical characteristics of normal and pathological pregnancies).

Specific exclusion criteria for the control group included a history of hypertension, diabetes mellitus, cardiac disease, renal disease, thyroid and immunological disease and congenital or acquired thrombophilia disorders, and the presence of chromosomal and other foetal anomalies. Immediately after delivery and gross examination of the placentas, three zones were identified: the central one (near the umbilical cord insertion), the peripheral one (the most distal from the umbilical cord) and the intermediate one (between the others). Two placental tissue samples from each zone were then taken: - three placental tissue samples of approximately 2x2.5 cm were collected for immunohistochemistry and morphological analysis; - three placental tissue samples, were collected and immediately frozen in liquid nitrogen and stored at -70°C until molecular biology analysis.

Samples for immunohistochemistry and morphology were fixed in 4% neutral buffered formalin at 4°C for 12 h then washed in cold phosphate buffer pH 7,4 for 30 min. Thereafter the specimens were dehydrated via a graded series of ethyl alcohol (50°C for 30 min, 75°C for 30 min, 2 × 96°C for 75 min, 3 × 100°C for 75 min), and two steps in xylene for 60 min., at Room Temperature (RT). Then, samples were processed for paraffin embedding at 56°C. Paraffin-embedded tissue sections (4 μm) were cut and stretched at 45°C, allowed to dry and stored at 4°C until use.

Immunohistochemical analysis

Immunohistochemical staining was performed as previously described (Altobelli et al. 2017; Tossetta et al. 2016). Briefly, after dewaxing and rehydrating the paraffin sections via xylene and a graded series of ethyl alcohols (from 100 % to 50 %) at RT, sections were rinsed in phosphate buffered saline (PBS) and immersed in 0.1M Citrate buffer pH 6 and subjected to high temperature treatment for 10 min at 98°C. To inhibit endogenous peroxidase activity, sections were incubated with 3 % hydrogen peroxide (in deionized water) for 30 min at RT, rinsed with PBS and incubated with normal goat serum (Vector laboratories, Burlingame, CA) diluted 1:75 in PBS for 30 min at RT.

Sections were then incubated with the primary anti-ARID1A rabbit polyclonal antibody (#HPA005456, Sigma- Aldrich, St. Louis, MO) diluted 1:150 in PBS, incubated overnight at 4°C. After a thorough rinse in PBS, the antigen was visualized by using the streptavidin-biotin-peroxidase complex method (Vector Laboratories, Burlingame, CA, USA) using a biotinylated goat anti-rabbit secondary antibody diluted 1:200 in PBS for 30 min. 3', 3'- diaminobenzidine hydrochloride (Sigma-Aldrich, St Louis, MO, USA) was used as the chromogen. Sections were counterstained with

Mayer's haematoxylin, dehydrated, and mounted using Eukitt solution (Kindler GmbH and Co., Freiburg, Germany). Human prostate cancer tissue was used as positive control (Kim et al. 2012). For negative controls, nonimmune rabbit serum was used in the same way as the respective primary antibody (dilution, volume, incubation conditions).

Rabbit IgG Isotype Control (#NBP2-24891, Novus Biologicals, Colorado, USA) was used as an isotype controls at the same dilution and condition of the primary antibody. In addition, Sections were observed under the optical microscope Eclipse E600 (Nikon Instruments, Melville, USA) by using the following objective lens (Nikon);

- Plan Fluor 20X NA=0.50 WD= 2.1 mm Correction Cover Glass Thickness = 0.17; Infinity Corrected
- Plan Fluor 40X NA= 0.75 WD= 0,72 mm Correction Cover Glass Thickness = 0.17; Infinity Corrected
- 100X Oil NA= 1.25 WD= 0.18 mm Correction Cover Glass Thickness = 0.17; Infinity Corrected

Images were acquired by using Digital Sight Camera DS-Fi2 (5 Megapixels color CCD) (Nikon) and processed by DS-L3 stand-alone control unit (software ver. 140.3).

Semi-quantitative and statistical analysis of samples

Immunostaining analysis was performed as previously described (Goteri et al. 2015). Briefly, immunohistochemical evaluations were performed independently by two morphologists (M.D. and T.G). Staining was scored as positive when a brown colour was present in the nuclei. Percentages were determined by randomly counting positive nuclei of at least 100 nuclei on three different microscopic fields from each placental sample. In summary we counted 300 nuclei/cells for each sample using an image analysis software (ImageJ ver 1.52). A semi-quantitative data was obtained as a mean of positive nuclei of the three microscopic field analyzed. The researchers independently reviewed all slides in blind. The level of concordance, expressed as the percentage of agreement between the observers was 94%.

Positive nuclei are presented as means ± standard deviation (SD). Data were analysed using Student's t test and P values <0.05 were considered statistically significant. All analyses were carried out using GraphPad Prism (ver 8) statistical software.

Results and discussion

Many studies support the idea that ARID1A play a pivotal role in regulating normal tissues homeostasis and cell differentiation trough regulating chromatin remodeling and transcription factors recruitment (Hiramatsu et al. 2019; Lei et al. 2012; Qu et al. 2019). In particular, it has been shown that ARID1A plays an important role in regulating stem cell differentiation and self-renewal in normal tissues (Hiramatsu et al. 2019). Cytotrophoblastic cells function as stem cells that proliferate, differentiate and finally fuse with the syncytiotrophoblast overlying generating a multinuclear syncytium (Turco and Moffett 2019). Cell proliferation and differentiation are characterized by an inverse relationship because

precursor cells need to arrest cell proliferation to acquire a fully differentiated state otherwise tissue homeostasis is compromised (Ruijtenberg and van den Heuvel 2016). It follows that temporal synchronization between cell cycle arrest and differentiation play a pivotal role in normal tissue growth and development, becoming crucial for tissue homeostasis. Concerning the role of extravillous cytotrophoblast cells in PE, it is still matter of debate. In fact, some author suggest that it is not only the compromised invasiveness of extravillous trophoblast (EVT) that causes PE but an impaired interplay between the decidual stroma cells and EVT (Ridder et al. 2019). Moreover, other studies suggest new routes of trophoblast invasion according to the subtypes of EVT (endo-arterial, endo-glandular, interstitial, endo-venous) suggesting that impairment of specific subtypes of EVT may lead to specific placental pathology such as IUGR, PE, spontaneous abortion and pregnancy loss (Huppertz 2019). Although there are these conflicting theories, there are many studies focused on the impairment of the EVT in preeclamptic pregnancies and the role of these cells is still to be determined. Our immunohistochemical analysis showed a specific expression of ARID1A as shown in Figures 1 and 2. In particular, ARID1A was highly expressed in the nuclei of villous cytotrophoblastic cells (V Cy, Figure 1a and 2a) and in the EVT of placental columns (Figure 1,a**) in first trimester placentas. Interestingly, the syncytiotrophoblast (Sy) was negative suggesting that this protein has a role in modulating proliferative processes present in the cytotrophoblast and not in the syncytiotrophoblast. In addition, ARID1A immunostaining was also present in the nuclei of stromal cells and endothelial cells of foetal vessels (Figure 2a) suggesting a role in remodelling of the placental villous core during first trimester placental development.

This protein was also expressed in cytotrophoblast cells of third trimester placental villi (Figures 1b and 2b, FV) indicating that this protein is important in maintaining placental homeostasis from first to third trimester of pregnancy. Interestingly, in placenta affected by PE (Figures 1c and 2c) and PE-IUGR (Figures 1d and 2d) we found ARID1A expressed in both villous cytotrophoblastic cells and syncytiotrophoblast. Semi-quantitative analysis did not show any significant statistical difference in nuclear expression of ARID1A in cytotrophoblast cells among the groups (see Figure 3). However, as shown in Figure 3, a statistically significant increase of nuclear expression of ARID1A in syncytiotrophoblast, stromal and endothelial cells was detected in PE and PE-IUGR placental samples compared to normal third trimester placental samples. These data could indicate a poor differentiation of syncytiotrophoblast in PE and PE-IUGR placental samples because cytotrophoblast cells fuse with syncytiotrophoblast overlaying before arrest their cell cycle. Our hypothesis is supported by many studies indicating an increase in trophoblast proliferation rate in preeclampsia compared to normal term placentas (Arnholdt et al. 1991; Kaya et al. 2015; Unek et al. 2014). In particular, Unek and colleagues showed an increase of PCNA and Ki67 staining intensities in villous parts of PE placentas (Unek et al. 2014). This may be due to the fact that syncytiotrophoblast is damaged in preeclampsia (Jones and Fox 1980) then cytotrophoblast cells try to compensate by increasing their proliferation rate leading to a poor differentiation of syncytiotrophoblast. Preeclamptic

placentas are also characterized by an increased production of syncytial knots (Heazell et al. 2007), aggregates of nuclei of syncytiotrophoblast that get detached and released into maternal circulation. Interestingly, we found ARID1A expression also in nuclei of syncytial knots of placentas affected by PE and PE-IUGR (Figure 1c, Figure 2d; Sy Knots) indicating a poor differentiation of syncytiotrophoblast also in this structure.

In conclusion, ARID1A is significantly high expressed in syncytiotrophoblast, in villous stromal and endothelial cells of placenta affected by PE and PE-IUGR indicating this protein as a good marker of poor placental tissues differentiation.

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Figure legends

Figure 1 ARID1A immunolocalization in normal first and third trimester placentas and in placentas affected by PE and PE–IUGR. First trimester (a) and third trimester (b) of normal placental villi show ARID1A highly expressed in the villous and extravillous (**) cytotrophoblastic cells, whereas the syncytiotrophoblast was negative. In placentas complicated by PE (c) and PE-IUGR (d) ARID1A was expressed in the villous cytotrophoblastic cells and in the syncytiotrophoblast. ARID1A immunostaining was present in stromal cells and endothelial cells of normal and pathological placental samples. Note syncytial knots (Sy Knots) positive for ARID1A in placental affected by PE (c). Prostate cancer tissue (e) was used as positive control while first trimester placenta (f) incubated with isotype control antibody was used as negative control. f: Bar= 60 µm; a, b, c, d, e: Bar=25 µm.

Figure 2 High magnification of ARID1A immunolocalization in normal first and third trimester placentas and in placentas affected by PE and PE–IUGR. First trimester (a) and third trimester (b) of normal placental villi show ARID1A highly expressed in the villous cytotrophoblastic cells (V Cy), whereas the syncytiotrophoblast (Sy) was negative. In placentas complicated by PE (c) and PE-IUGR (d) ARID1A was expressed in both cytotrophoblastic cells and syncytiotrophoblast. Only few nuclei of syncytiotrophoblast of pathological placentas were negative for ARID1A expression (*). ARID1A immunostaining was present in stromal cells and endothelial cells of normal and pathological placental samples. Note

syncytial knots (Sy Knots) positive for ARID1A in placental affected by PE-IUGR (d). FV= Fetal Vessel. a: Bar= 15 µm; b, c, d: Bar=25 µm.

Figure 3 Semi-quantitative analysis of placental ARID1A expression from 10 first trimester (I Trim.), 10 third trimester (III Trim.), 9 Preeclamptic (PE), and 9 Preeclamptic complicated by IUGR (PE-IUGR) pregnancies. Data are represented as mean ± SD. **p < 0.01, ***p < 0.001, ****p <0.0001.

Table 1 Clinical characteristics of normal and pathological pregnancies

	I trim.	III trim.	PE	PE-IUGR
N	10	10	9	9
Gestational age at delivery (weeks) mean±SEM	10 ± 1.8	34 ± 2.2	34 ± 2.8	32 ± 3.2
Neonatal weight (g) mean±SEM	n.a	2425 ± 530 >50th percentile for GA	1925 ± 653 <50th percentile for GA	1206 ± 310 <5th percentile for GA
Blood pressure (mmHg) mean±SEM				
Systolic	n.a	110 ± 10.5	150± 7.2	148 ± 8.6
Diastolic	n.a	70 ± 10	95 ± 5	94.5± 5.3
Fetal gender	n.a	Male (6), Female (4)	Male (6), Female (3)	Male (4), Female (5)

GA= Gestational Age
I Trim.= First Trimester
III Trim. = Third Trimester
PE = Preeclampsia
PE-IUGR = Preeclampsia with IntraUterine Growth Restriction
n.a = Not available

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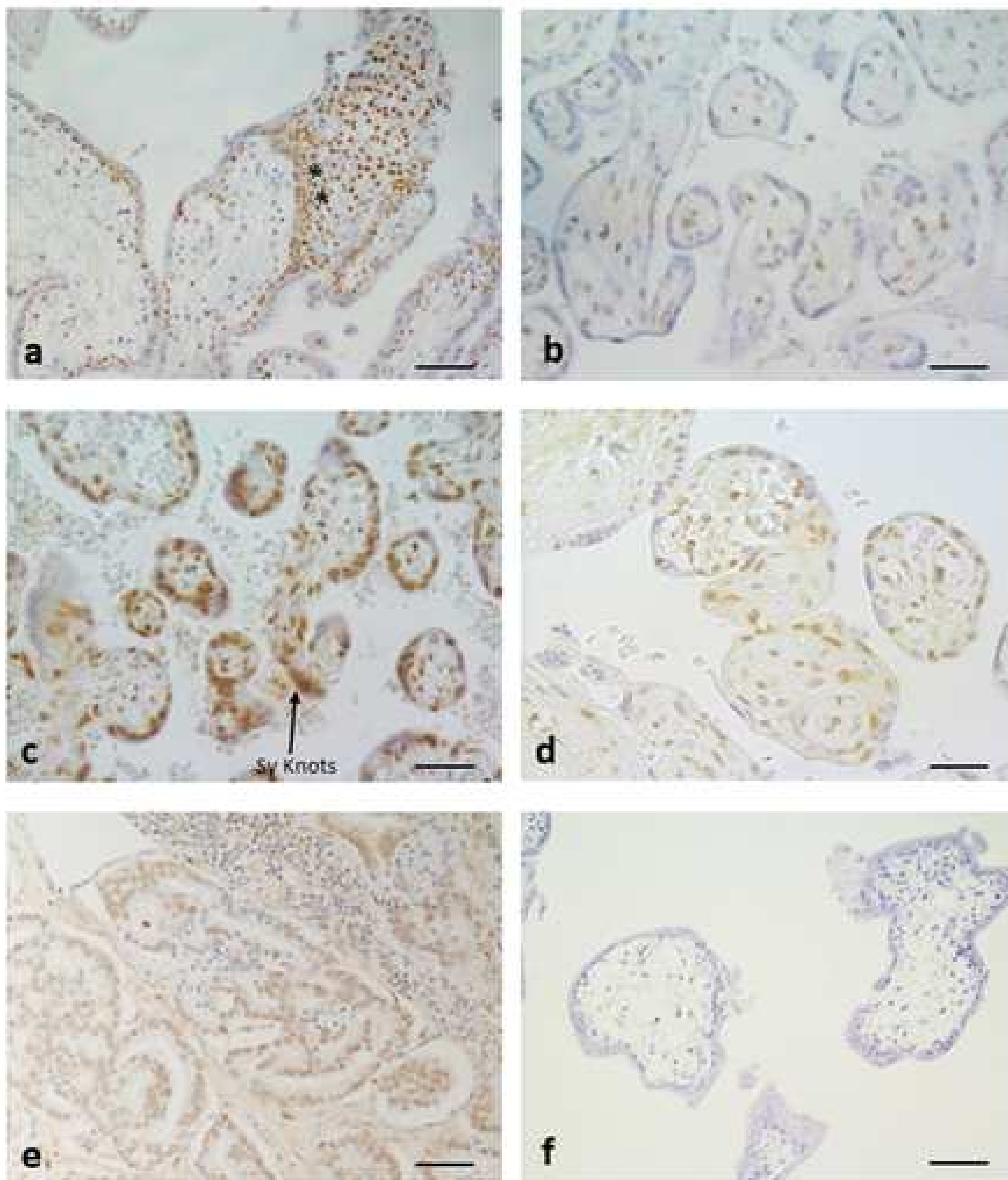

Figure 1

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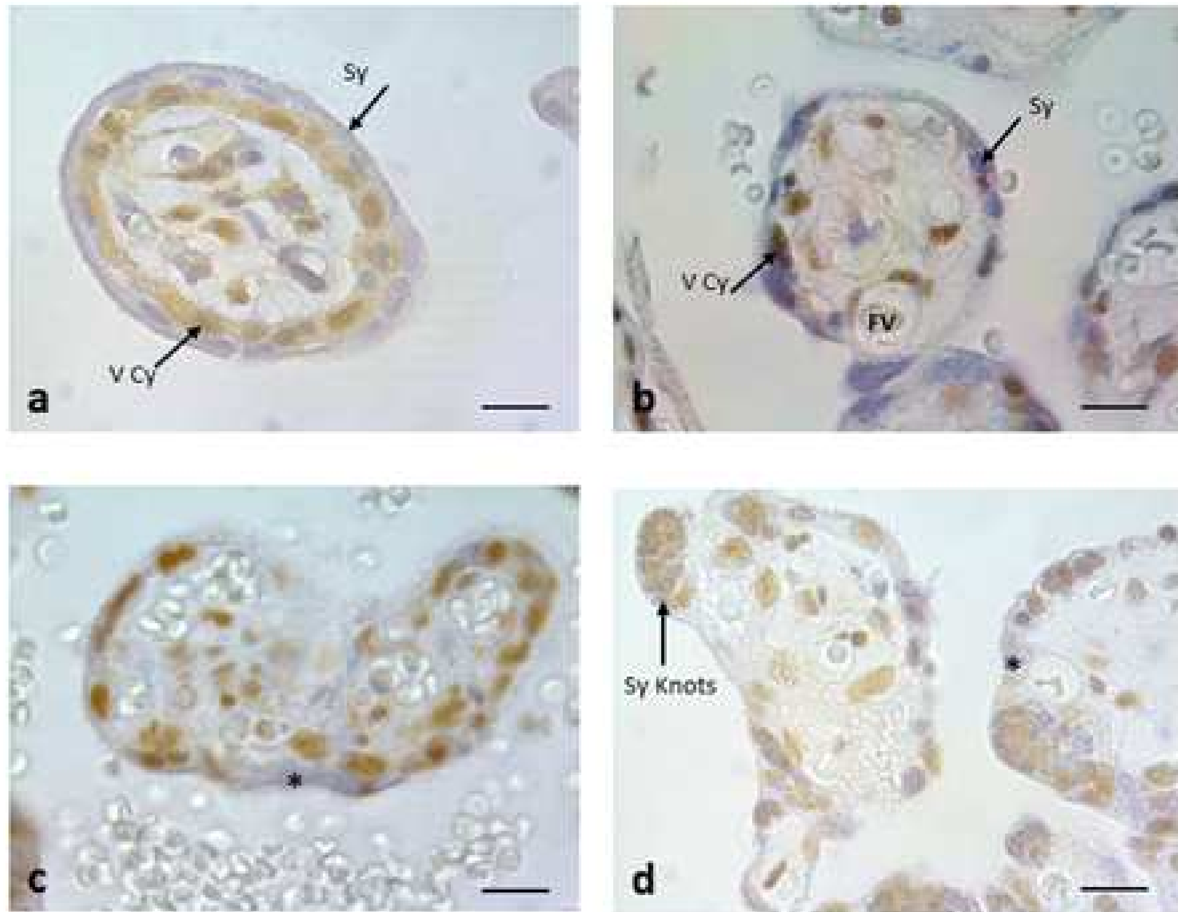

Figure 2

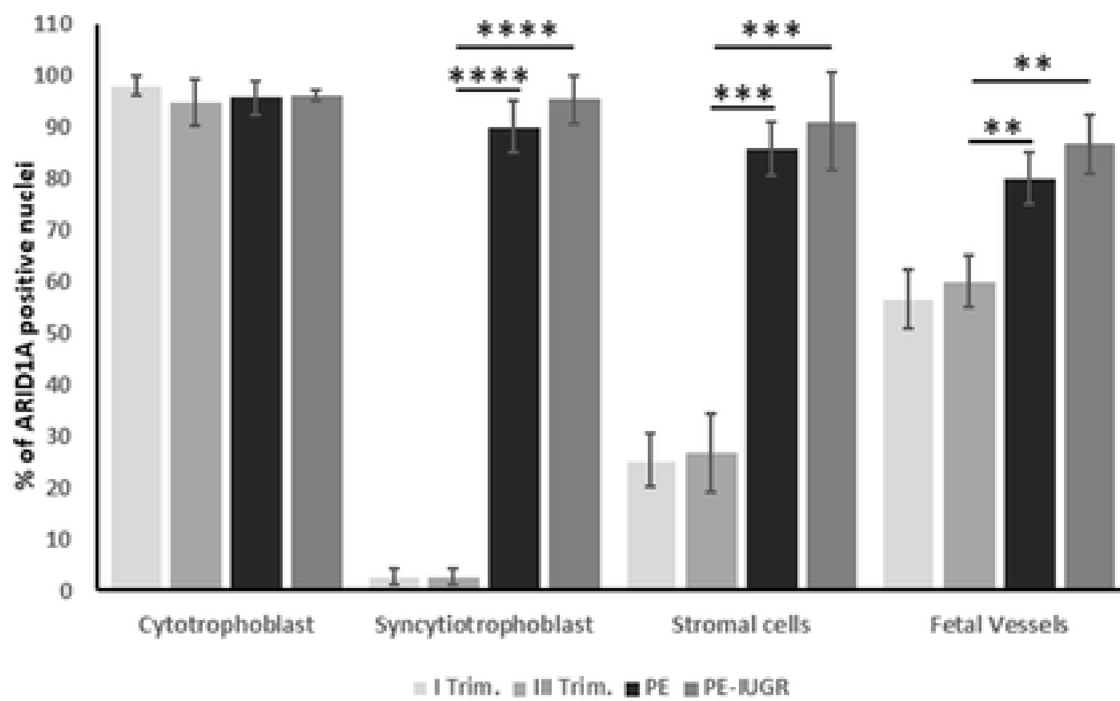

Figure 3