Gene expression during pre- and peri-implantation embryonic development in pigs

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Embryo technological procedures such as in vitro production and cloning by nuclear transfer are not as advanced in pigs as in cattle and cannot yet be applied under field conditions. The present paper focuses on genome activation in in vivo-derived, in vitro-produced and nuclear transfer pig embryos with special emphasis on the development of embryonic nucleoli, where the ribosomal RNA (rRNA) genes transcribed can be used as markers for genome activity. In addition, contemporary data on gene expression in in vivo-derived pig embryos are reviewed. In in vivo-derived pig embryos, pronounced transcription is initiated at the four-cell stage (the third cell cycle after fertilization), when nucleoli develop. In parallel with the development of the nucleoli as a result of rRNA gene activation, a cascade of other genes is also likely to be transcribed. However, apart from identification of transcripts for the oestrogen receptor at the blastocyst stage, reports on mRNAs resulting from initial transcription of the pig embryonic genome are lacking, in contrast to the situation in cattle and, in particular, mice. More information is available on gene expression during elongation of pig conceptuses, when the genes for steroidogenic enzymes, extracellular matrix receptors, oestrogen receptors, growth factors and their receptors, as well as retinol binding protein and retinoic acid receptors, are expressed. Nucleolus development appears to be disturbed in in vitro-produced pig embryos and in pig embryos reconstructed by nuclear transfer of granulosa cells to enucleated metaphase II oocytes produced by oocyte maturation in vivo or in vitro, which is indicative of disturbances in activation of rRNA genes.

Introduction

Pig embryos present great challenges from an embryo technological viewpoint. In vitro production (IVP) of pig embryos by in vitro maturation (IVM) of oocytes followed by in vitro
fertilization (IVF) and in vitro culture (IVC) of the resultant embryos resulted in live offspring only recently (for reviews see Abeydeera et al., 1998; Rath et al., 1999; Day, 2000) and is still very inefficient. Successful cryopreservation of pig embryos has been accomplished using the open pulled straw method for vitrification (Vajta et al., 1997; Berthelot et al., 2000) and cytoskeletal stabilization (Dobrinsky et al., 2000). Cloning of pigs by nuclear transfer of somatic cells has also presented a challenge that has been overcome only recently (Betthauser et al., 2000; Onishi et al., 2000; Polejaeva et al., 2000). These difficulties indicate that pig embryos have a particular biology that is likely to differ in several respects from that of both ruminant domestic species and small rodents.

In cattle it is clear that IVP is associated with an increased incidence of calving problems and oversized calves (Kruip and den Dass, 1997). A potential background for this observation is that the pattern of expression of several genes of importance for initial embryonic development deviates in in vitro-produced bovine embryos (for review see Niemann and Wrenzycki, 2000). Likewise, it has been demonstrated that bovine embryos produced by nuclear transfer from somatic cells display deviating patterns of gene expression (Daniels et al., 2000) that may be involved causally in the even more pronounced loss of both embryos and late fetuses that is associated with this procedure. Thus, basic knowledge of the patterns of gene expression during initial embryonic development under normal in vivo conditions has become an important tool in understanding how embryo technological procedures may cause deviations in gene expression that can be detrimental for further embryonic development.

In the present article, the data available on gene expression in pig embryos are reviewed and new data on the use of the development of the nucleolus as a marker for embryonic genome activation in pig embryos derived from IVP and nuclear transfer of somatic cells are presented.

**Maternal to embryonic transition**

The initial development of mammalian pre-implantation embryos is governed by gene transcripts and polypeptides produced by, and stored in, the oocyte, and the transition from maternal to embryonic control of development is a gradual event (for review see Thompson, 1996). Accordingly, using long-term incubation with [3H]uridine, transcription has been observed during the first (Hay-Schmidt et al., 2001) and second cell cycles in cattle (Plante et al., 1994; Viuff et al., 1996), well before the major transcriptional activation that occurs during the fourth cell cycle (Camous et al., 1986). In pigs, the major transcriptional activation is observed during the third cell cycle (the four-cell stage; Tomanek et al., 1989; Jarrel et al., 1991; Hyttel et al., 2000), but whether this activation is preceded by an earlier minor activation has not been investigated.

A more precise chronological description of the major transcriptional activation during the third cell cycle has been obtained by analyses of embryonic DNA and protein synthesis throughout this cell cycle (Schoenbeck et al., 1992). The third cell cycle more or less lacks a G1 phase, as DNA synthesis (the S phase) commences within the first 2 h after cleavage to the four-cell stage; the S phase is completed at 16 h after cleavage and the prolonged G2 phase results in a total duration of the cell cycle of about 50 h. The synthesis of a number of proteins of maternal origin ceases at 10–14 h after cleavage to the four-cell stage and new proteins derived from embryonic transcripts appear at 16 h and, in particular, at 24 h after cleavage. Culture of four-cell; embryos with α-amanitin from early during the third cell cycle blocked the synthesis of the embryonic proteins from 16 h after cleavage, thus verifying their embryonic origin, but also resulted in persistence of maternal protein synthesis, indicating that embryonic transcription is required for downregulation of the maternally derived translation. Moreover,
embryos cultured with α-amanitin from early during the third cell cycle did not cleave to the eight-cell stage, whereas embryos cultured with this substance from 24 h or 30 h after cleavage cleaved in 40 and 100% of cases, respectively. Hence, the embryonic protein synthesis occurring from 16 h to 30 h after cleavage is essential for further development. In accordance with these data on protein synthesis, incubation of embryos for 20 min in $^3$H-uridine followed by autoradiography has demonstrated that the major embryonic transcription is initiated between 10 h and 30 h after cleavage to the four-cell stage (Hyttel et al., 2000).

**Gene activation and expression in pig embryos**

**Pre-hatching development**

There is little information on which genes are activated during pre-hatching development of pig embryos. As described above, significant α-amanitin-sensitive transcription for embryonic development occurs during the third cell cycle after fertilization (Schoenbeck et al., 1992). The qualitative aspects of this transcription have not been investigated extensively. One candidate gene for transcription is cyclin B1, which forms part of the maturation-promoting factor (MPF; Jacobs, 1992) required for the G2 to M phase transition. The content of cyclin B1 transcripts has been examined in both in vivo-developed and in vitro-produced pig embryos throughout the third cell cycle after fertilization by RT-PCR (Anderson et al., 1999). In both categories of embryos, there was a decrease in the amount of cyclin B1 transcripts during the cell cycle, and it was concluded that there is a continuous degradation of maternal cyclin B1 transcripts during the third cell cycle after fertilization without any detectable formation of embryonic transcripts. Thus, it appears that the cyclin B1 gene is not included in the cascade of gene activation that occurs during the third cell cycle after fertilization.

Oestrogen is important for transformation of pig morulae to the cavitated blastocyst stage (Niemann and Elsaesser, 1986) and administration of anti-oestrogen to the culture medium impaired the morulae to blastocyst transformation in vitro (Niemann and Elsaesser, 1987). Ying et al. (2000) investigated the presence of oestrogen receptor mRNA in pre-implantation pig embryos by RT-PCR. The transcript was identified in one-, two- and four-cell embryos, whereas it was not detected in five- to eight-cell embryos and morulae, but reappeared at the blastocyst stage. Ying et al. (2000) also localized the oestrogen receptor protein in one- and four-cell embryos by immunocytochemistry with a reduced staining intensity at the five- to eight-cell stage. However, the blastocysts did not display any immunoreactivity, indicating that there may be a lag phase before translation of the mRNA. It is thought that the transcripts found in the one- to four-cell embryos are of maternal origin, whereas in the blastocysts they are probably of embryonic origin.

**Nucleolus development in pig embryos**

It is common for the major transcriptional activation to occur in parallel with nucleolar formation, thereby indirectly signalling transcription of the ribosomal RNA (rRNA) genes (Camous et al., 1986; Tomanek et al., 1989). Transcription of the rRNA genes and subsequent processing of the transcripts result in formation of the nucleolus, which is the most prominent nuclear organelle and the site of formation of the ribosomal subunits. In pig embryos, nucleoli form towards the end of the third cell cycle after fertilization (Tomanek et al., 1989; Hyttel et al., 2000), whereas in cattle, nucleolar formation occurs towards the end of the fourth cell cycle (Camous et al., 1986; Laurincik et al., 2000). A minor activation of the rRNA genes,
which is not associated with nucleolar development, has been demonstrated by fluorescence in situ hybridization (FISH) to occur during the third cell cycle in cattle (Viuff et al., 1998).

The active ribosome-synthesizing nucleolus contains three main ultrastructural components: the fibrillar components, consisting of the fibrillar centres and the dense fibrillar component, and the granular component (for review see Wachtler and Stahl, 1993). These components of the fibrillar–granular nucleolus reflect the steps in the biosynthesis of ribosomes according to the following model: the fibrillar centres contain the enzymatic apparatus for transcription; the dense fibrillar component carries the primary unprocessed transcripts; and the granular component represents processed transcripts associated with proteins in the form of pre-ribosomal particles. As rRNA gene transcription is expressed in the ultrastructure of the nucleolus, the development of this organelle may serve as an indirect morphological marker of activation of the embryonic genome (Kopecny and Niemann, 1993).

Pig embryos display the first signs of development of fibrillar centres in the developing nucleoli towards the end of the third cell cycle (Tomanek et al., 1989). Hyttel et al. (2000) analysed in vivo-developed pig zygotes and embryos by transmission electron microscopy to elucidate the chronology of this event in greater detail. After flushing from the oviducts, the zygotes and embryos were cultured into the subsequent cell cycle in vitro and were fixed at different intervals during the one-cell stage (first cell cycle; the zygotes were not cultured in vitro), the two-cell stage (second cell cycle), the four-cell stage (third cell cycle), the eight-cell stage (fourth cell cycle) and the 16-cell stage (fifth cell cycle).

The most prominent structural nuclear entities observed during the first two cell cycles were electron-dense spheres of packed fibrillar material, the nucleolus precursor bodies (NPBs; Fig. 1). NPBs were observed up to 10 h after cleavage to the four-cell stage, but at 20 and 30 h after cleavage, the nuclei displayed different stages of nucleolus formation, ranging from inactive NPBs to fibrillar–granular nucleoli presenting a dense fibrillar component, fibrillar centres and a granular component. Nucleolus formation was apparently initiated by the formation of the dense fibrillar component and the granular component, and later by the fibrillar centres on the surface of the NPBs. Fibrillar–granular nucleoli were formed at the start of the fifth cell cycle.

The nucleolus consists of the rRNA genes and their transcripts associated with proteins that play different roles in rRNA transcription and processing. We have analysed in vivo-developed pig zygotes and embryos fixed at different times during the first five cell cycles after fertilization to elucidate the protein composition of the nucleolus before and during activation of the rRNA genes. Antibodies against six important nucleolar proteins were applied on whole mounts that were analysed subsequently by confocal laser scanning microscopy (Hyttel et al., 2000). The nucleolar proteins against which antibodies were applied were: topoisomerase I, RNA polymerase I, upstream binding factor (UBF), fibrillarin, nucleolin and nucleophosmin. Each of these proteins has a fairly well defined role in rRNA gene transcription and subsequent processing of the transcripts (Hyttel et al., 2000). In brief, topoisomerase I uncoils the supercoiled DNA allowing transcription. RNA polymerase I, the binding of which to the rRNA genes is mediated by the transcription factor UBF, drives the actual transcription. Fibrillarin is probably involved in early processing of the transcripts, whereas nucleolin and nucleophosmin are involved in later processing. According to these functions, topoisomerase I, RNA polymerase I, UBF and fibrillarin have been localized to the fibrillar centres and the dense fibrillar component of the nucleolus in somatic cells, whereas nucleolin and nucleophosmin have been localized mainly to the granular component.

During the first cell cycle, labelling of nucleophosmin was localized to large shell-like bodies (Fig. 2). These bodies may be identical to the NPBs observed by transmission electron microscopy. The remaining proteins were not localized to nuclear entities. None of the
Fig. 1. Schematic illustration of the maternal–embryonic transition in a pre-implantation pig embryo as visualized using nucleolus development. Transcription, as evaluated by $^3$H]uridine incorporation after 20 min incubation followed by autoradiography, is initiated during the third cell cycle, where silver grains become localized over both the nucleoplasm and the nucleolus. In parallel, the dense nucleolus precursor body is transformed gradually into a ribosome-synthesizing nucleolus with fibrillar centres, a dense fibrillar component and a granular component.

Proteins were localized to nuclear entities during the second and early third cell cycles. The nucleophosmin observed during the first cell cycle is probably maternal proteins that target to the pronuclei and are degraded before or during the second cell cycle. RNA polymerase I was localized to discrete foci arranged in a shell-like pattern towards the end of the third cell cycle. This observation is in accordance with the observation by transmission electron microscopy of the first fibrillar–granular nucleoli established on the surface of the NPBs towards the end of this cell cycle. None of the remaining proteins were localized to nuclear entities. Early during the fourth cell cycle, labelling of topoisomerase I, RNA polymerase I, UBF and fibrillarin was localized to small foci arranged in a shell-like pattern, whereas labelling for nucleolin and
Fig. 2. Examples of the tentative localization of nucleolar proteins to the fibrillar nucleolar components (RNA polymerase I) and the granular nucleolar component (nucleolin and nucleophosmin). Each image represents an optical confocal section of a single nucleus of the developmental stage as indicated at the top, except for the images of the one-cell stage of RNA polymerase I and nucleolin, where two pronuclei are shown. Note the nucleolar labelling of RNA polymerase I to the presumptive fibrillar components at 30 h after cleavage to the four-cell stage and the labelling of nucleolin and nucleophosmin to the presumptive granular component at the eight-cell stage.
nucleophosmin was localized to more or less shell-like bodies. Again, this labelling pattern is compatible with the presence of fibrillar–granular nucleoli on the surface of the NPBs with topoisomerase I, UBF, RNA polymerase I and fibrillarin being confined mainly to the fibrillar centres and the dense fibrillar component, and nucleolin and nucleophosmin to the dense fibrillar component and the granular component. Similar observations were made during the fifth cell cycle. The labelling patterns described above were very consistent within each developmental group. No embryos lacked labelling and unlabelled blastomeres were found in only a few embryos of the later stages.

In conclusion, the labelling patterns observed by immunocytochemistry are compatible with the formation of fibrillar–granular nucleoli towards the end of the third cell cycle.

**Nucleolus development in in vitro-produced pig embryos**

IVP of pig embryos resulted in live offspring only recently (for reviews see Abeydeera et al., 1998; Rath et al., 1999; Day, 2000) and the technique is still hampered by great inefficiencies. In general, the procedure results in development of about 35% of the collected immature oocytes to blastocysts; however, these blastocysts are characterized by variable and low numbers of cells. Moreover, pig IVF is associated with various rates of polyspermy (Wang et al., 1998) and low rates of male pronucleus formation (Laurincik et al., 1994; Prather and Day, 1998), and the process of IVC is associated with decreased cell numbers in the whole embryo as well as in the inner cell mass (Macháty et al., 1998).

Proper activation of the embryonic genome is crucial for initial development and it is possible that this process is affected adversely in in vitro-produced pig embryos, as it is in cattle (for review see Niemann and Wrenzycki, 2000). Therefore, we aimed to evaluate the normality of genome activation in in vitro-produced pig embryos using nucleolus development as an indirect model.

The same panel of antibodies against key nucleolar proteins as was used for in vivo-derived embryos was used as a marker for nucleolus development and, hence, the activation of the rRNA genes (J. Laurincik, D. Rath, H. Niemann and P. Hyttel, unpublished). The embryos were produced according to the description of Rath et al. (1997, 1999). In brief, ovaries were obtained from a local abattoir and the contents of follicles 2–5 mm in diameter were aspirated and cumulus–oocyte complexes (COCs) with at least three cumulus cell layers were selected and placed into maturation medium (North Carolina State University (NCSU) 37 medium (Funahashi et al., 1994, 1997) supplemented with dbcAMP, eCG, hCG and mercaptoethanol). After 24 h of incubation at 38.5°C and 5% CO₂ in humidified air, the COCs were placed into NCSU 37 medium without hormonal supplements and incubated for a further 24 h. For IVF, mature COCs were denuded mechanically and placed into Tyrode's albumin lactate pyruvate (TALP) fertilization medium (Bavister, 1981) supplemented with 3% bovine serum albumin and 1 μg sodium pyruvate ml⁻¹. Frozen epididymal semen was thawed, washed twice in TALP and the final concentration was set to 1500 spermatozoa per oocyte dissolved in 10 μl TALP. Spermatozoa were added to the oocytes and the resultant embryos were cultured for different times depending on the experimental design, to obtain different developmental stages.

The in vitro-produced embryos lacked labelling of nuclear entities for any of the proteins investigated during the first, second and third cell cycles after fertilization except for a few embryos showing labelling for fibrillarin and nucleolin towards the end of the third cycle. No labelling was observed during the fourth cycle, when strong labelling of the nucleolar compartment by all antibodies was expected from the results of studies of in vivo-developed embryos (Hyttel et al., 2000). During the fifth cell cycle, about half of the embryos showed labelling of RNA polymerase I (Fig. 3) and a few had labelling for nucleolin, whereas no
Fig. 3. Localization of RNA polymerase I in in vivo-derived, in vitro-produced and nuclear transfer pig embryos. Each image represents an optical confocal section of a single nucleus of the developmental stage indicated at the top. Note that the nucleolar localization of the protein in the in vitro-produced embryos is delayed until the 16-cell stage, whereas no localization is visible in the nuclear transfer embryos, which were analysed only up to the eight-cell stage. Nuclear transfer embryos were fixed at one time point only during the third cell cycle.
labelling was observed for the other proteins examined. These data show that nucleolus development is disturbed, indicating deviations in rRNA gene activation in in vitro-produced pig embryos.

**Nucleolus development in pig nuclear transfer embryos**

Except for the birth of a single piglet resulting from nuclear transfer from the blastomere of a four-cell embryo to an enucleated oocyte (Prather et al., 1989), cloning by nuclear transfer was not accomplished until recently, when a litter of five piglets was born (Polejaeva et al., 2000). Polejaeva et al. (2000) applied a double reconstruction technique in which the granulosa cell was first electrofused with a pre-activated in vivo-matured enucleated metaphase II oocyte and the reconstructed embryo was subsequently fused with a fertilized zygote enucleated at the pronuclear stage. Only 4% of the total number of fused reconstructed embryos in this trial and fewer from other trials survived to birth as live piglets. Nevertheless, other workers have obtained cloned piglets without serial nuclear transfer. Onishi et al. (2000) injected fetal fibroblast cells into the cytoplasm of in vivo-derived enucleated oocytes and obtained one live piglet. Bethhauser et al. (2000) used in vitro-matured oocytes as an economical source of recipient oocytes, an improved activation method and a short in vitro culture of the cloned embryos to obtain four piglets from fetal body and genital ridge cells. In this case, activation was performed about 4 h after fusion. Two other studies have demonstrated that the use of in vitro-produced cytoplasts in combination with activation of cytoplasts before electrofusion and a blastocyst-derived cell line (Miyoshi et al., 2000) or fetal fibroblasts (Koo et al., 2000) can result in development of blastocysts in vitro. However, the efficiency of all these methods is low.

In cattle, fine structural investigations revealed only minor differences between somatic cell nuclear transfer embryos and in vitro-produced control embryos except that there was more cellular debris in the former (Heyman et al., 1995). However, transcripts for certain genes of significance for embryonic development and implantation were lacking in a major proportion of nuclear transfer embryos (Daniels et al., 2000). Very little is known about genome reactivation at nuclear transfer in pigs. Ouhibi et al. (1996) reported that nuclear transfer of pig ectodermal cells to enucleated metaphase II oocytes results in either complete or incomplete nucleolar remodelling. Thus, in some instances the active fibrillo–granular nucleolus of the ectodermal cell was transformed into a compact dense nucleolus precursor body, whereas in other cases it maintained the fibrillo–granular appearance. The reconstructed embryos were apparently transcriptionally quiescent in both cases. These data partially support the contention that after nuclear transfer the nucleolus is remodelled to an inactive entity in reconstructed embryos from cattle (Kanka et al., 1991, 1999; King et al., 1996; Lavoir et al., 1997) and rabbits (Kanka et al., 1996). The reformation of a functional nucleolus, indicating the reactivation of the rRNA genes, has been described less thoroughly. In rabbit embryos, fibrillo–granular nucleoli are established during the fourth cell cycle, corresponding to the normal rate of embryonic development in this species (Kanka et al., 1996), whereas in bovine embryos fibrillo–granular nucleoli were reported during the third cell cycle, which is one cell cycle earlier than expected (Kanka et al., 1999; Laurincik et al., (in press)).

We have studied nucleolus development as an indirect model to elucidate the normality of genome reactivation in pig embryos reconstructed by nuclear transfer. Antibodies against RNA polymerase I and UBF as described earlier for the in vivo-derived embryos were used as markers for nucleolus development (Hyttel et al., 2000). These two candidates were chosen as they, in particular UBF, were lacking during nucleolus reactivation in reconstructed embryos.
in a previous study of bovine somatic nuclear transfer embryos (Laurincik et al., in press). Pig nuclear transfer embryos were produced by a relatively simple technique developed at the Roslin Institute (P. A. De Sousa, J. R. Dobrinsky, J. Zhu, A. Archibald, A. Ainslie, W. Bosma, J. Bowering, J. Bracken, P. Ferrier, J. Fletcher, B. Gasparrini, L. Harkness, P. Johnston, M. Ritchie, W. A. Ritchie, A. Travers, D. Albertini, A. Dinnyes, T. J. King and I. Wilmut, unpublished). In brief, in vivo-derived oocytes from superovulated gilts that ovulated 42–48 h after hCG administration or oocytes submitted to IVM for 42–44 h were denuded from cumulus cells, exposed to 7.5 μg cytochalasin B ml⁻¹ and 5 μg Hoechst 33342 ml⁻¹, and enucleated at 39°C in Ca²⁺-free Hapes-buffered NCSU 23 medium (Peters and Wells, 1993) containing 7.5 μg cytochalasin B ml⁻¹. Enucleation success was confirmed by ultraviolet irradiation. Enucleated oocyte cytoplasts were reconstructed with fetal fibroblast cells (serum-starved for 5 days) in Ca²⁺-free Hapes-buffered NCSU 23 at 39°C, and fused by 3 x 80 μs electric pulses of 1.25 kV cm⁻¹ in Ca²⁺-free 0.3 mol mannitol l⁻¹ and 100 μmol MgCl₂ l⁻¹. A delay of 2 h was applied between fusion and activation during which the reconstructed embryos were held in Ca²⁺-free NCSU 23 supplemented with 7.5 μg cytochalasin B ml⁻¹ at 39°C, in 5% CO₂ in air. Activation was achieved by 3 x 80 μs electric pulses of 1.0 kV cm⁻¹ in 0.3 mol mannitol solution l⁻¹ supplemented with 100 μmol MgCl₂ l⁻¹ and 50 μmol CaCl₂ l⁻¹. Reconstructed embryos were cultured in NCSU 23 in groups of 20–40 per 500 μl medium under mineral oil in 5% CO₂ in air for 7 days, or transferred into synchronized recipients shortly after activation. In vitro culture of the fused embryos obtained from in vivo- or in vitro-derived oocytes using this method resulted in 58 and 41% cleavage, and 10 and 5% blastocyst development rates, respectively. Furthermore, the method was suitable for producing a healthy cloned piglet after co-transfer with parthenogenetically activated pig embryos (P. A. De Sousa, J. R. Dobrinsky, J. Zhu, A. Archibald, A. Ainslie, W. Bosma, J. Bowering, J. Bracken, P. Ferrier, J. Fletcher, B. Gasparrini, L. Harkness, P. Johnston, M. Ritchie, W. A. Ritchie, A. Travers, D. Albertini, A. Dinnyes, T. J. King and I. Wilmut, unpublished). Pig nuclear transfer embryos produced as described above were harvested for immunocytochemistry during the first, second, third and fourth cell cycle after fusion. None of the embryos produced from either in vivo- or in vitro-matured oocytes displayed labelling of either of the two proteins during these cell cycles. Again, these data show that nucleolus development is disturbed, indicating aberrations in the activation of the rRNA genes. Moreover, about one third of the reconstructed four- and eight-cell embryos derived from in vivo-matured cytoplasts presented one or more anucleated blastomeres, whereas this proportion was increased to about half in those originating from in vitro-matured cytoplasts.

Gene expression in pig conceptuses

During post-hatching and peri-implantation development

In pigs, the incidence of prenatal mortality ranges from 20% to 46% at term (Pope and First, 1985). Most of this loss occurs before day 20 of gestation (Geisert et al., 1982) and, in particular, the period between day 11 and day 12, during which the peri-implantation conceptus undergoes rapid differentiation and expansion of the trophectoderm, appears to be critical (Geisert et al., 1982; Stroband and Van der Lende, 1990). During elongation of the trophectoderm, pig conceptuses synthesize and secrete large amounts of oestrogen, which is probably the signal for recognition of pregnancy in pigs (Ford et al., 1982; Geisert et al., 1982, 1990). This important period of conceptus development is controlled by well-synchronized gene expression that has received greater attention than that in the pre-hatching embryo.

In a comprehensive study, Yelich et al. (1997a) investigated the content of mRNA encoding
Gene expression in pig embryos

Gene expression in pig embryos in the period about the time of elongation by RT-PCR. The work included analyses of embryos from the spherical 2 mm stage of development up to the > 100 mm filamentous stage for mRNAs of the steroidogenic enzymes 17α-hydroxylase and aromatase, which are of importance for oestrogen synthesis, of brachyury, a transcription factor that can be used as a marker of mesoderm formation (Herrmann et al., 1990), of the extracellular matrix receptor integrin β1, which is thought to be of importance for the morphological restructuring of the conceptus during rapid elongation, and a number of receptors for oestrogen, progesterone, oxytocin, PGF$_{2α}$ and leukaemia inhibitory factor (LIF).

Conceptuses at all developmental stages from the 2 mm spherical stage onwards expressed 17α-hydroxylase and aromatase. Ko et al. (1994) demonstrated that transcripts for cytochrome P450 aromatase are present in high concentrations at days 10, 11 and 12 in pig conceptuses. The expression of the steroidogenic enzymes is in accordance with the need for oestrogen synthesis for maternal recognition of pregnancy.

Brachyury was not expressed in the small 2–4 mm spherical conceptuses, but was clearly evident from the 6 mm stage of development. Brachyury expression, which is a marker of mesoderm formation, appears to precede the morphological recognition of mesoderm, which is reported to occur at the 10 mm stage (Conley et al., 1994).

Integrin β1 expression was evident from the 2 mm stage of development. Integrins serve as plasma membrane receptors for laminins (Rouslahti and Pierschbacher, 1987) and fibronectins (Chen et al., 1985; Knudson et al., 1986), both of which have roles in assembly of the extracellular matrix and, thus, for changes in cell shape and cell migration (Horwitz et al., 1986; Tamkun et al., 1986), which are important for elongation of pig conceptuses.

LIF receptor expression was evident in particular from the 6 mm stage of development onwards. In addition to its effect on cell differentiation, LIF may also stimulate cell proliferation in the inner cell mass and trophoderm (Stewart, 1994; Lavranos et al., 1995; Eckert et al., 1997). Another possible role of LIF on elongating pig conceptuses is to enhance protease production (Harvey et al., 1995); proteases serve in the modification of the extracellular matrix, which is required during the extensive morphological remodelling of conceptuses during elongation. mRNAs encoding the receptors for oestrogen, progesterone, PGF$_{2α}$ and oxytocin were not detected in pig conceptuses at any stage of development.

Yelich et al. (1997b) investigated the possible role of retinoic acid for conceptus elongation by RT-PCR. This substance, which is a metabolite of retinol, is a powerful morphogen in early embryonic development. The conceptuses displayed mRNA for retinol binding protein (RBP) from the 2 mm stage of development and onwards. Accordingly, Trout et al. (1991) demonstrated that day 13–17 pig conceptuses present transcripts for RBP and in situ hybridization has shown that the transcripts are distributed uniformly in the inner cell mass and the trophectoderm. The transcripts for retinoic acid receptor α (RARα), RARβ and RARγ were evident from the 2 mm stage of development onwards.

Pig conceptuses also display transcripts for several growth factor receptors during peri-implantation development as demonstrated by Vaughan et al. (1992) using RT-PCR. Epidermal growth factor receptor (EGF receptor) was detected at all developmental stages from day 7 to day 22 of pregnancy. Transforming growth factor α (TGF-α) was expressed on days 8–12 only. In contrast, epidermal growth factor (EGF) was first expressed by the post-elongation conceptus at about day 15 of pregnancy. The expression pattern of TGF-α indicates a possible role for this factor during cellular remodelling of the blastocyst during elongation, whereas EGF is more likely to be involved in initial organ development.

Recently, it has also been reported that one of the important genes for organizing the longitudinal axis of the embryo, goosecoid, is expressed in the embryo of the pig conceptus...
(as opposed to the fetal membranes) during peri-implantation development (Meijer et al., 2000). Goosecoid is a homeobox gene and is one of the first genes to be expressed in the vertebrate organizer regions and future dorsal regions of the embryo along the longitudinal axis (De Robertis et al., 1994; Bouwmeester and Leyns, 1998). Meijer et al. (2000) found that goosecoid was expressed at all stages of pig embryonic development from day 9 to day 12 of pregnancy. On day 10, the transcripts were localized by in situ hybridization to one side of the epiblasts and expression was localized to a region anterior to the primitive streak on day 13.

Conclusion

Information about the patterns of gene expression during normal embryonic development in vivo is crucial to optimize the outcome of different embryo technological procedures. In pigs, there is a lack of knowledge about the qualitative aspects of gene expression during pre-hatching embryonic development, whereas more information is available on gene expression in relation to conceptus elongation and oestrogen secretion. Data presented here indicate that the pattern of embryonic nucleolus development may be disturbed in pre-implantation pig embryos produced either in vitro or by nuclear transfer, indicating that there are deviations in the activation of rRNA genes. In the near future, the application of cDNA array technology to analysis of embryonic gene expression will probably allow studies of the expression of thousands of genes and, thus, lead to a rapid expansion of the understanding of the sequential activation of the embryonic genome. However, it is of crucial importance to enter the field of proteomics to understand the significance of such data on functional genomics. After all, it is the proteins that determine the character of the cells and cell populations involved in initial embryonic development.

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