Developmental Competence of HMC\textsuperscript{TM} Derived Bovine Cloned Embryos Obtained from Somatic Cell Nuclear Transfer of Adult Fibroblasts and Granulosa Cells

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Abstract. To enable us to handle a large number of oocytes at a given time and to have an increased throughput of cloned embryos, we attempted the Handmade cloning (HMC\textsuperscript{TM}) technique, a zona-free method of bovine somatic cell nuclear transfer. Our objective was to study the developmental competence of the HMC\textsuperscript{TM} derived embryos obtained using different types of somatic cells. A total of 6,874 cumulus-oocyte-complexes were used with either 7th or 11th passage fibroblasts (1st and 2nd groups, respectively), which were prepared from male animals, or granulosa cells (3rd group) as nuclei donors. The average cleavage rate was 65\%, accompanied by a blastocyst rate of just 2\% for the cleaved products and 5\% for the > 8-cell embryos, and there was no significant difference between the three groups. Out of 27 blastocysts recovered, 22 blastocysts were transferred to 22 recipients, resulting in two pregnancies. One pregnancy was lost after the fourth week while the other progressed to full term with the birth of a male calf. This first successful cloning of a male calf with the HMC\textsuperscript{TM} technique in Europe indicates the successful adoption and establishment of this technique in our laboratory, and that this technique can be successful in producing viable embryos.

Key words: Bovine, Fibroblast, Granulose cell, Somatic cell nuclear transfer

Since the first report of live mammals produced by nuclear transfer from a cultured differentiated cell population in 1995, cloning has been successfully achieved in different species of animals using a variety of somatic cell types as nuclear donors. In spite of its low efficiency, experimental animal cloning is being widely conducted in laboratories throughout the world because of the promise it holds in the fields of therapeutic cloning and endangered breed and species preservation [1], as well as in the production of transgenic animals [2]. The factors that probably contribute to the low level of efficiency in cloning include laboratory to laboratory variation, oocyte source and quality, methods of embryo culture, donor cell type, possible loss of somatic imprinting in the nuclei of the reconstructed embryo, failure to reprogram the transplanted nucleus adequately, and the failure of artificial methods of activation that are supposed to mimic the normal events accompanying...
fertilization.

In the past, nuclear transfer has mainly relied on micromanipulation-assisted enucleation followed by injection of one donor cell into the perivitelline space, thus requiring sophisticated and expensive micromanipulation equipment and considerable skills from the operator. The first published attempt to exclude micromanipulators was the bovine zona-free embryonic cell nuclear transfer method [3], which was, subsequently, slightly modified [4] for carrying out somatic cell nuclear transfer. We also made an attempt to use the Handmade Cloning (or HMC™) method, which is a 'zona-free' method of somatic cell nuclear transfer [5] in cattle. HMC™ bypasses the use of micromanipulators and involves removal of the zona pellucida of bovine oocytes by pronase, manual bisection of the oocytes, selection of cytoplasts by Hoechst staining, and two-step fusion of one somatic cell with two cytoplasts. The fused reconstructed embryos are then activated by calcium ionophore and dimethylaminopurine and subsequently cultured in the well-of-the-wells (WOW) system [6]. Blastocysts are collected on day 7 of culture.

We made an attempt to study the developmental competence of HMC™ derived embryos obtained by using different types of somatic cells (7th and 11th passage fibroblasts and granulosa cells) as nuclei donors, and to determine the viability of cloned embryos derived by the HMC™ technique when transferred at the blastocyst stage.

**Materials and Methods**

Unless otherwise indicated, all plasticware i.e. culture vessels and dishes used in our experiments were obtained from Nunc (Wiesbaden, Germany), while all chemicals and medium, etc. were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA).

*Nuclear donor cells, cell culture and passages*

A cell line of bovine fibroblasts was established using mechanically isolated sections of ear skin isolated from an adult elite bull. Small tissue sections (approximately 1 mm in diameter) were cultivated in TCM-199 medium containing 10% FCS (fetal calf serum), 1 mM sodium pyruvate, and antibiotics. After one to two weeks, the culture vessels were washed with medium to remove the tissue sections and dead cell material. The cells growing in the monolayer were cultivated up to confluence. Passages were performed using trypsination (0.02–0.05% trypsin-EDTA) and recultivation. Aliquots were stored by cryopreservation (20% FCS and 1% DMSO in TCM-199; shock freezing, −96 C) after each passage.

Fibroblast cells from passage 7 and 11 and freshly isolated bovine granulosa cells (ovarian follicles > 5 mm of ovaries collected from a commercial slaughterhouse were punctured) were cultivated in TCM-199 (10% FCS, 1 mM sodium pyruvate and antibiotics) up to confluence. Cells in the G0/G1 phase of the cell cycle were enriched for the purpose of nuclear transfer by the method of serum deprivation in culture medium (0.5% FCS, 2 days).

**Cell cycle analysis**

Adherent cells of confluent cultures were washed gently twice with HBSS (Hank's balanced salt solution without Ca²⁺ and Mg²⁺). Ten milliliters of trypsin-EDTA (0.02–0.05%) in HBSS were added to a 10 ml flask. After 10 min, the cells were washed twice with HBS (Hank's balanced salt solution with

![Flow cytometric histogram of cultured bovine fibroblasts (11th passage) after serum starvation, trypsination and propidium iodide staining. H: G1/G0 peak, I: S phase, J: G2/M peak, and G: apoptotic and necrotic cells and cell debris.](image)
Ca\(^{2+}\) and Mg\(^{2+}\)) and resuspended in HBS. Cells were fixed in ethanol (70%), washed, and treated with RNAse solution (100 units ml\(^{-1}\) in HBS, 37 C, 30 min). Prior to that, the RNAse solution was heated (85 C, 60 min) to denature DNase contamination. After incubation for 30 min with propidium iodide (70 \(\mu\)M) in HBS (5 mmol l\(^{-1}\) Hepes, pH 7.3, 150 mmol l\(^{-1}\) NaCl), flow cytometry was performed (Coulter Elite) and the cell cycle was analysed by the computer-aided Multicycle Program (Phoenix) of the flow cytometer program package.

**Cytogenetic analysis**

Fibroblast cells from passage 10 were used for metaphase preparation using standard procedures. Cells were grown in MEM supplemented with 10% FCS and antibiotics till confluency. Afterwards, the passaged cells were treated with 10\(^{-7}\) mmol/ml MTX to block mitotic activity for 18 h. The block was released by washing in PBS and feeding with medium containing 1.6 mM thymidine. After 5 h, cells were treated with 0.001% Colchicine for 30 min and harvested. After hypotonic treatment in 0.075 M KCl for 15 min cells were fixed in 3:1 methanol-glacial acetic acid for 20 min. After changing the fixative 2 times, cells were dropped onto ice-cold wet slides and air dried. For overview analysis, slides were stained with 4% Giemsa solution (Merck: Azur Eosin). At least 30 metaphase spreads were examined at a magnification of 1,600 \(\times\). Chromosomal abnormalities, defined as deletions, breaks, fragments and gaps [7], were analysed.

For G-banding, 4-day-old slides were treated with 0.2 N HCl for 10 min followed by a short rinse in 0.9% NaCl. After digestion in 0.025% trypsin (GIBCO BRL-in 0.9% NaCl) at 37 C for 25–45 sec, slides were incubated in 2xSSC at 60 C for 10–15 min and stained with 5% Giemsa (Merck: Azur Eosin) in Sorensen’s buffer, pH 6.8, for 7–8 min. Karyotyping was performed using the MacKType image analysis system (Perceptive Scientific Instruments) [8]. Ten karyograms were examined for visible abnormalities.

**Oocyte collection and in vitro maturation (IVM)**

Oocytes were collected by the ovarian slicing method [9–11] from abattoir-derived ovaries 4 to 6 h after slaughter. Within 3 h of slaughter, the ovaries were transported in physiological saline (0.9% NaCl) to the cell culture laboratory where they were washed in phosphate buffered saline (PBS). Prior to slicing, each ovary was placed in a Petri dish and covered with TCM-199 [with Hank’s salts and 25 mM N-2-hydroxyethyl piperazine-N’-2-ethanesulfonic acid (HEPES) buffer] containing 0.5 IU/ml heparin. A stereo dissecting microscope at a magnification of up to 120 \(\times\) was used to locate the oocytes within the dish. The oocytes were then collected in a sterile 4-well culture dish containing 500 \(\mu\)l TCM-199. Oocytes with a compact cumulus investment were selected for further culture and were designated as cumulus-oocyte-complexes (COCs). The collected COCs were washed twice in maturation medium [medium TCM 199 supplemented with 20% (v/v) heat-treated FCS (F 7425) and 10 \(\mu\)g/ml follicular stimulating hormone (FSH, Ovagen, New Zealand)] and then incubated in maturation medium (in 4-well dishes with 500 \(\mu\)l of medium per well) for 21 h at 38.5 C in 5% CO\(_2\) in air.

**Nuclear transfer (NT)-handmade cloning (or HMC\(^{TM}\) method**

The HMC\(^{TM}\) process involved in vitro maturation of bovine oocytes, removal of the zona pellucida by pronase, manual bisection of the oocytes, selection of cytoplasts by Hoechst staining, and two-step fusion of one somatic cell with two cytoplasts [5]. Briefly, at 21–22 h after the start of maturation, 100–150 COCs were vortexed for 3 min in 0.5 mg/ml hyaluronidase dissolved in 500 \(\mu\)l T0 (T indicates Hepes-buffered TCM 199 medium, and the number indicates the percentage of calf serum or CS supplementation). After cumulus removal, oocytes were transferred for 8 min into 1.5 mg/ml pronase dissolved in 600 \(\mu\)l T10 to remove the zona. Oocytes were then transferred to a 35-mm Petri dish with T20 containing the appropriate concentration of cytochalasin B and 10 \(\mu\)g/ml fluorochrome Hoechst 33342 dissolved in T2 where they were manually bisected under stereomicroscopic control (Fig. 2 A) using Ultra Sharp Splitting Blades (AB Technology, Pullman, WA). All stained demioocytes were then placed into 3-\(\mu\)l drops of T2 (three per drop) and positions of half-oocytes without chromatin staining (cytoplasts) were registered under ultraviolet light (Fig. 2 B). The cytoplasts were then collected under a stereomicroscope and transferred into 800 \(\mu\)l T20 for temporary storage.
The somatic cells (in a 4-well cell culture dish) were repeatedly washed with Ca\(^{2+}\)– and Mg\(^{2+}\)–free PBS and incubated at 39°C for 5 min in 100 µl of 0.05% trypsin dissolved in PBS. The well was then filled with 800 µl of T20, and the cells were separated by vigorous pipetting and stored in 2 ml Eppendorf tubes at room temperature until fusion.

For the first fusion, half of the total quantity of prepared cytoplasts was transferred into the first well of a four-well dish containing 800 µl T20. Five microliters of the somatic cell suspension was sedimented to the bottom of the middle compartment filled with 4 ml T2. Cytoplasts were then individually transferred to the second well which contained 500 µg/ml phytohemagglutinin dissolved in 400 µl T2 for 3 sec and then quickly dropped onto a single somatic cell. Following attachment, the cytoplast-somatic cell pair was transferred to a fusion chamber (0.5-mm fusion chamber of an electro cell fusion machine; CFA 500 Krüss, Hamburg, Germany) with 0.5 mm separation of wires. Wires were covered with 1 ml of fusion medium (0.3 M Mannitol, 0.1 mM MgSO\(_4\), 0.05 mM CaCl\(_2\)) at 26–27°C. The pair was attached to the north wire using 15 V AC at 700 KHz (Electro cell fusion Machine; CFA 500 Krüss, Hamburg, Germany) in such a way that the somatic cell was positioned furthest from the wire. Fusion was performed with a double DC pulse of 65 V, with each pulse being 20 µsec in length and 0.1 sec apart. The pair was then carefully transferred to the third well of the four-well dish which contained 800 µl T20, and incubated for 15–30 min to determine successful fusion.

For the second fusion, all remaining cytoplasts and fused pairs were transferred to fusion medium.
covering the fusion chamber. Ten cytoplasts at a
time were aligned to one electrode using the same
AC as for the first fusion. One fused pair was then
attached to each cytoplast (Fig. 2 C). A double
fusion pulse with the same parameters as used
previously but at 45 V DC was applied, and the
reconstructs were incubated in T20 for 20 min.
These fused reconstructed embryos were then
transferred into a four-well dish containing 400 µl
culture medium, covered with oil, and incubated in
5% CO2 in air at 39 C.

Activation was initiated approximately 4 h after
the second fusion. Reconstructed embryos were
first incubated in 1 ml T20 containing 2 µM Ca
ionophore A23187 for 5 min at room temperature.
After two subsequent washings in T20,
reconstructed embryos were incubated
individually in 5-µl droplets of culture medium
containing 2 mM 6-dimethylaminopurine (6-
DMAP), covered with oil, and incubated in an
atmosphere of 5% CO2 in air at 39 C for 6 h.
Embryos were then washed twice in culture
medium, cultured individually in well of the wells
(WOWs) [6] in SOF medium (Minitüb, Germany)
supplemented with 10% estrous cow serum, and
covered with mineral oil. Embryo culture was
performed at 39 C in 5% CO2, 5% O2, and 90% N2.

Developmental potential of zona-free cloned bovine
embryos
The developmental competence of the cloned
bovine embryos was assessed by placing the
reconstructed embryos in two different groups—(i)
Group A, where the culture of the cloned embryos
was interrupted on day 4 (day 0 being the day of
the cloning experiment) to determine the number of
cleaved embryos in different stages of cleavage,
and, (ii) Group B, where the culture of the cloned
embryos after reconstruction was allowed to carry
on uninterrupted for the next seven days,
subsequent to which the number of blastocysts was
determined using a stereomicroscope.

Blastocyst transfer, pregnancy monitoring, and birth
Embryo (Blastocyst) transfers (ET) were carried
out in a large herd of recipients on the day of
embryo collection. Synchronized (by application of
PGF2α) heifers or animals after natural heat were
selected for transfer. The animals were at least 15
months old, healthy, cycling regularly, and
weighed a minimum of 350 kg at the time of
transfer. They were checked for an active corpus
luteum. Non-surgical transfers of the embryos
were made by passing a miniature ET gun through
the cervix and into the upper one-third of the
uterine horn, ipsilateral to the ovary bearing the
CL. In recipients where cranial placement of the ET
gun was difficult, a rapid placement of the embryo
in a more caudal position was preferred.

Pregnancy diagnosis was carried out on day 30 by
ultrasonography using a 5 MHz linear probe.
Rectal examinations were done on days 42 to 45, in
the third and sixth months, and immediately before
birth. A calf was born after cesarean section on day
280 of pregnancy. Cesarean section was
performed via left paralumbar fossa with the
recipient standing.

Results

Cell cycle analysis of donor cells
Analysis of the cell cycle stages (Fig. 1) revealed
that after serum starvation, approximately 82% (7th
passage fibroblasts: 81%, 11th passage fibroblasts:
80%, granulosa cells: 87%) of the cells were at the
G0/G1 phase of cell division, and, therefore, were
suitable for use as nuclei donors. Without serum
starvation, the respective figures stood at 60, 69,
and 70%. Flow cytometric analyses was repeated
three times. No chromosomal abnormalities were
detected until the 11th passage (cytogenetic
overview and banding studies, data not shown).

Cleavage rate and fusion efficiency
We experimented with a total of 6,874 COCs
using three different sources of somatic cells as
nuclei donors, i.e. either 7th or 11th passage
fibroblasts from an elite high performing bull’s ear
skin or granulosa cells from a cow. The overall
average efficiency of formation of the reconstructed
embryos was 94%, with a 61% average cleavage
rate. The efficiency of obtaining the first fusion
products was 99%. Of these, 96% could be
successfully fused for the second time to obtain
reconstructed embryos. A further 2% lysis rate
during activation with calcium ionophore and
DMAP reduced the efficiency of embryo
production to 94%.

Development of reconstructed embryos
In group A (Table 1), only two types of somatic
cells were used as nuclei donors: fibroblasts from the 7th passage and granulosa cells. A total of 1,802 COCs were assessed for their developmental competence up to the 8-cell stage. An average cleavage rate of 57% was obtained, with 24% of the reconstructed embryos being able to reach the 8-cell stage. It was observed that an average of 34% of the cleaved embryos reached the 8-cell stage of development. No significant differences were observed between the two groups of somatic cell donors in terms of cleavage rate and number of > 8-cell stage embryos.

In group B (Table 2), all three types of somatic cells were used as nuclei donors - fibroblasts from the 7th and the 11th passages and granulosa cells. A total of 5,072 COCs yielded an average cleavage rate of 65%, with 26% of the reconstructed embryos being able to reach the 8-cell stage and beyond. Of the cleaved embryos, 43% progressed up to the 8-cell stage and beyond, with an overall blastocyst rate of just 2% of cleaved embryos and 5% of the > 8-cell embryos. No significant differences were observed between the three groups of somatic cell donors in terms of cleavage rate, number of >8 cell stage embryos, and blastocyst rates.

Table 1. Developmental competence of cloned embryos up to the 8-cell stage (group A) assessed using 7th passage fibroblasts and granulosa cells

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Fibroblasts 7th passage</th>
<th>Granulosa cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cumulus oocyte complexes utilised (n)</td>
<td>1582</td>
<td>220</td>
</tr>
<tr>
<td>Reconstruction efficiency (% of COCs used)</td>
<td>86</td>
<td>99</td>
</tr>
<tr>
<td>Cleavage rate (% of fused products)</td>
<td>62</td>
<td>52</td>
</tr>
<tr>
<td>8-cell+ embryos (% of total fused products)</td>
<td>23</td>
<td>25</td>
</tr>
</tbody>
</table>

Table 2. Developmental competence of cloned embryos up to the blastocyst stage (group B) assessed using 7th and 11th passage fibroblasts and granulosa cells

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Fibroblasts 7th passage</th>
<th>Fibroblasts 11th passage</th>
<th>Granulosa cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cumulus oocyte complexes utilised (n)</td>
<td>1370</td>
<td>2962</td>
<td>740</td>
</tr>
<tr>
<td>Reconstruction efficiency (% of COCs used)</td>
<td>95</td>
<td>96</td>
<td>94</td>
</tr>
<tr>
<td>Cleavage rate (% of fused products)</td>
<td>60</td>
<td>70</td>
<td>65</td>
</tr>
<tr>
<td>8-cell+ embryos (% of total fused products)</td>
<td>23</td>
<td>28</td>
<td>28</td>
</tr>
<tr>
<td>No. of blastocysts produced (n)</td>
<td>4</td>
<td>19</td>
<td>4</td>
</tr>
<tr>
<td>Blastocyst rate (% of total fused products)</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

Embryo transfers and birth of the cloned calf

A total of 27 transferable blastocysts were recovered out of which 4 were produced using the granulosa cells. Of the remaining 23 blastocysts produced using the bull’s fibroblasts as nuclei donors, 22 blastocysts were transferred to 22 synchronized recipients, resulting in two pregnancies. One pregnancy was lost after the fourth week while the other progressed to full term. The decision was made to use a surgical procedure to secure the survival of the calf. Caesarian section was carried out on day 280 of pregnancy, resulting in the birth of a cloned male calf (Fig. 2 F) that was apparently healthy, but overweight (i.e. approx. 73 kg). DNA fingerprinting of the cloned male calf, conducted by an independent institute, IFN (Schoenow, Germany), confirmed its genetic identity in relation to the parent bull. The genetic match between the calf and the parent bull was performed with the help of a marks Stock II kit for cattle (Perkin Elmer Co., Boston, USA) by referring to 11 marker loci (ISAG database) on two tissue samples (muscle and ear skin). Unfortunately, the calf died 52 h after birth, with the post mortem pathological findings indicating colitis, pneumonia, and liver fibrosis as the cause of death.

Discussion

The primary aim of our present investigation was to successfully establish the zona-free somatic cell nuclear transfer technology in our laboratory, and to study the developmental potential of zona-free...
cloned bovine embryos. Although the number of blastocysts obtained in these investigations is too low to arrive at any definitive conclusions, the initial results are nevertheless promising. It should be borne in mind that this zona-free method tends to waste 50% of the oocyte starting material due to the requirement of fusion of two half cytoplasts in order to restore the original cytoplasmic volume. Since the cloned embryos could consequently contain up to three different genotypes of mitochondrial DNA owing to random fusion between one donor nucleus and two half cytoplasts, the possibility of mitochondrial heteroplasmy, therefore, exists, at least theoretically.

The efficiency of reprogramming by nuclear transfer to enucleated oocytes varies considerably among the different types of somatic cells used, which may originate from a variety of tissues such as mammary gland cells, fibroblasts, cumulus cells, lymphocytes or granulosa cells. Both, fetal and adult, fibroblasts have been successfully used for nuclear transfer, indicating that these tissues, even in the older animals, might contain progenitor cells that are in a relatively undifferentiated state and, therefore, are capable of division. It has been hypothesized that successful reprogramming might only occur in the nuclei of these progenitor cells, leading to the varied results observed [12]. Although cloning using skin cell nuclei offers the advantages of easy accessibility, non-invasiveness, and successful serial passages without the risk of aneuploidy, the developmental or cloning competence of the bovine fibroblasts seem to be affected not only by the genotype of the donor animal but also by the culture conditions used to derive the cell lines [12].

We observed no significant differences between the various somatic cell lines (fibroblasts or granulosa cells) or the different passages (7th vs. 11th) as far as the blastocyst rate was concerned. In a previous study as well, no significant differences in blastocyst development rates were found using various somatic cell types from adult, newborn, and fetal female and male donor cattle [13]. Although no differences in the development of nuclear transfer embryos were observed when using adult fibroblasts that ranged in passage numbers from 5 to 15 [14], a significant decrease in the rate of nuclear remodelling was encountered when using fetal fibroblasts of increasing passage numbers [15]. The cultured cells were unable to direct embryonic development as compared to those derived from a primary culture, possibly due to accumulation of genetic or epigenetic alterations [16]. Somatic cells during in vitro culture are usually prone to undergoing mutations or loss of imprinting and alteration in methylation status, which may account for the abnormal embryo development and perinatal mortality frequently associated with somatic cloning [17].

Enucleated metaphase II stage oocytes are almost exclusively selected as recipient cytoplasts as an obvious choice over the pronuclear zygotes because of their better support to the reprogramming of the donor nuclei [18]. Enucleation tends to remove the metaphase spindle together with some surrounding cytoplasm, which can vary between 5% and 50%, resulting, perhaps, in a corresponding loss of cytoplasmic factors essential for successful nuclear remodelling [19]. It is presumed that some of these cytoplasmic factors may be located in the vicinity of the metaphase plate, resulting in their removal during enucleation. Recent suggestions [20] that the presence of maternal nucleus-associated material may play a role in donor nucleus reprogramming indicate the importance of these factors.

During embryo reconstruction, correct ploidy can be maintained either by transferring nuclei at a defined cell cycle stage (for eg. G0/G1 stage) or by transferring the donor nucleus (G1/S/G2 stage) in preactivated recipient oocytes. In our experiment, we used enucleated non-activated metaphase II arrested oocytes, supposedly having high MPF (maturation promoting factor) activity [21], as cytoplasmic recipients and G0/G1 stage somatic cells as nuclei donors. After completion of second fusion and prior to activation, we incubated the embryo reconstructs for 3 to 4 h in 5% CO₂, 5% O₂, and 90% N₂. It has been suggested [22] that this extension of the exposure period of the donor nucleus to the high MPF activity of the oocyte (and thereby oocyte cytoplasmic factors) to 3 to 4 h before activation may either improve subsequent development or promote development from more differentiated cell types.

Contemporary protocols for the activation of reconstructed bovine nuclear transfer embryos commonly use a sequential treatment that is often comprised of calcium ionophore, ionomycin, or electropulse as the primary treatment designed to
generate an intracellular initial calcium transient [23], followed by a secondary treatment using either cycloheximide (CHX) [24] as a non-specific inhibitor of protein synthesis [25] or dimethylaminopurine (DMAP) [26] as a protein phosphorylation inhibitor [27]. In our experiments, we induced activation of the reconstructed bovine embryos by sequential treatment with calcium ionophore and DMAP. It has been concluded in other studies that cell fusion and activation with 6-DMAP are the most successful procedures to achieve a high rate of blastocyst development following somatic cloning in cattle [28].

Although we did have some success in the production of blastocysts in all three groups of somatic cell donors, the number was rather low to allow us to make any concrete comparisons. Still, there were no significant differences observed in terms of blastocyst yield between all three groups. Although a number of tests are available for assessing the developmental viability of blastocysts, we opine that the best test is to check for pregnancies that may arise from the transfer of the cloned blastocysts. The low overall yield of blastocysts in our nuclear transfer experiments restricts us from being able to make any significant comparisons, although we did find that the blastocysts delivered by zona-free cloning were viable in that two pregnancies resulted from 22 transfers out of which one led to the birth of a viable cloned male calf. In vitro production (IVP) experiments running concurrently with the nuclear transfer experiments in our laboratory yielded a blastocyst rate of 28 to 30%. Similar experiments conducted earlier [9] in our laboratory have yielded a 34% blastocyst rate with IVP, showing that the present blastocyst rate of 28 to 30% was well within acceptable limits within our laboratory. This IVP blastocyst rate is in accordance with other reports published by many international groups. For example, there is a report in which a 29% IVP blastocyst rate was claimed [29]. All of this is an indication of the optimal culture conditions that exist in our laboratory. However, an average blastocyst rate of 51% with the HMC™ in bovines was obtained [5], with no significant difference in developmental rates achieved between granulosa cell and fetal fibroblast cell donors (49% versus 52%, respectively). The total cell number of cloned blastocysts was determined as a measure of embryo quality [30], and it was concluded that nuclear transfer embryos were indistinguishable from in vitro produced embryos using this criteria [30].

Out of the two pregnancies that we could obtain upon transfer of cloned blastocysts, one was lost after the fourth week. This is in conformation to reports that in bovine somatic cloning, most of the pregnancy losses after transfer of cloned blastocysts occur during the second month of pregnancy amounting to a loss of 50% of these pregnancies in cattle [26, 31]. Early loss is frequently associated with functional deficiencies occurring at the onset of placentation as evidenced in sheep [32] and cattle [33] by the lack of placentome development and abnormal vascularization of extraembryonic tissues. These placental abnormalities lead to severe nutrient deficiencies affecting the fetus and induce growth retardation, ultimately causing perimplantation loss. High rates of late abortion in recipients carrying somatic clones has also been observed [26, 31]. The calving and the postnatal period were also critical, with prolonged gestation and dystocia being frequent after somatic cloning in cattle, often requiring the use of corticosteroids or cesarian section for calf delivery [26, 31].

The fact that our cloned male calf was overweight supports the observation of LOS (Large Offspring Syndrome) in bovine and ovine ofsprings following transfer of IVP or cloned embryos [34], resulting, in particular, in increased birth weights. A calf is considered to be suffering from LOS if its birth weight is higher than the mean birth weight of the breed type plus two standard deviations [12]. This problem has often been encountered in cloned and in vitro produced ofsprings and has been correlated with considerable fetal and postnatal losses. It has been demonstrated that in vitro culture of bovine embryos in the presence of high levels of proteins alters the kinetics of embryo development, the expression of developmentally important genes, and ultimately the weight of calves at birth [35]. The occurrence of LOS was reported in 13% of somatic cloned calves born [12], whereas higher proportions (up to 47%) have also been reported [13]. Extended exposure to in vitro conditions may lead to epigenetic modifications of expression patterns in developmentally important genes, which may be regarded as an effort of the embryo to compensate for sub-optimal culture conditions [36]. These errors are likely to contribute to the incidence of developmental anomalies in cloned
It has been reported that the proportion of somatic cloned calves born that are ultimately able to develop normally into adults is limited to 50–60% [12]. Postnatal mortality of cloned calves is increased in the first two days after delivery, and a variety of dysfunctions and anomalies have been observed and confirmed during necropsy, such as respiratory distress, hypertrophic liver, and cardiopathology [37]. Our own observation regarding the death of our cloned male calf 52 h after birth seems to conform to these earlier findings. Our cloned calf also exhibited pneumonia and liver fibrosis, although the only difference seems to be that it did not exhibit any cardiopathological symptoms on post mortem.

In conclusion, technical developments over the years have improved the developmental rates of cloned embryos, although the efficiency still remains low. The only true measure of the efficiency of the nuclear transfer process is the production of viable offspring. The successful cloning of a male calf from an elite high performing bull for the first time with the HMC™ technique in Europe indicates the successful adoption and establishment of this technique in our laboratory. The birth of the cloned calf can also be regarded as an indication that the HMC™ technique is capable of producing viable embryos. We presume that in the future, two approaches would be concentrated upon to increase the efficiency of nuclear transfer: (1) unfolding the mechanisms of reprogramming and the factors that affect it in order to increase the yield of successfully reprogrammed cloned embryos, and (2) selection of somatic cells and recipient oocytes to facilitate an increase in the successfully reprogrammed nuclear transfer embryos after activation. It is also presumed that improvement in activation and culture regimes would help improve the overall success rate of the somatic cell nuclear transfer technology.

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References

10. Roca J, Martinez E, Vazquez JM, Lucas X. Selection


35. Lazzari G, Wrenzycki C, Herrmann D, Duchi R,
