Title: Embryo gene expression in pig pregnancy

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Summary: Pregnancy is a complex process which significant changes occurring continually in both the corpora lutea and in the endometrium of the females and varies depending on the embryonic, pre-implantation, or foetal stages. In the embryonic stages, the majority of genes expressed in the pig embryo correspond to the loss of cellular pluripotency. In contrast, the implantation consists of three phases: elongation of the conceptus, adhesion, and union of the embryo to the endometrial epithelium. During these phases, many factors are expressed, including growth factors, molecules that facilitate adhesion, and cytokines, among others. All these changes are ultimately regulated by different lipid and hormonal substances, specifically by progesterone, oestradiol, and prostaglandins, which regulate the expression of many proteins necessary for the development of the embryo, endometrial remodelling, and embryo-maternal communication. This paper is a review of primary gene regulatory mechanisms in pigs during different stages of implantation.

Keywords: gene expression, molecular mechanisms, reproduction

Main text

Pluripotency transcription factors

The most critical transcription factors related to pluripotency in all mammals species are the *Oct4* transcription factor (belonging to the *POU* gene family), *Nanog* and *SOX2* transcription factor, expressed predominantly in pluripotent cells (Boyer et al., 2006). Among these, previous studies have shown *Oct4* is required to cell differentiation processes in different mammalian species, such as human, mice, rabbit and pig, and is expressed earlier, and required for embryonic cells differentiation (Assadollahi et al., 2019; De Los Angeles et al., 2019; Dode et al., 2006; Fair et al., 2004; Llobat et al., 2012; Shen et al., 2019). *Oct4* transcription factor binds to DNA during embryonic development and acts as a gene activator or repressor during cell differentiation and early embryonic development (Smith et al., 2007). In pigs, *Oct4* expression is present in trophoblast and inner cell mass (ICM) (Hall et al., 2009; Vejlsted et al., 2006). Both *Nanog* and *SOX2* are expressed in swine ICM, and are also detected on day 8.5 in the early epiblast, whereas *Oct4* seems to start on day 10 (du Puy et al., 2011; Hall et al., 2010; Shen et al., 2019; Yoon et al., 2019)

However, in porcine and mouse ICM, other transcription factors related to pluripotency such as *GATA6* have been detected (Kuijk et al., 2008; Meng et al., 2018; Schrode et al., 2014).. Nevertheless, Hall (2012) indicate a possible entry of the embryo at rest due to the lack of genes expressed in the ICM, while during the same stage, the epiblast expresses several genes, such as *SMAD* (1, 2, 3, 4 and 5) or *BMP4*, demonstrates higher pluripotent activity in porcine epiblast than in ICM, which exhibits a very premature pluripotency (Hall et al., 2010; Hall & Hyttel, 2014; Kuijk et al., 2008; Wolf et al., 2011). Furthermore, recent studies in cloned embryos showed the dependence of pluripotency-related and apoptosis gene expression on epigenetic transformations (Samiec et al., 2019). The single-cell expression analysis technique pluripotency-related genes in pig embryos, such as paired box 6 (*PAX6*) and aquaporin 3 (*AQP3*), and, in late blastocysts, clathrin adaptor protein (*DAB2*), platelet-derived growth factor receptor alpha (*PDGFRA*), fibronectin 1 (*FN1*), hepatocyte nuclear factor 4 alpha (*HNF4A*), goosecoid homeobox (*GSC*), nuclear receptor subfamily 5 group A member 2 (*NR5A2*) and lysine acetyltransferase 6A (*KAT6A*) (Wei et al., 2018). However, the underlying factors involved in pluripotency and its regulation require further study.

Vascular endothelial and transforming growth factors

The adhesion process can be affected by growth factors that regulate vascularisation and cell motility. One such factor the vascular endothelial growth factor (*VEGF*), is associated with *de novo* vascularisation during processes such as implantation, embryogenesis, menstrual cycle, development of luteal bodies, development of ovarian follicles and tumorigenesis (Ferrara et al., 1998; Valdés et al., 2008). In pigs, *VEGF* expression has recently been associated with foetal weights at 80 and 105 days of pregnancy (Guimarães et al., 2017). Moreover, studies in vitro shows an increase development of porcine embryos *VEGF* dependent, suggesting *VEGF* functions related not only to vascularization, but also to development and growth (Biswas et al., 2018).

Transforming growth factor superfamily (*TGF*) is another group of transcription factors present in embryos of different species both before and during implantation. The *TGF-β* regulates blastocyst differentiation and maturation events, including modulating the interactions between the uterus and embryo during implantation (Paria and Dey, 1990; Pauken and Capco, 1999). In pigs, the expression of integrin-mediated *TGF-β* increases at the time of embryonic elongation and pre-implantation. This increase is related to several functions of *TGF-β* in the maternal-embryonic interface, such as communication between the endometrium and conceptus (Jaeger et al., 2005; Li et al., 2019). Furthermore, recent studies have shown that *GFD8* (member of *TGF-β*) is involved in the expression of ICM marker *SOX2* during embryo in vitro development, indicating their role in preimplantation embryonic development (Yoon et al., 2019). Other growth factors, such fibroblast growth factor 2 (*FGF2*) and angiopoietins (*ANGPTs*), has been related to vascularization during peri-implantation process, since it has recently been shown that prostaglandin increases the expression of *VEGF* in trophoblast and *FGF* and *ANGPTs* in swine endometrium on days 15 and 20 (Kaczynski et al., 2019). These results suggest an important function of different growth factors mediated by prostaglandins in embryo development and creation of new blood vessels between endometrium and trophoblast in pigs.

Family of Integrins

Integrins are adhesion molecules involved in the maternal-embryonic interaction in different species. Pigs show increased expression of different integrin subunits in the endometrium on day 18 of pregnancy (*αVβ3*). However, integrin expression decreases on day 25 before implantation, which indicates a critical role for integrins during elongation and implantation stages (Lin et al., 2007). Among the group of integrins and their subunits, osteopontin (*SSP1*) is a phosphoprotein secreted by the matrix that binds integrin heterodimers *αV* and *β6* subunits and promotes the migration and binding of the trophectoderm to the endometrium (Erikson et al., 2009). *SSP1* is involved in the regulation of signalling events related to adhesion, including invasion by the trophoblast and its migration (Johnson et al., 2003). In pigs, it was shown to contain an Arg-Gly-Asp (RGD) peptide sequence that joins the surface of the endometrium with the trophectoderm. *In vitro* studies showed that this peptide sequence is essential for both the elongation of the blastocyst and later stages of pregnancy, since it activates the ion transporters, thereby increasing nutrient transport (Laughlin et al., 2017). Furthermore, the mechanical forces from the union of the conceptus to the endometrium appear to be generated from the focal adhesions created during implantation and formed by *SSP1* and the *αV* subunit of the integrin. These focal adhesions are lost as placentation proceeds (Frank et al., 2017). The *SSP1* protein present on the entire apical surface of the uterine cells and trophectoderm Nevertheless its expression is limited to the endometrium. Endometrial expression begins on day 11 and is induced by oestradiol to regulate maternal embryonic recognition (Burghardt et al., 2002; Johnson et al., 2003). Besides, increased expression of *SSP1* in the porcine endometrium has been observed between days 25-30, and remains until day 85, indicating the role of *SSP1* not only in implantation, but also in later stages of pregnancy (Garlow et al., 2002). In pigs, the placenta is epitheliochorial, so that the placental barrier includes both the trophectoderm and the uterine epithelium (Wildman et al., 2006). Therefore, the factors that regulate or are related to the process of implantation are of great importance in the placentation. Recent studies showed relationship between foetal size and integrin expression, since regulate adhesion and foeto-maternal interface by interacting with *SSP1* (Stenhouse et al., 2019). Concretely, *SSP1* has a fundamental role, primarily in the non-invasive epitheliochorial placentation, and similar relates to processes that are occurring in pigs (Garlow et al., 2002; Rashev et al., 2005).

Cytokines

Cytokines are a group of proteins and glycoproteins (interleukins (*IL*), tumour necrosis factors (*TNF*), interferons (*IFN*), colony-stimulating factors (*CSFs*), and chemokines) produced by different cell types that act primarily as regulators of immune and inflammatory responses and are essential for maternal-embryonic recognition (Sharkey, 1998). Aproximately at day 12, pig embryo secretes *IFNs* (γ and δ), and *IL* (1B and 6) (Bazer, 2013). Specifically, the expression of interleukin 1β, *IL1B2* increases in the pig embryo around day 14, indicating cytokine requirement for conceptus elongation and attachment to the uterine wall (Whyte et al., 2018). Other interleukins, such as *IL-2* or *IL-4,* are produced by the foetal and maternal placentas from day-30, suggesting their role in maternal-foetal recognition (Vélez et al., 2019). Other important cytokines for successful pregnancy are interferons (*IFN*), which are classified into two families, type I and II interferons. The type II interferon family is composed of a single known gene, whose product is γ interferon (De Maeyer and De Maeyer-Guignard, 1992), the primary product of T cells, and is found in different placental cell types, and human embryonic membrane and porcine trophoblast cells (Bazer et al., 1997). The other group of interferons, type I, is composed of different subtypes with similar biological properties, and interaction with the same receptor. This group includes interferons α, β, δ, ω and interferon τ. Each subtype is different from each other in their amino acid sequence and serological properties, although all are involved in maternal-embryonic recognition (Aboagye-Mathiesen et al., 1995; Charlier et al., 1993; Charpigny et al., 1988; Cross and Roberts, 1989; Fung et al., 2004; Godornes et al., 2007; Imakawa et al., 1987; Kawasaki et al., 1992; Li et al., 2007; Muscettola et al., 2003). During pregnancy in pigs, interferons exert both paracrine and autocrine effects; however, the effects in the uterus are not well understood, although expression of interferon γ type 2 has been observed in the trophectoderm cells (Lefèvre et al., 1998). In addition, the conceptus also expresses interferons, specifically γ and δ interferons between days 12-20 of gestation (Cencic et al., 2003; Cencic and La Bonnardière, 2002; Joyce et al., 2007a, 2007b). The expression of interferon-stimulated genes (*ISG*), including *Mx, ISG15/17, IRF1, STAT1,* and *STAT2*, is limited to specific uterine cells in pigs between days 14-18 of pregnancy (Hicks et al., 2003; Joyce et al., 2007a). The induction of *ISG* expression occurs, not only in pigs, but also in other mammalian species such as sheep, cows, mice, rat, primates, and humans, suggesting that the induction of *IFN* promotes the gene expression in the uterine epithelium to facilitate implantation, placentation, and foetal development (Bazer et al., 2011). Recently, it has been shown that the *IFN-γ* in the porcine trophoblast influences the expression of specific chemokines (*CCL2, CCL5, CCL11,* and *CXCL12*) required for endometrial communication with the trophoblast or recruitment of immune cells and establishment of an immunotolerant environment (*CXCL9, CXCL10*) for the embryo (Złotkowska and Andronowska, 2019).

Insulin-like growth factors

Insulin-like growth factors (*IGFs*) are polypeptides with insulin-like sequences with mitogenic properties, for inducing proliferation and growth of somatic cells (Rinderknecht and Humbel, 1978). *IGFs* are also required for the regulation of amino acid and glucose transport in the placenta (Ashton and Spencer, 1983; Kniss et al., 1994). Type I receptor (*IGF-IR*) is a transmembrane tetrameric glycoprotein that resembles the insulin receptor and has a high affinity for both *IGF-I* and *IGF-II* (Germain-Lee et al., 1992; Ullrich et al., 1986). In contrast, the type II receptor (*IGF-IIR*) is a single chain polypeptide with a high affinity for *IGF-II* and is unable to bind *IGF-I* or insulin (Liu et al., 1993). *IGF* deficiencies exhibit distinct functional differences, and studies with *IGF-IIR* knock-out mice showed excessive placental and foetal growth (Kitamura et al., 2003). Studies in humans showed that mutations in the *IGF-IR* gene resulted in reduced functionality associated with low pre- and post-natal growth, or that excessive foetal growth occurs when *IGF-II* is overexpressed (Abuzzahab et al., 2003; Lau et al., 1994; Murrell et al., 2004; Wang et al., 2017). These results demonstrate that *IGFs*, together with their receptors, have an important role in the regulation of foetal and placental growth in most species (Wilson et al., 1982). Also, Fant and colleagues (1986) showed that the placenta produces both *IGF-I* and *IGF-II*, which act as local growth regulators in human. Specifically, *IGF-II* is expressed predominantly in the placenta, with both paracrine and autocrine functions, which are especially important during implantation and trophoblastic invasion (Giudice, 1997; Hamilton et al., 1997). However, the *IGFs* are not only related to the foetal and placental growth but also regulate different signalling cascades to promote both cell proliferation and differentiation (Clemmons & Maile, 2005; Kitamura et al., 2003). Studies with preimplantation mouse embryos showed that decreased *IGF-IR* induced apoptosis through a cascade of signal transduction pathways and enhanced embryonic resorption (Chi et al., 2000). Similar studies demonstrated the relationship between *IGFs* and embryonic losses in rat, pig, or humans (Katagiri et al., 1997; Pinto et al., 2002; Sferruzzi-Perri et al., 2007, 2006). The final group in the *IGF* family is the IGF-binding protein group (*IGFBP*), a large group in humans consisting of six different proteins (Denley et al., 2005). Of these, dephosphorylated *IGFBP-1* is found in the serum of pregnant women (Westwood et al., 1994), while *IGFBP-3* is produced by the placenta and foetal membranes (Han, 1996; Rogers et al., 1996). *IGFBP-1* is involved in the regulation of *IGFs* by inhibiting their functions, such as cell proliferation and differentiation, and trophoblastic migration (Gleeson et al., 2001; Hamilton et al., 1997; Irving et al., 1995; Ritvos et al., 1988). In pigs, the expression of *IGF-I* is explicitly observed in both the uterine lumen and glandular epithelium of pregnant pigs, while the *IGF-IR* is expressed in endometrial cells and the embryo, indicating the presence of both paracrine and autocrine functions (Letcher et al., 1989).

Conclusions

During different pregnancy stages in pigs, several cellular and molecular mechanisms are activated, each involving different transcription factors, growth factors, cytokines and others,related to cell differentiation, implantation, placentation, vascularisation and maternal-embryonic recognition. Despite extensive knowledge of these factors, the interaction of these factors with each other and the metabolic pathways involved remain to be clarified. The use of new technologies, such as single-cell gene expression, could help reveal the genes involved and their interactions. However, many questions about these and other molecules, as well as the interactions between them, remain to be discovered.

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Conflict of Interest Statement

The author are not conflict of interest.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analysed in this study.

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