# Production of Boer goat (*Capra hircus*) by nuclear transfer of cultured and cryopreserved fibroblast cells into slaughterhouse-derived oocytes

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**ABSTRACT**: The present study reports a birth of goat clone produced by nuclear transfer of cultured and cryopreserved fibroblast cells into slaughterhouse-derived oocytes. The donors of somatic cells were fibroblasts derived from the ear skin of a Boer goat while the recipient ooplasm was *in vitro* matured oocytes of Huanghuai white goat, an Anhui native goat species. The reconstructed embryos were cultured *in vitro* and then the morphologically normal embryos were transferred to the surrogates. The reconstructed embryos were surgically transferred into 37 recipient surrogates, Huanghuai white goats with natural oestrus. Five of them were treated with hCG after transfer. Among them, one was pregnant and gave birth to a live kid. Due to the improper delivery aid, the cloned kid died accidentally after birth. The cloned kid was then anatomised to observe the viscera development, and the results showed that the organs were normal. Paraffin tissue slices were prepared and stained to ensure the viscera development further, and the results suggested that the organs also developed well in spite of incipient hydropericardium. The microsatellite analysis identified the cloning. It is suggested that the optimised nuclear transfer protocol and proper hCG treatment lead to the successful birth of a goat clone.

**Keywords**: goat; fibroblast; clone; hCG

The cloning of domestic animals by somatic cell nuclear transfer (SCNT) holds great potentials for fundamental science as well as for the production of genetically modified animals for biomedical research, and it may also find use in endangered wild life protection. Since 1997 when the first SCNT sheep was announced (Wilmut et al., 1997), offspring have been produced in a number of different species (Vajta and Gjerris, 2006). The cloning of goats by SCNT has also been reported (Baguisi et al., 1999; Keefer et al., 2001, 2002; Reggio et al., 2001; Zou et al., 2001, 2002; Behboodi et al., 2004; Chen et al., 2006; Lan et al., 2006; Shen et al., 2006).

It is, however, clear that SCNT is associated with a substantial occurrence of foetal losses (Heyman et al., 2002). The success or failure of somatic cell nuclear transfer depends upon a number of factors including the source of oocytes. More studies

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are needed to elucidate to what extent the origin of the oocyte affects the development of resultant reconstructed embryos into foetuses and possibly offspring in the goat.

The administration of exogenous hormones like human chorionic gonadotropin (hCG) was used in cattle, sheep and goat (Fonseca and Torres, 2005). This hormonal administration can efficiently promote the ovulation of the first-wave dominant follicle forming the accessory corpus luteum.

The Boer goat, a famous subspecies originated from South Africa, has advantages primarily for meat production. In addition to its docility and adaptability to various environments and feeds, this breed grows faster and its meat is popular among consumers. Moreover, the Boer goat phenotype including a brown and white head with big ears may be utilised as a marker in genetic studies.

The present study was therefore undertaken to initiate Boer goat cloning by SCNT using native goats as oocyte donors and foster mothers to examine whether the hCG injection to recipients after embryo transfer improves the success rate.

## MATERIAL AND METHODS

### Selection of animals

An adult Boer goat was used as a donor of somatic cells. Huanghuai white goat, an Anhui native goat species, was used as donors of oocytes as well as recipients of SCNT embryos. More than 70 goats were raised on a farm as recipient candidates, receiving general health care, and their oestrus was also observed. Exclusively healthy individuals were selected for the present experiments. Thirty-seven recipient surrogates were used altogether in the present study.

Blood samples were obtained by jugular venipuncture using heparin-vacutainer from the somatic cell donor, six age-matched companions of the donor, and the doe which delivered the cloned kid. A piece of ear tissue was collected from a cloned goat generated in this study. Body weight of the cloned goat was measured after birth.

### Chemicals and reagents

All reagents and chemicals used in this study were obtained from Sigma Chemical Co. (Sigma, St. Louis, MO) unless stated otherwise.

### Preparation of donor cells

Fibroblasts, which were used as donor cells, were prepared from ear skin biopsies taken from a 3-year-old, white-haired female Boer goat, as described in our previous reports (Liu et al., 2003, 2004). Briefly, the biopsies were collected, sterilized and immediately transported to the laboratories. Then the biopsies were finely minced into pieces of about 1 $mm^3$ and seeded in 25 $cm^2$ flasks. They were desiccated in 5% $CO_2$ at 37.5°C for 4 h, and then it was cultured with Dulbecco's modified Eagle's medium/F12 (DMEM/F-12) supplemented with 20% foetal bovine serum (FBS, GIBCO, Life Technologies, Rockville, MD) and 100 mg/ml penicillin G potassium (Sigma, P-3032) and 100 mg/ml streptomycin sulphate (Sigma, S-1277) in a humidified atmosphere of 5% $CO_2$ at 37.5°C for 10 days until a confluent primary fibroblast monolayer was established.

The fibroblasts were cryopreserved and thawed according to our previous report with minor modification (Liu et al., 2004). Briefly, after reaching 90% confluency, the cells were digested with 0.1% (w/v) trypsin and 0.02% EDTA for 90 s and then they were resuspended in DMEM containing 20% FBS and 7% dimethyl sulphoxide (DMSO, D-5879). Finally, the cells were equilibrated at 4°C for 30 min and then preserved in liquid nitrogen. The fibroblasts were thawed and inoculated in 24-well dishes (Corning, New York, US) at a density of $6 \times 10^4$ cells/ml in DMEM/F12 and 20% FBS in 5% $CO_2$ at 37.5°C. Before use, the fibroblasts stored in liquid nitrogen were thawed at 37°C by water bath, rinsed in Dulbecco's phosphate buffered saline (DPBS), and resuspended in DMEM supplemented with 10% FBS and cultured at 37.5°C, 5% $CO_2$ in humidified air.

The fibroblasts at fourth to ninth passage were used as somatic cell donors. Before nuclear transfer, the donors were serum-starved for 2–5 days. The fibroblasts were trypsinised 1 h before microinjection.

### *In vitro* maturation of oocytes

Goat ovaries were collected at the abattoir and transported to the laboratory in a thermal container containing sterile saline within 5–8 h after slaughter. The ovaries were rinsed with 75% ethanol and immediately with the same saline four times. The antral follicles of 1–6 mm in diameter on the

ovarian surface were punctured under a dissecting microscope (Olympus, TRPT-4045) using a blade, and the cumulus-oocyte complexes (COCs) were then harvested from the follicles. Only the oocytes well enclosed by compact cumulus cells were picked up with pipette and rinsed four times in fresh M199 containing Earle's salt and $NaHCO_3$ (Sigma, M-7653). The oocytes were washed three times in DPBS.

The basic culture medium was M199 containing Earle's salt (Sigma, M-4530), and it was supplemented with 10 μg/ml follicle stimulating hormone (FSH, No 2. Hormone Co. Ltd., Ningbo, China), 10 μg/ml luteinizing hormone (LH, No 2. Hormone Co. Ltd., Ningbo, China), 1 μg/ml 17 β-estradiol (Sigma, E-8875), 10% FBS, 0.3mM sodium pyruvate, 20 ng/ml epidermal growth factor (EGF, Sigma, E9644) and 75 IU/ml penicillin and streptomycin (North China Pharmaceutical Group Co., China). A total of 14 droplets of 50 μl were prepared in a 60 mm plastic dish (Corning, New York, US) and covered by sterile mineral oil (Sigma, M-8410). The culture was carried out at 38.5°C, 100% humidity, in an atmosphere with 5% $CO_2$ in air without any change of the medium in a carbon dioxide incubator (MCO-15A, Sanyo Electric Co., Ltd., Japan) for 19–24 h.

After *in vitro* maturation, cumulus cells surrounding the oocytes were removed by immersing the COCs in 0.5% hyaluronidase (Sigma, H-3506) in DPBS for 3 to 5 min. Isolated oocytes were washed three times in DPBS/10% FBS, after which only morphologically normal oocytes exhibiting homogeneous ooplasms and also clearly visible first polar body (PB1) were selected for cytoplast production.

## Somatic cell nuclear transfer protocol

The micromanipulation was carried out according to our previously established protocol with minor modifications (Liu et al., 2003; Zhang et al., 2004; Zhang et al., 2005; Zhang et al., 2006). In brief, the enucleation of oocytes was completed within 1 h after collecting the oocytes from *in vitro* maturation. The oocytes were primed in M2 medium (Sigma, M-7167) containing 7.5 mg/ml cytochalasin B (CB, Sigma, C-6762). Subsequently, a hole was made in the oocyte zona pellucida (ZP) on the stage of an inverted microscope (Nikon, Japan) equipped with a micromanipulator (Narishige Co., Ltd., Japan). The polar body and the adjacent cytoplasm were removed by suction through the partially drilled hole using a 20 to 30 mm (outer diameter) micropipette. A single fibroblast, which had been cultured and loaded within the micropipette in advance, was subsequently inserted into the perivitelline space through the already prepared hole in the ZP.

According to the previous report (Shen et al., 2006), the chemical fusion solution consisted of 0.32M mannitol, 100μM $CaCl_2$, 100μM $MgSO_4$, 0.5mM Hepes, and 0.01 mg/ml BSA. According to our previously established method (Liu et al., 2004), electrofusion and activation were undertaken after chemical fusion. Briefly, 30 min after the operation, the enucleated oocyte (cytoplast)-fibroblast (karyoplast) couplet was fused by the electrical pulse using an electrocell manipulator (Kefa, Institute of Development, CAS, China). The couplets were equilibrated in a fusion solution for 3–5 min and then electrofused by a single direct currency pulse (2.30~2.45 kv/cm, 10 μs), 1 s of interval. The reconstructed embryos were then incubated in the embryo culture medium for 30 min incubation. Couplets, which had not fused after the initial pulse, received a second pulse under the same conditions.

The activation of reconstructed oocytes was performed in 5mM ionomycin for 4–5 min and 2mM 6-dimethylaminopurine (6-DMAP, Sigma, D-2629) and 5 μg/m CB for 4–6 h. The oocytes were then rinsed three times before *in vitro* culture.

## *In vitro* culture of embryos

The reconstructed embryos were transferred to CR1aa medium containing 0.3% BSA and incubated at 38.5°C in 5% $CO_2$ in air for 12 to 20 h until they developed to the 2- to 4-cell stage. Cleavage was checked in 36–48 h after activation. Three days later, the embryos were co-cultured with feeding monolayer cells prepared in advance. A half of the medium was refreshed every 48 h. The embryos from 2-cell stage to morula were transferred to the recipients.

## Embryo transfer and hCG treatment

Sexually mature Huanghai white goats were used as recipients for the reconstructed embryos. The oestrus cycles of primiparous or biparous surrogate does were observed and recorded. Just before

Table 1. The design of the primers

| Code | Forward | Backward |
|---|---|---|
| 1 CHINRA5C | 5'-CAATCTGCATGAAGTATAAAT-3' | 5'-TGGTGTAGGGTATGCCGTAAG-3' |
| 2 CHAST008C | 5'-TAGCAGTGAGTNAGGTTGG-3' | 5'-CCCCAHAAAAATCCATGATTC-3' |
| 3 CHSAT011C | 5'-CGAGTTTCTTTCCTCGTGGTAGGC-3' | 5'-AGTTGCTAAGGAAGATGTGCCGAGC-3' |
| 4 CHMICROS | 5'-CCAGAAAGAAATAGAATGGACAGA-3' | 5'-ATTCAAGAACCAGCTGACATCTTT-3' |
| 5 CHSAT063C | 5'-ATTTGCACAAGCTAAATCTAACCA-3' | 5'-TTTCTTCCAAGCATTTCTGTGGTTT-3' |
| 6 CHSAT040C | 5'-CTCATTCTCCAGGAGAGAAAACGT-3' | 5'-CACAATACACATCCTCAGGGCAGA-3' |
| 7 CHSAT023C | 5'-TAACTACAGGGTGTTAGATGAACT-3' | 5'-GGAAGTTTATCTTGTAGCTCTACTC-3' |
| 8 INRA006 | 5'-AGGAATATCTGTATCAACCTCAGTC-3' | 5'-CTGAGCTGGGGTGGGAGCTATAAATA-3' |

the identity of the PCR product was confirmed by microsatellite in order to identify the relationship between animals

the surgical operation, the ovaries of the recipients were checked with celioscope for the corpora lutea development. Only those with at least one well developed corpus luteum were selected for embryo transfer. Each recipient received embryos two or three days after the beginning of oestrus. The embryos were surgically transferred into the oviducts or uterine horns after 24 h fasting. Totally, 37 recipients received reconstructed embryos by surgical operation.

After embryo transfer, all the recipients were housed in a special pen and examined twice daily by adult rams that were strictly controlled to avoid insemination. Does exhibiting oestrus were removed from the pen. Five of the recipients were treated with hCG (Ningbo No. 2 Hormone Campany, Ningbo, China) once or twice by intramuscular injection. The dose varied from 200 IU to 500 IU. The feed and nutrition of the recipients was adjusted to facilitate the pregnancy.

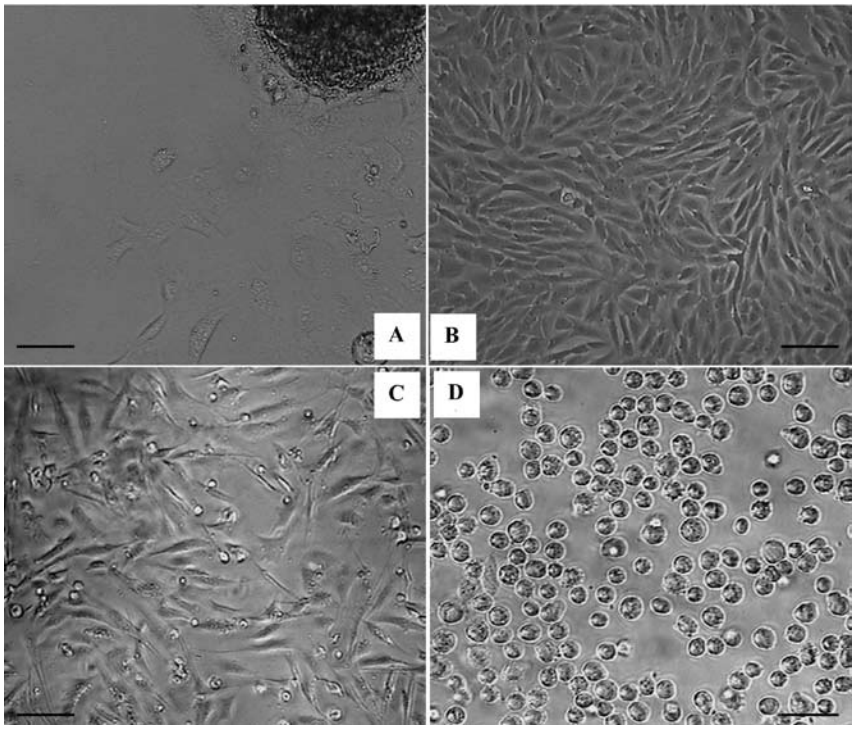


Figure 1. Growth of Boer goat ear skin fibroblasts

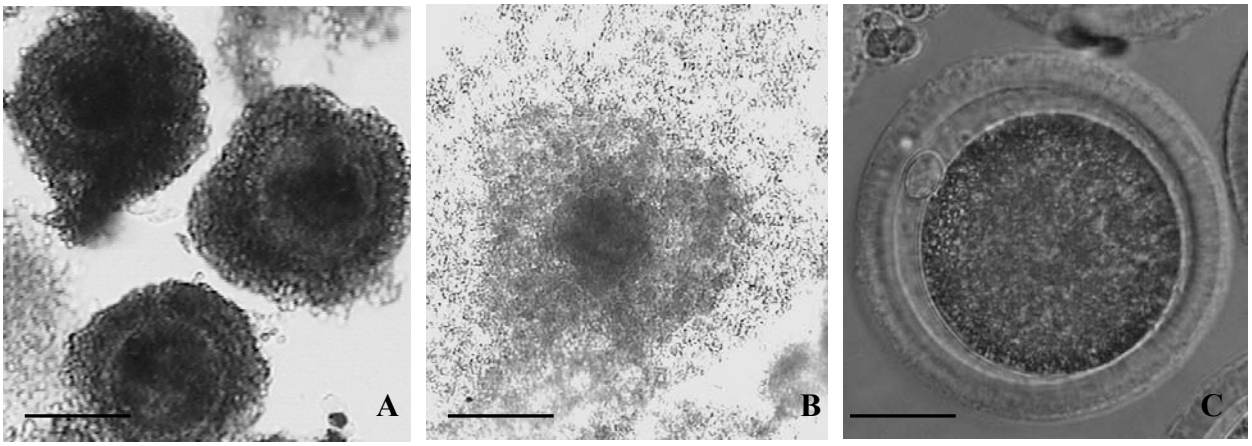


Figure 2. Goat oocytes cultured *in vitro*

## Birth and tissue observation of the clone

The birth occurred 152 days after embryo transfer but the cloned kid died 8 h later accidentally. Autopsy was undertaken by an expert. Afterwards, the organs were fixed in formaldehyde, including larynx, thymus, lymph, heart, liver, spleen, lung, kidney, adrenal gland, ovary, and uterus. Paraffin sections were prepared and stained with haematoxylin and eosin. The slides were observed using bright field microscopy.

## Polymerase chain reaction

Genomic DNA was isolated (Tianwei Shidai DNA Blood Mini Kit, China) from the cloned kid, the surrogate doe, the biological does, and the cell donor doe. Each sample was analysed by polymerase chain reaction (PCR)-single strand confirmation polymorphism (SSCP) to confirm that the clone was derived from the NT procedure. Eight sets of primers were used as listed in Table 1.

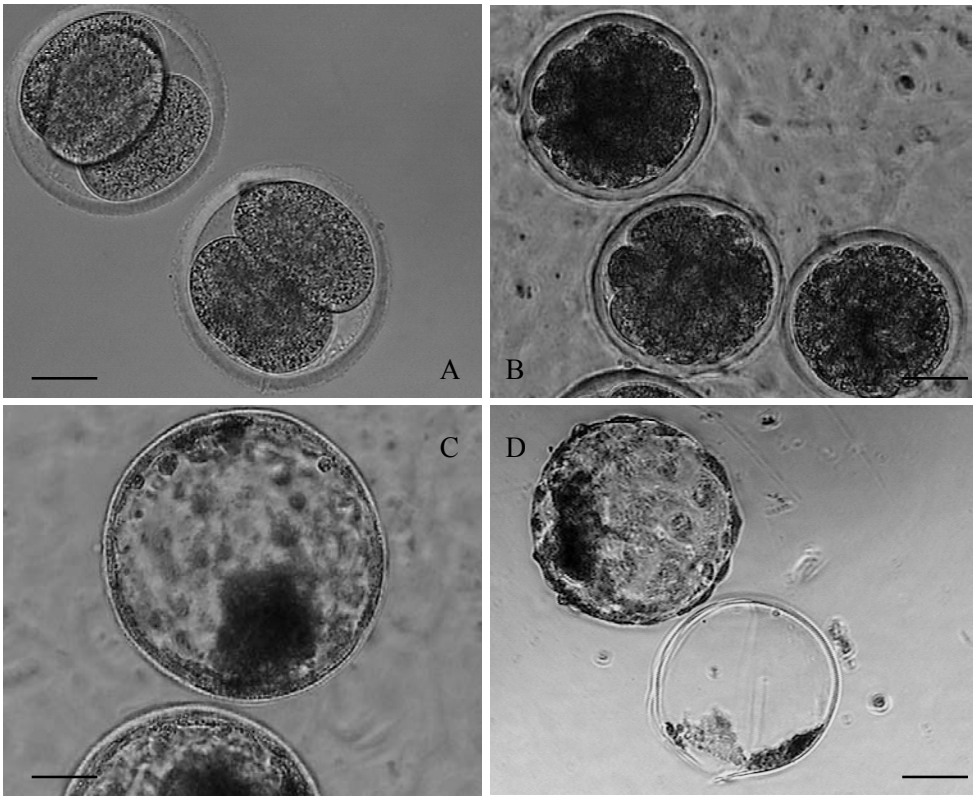


Figure 3. Development of goat reconstructed embryos

Figure 4. A cloned female Boer Goat "Anim8"

## RESULTS

### Donor cell growth and oocyte maturation

Fibroblasts started to attach to the culture dish after 4 days of culture of the ear skin tissue (Figure 1A). The cells reached 100% confluency after 8 days of culture (Figure 1B) after which they could be cryopreserved and prepared for nuclear transfer (Figures 1C, D).

Oocytes were matured *in vitro* and those presenting the first polar body were used for cytoplasm production (Figure 2).

### Embryo and kid cloning

The embryos were reconstructed and developed to blastocysts after *in vitro* culture (Figure 3). The reconstructed embryos from the donor Boer goat (Figure 4A) were transferred to Huanghuai White goat recipients. Nearly 600 embryos were cloned altogether, and most of the morphologically nice embryos were transferred to 37 surrogate recipients, among which only one pregnancy developed to term and the gestation was 153 days. The surrogate delivered a female Boer kid who weighed 4 kg. Its exterior tallies were coincident with the typical colour character of Boer goat. She blatted silvery, micturated normally, and acted normally after birth (Figure 4B). The other recipients failed to gestation with ultrasound-assisted pregnancy diagnosis, and the oestrus appeared again after the experiment.

### The effect of hCG treatment

Altogether, 491 reconstructed embryos were transferred into 37 recipients, and five accepted hCG. Only the one that accepted 400 IU hCG twice

Table 2. The hCG treatment after cloned embryo transfer

| Surrogate | No. of corpora lutea | hCG timing[a] and dose | Pregnancy | Birth |
|---|---|---|---|---|
| 32[b] | – | – | none | none |
| S25 | 2 | D3, 500 IU | no | no |
| H029 | 1 | D3, 500 IU; D4, 500 IU | no | no |
| H025 | 1 | D3, 200 IU | no | no |
| H009 | 1 | D4, 400 IU | no | no |
| H036 | 2 | D28, 400 IU; D32 400 IU | yes | yes |

the surrogates accepted hCG by intramuscular injection;

[a]the beginning day of oestrus was regarded as D1;

[b]among 33 fosters, 21 of them fosters the oestrus appearance at the first oestrus cycle, about 17 days after embryo transfer

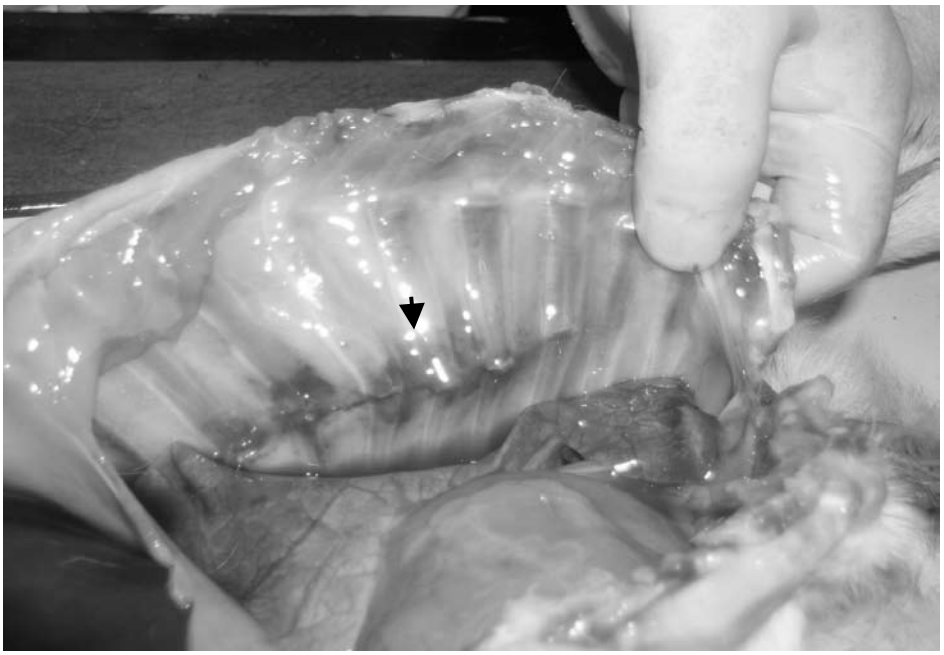

Figure 5. Anatomy of the cloned kid

at D 28 and D 32, respectively, was pregnant and gave birth 152 days after embryo transfer (Table 2). In 2 months, the other 36 recipients showed oestrus appearance again. After that, most of them were pregnant after artificial insemination and gave normal birth.

## Anatomy and histostaining of the clone

The newborn goat clone weighed 4.0 kg with normal cry after birth. But its breath and heartbeat showed an abnormal rhythm shortly after birth. The medical treatment was taken for the emergency. However, its heart stopped beating 8 hours later. By surgical dissection, the viscera of the cloned kid developed normally, including heart, lung, liver, spleen, kidney, adrenal thymus, lymph and ovary. But seven of the right ribs ($4^{th}$ to $10^{th}$) were broken mechanically (Figure 5). It was speculated that this mechanical trauma might result from the carelessness of a deliver assistant who afterwards most likely caused the gas chest and finally the death of the clone.

The tissues of the viscera were examined by haematoxylin-eosin staining, and it was found that none of the organs showed an abnormality, including heart, lung, liver, spleen, kidney, adrenal thymus, lymph and ovary (Figure 6).

Table 3. Boer goat microsatellite STR sites and replicates of the clone

| No. of SRT site | STR site | Replicates |
|---|---|---|
| 1 | TG | 9 |
| 2 | TG | 19 |
| 3 | AGAC | 5 |
| 4 | AC | 7 |
| 5 | GT | 16 |
| 6 | GT | 9 |
| 7 | GT | 4 |
| 8 | TG | 6 |

STR sequencing of cloned kid

## Identification of the clone

Immediately after the death, a liver sample was collected for microsatellite DNA analysis in Shanghai Zhijiang Bio-technology Corporation Limited. Meanwhile, the donor, the recipient, and an unrelated female goat were also sampled for comparison. The STR sequencing of the clone is shown in Figure 7 and summarised in Table 3.

The suspected goats were also subjected to the STR sequencing and the results and the comparison with the clone are listed in Table 4.

The results strongly suggested that the birth was the clone from the somatic cell donor goat.

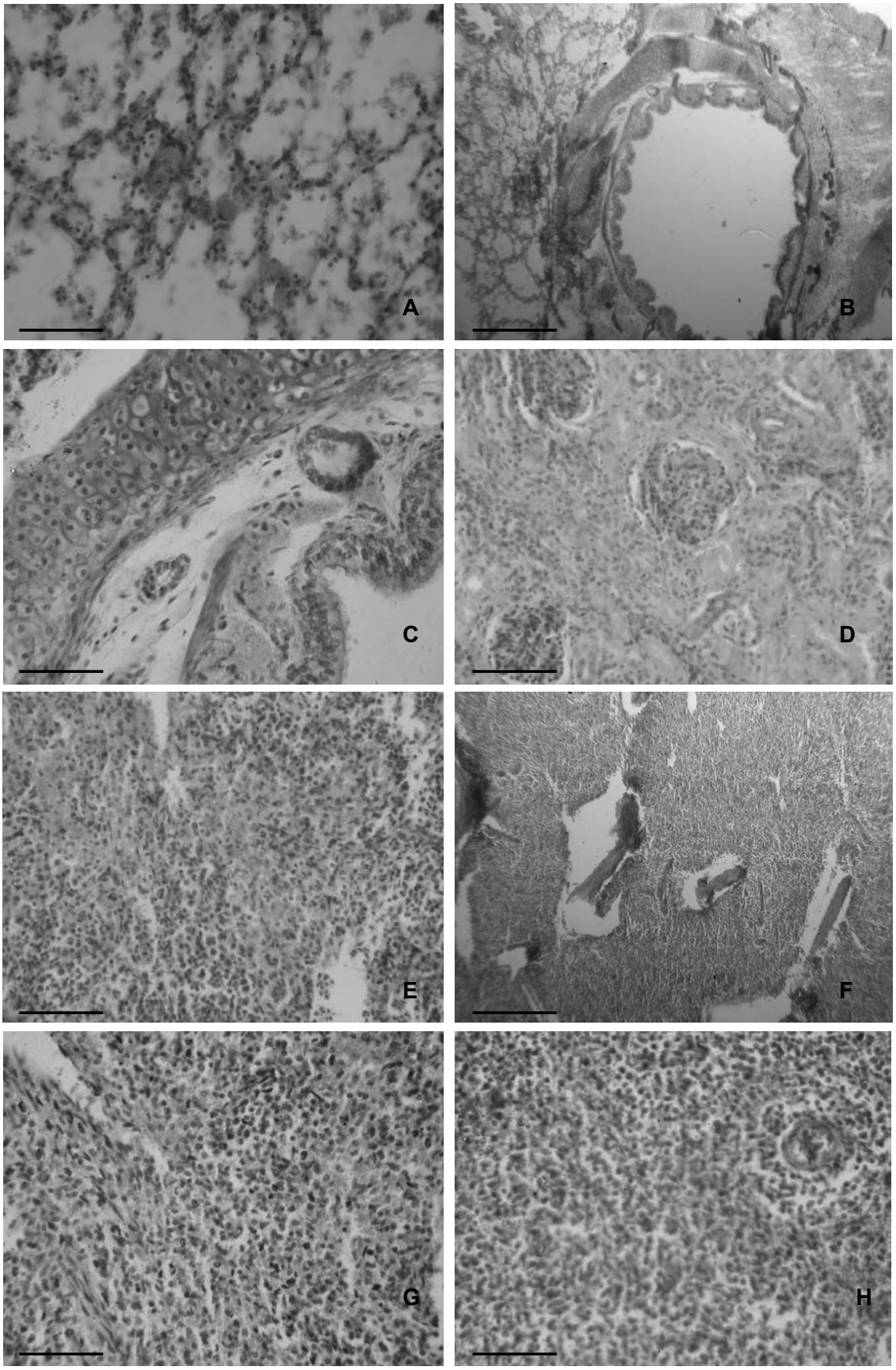


Figure 6. Tissue observation for the cloned kid viscera

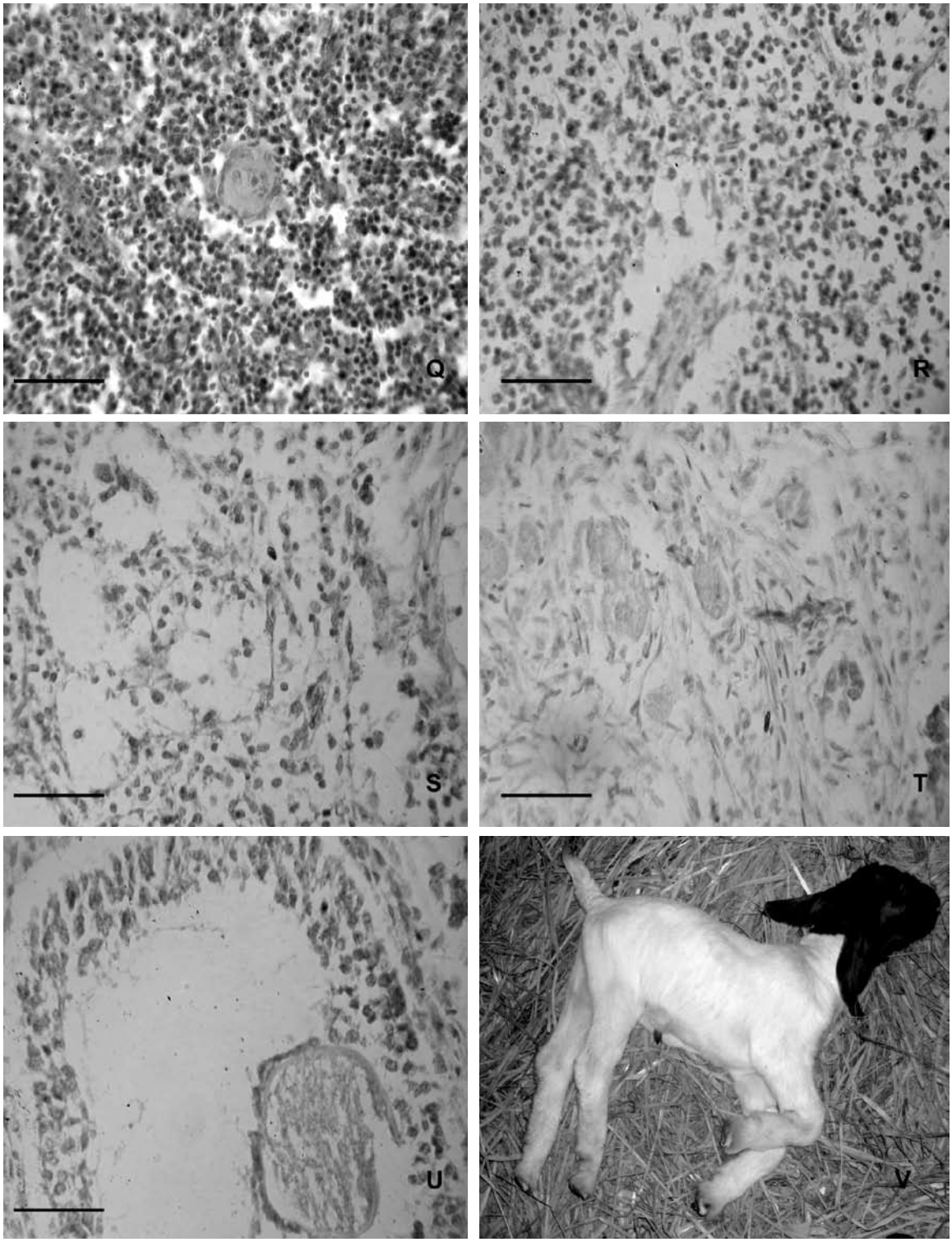


Figure 6. to be continued

## DISCUSSION

The present study indicates that the slaughterhouse-derived and *in vitro* matured oocytes could properly direct the reprogramming of the genome of the cryopreserved adult fibroblast cells, produce Boer goat cloned embryos and healthy kid with a small difference in appearance. The optimised nuclear transfer protocol and hCG treatment led to the successful birth of a goat clone.

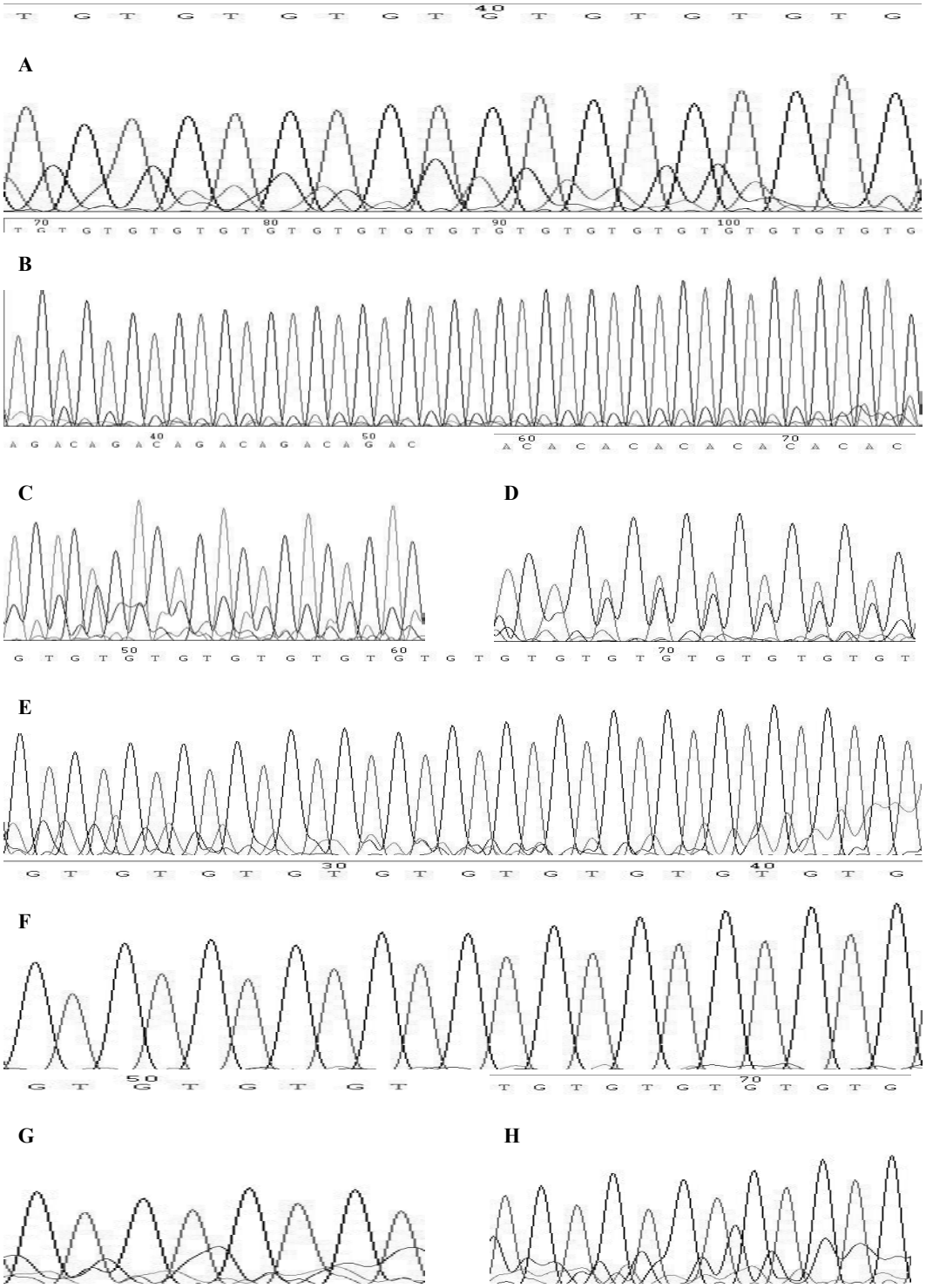


Figure 7. STR sequencing of the cloned kid

Previously, goat cloning by somatic cell nuclear transfer was reported (Baguisi et al., 1999; Zou et al., 2001, 2002; Behboodi et al., 2004; Chen et al., 2006; Lan et al., 2006; Shen et al., 2006). But the success rate has never been desirable. There are too many factors affecting the results, almost including all the steps of the technique. It was known that the foetal loss occurred after cloned embryo transfer (Taverne et al., 2002). In cattle, for instance, the incidence of a loss between Day 90 of gestation and calving was

Table 4. Identification of the Boer goat clone by microsatellite DNA

| Sample number of suspected goats | STR sites with replicates from the clone | Determination |
|---|---|---|
| BG03-110 | TG1TG2GT6 | eliminated |
| 03-032 | TG1AGAC3AC4GT5GT6 | eliminated |
| BG05-308 | none | identical to the donor |
| 04-4-10 | GT5GT6 | eliminated |
| BG05-2061 | AC4GT6 | eliminated |
| BG05-2062 | TG2GT5 | eliminated |
| 05-414 | TG2AGAC3 | eliminated |
| H036 | TG2 | eliminated |

the identification was performed and ensured by Shanghai Zhijiang Bio-technology Corporation Limited

43.7% for adult somatic clones and 33.3% for foetal somatic clones, 4.3% for embryo cloning and 0% for IVF (Heyman et al., 2002). The loss of cloned foetuses might be associated with the endocrine pattern change (Gootwine, 2004). We speculated that the surrogate endocrine condition after embryo transfer might change and be different from the normal hormonal pattern. Therefore, in our study, in order to minimise the impact of hormone stimulation, we established a large scale of foster population, and only those with natural oestrus accepted the cloned embryo when meeting the time of embryo development. Furthermore, some of the surrogates were treated with hCG after embryo transfer to induce the progesterone secretion and then to facilitate the pregnancy at the early stage. Because of the stimulation of the surgical operation and particularity of the cloned embryo, the endocrine condition might change improperly and cease to meet the demand of cloned embryo implantation and/or subsequent development. Maybe, this can partly explain the pregnancy failure of 32 fosters. In order to help the implantation and early embryo/foetus development, hormones should be used, such as progesterone, LH and hCG. In the present study, hCG was chosen. Specifically, 4 surrogates were treated immediately after embryo transfer (D3–4). Such treatment was to induce the progesterone secretion of the surrogates and then to facilitate the implantation. The failure might indicate that at this stage the artificial endocrine intervention might not be necessary. Another surrogate was treated at Day 28 and Day 32 when the abortion tended to occur, and the pregnancy was established and the birth occurred. This might suggest the weakness of the reconstructed goat embryo development at this stage and the importance of exogenous hormone help. But this speculation needs to be further investigated with more surrogates. It was reported that the clones born to different recipients might have different endocrine patterns (Landry et al., 2005).

Reggio et al. (2001) and Keefer et al. (2001, 2002) reported that *in vitro* matured oocytes supported full-term development in goats, whereas in the study of Behoodi et al. (2004), *in vivo* matured oocytes produced the pregnancy. Our study also confirmed the feasibility of cryopreserved somatic cells as donors and slaughterhouse-derived oocytes as recipients after *in vitro* maturation. Interestingly, we found that the cloned kid had a little different black-and-white fur profile on the head, which might be due to the cytoplasm effect.

In summary, the slaughterhouse-derived and *in vitro* matured oocytes support the reprogramming of the cryopreserved fibroblast cells after nuclear transfer in goat, and it appears that the optimised nuclear transfer protocol and proper hCG treatment led to the successful birth of a goat clone.

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