# ABSTRACTS FOR POSTER PRESENTATION

*Transgenesis*

## 340 PRODUCTION OF TRANSGENIC PORCINE BLASTOCYSTS BY HANDMADE CLONING

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The present study demonstrates the application of the recently developed handmade cloning (HMC) technique in production of transgenic porcine blastocysts. The HMC technique was originally established for bovine nuclear transfer (Vajta *et al.*, 2003, Biol. Reprod. 68, 571–578), and has the advantages of being less demanding and more productive than traditional nuclear transfer techniques. Cumulus-oocyte complexes were aspirated from slaughterhouse ovaries and matured for 41 h. Subsequently, the cumulus cells were removed by pipetting in 1 mg mL$^{-1}$ hyaluronidase in HEPES-buffered TCM-199; zonae pellucidae were removed by incubation in 2 mg mL$^{-1}$ pronase in HEPES-buffered TCM-199 supplemented with 2% cattle serum (T2) for 1 min. Bisection was performed by hand under a stereomicroscope using a microblade in 5 μg mL$^{-1}$ cytochalasin B in TCM-199 supplemented with 20% cattle serum (T20). Demi-oocytes were incubated in 5 μg mL$^{-1}$ Hoechst 33342 in T20 for 10 min, followed by examination under UV light to select the halves containing no chromatin, i.e., the cytoplasts. Porcine fibroblasts harvested from an ear skin biopsy were transfected with pN1-EGFP (Clontech) using Lipofectamine (Gibco, Life Technologies). G418 selection (0.8 mg mL$^{-1}$) was applied 48 h after transfection, and well separated G418-resistant cell colonies originating from a single transfected cell were isolated, expanded, and cryopreserved. Days before, nuclear transfer cells were grown to a confluent monolayer in DMEM supplemented with 10% FCS. Fusions were performed 43 h after start of maturation. One cytoplast was attached to one fibroblast in 500 μg mL$^{-1}$ phytohemagglutinin dissolved in T2. In the fusion chamber, covered with fusion medium (0.3 M mannitol, 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$, and 0.01% PVA), one cytoplast-fibroblast pair was fused with one cytoplast in a single step. The fusions were performed with a double DC pulse of 65 V, each pulse for 20 μs and 0.1 s apart from each other. Successfully fused embryos were activated 1 h after the end of fusion by incubation in 2 μM calcium ionophore A23187 in T20 for 5 min followed by 3-h incubation in microdrops of culture medium (NCSU-23 with 4 mg mL BSA) containing 2 mM 6-dimethylaminopurine. Activated embryos were cultured individually in microdrops of culture medium for 7 days. In four independent experiments, 93% of attempted reconstructed embryos fused and survived activation (31/31, 15/23, 28/28, and 37/37, respectively). On Day 7 after activation, the blastocyst rates (per successfully reconstructed embryos) were 6% (2/31), and 7% (1/15), 7% (2/28), and 3% (1/37), respectively. Green Fluorescent Protein was expressed in all cells of the developing blastocysts. The results show that transgenic porcine blastocysts can be produced using HMC, and the technique may also be applied for the production of transgenic pigs.

## 341 APOPTOSIS IN SOMATIC CELL CLONED AND EGFP TRANSGENIC BOVINE EMBRYOS

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The overall success rate achieved by cloning techniques to date is low, mainly due to deficiencies in nuclear reprogramming, gene expression and DNA fragmentation, which result in early and late embryonic losses. This study was carried out to compare the incidences of DNA fragmentation during development of IVF, parthenote (PT), nuclear transfer (NT) and transgenic-cloned (TG) embryos. Terminal deoxynucleotidyl transferase (TdT) nick-end labelling (TUNEL) with propidium iodide counterstaining was used for determination of DNA fragmentation and total cell number. Donor cells were fetal fibroblasts with or without transfection with EGFP, and cultured in DMEM + 15% FCS until confluent, for up to 5 days. At 19 h post maturation (hpm), enucleated oocytes were reconstructed with donor cells and activated at 24 hpm with the combination of ionomycin (5 μM, 5 min) and cycloheximide (10 μg/mL, 5 h) after electric fusion by a single DC pulse (1.6 KV/cm, 60 μs) delivered by a BTX 200. Parthenotes were produced by the same activation protocol at 24 hpm. The eggs and control IVF embryos were cultured in CR1aa at 39°C in a humidified atmosphere of 5% $CO_2$ in air. Differences among groups were analyzed using one-way ANOVA after arc-sine transformation of proportional data. Embryos at the 8-cell stage in all treatments, IVF, PT, NT and TG, showed DNA fragmentation. The apoptotic cell index (total number of apoptotic nuclei/total number of nuclei) of Day 7 blastocysts was significantly ($P < 0.05$) higher in TG and NT embryos (17/91, 18.6 ± 4.0% and 13/94, 13.8 ± 4.7%, respectively) compared to IVF and PT embryos (9/122, 7.4 ± 3.4% and 8/93, 8.6 ± 2.9%, respectively). TUNEL positive cells were detected in almost all blastocysts at Day 7 and were mainly observed in the ICM. The DNA fragmentation ratio of the ICM in the blastosysts at Day 7 (number of apoptotic nuclei in the ICM/total number of apoptotic nuclei in the blastocyst) was significantly ($P < 0.05$) higher in TG embryos (64.7 ± 21.4%) than in IVF (44.4 ± 28.0%), PT (50.0 ± 18.6%) or NT (53.0 ± 32.5%) embryos. These results indicate a higher occurrence of DNA fragmentation observed in NT and TG embryos when compared to IVF and PT embryos. In addition, ICM of TG blastocysts revealed a high DNA fragmentation ratio, which may be related to early embryonic loss after transfer of the resulting embryos. [Supported by High Technology Development Project for Agriculture and Forestry Korea, MAF-SGRP, 300012-05-3-SB010 and Cho-A Pharm. Co. LTD.]

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## 342 EFFECT OF INCREASED SUCKLING STIMULATION ON PIGLET WEIGHT GAIN AND DAILY MILK YIELD OF SOWS TRANSGENIC FOR BOVINE α-LACTALBUMIN

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The objective of the present study was to determine the effects of over-expression of a mammary-specific transgene, bovine α-lactalbumin, and increased suckling stimulation on milk yield in sows and resultant piglet weight gain. Lactational response to increased suckling stimulation was determined by fostering piglets of either the same age (D1) or seven days older (D7) than sow day of lactation to Yorkshire sows either non-transgenic (C) or transgenic for bovine α-lactalbumin (bALA). Twenty first-parity Yorkshire sows were allocated between four treatments dependent on the combination of sow genotype and age of litter fostered (bALA-D1, bALA-D7, C-D1, C-D7). Litters were standardized to 10 piglets each and fostered to subject sows at approximately 36 hours postpartum. All D1 and D7 litters were not significantly different in mean piglet weight within age groups. Sow milk yield (kg) was determined by the weigh-suckle-weigh method on Days 6, 9, 12, 15, and 18 postpartum. Piglet weight gain (g) was determined by calculating the difference between individual piglet weights on Days 3 and 18 of sow lactation. Least-squares means and standard errors for overall daily milk yield of bALA sows with D1 and D7 foster piglets were $7.1 \pm 0.4$ and $9.1 \pm 0.4$ kg, respectively, reflecting a significantly greater milk yield (28% increase, $P < 0.05$) in response to increased suckling stimulation. Least-squares means and standard errors for overall daily milk yield of control sows with D1 and D7 foster piglets were $6.7 \pm 0.4$ and $7.0 \pm 0.4$ kg, respectively, reflecting a non-significant change in milk yield (4% increase, $P > 0.05$) in response to increased suckling stimulation. The bALA-D7 treatment resulted in a 31% increase in mean daily milk yield as compared to the combined mean daily milk yield of the three remaining treatments. Cumulative weight gain of piglets suckling bALA sows resulted in least-squares means and standard errors of $3411 \pm 156$ g for D1 and $3458 \pm 202$ g for D7, and piglets suckling control sows had cumulative weight gains of $2944 \pm 143$ g for D1 and $3016 \pm 175$ g for D7. Overall effect of treatment in the model was significant ($P < 0.05$) through Day 12 of sow lactation. Piglets that suckled bALA sows, regardless of age of litter when fostered, grew significantly faster than piglets that suckled control sows for the entire 15 days of lactation studied ($P < 0.05$), resulting in a cumulative average of 455 grams of additional gain per piglet. First-parity bALA sows were more able to respond to increased suckling stimulation by a resultant increase in milk yield than control sows under equivalent conditions. Similarly, piglets suckling bALA sows gained weight faster than piglets suckling control sows. Over-expression of the bovine α-lactalbumin transgene in sows enhances mammary response to regulators of milk yield, such as level of suckling stimulation. (Supported by the Illinois Council for Food and Agricultural Research Sentinel Grant).

## 343 NUCLEAR LOCALIZATION SIGNAL AND CELL SYNCHRONY ENHANCES GENE TARGETING EFFICIENCY IN FETAL BOVINE FIBROBLASTS

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The use of primary somatic cells for nuclear transfer has facilitated the manipulation of the domestic animal genome via homologous recombination. Yet, the absolute frequency of homologous recombination (HR) in somatic cells is two orders of magnitude lower than in ES cells whereas frequencies of non-homologous end joining are higher. While a few loci have been targeted in somatic cells using enrichment strategies similar to those used in mouse ES cells, there have been problems of low efficiency, mixed targeted and non-targeted cells, and difficulties in cloning the cell after targeting. We present evidence that the use of a nuclear localization signal (nls) and S-phase cell cycle synchronization by thymidine block enhances targeting efficiency at the hypoxanthine phosphoribosyl transferase locus in primary fetal bovine fibroblasts. We designed two hypoxanthine phosphoribosyl transferase (HPRT)-targeting constructs, HPRT-DEx6 and HPRT-DEx6-nls. Both constructs have a 31-bp deletion and a PGK-puro insertion in exon 6 to ensure inactivation of the HPRT locus. Additionally, the HPRT-DEx6-nls construct contains a 180-bp cassette comprised of two 72-bp tandem repeats from SV40 enhancer known to act as nuclear localization signal. Diploid male cells that undergo targeted gene disruption at the single copy X-linked HPRT locus can be selected with 8-Azaguanine (8-AG) as HPRT cells are resistant to 8-AG; all transformants, random and targeted, can be selected in puromycin. Male primary bovine fibroblasts were electroporated with linearized targeting constructs, and plated in media containing puromycin or puromycin plus 8AG. All experiments were done in triplicate and data were analyzed by two-way ANOVA with NLS and cell synchrony as the two factors. Significance was set at $P < 0.01$. While the total number of insertions (random plus targeted) with both constructs were equivalent, the HPRT-DEx6-nls construct produced a significantly higher number of targeted colonies (1–2 8AG-resistant colonies per $9.5 \times 10^6$ cells) than HPRT-DEx6 where no targeted events were seen. Cells were synchronized in the S-phase of the cell cycle by a 2 mM thymidine treatment for 24 hours and electroporated with the linearized targeting constructs. Compared to non-synchronous cells, the total number of insertions (random plus targeted) was reduced by 59-fold in constructs with or without nls ($P < 0.01$), while targeted insertions increased 6-fold in the HPRT-DEx6-nls construct, from an average of 10 per million cells without nls to 7.6 per 10 million with nls ($P < 0.01$). All 8AG-resistant colonies were verified by Long Range-PCR, and PCR products confirmed by end sequencing. This finding has important implications for targeting in somatic cells, as with a drastic reduction in the number of random insertions, and increased targeting due to the presence of the nls, identification of a targeted colony is greatly facilitated even in cases where no enrichment protocols are available.

## 344 ICSI-MEDIATED GENE TRANSFER SKEWS SEX RATIO AGAINST FEMALE BIRTHS IN MICE

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ICSI-mediated gene transfer has been used as an alternative method to pronuclear microinjection for the genomic modification of many species. With this method, transgenic embryos are produced by the microinjection of metaphase II oocytes with spermatozoa previously incubated with foreign DNA. Recently, it was shown in mice that the low percentage of transgenic animals produced from injected oocytes, results from the fact that the expression of foreign DNA is associated with paternal chromosome degradation (Szczygiel M.A. *et al.*, 2003 Biol. Reprod. 68, 1903–1910). It is also known that sex chromosomes localize preferentially, at least in humans, on the periphery of the sperm nucleus on sub-acrosomal regions (Sbracia M. *et al.*, 2002 Hum. Reprod. 17, 320–324), suggesting a high level of interaction with foreign DNA molecules with possible impact on the sex ratio of the offspring. In order to test this hypothesis we have compared ICSI (no DNA), and with ICSI-mediated EGFP (5 Kb plasmid DNA from Clonetech, Spain) transfer, with ICSI-mediated YRT3 (a mouse tyrosinase gene derivative YAC-DNA with 100 Kb; Montoliu L. *et al.*, 1996 EMBO) transfer. Gametes were from 6–8 weeks old CD1 mice. ICSI-mediated gene transfer with post-thawed immotile spermatozoa, extended in M2 medium in the absence of ion chelators (EDTA and EGTA), was done as previously described (Szczygiel M.A. *et al.*, 2003 Biol. Reprod. 68, 1903–1910). Table 1 below summarizes the data collected. Relative to our control, sex ratio deviation was a consequence of the coinjection of DNA. Forty-three percent of males were obtained with regular ICSI, whereas 64% and 65% were the respective percentages when EGFP or YRT3 DNA was coinjected with spermatozoa. This statistically significant ($P < 0.05$, z-test, Sigma Stat, Jandel Scientific, USA) sex ratio deviation, favoring male ICSI offspring when foreign DNA is coinjected, may result from a higher female embryo susceptibility to parental sex chromosome fragmentation induced by the interaction with foreign DNA molecules. Possible impairment of X chromosome inactivation and dosage compensation resulting from the fragmentation of the sex chromosome on X-carrying spermatozoa could explain this female embryo degeneration. Supporting this view, it was recently shown in mice that sex ratio can be skewed against female births by a mutation in a single gene of the X chromosome (Tsix) involved in such mechanisms (Lee J.T., 2002 Nat. Genet.). In conclusion, mouse ICSI-mediated gene transfer induces sex ratio deviation favoring male offspring.

**Table 1. Sex ratio of the offspring obtained with ICSI, ICSI-mediated EGFP transfer, and ICSI-mediated YRT3 transfer**

| Technique employed ([DNA] in ng $\mu L^{-1}$) | Embryos transferred | Offspring (%) | Sex ratio (male/female) |
|---|---|---|---|
| ICSI (0) | 98 | 21 (21.4)[a] | 0.43[a] (9/12) |
| ICSI-EGFP (4–8) | 223 | 22 (9.9)[b] | 0.64[b] (14/8) |
| ICSI-YAC (0.2–8) | 659 | 52 (7.9)[b] | 0.65[b] (34/18) |

[a,b]Values with different superscripts are significantly different, ($P < 0.05$).