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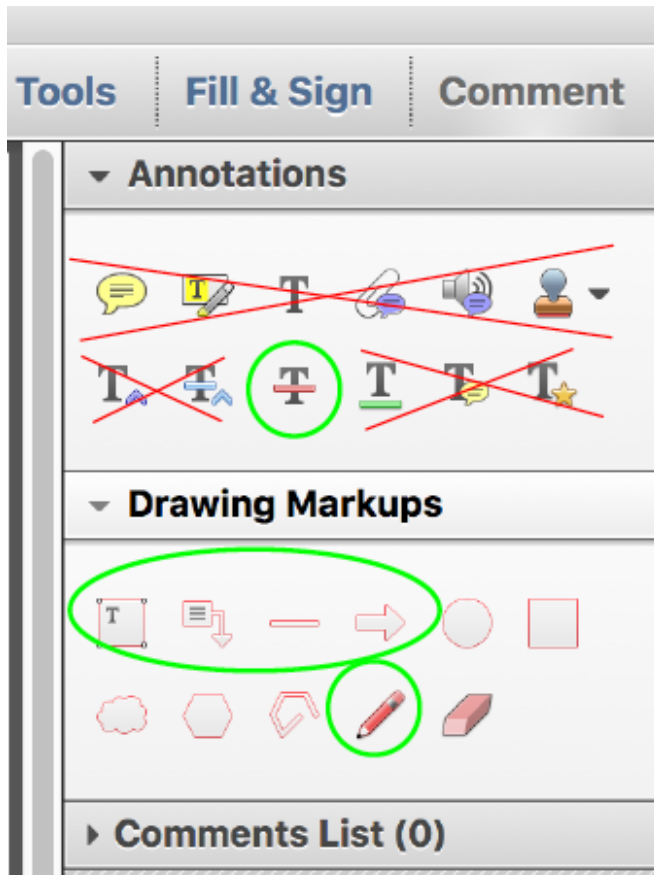
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Remodeling somatic nuclei via exogenous expression of protamine 1 to create spermatid-like structures for somatic nuclear transfer

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Q2 This protocol describes how to convert the chromatin structure of sheep and mouse somatic cells into spermatid-like nuclei through the heterologous expression of the protamine 1 gene (*Prm1*). Furthermore, we also provide step-by-step instructions for somatic cell nuclear transfer (SCNT) of *Prm1*-remodeled somatic nuclei in sheep oocytes. There is evidence that changing the organization of a somatic cell nucleus with that which mirrors the spermatozoon nucleus leads to better nuclear reprogramming. The protocol may have further potential application in determining the protamine and histone footprints of the whole genome; obtaining 'gametes' from somatic cells; and furthering understanding of the molecular mechanisms regulating the maintenance of DNA methylation in imprinted control regions during male gametogenesis. The protocol is straightforward, and it requires 4 weeks from the establishment of the cell lines to their transfection and the production of cloned blastocysts. It is necessary for researchers to have experience in cell biology and embryology, with basic skills in molecular biology, to carry out the protocol.

INTRODUCTION

The SCNT technique was developed in the 1950s as a tool for studying the mechanisms of cell differentiation¹.

Later, SCNT was used to demonstrate the possibility of producing viable animals by the transfer of a somatic cell, with the subsequent birth of live offspring, a sheep named Dolly². The technique used by Dolly's team involved the transfer of a somatic cell (from a mammary gland cell line) into an enucleated oocyte by electro-mediated membrane fusion. The 'reconstructed' embryos were cultured in the oviduct of a temporary sheep foster mother for 7 d, and they were finally transferred for development to term into another ewe, whose uterus had been previously synchronized with the developmental stage of the embryos (blastocyst stage, 7 d after fertilization).

However, although 20 years have now passed since the production of the first mammal cloned by SCNT (<http://www.nature.com/news/dolly-at-20-the-inside-story-on-the-world-s-most-famous-sheep-1.20187>), the use of technology to produce healthy offspring is still limited³. The limited efficiency results from an incomplete nuclear reprogramming of the somatic nucleus. Nuclear reprogramming denotes the molecular mechanisms, operating in the oocyte, that wipe out the epigenetic changes established throughout cell differentiation, restoring a condition of 'totipotency' (i.e., the ability to generate all types of somatic and extra-embryonic cell types). Unfortunately, complete nuclear reprogramming remains an unpredictable event when current methodology is used. Although progress has been achieved in reprogramming to produce laboratory mice⁴, the efficiency of SCNT in large animals remains low³. The most effective nuclear reprogramming approaches in the mouse rely on targeted intervention, such as *Xist* RNAi-mediated knockdown in SCNT⁵, or genome-wide approaches, such as treatment with histone deacetylase inhibitors (Trichostatin A (TSA))⁶, or the removal of epigenetic marks inhibiting nuclear reprogramming⁷. *Xist* knockdown and H4K9TriMe depletion are not translatable to other species. Thorough genetic characterization and the availability of the

full spectrum of 'omics' tools are possible only in the laboratory mouse. TSA treatment, although straightforward, unfortunately produces contradictory results in other species⁸. SCNT is a tool that can be used for asexual reproduction in all species, including amphibians, mammals, fish and insects⁹. However, a protocol for nuclear reprogramming that is applicable to all species is unavailable. We therefore decided to develop a protocol for nuclear reprogramming, based on the knowledge that the spermatozoon is the best nuclear transfer device.

Spermatogenesis is largely conserved from flies to mammals¹⁰. The postmeiotic phase of spermatids is characterized by the timely translation of mRNA, starting with the expression of testis-specific histone variants, which transform spermatids into mature spermatozoa. The incorporation of testis-specific linker (H1t, H1t2 and HILS1)¹¹ and core histone variants (H2A-H2B, H3 and H4)¹¹, followed by their post-translational modification⁴, destabilizes the nucleosomes in preparation for the incorporation of transition proteins (TPs)¹³. Finally, protamine binds with high affinity to all DNA, conferring the typical toroid structure found in mature spermatozoa¹⁴, while a small proportion of the genome (1–10%, according to the species¹⁵)—maintains nucleosomal organization, particularly in centromeres¹⁶, telomeres, and in crucial developmentally relevant genes^{17–19}. Upon fertilization, the remodeling is completely reversed. Paternal chromosomes exposed to the oocyte cytoplasm rapidly lose the protamine- and testis-specific histones^{20,21} and regain a nucleosomal organization that is built on maternally provided histones. Hira²² and members of the nucleoplasmin/nucleophosmin family²³ are actively involved in the protamine-to-histone transition in the oocyte.

The remodeling of the spermatozoon nucleus into the paternal pronucleus releases the intrinsic totipotency of its genome, leading to the development of a normal individual. When a somatic cell is used to 'fertilize' an oocyte, as accomplished in SCNT, the nucleosome organization of the chromatin is a formidable obstacle for the reprogramming machinery. In this case, the process is

PROTOCOL

not efficient, leading to the developmental abnormalities commonly reported in SCNT clones²⁴.

Given that the oocyte reprogramming machinery is evolutionarily adapted to the chromosome configuration of the spermatozoa, the ‘reprogrammability’ of somatic cells might be improved by imposing a protamine-based DNA organization on the somatic cell nucleus.

The nuclear remodeling that takes place during spermatozoa maturation in somatic cells is not possible. However, in several organisms, such as mollusks, the nucleosomal chromatin organization in spermatids is converted to a Prm-based toroidal structure through the direct assembly of Prm, or simil-Prm proteins, on DNA²⁵. Therefore, we established a system in which we could reproduce this conversion in somatic cells. Most mammalian species express only Prm1, whereas humans and mice express both Prm1 and Prm2 (ref. 26). Because ram spermatozoa (our main experimental model) contain Prm1 only, we selected Prm1 for our nuclear remodeling protocol. In addition, Prm1 binds to DNA in its native form, in contrast to Prm2, which needs to be post-transcriptionally processed before DNA binding²⁷. The pathway required to post-transcriptionally process Prm2 is not present in a somatic cell.

This protocol transforms interphase nuclei into ‘spermatid-like’ structures. It has already been shown, in our previous work²⁸, that the nucleus of a somatic cell can be formatted into a spermatid-like structure, simply by the heterologous expression of a single gene, *Prm1*. The radical reorganization of somatic chromatin from histone to protamine resulted in significantly greater numbers of somatic cells reaching the blastocyst stage of embryonic development²⁸. This protocol describes how to reconfigure a somatic cell nucleus to a protamine-based toroid organization with 50–80% efficiency, resulting in improved nuclear reprogramming of SCNT embryos to blastocyst stage. The functionality of the protocol has been confirmed in sheep (Fig. 1a,b) and mouse (Fig. 1c,d) adult fibroblasts using human (Fig. 1a,c) or mouse (Fig. 1b,c) protamine plasmids.

Development of the protocol

The technique that we have developed is based on the transient, heterologous expression of the main testis-specific nuclear remodeling proteins in somatic cells (fibroblasts).

Our first approach to remodeling somatic cell nuclei was to induce the coexpression of the main testis-specific genes in a primary culture of sheep fibroblasts, with the aim of producing a physiological remodeling similar to that occurring in spermatids. Expression vectors for the bromo domain testis-specific (Brdt-GFP tag), TPs I and II (HA tag) and Prm1 and RFP (Prm1-RFP tag) were generated. Proliferating cells were liposome-transfected with a cocktail of the four transfection vectors. Transfected cells were monitored every 24 h; however, no phenotypic changes were observed after 7 d of monitoring.

We therefore opted for a stepwise transfection, starting with the Brdt-GFP tag. Expression of the vector was ~40%, and a reorganization of the nuclear structure, with evident chromatin compaction, was observed 24 h post transfection, followed by rounding up of the cells. Nuclear condensation of Brdt-GFP in sheep fibroblasts was comparable to that seen previously in mouse fibroblasts expressing Brdt²⁹. The treatment of cells with the histone deacetylase inhibitor TSA augmented the extent of

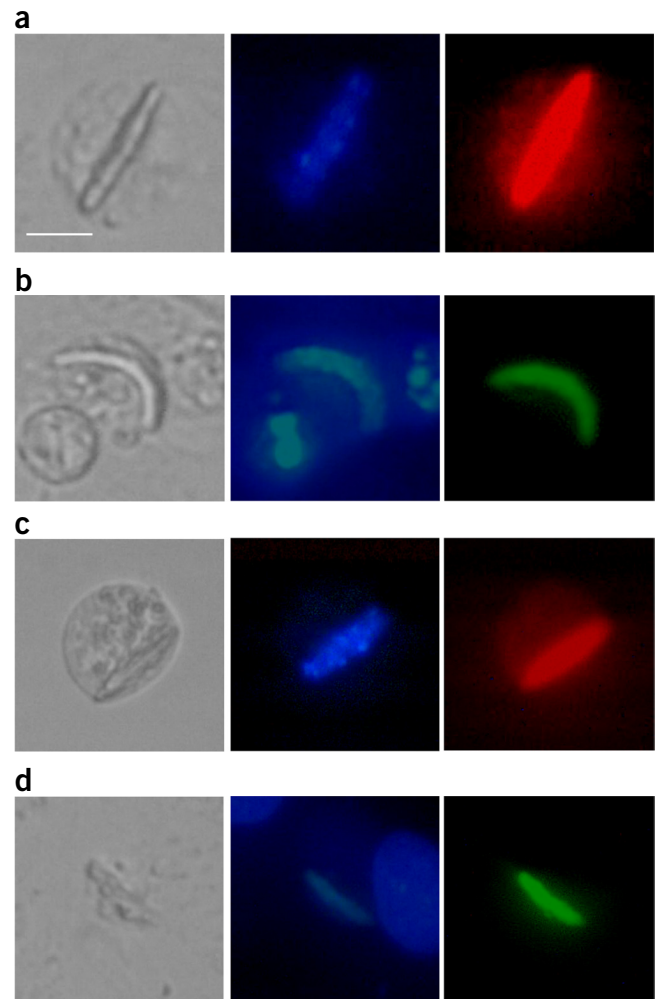


Figure 1 | Protaminized adult fibroblasts in sheep and mouse. Fully protaminized adult fibroblasts in sheep (a,b) and mouse (c,d) using human (a,c) or mouse (b,d) protamine 1. Scale bar, 5 μ m.

nuclear compaction, as expected. However, Brdt-GFP-expressing cells did not take up TPs I and II vectors (HA tag) in the following transfection round, and showed signs of degeneration 96 h after transfection. It is very likely that the Brdt-acetylation-induced chromatin compaction shuts down the bulk cellular transcription, with negative effects on cell homeostasis.

Thus, our attempts to induce chromatin remodeling of somatic cells—following either co-transfection or stepwise transfection with all four expression vectors—were ineffective.

The nuclear reorganization occurring in round and elongating spermatids relies on a fine hierarchic translation of previously synthesized mRNA, and each of the proteins prepares the ground for translation of the next mRNA³⁰, a scenario that we failed to repeat in cultured somatic cells.

However, given that in several animal models, male germ cells compact DNA through the expression of protamine only²⁵, we decided to induce a nuclear compaction in somatic cells through the sole expression of Prm1. In this case, sheep were a perfect model for the study of protaminization because ram spermatozoa express only Prm1, whereas the laboratory mouse, a better model for SCNT, requires both Prm1 and Prm2 for proper

nuclear compaction and fertility. Indeed, this approach skips all other relevant steps but fulfills our goal to impose a male germ cell's nuclear structure onto somatic cells. The nuclear compaction shuts down all transcription activity, leading to cell death. However, this is not an issue, as we proved that nonviable cells are successfully reprogrammed by the oocyte³¹. We had previously demonstrated that granulosa cells heated to nonphysiological (55 or 75 °C) temperatures and injected into enucleated metaphase II oocytes were able to develop into viable offspring³¹.

In this protocol, we also provide step-by-step instructions for the reversal of nuclear remodeling through the nuclear transfer (NT) of protaminized cells into enucleated oocytes, as shown in our previous publication, Iuso *et al.*²⁸.

The present protocol allows scientists working in different fields to reproduce the nuclear remodeling that occurs in the final phase of spermatocyte maturation *in vitro*.

Applications of the protocol and the advantages and limitations

The biological implications of our findings primarily concern SCNT. Our recent work has shown statistically higher percentages of embryonic development to the blastocyst stage when 'protaminized' cells, as compared with control, untransfected fibroblasts (14% versus 7%), were used for SCNT²⁸. In addition to the applications to SCNT, the protocol might be useful for scientists interested in understanding the following biological issues:

- Use as a simplified model for whole-genome protaminization. Data from human spermatids show that chromatin remodeling invariably starts from a definite doughnut¹⁵-shaped site and then spreads to the entire nucleus, conferring the universal, hydrodynamic shape of all male gametes. Given the lack of a complete *in vitro* model of spermatogenesis in mouse and human, our system would allow the identification of the very early genomic domain(s) targeted by protamine, by picking up cells transfected early in the process (when the first Prm1 foci appear). Running genome-wide ChIP-seq analysis may allow researchers to identify the very early DNA domains targeted by Prm1.
- Determination of the protamine/histone footprints on the whole genome. The question of nucleosomal retention during male gamete maturation remains a controversial issue¹⁹. Our protocol will provide a unique tool for mapping whole-genome protamine versus histone footprints in somatic cells, and comparing protaminized cells with male gametes.
- The protocol could improve our understanding of the molecular mechanisms regulating the maintenance of DNA methylation in imprinted control regions during male gametogenesis. Our whole-genome protaminization protocol will allow researchers to monitor the maintenance/removal of imprinted marks during nuclear compaction.

The main advantage of our somatic cell protaminization technique is that is straightforward, fast (only 48 h for full protaminization of the nucleus) and highly efficient. Moreover, no advanced instruments or particular molecular biology skills are required to carry out the protocol.

Besides those advantages, there are few limitations of the protaminization procedure. Although Prm1 expression leads to morphological changes in the nuclei, more work is needed to determine whether the protaminized chromatin obtained is truly similar to that of the sperm at the chromatin level. Moreover, the effective improvement of nuclear reprogramming in protaminized cells has to be demonstrated by improved proportions of healthy cloned offspring.

Our protaminization protocol could be useful to researchers investigating nuclear remodeling in male germ cells, but the limitation is that it is a radical shortcut that excludes the preceding steps, providing only a limited portion of the entire process of nuclear maturation in male sperm cells. Another limitation is that protaminization of the cell nuclei precludes their viability (fully protaminized cells showed signs of degeneration 50–55 h after transfection); therefore, long-term studies cannot be conducted.

The last limitation is that in some species (mouse and human) Prm1 and Prm2 are both essential for proper chromatin compaction and fertility. Even if our protaminization procedure is confirmed in a mouse model (in which both protamines are expressed), we cannot exclude the possibility that the expression of both protamines is necessary.

Overview of the procedure

We present a protocol for the conversion of interphase somatic nuclei into 'spermatid-like' structures, which we have termed protaminization, by the exogenous expression of a single gene—*Prm1*. We describe how to use these protaminized cells in sheep SCNT.

The technique involves five major stages, and a summary of our protocol can be seen in **Figure 2**. Stages one to three can be applied to mouse or sheep cells. Stage one (Steps 1–19) describes how to establish primary fibroblast cell culture from adult/embryonic tissue (Steps 1–13), or how to proceed with commercially available

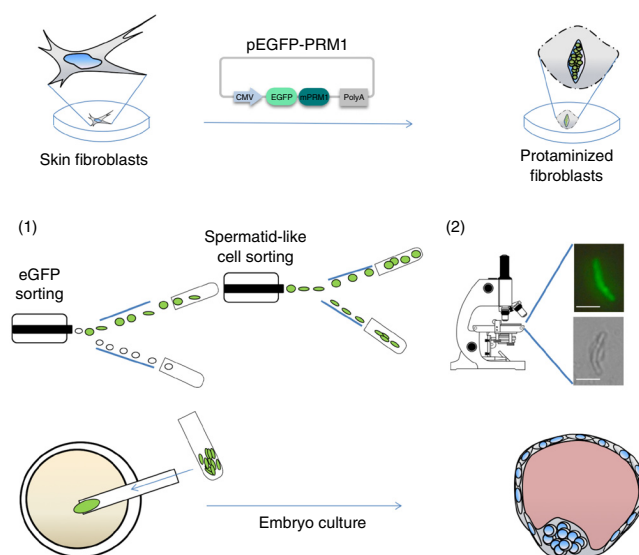


Figure 2 | Schematic diagram of the protaminization of somatic cell nuclei, isolation and use of donors for the somatic cell nuclear transfer (SCNT) procedure. **(a)** The first steps of the protocol (establishment of primary cell line and plasmid transfection). **(b)** Two different methods for choosing protaminized cells by (1) FACS sorting or (2) manual picking. Scale bars, 5 μ m. **(c)** Protaminized cells are used for SCNT.

PROTOCOL

dermal cells (Steps 14–19). Stage two (Steps 20–28) shows protaminization of somatic cells by transient expression of the vector (pAcGFP1-C1)—protamine tagged with GFP (mPrm1-GFP). The protocol explains how to induce transient expression of mPrm1-GFP in adult fibroblasts, with 80% efficiency (Steps 20–27). In the next steps (28A, B, C), assessment of transfection efficiency and monitoring of the dynamics of nuclear remodeling are described. Positive expression of mPrm1-GFP is assessed in growing (Step 28A), attached (Step 28B) and detached (Step 28C) cells. Stage three (Step 29) describes picking the protaminized cells by sorting a 100% enriched fraction of mPrm1-GFP-positive cells (Step 29A). This approach is recommended when large numbers of cells are required for molecular analysis. Alternatively, for SCNT, we recommend selecting the protaminized cells using light and/or fluorescence microscopy based on cell morphology (Step 29B). In stage four (Steps 30–56), SCNT in sheep using protaminized cells is explained in detail, from *in vitro* maturation (Steps 30–33) to the preparation of oocytes for SCNT (Steps 34 and 35), oocyte enucleation (Steps 36–42), preparation of donor cells (Steps 43 and 44), donor cell injection (Steps 45–54) and, finally, oocyte activation (Steps 55 and 56). In stage five (Steps 57–61), assessment of *in vitro* embryonic development of the NT embryos is detailed (Steps 57–61).

Experimental design

As anticipated in the previous section, our aim was to develop a straightforward protocol for nuclear remodeling of somatic cells before SCNT, which can easily be applied to all species by relatively inexperienced researchers and technicians. Therefore, there are no particular experimental aspects that need to be discussed/prepared before applying the protocol.

Source of cells. For protaminization of somatic cells, it is possible to use commercially available or primary cell lines established from adult or embryonic tissue. The advantage of using primary cell lines established from adult tissues is that they have been studied extensively for SCNT, and cells can be collected and protaminized from the same animal that will be cloned by SCNT. Our protocol for somatic cell protaminization was tested on sheep and mouse. In both models, somatic nuclei were protaminized with the same timing and efficiency.

Plasmid delivery. Prm1 plasmids tagged with GFP and RFP were used in both the mouse and sheep models. The results show that the tags do not have an effect on the protaminization efficiency of the somatic nucleus. We suggest using the RFP plasmid to follow the dynamics of Prm1 nuclear localization. However, the RFP tag might create a problem if flow cytometry is used to separate the cells because of the need to use distinctive excitation and fluorescence detection conditions. The advantage of using the GFP tag is that it can be used for microscopy and flow cytometry analysis.

It is important to bear in mind that many animal species required both Prm1 and Prm2 for proper nuclear compaction and fertility. Although we have confirmed nuclear compaction in mouse cells (both protamines are expressed in spermatids) using only Prm1, more work is needed to determine whether Prm2 can be excluded or is instead needed for proper nuclear remodeling.

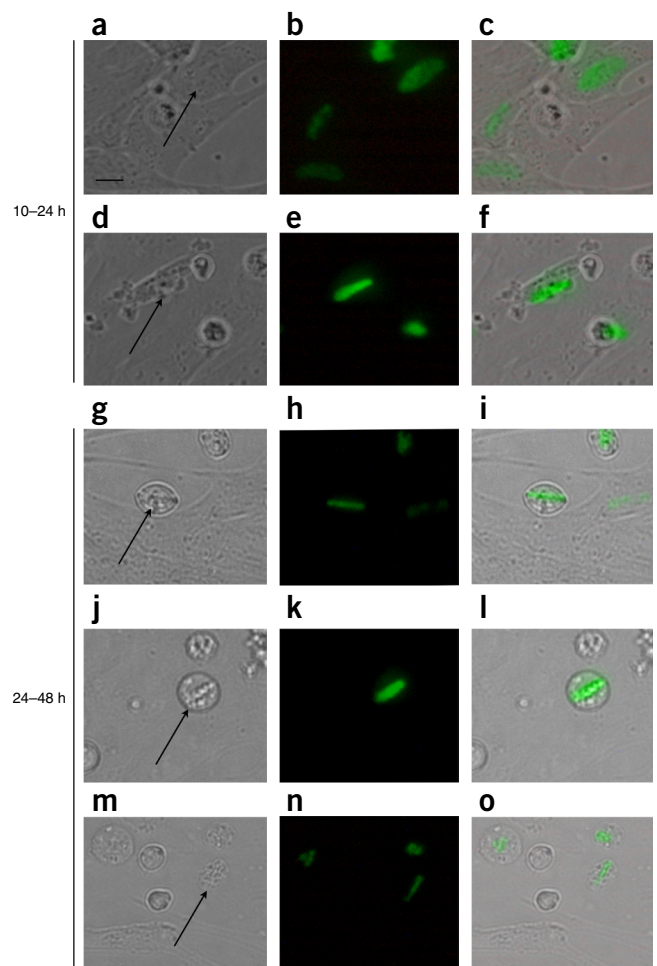


Figure 3 | Visualization of protaminized nuclei of fresh somatic cells using a Nikon Eclipse TE 300 fluorescence microscope. (a–f) Somatic cells 10–24 h post protaminization. (g–o) Somatic cells 25–48 h post protaminization. Arrows indicate protaminized nuclei at different stages of protaminization. (a,d,g,j,m) Somatic cells in contrast phase. (b,e,h,k,n) GFP fluorescence (cells expressing protamine tagged with GFP). (c,f,i,l,o) Merge. Scale bar, 5 μ m.

The incubation with TSA is crucial for protaminization of the somatic nucleus. Accordingly, cells treated with TSA incorporated protamine more efficiently than control cells cultured without TSA²⁸. It has been observed that the concentration of TSA affects protaminization efficiency. Our protocol has been tested on only two animal species (mouse and sheep), in which a 25–50 nM concentration of TSA worked best. It is recommended that the optimal TSA concentration be established if different cell lines or cells from other species are used.

Controls. As a negative control for SCNT technique (mock-transfected cells) with Prm1, we recommend transfection without the addition of DNA (i.e., cells are treated with transfection reagent only). This control can be used to determine any nonspecific effects that may be caused by the transfection reagents or process.

As a negative control for FACS analysis, transfect cells with a pDNA that contains only GFP plasmid, and also use nontransfected cells. Alternatively transfect cells with pDNA that does not contain GFP (null) and Prm1. This is critical to enable the setup of FACS sorting of the cells. Toxicity

can induce autofluorescence and result in the overestimation of protaminization efficiency.

Visualizing and selecting protaminized cells. Recognizing fully protaminized nuclei will come with experience and time. First, we recommend running a few preliminary experiments to check the appearance of somatic cells with protaminized nuclei. **Figure 3** shows how compaction (a dynamic of protaminization) of somatic nuclei in cultured cells appears over time.

It is important to note that the proposed procedure does not offer 100% protaminized somatic cells; only ~40–50% of the

cells become fully protaminized. We propose two methods for harvesting protaminized cell, depending on their final use.

For SCNT, we recommend picking up the protaminized cells, identified by their unique nuclear structure, with an injection pipette connected to a microsyringe. However, it is important to remember that UV is harmful to DNA and proteins; hence, we do not recommend selecting cells for SCNT by UV exposure, for it may negatively affect embryo development and final SCNT efficiency³².

If large numbers of cells are needed for molecular analysis, a FACS sorter is recommended.

MATERIALS

REAGENTS

- Sodium chloride (Sigma-Aldrich, cat. no. S7653)
- Dulbecco's PBS (D-PBS; Sigma-Aldrich, cat. no. D8662)
- L-Glutamine (Sigma-Aldrich, cat. no. G3126)
- ddH₂O (Gibco, cat. no. 15230-089)
- Gentamicin solution (Sigma-Aldrich, cat. no. G1272)

Somatic cell preparation and propagation

- NIH/3T3 mouse embryonic fibroblasts (ATCC, cat. no. 1658), 3T3 MEF's wt (ATCC, cat. no. 2752) **! CAUTION** The cell lines used in your research should be regularly checked to ensure that they are authentic and are not infected with mycoplasma.
- Minimum essential medium Eagle (MEM; Gibco, cat. no. 11700)
- FBS (heat-inactivated FBS; Gibco, cat. no. 10500)
- Gelatin solution (0.1% (wt/vol) in ddH₂O; Sigma-Aldrich, cat. no. G9136)
- Ethanol (Sigma-Aldrich, cat. no. 02856)
- Trypsin-EDTA solution (0.25% (wt/vol) trypsin/1 mM EDTA-4Na (1×), liquid; Gibco, cat. no. 25200-072)
- DMSO (Sigma-Aldrich, cat. no. D1435)
- Mycoplasma detection kit (InvivoGen, cat. no. rep-pt1)
- Liquid nitrogen

Protaminization of somatic cells and visualization

- Opti-MEM reduced serum medium (Gibco, cat. no. 11058-021) or, alternatively, serum-free culture medium
- DNA: pPrm1-GFP and GFPtag (pEGFPC2 vector; Clontech)
- Trichostatin A (TSA; Sigma-Aldrich, cat. no. T8552)
- Lipofectamine 2000 (Invitrogen, cat. no. 11668-019)
- Bisbenzimidazole H33342 (Hoechst 33341; Fluka, cat. no. 14533)
- Paraformaldehyde (PFA; Sigma-Aldrich, cat. no. P6148) **! CAUTION** Avoid inhalation. PFA is a teratogen, and it is carcinogenic.

SCNT

- Oocytes isolated from sheep ovaries (*Ovis aries*) sacrificed at a slaughterhouse **! CAUTION** Experimental procedures involving animals must be carried out according to relevant national and institutional regulations.
- H-199 medium (Sigma-Aldrich, cat. no. M5017) or TCM medium (Gibco, cat. no. 31100-027)
- Heparin (Sigma-Aldrich, cat. no. H3149)
- Sodium bicarbonate (Sigma-Aldrich, cat. no. S5761)
- Sodium pyruvate (Sigma-Aldrich, cat. no. P4562)
- Cysteamine (Sigma-Aldrich, cat. no. 30070)
- Follicle-stimulating hormone from sheep pituitary (FSH; Sigma-Aldrich, cat. no. F8174)
- β-Estradiol (Sigma-Aldrich, cat. no. E2758)
- Embryo-tested bovine testis hyaluronidase (Sigma-Aldrich, cat. no. H3506)
- Bis-benzimidazole (Hoechst 33412; Sigma-Aldrich, cat. no. B2261)
- Cytochalasin B (CB; Sigma-Aldrich, cat. no. 6726)
- Polyvinylpyrrolidone (PVP 360 (average MW = 360,000); Sigma-Aldrich, cat. no. PVP360)
- Mineral oil (Sigma-Aldrich, cat. no. M8410)
- Elemental mercury (Hg⁰) **! CAUTION** Hg⁰ is a cumulative neurotoxin that is absorbed through the skin. Handle it with gloves and dispose of waste according to the local institutional guidelines.
- Fluorinert (FC40; Sigma-Aldrich, cat. no. F9755)
- Ionomycin (Sigma-Aldrich, cat. no. I0634)

- 6-Dimethylaminopurine (6-DMAP; Sigma-Aldrich, cat. no. D2629)

In vitro embryo culture

- Potassium chloride (KCl; Sigma-Aldrich, cat. no. P5405)
- HEPES (Sigma-Aldrich, cat. no. H6147)
- Potassium phosphate monobasic (KH₂PO₄; Sigma-Aldrich, cat. no. P5655)
- Magnesium chloride (MgCl₂; Sigma-Aldrich, cat. no. M4880)
- L-Sodium lactate (Sigma-Aldrich, cat. no. L7900)
- Phenol red (Sigma-Aldrich, cat. no. P3532)
- Sodium pyruvate (Sigma-Aldrich, cat. no. P4562)
- Calcium chloride (CaCl₂; Sigma-Aldrich, cat. no. C5670)
- BSA (Sigma-Aldrich, cat. no. A3311)
- BME Amino Acids Solution 50× (BME; Sigma-Aldrich, cat. no. B6766)
- Minimum essential medium (MEM), non-essential amino acids (Gibco, cat. no. 11140-035)
- M199 medium (Sigma-Aldrich, cat. no. M5017; or Gibco, cat. no. 31100-027)

EQUIPMENT

- Polypropylene conical tubes (15 ml; BD Falcon, cat. no. 352096)
- Polypropylene conical tubes (50 ml; BD Falcon, cat. no. 352077)
- Tissue culture dish (60 mm; CytoOne, cat. no. CO 7672-3559)
- Tissue culture dish (90 mm; Corning, cat. no. 353003)
- Serological disposable pipette (2 ml; StarLab, cat. no. T14)
- Serological disposable pipette (10 ml; StarLab, cat. no. E4860-1011)
- Serological disposable pipette (25 ml; Falcon, cat. no. 357525)
- Pipette (2 µl; StarLab, cat. no. S1111-0000)
- Pipette tips (20 µl; StarLab, cat. no. S1111-0000)
- Pipette tips (200 µl; StarLab, cat. no. S1111-0000)
- Pipette tips (1,000 µl; StarLab, cat. no. S1111-0000)
- Filtered pipette tips (10 µl; TipOne, cat. no. s1121-3810)
- Filtered pipette tips (20 µl; Gilson, cat. no. F171303)
- Filtered pipette tips (200 µl; Gilson, cat. no. F171503)
- Filtered pipette tips (1,000 µl; Neptune, cat. no. BT20)
- Pasteur pipette (5 inch; StarLab, cat. no. T0407A)
- Microcentrifuge tubes (1.8 ml; StarLab, cat. no. S1615-5500)
- Cryo-vials (1.8 ml; StarLab, cat. no. e 3110-6122)
- Filter (0.22 µm; Sartorius, cat. no. 16532-K-F0760)
- Nylon mesh filter (Corning cell strainer, size 70 µm; Sigma-Aldrich, cat. no. CLS431751)
- 4-Well Nunc Lab-Tek Chamber Slide system (Sigma-Aldrich, cat. no. C7182)
- Fluoroshield with PI (propidium iodide) histology (mounting medium; Sigma-Aldrich, cat. no. F5932)
- Poly-Prep Slides (poly-L-lysine-coated glass slides; Sigma-Aldrich, cat. no. P0425)
- 10-ml Syringe (Kruuse, cat. no. 132030)
- 21-gauge needle (Sutter Instrument, cat. no. P-87)
- Stereomicroscope stage with heated platform, such as Nikon SMZ645 or equivalent
- NanoDrop Spectrophotometer (Thermo Fisher Scientific)
- Fridge set to 4 °C
- Freezer set to -4 °C
- Deep freezer set to -80 °C
- CO₂ incubator (for cell culture) set to 5% CO₂ and 37 °C (for mouse) or 38.5 °C (for sheep) (Thermo Fisher Scientific)
- CO₂/O₂ incubator (for embryo culture) set to 5% CO₂, 7% O₂ and 38.5 °C (Thermo Fisher Scientific)

Q13
Q14
Q15
Q16

PROTOCOL

- Tissue culture hood such as Thermo Fisher Scientific 1300 Series Class II, Type A2 Biological Safety Cabinet Packages, or equivalent
- Cell culture centrifuge (Eppendorf Centrifuge, model no. 5804R)
- Mr. Frosty Freezing Container (Thermo Fisher Scientific, cat. no. 5100-0001)
- Microcentrifuge (Eppendorf Centrifuge, model no. 5424R)
- Heated work stage composed of an inverted microscope, such as a Nikon Eclipse TE300 or equivalent, equipped with a phase-contrast objective (phase $\times 4$, $\times 10$ and $\times 20$), a charge-coupled device optical system (e.g., Jenoptik), and a Narishige MM188 micromanipulator with micro-injectors (e.g., Nikon, Narishige)
- Eppendorf PiezoXpert (Eppendorf)
- Water bath set to 37–38.5 °C
- Fluorescence-activated cell sorter, such as a FACS Aria III cell sorter, that can measure 16 colors—(BD Biosciences)
- Transfer glass pipettes (Volac, cat. no. D812)
- Borosilicate glass capillaries, 10 cm \times 1 mm (outer diameter), 0.78 mm (inner diameter) (Harvard, cat. no. GC 1005-15)
- Microforge (Narishige, cat. no. MF-900)
- Micropipette puller (Sutter Instrument, Model P-87, flaming/brown micropipette puller)

REAGENT SETUP

Gelatin solution Dissolve 0.1 g of gelatin in 100 ml of D-PBS to obtain a 0.1% (wt/vol) gelatin solution. Store the solution at 4 °C for up to one month.

Minimal essential medium Add 800 ml of distilled water to a 1-liter glass beaker. Weigh 9.4 g of minimal essential medium (MEM), 292 mg of glutamine and 2.2 g of sodium bicarbonate, and transfer them to a beaker. Add water to a final volume of 1 liter. Add 5 ml of gentamicin solution. Check the osmolarity and adjust it to 280 mOsm/kg. Pass the solution through a 0.22- μ m filter and store it at 4 °C for up to 2 months.

Cell culture medium To prepare a total of 50 ml of culture medium, mix 45 ml of MEM with 5 ml of FBS to obtain cell culture medium containing 10% (vol/vol) FBS. Store the solution at 4 °C for up to 1 week.

Paraformaldehyde 4% (wt/vol) To prepare the fixative, dissolve 4 g of paraformaldehyde (PFA) in 50 ml of ddH₂O in a water bath set to 80 °C. Titrate with several drops of 2 N sodium hydroxide solution to dissolve the PFA. After dissolving, add ddH₂O to make up the total volume to 100 ml. Store the solution at 4 °C until use (up to 1 month).

Freezing solution To prepare 10 ml of solution for cell freezing, dilute 2 ml of DMSO in 6 ml of MEM medium and 2 ml of FBS. Store the solution at –20 °C for up to 1–2 weeks.

Trichostatin A Add 1 ml of 100% (vol/vol) ethanol to a 1-mg vial of TSA and shake it up and down 2–3 times to dissolve well. Work on ice, because ethanol evaporates at room temperature (RT, 23–27 °C), and this may change the final concentration of TSA. Prepare 0.75- μ l aliquots and store them at –20 °C for up to one year. To make a final solution, take one aliquot and dilute it in 15 ml of culture medium to obtain a final concentration of 50 nM TSA.

Polyvinylpyrrolidone solution Dissolve 1.2 g of polyvinylpyrrolidone (PVP) in ~8 ml of high-quality, ddH₂O in a 50-ml tube at RT. Transfer the solution to a water bath or an incubator set to 37 °C. The PVP takes ~1 h to dissolve. Add ddH₂O to make up the total volume to 10 ml, pass this solution through a 0.45- μ m filter and store 0.2- to 1-ml aliquots at –20 °C for up to 6 months.

Handling medium (H-199) Add 800 ml of ddH₂O to a 1-liter glass beaker. Weigh 9.5 g of TCM 199 medium, 4.7 g of HEPES, 143 mg of glutamine and 2.2 g of sodium bicarbonate, and transfer them to a beaker. Add 5 ml of gentamicin solution. Weigh 4 g of BSA and completely dissolve it in the mixture. Transfer the medium to a 1-liter cylinder and add water up to a total volume of 1 liter. Check the osmolarity and adjust it to 280 mOsm. Pass the solution through a 0.22- μ m filter and store it at 4 °C for up to 1 week.

Basic medium for *in vitro* maturation (M-199) Add 800 ml of ddH₂O to a 1-liter glass beaker. Weigh 9.5 g of TCM-199 medium, 143 mg of glutamine and 2.2 g of sodium bicarbonate and transfer them to a beaker. Mix gently. Add water up to a total volume of 1 liter. Check the osmolarity and slowly adjust it to 280 mOsm/kg. Pass the solution through a 0.22- μ m filter and store it at 4 °C for no more than 1 week.

***In vitro* maturation medium** Add 2 mM glutamine, 0.3 mM sodium pyruvate, 100 μ M cysteamine, 5 μ g/ml FSH, 5 μ g/ml LH, 1 μ g/ml estradiol,

0.04% (vol/vol) gentamicin solution and 10% (vol/vol) FBS to basic medium (M-199). **! CAUTION** *In vitro* maturation (IVM) medium should be freshly made before use.

Medium for oocyte enucleation Dissolve 7.5 μ g/ml cytochalasin B (CB) in H-199 medium. Warm and equilibrate in an incubator before use. Freshly prepare enucleation medium before use.

Medium for donor cell injection The procedure is the same as that for H-199 medium preparation, except that the injection medium should be warmed and equilibrated in the incubator before use, and should be freshly prepared before use.

Hyaluronidase Weigh 10 mg of hyaluronidase and dissolve it in 1 ml of distilled water. Prepare stock solution aliquots of 30 μ l and store them at –20 °C for up to 1 year. For the working solution, dilute one aliquot in 1 ml of H199 medium to obtain a final concentration of 0.3 mg/ml.

Hoechst 33341 Weigh 5 mg of Hoechst 33341 and dissolve it in 1 ml of D-PBS. Prepare stock solution aliquots of 10 μ l and store them at –20 °C in the dark for up to 1 year. For the working solution, dilute one aliquot in 10 ml of H199 medium to obtain a final concentration of 5 μ g/ml.

Ionomycin Dissolve the contents of the vial (1 mg) in pure ethanol. Work on ice because ethanol evaporates at RT and might change the final concentration of ionomycin. Prepare stock solution aliquots of 10 μ l and store them at –20 °C in the dark. Dilute one aliquot in H199 medium to obtain a final concentration of 5 μ M. **! CAUTION** Ionomycin is light-sensitive. Keep it in the dark.

Basic medium for sheep embryo culture: synthetic oviductal fluid– (10 \times)

To prepare synthetic oviductal fluid (SOF)– (10 \times), add 80 ml of ddH₂O to a 100-ml glass beaker. Weigh 6.294 g of NaCl, 0.5338 g of KCl, 0.1619 g of KH₂PO₄, 0.04665 g of MgCl₂ and 0.616 g of L-sodium lactate and transfer them to the beaker. Add 5 ml of gentamicin solution. Mix gently and add water to a final volume of 100 ml. Pass the solution through a 0.22- μ m filter and store it at 4 °C for up to 3 months.

6-Dimethylaminopurine Weigh 4 mg of 6-dimethylaminopurine (6-DMAP) and dissolve it in 10 ml of SOF to obtain a working solution of 2 mM 6-DMAP. Freshly prepare the solution each time, and equilibrate it for at least 1 h in an incubator before use.

Basic medium for sheep embryo culture: SOF+ (10 \times) Add 80 ml of ddH₂O to a 100-ml glass beaker. Weigh 6.294 g of NaCl, 0.5338 g of KCl, 0.1619 g of KH₂PO₄, 0.04665 g of MgCl₂, 0.616 g of L-sodium lactate and 0.2703 g of glucose and transfer them to the beaker. Add 5 ml of gentamicin solution. Mix gently and add water to a final volume of 100 ml. Pass the solution through a 0.22- μ m filter and store it at 4 °C for up to 3 months.

Sodium bicarbonate (10 \times) Add 80 ml of distilled water to a 100-ml glass beaker. Weigh 2.1106 g of sodium bicarbonate and 1.3 mg of phenol red, and transfer them to the beaker. Mix gently and add water to a final volume of 100 ml. Pass the solution through a 0.22- μ m filter, prepare 5-ml aliquots and store them at –20 °C for no more than 3 months.

Sodium pyruvate (100 \times) Add 8 ml of ddH₂O to a 10-ml glass beaker. Weigh 36.3 mg of sodium pyruvate, transfer it to the beaker and add water to a final volume of 10 ml. Pass the solution through a 0.22- μ m filter and store it at 4 °C for no more than 3 weeks.

Calcium chloride (100 \times) Add 8 ml of distilled water to a 10-ml glass beaker. Weigh 0.1898 g of calcium chloride and transfer it to the beaker. Add water to a final volume of 10 ml. Pass the solution through a 0.22- μ m filter and store it at 4 °C for no more than 3 weeks.

SOF– medium Add 5 ml of SOF – (10 \times), 5 ml of 10 \times sodium bicarbonate, 0.5 ml of 100 \times sodium pyruvate, 0.5 ml of 100 \times calcium chloride and 7.3 mg of glutamine to a 50-ml glass beaker. Add water to a final volume of 50 ml. Check the osmolarity and adjust it to 280 mOsm/kg. Pass the solution through a 0.22- μ m filter and store it at 4 °C for no more than 1 week.

Embryo culture medium, day 0 Add 2% (wt/vol) (BME essential amino acids and 1% (wt/vol) MEM non-essential amino acids to SOF– medium. Weigh 8 mg/ml BSA and gently transfer it to a glass beaker. When the BSA is completely dissolved, pass the solution through a 0.22- μ m filter. Equilibrate the medium in the incubator 1 h before use. Always freshly prepare the embryo culture medium for use on day 0.

SOF+ medium 1 Add 5 ml of SOF+ (10 \times), 5 ml of 10 \times sodium bicarbonate, 0.5 ml of 100 \times sodium pyruvate, 0.5 ml of 100 \times calcium chloride and 7.3 mg of glutamine to a 50-ml glass beaker. Add water to a final volume

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TABLE 1 | Required amount of gelatin for dish coating.

Dish size	Volume of gelatin solution (per dish or well)
90-mm	3 ml
60-mm	1 ml
35-mm	500 μ l
6-well	200 μ l
12-well	100 μ l

of 50 ml. Check the osmolality, and adjust it to 280 mOsm/kg. Pass the solution through a 0.22- μ m filter and store it at 4 °C for no more than 1 week.

Embryo culture medium, day 3 Add 2% (wt/vol) BME essential amino acids and 1% (wt/vol) MEM nonessential amino acids to SOF+ medium. Weigh 8 mg/ml BSA and gently transfer it to a glass beaker. When the BSA is completely dissolved, pass the solution through a 0.22- μ m filter. Equilibrate the mixture in the incubator 1 h before use. Always freshly prepare embryo culture medium for use on day 3.

Q25 **SOF+ medium 2** Add 5 ml of SOF+ (10 \times), 5 ml of sodium bicarbonate (10 \times), 0.5 ml of sodium pyruvate (100 \times), 0.5 ml of calcium chloride (100 \times) and 7.3 mg of glutamine to a 50-ml glass beaker. Add water to a final volume of 50 ml. Check the osmolality and adjust it to 280 mOsm. Pass the solution through a 0.22- μ m filter and store it at 4 °C for no more than 1 week.

Embryo culture medium, day 5 Add 1% (wt/vol) BME essential amino acids, 1% (wt/vol) MEM nonessential amino acids and 10% (vol/vol) FBS to SOF+ medium. Pass the solution through a 0.22- μ m filter. Equilibrate the medium in the incubator 1 h before use. Always freshly prepare embryo culture medium for use on day 5.

Q26 **Blastocyst medium** To prepare 10 ml of blastocyst medium, mix 8.9 ml of MEM/M-199 (1:1 vol/vol), 1 ml of FBS and 100 μ l of sodium pyruvate (10 \times). Equilibrate the medium in the incubator 1 h before use. Always freshly prepare the blastocyst medium.

TABLE 2 | Parameters for micropipette preparation for sheep oocyte manipulation.

Pipettes	Puller parameter	Diameter (μ m)	Bend
Holding	Heat = 750, Pull = 150	Outside (o.d.) = 70 Inside (i.d.) = 23–25	30°
Enucleation	Heat = 750, Pull = 150	Outside (o.d.) = 20–22 Inside (i.d.) = 15–20	30°
Injection	Heat = 730, Pull = 180, Vel = 45 Time = 100	Outside (o.d.) = 10 Inside (i.d.) = 8–9	20–25°

EQUIPMENT SETUP

Coating of the culture dish with 0.1% (vol/vol) gelatin Add 2 ml of sterile gelatin solution to the 60-mm culture dish. Rock it gently to ensure even coating of the culture surface. To culture cells in a different tissue culture format, you must add a different amount of gelatin solution—enough to cover the culture surface (**Table 1**). Incubate the gelatin-covered dishes for 1 h at RT, and then remove the solution by aspiration and thoroughly rinse the surface with sterile tissue-culture-grade water. Allow the dishes to dry for at least 1 h before introducing cells and medium. **! CAUTION** Coated culture dishes should be stable for one year if they are protected from dust. There is no need to sterilize coated gelatin dishes by autoclaving; simply expose the dishes to UV light overnight.

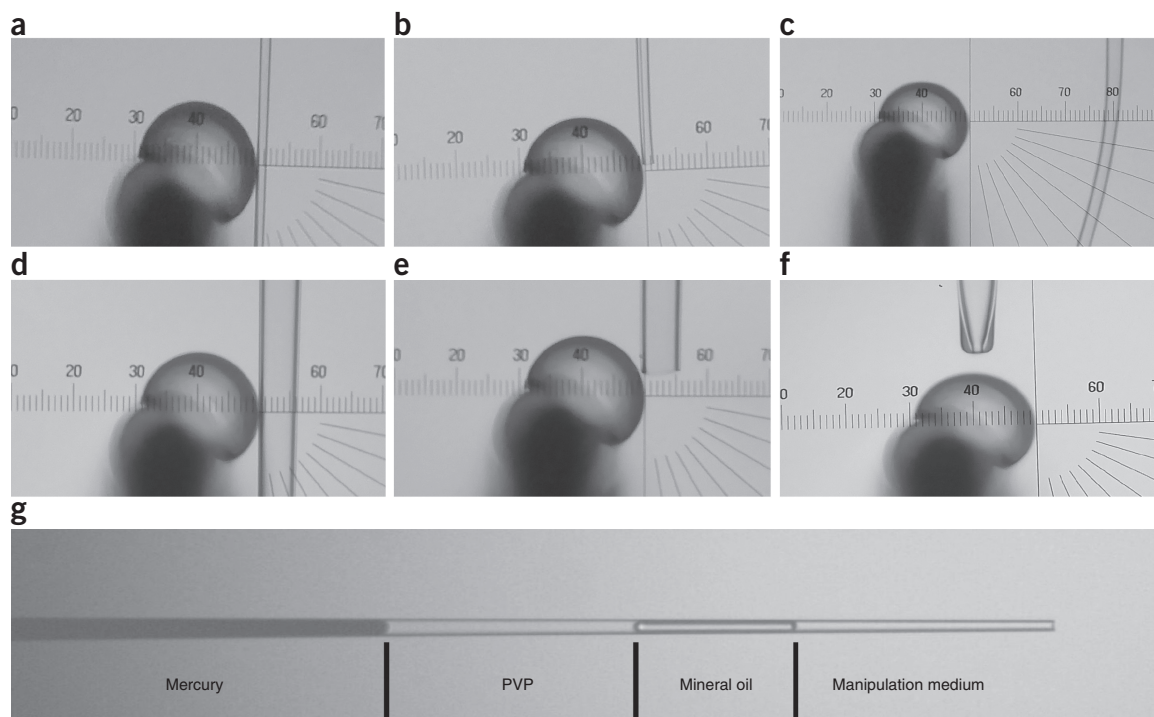


Figure 4 | Piezo micro-tool fabrications. The glass capillary before cutting for (a) enucleated/injection or (d) the holding micropipette; (b) enucleation/injection and (e) holding pipettes after cutting. (c) Banding micropipette; (f) blinding of the holding pipette. (g) Enucleation micropipette loaded with mercury, PVP, mineral oil and manipulation medium, and ready to be used.

PROTOCOL

Pulling of the glass micropipettes The first step of all types of micropipette preparation is to pull down a glass capillary. To do that, turn on the puller and select the correct program on the keyboard (set up the three programs with puller parameters as described in **Table 2**). Next, take the glass micropipette very gently and insert it into the indicated place. Press and hold the two springs and slide it into the middle. Firmly secure the micropipette into the holder from the right and left. Push the PULL button on the keyboard and wait for a few seconds while the micropipette is pulled. **! CAUTION** The diameters listed in **Table 2** are appropriate for sheep oocyte (120 μm) manipulation.

Cutting of the micropipettes using a microforge Place the pulled micropipette into the holder. Focus on the glass sphere of the forge and place it at the center of the visual field. Depending on the micropipette type needed, choose the diameter using the ruler of the lens: 6–7 for a holding micropipette (60–70 μm), 1.5–2 for an enucleating micropipette (15–20 μm) and <1 for an injection micropipette (8 μm).

Q29 Adhere the micropipette to the glass sphere (Fig. 4a shows how to do this for an enucleating or injection micropipette; Fig. 4d shows how to do this for a holding micropipette) and gradually increase the heat. When the micropipette starts to melt, release the heat by lifting your foot off the pedal (Fig. 4b shows how to do this for an enucleating or injection micropipette; Fig. 4e shows how to do this for a holding micropipette). Make sure that the cut is clean; if not, repeat the operation. **▲ CRITICAL** The micropipette tips should appear regular and flush-ended. Notched tips often kill the oocyte during the enucleation or injection step. Good needles are essential for success in piezo functions and for the survival rate of manipulated oocytes.

Bending of the micropipettes using a microforge Position the micropipette that has previously been cut, without letting it stick to the glass ball, and bring it down until the cut end does not disappear from view (Fig. 4c). At this point, increase the heat gradually until the micropipette starts to bend (30° for holding and enucleating micropipettes; 20–25° for injection micropipettes). After completing the operation, release the pedal (Fig. 4c). Transfer the pipettes to a pipette box to store them until use. **! CAUTION** Prepare capillaries just before manipulation, or keep them in a closed, clean box. They can be kept in a clean, closed box for a few years.

Preparation of the holding micropipette After pulling and cutting the holding micropipette, place the pipette above the glass sphere without letting it stick. Increase the heat gradually until the edges of the micropipette start to melt and close. Continue with gradual heating until 50–60% of the capillary diameter end rounds up (Fig. 4f). Next, bend the micropipette as described and shown in **Figure 4c**. Store the micropipette until use in a clean box. Pipettes can be stored for a few years.

Preparation of the enucleating and injection micropipettes After pulling, cutting (Fig. 4a,b) and bending the micropipettes (Fig. 4c), load a small amount of mercury (~3 mm long inside the pipette) into the enucleating and injection pipettes using a 1-ml syringe, and store the pipettes in a 10-cm dish at RT until use (even for a few years). **! CAUTION** Mercury is toxic if it is absorbed through inhalation or through the skin. Wear appropriate gloves and handle the compound in a chemical fume hood. **▲ CRITICAL** Mercury inside the pipette is essential for manipulation because it enhances the intensity of Piezo pulse's electric power and ensures better control.

PROCEDURE

Preparation of primary cell line from adult or embryonic tissues ● TIMING 2–3 weeks

▲ CRITICAL If you are protaminizing somatic cells to be used for SCNT, follow Steps 1–13 for the preparation of skin fibroblasts from adult or embryonic tissue. Cells must be prepared at least 3 weeks before planned protaminization and/or a SCNT experiment. For other types of analyses such as chromatin remodeling studies or protamine/histone footprints, it is fine to use commercially available adult skin cell lines and begin your experiment at Step 14.

1| Isolate a small piece of tissue (tail, ear or skin) from the chosen animal and wash it in 70% (vol/vol) ethanol.

! CAUTION Procedures must be carried out according to relevant national and institutional regulations.

2| In a sterile tissue culture hood, remove the skin and/or hair, press delicately to remove blood (if you use tail tissue) and cut the tissues into small fragments, at least 0.5–1 cm long. Place tissue pieces in a 60-mm plastic dish in 1 ml of trypsin-EDTA for at least 10 min. Transfer the tissue fragments to a 15-ml conical tube and add 5 ml of MEM containing 10%

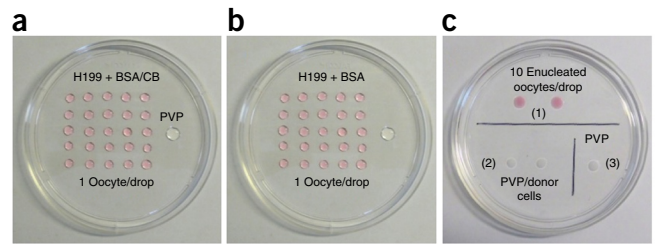


Figure 5 | Suggested layout of droplets in the manipulation chambers of the 10-cm dish. (a,b) Enucleation dish for processing 25 oocytes each time. (c) Injection dish for processing 20–30 oocytes each time.

Preparation of the manipulator for enucleation or injection Take the top of a 90-mm dish and position it at the center of the manipulator table. Insert the holding micropipette into the holder, taking care to avoid the formation of air bubbles. Use the macro-meter joystick to position the holding micropipette at the center of the visual field. Bring down the holding micropipette to the bottom of the Petri dish and position it just above its stopper surface. Focus the holding micropipette.

Next, insert the enucleation or injection micropipette into the holder (again, avoiding the formation of air bubbles). Use the joystick to position the micropipette at the center of the visual field. Focus the micropipette in relation to the holding pipette. Add ~200 μl of H199 medium to the center of the Petri dish. Slightly below the center of the Petri dish, place four drops of 10 μl of PVP and cover the entire dish with mineral oil. Place the tip of the enucleation or injection micropipette into one of the drops of PVP (washing drop). Remove all of the air from the micropipette up to the mercury level, and expel several drops of mercury (Hg^0); repeat this process to wash and lubricate the interior and make sure that all of the air has been removed. Aspirate a small amount of PVP (~2 mm long inside the pipette).

For the enucleation pipette, beyond the PVP, aspirate small amounts of mineral oil (~3 mm long inside pipette) and H199 medium (~3 mm long inside pipette), as shown in **Figure 4g**.

For the injection pipette, do not aspirate any solution besides PVP. It is important to prevent adhesiveness of the donor cells. **▲ CRITICAL** The holding pipette should never come into contact with the PVP.

Preparation of the enucleation dish To enucleate sheep oocytes without UV exposure, you will need to prepare two manipulating dishes (Fig. 5). Dish A (Fig. 5a) should contain 25 10- μl drops of enucleation medium and one 20- μl drop of PVP on the right side of the dish, whereas dish B (Fig. 5b) should contain 25 10- μl drops of H199 medium. Cover all of the drops with mineral oil and transfer the dishes into an incubator for equilibration at least one hour before use.

Preparation of the injection dish Place two to three 20- μl drops of injection medium (see Reagent Setup) into the injection dish (Fig. 5c, drop 1) for reconstruction of oocytes. Then, place two 10- μl drops of PVP solution for donor cells (Fig. 5c, drop 2) and one to two 10- μl drops of PVP solution for pipette wash (Fig. 5c, drop 3). Cover the drops with mineral oil and keep the dish in an incubator until use.

(vol/vol) FBS. Centrifuge the mixture at 420g for 10 min and place the pieces of tissue on 60-mm gelatin-coated dishes. Leave the tissue (without any culture medium) for 1 h at RT to allow it to attach to the surface of the dish.

3| After incubation, add 2 ml of MEM containing 10% (wt/vol) FBS dropwise and culture for 24 h in an incubator set to 5% CO₂ and a temperature adapted for the selected animal species.

▲ CRITICAL STEP Add the culture medium very gently; do not pour it directly on tissue fragments. This is crucial for further cell growth.

4| The next morning, remove the culture medium and any tissue fragments that did not attach to the dish, and add 3 ml of fresh MEM medium containing 10% (vol/vol) FBS. Culture the tissue fragments under the same conditions for 3–4 d. After 2–3 dof culture, cells should start to grow on the dish surface (**Fig. 6a**).

▲ CRITICAL STEP Genetic background and age of the donor animal will affect the success rate. It is important to note that there are differences between species regarding cell growth. Mouse dermal cells attach to the dish surface in 2–3 d, whereas bovine/ovine/horse/pig dermal cells attach after 3–4 d.

? TROUBLESHOOTING

5| When cells reach 40–50% confluence (**Fig. 6b**), gently remove the remaining tissue fragments using sterile tweezers.

6| When cells reach 90% confluence (**Fig. 6c**), aspirate the culture medium and add 5 ml of D-PBS to the culture dish for washing. Discard the D-PBS and add 1 ml of trypsin-EDTA solution. Incubate the culture dish for 2 min in an incubator set to 5% CO₂ and temperature adapted for the animal species used.

▲ CRITICAL STEP Do not incubate the culture for more than 5 min.

7| After 2–3 min of incubation, observe the cells under a phase-contrast microscope to confirm detachment from the surface of the culture dish. When more than 90% of cells are detached, add 8 ml of MEM medium containing 10% (vol/vol) FBS to stop the trypsin reaction, and transfer all of the solution containing the dissociated cells to a 15-ml conical tube. Centrifuge the tube at 420g for 5 min.

8| Discard the supernatant and add 5 ml of MEM containing 10% (vol/vol) FBS; pipette the mixture up and down a couple of times to dissociate the cells, and add 2.5 ml of the solution containing the dissociated cells to each of the two individual 60-mm tissue culture dishes, and then add 2.5 ml of fresh MEM containing 10% (vol/vol) FBS to each dish. Incubate the culture dishes in an incubator set to 5% CO₂ and temperature adapted for the animal species used.

9| The next day, if many floating cells are observed, wash the dish twice with prewarmed D-PBS and add 5 ml of fresh MEM containing 10% (vol/vol) FBS.

10| Usually, the cells reach 90% confluence within 4 d, after which either repeat Steps 6–8 to continue to the next passage or make frozen cell stocks (Steps 11–13). When you wish to protamine the cells, you can proceed to Step 20.

! CAUTION The cell lines should be regularly checked to ensure that they are not infected with mycoplasma (using a standard mycoplasma detection kit).

? TROUBLESHOOTING

11| Freezing. Follow Steps 6 and 7 and remove the supernatant; add 5 ml of D-PBS and wash the cells by aspirating up and down a couple of times. Centrifuge the tube at 420g for 5 min. Remove the supernatant and suspend the cells in 1 ml of MEM serum-free medium.

12| Transfer 500 µl of medium containing the cells to 2 cryovials, and add 500 µl of freezing medium dropwise to each cryovial. Close the lid tightly to avoid contamination.

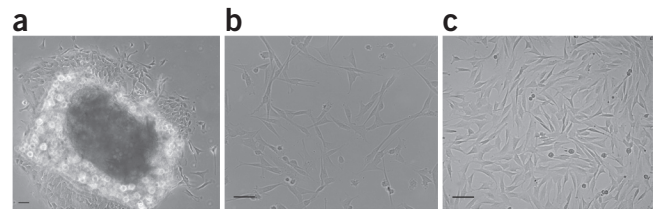


Figure 6 | Primary cells isolated from adult ear tissue. (a) Cells after 48 h, (b) 4 dof (c) 5–6 dof culture. Scale bars, 100 µm.

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PROTOCOL

13| Put the cryovials into a Mr. Frosty freezing container and freeze them at -80°C for 24 h. The next day, transfer the cryovials from the freezing container to a liquid nitrogen refrigerator for long-term preservation.

▲ **CRITICAL STEP** The Mr. Frosty freezing container allows cells to achieve a rate of cooling very close to $-1^{\circ}\text{C}/\text{min}$, which is the optimal rate for cell preservation and gives the best results in term of cell viability.

■ **PAUSE POINT** Cells can be stored in liquid nitrogen indefinitely.

Preparation of adult skin fibroblasts ● **TIMING 1–2 h**

14| Prepare the following solutions in 15-ml conical tubes: 2×10 ml of MEM containing 10% (vol/vol) FBS for thawing frozen fibroblast cells and culturing cells, and 10 ml of D-PBS. Solutions should be prewarmed in a water bath set to 37°C . In addition, prepare 70% (vol/vol) ethanol for sterilization of the cryovial.

15| In a sterile tissue culture hood, add 1 ml of warmed MEM + 10% (vol/vol) FBS medium to the cryovial containing frozen cells. Tighten the lid of the cryovial and thaw the cells for 1 min in a water bath set to 37°C .

▲ **CRITICAL STEP** Make sure that the cryovial lid does not touch the water in the water bath. This is crucial to avoid contamination.

16| Remove the cryovial from the water bath. Disinfect the cryovial by spraying it with 70% (vol/vol) ethanol to protect it from contamination. Transfer the cell solution to a 15-ml conical tube containing 9 ml of MEM with 10% (vol/vol) FBS, and centrifuge it at 420g for 5 min.

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17| Discard the supernatant and add 1 ml of fresh MEM containing 10% (vol/vol) FBS, and suspend the cell pellet by tapping gently ~10 times. Add 9 ml of MEM containing 10% (vol/vol) FBS, count the cells, and transfer them to an appropriate culture dish. Incubate the cells overnight in an incubator set to 5% CO_2 and temperature adapted for the animal species used.

18| The next day, if many floating cells are observed, wash the cells twice with prewarmed D-PBS and add 5 ml of fresh MEM containing 10% (vol/vol) FBS. The cells usually reach 90% confluence within 4 d, after which they can be passaged.

19| Passage the cells 2–4 times (as described in Steps 6–8) before continuing to protaminization.

▲ **CRITICAL STEP** The age of the cells may effect protaminization. Frozen cells should not be directly applied to protaminization. Thus, the cells should be passaged at least once before protaminization.

Protaminization of somatic cells ● **TIMING 48 h**

▲ **CRITICAL** All medium, solution and reagent volumes are given for a 60-mm culture dish. To protaminize cells in different tissue culture formats, follow **Table 3** for guidance on using different amounts of transfection complex and medium.

20| Prepare the following solutions and reagents in 15-ml conical tubes: 5 ml of D-PBS, 5 ml of Opti-MEM (or, alternatively, serum-free medium can be used), and 5 ml of MEM containing 10% (vol/vol) FBS with 50 nM TSA. Solutions should be prewarmed in a water bath set to 37°C . In addition, Lipofectamine 2000 and 3 μg of Prm1-GFP plasmid will be needed.

21| One hour before protaminization, take the cells grown to 80–90% confluence in a 60-mm culture dish, aspirate the culture medium from the cells, and wash them once with D-PBS. Add 500 μl of Opti-MEM medium.

▲ **CRITICAL STEP** 100% confluent cells are best for SCNT^{33–35}, but they are not optimal for the protaminization procedure. For protaminization of somatic cells that are going to be used for SCNT, we suggest blocking cells at the G0 stage by culturing them in MEM medium containing 0.1% (vol/vol) FBS for 24–36 h before protaminization.

22| Prepare solution A (lipid complex) in a 1.8-ml microcentrifuge tube by adding 7 μl of Lipofectamine 2000 to 250 μl of Opti-MEM.

23| Mix gently by pipetting up and down, and incubate at RT for 10 min. Proceed with the next step during the incubation time.

▲ **CRITICAL STEP** Do not incubate for longer than 20 min.

24| During the incubation time, prepare solution B (DNA complex) in a 1.8-ml microcentrifuge tube by diluting 3 μg of Prm1-GFP plasmid (or GFP plasmid only, empty vector or alternatively the same volume of Opti-MEM medium, for transfection control) in 250 μl of Opti-MEM medium. Incubate the mixture for 5 min at RT.

▲ **CRITICAL STEP** The DNA/Opti-MEM solution is more stable and can be prepared up to 4 h in advance.

▲ **CRITICAL STEP** The supplier of Lipofectamine 2000 claims that serum may be used during transfection, but our personal experience has shown a higher protein expression when transfection is done in the absence of serum.

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TABLE 3 | Transfection conditions in different culture dishes.

Components	12-well	6-well	35-mm	60-mm	90-mm
Prm1-GFP	0.5 µg	1 µg	1.5 µg	3 µg	4 µg
Lipofectamine	1 µl	3 µl	5 µl	7 µl	9 µl
Q68 Opti-MEM in Eppendorf(s)	50 µl	90 µl	125 µl	250 µl	500 µl
Opti-MEM in culture dish	100 µl	180 µl	250 µl	500 µl	1000 µl

25 | Spin both solution A (lipid complex) and solution B (DNA complex) briefly and add solution B (DNA complex) to solution A (lipid complex) to make a total volume of 500 µl. Mix by pipetting up and down 3–4 times, flicking the bottom of the tube or briefly vortexing. Incubate solution AB (DNA/lipid complex) for 30 min at RT.

26 | After incubation, briefly spin solution AB and add it dropwise to the culture dish containing the cells and Opti-MEM medium. Gently rock the plate after adding. Avoid forceful dispensing of solution AB into the culture dish, because it may displace cells.

! CAUTION Do not incubate for more than 4 h because the DNA/lipid complex may be toxic to the cells.

▲ CRITICAL STEP Before transferring the culture dishes to the incubator, mix to ensure that solution AB (DNA/lipid complex) is well distributed. It is important to have uniform transfection. Incubate the cells with DNA/lipid complex for 3.5/4 h in an incubator set to 5% CO₂ and temperature adapted for the animal species used.

27 | After the incubation time, aspirate the medium containing solution AB and add 5 ml of MEM containing 10% (vol/vol) FBS and 50 nM TSA. Incubate the mixture for 16–20 h in a CO₂ incubator set to 5% CO₂ and temperature adapted for the animal species used. The next morning, aspirate the culture medium containing 50 nM TSA and wash it once with D-PBS. Add 3 ml of fresh MEM containing 10% (vol/vol) FBS and culture.

▲ CRITICAL STEP 16 h after transfection, some cells may already be protaminized. When cells are fully protaminized, they detach from the surface and float in the medium. Do not discard the supernatant because the highest percentages of fully protaminized cells are detached and floating in culture medium. Transfer the supernatant to the 15-ml conical tube, spin it at 420g for 5 min, **Q41** and discard the supernatant. Resuspend the pelleted cells in fresh culture medium and transfer the mixture to the same culture dish or use for analysis.

? TROUBLESHOOTING

Evaluation of protaminization of somatic nuclei

28 | To evaluate cell protaminization, visualize positively transfected cells under a fluorescence/confocal microscope. You can do that by examining growing cells (option A) or fixed cells that are still attached to the culture surface (10–20 h post protaminization) (option B) or fixed cells that have already detached from the culture dish (~25–48 h post protaminization) (option C).

(A) Visualization of protaminization using growing cells ● TIMING 2 h

- Place the culture dish with cells 16–48 h post protaminization under a fluorescence microscope, and observe the cells using a green filter (FITC) if protamine is tagged with GFP.

(B) Visualization of protaminization in cells attached to the dish surface ● TIMING 2–3 h

▲ CRITICAL To perform this analysis, protaminized cells must be grown in 4-well chamber slides.

- Prepare the following chemical agents in individual 15-ml conical tubes: 15 ml of D-PBS, 2 ml of 4% (wt/vol) PFA and mounting medium (with DAPI or PI). Also prepare cover glasses.
- Aspirate the culture medium from every well of the chamber slide and add 1 ml of D-PBS. Wash the slide by gently turning it to the left and right 2–3 times. Discard D-PBS and add 500 µl of 4% (wt/vol) PFA slowly into each well of the slide chamber with protaminized cells and incubate for 20 min at RT.
- After incubation, gently aspirate the PFA and wash the slide with 1 ml of D-PBS for 5 min. Repeat this washing procedure 3–4 times, each time with 1 ml of fresh D-PBS.
- Aspirate the D-PBS gently and mount the slide with mounting medium and a cover glass. **Q42** Observe the protaminized somatic cells under a fluorescence/confocal microscope (**Fig. 7**).

(C) Visualization of protaminization in cells detached from the dish surface ● TIMING 2–3 h

▲ CRITICAL STEP Protaminized cells grown in a 60-mm dish can be used in this option.

- Prepare the following chemical agents in individual 15-ml conical tubes: 15 ml of D-PBS, 2 ml of 4% (wt/vol) PFA and mounting medium (with DAPI or PI); also prepare poly L-lysine-coated glass slides and cover glasses.

PROTOCOL

- (ii) Aspirate the culture medium from the 60-mm culture dish, transfer it to a 15-ml conical tube and centrifuge at 420g for 5 min. Discard the supernatant and add 5 ml of D-PBS to the protaminized pelleted cells. Wash by pipetting up and down twice, and centrifuge the tube at 420g for 5 min.
- (iii) Aspirate the D-PBS and add 2 ml of 4% (wt/vol) PFA slowly to the 15-ml conical tube with the protaminized cells. Suspend the cells and incubate them for 20 min at RT. Centrifuge the tube at 420g for 5 min.
- (iv) After centrifugation, aspirate the PFA gently and wash it with 1 ml of D-PBS for 5 min. Repeat this washing procedure 3–4 times, each time with fresh D-PBS (1 ml). After the last wash, resuspend the pellet in a very small volume (~10–20 μ l) of D-PBS and drop the cells onto a poly L-lysine-coated glass slide.
- (v) Wait for 2–3 min for the cells to attach to the glass surface, and then add a small amount of mounting medium. Cover the cells very gently with a cover glass and observe the protaminized somatic cells under a fluorescence or confocal microscope.

? TROUBLESHOOTING

Picking protaminized somatic cells

29 | For chromatin remodeling, protamine/histone footprints or molecular study, follow option A to isolate protaminized cells with a FACS. If you are proceeding to SCNT, follow option B for manual sorting.

(A) Isolation of protaminized cells by FACS **● TIMING 5–7 h**

- (i) Prepare the following chemical reagents individually in 15-ml conical tubes: 2 \times 10 ml of D-PBS, for washing cells in culture and for washing cells after centrifugation; 1 ml of trypsin-EDTA solution, for blocking the trypsin reaction; 8 ml of MEM containing 10% (vol/vol) FBS or alternatively 1 ml of FBS. Reagents should be warmed in a water bath set at 37 °C.
- (ii) Aspirate the culture medium with floating protaminized cells, and transfer it to a 15-ml conical tube. For cells attached to a dish surface, proceed as described in Steps 6–8.
- Q45** (iii) Centrifuge the tube containing protaminized cells at 420g for 5 min, discard the supernatant, and suspend the cells in FACS buffer or D-PBS at $<1.0 \times 10^6$ cells per 100 μ l. Pass the cells through the 70- μ m nylon mesh filter to avoid cell cluster formation. Turn on and set up the FACS machine.
- (iv) Place the tube containing the sample cells and two new 10-ml tubes for collection of the sorted cells in the FACS machine.
- Q46** (v) Determine the protamine cell fraction using control samples, and then sort for protamine cells using the FACS machine. Excite the GFP with an argon laser (488 nm) and detect protaminized cells (positive for GFP) in two channels: P2—‘dim’, having average expression of GFP ($\sim 10^3$) (cells in the first step of protaminization, not fully protaminized); and P3—‘bright’, having high expression of GFP ($\sim 10^4$ – 10^5), for fully protaminized cells.
- Q47** (vi) Centrifuge the 10-ml tubes containing the sorted cells (dim and bright populations separately) at 420g for 5 min, and resuspend the cells in freezing medium (20% DMSO in MEM medium containing 20% (vol/vol) FBS). Freeze the protaminized cells in two separate cryo-vials, as described in Steps 11–13, for further analysis.

! CAUTION Note that selection of protaminized cells using FACS is recommended when cells are to be used for molecular analysis (such as footprint, whole-genome analysis or DNA methylation).

(B) Manual sorting for SCNT **● TIMING 1 h**

▲ CRITICAL STEP This option relies on the operator’s experience. The operator needs to observe many transfected cells to become familiar with the proper shape of fully protaminized cells. Before using protaminized cells for SCNT, we suggest performing preliminary experiments to become familiar with the shape of fully protaminized somatic nuclei.

▲ CRITICAL STEP If you are manually sorting cells for use in SCNT, these steps should be carried out after Steps 30–46 and during Step 47.

- (i) Use a light microscope to choose protaminized cells (**Fig. 8a**; **Fig. 1**). The cells that perform best in SCNT development are those that are fully protaminized (**Fig. 3g–o**) 40–48 h post protaminization. Identify protaminized cells by observing the nuclear shape, which is drastically elongated and looks like a spermatid nucleus (**Figs. 1** and **8b**).

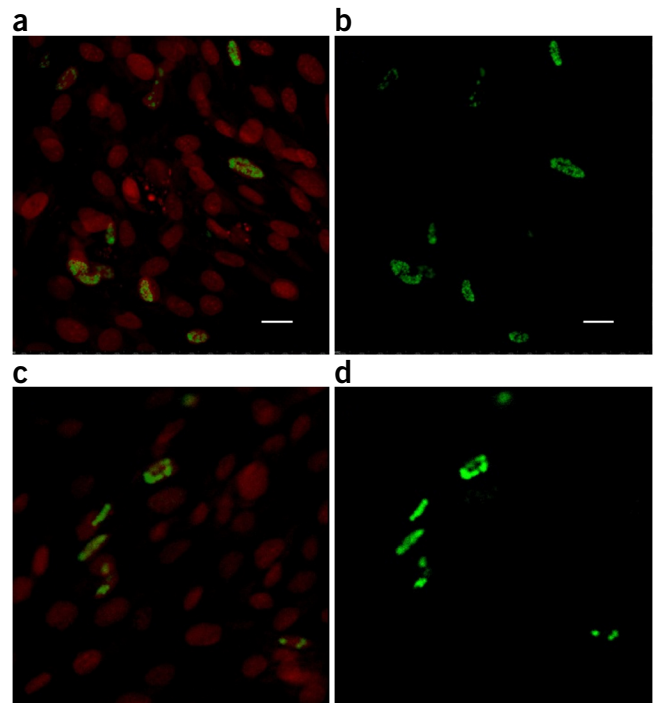


Figure 7 | Visualization of protaminized nuclei of fixed somatic cells. (a,b) Cells growing on the cell surface observed under a fluorescence microscope 24 h post protaminization. (c,d) Somatic cells 30–40 h post protaminization. Green: somatic cells expressing Prm1-GFP; red: nuclei of somatic cells stained with PI. (a,c) Merge. (b,d) Protaminized somatic cells. Scale bars, 5 μ m.

- (ii) Use selected protaminized cells directly for SCNT. In addition, cells can be selected and frozen (Steps 11–13), but using cells that have undergone multiple freeze–thaw cycles for SCNT may affect embryo viability.

Q49

IVM of sheep oocytes ● TIMING 24 h

▲ **CRITICAL** The procedure from this point on can only be carried out using sheep cells.

30 Prepare the following reagents in 15-ml conical tubes: 10 ml of H-199 medium, 10 ml of H-199 medium containing 0.005% (wt/vol) heparin and IVM medium (see Reagent Setup). Warm and equilibrate all solutions in an incubator set to 38.5 °C, with a humidified atmosphere of 5% CO₂, at least one hour before use.

31 Collect sheep ovaries from local slaughterhouses and transfer the ovaries to the laboratory within 1–2 h at 37–38 °C.

32 Aspirate 500 µl of H-199 medium containing 0.005% (wt/vol) heparin into a sterile 5-ml syringe and aspirate the follicular fluid within oocytes from 3–6 mm antral follicles using a 21-gauge needle. Using a stereomicroscope at ×15–×20 magnification, select oocytes surrounded by at least two layers of granulosa cells, with evenly granulated cytoplasm (**Fig. 9a,b**).

Q50

Q51

33 Wash oocytes once in 2 ml of H-199 medium and twice in IVM medium to equilibrate. Transfer up to 30 oocytes per well to a 4-well Nunc dish containing 500 µl of IVM medium. Incubate the dish with the oocytes in a humidified atmosphere of 5% CO₂ at 38.5 °C for 22 h.

Preparation of sheep oocytes for SCNT ● TIMING 30 min

34 After 22 h of IVM, select oocytes with expanded and uniform cumulus (**Fig. 9c,d**). Remove granulosa cells by adding H-199 with 0.3 mg/ml hyaluronidase to the oocytes and gently pipetting to allow the hyaluronidase to digest the intercellular matrix.

▲ **CRITICAL STEP** Do not incubate the oocytes for more than 2 min in hyaluronidase in order to avoid their self-activation.

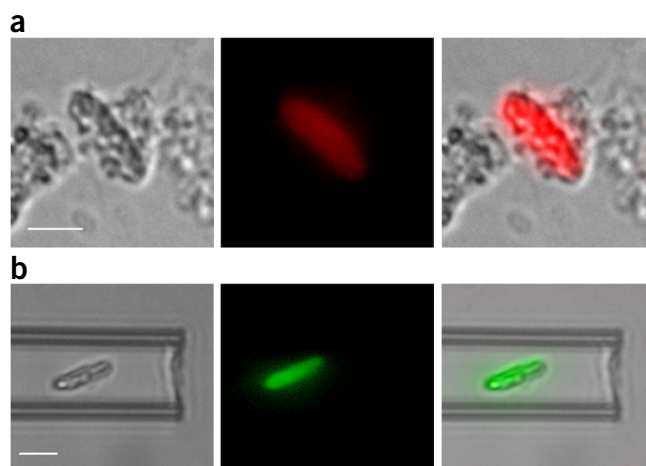
35 Wash the oocytes by pipetting them in H-199 medium 4–6 times, and incubate them in H-199 medium containing 5 µg/ml Hoechst 333412 for 10 min. Then, wash the oocytes once in H-199 medium and place the first group of oocytes in H199 medium drops in the enucleation dish (1 oocyte/drop) (**Fig. 5a**), and wait for ~3–5 min before beginning enucleation (to allow CB to work).

Q52

Enucleation of sheep oocytes ● TIMING 1–1.5 h

36 Hold the oocyte with the holding pipette and use the enucleation needle to orientate the oocyte so that its metaphase plate (MII plate) and polar body are located between 9 and 11 o'clock (**Fig. 10a**, arrow points to MII plate). Bring the oocyte's plasma membrane into sharp focus. Use the fine z-axis control to move the micro-tools up and down so that the ends of the enucleation and holding pipettes are in focus.

▲ **CRITICAL STEP** Accurate focus is crucial for all the micromanipulation steps.



Q65

Figure 8 | Fully protaminized somatic nucleus ideal for nuclear transfer (NT). (a) Sheep somatic cell transfected with hPrm1-RFP. (b) Sheep somatic cell transfected with mPrm1-GFP and aspirated by injection micropipette. Scale bars, 5 µm.

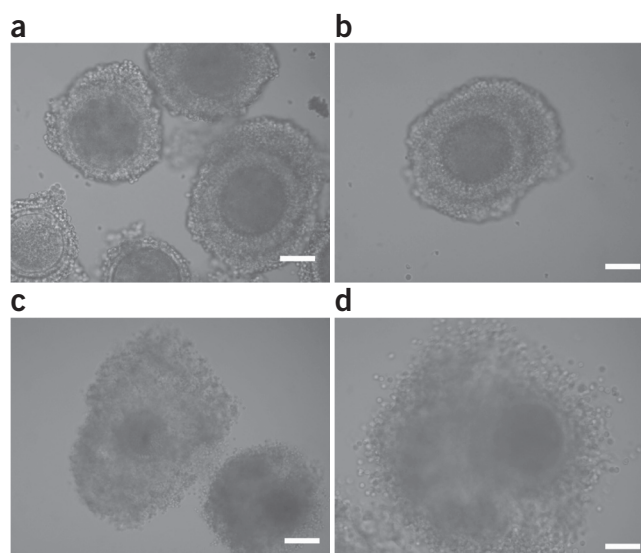


Figure 9 | Microscopy images of sheep oocytes. Representative microscopy images of sheep oocytes before (a,b) and after (c,d) *in vitro* maturation. Scale bars, 50 µm (a,b,d); 80 µm (c).

Q76

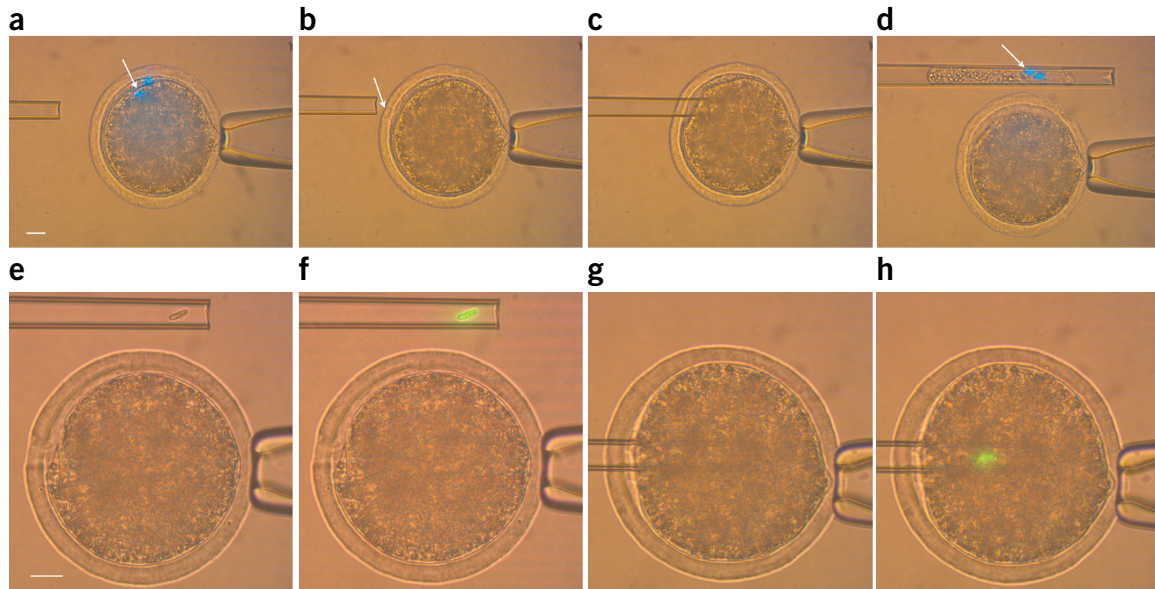


Figure 10 | Enucleation of sheep oocytes and injection of protaminized somatic nucleus using the Piezo unit. **(a)** The oocyte is rotated, and the metaphase II spindle is located and placed between the 9 o'clock and 11 o'clock positions (arrow). **(b)** The zona pellucida is cut off by applying a piezo pulse (arrow). **(c)** The spindle is removed by suction without breaking the plasma membrane. **(d)** The pipette is gently pulled away from the oocyte, allowing the experimenter to check for proper enucleation (arrow). **(e)** The protaminized cell/nucleus is aspirated into the injection micropipette. Donor nuclei are gently aspirated in and out of the injection pipette until the nuclei are largely devoid of visible cytoplasmic material. **(f)** Somatic nucleus expressing mPrm1-GFP. **(g)** The injection pipette is inserted into the enucleated oocyte, a single piezo pulse is applied to break the membrane, and the donor nucleus is injected immediately. **(h)** Finally, the experimenter checks that the donor nucleus was injected properly. Scale bars, 20 μm . Isolation of sheep oocytes for the experiments was carried out according to national and institutional regulations (DPR 27/1/1992, Animal Protection Regulations of Italy) in concordance with European Community regulation 86/609 and were approved by CEISA (Inter-Institutional Ethics Committee for Animal Experimentation) Prot. 79/2013/CEISA Prog. 58.

37 | Apply gentle suction with the holding pipette. Apply piezo pulses to cut the zona pellucida (ZP) (**Fig. 10b**, arrow). To avoid damaging the oocyte, ensure that there is a large space between the ZP and the oolemma.

? TROUBLESHOOTING

38 | The tip should rapidly but gently pass through the ZP without breaking the oolemma. Gently increase positive pressure in the enucleation pipette and aspirate a very small amount of the cytoplasm below the polar body (**Fig. 10c**) without breaking the oolemma. Remove the MII spindle by aspiration with a minimal volume of cytoplasm. The oocyte membrane and spindle must be pinched off slowly; do not apply piezo pulses to cut the membrane.

39 | Withdraw the enucleation pipette smoothly from the oocyte, and release the removed MII plate into the same drop (**Fig. 10d**). Try to remove as little cytoplasm as possible with the MII plate.

? TROUBLESHOOTING

40 | Release the enucleated oocyte by gently applying positive pressure within the holding pipette. Repeat the procedure with the other oocytes (Steps 36–39). When all oocytes are enucleated, transfer them from dish A (**Fig. 5a**) to the same drop order in dish B, containing only H-199 medium (**Fig. 5b**), and incubate in a 5% CO_2 incubator at 38.5 $^{\circ}\text{C}$ for 20–30 min before starting injection of the protaminized cells.

! CAUTION Be careful to avoid aspirating the metaphase II plate removed from the oocyte.

Q53

41 | Place dish A (**Fig. 5a**) with released enucleated cytoplasm with MII under UV light to verify that the enucleation step has been performed successfully. Repeat the enucleation Steps 36–41 with oocytes that were not enucleated correctly.

42 | Transfer the second group of oocytes to the enucleation chamber, and repeat the entire enucleation procedure with the rest of the oocytes (Steps 36–41).

▲ CRITICAL STEP The entire enucleation step should not take longer than 1 to 1.5 h.

Preparation of protaminized donor cells ● **TIMING** 20–30 min

▲ **CRITICAL** Collect the protaminized cells immediately before nuclear transplantation.

43| Transfer the supernatant from the dish with the protaminized cells to a 15-ml conical tube (fully protaminized cells detach from dish surface and float in the supernatant).

44| Proceed as described in Steps 6–8 to passage the remaining cells that are attached to the dish surface. Discard the supernatant and suspend the cells in 200–500 μ l (depending on cell numbers) of MEM containing 10% (vol/vol) FBS.

Donor cell injection ● **TIMING** 1–2 h

45| Place 1–2 μ l of harvested protaminized donor cells into PVP drops (**Fig. 5c**, drop 3) in the injection chamber. Mix gently until cells are mixed with the solution.

▲ **CRITICAL STEP** PVP is essential to prevent the donor cells from sticking to the microinjection pipette.

46| Place the enucleated oocytes (10 oocytes/drop) into H199 medium in the injection chamber (**Fig. 5c**, drop 1). The number of oocytes depends on the operator's skill level. Each group of oocytes should be injected within 15–20 min.

47| Using the procedures described in Step 29B (manual sorting for SCNT), choose protaminized cells and use them directly for donor cell injection.

48| Bring the cells into sharp focus and draw the fully protaminized cell (**Fig. 10e**) into the microinjection pipette. Although the cell is in the injection pipette, apply several pulses with the piezo unit foot pedal until the cellular membrane breaks (**Fig. 10e,f**). Alternatively, aspirate the cells gently in and out of the injection pipette until the nucleus is clearly separate from any visible cytoplasmic material.

▲ **CRITICAL STEP** The number of donor cells to be loaded into the injection pipette depends on the operator's skill. With experience, multiple cells (3–5 cells) can be accumulated within a pipette at ~100- μ m intervals.

? **TROUBLESHOOTING**

49| Apply gentle suction within the holding pipette to hold the enucleated oocyte. Drill the ZP using piezo pulses, or, alternatively, use the previous hole opened during enucleation. Reduce the power level of the piezo unit (power level 1–2 and speed 1).

▲ **CRITICAL STEP** The power level of the piezo unit must be reduced before continuing to the next step because the oolemma is more fragile than the ZP.

50| Push one nucleus forward until it is near the tip of the pipette. Apply a piezo pulse to break the oolemma (indicated by a rapid relaxation of the oocyte membrane) (**Fig. 10g**). By application of low positive pressure in the microinjection pipette, deposit the donor nucleus into the cytoplasm of the enucleated oocyte. Introduce a minimal amount of extraneous medium and do not suck cytoplasm into the pipette (**Fig. 10h**).

▲ **CRITICAL STEP** Try to inject as little PVP solution as possible. PVP may negatively affect oocyte survival and further embryo development.

? **TROUBLESHOOTING**

51| Withdraw the injection pipette from the oocyte very gently. Release the reconstructed oocytes by gently applying positive pressure within the holding pipette. Repeat the procedure with other enucleated oocytes (Steps 46–51).

52| After reconstruction of 3–5 oocytes, move the injection micropipette to drop 3 (**Fig. 5c**) and wash the injection pipette with PVP solution. Wash the injection pipette twice by aspirating PVP solution up and down and by expelling some mercury and applying power from the piezo unit.

▲ **CRITICAL STEP** This washing step is essential to avoid donor nuclei becoming stuck in the injection pipette.

53| After injection of the first group of oocytes, transfer 1–2 μ l of fresh protaminized cells to a new PVP drop and continue the injections using fresh cells.

54| Place the second group of oocytes into the injection chamber (drop 1, **Fig. 5c**) and repeat the entire procedure with the remaining oocytes (Steps 46–52). When all oocytes are reconstructed with protaminized nuclei, incubate them in a 5% CO₂ incubator at 38.5 °C for 30–40 min before activation.

PROTOCOL

Activation of sheep oocytes reconstructed with protaminized nuclei ● TIMING 5 h

55| Incubate reconstructed oocytes with protaminized nuclei for 5 min with 5 μM ionomycin in H-199 medium containing 5 μM ionomycin. Wash the oocytes by pipetting them in H-199 medium 4–6 times. Repeat this washing procedure 3–4 times, each time in a fresh droplet of H-199 medium.

56| Incubate the oocytes with SOF– medium containing 2mM 6-DMAP and 7.5 μg/ml CB for 3–5 h.

Assessment of *in vitro* embryonic development of the NT sheep embryos ● TIMING 7–8 d

57| Wash activated oocytes twice in SOF– medium for 5 min to completely remove the CB.

58| Transfer a group of 5 potential zygotes in a drop (20 μl) of SOF– medium (day 0) and incubate in a humidified atmosphere in 5% CO₂, 7% O₂ and 88% N₂ at 38.5 °C for 48 h.

59| On day 3, refresh the medium under sterile conditions using a stereomicroscope with a warm surface to maintain the temperature of the zygotes. Remove 10 μl of medium from each drop, and add 10 μl of SOF+ medium 1 (day 3) (see Reagent Setup) and incubate in a humidified atmosphere in 5% CO₂, 7% O₂ and 88% N₂ at 38.5 °C for another 48 h.

60| On day 5, refresh the medium under sterile conditions using a stereomicroscope with a warm surface to maintain the temperature of the zygotes. Remove 10 μl of medium from each drop and add 10 μl of SOF+ medium 2 (day 5) (see Reagent Setup) and incubate in a humidified atmosphere in 5% CO₂, 7% O₂ and 88% N₂ at 38.5 °C until day 6 of culture.

61| On day 6, transfer morula- and blastocyst-stage embryos from 20 μl of day 5 medium to a 4-well Nunc dish containing 500 μl of blastocyst medium (see Reagent Setup) and culture for an additional 24–48h.

? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 4.

TABLE 4 | Troubleshooting table.

Step	Problem	Possible reason	Solution
Q69	4	No proliferation of cells after 2–3 days of culture	Tissue was not sufficiently attached to the culture dish Incubate the tissue for 1.5 h before adding the culture medium. This allows attachment of tissue and further cell proliferation Alternatively, add 15% FBS instead of 10% (vol/vol) to culture medium to increase proliferation
	10	Cells are growing slowly	Seeding density is too low Cell-to-cell contact is required for efficient growth. If initial seeding density falls below 20%, the growth rate may be compromised. Try lowering the split ratio to increase the cell concentration
	27	Toxicity after overnight protaminization	Transfection conditions and/or TSA treatment are toxic for cells The incubation time with the DNA/lipid complex should not exceed 4 h. Reduce incubation time with the DNA/lipid complexes Perform optimization by testing various concentrations of TSA to determine an acceptable range Supplement the transfection medium with 1% (vol/vol) FBS
	Low transfection efficiency	The transfection protocol is not optimized Cells were passaged too many times	The following factors can be optimized: cell density at the time of transfection, the ratio of DNA to transfection reagent, time of incubation with the transfection complex Fibroblasts (at passages higher than 4) are less metabolically active and start becoming senescent. Obtain a new batch of cells or use a lower passage number

Q70

(continued)

TABLE 4 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
28C(v)	No protaminized cells are observed	Cells may have been aspirated during the procedure	Visualization of protaminized cells in suspension requires a high starting number of cells because many cells are lost during the procedure steps. If too many have been lost, prepare cells in 2 to 3 different dishes and collect cells for one experiment. Be careful during every step of the procedure
37	Cannot cut the zona pellucida	The pipette tip may not be in full contact because the tip surface is sharp PiezoXpert parameters are not set up correctly (too low) Pressure inside the pipette	Change the enucleation needle for one with a smoother tip Increase PiezoXpert pulse parameters To enhance the piezo power, there should be a slight negative pressure inside the enucleation pipette
39	Oocytes lyse during the enucleation step	The enucleation micropipette is too big or too sharp	Prepare a smaller micropipette
48	It is difficult to pick up donor cell nuclei	The donor cell is incompletely mixed with PVP solution	When placing donor cells into PVP, mix the solution completely. Somatic cells are fragile in PVP medium. Make a new drop every 20 min
50	It is difficult to release nuclei from the pipette	Dirty injection micropipette	The injection pipette must be washed frequently using PVP solution by expelling several drops of Hg ⁰ and simultaneously applying few piezo pulses or changing the needle
61	No embryo development occurs	Culture conditions are not adequate	Ensure that <i>in vitro</i> culture conditions are optimal, because cloned embryos are very sensitive to any contaminant or toxin in the medium. Therefore, inspect the media and their supplements, and replace the mineral oil Check the osmolarity of all <i>in vitro</i> culture media and slowly adjust to 280 mOsm/kg Check the quality of all media and reagents for embryo production and culture on fertilized or parthenogenetic embryos

Q71

● TIMING

Steps 1–13, preparation of primary cell line from adult or embryonic tissues: 2–3 weeks

Steps 14–19, preparation of adult skin fibroblasts: 1–2 h

Steps 20–27, protaminization of somatic cells: 48 h

Step 28A(i), visualization of protaminization using growing cells: 2 h

Step 28B(i–iv), visualization of protaminization in cells attached to the dish surface: 2–3 h

Step 28C(i–v), visualization of protaminization in cells detached from the dish surface: 2–3 h

Step 29A(i–vi), isolation of protaminized cells by FACS: 5–7 h

Step 29B(i–ii), Manual sorting for SCNT: 1 h

Steps 30–33, IVM of sheep oocytes: 24 h

Steps 34 and 35, preparation sheep oocytes for SCNT: 30 min

Steps 36–42, enucleation of sheep oocytes: 1–1.5 h

Steps 43 and 44, preparation of donor cells: 20–30 min

Steps 45–54, donor cell injection: 1–2 h

Steps 55 and 56, activation of sheep oocytes reconstructed with protaminized nuclei: 5 h

Steps 57–61, assessment of *in vitro* embryonic development of the NT sheep embryos: 7–8 d

Q54

ANTICIPATED RESULTS

The protocol converts interphase nuclei from somatic cells into spermatid-like structures. Protaminization of the nucleus can be visualized incrementally over time beginning with a few focal points within the nucleus, starting 10–15 h post pro-

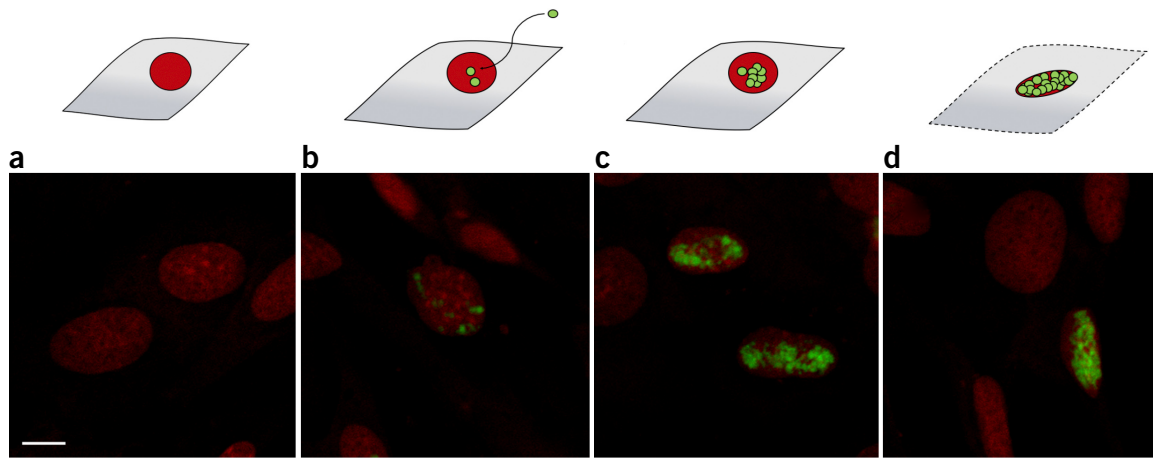


Figure 11 | Gradual protamine incorporation into the somatic nucleus. **(a)** Somatic nuclei before protaminization. **(b)** Somatic nuclei 10 h post protaminization. **(c)** Protamine foci in somatic nuclei 24 h post protaminization. **(d)** Fully protaminized somatic nuclei 48 h post protaminization. Top pictures present schematics of protamine incorporation. In the bottom panels, green represents somatic cells expressing Prm1-GFP; red represents nuclei stained with propidium iodide (PI). Scale bar, 5 μm .

taminization (**Fig. 11**), with the number of foci increasing and scattering throughout the entire nucleus 20 h later (**Fig. 11c**). Finally, a radical nuclear remodeling is achieved within 48 h, with the protaminized nuclei acquiring an elongated structure (**Figs. 1** and **11d**). This pattern has been confirmed in fibroblasts from the following animal models: sheep (**Fig. 1a,b**) and mouse (**Fig. 1c,d**) using human (**Fig. 1c**) or mouse (**Fig. 1b,c**) Prm1.

The size of the nucleus of a protaminized cell is larger than that of a spermatid, because the nucleus contains diploid DNA (**Fig. 12**). A fully protaminized, toroidal nucleus of a spermatozoon viewed from the front and the side looks exactly the same as a protaminized somatic nucleus (**Fig. 12**). **Figure 12** shows the similarity between protaminized and spermatid nuclei as compared with a nontransfected somatic nucleus.

10–20 h after protaminization, most of the cells are still attached to the dish culture surface, with some protamine spots in the nuclei (**Fig. 3a–c**). At this point, it is impossible to recognize protaminized cells (arrow, **Fig. 3a**) without a fluorescence microscope. They do not differ from control (nonprotaminized) cells.

With time, somatic cells with protaminized nuclei start to detach from the surface (**Fig. 3d–f**); in this phase, a protaminized cell can be clearly identified without using fluorescence exposure (**Fig. 3d**, arrow). Under a fluorescence microscope, the elongated nuclei with more compact spots of protamine inside are visible (**Fig. 3e,f**).

Around 30 h post protaminization, most of the somatic cells expressing Prm1 detach from the dish culture surface and float in the culture medium (**Fig. 3g**, arrow). Protaminized somatic nuclei are elongated, sometimes bent, and strongly compact (**Fig. 3h–i**).

Somatic cells radically change their nuclear shape 40 h post transfection (**Fig. 3j–l**). The nuclei of somatic cells elongate into spermatid-like structures (**Fig. 3j**, arrow), and they are easily recognizable without fluorescence at this stage. Full protaminization of somatic nuclei are observed at 48 h post transfection, and this is accompanied by the loss of cellular membrane (**Fig. 3m**, arrow). In this step, the somatic nucleus is strongly elongated and looks like a nucleus from a spermatozoon (**Figs. 1** and **3m–o**, **7d**, **8** and **12**).

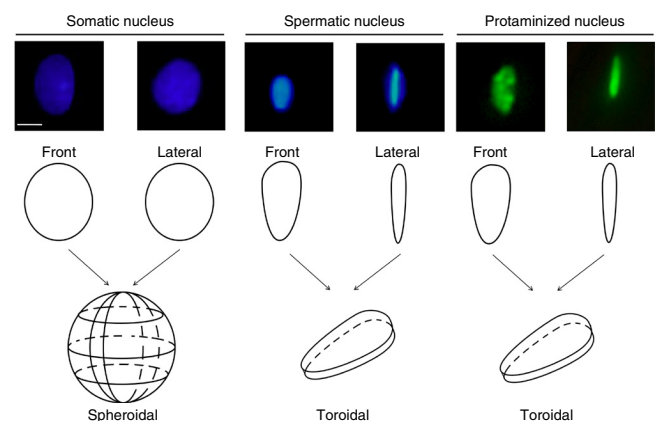


Figure 12 | Photographic (top) and schematic (bottom) representation of overlapping of spermatid and protaminized nuclei. Scale bar, 5 μm .

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Q74 **COMPETING FINANCIAL INTERESTS** The authors declare no competing financial interests.

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EDITORIAL SUMMARY

AOP: This protocol describes how to remodel sheep and mouse fibroblast nuclei into spermatid-like structures by exogenous expression of the protamine 1 gene. Blastocyst formation following somatic cell nuclear transfer in sheep oocytes is also described.

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AQ1	Please carefully check the spelling and numbering of all author names and affiliations.
AQ2	Per journal style, genes are shown in italics and proteins are shown in regular type; please check throughout the paper and confirm that this has been done.
AQ3	Reference [12] is not cited in the text. Please cite between first citations of refs. 11 and 13, or delete the reference and renumber subsequent references.
AQ4	Please check the changes made to the sentence 'Finally, protamine binds with ...'
AQ5	Per journal style, we reserve use of "significantly" for mentions of statistical significance. If you are referring to statistical significance here, please provide a <i>P</i> value. If not, please revise to "substantially," etc. Also, please check the changes made to the sentence.
AQ6	Please check the changes made to the sentence "Expression vectors for the bromo..."
AQ7	Please confirm that the edits to the sentence 'We therefore opted for.....' preserve the originally intended meaning.
AQ8	Please confirm whether the change of 'protamine 1 or 2' to 'Prm1 or 2' throughout the article to maintain consistency is ok.
AQ9	Please check the changes made to the sentence "Given the lack of a complete..."
AQ10	Can "the sperm at the chromatin level" be changed to "naturally occurring sperm" or something similar? "at the chromatin level" appears to be redundant.
AQ11	Please check the changes made to the sentence "It is recommended that the optimal..."
AQ12	Should "does not contain GFP (null) and Prm1" be "does not contain GFP (null) OR Prm1"? If not, change to "with Prm1 and pDNA that does not contain GFP (null)"

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AQ14	Please check the changes made to the BME Amino Acids Solution entry per the Sigma-Aldrich website.
AQ15	Please provide the supplier for 25-ml Falcon serological disposable pipette.
AQ16	Please check the changes made to the “Fluoroshield with PI” entry.
AQ17	Please check the changes made to the entry beginning “Heated work stage...”
AQ18	Please note that “to obtain MEM” was changed to “to obtain cell culture medium”. Please check all occurrences of “MEM” to see if they need to be changed to “cell culture medium”.
AQ19	Is the change from “eventually adjust it” to “slowly adjust it” OK?
AQ20	Please spell out “LH” here.
AQ21	Please specify what temperature the medium should be warmed to.
AQ22	Please check the changes made to the “Medium for donor cell injection” entry and provide the temperature to which the medium should be warmed.
AQ23	Please specify how much pure ethanol should be used. Also, please specify how long the solution can be stored.
AQ24	Please note that the 6-Dimethylaminopurine entry was moved to after the SOF entry because SOF was listed as a component.

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AQ25	Please confirm that the edits to the sentence 'Add 5 ml of...' preserve the originally intended meaning.
AQ26	Please confirm that the edits to the sentence 'To prepare 10 ml.....' preserve the originally intended meaning.
AQ27	Should "0.1% (vol/vol) gelatin" be "0.1% (wt/vol) gelatin" as it is in all other instances? Please check.
AQ28	Please confirm that the edits to the sentence 'There is no need.....' preserve the originally intended meaning.
AQ29	Per journal style, figure panels should be cited in alphabetical order or figures should be called out in their entirety at first mention. Please cite the Figure 4 panels in alphabetical order or cite the figure as a whole at first mention.
AQ30	Should "until the cut end does not disappear from view" be "until the cut end almost disappears from view"? If not, please reword.
AQ31	Can "until 50–60% of the capillary diameter end rounds up" be changed to "until the capillary diameter shrinks to 50–60% of its original size"?
AQ32	By "place four drops of 10 µl of PVP", do you mean "place four 10-µl drops of PVP" or "place four drops (totaling 10 µl) of PVP"?
AQ33	Please check the changes made to the paragraph beginning "To enucleate sheep oocytes without..."
AQ34	Please check the changes made to the paragraph beginning "Place two to three 20-µl drops..."
AQ35	Please provide the exact temperature of centrifugation in Steps 2 and 7.
AQ36	Should "MEM containing 10% (wt/vol)" be "MEM containing 10% VOL/vol)", as in all other instances?
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AQ38	Please provide the exact temperature of centrifugation for Steps 11 and 16.
AQ39	Please provide the exact temperature for centrifugation.
AQ40	Can “The DNA/Opti-MEM solution is more stable” be changed to “Solution B is more stable than solution A”?
AQ41	Please provide the exact temperature of spin for Step 27.
AQ42	Please confirm that the edits to the sentence ‘Aspirate the D-PBS.....’ preserve the originally intended meaning.
AQ43	Please provide the exact temperature of centrifugation for Step 28C(ii) and Step 28C(iii).
AQ44	Please check the changes made to Step iv.
AQ45	Please provide the exact temperature of centrifugation for Step 29A(iii) and Step 29A(vi).
AQ46	Are units needed with “ $\sim 10^3$ ” and “ $\sim 10^4$ – 10^5 ”?
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AQ48	Please confirm that the edits to the sentence ‘If you are manually.....’ preserve the originally intended meaning.
AQ49	Please confirm that the edits to the sentence ‘In addition, ...’ preserve the originally intended meaning.
AQ50	In “aspirate the follicular fluid within oocytes from 3–6 mm antral follicles”, do you mean “aspirate the follicular fluid containing oocytes from 3–6 mm antral follicles”? Please clarify.
AQ51	Please check whether the change of ‘granulose’ to ‘granulosa’ is correct in this sentence.

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AQ52	Please check the text “to allow CB to work”: CB is not mentioned in this step.
AQ53	Please confirm that the edits to the sentence ‘Be careful.....’ preserve the originally intended meaning.
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AQ55	Please cite Figure 11 as a whole at first mention or cite the figure panels in alphabetical order.
AQ56	Please confirm that the edits to the sentence ‘10–20 h after.....’ preserve the originally intended meaning. Also, is “some protamine spots in the nuclei” correct?
AQ57	Please check the changes made to the Acknowledgments.
AQ58	Please check that all funders have been appropriately acknowledged and that all grant numbers are correct.
AQ59	Please check figures titles added for Figures 1 and 9.
AQ60	Explain what fluorescence you have used/what the colors represent.
AQ61	Please check the changes made to the organization of Figure 2. Also, please provide the expansions of “CMV” and “PolyA”.
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AQ63	Please note that “a”, “b” and “c” in Figure 5c have been changed to “1”, “2” and “3”, respectively to avoid confusion with the panel letters; corresponding changes have been made in the text where the figure is discussed.

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AQ67	Please check “overlapping”: nothing appears to be overlapping in the figure.
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