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Immunohistochemical determination of Ang-1, Ang-2 and Tie-2 in placentas of sows at 30, 60 and 114 days of gestation and validation through a bioinformatic approach

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ABSTRACT

Angiopoietins (Ang-1, Ang-2) participate in vascular development and placental growth, both bind to Tie-2. This study aimed to determine the localization of angiopoietins in placental development of sows by immunohistochemistry and to validate the gene expression during gestation through a bioinformatic approach. Samples were collected from fifteen maternal-fetal interface from approximately 30 (n = 5), 60 (n = 5) and 114 (n = 5) days of gestation for immunohistochemistry. A bioinformatic approach was performed by re-analysis of public datasets to determine the increase or decrease of genes involved in angiogenesis during pregnancy. There was no significant statistical difference of Ang-1 during gestation, although there was a tendency to increase from mid- to term-gestation (P = 0.7680). A notable decrease of Ang-2 was observed from early- to term-pregnancy ($P \leq 0.05$), consistent with the gene expression determined through bioinformatics. Furthermore, there were greater abundances of Tie-2 at both early and at term periods, but lesser abundances at mid-gestation ($P \leq 0.05$). The bioinformatics approach indicated that genes related to biological processes such as angiogenesis (*i.e.*, development and morphogenesis of blood vessels) were expressed to a greater extent in early gestation as compared with later in gestation. The Ang-1 gene expression related to cell maturation, response to hypoxia and apoptosis, however, increased as gestation period advanced. In conclusion, angiopoietins may have an important role in the vascular development thus ensuring adequate placental growth in sows. The presence of angiopoietins in the trophoblast suggests a specific role for these pro-angiogenic factors in the tissue formation at the maternal-fetal interface.

1. Introduction

Physiological angiogenesis in the placenta of sows is critical for peri-attachment development at the maternal-fetal interface, and an essential developmental stage for efficient placental function (Kridli et al., 2016). Thus, angiogenesis is tightly controlled by proangiogenic and antiangiogenic factors at the maternal-fetal interface (Krawczynski et al., 2015). During pregnancy, these pro-angiogenic factors, such as angiopoietin-1 and -2 (Ang-1 and Ang-2), are mainly produced by the placenta and have an important role

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in endothelial cell survival and vessel remodeling (Eklund and Olsen, 2006; Fagiani and Christofori, 2013; Kappou et al., 2015). Ang-1 and Ang-2, also known as ANGPT-1 and ANGPT-2 (Augustin et al., 2009), are ligands for Tie-2 (TEK), one the tyrosine kinase receptors of vascular endothelial cells, with similar affinity for both ligands (Gale et al., 2002; Thurston, 2003; Fiedler and Augustin, 2006; Thomas and Augustin, 2009; Linares et al., 2014; Wang and Lash, 2017). The Tie-2 gene expression, however, does not only occur in the vascular endothelium, but also in the placental tissue of humans (Tseng et al., 2006; Seval et al., 2008).

The angiopoietins together with vascular endothelial growth factors (VEGFs) and the relevant receptors, form the two signaling pathways that are almost exclusively endothelial cell (EC) specific (Eklund and Saharinen, 2013). The *in vivo* biologic effects of the angiopoietins also depend on concentrations of VEGFA in tissues having receptors of the angiopoietins (Wang and Lash, 2017). The angiogenic phase might be initiated by an increase in Ang-2 and VEGF, while the microvessel maturation phase might be initiated by a relative increase in Ang-1 and a decrease in VEGF (Lobov et al., 2002; Wakui et al., 2006; Yuan et al., 2007; Thurston and Daly, 2012; Biel et al., 2014). Sanchis et al., (2015) investigated the temporal and spatial localization of VEGFA in the sow placenta and this factor was detected in the endothelium, stroma, fetal mesenchyme, trophoblast and myometrium during gestation. In other studies, the binding site of Ang-1 and Ang-2 and the Tie-2 receptor for these angiopoietic factors have been detected in the human (Zhang et al., 2001; Dunk et al., 2000; Geva et al., 2002; Tseng et al., 2006; Seval et al., 2008; Schiessl et al., 2009) and baboon placenta (Babischkin et al., 2007). It was reported that changes in the specific gene expression patterns of Ang-1, Ang-2 and Tie-2 are the leading cause of alterations in abnormal placental development. The implication of these angiopoietins in vascular bed formation and placental development in sows, however, is still unknown. In addition, it is important to note that these biological processes are closely regulated by the differential expression of specific genes during placental development.

There has been investigations of differential gene expression resulting in production of microRNAs in the trophoblastic elongation and adhesion to the sow endometrium from days 10 to 11 post-fertilization (Ross et al., 2009; Krawczynski et al., 2015), and at the onset of placentation around days 20 to 26 and days 50 to 90 of gestation (Su et al., 2010; Wessels et al., 2013; Su et al., 2014). The study of placental gene expression has become an important aspect on days 12 to 14 post-fertilization (Østrup et al., 2010; Gu et al., 2014), and subsequent development until days 75 and 90 of gestation (Zhou et al., 2009). Through use of bioinformatics, gene expression datasets from different studies that are available in public databases can be combined and re-analyzed as one dataset to identify times when gene expression is changed during the sow gestation period.

The hypothesis in the present study is that abundance of the angiopoietin synthesis in the sow placenta is modified from early to late gestation and that these modifications correspond with changes in gene expression during these periods. The aims of the present study, therefore, were to determine the localization of angiopoietins (Ang-1, Ang-2) and Tie-2 during placental development in sows by utilization of immunohistochemistry and to validate gene expression during gestation through a bioinformatics approach.

2. Material and methods

2.1. Animals and tissue collection

All procedures were approved by the National University of Río Cuarto Ethical Committee of Research in animals (CoEdI), Res. 186/2016. Reproductive tracts of healthy crossbred gilts (Yorkshire x Landrace) from different slaughterhouses from Río Cuarto, Argentina (33.11 °S; 64.3° O) were used.

Tissues ($n = 15$) at the maternal-fetal interface were obtained from the reproductive tracts immediately after slaughter or after parturition, washed with Hank's saline solution (SSH) containing sodic penicillin G, streptomycine sulphate and fungizone (Gibco, Grand Island, NY USA) and maintained at 4 °C until processing within 30 min (Sanchis et al., 2015).

The lumen of the uterine horns was accessed longitudinally by making an incision on the anti-mesometrial edge. Fetuses were removed and gestational age was determined according to the crown-rump length of the litter using the methods previously reported by Marrable (1971).

The periods evaluated in the present study were: early (approximately day 30 of gestation; $n = 5$ placentas), mid (approximately days 60 of gestation; $n = 5$ placentas), and term gestation (approximately days 114; $n = 5$ placentas). Samples were collected from maternal-fetal interface. The tissues were processed using the conventional histological technique for immunohistochemistry.

2.2. Conventional histological technique

Portions of approximately 6 mm^3 of placental tissue were fixed by immersion in 10% (v/v) buffered-saline formaldehyde, pH 7.2–7.4 at 4 °C, dehydrated with alcohol and embedded in paraffin. Tissues were subsequently dissected into 4 µm histological sections with a microtome (Micron, Germany) and mounted on slides. Paraffin embedded sections were used for immunohistochemistry.

2.3. Immunohistochemistry

Tissue sections were deparaffinized in xylol and then hydrated in alcohol of decreasing concentrations (100%, 90%, 80% and 70%) and distilled water. Antigenic retrieval was performed in which the slides were treated in a microwave oven in 10 mM citrate buffer, pH 6.0, for 15 min and left to cool for 20 min. After three washes in phosphate buffered saline (PBS), endogenous peroxidase activity was quenched by use of 3% hydrogen peroxide in PBS for 20 min and subsequently there were three washes with PBS.

After incubating for 1 h at room temperature with 5% horse serum (Ang-1 and Tie-2) or goat serum (Ang-2) to inhibit non-specific