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Carry-over of mRNA during nuclear transfer in pigs

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Summary — This research was designed to evaluate the changes that might occur to protein production after nuclear transfer. Eight-cell stage pig embryo nuclei were transferred to enucleated metaphase II oocytes. Twelve hours after nuclear transfer the nuclear transfer embryos were labeled with L-[^{35}S] methionine and subjected to 1-dimensional polyacrylamide gel electrophoresis. During normal pig embryo development, embryonic RNA is produced by the 4-cell stage and results in the production of a 51 kDa band. This 51 kDa band persists through the 8-cell and compact morula stages. Eight-cell stage blastomeres of reconstituted embryos continued the production of the 51 kDa band even after treatment with α-amanitin. Since α-amanitin should block new mRNA synthesis, the production of this 51 kDa band is likely the result of a carry-over of mRNA coding for this protein in the blastomere cytoplasm.

nuclear transfer / cloning / swine / embryo / protein synthesis

Résumé — Transfert d'ARNm pendant le transfert nucléaire chez le porc. *Cette étude a pour but d'évaluer les modifications de synthèse protéique qui peuvent se produire après transfert nucléaire. Des noyaux d'embryons de porc au stade 8 cellules sont transférés à des ovocytes «énucléés» en métaphase II. Douze h après transfert nucléaire, les embryons obtenus sont marqués avec la L-méthionine ^{35}S et les protéines analysées par électrophorèse mono-directionnelle sur gel de polyacrylamide. Pendant le développement embryonnaire normal, la synthèse d'ARN au stade 4 cellules aboutit à l'apparition d'une bande protéique de 51 Kd. Cette bande persiste pendant les stades 8 cellules et morula compacte. Après transfert nucléaire, les embryons du stade 8 cellules continuent à produire la bande protéique de 51 Kd après traitement par l'α-amanitine. Comme cet inhibiteur bloque la synthèse d'ARNm, la production de la bande protéique résulte du transport de l'ARNm correspondant présent dans le cytoplasme du blastomère transféré.*

transfert nucléaire / clonage / porc / embryon / synthèse protéique

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INTRODUCTION

Nuclear reprogramming can be described as the event in which a transplanted nucleus is coerced to go through morphological events that it previously completed. Without this reprogramming serious developmental consequences would result (Prather and Robl, 1991). For example, if an 8-cell blastomere was transferred to a recipient enucleated metaphase II oocyte and compaction occurred at the next cell stage, *ie* the 2-cell stage, too few cells will be present to form a normal embryo. In the mouse such an embryo would fail after implantation because a minimum of 8–16 cells at compaction is required to form a competent blastocyst, containing both an inner cell mass and a trophoblast (Tarkowski and Wroblewska, 1967). When nuclei are transferred to enucleated oocytes and the early developmental events are recapitulated, it might be concluded that reprogramming occurred. Smith and Wilmut (1989) demonstrated this by transferring inner-cell-mass cells of sheep into recipient enucleated oocytes. They observed early cleavage stages and then compaction, blastocoel formation and development to term. Theoretically, if the nuclei had not been reprogrammed to act as a zygote nucleus, then the conceptus would not have gone to term. Therefore, the presence of blastocyst stage embryos from nuclear transfer procedures at a time coincident with normal development of fertilized embryos indicates that the transplanted nucleus has, to some degree, been reprogrammed (Willadsen, 1986; Prather *et al,* 1987, 1989a; Stice and Robl, 1988; Young *et al,* 1991).

In an attempt to begin to study the biochemical events that would be indicative of nuclear reprogramming, we focused on the 51 kDa band produced by the 4-cell stage pig embryo. Sixteen-hours after cleavage to the 4-cell stage RNA polymerase II-dependent RNA synthesis results in the appearance of at least 3 new proteins (26, 42, and 51 kDa) (Schoenbeck *et al,* 1992). The 51 kDa band increases in intensity and became most prominent at 24 h post-cleavage, and persists into the 8- and 16-cell stage. Thus, we wanted to test whether the 51 kDa band would disappear after a blastomere from an 8-cell stage embryo was transferred to an enucleated metaphase II oocyte.

MATERIALS AND METHODS

Oocyte collection and selection

Ovaries were collected and transported from a commercial slaughterhouse to the lab at 39°C. Follicles 3–6 mm in diameter were aspirated from oocytes and then rinsed in TL Hepes (Prather *et al,* 1989a) supplemented with 0.3% BSA. Oocytes with a uniform cytoplasm and a thick complete cumulus cell mass were selected and matured in Waymouth MB 752/1 (Sigma Chemical Co, Saint Louis, MO) supplemented with 10% fetal calf serum, 10% follicular fluid, 10 IU/ml PMSG and 10 IU/ml hCG for 20 h, and then in hormone-free Waymouth medium for 24 h (Funahashi and Day, 1993). Fifty microliter drops of medium were placed in polystyrene culture dishes (Becton Dickinson Labware, Oxnard, CA), and overlaid with paraffin oil that had been pre-equilibrated in Whitten's medium salts and sterilized. Maturation occurred at 39°C in a humidified atmosphere of 5% CO_2 in air with 10 oocytes per drop of medium.

Embryo collection

Crossbred gilts were monitored for estrus twice a day, and inseminated at 12 and 24 h post-estrus detection. Four days after estrus detection, gilts were anesthetized and a midventral laparotomy was performed to exteriorize the reproductive tract. The oviducts and uterus were flushed retrograde with TL Hepes. Embryos were then recovered and placed in 50 μl of TL Hepes and placed in a 39°C incubator with a humidified air atmosphere until manipulation.

Manipulation

Nuclear transfer was conducted as described by Prather *et al* (1989a). Eight-cell stage embryos and unfertilized recipient oocytes were treated with cytoskeltal inhibitors (7.5 μg/ml cytochalasin B and 0.1 μg/ml demecolcine; Sigma, Saint Louis) and the DNA-specific fluorescent dye bisbenzimide (2 μg/ml, Sigma). The recipient oocyte was flashed with ultraviolet light in order to locate the metaphase chromosomes; a micropipette was then inserted into the oocyte, and the metaphase chromosomes were removed with a portion of membrane-bound cytoplasm. One blastomere from the donor embryo was inserted in the perivitelline space, against the plasma membrane of the enucleated metaphase II oocyte. The nuclear transfer embryo was then exposed to 5 V/mm AC for 10 s, followed by 120 V/mm DC for 30 μs in electroporation medium (0.3 M mannitol + 5% TL Hepes: Prather *et al*, 1991). This both activated the oocyte and induced cell-cell fusion. Thirty minutes post-electrostimulation, samples were evaluated for fusion or non-fusion.

Culture and radiolabeling

Embryos, oocytes and nuclear transfer embryos were cultured in TL Hepes *(Experiment 1)* or TL Hepes containing 20 μg/ml α-amanitin *(Experiment 2)* in a humidified atmosphere of air at 39°C for 12 h, then were placed in 26 μl of TL Hepes containing 3 μl L-[^{35}S] methionine (Du Pont Company, Wilmington, DE) for 3.5 h. The samples were then rinsed through six 50 μl drops of non-radioactive TL Hepes with a final rinse in phosphate-buffered saline (BSA-Free). Samples were then placed in microcentrifuge tubes with 15 μl of SDS lysis buffer and stored at –80°C.

Protein profiles

Radiolabeled samples and prestained molecular mass markers (Rainbow Protein Molecular Weight Markers, Amersham) were boiled for 1 min. Samples were subjected to 10% 1-dimensional SDS-PAGE and run in parallel with molecular mass markers. After electrophoresis, gels were fixed and treated with a fluorite (En^{3}Hance; New England Nuclear, Boston, MA). Gels were dried under vacuum at 70°C for about 1 h, and exposed to Kodak X-OMAT AR film (Eastman Kodak, Rochester, NY) for 2–6 d.

Statistics

Differences in the presence or absence of the 51 kDa band were evaluated by constructing 95% confidence intervals about the mean (Snedecor and Cochran, 1980).

RESULTS

After 12 h, the 51 kDa band was present in all donor 8-cell stage embryos (7/7), while it was not detectable in oocytes (0/7) or enucleated oocytes (0/7). The 51 kDa band was present in the non-fused nuclear transferred embryos 66% (8/12) of the time, while in the fused nuclear transferred embryos it was present 43% (9/21) of the time ($P > 0.05$).

Since the 51 kDa band remained even after nuclear transfer, it was decided to block new mRNA synthesis with α-amanitin. In the second experiment, embryos were placed in α-amanitin after cell fusion. The 51 kDa band could be detected in all of the donor 8-cell stage embryos (8/8) while it was not detectable in the oocytes (0/6) or the enucleated oocytes (0/6). Interestingly, the 51 kDa band was still present in the non-fused (5/7) as well as the fused (7/7) nuclear transfer embryos ($P > 0.05$) (fig 1).

DISCUSSION

Nuclei from early cleavage stage mammalian embryos other than mouse embryos, have been reprogrammed to behave as a 1-cell embryos by transfer to enucleated activated meioctic metaphase II oocyte (First and Prather, 1991). In efforts to test the idea of reprogramming we placed a blastomere

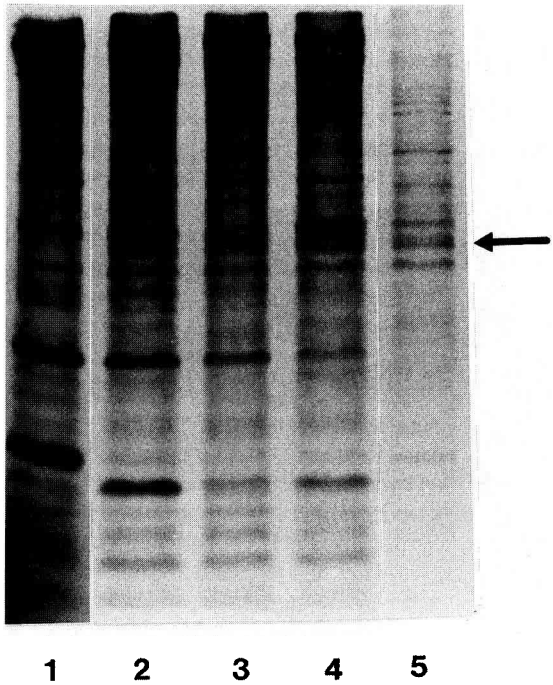

Fig 1. Protein profiles of nuclear transfer and control embryos. Embryos were cultured for 12 h in the presence of α-amanitin and then labeled with L-[^{35}S] methionine for 3.5 h, rinsed and individually lysed. Individual embryos were placed on each lane; lane 1, metaphase II oocyte; lane 2, non-fused nuclear transfer embryo; lanes 3 and 4, fused nuclear transfer embryo; lane 5, donor 8-cell stage embryo. Arrow denotes the 51 kDa band.

from an 8-cell stage embryo in an enucleated metaphase II oocyte and evaluated the protein profiles. Since the late 4-cell stage to blastocyst stage pig embryo produces a 51 kDa band, we hypothesize that the disappearance of this band after nuclear transfer would indicate reprogramming. It was found that half of the fused nuclear transferred embryos were still producing the 51 kDa band, which was not different from the non-fused nuclear transfer embryos. In the next experiment the production of new mRNA was blocked with α-amanitin. After culturing the fused nuclear transferred embryos in α-amanitin, the 51 kDa band was detected 100% of the time. While the 51 kDa band was present in all of the nuclear transfer embryos, this was no different from the non-fused controls; a similar relationship held for both experiments. Unfortunately, this did not answer the question of reprogramming but does suggest that there is mRNA carry-over from the cytoplasm of the transferred blastomere. When cell fusion was initially used to facilitate nuclear transfer little concern was given to the possibility of message carry-over. This is the first study clearly demonstrating that a message present in the donor blastomere can result in protein production in the resulting nuclear transfer embryo.

Nuclear remodeling is suggested by previous studies showing an exchange of protein between the cytoplasm of the oocyte to the transferred nucleus and from the transferred nucleus to the cytoplasm of the oocytes (Prather and Robl, 1991). A specific example in the pig of the acquisition of oocyte protein by the transferred nucleus is the nucleus lamins (Prather *et al,* 1989b), while the release of nuclear protein into the cytoplasm is suggested with the I1 antigen (Prather *et al,* 1992). This exchange of protein is thought to be responsible for the

structural remodeling needed for reprogramming the transferred nucleus. Furthermore, results obtained with electron microscopy demonstrate that after nuclear transfer, nucleoli appear morphologically similar to zygotic nucleoli. This suggests that due to the morphologic change the nucleoli of the nuclear transfer embryo are not synthesizing rRNA (Mayes *et al,* 1994). In addition, data gathered by Prather and Rickords (1992), using a monoclonal antibody to proteins involved in splicing RNA, suggest that RNA processing does not occur immediately after nuclear transfer.

Similar suggestions of reprogramming in other domestic species have been observed with the TEC antigen (Van Stekelenburg-Hamers *et al,* 1994) as well as the attainment of specific developmental stages after nuclear transfer (reviewed by First and Prather, 1991). Therefore, since we detected the 51 kDa band in the α-amanitin-treated fused nuclear transferred embryos, we cannot conclude that nuclear reprogramming had occurred, while we can conclude that there is mRNA carry-over from the donor blastomere. One way to circumvent this carry-over of RNA would be to transfer only the nucleus as in amphibian nuclear transfer (Gurdon and Laskey, 1970). Such a procedure has recently been reported in cattle (Collas and Barnes, 1994), but unfortunately has not dramatically increased the subsequent rates of development. If message carry-over has an affect on the ability of the nuclear transfer embryo to develop, there may actually be an advantage to using donor cells that are more advanced in development, as they would also be smaller in size and possibly contain less RNA to carry-over.

Further studies are needed to evaluate the effects to these messages on development and evaluate specific messages for reprogramming. Such experiments will be much easier to conduct and much more meaningful when the 51 kDa protein(s) is identified and the sequence of the mRNA elucidated. Such knowledge will reduce the problems of identifying maternal, zygotic or carry-over RNA.

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