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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*

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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 [^{3}H]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 fibrillo–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 fibrillo–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. Fibrillo–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

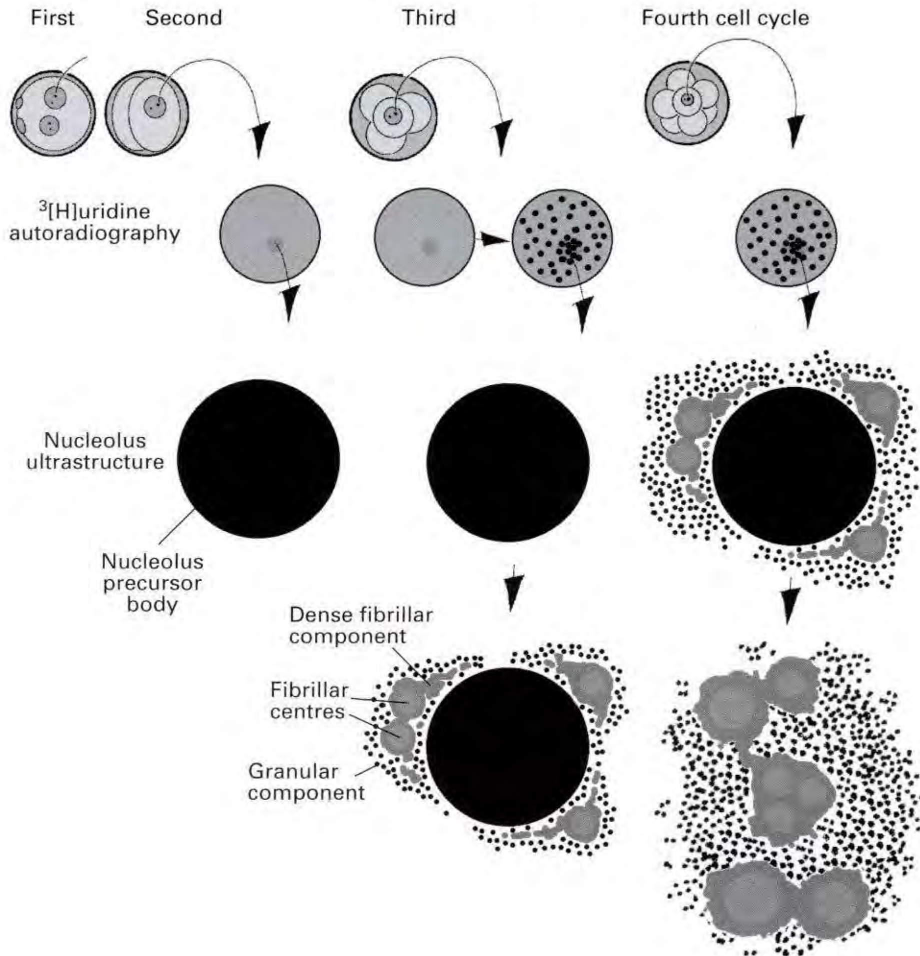

Fig. 1. Schematic illustration of the maternal–embryonic transition in a pre-implantation pig embryo as visualized using nucleolus development. Transcription, as evaluated by ^{3}[H]uridine incorporation after 20 min incubation followed by autoradiography, is initiated during the third cell cycle, where 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 fibrillo–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

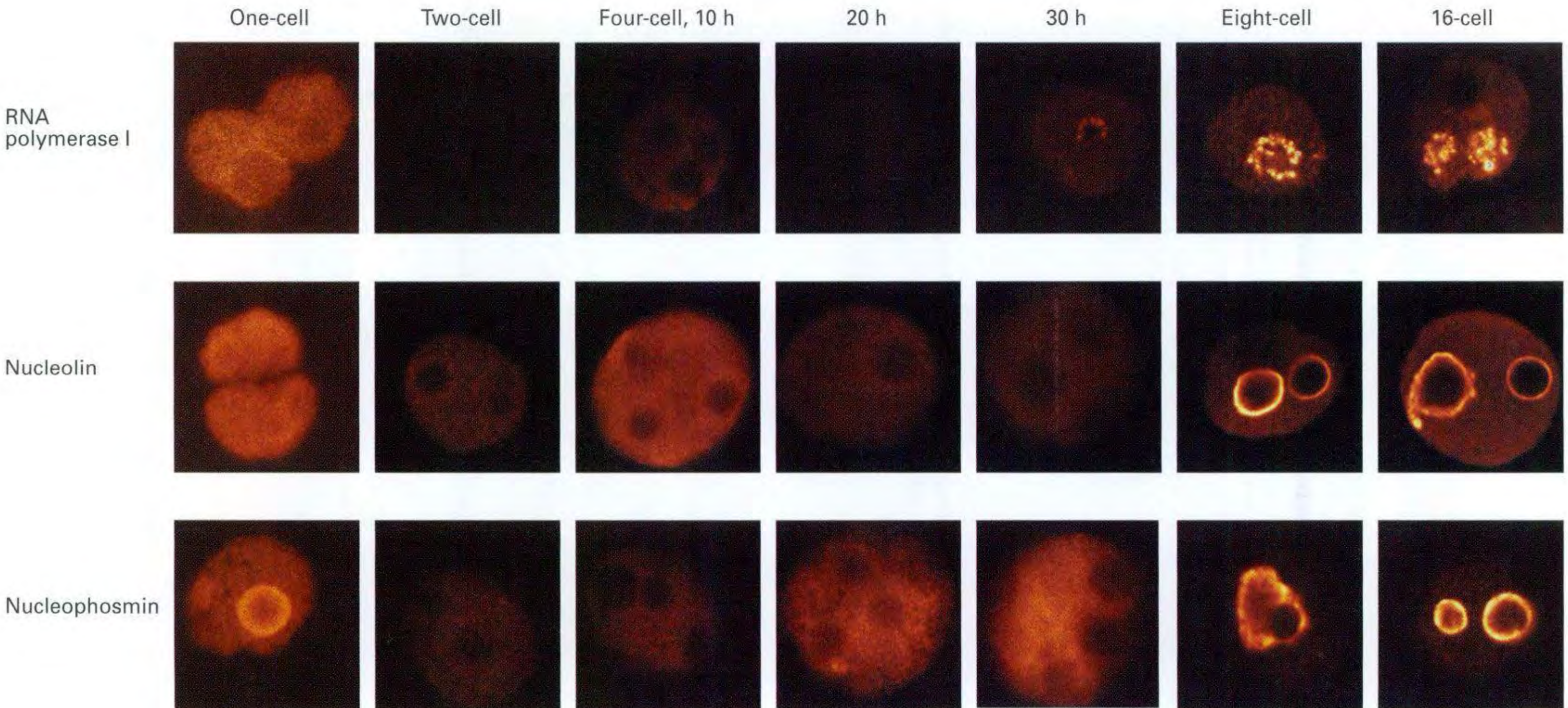

Fig. 2. Examples of the tentative localization of nucleolar proteins to the fibrillar nucleolar components (RNA polymerase I) and the granular nucleolar component (nucleolin and nucleophosmin). Each image represents an optical confocal section of a single nucleus of the developmental stage as indicated 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 fibrillo–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 fibrillo–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% CO_2 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

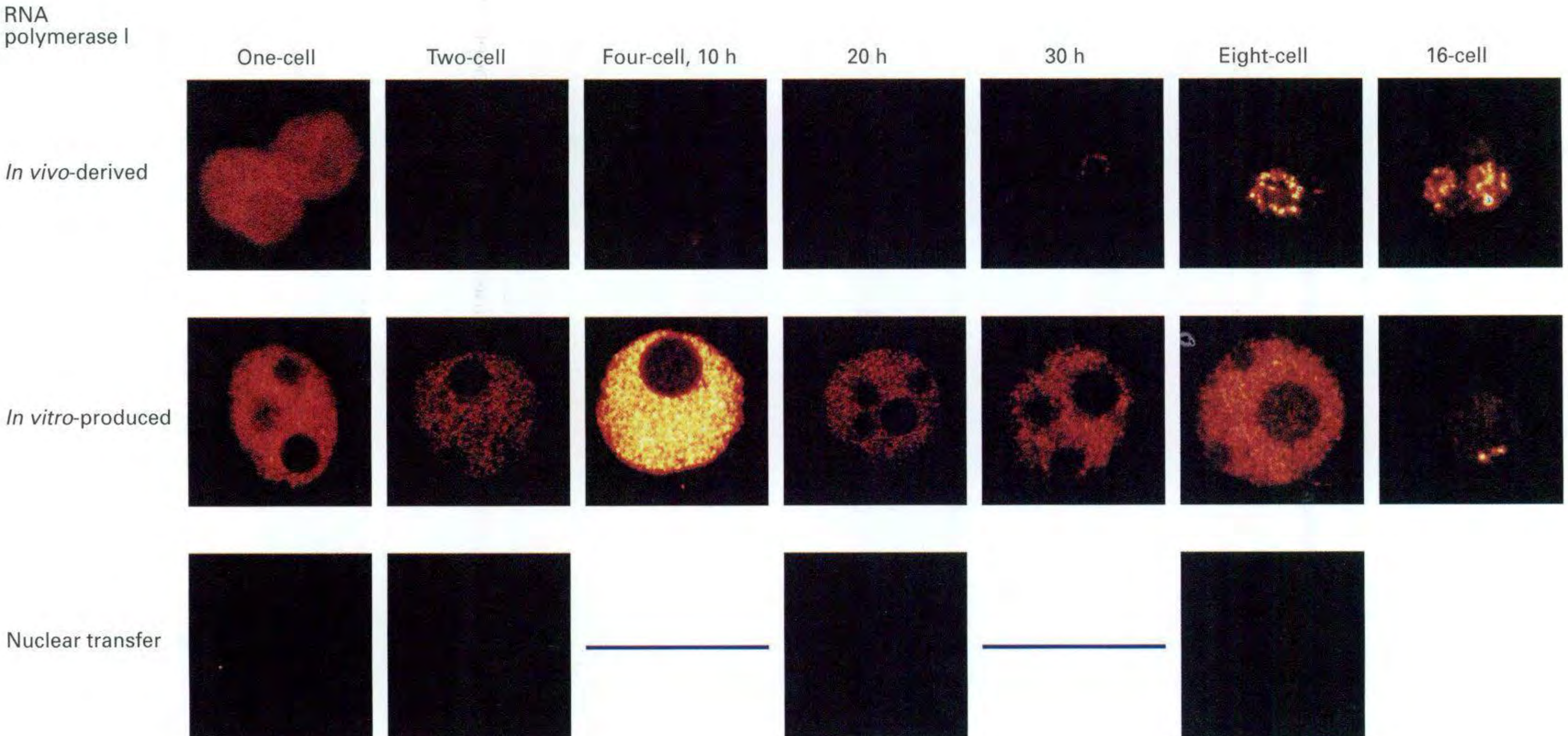

Fig. 3. Localization of RNA polymerase I in *in vivo*-derived, *in vitro*-produced and nuclear transfer pig embryos. Each image represents an optical confocal section of a single nucleus of the developmental stage indicated at the top. Note that the nucleolar localization of the protein in the *in vitro*-produced 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. Betthauser *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 Ca^{2+}-free Hepes-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 Ca^{2+}-free Hepes-buffered NCSU 23 at 39°C, and fused by 3 × 80 μs electric pulses of 1.25 kV cm^{-1} in Ca^{2+}-free 0.3 mol mannitol l^{-1} and 100 μmol $MgCl_2$ l^{-1}. A delay of 2 h was applied between fusion and activation during which the reconstructed embryos were held in Ca^{2+}-free NCSU 23 supplemented with 7.5 μg cytochalasin B ml^{-1} at 39°C, in 5% CO_2 in air. Activation was achieved by 3 × 80 μs electric pulses of 1.0 kV cm^{-1} in 0.3 mol mannitol solution l^{-1} supplemented with 100 μmol $MgCl_2$ l^{-1} and 50 μmol $CaCl_2$ l^{-1}. Reconstructed embryos were cultured in NCSU 23 in groups of 20–40 per 500 μl medium under mineral oil in 5% CO_2 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

a number of developmentally important proteins 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, $PGF_{2\alpha}$ 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 trophectoderm (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, $PGF_{2\alpha}$ 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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References

Abeydeera LR, Johnson LA, Welch GR, Wang WH, Boquest AC, Cantley TC, Rieke A and Day BN (1998) Birth of piglets preselected for gender following *in vitro* fertilization of *in vitro* matured pig oocytes by X and Y chromosome bearing spermatozoa sorted by high speed flow cytometry *Theriogenology* **50** 981–988

Anderson JE, Matteri RL, Abeydeera LR, Day BN and Prather RS (1999) Cyclin B1 transcript quantitation over the maternal to zygotic transition in both *in vivo*- and *in vitro*-derived 4-cell porcine embryos *Biology of Reproduction* **61** 1460–1467

Bavister BD (1981) Substitution of a synthetic polymer for protein in a mammalian gamete culture system *Journal of Experimental Zoology* **217** 45–51

Berthelot F, Martinat-Botté F, Locatelli A, Perreau C and Terqui M (2000) Piglets born after vitrification of embryos using the open pulled straw method *Cryobiology* **41** 116–124

Betthauser J, Forsberg E, Augenstein M *et al.* (2000) Production of cloned pigs from *in vitro* systems *Nature Biotechnology* **18** 1055–1059

Bouwmeester T and Leyns L (1998) Vertebrate head induction by anterior primitive endoderm *BioEssays* **19** 855–863

Camous S, Kopecny V and Fléchon JE (1986) Autoradiographic detection of the earliest stage of [3H]-uridine incorporation into the cow embryo *Biology of the Cell* **58** 195–200

Chen WT, Hasegawa E, Hasegawa T, Weinstock C and Yamada KM (1985) Development of cell-surface linkage complexes in cultured fibroblasts *Journal of Cell Biology* **100** 1103–1114

Conley AJ, Christenson LK, Ford SP and Christenson RK (1994) Immunocytochemical localization of cytochromes P450 17α-hydroxylase and aromatase in embryonic cell layers of the elongating porcine blastocyst *Endocrinology* **135** 2248–2254

Daniels R, Hall V and Trounson AO (2000) Analysis of gene transcription in bovine nuclear transfer embryos reconstructed with granulosa cells *Biology of Reproduction* **63** 1034–1040

Day BN (2000) Reproductive biotechnologies: current status in porcine reproduction *Animal Reproduction Science* **60–61** 161–172

De Robertis EM, Fainsod A, Gont LK and Steinbeisser H (1994) The evolution of vertebrate gastrulation *Development Supplement* 117–124

Dobrinsky JR, Pursel VG, Long CR and Johnson LA (2000) Birth of piglets after transfer of embryos cryopreserved

by cytoskeletal stabilization and vitrification *Biology of Reproduction* 62 564–570

Eckert J, Tao T and Niemann H (1997) Ratio of inner cell mass and trophoblastic cells in blastocysts derived from porcine 4- and 8-cell embryos and isolated blastomeres cultured *in vitro* in the presence or absence of protein and human leukemia inhibitory factor *Biology of Reproduction* 57 552–560

Ford SP, Christenson RK and Ford JJ (1982) Uterine blood flow and uterine arterial, venous and luminal concentrations of oestrogens on days 11, 13 and 15 after oestrus in pregnant and non-pregnant sows *Journal of Reproduction and Fertility* 64 185–190

Funahashi H, Cantley TC, Stumpf TT, Terlouw SL and Day BN (1994) Use of low salt culture medium for *in vitro* maturation of porcine oocytes is associated with elevated oocyte glutathione levels and enhanced male pronuclear formation after *in vitro* fertilization *Biology of Reproduction* 51 633–639

Funahashi H, Cantley TC and Day BN (1997) Synchronization of meiosis in porcine oocytes by exposure to dibutyryl cyclic adenosine monophosphate improves developmental competence following *in vitro* fertilization *Theriogenology* 47 679–686

Geisert RD, Brookbank JW, Roberts RM and Bazer FW (1982) Establishment of pregnancy in the pig. II. Cellular remodelling of the porcine blastocyst during elongation on day 12 of pregnancy *Biology of Reproduction* 27 941–955

Geisert RD, Zavy MT, Moffat RJ, Blair RM and Yellin T (1990) Embryonic steroids and the establishment of pregnancy in pigs *Journal of Reproduction and Fertility* 40 293–305

Harvey MB, Leco KJ, Arcellana-Panlilio MY, Zhang X, Edwards DR and Schultz GA (1995) Roles of growth factors during peri-implantation development *Molecular Human Reproduction* 10 712–718

Hay-Schmidt A, Viuff D, Greve T and Hyttel P (2001) Transcriptional activity in *in vivo* developed early cleavage stage bovine embryos *Theriogenology* 56 167–176

Herrmann BG, Labiet S, Poustka A, King TR and Lerach H (1990) Cloning of the T gene required in mesoderm formation in the mouse *Nature* 343 617–622

Heyman Y, Degrolard J, Adenot P, Chesne P, Flechon B, Renard JP and Flechon JE (1995) Cellular evaluation of bovine nuclear transfer embryos developed *in vitro*. *Reproduction, Nutrition and Development* 35 713–723

Horwitz A, Duggan K, Buck RC, Beckerle MC and Burridge K (1986) Interaction between plasma membrane fibronectin receptor with talin, a transmembrane linkage *Nature* 320 531–533

Hyttel P, Laurincik J, Rosenkranz C, Rath D, Niemann H, Ochs RL and Schellander K (2000) Nucleolar proteins and ultrastructure in pre-implantation porcine embryos developed *in vivo*. *Biology of Reproduction* 63 1858–1866

Jacobs T (1992) Control of the cell cycle *Developmental Biology* 153 1–15

Jarrel VL, Day BN and Prather RS (1991) The transition from maternal to zygotic control of development occurs during the 4-cell stage in the domestic pig, *Sus scrofa*: quantitative and qualitative aspects of protein synthesis *Biology of Reproduction* 44 62–68

Kanka J, Fulka J, Jr and Petr J (1991) Nuclear transplantation in bovine embryos: fine structural and autoradiographic studies *Molecular Reproduction and Development* 29 110–116

Kanka J, Hozak P, Heyman Y, Chesne P, Degrolard J, Renard JP and Flechon JE (1996) Transcriptional activity and nucleolar ultrastructure of embryonic rabbit nuclei after transplantation to enucleated oocytes *Molecular Reproduction and Development* 43 135–144

Kanka J, Smith SD, Soloy E, Holm P and Callesen H (1999) Nucleolar ultrastructure in bovine nuclear transfer embryos *Molecular Reproduction and Development* 52 253–263

King WA, Shepherd DL, Plante L, Lavoir MC, Looney CR and Barnes FL (1996) Nucleolar and mitochondrial morphology in bovine embryos reconstructed by nuclear transfer *Molecular Reproduction and Development* 44 499–506

Knudson K, Horwitz AF and Buck C (1986) A monoclonal antibody identifies a glycoprotein complex involved in cell–substratum adhesion *Experimental Cell Research* 157 218–226

Ko Y, Choi I, Green ML, Simmen FA and Simmen RC (1994) Transient expression of the cytochrome P450 aromatase gene in elongating porcine blastocysts is correlated with uterine insulin-like growth factor levels during peri-implantation development *Molecular Reproduction and Development* 37 1–11

Koo D-B, Kang Y-K, Choi Y-H *et al.* (2000) *In vitro* development of reconstructed porcine oocytes after somatic cell nuclear transfer *Biology of Reproduction* 63 986–992

Kopecny V and Niemann H (1993) Formation of nuclear microarchitecture in the preimplantation bovine embryo at the onset of transcription: implications for biotechnology *Theriogenology* 39 109–119

Kruip TAM and den Dass JHG (1997) *In vitro* produced and cloned embryos: effects on pregnancy, parturition and offspring *Theriogenology* 47 43–52

Laurincik J, Rath D and Niemann H (1994) Pronucleus formation and early cleavage following *in vitro* fertilization of porcine oocytes matured *in vivo* and *in vitro*. *Journal of Reproduction and Fertility* 102 277–284

Laurincik J, Thomsen PD, Hay-Schmidt A, Avery B, Greve T, Ochs RL and Hyttel P (2000) Nucleolar proteins and nuclear ultrastructure in pre-implantation bovine embryos produced *in vitro*. *Biology of Reproduction* 62 1024–1032

Laurincik J, Zathartchenko V, Stozkovic M, Brem G, Wolf E, Müller M, Ochs RL and Hyttel P Nucleolar protein allocation and ultrastructure in bovine embryos produced by nuclear transfer from granulosa cells (in press)

Lavoir MC, Kelk D, Rumph N, Barnes F, Betteridge KJ and King WA (1997) Transcription and translation in bovine nuclear transfer embryos *Biology of Reproduction* 57 204–213

Lavranos TC, Rathjen PD and Semark RF (1995) Trophic effect of myeloid leukemia inhibitory factor (LIF) on

mouse embryos *Journal of Reproduction and Fertility* **105** 331–338

Macháty Z, Day BN and Prather RS (1998) Development of early porcine embryos *in vitro* and *in vivo*. *Biology of Reproduction* **59** 451–455

Meijer HA, Van De Pavert SA, Stroband HW and Boerjan ML (2000) Expression of the organizer specific homeobox gene goosecoid (gsc) in porcine embryos *Molecular Reproduction and Development* **55** 1–7

Miyoshi K, Taguchi Y, Sendai Y, Hiroyoshi H and Sato E (2000) Establishment of a porcine cell line from *in vitro*-produced blastocysts and transfer of the cells into enucleated oocytes *Biology of Reproduction* **62** 1640–1646

Niemann H and Elsaesser F (1986) Evidence for estrogen-dependent blastocyst formation in the pig *Biology of Reproduction* **35** 10–16

Niemann H and Elsaesser F (1987) Accumulation of oestrone by pig and its potential physiological significance for blastocyst development *in vitro*. *Journal of Reproduction and Fertility* **80** 221–227

Niemann H and Wrenzycki C (2000) Alterations of expression of developmentally important genes in preimplantation bovine embryos by *in vitro* culture conditions: implications for subsequent development *Theriogenology* **53** 21–34

Onishi A, Iwamoto M, Akita T, Mikawa S, Takeda K, Awata T, Hanada H and Perry AC (2000) Pig cloning by microinjection of fetal fibroblast nuclei *Science* **289** 1188–1190

Ouhibi N, Fulka J, Jr, Kanka J and Moor RM (1996) Nuclear transplantation of ectodermal cells in pig oocytes: ultrastructure and autoradiography *Molecular Reproduction and Development* **44** 533–539

Peters RM and Wells KD (1993) Culture of pig embryos *Journal of Reproduction and Fertility Supplement* **48** 61–73

Plante L, Plante C, Shepherd DL and King WA (1994) Cleavage and ^{3}H-uridine incorporation in bovine embryos of high *in vitro* developmental potential *Molecular Reproduction and Development* **39** 375–383

Polejaeva IA, Chen S-H, Vaught TD *et al.* (2000) Cloned pigs produced by nuclear transfer from adult somatic cells *Nature* **407** 86–90

Pope WF and First NL (1985) Factors affecting the survival of pig embryos *Theriogenology* **23** 91–105

Prather RS and Day BN (1998) Practical considerations for the *in vitro* production of pig embryos *Theriogenology* **49** 23–32

Prather RS, Sims MM and First NL (1989) Nuclear transplantation in early pig embryos *Biology of Reproduction* **41** 414–418

Rath D, Johnson LA, Dobrinsky JR, Welch GR and Niemann H (1997) Production of piglets preselected for sex following *in vitro* fertilization with X and Y chromosome bearing spermatozoa sorted by flow cytometry *Theriogenology* **47** 795–800

Rath D, Long CR, Dobrinsky JR, Welch GR, Schreier LL and Johnson LA (1999) *In vitro* production of sexed embryos for gender preselection: high speed sorting of X-chromosome bearing sperm to produce piglets after embryo transfer *Journal of Animal Science* **77** 3346–3352

Rouslahti E and Pierschbacher MD (1987) New perspectives in cell adhesion: RGD and integrins *Science* **238** 491–497

Schoenbeck RA, Peters MS, Rickords LF, Stumpf TT and Prather RS (1992) Characterization of deoxyribonucleic acid synthesis and the transition from maternal to embryonic control in the 4-cell porcine embryo *Biology of Reproduction* **47** 1118–1125

Stewart CL (1994) Leukemia inhibitory factor and the regulation of preimplantation development of the mammalian embryo *Molecular Reproduction and Development* **39** 233–238

Stroband HWJ and Van der Lende T (1990) Embryonic and uterine development during early pregnancy *Journal of Reproduction and Fertility Supplement* **40** 261–277

Tamkun JW, DeSimmone DW, Fonda D, Patel RS, Buck C, Horwitz AF and Hynes RO (1986) Structure of integrin, a glycoprotein involved in the transmembrane linkage between fibronectin and actin *Cell* **46** 271–282

Thompson EM (1996) Chromatin structure and gene expression in the preimplantation mammalian embryo *Reproduction, Nutrition and Development* **36** 619–635

Tomanek M, Kopecny V and Kanka J (1989) Genome reactivation in developing early pig embryos: an ultrastructural and autoradiographic analysis *Anatomy and Embryology* **180** 309–316

Trout WE, McDonnell JJ, Kramer KK, Baumbach GA and Roberts RM (1991) The retinol-binding protein of the expanding pig blastocyst: molecular cloning and expression in trophectoderm and embryonic disc *Endocrinology* **5** 1533–1540

Vajta G, Holm P, Greve T and Callesen H (1997) Vitrification of porcine embryos using the open pulled straw (OPS) method *Acta Veterinaria Scandinavica* **38** 349–352

Vaughan TJ, James PS, Pascall JC and Brown KD (1992) Expression of the genes for TGF alpha, EGF and the EGF receptor during early pig development *Development* **116** 663–669

Viuff D, Avery B, Greve T, King WA and Hyttel P (1996) Transcriptional activity in *in vitro* produced bovine two- and four-cell embryos *Molecular Reproduction and Development* **43** 171–179

Viuff D, Hyttel P, Avery B, Vajta G, Greve T, Callesen H and Thomsen PD (1998) Ribosomal ribonucleic acid is transcribed at the 4-cell stage in *in vitro* produced bovine embryos *Biology of Reproduction* **59** 626–631

Wachtler F and Stahl A (1993) The nucleolus: a structural and functional interpretation *Micron* **24** 473–505

Wang WH, Abeydeera LR, Prather RS and Day BN (1998) Morphological comparison of ovulated and *in-vitro* matured porcine oocytes, with particular reference to polyspermy after *in vitro* fertilization *Molecular Reproduction and Development* **49** 308–316

Yelich JV, Pomp D and Geisert RD (1997a) Ontogeny of elongation and gene expression in the early developing porcine conceptus *Biology of Reproduction* **57** 1256–1265

Yelich JV, Pomp D and Geisert RD (1997b) Detection of

transcripts for retinoic acid receptors, retinol binding protein, and transforming growth factors during rapid trophoblastic elongation in the procine conceptus *Biology of Reproduction* 57 286–294

Ying C, Hsu WL, Hong WF, Cheng WT and Yang Y (2000) Estrogen receptor is expressed in pig embryos during preimplantation development *Molecular Reproduction and Development* 55 83–88