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Improvement of mouse and sheep cloned blastocysts' quality upon nuclear transfer of BRDT - expressing somatic cells*

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Somatic cell nuclear transfer (SCNT) has been achieved in many species by transplanting differentiated cell nuclei into enucleated oocytes. However, the low efficiency of cloning has remained an unresolved issue. Incomplete nuclear reprogramming is considered to be a causal reason for the current insufficient cloning effectiveness. Here, we wanted to know whether the forced expression of testis specific nuclear remodeling factor [BRomo Domain Testis-specific (BRDT)] in donor somatic cells improve nuclear reprogramming.
Mouse and sheep adult skin fibroblast were transfected with BRDT-GFP plasmid and treated with 100nM of Trichostatin A (TSA) for 16h. Cells were checked under confocal microscope for nuclear remodeling, then BRDT transfected and CTR (non transfected) cells were used as a donor for SCNT. Vectors were expressed in approximately 40-65% of the somatic cells. Reorganization of the nuclear structure, with an evident chromatin compaction was observed 24 hours post transfection, cells

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lost fibroblast cell structure and they become rounded. Moreover, we have shown that donor cells expressed BRDT protein used as a donor for SCNT improved quality of the cloned blastocysts as well as efficiency of the cloned embryos but remodeling using BRDT was not enough to improve nuclear reprogramming. RT-PCR of major pluripotency markers shown no difference between BRDT and CTR cloned. Our results clearly indicate that BRDT treatment can be used as a first step of chromatin reorganization of donor cells.

KEY WORDS: BRDT / Somatic cell nuclear transfer / cloning / chromatin

Somatic cell nuclear transfer (SCNT) is one of the most powerful tool used to study events that occur during reprogramming and cellular differentiation [Lagutina *et al.* 2013]. Unfortunately, current cloning efficiency is low, only 1-6% of the offspring survived to term [Ogura *et al*. 2013, Loi *et al*. 2004]. Many cloned embryos die at different stages of development due to incomplete nuclear reprogramming (NR) of donor cell. The reduced development of clones results from the oocyte's failure to restore a totipotent state in the transplanted nucleus through the nuclear reprogramming process [Meissner *et al*. 2006]. Various approaches have been proposed to improve nuclear reprogramming [Inoue *et al.* 2010, Matoba *et al*. 2014], but they are still not satisfactory. Despite expanding knowledge about mechanisms affecting nuclear reprogramming, we are still searching for more effective procedures.

Findings related to SCNT and NR in cloned embryos unequivocally demonstrate that the nuclear organization of somatic cells is rarely reset by the oocyte. Now, sperm chromatin is completely remodelled during fertilisation with an efficiency close to 100% (in mouse). Testis- specific factors mediating the transition from somatic / sperm specific conformation of chromatin might be potential candidates for improving SCNT by the directing genome remodeling [Cantone and Fisher 2013]. During post-meiotic maturation, male germ cells undergo a huge reorganization and condensation of their chromatin [Teperek *et al.* 2016]. During this phase, most histones are acetylated and then replaced by sperm-specific basic proteins, which compact the genome into a specific structure - protamines pack of the spermatozoid genome [Rosseaux *et al.* 2005, 2008], suggesting the existence of specific genome organizing programs. The molecular effectors and regulators of this event are still mostly unknown. Recent studies suggested the existence of another level of organization involving testis specific histone variants, which interact with acetylated chromatin. They are therefore promising candidates for directing the genome reprogramming events triggered by the wave of histone hyper-acetylation. One of the most interesting consequences of this process is the reprogramming of chromocenter [Govin *et al.* 2007]. BRomo Domain Testis-specific (BRDT) was found to be one of these factors. Bromodomains are conserved modules present in many chromatin- and transcription-related proteins, like histone acetyltransferases and chromatin remodeling factors. They are found to be important for the recognition of acetylated lysine's in non-histone proteins and they play an essential role in establishing new acetylation-dependent functions in isolated nuclei [Dorr *et al.* 2002, Mujtaba *et al.* 2002, Govin *et al*. 2007]. It was suggested that BRDT could play a similar role in spermiogenesis, functioning in elongating spermatids

by binding to acetylated histones or other acetylated nuclear proteins and compacting the chromatin [Govin *et al.* 2006]. Interestingly, in somatic cells, although it had no dramatic effect on the organization of chromatin, the protein was capable of inducing, *in vivo* and *in vitro,* spectacular chromatin remodeling when histone hyperacetylation was induced [Pivot-Pajot *et al.* 2003]. Here we have shown that BRDT induces chromatin reorganization in mouse and sheep fibroblasts and interphase chromatin of somatic cells responds to the BRDT protein by the acquiring a compact structure upon global histone acetylation. Moreover, oocyte reconstructed with BRDT positive fibroblasts developed to blastocysts stage in higher proportion comparing to controls ones (36,5% vs 12.8 % (mouse); 9,7% vs 3,6% (sheep) respectively $p<0,0026$). Preliminary results shown that transfer of donor cells expressing BRDT protein improve the developmental competence and quality of cloned embryos.

Material and methods

All inorganic and organic compounds were purchased from Sigma unless otherwise stated

Animals and ethics statements

Mouse. All animal care and experiments were carried out in the Institute of Genetics and Animal Breeding of the Polish Academy of Sciences. Mature F1 (C57Bl10 x CBA/H) hybrid mice approximately 3-month-old were used as donors of metaphase II oocytes. All animals were housed in 30.5 × 13 × 11 cm cages, kept in a temperature-controlled room with a 12h light: 12 h darkness cycle (light on from 06.00 to 18.00) Food (Labofeed H, Kcynia, Poland; metabolic energy of 13.0 MJ/kg) water were available *ad libitum*. The experiments were performed according to the rules of the Polish Governmental Act for Animal Care and were approved (No **53/2011**) by the II Local Ethics Committee for Animal care at Warsaw University of Life Sciences.

Sheep. Animal work on sheep model was carried out in Experimental Embryology Laboratory in Teramo University in Italy and has been approved by the Italian Ministry of Health, upon the presentation of the research description prepared by the Ethics Committee of the Istituto Zooprofilattico Sperimentale di Teramo (Prot. 944F0.1 del 04/11/2016). The number of the authorization granted by the Italian Ministry of Health is n° 200/2017-PR.

Collection of oocytes

Mouse. For oocytes collection, donor F1 females were stimulated for ovulation by intraperitoneal injection of 5 IU (0.1 mL) PMSG (Pregnant Mare Serum Gonadotropin; Folligon, Intervet, Netherlands) followed 48 hr later by 5 IU (0.1 mL) of hCG (human Chorionic Gonadotropin; Chorulon, Intervet, Netherlands). Donor females were killed by cervical dislocation about 14 hrs after hCG injection and metaphase II (MII)

oocytes were collected from oviducts by tearing of the ampulla, devoid of cumulus cells by treatment with hyaluronidase (150 IU/ml PBS), washed three times in KSOM medium and kept prior to manipulation in KSOM medium in an atmosphere of 5% CO_2 in air at 37.5°C

Sheep. Sheep ovaries were collected from local slaughterhouses and transferred to the laboratory within 1±2 hours. Oocytes were aspirated with 21 G needles in presence of TCM-199 medium (Gibco, Milan, Italy) containing Hepes and 0.005% (w/v) Heparin. Then, all oocytes with an unexpanded cumulus and uniform cytoplasm were *in vitro* maturated (IVM) in bicarbonatebuffered TCM-199 medium (Gibco Milan, Italy) containing 2 mM glutamine, 0.3 mM sodium pyruvate, 100 μM cysteamine, 10% fetal bovine serum (FBS) (Gibco Milan, Italy), 5 μg/ml FSH (Ovagen, Glenfield, New Zeland), 5 μg/ml LH, and 1 μg/ml estradiol. Maturation was conducted into 4-well culture plates (Nunclon, Roskilde, Denmark) containing 0.4 ml of IVM medium per well and incubated in a humidified atmosphere of 5% CO_2 in air at 39°C for 24 h.

Mouse and sheep fibroblasts transfection and TSA treatment. Mouse and sheep fibroblasts were transferred with 4μg of GFP-BRDT plasmid using the Lipofectamine reagent (Lipofectamine™ LTX Reagent, Invitrogen and Obtimel, Gibco) at 80% of confluence. 24 hours post transfection, cells were treated with 100ng/mL of Trichostatin A (TSA) for 16h and then were culture for 48hours in low serum concentration (0.1% FBS). Un-transfected fibroblasts were cultured under the same conditions and use as control.

To evaluate transfection efficiency cells were stained with 5 μg/ml of Hoechst for 10min and GFP+ cells were then counted in relation to total cells count. At least 200 nuclei were analyzed for each group for statistical soundness.

Chromatin remodeling evaluation. To evaluate chromatin remodelling of BRDT-GFP expressed cells mouse and sheep fibroblasts were grown on a glass cover slip, and 24h post transfection were fixed with 4% Paraformaldehyde. DNA counterstaining was done with 5 μg/ml of DAPI. Cells were then mounted on slides with Fluoromount™ aqueous mounting medium and analysed on a confocal microscope (Nikon Eclipse Ti-E).

Somatic cell nuclear transfer

Mouse. Prior to micromanipulation MII oocytes were preincubated for 20-30 minutes in M2 medium with the addition of cytochalasin B (CB, 5 μg/ml) into micromanipulation chamber under paraffin oil. Metaphase II spindle inside oocyte were localized and then removed by aspiration with a minimal volume of cytoplasm using Piezo stepper (Piezo-micro Manipulator Prime Tech, Japan) connected with Nikon Eclipse T300 Inverted microscope (Nikon, Japan) After enucleation oocytes were incubated for 30 min KSOM medium (Simplex Optimized Medium (SOM) with enhanced concentration of NaCl and KCl - Specialty Media, Philipsburg, USA) before reconstruction. Enucleated oocytes were reconstructed using Sendai Virus

(GenomONE™ - CF EX Sendai virus (HVJ) Envelope Cell Fusion Kit; CosmoBio) with BRDT expressed cells (cells were selected by a short exposure to UV light with fluorescein filters) (BRDT-SCNT). Non-transfected cells were used as a control (CTR-SCNT). Reconstructed oocytes (CTR-SCNT and BRDT-SCNT) were cultured in a 5% CO_2 incubator at 37°C for 30 to 60 minutes and fusion were verified under standard microscope. The successfully reconstructed embryos were activated. Oocytes were incubated in activating media for 5h durations at 37.5C in a humidified atmosphere with 5% CO2in air. The activating media were Ca2+-free CZB supplemented with concentrations of 10 mM of SrCl2 (Sigma). After SrCl2 treatment reconstructed oocytes were cultured in standard medium KSOM (Simplex Optimized Medium (SOM) in an atmosphere of 5% CO_2 in air at 37°C till the blastocyst stage.

Sheep. SCNT was performed as previously described [Iuso *et al.* 2013]. Briefly, mature sheep oocytes were incubated in Hepes-buffered TCM-199 medium containing 4 mg/ml BSA, 7.5 mg/ml Cytochalasin B and 5 mg/ml Hoechst 33342 in an incubator for 15 minutes. Oocyte manipulation was facilitated by Piezo pulses (PiezoXpert, Eppendorf, Milan, Italy). Enucleation was carried out in Hepes-buffered TCM-199 medium with 0.4% (w/v) BSA and Cytochalasin B by using a Narishighe micromanipulator. Enucleated oocytes were allowed to recover from the Cytochalasin B treatment and then were directly injected, using Piezo step devise, with fibroblasts in PBS with 6% Polyvinylpyrrolidone. Reconstructed oocytes were activated in Hepes-buffered TCM-199 medium containing 5 mg/ml Ionomycin for 5 minutes and then incubated in SOF medium plus antibiotics and 0.8% BSA containing 10mM dimethylaminopurine and 7.5 mg/ml Cytochalasin B for 3±5 hours. Then embryos were cultured for 10±12 hours in SOF enriched with 1% (v:v) MEM nonessential amino acids (Gibco), 2% (v:v) basal medium Eagle (BME) essential amino acids, 1 mM glutamine, and 8 mg/ml BSA covered with mineral oil pre-washed in SOF.

Real-Time Polymerase Chain Reaction

Isolation of total RNA was performed using High Pure miRNA Isolation Kit (Roche Applied Science, Germany) according to manufacturer's protocol. Synthesis of cDNA was performed using Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science, Germany) according to manufacturer's protocol. For the reaction, we used 100 ng of RNA. qPCR reaction was performed using Light Cycler FastStart DNA Master SYBR Green I qPCR Kit (Roche Applied Science, Germany) according to manufacturer's protocol on Roche 96 thermocycler. The primer pairs (in Table 1) were designed using the Primer-BLAST tool (NCBI, USA) (www.ncbi.nlm.nih.gov/tools/primer-blast/) using the Ref Seq IV.80 database containing a complete list of known mouse transcripts. We used *H2afz* and *18S* as a reference genes.

Statistical analysis

Data were analyzed using GraphPad Prism (Version 6.01, GraphPad Software, Inc, CA, USA). The influence of the manipulation method on the developmental

potential of embryos was analyzed using the Fischer test. P <0.05 was accepted as the statistical significance. mRNA expression of pluripotent genes (Sox, Nanog, Oct4) in BRDT and CTR cloned blastocysts data were presented as means ± S.E.M.

Results and discussion

Morphology of somatic nucleus expressing BRDT – GFP protein

Our results shown that interphase chromatin of somatic cells responds to the BRDT protein by the acquiring a compact structure upon global histone acethylation (Fig. 1). Vectors were expressed in approximately 65% of mouse and 45% of sheep cells (Tab. 2) and reorganized the nuclear structure with an evident chromatin compaction observed 24 hours post transfection, followed by rounding up of the cells (Fig. 1). Additionally, we have treated cells with Histone Deacetylase inhibitor Trichostatin A (TSA) which augmented the extent of nuclear compaction as expected (Fig. 1D, H and Tab. 1).

Table 1. Primer pairs used to Real-Time Polymerase Chain Reaction

Gene	Sequence (5'=>3')	Product size	Tm (°C)	E%
Sox2	TTTGTCCGAGACCGAGAAGC CTCCGGGAAGCGTGTACTTA	146	60	102.9
Pou5f1	GGTGGAACCAACTCCCGAGG ACCTTTCCAAAGAGAACGCCC	150	60	96.4
Nanog	TGATTCAGAAGGGCTCAGCA GCCCCACATGGAAAGGCTTC	115	60	95.6
H2afz	CGCAGAGGTACTTGAGTT TCTTCATCTCCACGTATAGC	99	59	101.9
18S	CGCGGTTCTATTTGTTGGT AGTCGGCATCGTTTATGGTC	219	60	103.4

Table 2. TSA – dependent chromatin reorganization after expression of BRDT-GFP protein. Skin sheep and mouse fibroblast were transfected with GFP – BRDT plasmid, then cells were either treated with TSA (100 ng/mL) (+TSA) or not treated with TSA (-TSA)

Item	Mouse		Sheep	
	-TSA	+TSA	-TSA	+TSA
Efficiency of transfection	50%	65%	40%	45%
Reorganization chromatin (BRDT-expressing cells)	45%	85%	40%	90%
Reorganization chromatin (untransfected cells)	0%	1%	0%	0%

BDRT expressing donor cells improved cloned blastocyst quality

Treatment of donor cells with BRDT significantly improved the production of cloned embryos compared to control group. Table 3 shows that cleavage and blastocyst rates of BRDT clones were higher than CTRL (cleavage: 50,6% in BRDT-SCNT vs.

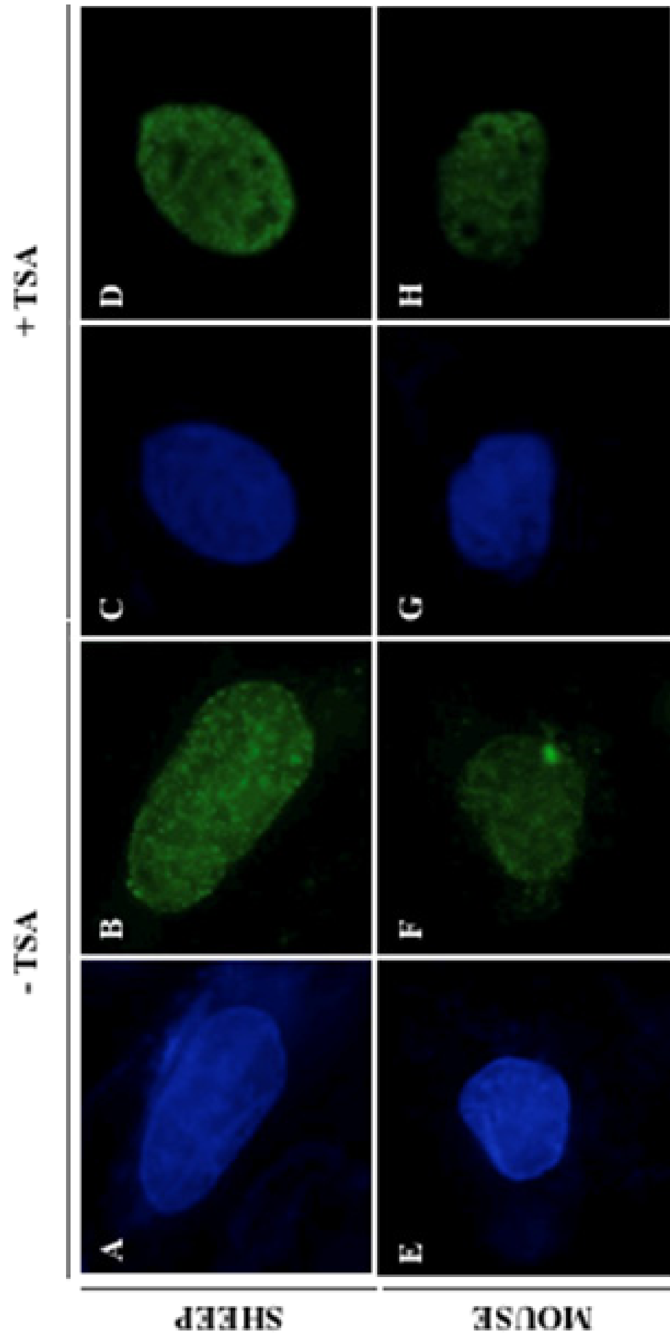

Fig. 1. Nuclear morphology of BRDT positive cells visualized by chromatin staining with BRDT-GFP (B, D, F, H) in sheep (A-D) and mouse (E-H) model; A, C, E, G - nuclei stained with Hoechst.

Table 3. Development of mouse and sheep oocytes reconstructed with BRDT expressing cell nuclei

Item			Number (%) of embryos that developed to		
MOUSE	# Oocytes	Fusion rate	2-cells (%)	4- cells (%)	Blastocysts
CTR-SCNT	86	53 (61.6%)	41 (47.6%)	27 (31.4%)	11 (12.8%)
BRDT-SCNT	150	87 (58%)	76 (50.6%)	71 (47.3%)	55 (36.6%)
SHEEP	# Oocytes	Fusion rate	4-cells (%)	Morula (%)	Blastocysts
CTR-SCNT	41	28	20 (48.8%)	2 (4.8%)	1/28 (3.6%)
BRDT-SCNT	255	134	154 (60.4%)	25 (9.8%)	13/28 (9.7%)

47,6% in CTR clones (mouse); 60,4% in BRDT-SCNT vs. 48,8% in CTR (sheep); blastocyst: 12,8% vs. 36,6% (mouse); 9,7% vs 3,6% (sheep) respectively). (Tab. 3).

To confirm that the *Brdt* gene introduced to oocytes were expressed, we observed 96h blastocysts under confocal microscope. To confirm protein expression in reconstructed oocytes, blastocysts were placed under confocal microscope, and expression were identified by positive GFP expression in blastocyst of experimental group (Fig 2., white arrow).

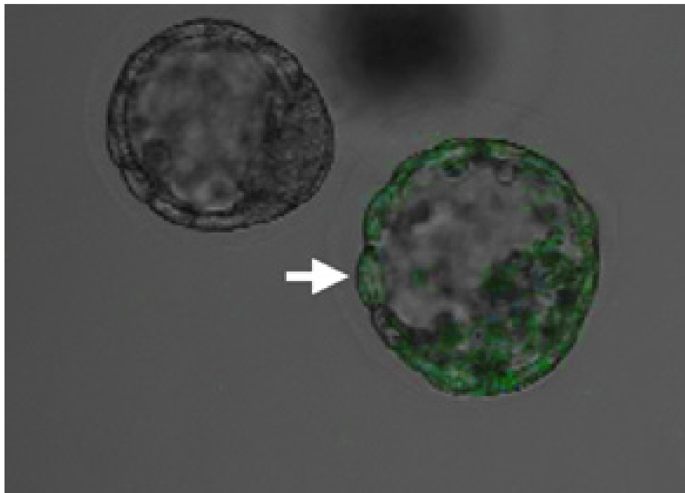

Fig. 2. Mouse BRDT- SCNT embryo at blastocyst-stage (white arrow).

Additionally, we also observed, that BRDT-SCNT blastocysts exhibited very robust, better-expanded morphologies in mouse (Fig. 3B) and sheep (Fig. 3D) compare to CTR-SCNT blastocyst (Fig. 3A – mouse; Fig. 3C – sheep) but mRNA of Sox2, POU5F1 (Oct4) and Nanog in BRDT-SCNT embryos do not shown any statistic differences compare to the control group.

In the present study, we aimed to elucidate whether BRDT can be used for pre-remodeling of fibroblasts nuclei hence improving quality of cloned embryos. In

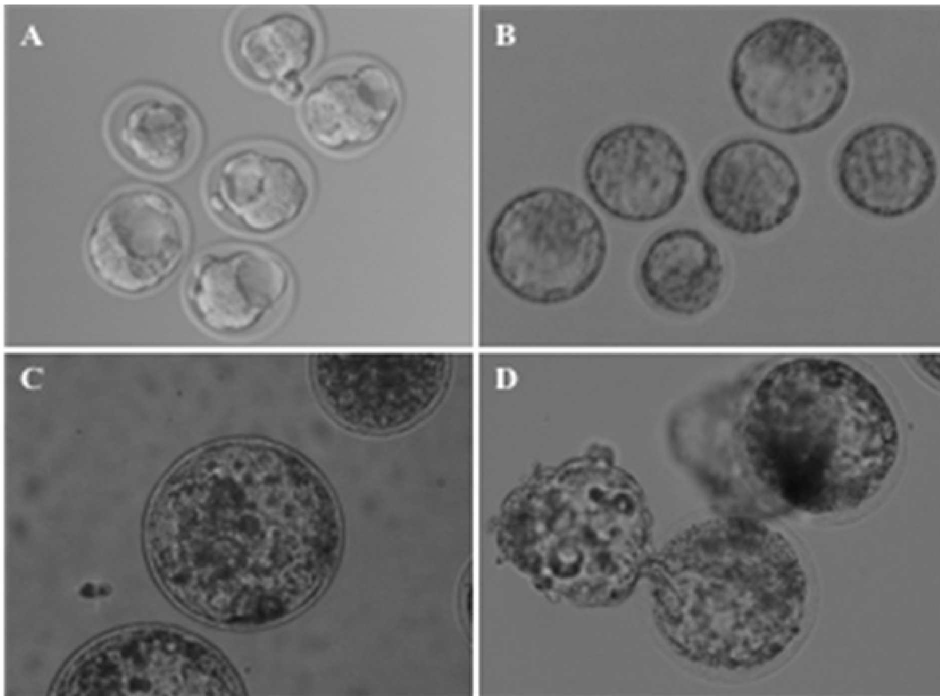

Fig. 3. Representative pictures of cloned blastocyst using CTR (A, C) and BRDT expressed cells (B, D) in mouse (A, B) and sheep (C, D) model.

order to evaluate this, we analyzed the effect of BRDT expressed donor cells on preimplantation embryo developmental rates.

Important factors for the development of reconstructed embryos are epigenetic modifications of the nuclei of somatic cell introduced into the recipient oocytes. The persistence of epigenetic marks on the somatic genome makes crucial developmental genes inaccessible for transcription, leading to abnormal gene expression patterns and developmental failure. This incomplete/abnormal NR explains the abnormal phenotypes of cloned embryos and offspring.

Spermatozoa are the perfect nuclear transfer devise. Sperm chromatin is completely remodelled during fertilisation with an efficiency close to 100%.

Nuclear remodelling during spermatid maturation occurs through a time-regulated translation of mRNAs for histone variants accumulated earlier in spermatogonia [Govine and Khochbin 2013]. The incorporation of such testis-specific histone variants into the chromatin leads to a destabilization of nucleosomes [Rathke *et al.* 2014]. Subsequent post-translation modifications of the histone variants further prepare the ground for the incorporation of transition proteins first, then protamines, that compact the nucleus [Shabazianet and Grunstein 2007]. Of course, it is impossible to repeat the stepwise nuclear remodelling that takes place in spermatids in a somatic cell.

We therefore opted for a step-wise transfection, starting with Bromo domain testis specific (Brdt-GFP tag). Expression of the vector was approximately 40-65%, and a reorganization of the nuclear structure, with an evident chromatin compaction observed 24 hours' post transfection, followed by rounding up of the cells. Nuclear condensation of BRDT-GFP in sheep and mouse fibroblasts was comparable with previous observation in mouse fibroblasts expressing Brdt [Pivot-Pajot *et al.* 2003]. Additionally, we have treated cells with Histone Deacetylase inhibitor Trichostatin A (TSA) which augmented the extent of nuclear compaction as expected. Moreover, we have observed that cells expressing BRDT die within 48 hours, very likely the BRDT-acetylation induced chromatin compaction reduced the bulk cellular transcription, with negative influence on cell homeostasis.

The reversibility of the differentiated status in somatic cells through SCNT has been experimentally demonstrated. In farm and companion animals the manipulation procedures for SCNT have been simplified; allowing the reconstruction of large number of embryos [Lagutina *et al.* 2005]. However, such technical progress does not match the knowledge acquired on basic mechanism that controls nuclear reprogramming. This is demonstrated by the low efficiency of offspring production from SCNT, further complicated by foetal losses, post -natal mortality, and the still debated reduced life span of clones [Tsunoda and Kato 2002, Rideout *et al*. 2001, Latham 2005, Loi *et al*. 2004, Ogura *et al.* 2002, Tamashiro *et al*. 2003]. Our data shown that embryos reconstructed with BRDT expressed fibroblast improved developmental rate and quality of cloned blastocysts in sheep and mouse models. However further studies and investigation need to be performed to fully confirm this data.

The altered epigenotype found in cloned embryos arises from an unbalanced nuclear reprogramming between parental chromosomes. Changes in the epigenotype observed in cloned embryos arise from unbalanced nuclear reprogramming between parental chromosomes. It is probable that the oocyte reprogramming machinery, devised for resident chromosomes, cannot target the paternal alleles of somatic cells. Loi *et al.* [2008] suggested that an "asymmetric" nuclear reprogramming is present in cloned embryos and that a reasonable approach to balance this asymmetry might involve the transient expression of chromatin remodeling proteins in donor cells. These proteins are physiologically expressed during spermatogenesis, in order to induce a male-specific chromatin organization in the somatic cells before nuclear transfer [Loi *et al.* 2008]. Such hypothesis is supported by an interesting experiment where nuclear reprogramming was assessed in somatic cells injected into Germinal Vesicles (GV) of Xenopus oocytes using as a marker the re-expression of OCT4 [Simonsson and Gurdon 2004]. It is likely that this enzyme is release to oocyte's cytoplasm following GV break down, where it might be responsible for the nuclear reprogramming of the transplanted somatic cell. In this perspective, it might be realistic that this enzyme, designed to target maternal chromosomes, doesn't the paternal one, leaving them unaffected.

In light of this study a possible strategy to give the chromatin of a somatic cell the same conformation of the sperm cells DNA. Such DNA configuration is readable by

the oocyte's reprogramming machinery. During male germ cell maturation, chromatin structures undergo dramatic changes leading to the exchange of somatic histones with sperm specific protamines [Rousseaux *et al*. 2005). Current knowledge implies a stepwise replacement of histones by transition proteins and protamines to pack the mature spermatozoid's genome [Rousseaux *et al*. 2008]. We therefore considered BRDT as a factor able to recognize acetylated chromatin and participate in the remodeling of acetylated chromatin. Our data suggested BRDT is able to induce global remodeling of chromatin in abut fibroblasts. TSA was added in order to induce histone hyperacetylation which was previously showed by Pivot-Pajot *et al.* [2003]. Our study indicate also that interphase chromatin of somatic cells responds to the BRDT protein by acquiring a compact structure upon global histone acethylation. These data might be relevant for in vitro "pre-reprogramming" of somatic cells prior to nuclear transfer. BRDT expressing fibroblasts can be used for SCNT, and the chromatin reorganization induced by BRDT might result in a more extensive nuclear reprogramming, and better development of both pre-and post implantation embryos. Therefore, BRDT protein could create a better physiological remodeling and improve nuclear reprogramming.

Epigenetic mechanisms have shown to critically influence embryonic development through the control of gene expression and chromatin packaging [Jones *et al.* 1999] and several genes are reported to be associated with the pluripotency of the embryos: Oct4 [Nicholas *et al.* 1998], Sox2 [Avilion *et al.* 2003] and Nanog [Chambers 2009] and with lineage segregation like Cdx2 [Strumpf et al. 2005]. In our study, we have shown no statistic difference in pluripotency markers genes between BRDT and CTR blastocyst. Those results suggest that additional nuclear remodeling need to be done to improve reprogramming of cloned embryos.

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