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Generation of Transgenic Rabbits by the Novel Technique of Chimeric Somatic Cell Cloning[1]

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ABSTRACT

A novel technique of chimeric somatic cell cloning was applied to produce a transgenic rabbit (NT20). Karyoplasts of transgenic adult skin fibroblasts with *Tg(Wap-GH1)* gene construct as a marker were microsurgically transferred into one, previously enucleated, blastomere of 2-cell non-transgenic embryos, while the second one remained intact. The reconstructed embryos either were cultured in vitro up to the blastocyst stage (Experiment I) or were transferred into recipient-females immediately after the cloning procedure (Experiment II). In Experiment I, 25/102 (24.5%) embryos formed blastocysts from whole embryos and 46/102 (44.12%) embryos developed to the blastocyst stage from single non-operated blastomeres, while the reconstructed blastomeres were damaged and degenerated. Thirteen (12.7%) embryos did not exceed 3- to 4-cell stages and 18 (17.7%) embryos were inhibited at the initial 2-cell stage. Out of 14 blastocysts which were subjected to molecular analysis, the transgene was detected in the cells of 4 blastocysts. In Experiment II, 163/217 (75.0%) embryos were transferred into 9 pseudopregnant recipient-rabbits (an average of 18 embryos per recipient). Four recipient-females (44.4%) became pregnant and delivered a total of 24 (14.7%) pups. Molecular analysis confirmed that two pups (1.2%), one live and one stillborn, showed a positive transgene signal. Live transgenic rabbit NT20 appeared healthy and anatomically as well as physiologically normal. The results of our experiments showed that transgenic adult skin fibroblast cell nuclei, which have been introduced into the cytoplasmic microenvironment of single enucleated blastomeres from 2-cell stage rabbit embryos, are able to direct the development of chimeric embryos not only to the blastocyst stage but also up to term.

embryo, assisted reproductive technology, developmental biology, early development

INTRODUCTION

The possible propagation of individuals by somatic cell cloning has important economic implications in biotechnology and biomedicine as has already been shown by the generation of cloned transgenic animals which are able to produce valuable xenogeneic (human) recombinant proteins (i.e., biopharmaceuticals) in their body fluids and are resistant to interspecies transmissible and heritable diseases. However, the overall efficiency of somatic cell cloning in mammals is still low and unsatisfactory. Nevertheless, nuclear transfer-derived offspring have already been obtained not only in livestock species or infertile crossbreds/bastards (cattle, sheep, goats, pigs, horses, and mules [1–6]), but also in laboratory animals (mice [7]), companion animals (cats, horses [8, 9]), and even endangered species living in the wild (gaur, mouflon, African wild cats [10–12]). In rabbits, particularly when compared to other farm livestock species, the number of somatic cell clones produced is significantly smaller. Although the birth of nuclear transfer-derived rabbits following embryo cell cloning was reported by Stice and Robl [13] over 18 years ago, the first rabbit somatic cell clones were generated by Chesné et al. [14] only 4 years ago. At the same time, Yin et al. [15] reported an improved procedure for the production of rabbit reconstructed embryos and fetuses following the nuclear transfer of fibroblast cells into in vitro and in vivo matured oocytes which had been previously enucleated using a chemically assisted microsurgical technique. However, the results of somatic cell cloning-associated experiments demonstrate that mammalian nuclear-transferred embryos rarely reach full postimplantation development, especially in relation to the number of reconstructed oocytes and to the number of embryos suitable for transplantation into surrogate recipient females.

Successful cloning of animals requires epigenetic reprogramming of the differentiated state of the donor cell nucleus to a totipotent embryonic state. This means that the donor nuclei must cease their own program of gene expression and restore a particular program of the embryonic genome expression (transcriptional activity) necessary for normal development [16–19]. Epigenetic modifications, such as donor genomic DNA methylation and its likely interaction with histone deacetylation and methylation, have been considered to be candidates regulating nuclear reprogramming [20–22]. In the early stages of mammalian cloned embryo development, two-step changes in the somatic tissue-specific pattern of donor genomic DNA and constitutive heterochromatin methylation occur, which are related to epigenetic nuclear reprogramming [23–25]. After reconstruction (by intraooplasmic karyoplast/

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whole cell microinjection or nucleus donor cell-ooplast couplet electrofusion) and artificial activation (chemical or physical) of nuclear transfer-derived cytoplasmic hybrids, advanced processes of somatic DNA active demethylation (replication-independent) and passive demethylation (replication-dependent) take place, which persist up to the blastocyst stage. This first phase of genome-wide reprogramming in preimplantation cloned embryos may be a prerequisite for removing somatic epigenetic information in order to allow embryonic gene expression and restore totipotency of cell nuclei. In turn, this last phenomenon is essential for the formation of pluripotent stem cells that are important for the later development and differentiation of many somatic cell lines in clonal embryos. After implantation of a reconstituted embryo, DNA hypomethylation status subsists in the cells of extraembryonic tissues derived from trophectoderm [17, 26–29]. In the second cycle of donor genome transcriptional reprogramming, the DNA of epiblast somatic cell lines is largely methylated de novo, during gastrulation. After an increase of the overall genomic methylation level, selective demethylation of DNA cytosine residues, characteristic of individual differentiating cell lines, sets in. This process is at least partially associated with subsequent selective gene expression in these cells [21, 25, 30–32].

We wanted to evaluate the capability of a new source of recipient cytoplasm, different from Metaphase II oocytes, to support the development of reconstructed embryos by donor nuclear genome of transgenic somatic cells. Therefore, we decided to use the enucleated intracellular environment (the so-called blastoplast) of rabbit 2-cell embryo-derived blastomeres in the somatic cell nuclear transfer procedure. In this paper, we describe a novel technique that led to the production of rabbit chimeric genetically engineered embryos and offspring because the donor nuclear transplantation took place within only one blastomere of 2-cell embryos while the second one remained intact. Our experimental system, which was designed for somatic cell cloning, involved the use of a transgenic animal as a donor of cell nuclei with an easily distinguishable recombinant protein marker, the human growth hormone gene. The transgenic rabbit that was previously created by standard zygote intrapronuclear microinjection of gene construct was fully described on the molecular and cytogenetic level [33]. The F2 generation heterozygous female was the donor of ear skin tissue for the establishment of an adult somatic cell line which was used subsequently as a source of nuclear donor cells in the novel chimeric cloning technique.

MATERIALS AND METHODS

Animals

Adult New Zealand White rabbits were purchased from a breeder colony of the Animal Breeding Experimental Center (Chorzelów, Poland) and maintained in the breeding facility at the National Research Institute of Animal Production, Balice/Kraków, Poland. Animal care and handling were conducted in accordance with the Poland Animal Protection Act (No 111 item 724 of 1997) and in conformity with the Local Ethical Committee for matters of experiments on animals. All animals were separately housed in stainless steel cages under controlled humidity, temperature, and light conditions, and were provided with standard rabbit fodder and tap water ad libitum.

Preparation of Ear Skin Fibroblasts

Fibroblast cells were collected from an ear-skin biopsy of a 6-month-old New Zealand White transgenic female with *Tg(Wap-GH1)* gene construct [33] as a marker (heterozygous founder rabbit 42). Tissue samples were cut into small pieces using a tissue chopper (0.5 mm) and tissue explants were placed in a culture flask with a small volume of Dulbecco Modified Eagle Medium (DMEM, Gibco Invitrogen Co., UK), enough to wet the bottom of flask but not too much to cause the tissue pieces to float. For the first 2 to 3 days of incubation, a few more drops of the medium were added every 2 to 3 hours, then gradually more medium was added when pieces had definitely attached. Cultures were replenished 2 to 3 times per week. For the primary cultures of dermal fibroblasts, modified Dulbecco Minimum Essential Medium, which had been supplemented with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO), 2 mM non-essential amino acids (NEAA, Sigma), 2 mM L-glutamine (Sigma), 0.36 mM sodium pyruvate (Sigma), and 1% antibiotic-antimycotic solution (Sigma), was used. After removal of the explants (Days 5 to 6), monolayers of fibroblast cells were harvested using DMEM supplemented with 0.25% trypsin-EDTA (Sigma). Cells were subsequently cultured up to a total confluency and then passaged at least three times. Cells harvested from flasks by trypsinization were washed in 10 mL HEPES-buffered Tissue Culture Medium 199 (TCM 199-HEPES, Sigma) with 10% FBS and centrifuged at 200 x g for 10 min. The cell pellet was then suspended in FBS containing 9% dimethyl sulfoxide (DMSO, Sigma) before freezing in a Minicool freezer. Cryopreserved donor cells were thawed at 37°C and 200 μL of FBS was added. The suspension was kept at room temperature for 10 min, then 800 μL of cell culture medium was added and the cells were centrifuged at 300 x g for 5 min. The supernatant was removed and 50 μL of manipulation medium was added. Frozen/thawed adult skin fibroblast cells, which had been cultured in vitro up to a total confluency state to synchronize their mitotic cycle at G0/G1 through contact inhibition of migration and proliferative growth, were used in the somatic cloning procedure.

Collection of Recipient 2-cell Embryos

Superovulation of postpubertal New Zealand White non-transgenic female rabbits was induced by i.m. injection of 100 IU of eCG (Serogonadotropin, BioWet, Poland). After 72 h, the females were i.v. injected with 100 IU of hCG (BioMed, Lublin, Poland) and mated with fertile non-transgenic males. The donor females were killed 24 to 26 hours after hCG administering/copulation and 2-cell stage embryos were flushed from the separated oviducts using TC 199 medium supplemented with 4 mg/mL bovine serum albumin (BSA, fraction V, Sigma).

Chimeric Embryo Production by Nuclear Transfer

Two-cell rabbit embryos were placed into a glass micromanipulation chamber filled with TCM 199 containing 4 mg/mL BSA and 7.5 μg/mL cytochalasin B (CB, Sigma). Enucleation of a single blastomere was accomplished by removing the interphase nucleus with a small amount of surrounding cytoplasm, while the second blastomere remained intact. The reconstruction of an enucleated blastomere-derived cell (i.e., blastoplast) was performed by introducing the karyoplast or sometimes, even the intact small fibroblast (if the diameter of the somatic cell was smaller than 15 μm; [4]) directly into the cytoplasm. Adult skin fibroblasts with a smooth, intact plasma membrane surface were chosen as donor nuclei. The somatic cell plasma membrane was broken by gentle, repeated aspiration of the fibroblast cell into and out of the pipette whose tip had an external diameter smaller than the diameter of the selected cells. In Experiment I, the partially reconstructed 2-cell rabbit embryos were cultured in 500 μL of B2 INRA medium (Laboratoire C.C.D, Paris, France), supplemented with 10% FBS at 38.5°C, in a 100% water-saturated atmosphere of 5% CO_2 in air. In Experiment II, the chimeric embryos were surgically transferred into the oviduct lumen of anesthetized pseudopregnant recipient females immediately after reconstruction. The pre- and postimplantation developmental competencies of partially reconstructed chimeric 2-cell embryos were evaluated for both blastocyst formation rate after 72 and 96 hours of in vitro culture and pregnancy status of recipient rabbits.

Screening for Chimeric Embryos and Offspring

Genomic DNA was isolated using the guanidinium isothiocyanate procedure combined with proteinase K (Sigma) digestion and phenol extraction. Two pairs of PCR primers were placed on both sides of the junction between the promoter and the hGH gene encompassing part of WAP promoter, six histidine tag sequences, thrombin recognition site, and the first hGH exon sequence. PCR reaction mixture contained in 20 μL: 100 ng of genomic DNA, 50 mM KCl (Sigma), 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$ (Sigma), 0.25 mM dNTP, 7.5 pmol of each primer, and 0.7 unit of Taq DNA polymerase. The reaction was performed using a cycling protocol of 94°C for 45 sec.; 55°C for 45 sec.; and 72°C for 90 sec., for 30 cycles. Prior to the first cycle an initial denaturation step of 94°C for 4 min and following the last cycle, an extension step of 5 min at 72°C was performed. For the first screening the primers WhGH2-F (5′-Cy5-AGTCTTCCTCCTGTGGGTC-3′) and WhGH2-R (5′-TCTCTCTCCATCCCTCCAG-3′) were used to amplify a 313

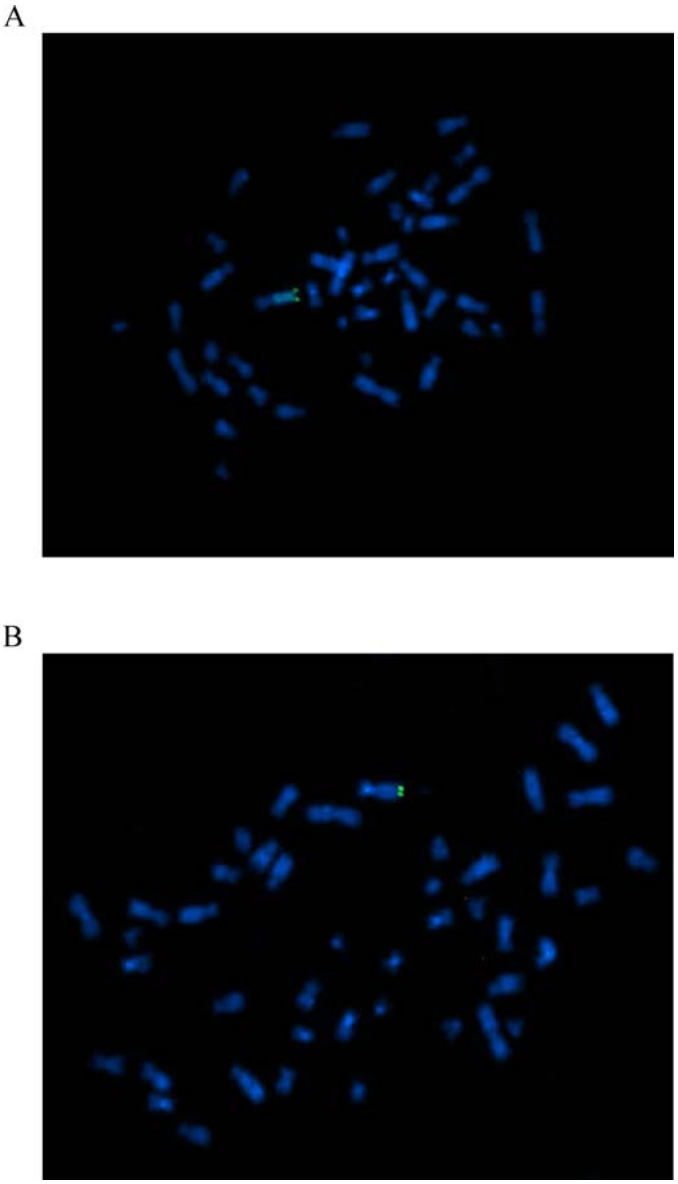

FIG. 1. Mapping of human growth hormone (hGH) gene in transgenic rabbits. **A**) Donor of cell nuclei for chimeric somatic cell cloning. **B**) The resultant nuclear transfer-derived rabbit NT20. The mapping of the hGH gene was performed by FISH analysis using the primary culture of ear skin-derived fibroblasts and the biotinylated gene construct as a molecular probe. The *Tg(Wap-GH1)* transgene was located on chromosome 7q of rabbits. One hundred percent of all karyotypes originating from dermal tissue of rabbit NT20 were positive for the transgene in comparison with the heterozygous founder transgenic rabbit 42 that was the nuclear cell donor. Original magnification ×1000.

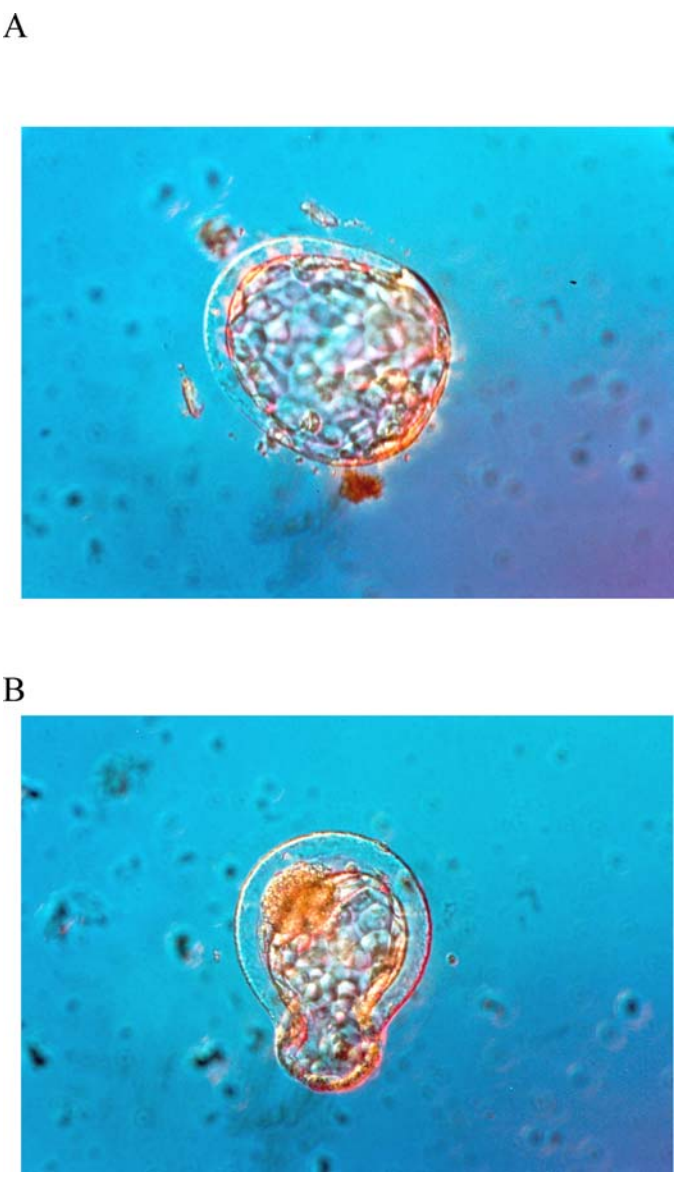

FIG. 2. Chimeric rabbit blastocysts derived from *in vitro* cultured 2-cell stage embryos whose one blastomere was subjected to somatic cell nuclear transfer. **A**) The mosaic blastocyst developing from a whole partially reconstructed embryo (both from enucleated/injected and non-operated blastomeres). **B**) The blastocyst developing only from a single non-manipulated blastomere while the second one remained uncleaved and became a degenerating cell. Original magnification ×200.

bp DNA fragment. In the second PCR screening, primers WhGH1-F (5′-Cy5-GTCCCAACCCAACCATTC-3′) and WhGH1-R (5′-TGGCGATACTCA-CATTCAGA-3′) were used to amplify a 524 bp DNA fragment.

Mapping of Transgene

The mapping of the *Tg(Wap-GH1)* transgene was performed by fluorescence *in situ* hybridization (FISH) on the chromosome preparations obtained from cell cultures of skin fibroblasts (Fig. 1A, B). Cells were cultured for 5 days in the modified DMEM at 37°C in an atmosphere of 5% CO_2. The standard method was applied for metaphase chromosome preparation. The DNA probe specific for the *Tg(Wap-GH1)* transgene consisted of the plasmid labelled with biotin-dUTP by a random-priming method and purified by ethanol precipitation. Hybridization with a molecular probe was performed for 17 hours at 37°C. For signal detection slides were incubated with the antibodies fluorescein avidin D (Vector Laboratories), biotinylated anti-avidin D (Vector Laboratories), and subsequently with fluorescein avidin D. Visualization of the transgene signals was achieved with a fluorescence microscope (Zeiss, Axiovert 200), after standard staining of the preparation with DABCO/DAPI.

RESULTS

The Developmental Potential of Chimeric 2-cell Embryos

The single blastomeres of 2-cell rabbit embryos were reconstructed with nuclei of transgenic adult dermal fibroblasts. The interphase nuclei of the 2-cell embryo-derived blastomeres were easily distinguishable, and the use of live-DNA fluorescent dye for its detection was not necessary. Therefore, the blastomere enucleation was a simple operation and the detrimental effects of the dye and exposure to UV light on the embryo was avoided. A total of 319 2-cell rabbit embryos was

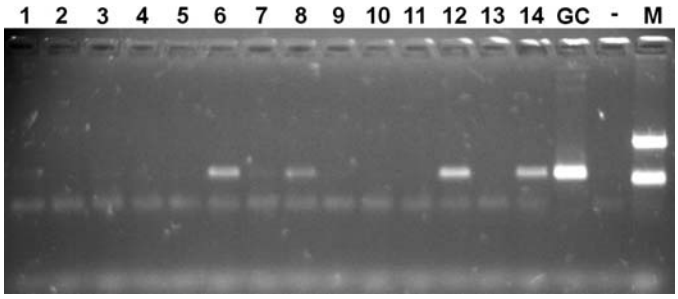

FIG. 3. Screening of chimeric nuclear transfer-derived (NT) blastocysts for the presence of *Tg(Wap-GH1)* transgene. Each lane represents the analysis of a single partially reconstructed blastocyst. Lanes 1–14, analysis of blastocysts 1NT to 14NT, respectively; lane GC, gene construct *Tg(Wap-GH1)* used as positive control; lane –, negative control, wild-type rabbit; lane M, size marker 267 and 745 bp. A positive signal of the expected size (313 bp) was detected in samples 6NT, 8NT, 12NT, and 14NT. Fractionation was performed in 1.5% agarose gel.

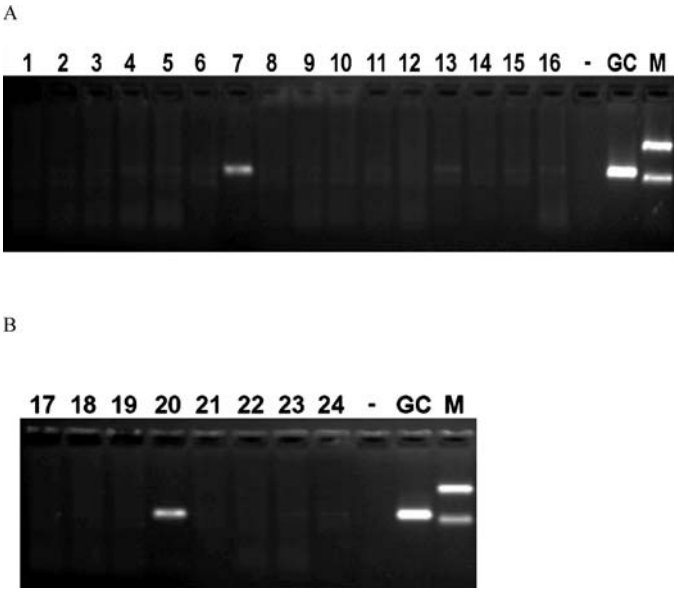

FIG. 4. Screening for ear-derived dermal tissues of the animals generated by chimeric somatic cell cloning using the nuclear transfer into one blastomere of a 2-cell embryo. Analysis for the presence of *Tg(Wap-GH1)* transgene with primers WHGH2 (PCR product size 313 bp). **A**) Lanes 1–16, rabbits NT1 to NT16, **B**) Lanes 17–24, rabbits NT17 to NT24, respectively; lane –, negative controls, without DNA; lane GC, positive controls, gene construct; lane M, size markers 267 and 745 bp. Fractionation was performed in 1.5% agarose gel. Positive signal is observed in lanes 7 and 20, rabbits NT7 and NT20, respectively.

used in the experiments. In Experiment I, 102 (32.0%) 2-cell embryos, whose single blastomeres had been reconstructed with cell nuclei of transgenic fibroblast cells, were cultured *in vitro*. The preimplantation development of partially reconstructed 2-cell embryos was evaluated on the basis of cleavage activity and blastocyst formation. Out of 102 cultured embryos, 84 (82.3%) embryos underwent at least one cell division. The potential of chimeric embryos to develop into blastocysts/hatching blastocysts was relatively high (71/102; 69.6%); however, out of 71 blastocysts obtained, only 25 (24.5%) embryos were formed from both blastomeres (Fig. 2A). For other blastocysts, as many as 46 (45.1%) embryos were developed from only intact non-reconstructed blastomeres (Fig. 2B). Out of 14 chimeric blastocysts, which were subjected to molecular analysis, 4 (28.6%) were positive for *Tg(Wap-GH1)* transgene (Fig. 3).

In Experiment II, one live transgenic and potentially chimeric NT20 female rabbit and one stillborn NT7 female rabbit were produced after transfer of partially reconstructed 2-cell embryos into synchronized recipient females. Out of 217 partially reconstructed embryos, 163 (75.0%) 2-cell chimeric embryos, immediately after manipulation, were transferred surgically into 9 recipient surrogates. Four of them (4/9; 44.1%) became pregnant and delivered 23 healthy pups (23/163; 14.1%) and one stillborn pup (1/163; 0.6%). Molecular analysis of ear skin-derived tissue samples of all the pups obtained confirmed that the transgenic donor cells were the source of the genetic material used to produce two cloned rabbits, which revealed the presence of the integrated *Tg(Wap-GH1)* transgene in the genomic DNA of both neonates, live NT20 and stillborn NT7 (Fig. 4). The rest of the tested pups were negative for the transgene; however, we cannot categorically exclude the possibility of a mosaic pattern of cells in other non-analyzed tissues.

Analysis of Chimerism in Cloned Offspring

The stillborn NT7 female, which was considered to be positive for the *Tg(Wap-GH1)* transgene presence in the ear skin-derived explants, was subjected to further examination. Tissue samples were collected from various organs such as the liver, kidneys, heart, lungs, and gonads and also from skeletal muscles. All analyzed tissue samples were found to be negative for the transgene (Fig. 5A). Our screening procedure for transgenes always includes two different transgene fragments, so the possibility of misclassification is very low. Also, *post mortem* initial transgene screening of various (the same as mentioned above) tissue samples originating from the next two females (NT4 and NT10) revealed that they were non-transgenic (data not shown). The female NT20 was the last to be evaluated (after death 9 months later, which was not the result of chimeric somatic cell cloning). Nuclear DNA samples isolated from heart, skeletal muscles, liver, kidneys, gonads, and lungs were subjected to the PCR analysis. The *Tg(Wap-GH1)* transgene was detected in all tissue biopsies (Fig. 5B). Unfortunately, the nuclear transfer-derived placentas were not diagnosed for the presence of transgenic chimerism marker because tissue samples were not collected *post partum*.

Cytogenetic Analysis of Transgenic Cells

Fibroblast cell lines derived from transgenic rabbit NT20 were established and subjected to cytogenetic analysis using FISH. The FISH analysis enabled specific DNA sequences to be mapped to chromosome 7 (Fig. 1A) of primary cultures. The results additionally confirm that the *Tg(Wap-GH1)* transgene became stable, integrated into the host genome and could be transmitted to the germline cells and expressed in offspring (Fig. 1B).

DISCUSSION

High mortality rates of nuclear-transferred embryos at both pre- and postimplantation stages as well as numerous malformations in resulting cloned fetuses and neonates are still common [34, 35]. That is why we proposed a novel somatic cell cloning technique that led to the production of rabbit chimeric embryos. Our experiments showed that the somatic cell nucleus, which has been exposed to cytoplasm of a 2-cell embryo-derived blastomere, is able to direct the

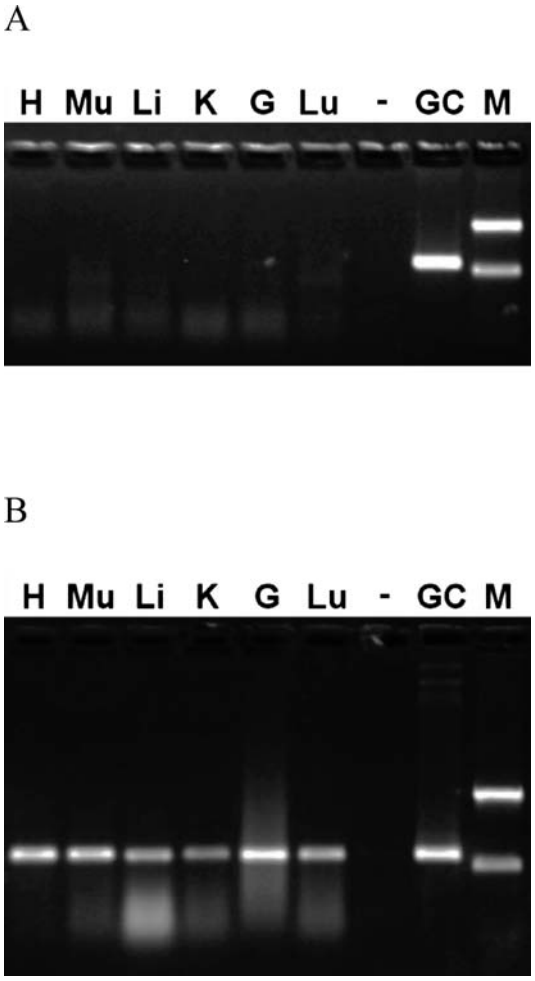

FIG. 5. Screening for tissues of two transgenic animals (NT7 and NT20) generated by chimeric somatic cell cloning using the nuclear transfer into one blastomere of a 2-cell embryo. Analysis for the presence of *Tg(Wap-GH1)* transgene with primers WHGH2 (PCR product size 313 bp). **A**) Tissues of rabbit NT7. **B**) Tissues of rabbit NT20. Lane H, heart tissue; lane Mu, muscle tissue; lane Li, liver tissue; lane K, kidney tissue; lane G, gonad tissue; lane Lu, lung tissue; lane –, negative controls, without DNA; lane GC, positive controls, gene construct; lane M, size markers 267 and 745 bp. Fractionation was performed in 1.5% agarose gel. Positive signals are observed for tissues of rabbit NT20.

development of the partially reconstructed embryo not only to the blastocyst stage but also up to term. This was confirmed by the generation of a live healthy transgenic rabbit NT20. Embryo reconstruction by somatic cell nuclear transfer demonstrated that donor genomic DNA that has been introduced into one cleavage division-descended cell of a 2-blastomere-stage embryo can dedifferentiate into a pluri-/totipotent state. Converting the somatic nuclear configuration defined as remodeling/reprogramming into an embryonic state is highly desirable for promoting events similar to those occurring during early embryo development. Replacement of the embryonic interphase nucleus with the nuclear genome from adult genetically transformed skin fibroblast-derived karyoplast or whole cell at the G0/G1 stage of mitotic cycle was performed within only one blastomere, while the second one was kept intact to improve the structuro-functional quality of the partially reconstructed embryo and consequently to support further pre- and/or peri-implantation embryogenesis. Therefore, we did not make any attempts to reconstruct both blastomeres in order to produce chimeric nuclear transfer-derived embryos of good morphological quality. The high percentage of destroyed/lysed or degenerated (necrotic or apoptotic) blastomeres as a result of microsurgical manipulation and, at the same time, the high rate of blastocysts which developed exclusively from the intact blastomeres, reflects the strong invasiveness of the karyoplast/tiny whole cell direct microinjection technique into the cytoplasm of 2-cell stage embryos. The effectiveness of our experiments remains to be improved, but is at a similar level to the overall efficiency of standard somatic cell cloning procedures. However, the essential difference between our study and other studies concerns the source of recipient cytoplasm used for exogenous donor nuclei. To date, in mammalian somatic cell nuclear transfers, in vivo or in vitro matured (meiotic Metaphase II-arrested) oocytes were used as a source of recipient cytoplasm for allogeneic cell nuclei [1–7, 14]. The somatic genomic DNA that had been introduced into a non-activated ooplast and had been exposed to the high activity of such ooplasmic factors as maturation/meiosis promoting factor (MPF) and the cytostatic factor (CSF) that stabilizes its functions, underwent a series of ultrastructural and biochemical transformations involving nuclear envelope breakdown (NEBD), dispersion of nucleoli, and premature chromosome condensation (PCC); then, after an artificial activation, further architectural alterations, including chromosome decondensation, nuclear envelope restoration, nucleologenesis and nuclear swelling occur [36]. According to Tani et al. [37] and Wakayama et al. [38], in cattle as well as in mice, this scenario of transformations assures exclusively effective reprogramming of the nuclear genome and guarantees success in somatic cell cloning. It appears that the non-activated ooplasm is the *sine qua non* condition of a proper and complete epigenetic transcriptional reprogramming of donor genomic DNA. The results of our experiment demonstrate that oocyte cytoplasm is not the only source of somatic genome reprogramming capability. Cytoplasm in 2-cell blastomeres is also able to reprogram somatic cell nuclei. Other studies by Tani et al. [39] demonstrate that the process of functional reprogramming of the bovine donor nuclear genome is not regulated by MPF or CSF activity-mediated mitogen-activated protein kinase (MAPK). Moreover, MPF is unnecessary for reprogramming of somatic cell nuclei when actively-dividing cells are in vitro synchronized at the M phase of the mitotic cycle. However in terms of the above-mentioned findings it cannot be excluded that some species-specific differences, which can be especially related to the preferential treatment of in vitro cultured somatic cells, exist in this area.

As a result of in vitro culture of partially reconstructed 2-cell embryos, the relatively high percentage (approximately 25%) of blastocysts, which developed from both blastomeres, was generated. However it was revealed that only 4 out of 14 genotypically analyzed blastocysts were positive for the *Tg(Wap-GH1)* transgene signalling/screening. In the other presumably chimeric blastocysts diagnosed, the transgene PCR screening turned out to be negative. This may be explained by the fact that sensitivity of our PCR genotyping was not high enough for the single blastocysts. Therefore, the PCR assays were not able to detect the *Tg(Wap-GH1)* transgene copies integrated into the genomic DNA of the separate blastocysts.

In our experiments, almost total synchrony between the nuclear donor cell and the recipient cytoplasm was preserved in enucleated/injected blastomere-derived nuclear-cytoplasmic hybrid cells of 2-cell embryos, which had been previously partially reconstituted by the novel technique of chimeric somatic cell cloning, because both somatic cell karyoplast and blastomere-descended cytoplast were at the interphase stages of the mitotic cell cycle. It is well known that the proper

coordination of the mitotic cycle phase of the genomic DNA donor cells with the meiotic cycle stage of the recipient oocytes may increase the ability of somatic nuclei to induce correct remodeling of chromatin spatial configuration and epigenetic reprogramming of the nuclear genome. According to Adenot et al. [40], the developmental failure in embryo cloning using the nuclear transfer technique was related to the physiological state of the donor nucleus, which was synchronized at the mitotic interphase and the recipient cytoplasm, which was arrested at the meiotic Metaphase II stage. The authors suggested that as the oocyte cytoplasm aged and approached the interphase state, the asynchrony between donor cells and the metaphase cytoplasm of recipient oocyte diminished. Moreover, the cytoplasm of the *in vivo* aged oocyte induced nuclear remodeling in spite of the fact that nuclear envelope of the transferred nucleus was not broken down [40]. This finding was reflected in experiments by Tsunoda and co-workers [41], because the transplantation of cell nuclei derived from blastomeres of 8-cell staged embryos, which were at the interphase, into the cytoplasm of two-blastomere mouse embryos, whose nuclei were also at the interphase of karyokinesis, resulted in the birth of live offspring.

We applied the karyoplast or whole-cell intracytoplasmic microinjection technique for the reconstruction of one enucleated blastomere in rabbit 2-cell embryos, instead of cell fusion induced in an electric field. The microsurgical deposition of the karyoplast or small plasma membrane-intact fibroblast cell into the 2-cell embryo blastomere-descended cytoplast allowed us to avoid the problems related to the need for extremely close surface contact between nuclear donor and recipient cell plasma membranes (which limits the effectiveness of electrofusion). It also eliminated the possibility of creating tetraploid one-cell embryos through the double fusion effect of plasma membranes derived not only from the somatic cell and the enucleated blastomere but also from the non-manipulated blastomere. The injection of only the karyoplast prevents the disadvantageous influence of somatic cytoplasmic components on the epigenetic remodeling and reprogramming of transferred somatic cell nucleus, and consequently, on the normal development of reconstructed embryos. On the other hand, the intracytoplasmic microinjection technique for 2-cell embryo-derived recipient blastomere reconstruction proved to be a rather invasive method. The addition of cytochalasin B to the manipulation medium partially reduced the detrimental effect of pipette insertion, and at the same time, a significant percentage of enucleated/injected blastomere-derived cells were destroyed. In turn, the developmental potential of single 2-cell stage reconstituted blastomere-derived cells, which remained undamaged or non-degenerated, was very high. This was not unexpected, since Seidel et al. [42] already found that the blastomere from a rabbit 2-cell embryo, in which one of the blastomeres had been destroyed by puncturing with a microneedle, was capable of forming viable young. This observation was further confirmed by Tarkowski [43] in his studies on murine embryos.

To date the technique of whole tiny cell microinjection for nuclear transfer in rabbits has not been attempted (excluding interspecies cloning by xenonuclear transfer of giant panda plasma membrane-intact somatic cells into enucleated rabbit oocytes [44]), due to concerns that the plasma membrane of the donor cell may persist in the cytoplasm of recipient ooplasts resulting in failure to release the somatic cell nucleus. In turn, in our experiments the exact mechanism for dissolution of the donor cell plasma membrane in the cytoplasmic microenvironment of the enucleated blastomere of 2-cell rabbit embryos is unclear. Two possible mechanisms may explain the plasmolemma dissolution process. First, the 2-cell embryo-derived blastomere might have actively recognized the plasmolemma of the nuclear donor cell or its specific surface antigen proteins as belonging to a foreign (allogeneic) cell, not to the cytosol of the embryo cell. This recognition (immunological response) in the cytoplasm would then lead to the active degradation of the somatic cell plasma membrane or transport it to the cell surface of the enucleated/reconstructed blastomere and subsequent rejection (removal) through exocytosis of plasmolemma-derived vesicles (the so-called plasma membrane recycling hypothesis). Afterwards, this active dissolution would release the cell nucleus for the processes of architectural remodeling and epigenetic reprogramming in the partially reconstituted chimeric embryo. The second possible mechanism for donor cell plasma membrane dissolution may be that the somatic cell plasmolemma was damaged during the microinjection of the whole tiny cell or live-membrane karyoplast and the leaky plasma membrane, which was not repaired in the cytoplasm of the recipient blastomere (the so-called blastoplast), then released the cell nucleus. This would result in its chromatin rearrangement, since the 2-cell embryo-descended blastomere could not recognize the injected whole cell or karyoplast as being ectopic (allogeneic). Regardless of the mechanism for donor plasma membrane dissolution, the injected whole tiny cell or karyoplast was able to support embryo development to the blastocyst stage under in vitro culture conditions.

In conclusion, the cytoplasmic microenvironment of enucleated blastomere (the so-called blastoplast) from 2-cell stage rabbit embryo was successfully used as a source of recipient cytoplasm for the somatic cell nuclear genome, enabling the development of the partially reconstructed embryo to be supported by it to term. On the one hand, these results could suggest that transgenic adult dermal fibroblast cell nuclei underwent the complete and correct epigenetic reprogramming in the cytoplasm of dividing blastoplast-derived cells of the 2-cell stage embryo. Nevertheless, on the other hand specific cytoplasmic factors, which have been descended from the good quality Metaphase II oocyte, may affect to the highest degree the donor nuclear genome remodeling and reprogramming capabilities (28, 45). It cannot be excluded that in the case of our experiment such 2-cell stage embryos were randomly selected, in the blastomeres of which the presumptive DNA rearrangement-related ooplasmic agents had not yet been biodegraded (46). For that reason, the ability of this partially reconstructed chimeric embryo to reach term could be preserved. Furthermore, leaving the second embryonic cell intact improved the structuro-functional quality of the partially reconstructed embryo and allowed the newborn female rabbit NT20 to be healthy. The novel technique of chimeric embryo production can also be considered as an alternative possibility for the generation of transgenic animals.

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