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**Title:** Cheetah interspecific SCNT followed by embryo aggregation improves in vitro development but not pluripotent gene expression

**Short title:** SCNT and embryo aggregation in felids

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## ABSTRACT

The aim of this study was to evaluate the capacity of domestic cat (Dc, *Felis silvestris*) oocytes to reprogram the nucleus of cheetah (Ch, *Acinonyx jubatus*) cells by inter-species SCNT (iSCNT), by using embryo aggregation. Domestic cat oocytes were *in vitro* matured and subjected to zona pellucida free (ZP-free) SCNT or iSCNT, depending on whether the nucleus donor cell was of domestic cat or cheetah, respectively. ZP-free reconstructed embryos were then cultured in microwells individually (Dc1X and Ch1X groups) or in couples (Dc2X and Ch2X groups). Embryo aggregation improved *in vitro* development obtaining 27.4%, 47.7%, 16.7% and 28.3% of blastocyst rates in the Dc1X, Dc2X, Ch1X and Ch2X groups, respectively ($p<0.05$). Moreover, aggregation improved the morphological quality of blastocysts from the Dc2X over the Dc1X group. Gene expression analysis revealed that Ch1X and Ch2X blastocysts had significantly lower relative expression of *OCT4*, *CDX2* and *NANOG* than the Dc1X, Dc2X and IVF control groups. The *OCT4*, *NANOG*, *SOX2* and *CDX2* genes were overexpressed in Dc1X blastocysts, but the relative expression of these four genes decreased in the Dc2X, reaching similar relative levels to those of domestic cat IVF blastocysts. In conclusion, cheetah blastocysts were produced using domestic cat oocytes, but with lower relative expression of pluripotent and trophoblastic genes, indicating that nuclear reprogramming could be still incomplete. Despite this, embryo aggregation improved the development of Ch and Dc embryos, and normalized Dc gene expression, which suggest that this strategy could improve full-term developmental efficiency of cat and feline iSCNT embryos.

**Key words:** nuclear reprogramming, interspecific SCNT, aggregation, felids.

## INTRODUCTION

Most of the 36 species of wild felids are considered to be threatened or endangered. One such species, the cheetah (Ch, *Acinonyx jubatus*), has suffered from a loss of habitat and a reduction in their prey. This has resulted in a reduced population that in turn has led to genetic inbreeding resulting in poor sperm quality (Wildt *et al.*1983; Wildt *et al.* 1988). Because of the difficulty in obtaining oocytes from wild felids, the domestic cat (Dc, *Felis silvestris*) has been used as a model to develop reproductive biotechnologies and to understand the cellular aspects of nuclear reprogramming in felids. Shin *et al.* (2002) reported the first birth of a cat produced by SCNT. Since then, attempts to improve the technique in cats has led to a study of different stages in the cloning procedure such the use of several nuclear donor cell types (Shin *et al.* 2005; Tomii *et al.* 2011), synchronization strategies (De Barros *et al.* 2010) and activation protocols (Wang *et al.* 2009). However, none of these studies revealed significant improvements either in embryo development or in pregnancy rates.

In addition to the studies in domestic cat SCNT, inter-species SCNT (iSCNT) has also been reported in felids (Gómez *et al.* 2004a,b). This technique involves embryo reconstruction by SCNT, using the enucleated oocyte from one species and the donor cell from another. This approach becomes relevant in those species for which oocytes are very difficult to obtain. The successful application of iSCNT in felids was demonstrated by the birth of

African wild cats (*Felis silvestris lybica*) (Gómez *et al.* 2004b) and sand cats (*Felis margarita*) (Gómez *et al.* 2008), as well as by pregnancies reported from embryos generated by the fusion of leopard cat (*Prionailurus bengalensis*) cells with domestic cat enucleated oocytes (Yin *et al.* 2006). Despite these achievements, pregnancies and births after iSCNT are still elusive, as shown in several reports (Thongphakdee *et al.* 2010; Gómez *et al.* 2011; Imsoonthornruksa *et al.* 2012)

Failure in embryo production by SCNT or iSCNT is usually associated with epigenetic problems and inadequate cellular reprogramming. As a result of the donor nucleus and recipient ooplast state, each reconstructed embryo is unique in terms of epigenetic marks and gene expression (Park *et al.* 2002). This characteristic affects embryo quality and consequently cloning success. It was suggested that genetically identical cloned embryos produced with epigenetically different cells and different ooplasts could be cultured together to generate one single embryo that would have a mixture of blastomeres differing in their reprogrammed nuclei. This mixture would compensate for epigenetic problems of one individual embryo. This approach which is called embryo aggregation, results in higher blastocyst cell numbers, normalization of pluripotent gene expression and higher *in vivo* development in the mouse and miniature pigs (Boiani *et al.* 2003; Balbach *et al.* 2010; Siriboon *et al.* 2014). Moreover, rates of blastocyst production improved for bovine and equine aggregated embryos as also did blastocyst cell numbers and pregnancy rates (Pedersen *et al.* 2005; Zhou *et al.* 2008; Ribeiro *et al.* 2009; Gambini *et al.* 2012). Despite these promising reports, embryo aggregation has not been evaluated in felids or in iSCNT.

The aim of the present study was to determine the capacity of the domestic cat oocyte to reprogram a cheetah cell and generate an embryo by iSCNT. Moreover, we propose embryo aggregation as a strategy to improve the cloning efficiency in felids.

## MATERIALS AND METHODS

### Ethics for use of research animals

Animal manipulation was done according to the rules of the Direction of National Wildlife. The standards established by the code of ethics of ALPZA (Latin American Association of Zoological Parks and Aquariums) were followed. The study design was approved by the Ethics and Animal Welfare Committee for the Faculty of Agriculture University of Buenos Aires under number CEyBAFAUBA2014/3.

### Reagents

Except as otherwise indicated, all chemicals were obtained from Sigma Chemical Company (St. Louis, MO, USA). Media were prepared weekly and filtered through 0.22 µm pores (#4192 Acrodisc; Pall Corp., Ann Arbor, MI, USA) into sterile tubes.

### Oocyte collection and *in vitro* maturation

Ovaries were recovered from queens subjected to ovariectomy and transported to the laboratory within 2 h. They were washed in Tyrode's albumin lactate pyruvate medium buffered with HEPES (TALP-H; Bavister and Yanagimachi 1977). The cumulus-oocyte complexes (COCs) were released from follicles by repeatedly puncturing and scraping the ovaries. The maturation medium was TCM 199 (31100-035; Gibco, Grand Island, NY, USA) containing 1 IU/mL hCG (Ovusyn, Syntex SA, Buenos Aires, Argentina), 10 ng/mL

112 eCG (Novormon 5000, Syntex SA), 2.2 mM calcium lactate (L2000), 0.3 mM pyruvate
113 (P2256), 0.3% w/v BSA (A6003) and 3% v/v antibiotic-antimycotic (ATB; penicillin,
114 streptomycin and amphotericin B; 15240-096; Gibco). *In vitro* maturation conditions were
115 5% $CO_2$ in humidified air at 39°C. The oocytes were incubated in 100 µL of medium
116 droplets covered with mineral oil (M8410).

117 **Preparation of oocytes**

118 After 22h of IVM the oocytes were denuded of cumulus cells by pipetting in hyaluronidase
119 solution (H4272, 1 mg⁄mL TALP-H) for 1 min and washed three times in TALP-H. Only
120 those oocytes with homogeneous cytoplasm and a visible polar body were used. For the
121 enucleation, matured oocytes were incubated in 1.5 mg/ml pronase (P-8811) in TALP-H for
122 3-8 minutes on a warm plate to remove the zona pellucida. After that, the zona free oocytes
123 were individually incubated with 4 µM demecolcine (D1925) for 1 h to induce protrusion
124 of the chromosome plate and with 20 µg/ml Hoechst bisbenzimide 33342 (H33342) for 15
125 min to stain the DNA. A closed holding pipette was used to support the oocyte during
126 enucleation and the metaphase plate was aspirated using a blunt pipette by
127 micromanipulation. Enucleation was confirmed by observing the stained metaphase plate
128 inside the pipette under UV light. Enucleated oocytes were individually kept in Synthetic
129 Oviductal Fluid medium (SOF; Tervit *et al.* 1972; Holm *et al.* 1999) supplemented with
130 2.5% v⁄v fetal bovine serum (FBS, 10499-044; Gibco) until nuclear transfer.

131 **Somatic cell culture**

Adult fibroblasts were obtained from the culture of minced tissue derived from skin biopsies of a domestic cat and a cheetah, both adult and male. The domestic cat sample was cultured in Dulbecco modified Eagle Medium (DMEM, 11885, Gibco) with 10% FBS and 1% ATB. The cheetah sample was cultured in DMEM medium supplemented with 10% FBS, 0.292 mg/ml L-glutamine (25030-149, Gibco), 2.5 µg/ml fungizone amphotericin B (15290-018, Gibco) and penicillin-streptomycin (100 µg/ml each). After the primary culture was established, fibroblasts were sub-cultured every 4–6 days, frozen in DMEM with 10% FBS and 10% dimethyl sulfoxide and stored in liquid nitrogen. Quiescence of donor cells was induced by growth to confluence for 3–5 days prior to SCNT or iSCNT. Populations of cells were prepared by trypsinization 30 minutes before SCNT or iSCNT, then washed and re-suspended in DMEM.

**Somatic cell nuclear transfer**

Enucleated oocytes were individually transferred to 50 µl drops of phytohemagglutinin (PHA, L8754, 1 mg/ml in TCM-199). After a few seconds, they were quickly dropped over a single donor cell (domestic cat or cheetah cell); consequently these two structures were paired. The couplets were placed in fusion medium [0.3 M mannitol (M9546), 0.1 mM MgSO4 (A665286 525, Merck, Darmstadt, Germany), 0.05 mM $CaCl_2$ (C7902), and 1 mg/ml polyvinyl alcohol (P8136)] for 30 s and then removed to a fusion chamber containing 2 ml of fusion medium. Membrane fusion was performed with two 30 µs DC pulses of 1.4 kV/cm, 0.1 s apart, and then the couplets were placed in SOF medium. Fusion was assessed after 20 min by confirming the absence of the fibroblast cell attached to the enucleated oocyte. Re-fusion was performed when necessary. Two hours after fusion, the

reconstructed embryos were activated with 5 μM ionomycin (I24222; Invitrogen, Carlsbad, CA,USA) in TALP-H for 4 minutes followed by culture, for 3 h, in 1.9 mM 6-dimethylaminopurine (6-DMAP; D2629) in SOF medium.

### *In vitro* fertilization

Epididymal domestic cat frozen sperm was thawed in a 37°C water bath for 30 s. Spermatozoa were centrifuged twice (490 g, 5 min) and re-suspended in Talp-fert medium (Parrish *et al.* 1988). Spermatozoa were then diluted to a final concentration of 1.5-2.5 x $10^6$/ml and co-incubated with COCs in 50 μl droplets, for 20 h at 39°C in a humidified atmosphere of 5% $CO_2$ in air. The presumptive zygotes were then washed three times in TALP-H and placed into embryo culture.

### Embryo culture

IVF embryos were cultured in 100 μL droplets of SOF medium supplemented with 2.5% v/v FBS. Reconstructed embryos were cultured using the same medium, but in microwells, as described previously (Gambini *et al.* 2012). The reconstructed embryos were cultured individually (1X groups) or in couples in each well (2X groups or aggregated groups). The experimental groups were Dc1X and Dc2X when the nuclear donor cells were of domestic cat, and Ch1X and Ch2X when the nuclear donor cells were of cheetah. The culture conditions were a humidified gas mixture of 5% $CO_2$, 5% $O_2$ and 90% $N_2$ at 39°C. The culture medium was changed on day 2 and then supplemented with 10% FBS on day 5. Cleavage, compacted morula formation and blastocyst development were assessed on day 2, day 5 and day 8 of culture, respectively. Blastocyst rates were calculated per embryo and

per oocyte to determine the efficiency of our method. Blastocysts were fixed for immunocytochemistry as described below or kept in 50 µl RNAlater (AM7020, Ambion, Austin, TX, USA) at -20°C until gene expression analysis.

**Mitotracker staining**

In order to assess embryo aggregation efficiency, day 0 clones were stained with either green or red mitotrackers. After 6-DMAP treatment, half of the total reconstructed Dc embryos were incubated with 20 µM green mitotrackers (M7514, Invitrogen) and the other half with 0.5 µM red mitotrackers (M7512, Invitrogen), for 45 min in humidified conditions at 39°C. Once stained, the clones were washed once in TALP-H and cultured as described above, placing two embryos together, one of each color in each microwell. Embryo development and mitotrackers fluorescence were evaluated at day 2, 5 and 7. The incorporation of both structures in one single embryo was determined in compact morulae and blastocysts (Fig. 1).

**Immunocytochemistry**

The blastocysts generated by SCNT, iSCNT and IVF were analyzed by immunocytochemistry to determine OCT4 expression. For aggregated embryos, the blastocysts selected were those that both reconstructed embryos were cleaved at day 2, so we enhanced the probability of analyzing blastocysts formed by the two embryos cultured together. Embryos were fixed for 20 min in 4% v/v paraformaldehyde (F1635) in DPBS (14287-072, Gibco) and permeabilized for 15 min with 0.2% v/v Triton X-100 (T9284) in DPBS. Non-specific immunoreactions were blocked by 30 min incubation with 3% v/v FBS and 0.1% v/v Tween-20 (Promega, H5152) in DPBS (blocking solution). Incubation

with the primary antibody against OCT4 (goat polyclonal IgG, SC-8628 Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:100 in blocking solution, was performed for 1 h at room temperature. Embryos were then rinsed in blocking solution for 15 min. Incubation with the secondary antibody (Alexa 488-donkey anti-goat IgG, A11055, Molecular Probes Inc. Eugene, OR, USA) diluted 1:1000 in blocking solution, was performed for 45 min at room temperature in the dark. Nuclei were counterstained with 30 µg/mL propidium iodide (P4170) for 20 min in the dark. Stained blastocysts were mounted on glass slides, in 70% v/v glycerol under a cover slip and stored at 4°C for 24 h before fluorescence microscopic evaluation. Negative controls for OCT4 were generated using only the secondary antibody.

**Confocal laser scanning microscopy**

Embryos were analyzed on a Nikon Confocal C.1 scanning laser microscope. An excitation wavelength of 488 nm was selected from an argon-ion laser to excite the alexa-conjugated secondary antibody and an excitation wavelength of 544 to excite propidium iodide. Complete Z series of 13–18 optical sections at 3-4 µm intervals were acquired from each blastocyst and three-dimensional images were constructed using the software EZ-C1 2.20. Total cell number and OCT4 positive cells (OCT4+) were counted (Fig. 2).

**Gene expression analysis**

For gene expression analysis, blastocysts were pooled as follows: Dc1X, four replicates of three blastocysts each; Dc2X, four replicates of three blastocysts each; Ch1X, two replicates of two blastocysts and one replicate of one blastocyst; Ch2X, four replicates of three blastocysts each; IVF three replicates of three blastocysts each. As mentioned before,

the blastocysts selected from aggregated embryos were those that both reconstructed embryos were cleaved at day 2. Embryos were treated with a Cells-to-cDNA TM II kit (Ambion Co., Austin, TX, USA) lyses buffer according to manufacturer's instruction. Briefly, embryos were washed twice in cold DPBS to eliminate the RNAlater; 100 µl of lyses buffer were added and incubated 10 min at 75°C. All the samples were treated with DNase I (0.04 U/µl) for genomic DNA digestion. For cDNA conversion, 10 µl of total RNA was used in a 20-µl final reaction containing 5 µM random primers, 10 mM each dNTP, 2 µl first strand buffer (10×), 10 U of RNase inhibitor, and 200 U/ml M-MuLV (Ambion). Cycling parameters were: 70°C for 3 min, 42°C for 60 min, and 92°C for 10 min. The produced cDNAs were kept frozen at −20°C until use in PCR experiments.

Gene expression analysis was performed by real-time qPCR using the standard curve method. A standard curve for each gene was prepared using PCR products excised and eluted from agarose gels using a gel extraction kit (Omega Biotek, Santiago, Chile) and quantified by Epoch. Serial tenfold dilution of PCR products were prepared. At least eight points were included in each standard curve to assure reaction efficiency within a range of 90 and 110 %. For qPCR, the samples and the dilutions of the standard curve were loaded as duplicates (technical replicates). The primers used and PCR conditions for each gene are listed in Table 1. The Crossing Point (CP) and the amplification efficiency were calculated by the built-in software. In all qPCRs, *GAPDH* was used as an internal control.

**Statistical analysis**

*In vitro* embryo development was compared by non-parametric Fisher's exact test. Differences in total cell number were analyzed using Proc Mixed, considering

heterogeneity of variances and setting degrees of freedom by Kenward-Roger. The analysis of gene expression data was performed using a non-parametric Kruskal–Wallis test. Statistical analysis was conducted using InfoStat software. For these statistical analyses, the SAS program was used (SAS Institute Inc. SAS/STAT 1989). In all cases differences were considered significant at $p<0.05$.

## RESULTS

### Effect of iSCNT and embryo aggregation on *in vitro* development of domestic cat and cheetah embryos generated by cloning

*In vitro* development of IVF, domestic cat and cheetah cloned embryos aggregated or not, is summarized in Table 2. Mitotracker analysis revealed that from a total of 74 aggregated embryos (148 reconstructed embryos), 67.6% (50/74) of double cleavage per microwell was obtained and we evaluated the capacity of these both embryos to form the final embryo. We determined that 61% (22/36) of the compacted morulae and 54.2% (13/24) of the blastocysts, both of them formed by two cleaving embryos, showed green and red cells after fluorescence microscopy analysis, confirming real aggregation (Fig. 1).

No effect of iSCNT was seen in cleavage rates with respect to homologous SCNT (87.6% and 87.2% for Dc1X and Ch1X, respectively), but an improvement in both species was observed with embryo aggregation (98.2% and 96.7% for Dc2X and Ch2X, respectively). Cleavage rates for IVF embryos were significantly lower than in the other groups (34.7%) because matured oocytes were not selected before fertilization. Despite the differences in cleavage, morula formation was similar among all the cloning groups independently of the species and whether or not reconstructed embryos were cultured together (Table 2). Lower

rates of blastocyst production were observed in cheetah embryos as compared to those of the domestic cat. However, blastocyst rates per embryo improved after aggregation in all the experimental groups, 27.4% *vs.* 47.7% for Dc1X and Dc2X, respectively; and 16.7% *vs.* 28.6% for Ch1X and Ch2X, respectively. Moreover, aggregation did not involve the use of additional oocytes to obtain blastocysts in the domestic cat or cheetah, as no significant differences were observed in blastocyst rates per oocyte (Table 2). We also observed that all morulae reached the blastocyst stage in IVF embryos.

Blastocysts were morphologically classified as grade 1 (expanded blastocysts with a well defined ICM), grade 2 (expanded blastocysts without a well defined ICM) and grade 3 (not expanded blastocysts, without a defined ICM and observable dead cells) (Fig. 3). This classification showed an increase in grade 1 domestic cat blastocysts when embryos were aggregated and more grade 3 cheetah blastocysts generated by iSCNT (Table 3).

Total cell number and OCT4 expression from day 8 cloned and IVF blastocysts are shown in Table 4. The group with the highest cell number was Dc2X and this differed from Ch1X and IVF ($p<0.05$). We observed that the OCT4+ cells were distributed heterogeneously in all the blastocysts (Fig. 2). No differences were observed among groups with respect to total cell number and OCT4+ cells. However, the average cell number of aggregated embryos from both species was almost double than that of non aggregated embryos. The percentage of OCT4+ cells was lower in aggregated than in non aggregated embryos and markedly higher in the interspecific embryos compared to those of the domestic cat (Table 4). Moreover, the IVF blastocysts showed the percentage of OCT4+ cells closer to cheetah blastocysts than to domestic cat blastocysts.

**Effect of iSCNT and aggregation on *OCT4*, *SOX2*, *CDX2* and *NANOG* gene expression of domestic cat and cheetah blastocysts generated by cloning**

In order to evaluate the effect of aggregation and interspecific nuclear transfer on cellular reprogramming, we measured the relative expression of mRNA of the pluripotency *OCT4*, *SOX2*, and *NANOG* and differentiation *CDX2* related markers. These results are shown in Fig. 4. As no data of the cheetah genome is available, sequence homology analysis between cat and cheetah genes confirmed that we were measuring the correct genes in both species with the same primers. The percentages of homology between the cat and the cheetah genes were: 93% for *OCT4*, 93% for *SOX2*, 98% for *NANOG*, 100% for *CDX2* and 89% for *GAPDH*.

We observed that the relative expression of the four genes was higher in the Dc1X blastocysts compared with the IVF control. In contrast, the relative expression of these genes was significantly reduced in the Dc2X blastocysts compared with the Dc1X blastocysts. The relative expression of *SOX2* and *NANOG* in the aggregated embryos was similar as the relative expression of these genes in the IVF control.

Regarding the interspecific embryos, the relative expression of *OCT4* and *CDX2* was also significantly reduced in Ch2X blastocysts compared with Ch1X blastocysts. The expression of *NANOG* was not affected by embryo aggregation whereas the expression of *SOX2* was enhanced.

A comparison of Dc1X and Ch1X blastocysts revealed that the relative expression of these genes were lower in cheetah embryos compared with those of the domestic cat. However when we compared Dc2X with Ch2X we observed a different pattern for the expression of

307 *SOX2*, as the relative level of this gene was higher in Ch2X blastocysts compared with
308 Dc2X blastocysts.

309 **DISCUSSION**

310 This study evaluated the capacity of domestic cat oocytes to reprogram cheetah cells and
311 generate embryos until the pre-implantation stage. Moreover, we studied the effect of
312 embryo aggregation in domestic cat SCNT and cheetah iSCNT to determine whether this
313 strategy improves embryo quality and the cloning efficiency.
314 Felid iSCNT has been applied in various species but still remains inefficient
315 (Thongphakdee *et al.* 2006; Thongphakdee *et al.* 2010; Imsoonthornruksa *et al.* 2011).
316 According to the International Union for Conservation of Nature (IUCN) the cheetah is
317 considered vulnerable to extinction globally (Durant *et al.* 2010). To our knowledge,
318 cheetah embryos have not previously been produced by cloning, and this species can be
319 used as a model of the big wild felids from different genera than the domestic cat (Johnson
320 *et al.* 2006). This was a big challenge considering that the ability of an interspecific embryo
321 to develop to the blastocyst stage decreases as the taxonomic distance between donor and
322 recipient species increases (Beyhan *et al.* 2007). Moreover, the present work is the first to
323 report embryo aggregation as a strategy to improve cloning efficiency in felids.
324

325 **Effect of iSCNT and aggregation on *in vitro* development of domestic cat and cheetah**
326 **embryos generated by cloning**
327 In this work, we achieved high rates of *in vitro* development of domestic cat and cheetah
328 embryos generated by cloning, especially when aggregation was applied. Cleavage was not

affected by the origin of the donor cell and was comparable with results previously reported in other felid species (Hwang *et al.* 2001; Gómez *et al.* 2004b; Lorthongpanich *et al.* 2004; Gomez *et al.* 2006; Yin *et al.* 2006; Wang *et al.* 2009). Moreover, it was enhanced by embryo aggregation as the rates of cleaved embryos improved nearly 10% in both aggregated groups when compared with their non aggregated counterparts. It was reported that both in ICSI and NT mouse embryos, cleavage is a collective mechanism, which means that if one cell divides the sister cell is likely to divide as well, with equal duration of cell cycle between them (Balbach *et al.* 2012). Thus, the higher initial number of embryos cultured together could increase the probability that one of them divides and promotes division of the other co-cultivated embryo.

We obtained similar or higher morula formation in domestic cat cloned embryos compared to other reports (Gómez *et al.* 2003; Thongphakdee *et al.* 2006; Imsoonthornruksa *et al.* 2012). Despite this fact, only 40-50% of cleaved embryos were able to reach this stage. This arrest before morula formation has been previously reported for cat embryos cultured *in vitro* (Kanda *et al.* 1995), but we did not observe this arrest for the IVF embryos. In the domestic cat, the arrest has been attributed to suboptimal culture conditions and a failure in the transition from maternal to embryonic control (Kanda *et al.* 1995). Another cause of embryo arrest in other species is the mitochondrial heteroplasmy generated by SCNT or iSCNT, possibly from insufficient mitochondrial respiration (Thongphakdee *et al.* 2008). The maternal mitochondrial inheritance that occurs in normal mammalian fertilization and embryo development does not apply in SCNT, and heteroplasmy is observed in most of the reconstructed embryos (Hiendleder *et al.* 2003; Yang *et al.* 2004; Burgstaller *et al.* 2007). These organelles are involved in cellular metabolism with ATP production, apoptosis,

352 regulation of calcium and cellular aging (Wang *et al.* 2009), so an inefficient nucleo-
353 cytoplasmic communication for the regulation of mtDNA transcription and replication can
354 lead to failure in embryonic development.
355 At this stage it was possible to confirm that aggregation has occurred as 61% of the
356 compacted morulae were formed by both founder embryos after mitotracker analysis. It
357 was expected that not all the 2X embryos resulted in real aggregation and it is possible that
358 aggregation of 8-cell stage or more advanced embryos increases this percentage. In the
359 mouse, it was shown that most of the resultant morulae and blastocysts were generated by
360 cells from three aggregated 4-cell stage embryos (Balbach et al. 2010), but no other
361 information about aggregation success of 1-cell stage embryos is available.
362 Blastocyst rates were also increased as a result of embryo aggregation as has previously
363 been demonstrated for other species (Pedersen *et al.* 2005; Zhou *et al.* 2008; Ribeiro *et al.*
364 2009; Gambini *et al.* 2012). A 24% increase in the capacity of morulae to develop into
365 blastocysts was observed in the Dc2X group and 34% in the Ch2X group, when compared
366 with their respective 1X groups. Therefore, embryo aggregation decreased the
367 developmental arrest at this stage. Blastocyst quality was also improved with this approach
368 and we obtained higher rates of grade 1 blastocysts in the domestic cat. With these findings
369 we have evidence that embryo aggregation has positive effects on the *in vitro* development
370 of feline embryos generated by SCNT and iSCNT. This phenomenon could be due to
371 higher cell numbers from the beginning of embryo culture, or to an epigenetic combination
372 within each embryo that compensates for inefficient cellular reprogramming of individual
373 embryos, or to a combination of both hypotheses. The epigenetic combination obtained
374 after the aggregation of two genetically identical reconstructed embryos that were

reprogrammed differently (Boiani *et al.* 2002; Park *et al.* 2002), could compensate for defective individual embryos enhancing developmental competence of aggregates (Eckardt and McLaughlin 2004; Balbach *et al.* 2012). In this manner, aggregation could make the development of a complete embryo possible even if one of the two contributing embryos may not have been competent alone.

**Effect of iSCNT and aggregation on expression of reprogramming factors**

To better understand the effect of interspecific cloning and embryo aggregation in nuclear reprogramming, we analyzed the relative expression of *OCT4*, *NANOG, SOX2* and *CDX2* in the blastocysts generated by IVF, domestic cat SCNT and cheetah iSCNT. We observed that domestic cat aggregated embryos decreased the relative expression levels of the four genes evaluated compared with non aggregated embryos, achieving similar relative levels of *NANOG* and *SOX2* as IVF embryos. Using embryo aggregation we could normalize the relative expression of these two genes and also approximate the relative expression levels of *OCT4* and *CDX2* to those obtained in IVF embryos. Moreover, both cheetah groups showed significantly lower expression of *OCT4*, *CDX2* and *NANOG* than the domestic cat groups, which suggests that the cat oocyte was not able to reprogram the cheetah somatic cell efficiently. We observed more cheetah than domestic cat embryos arrested at the morula stage, which means that the first cell fate differentiation did not happened efficiently. This observation is consistent with the low expression of the genes evaluated.

To be successful, clones have to reset the differentiated state of the cell and establish embryo-specific gene expression. In feline species many of the strategies suggested to improve SCNT have not had much effect (Yin *et al.* 2005; Gómez *et al.* 2011; Imsoonthornruksa *et al.* 2011; Yin *et al.* 2007; Gómez *et al.* 2012). The majority of the

cloned embryos that have been produced so far have shown deficient nuclear reprogramming leading to failures in development to term (Tamada and Kikyo 2004; Sawai 2009). Fetal abnormalities were reported in African wildcat and sand cat cloned fetuses (Gómez *et al.* 2006; Gómez *et al.* 2008); these may have been associated with alterations in the expression of several genes and epigenetic disorders in donor cells (Gómez *et al.* 2008). Balbach *et al.* (2010) reported that aggregation of mouse cloned embryos normalized the levels of CDX2 and this effect was attributed to higher cell numbers. In contrast to our findings, embryo aggregation in miniature pigs enhanced the expression of *OCT4* and *CDX2* (Siriboon *et al.* 2014).

The increasing application of assisted reproductive techniques in felids requires the understanding of the molecular mechanisms involved in regulating pre-implantation embryonic development. In the mouse it was demonstrated that *Oct4* in association with *Sox2* and *Nanog* forms a complex which maintains the pluripotent cells in the inner cell mass (ICM) of the embryos (Nichols *et al.* 1998; Mitsui *et al.* 2003; Rodda *et al.* 2005; Masui *et al.* 2007). Moreover, *Sox2* expression is necessary during embryogenesis to facilitate establishment of the yolk sac lineage, which is essential for gestation (Wicklow *et al.* 2014). In the mouse, the differentiation of the ICM and the trophectoderm (TE) is also directed by the antagonistic expression of *Oct4* and *Cdx2*. Failure in the expression of these genes leads to aberrant ICM and TE, which is common in cloned embryos (Amano *et al.* 2002). In species other than the mouse, there are differences in genes regulating pluripotency and early differentiation, which may reflect differences in embryonic development (Kirchhof *et al.* 2000; Kuijk *et al.* 2008). Therefore, each species must be studied in order to understand the mechanism of maintaining pluripotency and

differentiation in pre-implantation embryos, which may be useful to improve embryo development or establish stable embryonic stem cells lines in different species (Kirchhof *et al.* 2000; Kuijk *et al.* 2008).

The iSCNT also affected the relative expression of the *OCT4*, *NANOG*, *CDX2* and *SOX2* genes. We observed that interspecific blastocysts decreased the relative expression of these genes when we compared Ch1X *vs*. Dc1X. Abnormalities in the transcription of reprogramming genes were reported in several studies in which iSCNT was performed (Loi *et al.* 2011); these have included feline species as the marble cat (Imsoonthornruksa *et al.* 2010) and the black-footed cat (Gómez *et al.* 2011). The inefficient gene expression may be related to the lower blastocyst rates and blastocyst quality obtained using cheetah cells compared to domestic cat cells.

In addition to studying the gene expression, we evaluated the distribution of the OCT4 protein in blastocysts from all the groups. In the mouse model, the OCT4 protein is down regulated in the TE of blastocysts and is expressed mainly in the ICM. On the other hand, bovine, porcine and primate blastocysts do not possess this distinctive pattern and also stain positive for OCT4 protein in TE (Kirchhof *et al.* 2000; Harvey *et al.* 2009). These differences in OCT4 distribution may be related to interspecies variations in the placenta and embryonic development. We observed that OCT4 was not restricted to the ICM in any of the blastocysts analyzed (Fig. 2), but was also distributed to the TE, as was previously shown (Gómez *et al.* 2010). By comparing the results observed in Table 4 with gene expression results for *OCT4*, we can presume that cheetah blastocysts have more cells expressing OCT4 in lower level than domestic cat blastocysts, but no quantification of the protein was done.

In summary, our study demonstrated that domestic cat oocytes were able to reprogram cheetah cells and generate embryos to the blastocyst stage, but less efficiently than in homospecific SCNT. We also proved that embryo aggregation modifies gene expression and enhances *in vitro* embryo development in both felid species. In addition to providing a tool for studying nuclear reprogramming, iSCNT can potentially be used to isolate embryonic stem cells.

## DECLARATION OF INTEREST

None of the authors have any conflict of interest to declare.

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## FIGURE LEGENDS

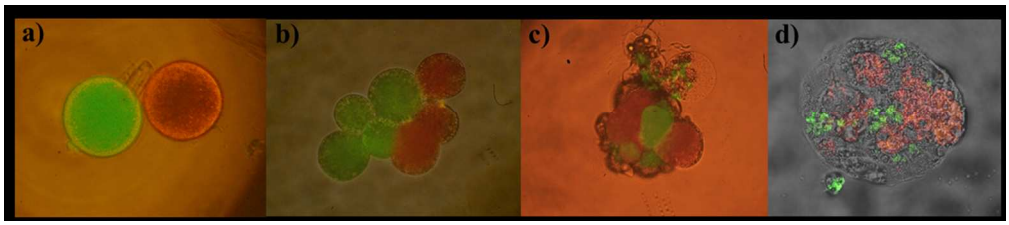
**Figure 1.** Embryo development and fluorescent analysis of one aggregated cat embryo (Dc2X group) stained with green and red mitotrackers. a) Aggregate of two 1-cell cloned embryos at the time of aggregation, day 0 of culture, b) Cleaved embryo after 2 days of culture, c) Aggregated morula at day 5 of culture, d) Aggregated blastocyst at day 7 of culture. (a-c) 40X zoom, d) 20X zoom.

**Figure 2.** OCT4 expression pattern of domestic cat and cheetah blastocysts generated by cloning (with or without aggregation) and IVF. Each picture represents one section of the total blastocyst. The nuclei are shown in red (propidium iodide) and OCT4 is shown in green (alexa fluor 488).

**Figure 3.** Representative photographs of blastocysts for morphological classification. Expanded blastocysts with a well-defined ICM were classified as grade 1 (a), expanded blastocysts without a well-defined ICM were classified as grade 2 (b) and not expanded blastocysts, without a defined ICM and observable dead cells were classified as grade 3 (c).

**Figure 4.** Relative transcript abundance of *OCT4*, *SOX2*, *NANOG* and *CDX2* genes in domestic cat and cheetah blastocysts generated by IVF, SCNT and iSCNT. All the genes were normalized with the *GAPDH* gene. (A, B) different letters are significantly different within each gene expression ($p<0.05$). Bars refer to SD between replicates. IVF, domestic cat blastocysts generated by in vitro fertilization; Dc1X, domestic cat blastocysts generated by SCNT; Dc2X domestic cat blastocysts generated by SCNT and aggregated during culture; Ch1X cheetah blastocysts generated by iSCNT with domestic cat oocytes; Ch2X

cheetah blastocysts generated by iSCNT with domestic cat oocytes and aggregated during culture.

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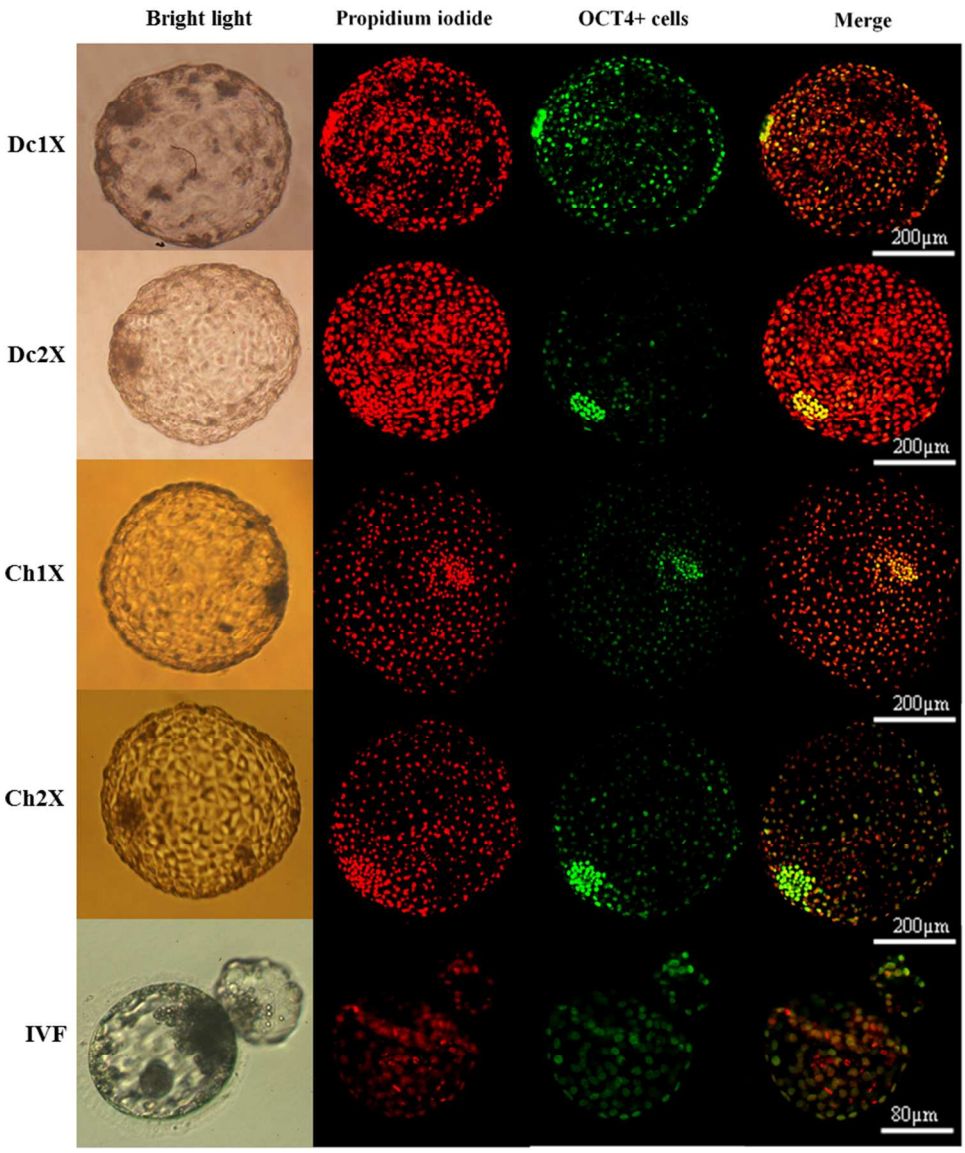


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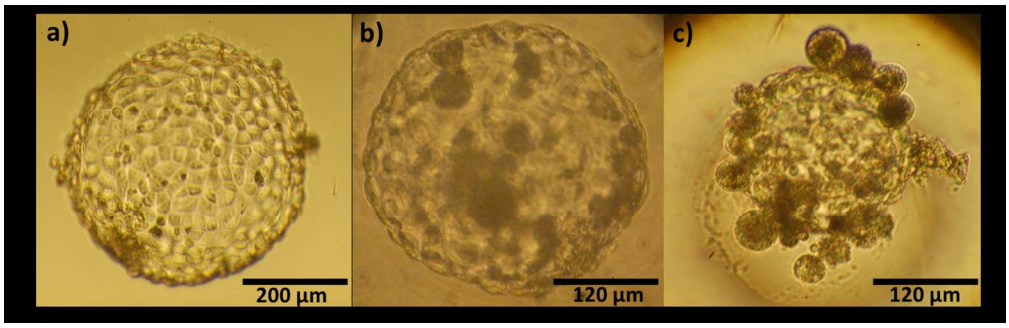


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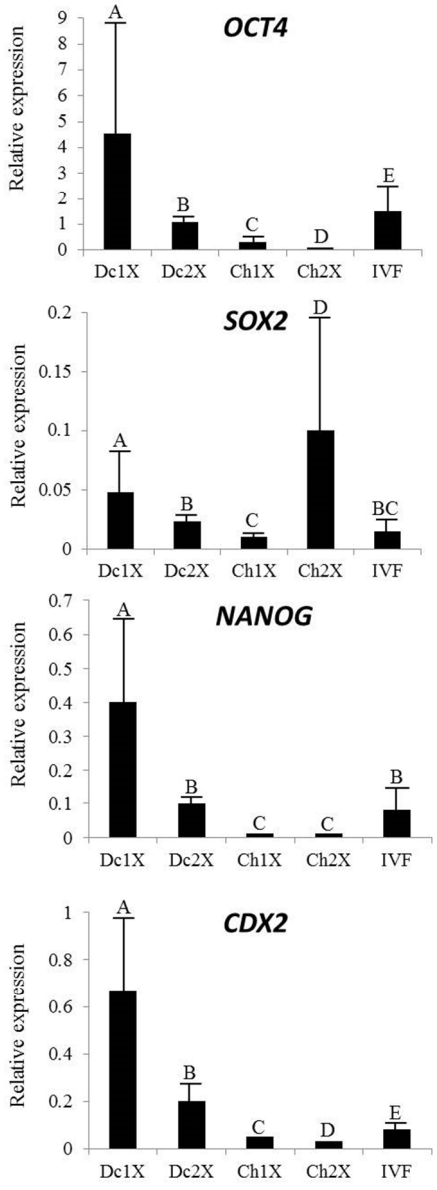


85x224mm (150 x 150 DPI)

**Table 1.** Primer sequences and conditions for qRT-PCR

| Gene name | Primer sequences | Annealing temperature (°C) | Product length (bp) | Accession No. |
|---|---|---|---|---|
| *OCT4* | F: 5′- CCGAAAGAGAAAGCGAACAAG 3′<br>R: 5′- GACCACATCCTTCTCCAGC 3′ | 58 °C | 136 | NM_001173441.1 |
| *NANOG* | F: 5′- CAGCCCCAGATACAGTTACAG 3′<br>R: 5′- GCTGGGCACTAAAATACTTGG 3′ | 58 °C | 115 | NM_001173442.1 |
| *SOX2* | F: 5′- ATGCACAACTCGGAGATCAG 3′<br>R: 5′- TTTATAATCCGGGTGCTCCTTC 3′ | 58 ºC | 132 | NM_001173447.1 |
| *CDX2* | F: 5′- CAGTGAAAACCAGGACGAAAG 3′<br>R: 5′- CCGGATGGTGATGTAACGAC 3′ | 55 ºC | 104 | XM_003980306.1 |
| *GAPDH* | F: 5′- AAGGCTGAGAACGGGAAAC 3′<br>R: 5′- CATTTGATGTTGGCGGGATC 3′ | 58 ºC | 80 | NM_001009307.1 |

**Table 2.** Effects of embryo aggregation on *in vitro* development of domestic cat and cheetah embryos.

| Groups | Replicates | Reconstructed embryos (R.E.) | Cultured embryos (wells) | Cleavage (%) | Compacted Morulae (%) | Blastocysts (%) | Blastocysts/ R.E. (%) |
|---|---|---|---|---|---|---|---|
| Dc1X | 7 | 113 | 113 | 99 (87.6)[a] | 43 (38)[ab] | 31 (27.4)[a] | 27.4[a] |
| Dc2X | | 218 | 109 | 107 (98.2)[b] | 54 (49.5)[a] | 52 (47.7)[b] | 23.8[a] |
| Ch1X | 4 | 102 | 102 | 89 (87.2)[a] | 39 (38.2)[ab] | 17 (16.7)[c] | 16.7[b] |
| Ch2X | | 182 | 91 | 88 (96.7)[b] | 34 (37.4)[ab] | 26 (28.6)[a] | 14.3[b] |
| IVF | 3 | - | 121 | 42 (34.7)[c] | 34 (28.1)[b] | 34 (28.1)[a] | - |

(a,b) Values with different superscripts in a column are significantly different ($p<0.05$, Fisher's exact test). Dc, domestic cat; Ch, cheetah; IVF, in vitro fertilization.

**Table 3.** Domestic cat and cheetah blastocyst quality classified by morphological characteristics at day 8 of embryo culture

| | | Morphological classification | | |
|---|---|---|---|---|
| **Groups** | **n** | **Grade 1 (%)** | **Grade 2 (%)** | **Grade 3 (%)** |
| Dc1X | 31 | 5 (16.1)[a] | 11 (34.5) | 15 (48.4)[ab] |
| Dc2X | 49 | 18 (36.7)[b] | 16 (32.6) | 15 (30.6)[b] |
| Ch1X | 17 | 2 (11.8)[a] | 4 (23.5) | 11 (64.7)[a] |
| Ch2X | 25 | 4 (16)[a] | 6 (24) | 15 (60)[a] |

(a,b) Values with different superscripts in a column are significantly different ($p<0.05$, Fisher's exact test). Dc, domestic cat; Ch, cheetah.

**Table 4.** Total cell number and OCT4 expression in aggregated and not aggregated domestic cat and cheetah blastocysts.

| | n | Cell number mean±SEM | $OCT4^+$ cells mean±SEM | $OCT4^+$cells/Cell number (%) |
|---|---|---|---|---|
| Dc1X | 10 | 385.1±127.4$^{ab}$ | 216.1±103.3 | 51$^{a}$ |
| Dc2X | 12 | 625.7±182.8$^{a}$ | 296.8±118.3 | 47.4$^{b}$ |
| Ch1X | 6 | 144.3±66.6$^{b}$ | 119±58.4 | 82.7$^{c}$ |
| Ch2X | 5 | 400.8±274.2$^{ab}$ | 321.4±96.6 | 80.2$^{d}$ |
| IVF | 8 | 140.7±14.5$^{b}$ | 105±15.8 | 74.6$^{e}$ |

(a,b,c,d,e) Values with different superscripts in a column are significantly different. For blastocyst cell number and OCT4+ cells Proc Mixed was applied ($p<0.05$). For OCT4+ cells/cell number the difference of proportions test was applied ($p<0.05$). Dc, domestic cat; Ch, cheetah; IVF, in vitro fertilization.