Summary of scientific achievement - Marta Czernik

Attachment 2 - (English)

1. *Name and Surname:*

   MARTA CZERNIK

2. *Diplomas and academic degrees (name, place, year of obtaining and the title of the doctoral dissertation):*

   2017 Italian National Scientific Qualification for **Associate Professor** of Veterinary Anatomy and Physiology (07/H1), Italian Ministry of Education;

   2015 **PhD in Veterinary Medicine**: "Development of lyophilization procedure for long-term storage of somatic cells and gametes" Faculty of Veterinary Medicine, University of Teramo, Teramo, Italy;

   2012 **PhD in Biology of Medicine**: "Rola wirusa antygenu T w procesie nowotworowym na przykładzie ludzkiego glioblastoma" in Jagiellonian University, Cracow, Poland based on results obtained in Department of Neuroscience, Centre for Neurovirology, Temple University, Philadelphia, USA;

   2004 **Master of Science**, Faculty of Biology, Jagiellonian University Cracow, Poland.

3. *Information on previous employment in scientific units:*

   2017 **up today** Institute of Genetics and Animal Breeding, PAS, Jastrzębiec, Poland in aim of the project, SONATA 11 (2016/21/D/ NZ3/0210) (Prof. Jacek A. Modliński);

   2007 **up today** Researcher, Faculty of Veterinary Medicine, University of Teramo, Teramo, Italy (Prof. Pasqualino Loi);

   2013 – 2014 Postdoctoral Research Fellow, RIKEN, Bioresource Engineering Division, Tsukuba, Japan (Dr Atsuo Ogura);

   2004 – 2007 Research Scholar in Department of Neuroscience, Centre for Neurovirology, Temple University, Philadelphia, USA (Prof. Kamel Khalili).

4. *Indication of achievement * resulting from z art. 16 ust. 2 ustawy z dnia 14 marca 2003 r. o stopniach naukowych i tytule naukowym oraz o stopniach i tytule w zakresie sztuki (Dz. U. 2016 r. poz. 882 ze zm. w Dz. U. z 2016 r. poz. 1311.)*

a) Title of scientific achievement:

New strategy of nuclear reprogramming in somatic cells before nuclear transfer

b) Publications that take part of the scientific achievement:

1. **Czernik M,** Anzalone DA, Palazzese L, Oikawa M, Loi P (2019). Somatic Cell Nuclear Transfer: Failures, Successes and the Challenges Ahead. ***Int. J. Dev. Biol.*** *63:123 – 130.*

   IF $_{2018}$= 2,4 (5 years IF=2,110); MNiSzW= 25; citation = 0; Q2, percentile: 6/17; 61%: Embryology

2. Palazzese L, **Czernik M**, Iuso D, Toschi P, Loi P (2018). Nuclear quiescence and histone hyper-acetylation jointly improve protamine-mediated nuclear remodelling in sheep fibroblasts. ***Plos One.*** Mar 15;13(3): e0193954.

   IF $_{2017/2018}$= 2,766 (5 years IF=3,352); MNiSzW= 40; Q1; citation = 0, percentile: 16/177; 91%: General Agricultural and Biological Sciences

3. **Czernik M**, Iuso D, Toschi P, Khochbin S, Loi P (2016). Remodelling somatic nuclei via exogenous expression of protamine 1 to create spermatid-like structures for somatic nuclear transfer. ***Nat Protoc.*** Nov;11(11):2170-2188.

   IF$_{2016}$=10,032 (5 years IF=15,269); MniSzW= 50; citation = 2; Q1; percentile: 5/187; 97%: General Biochemistry, Genetics and Molecular Biology

4. Loi P, Iuso D, **Czernik M,** Ogura A (2016). A New, Dynamic Era for Somatic Cell Nuclear Transfer?. ***Trends Biotechnol.*** Oct;34(10):791-797.

   IF$_{2016}$= 11,126 (5 years IF=14,635); MniSzW= 45; citation = 27; Q1; percentile: 5/246, 98%: Biotechnology

5. Iuso D*, **Czernik M***, Toschi P, Fidanza A, Zacchini F, Feil R, Curtet S, Buchou T, Shiota H, Khochbin S, Ptak GE, Loi P (2015). Exogenous Expression of Human Protamine 1 (hPrm1) Remodels Fibroblast Nuclei into Spermatid-like Structures. ***Cell Rep.*** Dec 1;13(9):1765-71. *co-first authorship

   IF$_{2015}$= 7,87 (5 years IF=8,7); MniSzW=40; cytowania = 12; Q1; percentile: 9/187; 95%: General Biochemistry, Genetics and Molecular Biology

6. **PATENT:** Loi P, Iuso D, **Czernik M**, Zacchini F, Ptak G, Khochbin S, Fidanza A (2015). Improved method for reconstructing a non-human animal embryo. (Unia Europejska and Israel) WO2015/162170 or PCT/EP2015/058701, given by European Patent Office.

Total MNiSzW = *200*;

Total IF =*34,194*

Total citations (without self-citations) = 41.

c) *Discussion of the scientific achievement/purpose of the work / and the results achieved, together with a discussion of their possible use:*

New strategy of nuclear reprogramming in somatic cells before nuclear transfer

ABSTRACT

Somatic Cell Nuclear Transfer (SCNT) has a broad spectrum of potential applications, ranging from therapeutic cloning, production of transgenic animals, drug production, regenerative medicine and the rescue of endangered species. It is already more than 20 years since Dolly, the first cloned mammalian was born, but despite several SCNT improvements, mostly technical, its efficiency is still disappointingly low. The low efficiency and the embryo/fetal abnormalities are due to incomplete Nuclear Reprogramming (NR) of the somatic nucleus. NR denotes the capacity of the oocyte to erase the epigenome of a differentiated cell restoring a condition of "totipotency", i.e., the capacity to generate all type of cells composing the body, including extra-embryonic ones. With the approach put forth in this habilitation application, I improved NR by mimicking Nature's nuclear transfer device, the spermatozoon, by conferring somatic cell nuclei the compact, hydrodynamic nuclear structure found in sperm cells what makes SCNT more efficient.

INTRODUCTION

The transplantation of differentiated cell nuclei into a previously enucleated oocyte, has been theorized by Hans Spemann in 1938 (*Spemann, 1938*). The "fantastic experiment", as cited by Spemann, would have been the final proof that cell differentiation does not foresees the progressive elimination of genes throughout development, but that the genome is equivalent in all cells (totipotent). The "fantastic experiment" was successfully carried out first from Brigg and King (*Brigg and King, 1952*) and next from Gurdon (*Gurdon et al., 1958*) twenty years later, when normal individual were produced following the nuclear

transfer of differentiated cells (intestine epithelial cells) info functionally (UV exposure) enucleated *Rana pipiens* and *Xenopus laevis* oocytes. However, nuclear transfer remained a "niche" of basic research mainly because of lack and complication of SCNT techniques.

In the case of mammals, the main restriction was the lack of techniques allowing both: removal of the nucleus from the mammalian oocytes (very small and delicate compared to the oocytes of amphibians), as well as the injection of an exogenous nucleus to it. These problems were overcome at the Department of Embryology of the University of Warsaw, in which the first successful attempts of micromanipulation on mammalian oocytes (mouse) were carried out. It has been shown that microsurgical removal nuclei from the zygote is possible (*Modliński, 1975*) as well as the injection of foreign nucleus (*Modliński, 1978, 1981*). In the Department of Embryology, the University of Warsaw also described, for the first time, the behavior of somatic cell nuclei (thymocytes) in the cytoplasm of activated and non-activated oocytes (*Czołowska et al., 1984*), as well as the method of germ cell fusion using the electric field (*Kubiak and Tarkowski, 1985*) used till today.

Thanks, those discovers reproductive biologists realized potential in SCNT technique as a tool for the multiplication of embryonic cells in farm animals. The first sheep cloned from embryonic cells have been produced by Willadsen (*Willadsen, 1986*); next, scientists optimized the efficiency by inducing cell cycle compatibility between the enucleated oocyte (the cytoplast) and the donor embryonic nuclei (*Smith et al., 1988; Smith and Wilmut, 1989*). Subsequently, work carried out at the Roslin Institute resulted in the production of viable lambs from the nuclear transfer of embryonic cells (*Campbell et al., 1996*), study that lead soon later to the production of the first mammalian cloned from a somatic cell: "Dolly", the lamb (*Wilmut et al., 1997*).

Fueled by these results, SCNT rapidly become a research priority, given its implication for animal breeding, endangered species salvation aging, differentiation, re-differentiation and the production of transgenic animals (*Loi et al., 2001; Galli et al., 2003; Loi et al., 2016;* ***work 4***). My latest survey indicates that 23 animal species, including amphibians, fish, insects, and mammals (14 species) have been cloned successfully so far. Thus, SCNT indeed represents a universal asexual reproductive tool, with important potential application. The problem is that the SCNT efficiency has remained the same of the original report, with some exception of the mouse (*Kishigami, et al., 2007; Ogura et al., 2013; Matoba et al., 2014*). In farm animals, only a small fraction of animals derived from the embryo transfer of cloned embryos develops into normal offspring (1–5%) (*Wilmut et al., 2015*). These findings unequivocally demonstrate that the nuclear organization of somatic

cells is rarely reset by the oocyte. Most embryos fail to develop at any stage of development. In addition, cloned animals adapt with difficulty to postnatal life, and a good proportion of them die within hours/weeks or months after birth (*Hill et al., 199, 2000; Wolf, 2001; Ogonuki et al., 2002*). The high pre- and postnatal mortalities arise largely from placental abnormalities (*Loi et al., 2006; Hill et al., 2014; Czernik et al., 2017*). These abnormal phenotypes derive from incomplete NR of the somatic cell nucleus by the oocyte. NR denotes the reversal of the epigenome established during cell differentiation, and it is normally conferred by the reprogramming factors of the oocyte. A complete NR restores the condition of totipotency, which is the capacity to generate all kinds of cell, including extra-embryonic ones, enabling the SCNT clone to develop into a normal adult. 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.
Thus, **the state of art of SCNT in animals (especially farm) is at stack, and no major solutions for the problem have been worked out.**

Findings related to SCNT and NR in cloned embryos unequivocally demonstrate that the nuclear organization of somatic cells is rarely reset by the oocyte. It is worthy to point out here, that oocytes molecular machinery is used to replace protamine (principal sperm nucleoprotein) with histones (principal somatic cell nucleoprotein), not the opposite. 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.

Now, the male gamete is the perfect nuclear transfer devise. Sperm chromatin is completely remodelled during fertilisation with an efficiency close to 100%. Its DNA is tightly packed around protamine proteins, which confer the sperm nucleus a hydrodynamic shape to easily reach and fertilize the female gamete (*Samans et al., 2014*). Upon entering the oocyte, the sperm genome "springs out" revealing its intrinsic totipotency.

In my opinion, any successful NR strategy must mimic the nuclear reorganization of the spermatozoon. In fact, this is the only nuclear formatting the oocyte has evolved to deal with, whereas 20 years of experiments following "Dolly", with hundreds of thousands of SCNT-derived embryos produced, have invariably shown that the nuclear organization of a somatic cells is hardly reset by the oocyte.
This observation contributed to the formulation of the research hypothesis, which later became the main achievement of the habilitation application, which assumes **that the nuclear**

reprogramming strategy will be effective if somatic nucleus will mimic the architecture of the sperm.

Protamine is the main nucleoprotein of sperm cells, its role in nuclear reprogramming (which occurs with ~ 100% efficacy in mouse fertilization) is not known, but it may significantly affect this process. Thus, to remodelling somatic cell nucleus into the sperm cell nucleus-like structure can significantly facilitate nuclear reprogramming, thus increasing the efficiency of somatic cloning.

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.

Spermatogenesis is perhaps the most complicated differentiation process in the body, and even though scientists have been struggling for over a century to develop a defined system to complete spermatogenesis *in vitro*, little success has been obtained so far. *In vitro* spermatogenesis has been achieved recently in the mouse through an organ culture system (*Sato et al., 2011, 2013; Kim et al., 2015; Reda et al., 2016; Sanjo et al., 2018).* Sato and co-workers show that neonatal mouse testes which contain only gonocytes or primitive spermatogonia as germ cells can produce spermatids and sperm *in vitro*. Authors maintained spermatogenesis over 2 months in tissue fragments positioned at the gas-liquid interphase. The obtained spermatids and sperm resulted in healthy and reproductively competent offspring through ICSI (*Intracytoplasmic Sperm Injection*) (*Sato et al., 2011*). Similar results based on tissue culture technique were shown in rat (*Reda et al., 2016*) and bovine (*Kim et al., 2015*) models. Latest, Sanjo et al., (2018) used for experiment testis fragments collected form adult tissue but unfortunately, the end product was round spermatids (*Sanjo et al., 2018*). But even if scientist would be able to produced functioned sperm *in vitro*, it is impossible to follow the nuclear/cytoplasmic remodelling steps which is crucial to understand spermatogenesis process (*Sato et al., 2015*). Our *in vitro* nuclear remodelling assay thus offers the unparalleled possibility to monitor in real time the nuclear dynamics of protamine-mediated nuclear compaction.

The new method of remodelling the somatic cell nucleus into the sperm cell nucleus *in vitro*, proposed as a my habilitation application, offers unparalleled real-time monitoring of the nuclear dynamics of protamine-mediated compaction.

RESULTS

The aim of the project in which I have been involved in Teramo University (Italy), we attempted to reproduce the nuclear remodelling taking place during spermatogenesis, using sheep fibroblasts as an experimental model.

My first approach to remodel somatic cell nuclei was to induce the co-expression of the main testis specific genes in primary culture of sheep fibroblasts, with the aim to repeat the physiological remodelling occurring in spermatids. To this extend, expression vectors for the bromo-domain testis specific (BRDT-GFP tag), Transition proteins I and II (HA tag), and Protamine 1 (Prm1 - red variant of GFP tag) were generated. Proliferating cells were lyposome-transfected with a cocktail of the 4-transfection vector. Transfected cells were monitored every 24 hours, but no phenotypic changes were observed following 7 days of monitoring.

We therefore opted for a step-wise transfection, starting with Bromo domain testis specific (BRDT-GFP tag). Expression of the vector was approximately 40%, 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 fibroblasts was comparable with previous observation in mouse fibroblasts expressing Brdt (*Pivot-Pajot et al., 2003*). Additional, we have treated cells with Histone Deacetylase inhibitor Trichostatin A (TSA) which augmented the extent of nuclear compaction as expected. However, BRDT-GFP expressing cells did not uptake Transition proteins I and II (HA tag) vectors in the following transfection round, and showed signs of degeneration 96 hours after transfection. Very likely the BRDT-acetylation induced chromatin compaction reduced the bulk cellular transcription, with negative fallout on cell homeostasis.

Thus, our attempts to induce chromatin remodelling of somatic cells following either a co- or a step wise transfection of all 4 expression vectors were ineffective.

The nuclear reorganization occurring in round and elongating spermatids relays on a fine hierarchic translation of previously synthetized mRNA, and each of the protein prepares the ground for the next one (*Barckmann et al., 2013*), a scenario we failed to repeat in cultured somatic cells.

There are exceptions to the canonical nucleosome disassembly pathway during spermatogenesis. In the male germ line of same species of fish, birds and invertebrates for instance, histones are replaced directly by protamine, or protamine-like proteins (*Martinez-Soler et al. 2007*).

Hence, we attempted to induce a direct nuclear remodelling is somatic cells through the transient expression of protamine 1 (Fig. 1). We named this process as "**protaminization of somatic cells**". We selected protamine 1 (Prm1) for the ensuing remodelling experiments firstly because ram spermatozoa contain Prm1 only; secondly, because Prm1 is synthetized as a mature protein, with no further processing (*de Mateo et al., 2011*).

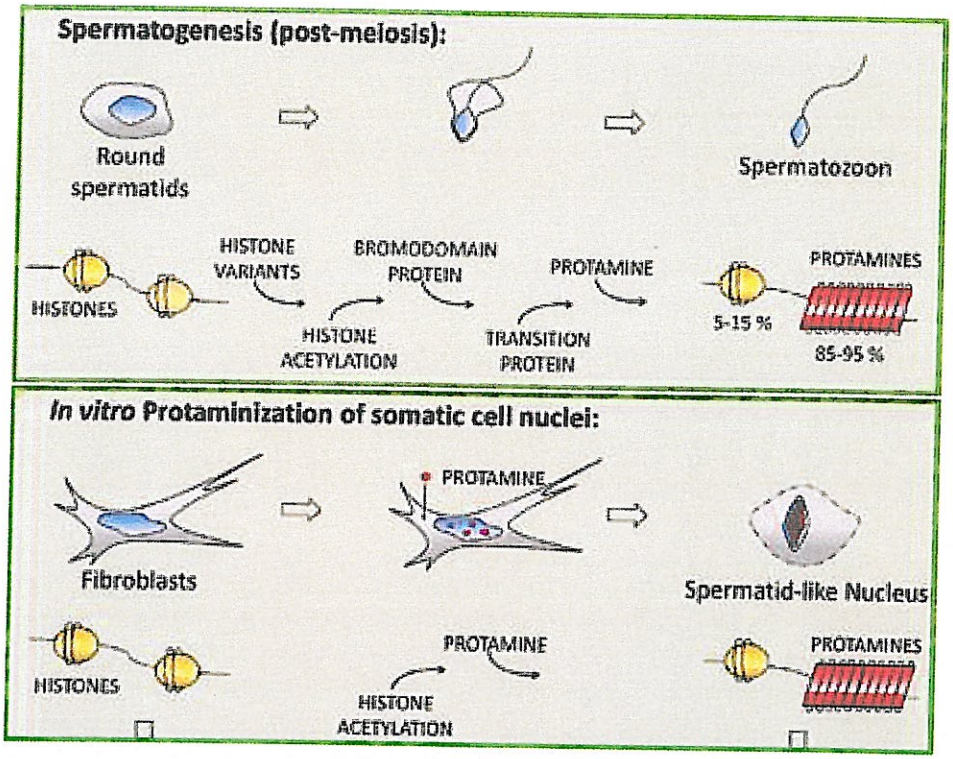


***Figure 1. Schematic representation of protaminization of nuclei of somatic cells** (Iuso, Czernik et al., 2015; **work 5**).*

Morphology of protaminized somatic cells

Very interestingly, we have observed that expression of Prm1 in somatic cells convert intephase nuclei into spermatid-like structure (Fig. 2E). Thanks to the time lapse analysis, we could have visualized incrementally incorporation of the protamine over time, beginning with a few focal points within the nucleus starting 3-4h post-protaminization (Fig. 2B), with the number of foci increasing, scattering throughout the entire nucleus 20h later (Fig. 2C). Finally, a radical nuclear remodelling is achieved within 48 h, with the protaminized nuclei

acquired an elongated structure (Fig. 2D, *Iuso, Czernik et al., 2015; Czernik et al, 2016*; **work 5 and work 3**).

Around 30 h post protaminization, most of the somatic cells expressing Prm1 detach from the dish culture surface and float in the culture medium. Protaminized somatic nuclei were elongated, sometimes hooked, and strongly compact. Somatic cells radically change their nuclear shape 40-43h post transfections (Fig. 2E). The nuclei of somatic cells elongate into spermatic-like structures and they were easily recognizable without fluorescence at this stage. Full protaminization of somatic nuclei were observed at 48 h post transfections, and this is accompanied by the loss of cellular membrane. In this step, the somatic nucleus was strongly elongated and looks like a nucleus of spermatozoon (*Iuso, Czernik et al., 2015; Czernik et al, 2016*; **work 5 and work 3**). Moreover, we have observed that cells expressing protamine die within 48 hours, likely as a consequence of a global transcriptional shutdown, exactly the same process which happened in nature in spermatogenesis process. Mature spermatozoa in fact shutdown transcription.

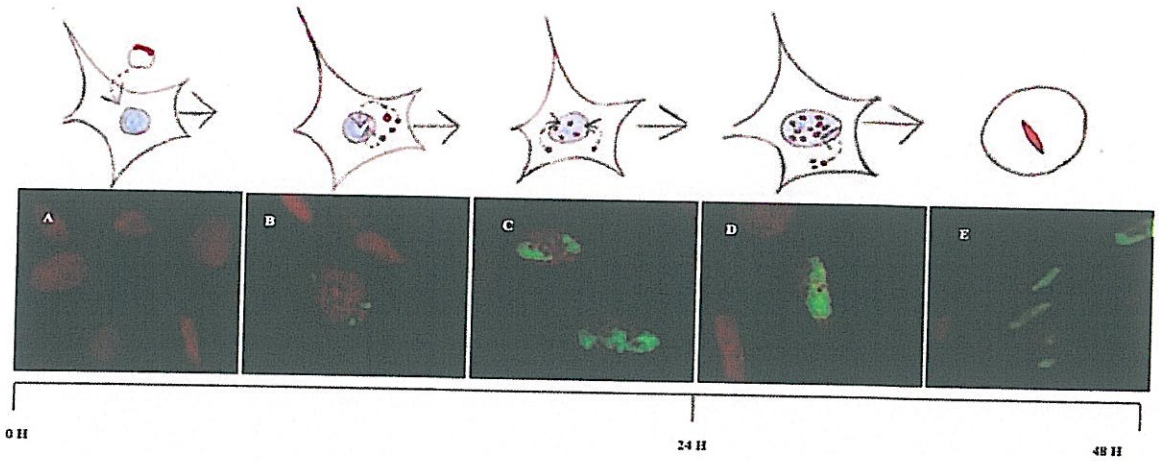


Figure 2. Progressive protamine incorporation into somatic nucleus. *(A) somatic nucleus before protaminization, (B) somatic nucleus after 3-4 hours post-protaminization; (C) protamine foci in somatic nucleus after 24h post-protaminization; (D) 40h post-protaminization; (E) fully protaminized somatic nucleus, 48h post-protaminization. Top pictures present schematic protamine incorporation; Bottom panel: green – somatic cells expressed Prm1-GFP; red - nucleus stained with Propidium iodide (PI)* (*Czernik et al., 2016*; **work 3**).

The protaminization process is rapid and is normally completed within 48 h in 30% of the transfected cells (*Iuso, Czernik et al., 2015; Czernik et al., 2016, **work 5 and work 3***) and pattern has been confirmed in fibroblasts from the following animal models: sheep (Fig. 3A, B) and mouse (Fig. 3C, D) using human (Fig. 3A, C) or mouse (Fig. 3B, C) Prm1 (*Iuso, Czernik et al., 2015; Czernik et al., 2016, **work 5 and work 3***).

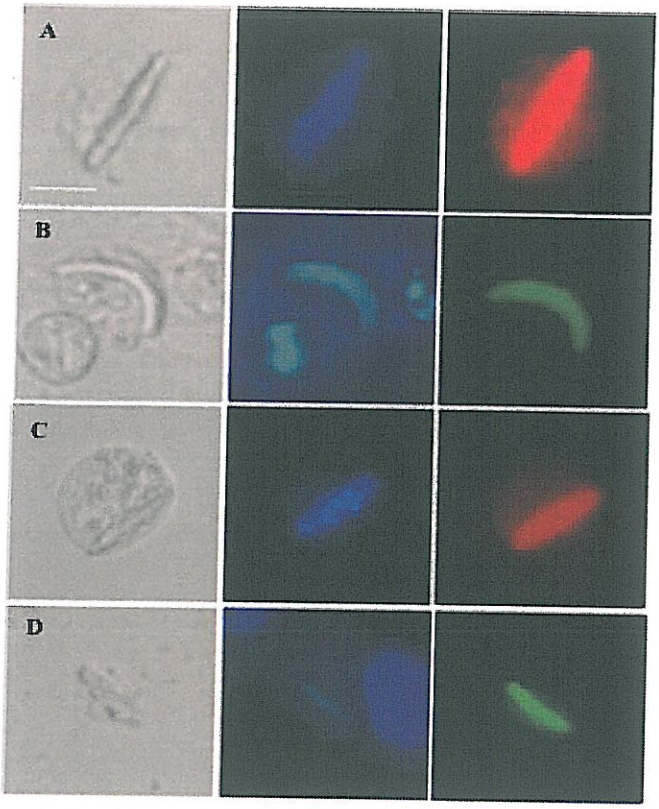


Figure 3. Protaminized adult fibroblasts in sheep and mouse. *Fully protaminized adult fibroblasts in sheep (A, B) and mouse (C, D) using human (A, C) or mouse (B, D) protamine 1. Green: somatic cells expressing Prm1-GFP; red: somatic cells expressing Prm1-RFP; blue: nuclei of somatic cells stained with Hoechst. Scale bar, 5 μm.* (*Czernik et al., 2016;* **work 3**)

The other proof that the protocol, presented as habilitation application, converts interphase nuclei from somatic cells into spermatid-like structures is the shape of protaminizated nucleus. The size of the nucleus of a protaminized cell is larger than that of a spermatid, because the nucleus contains diploid DNA. A fully protaminized, toroidal nucleus of a spermatozoon viewed from the front (Fig. 4C) and the side looks exactly the same as a protaminized somatic nucleus (Fig. 4). Figure 4 shows the similarity between protaminized and spermatic nuclei (Fig. 4B) as compared with a non-transfected somatic nucleus (Fig. 4A) (*Czernik et al., 2016*; **work 3**).

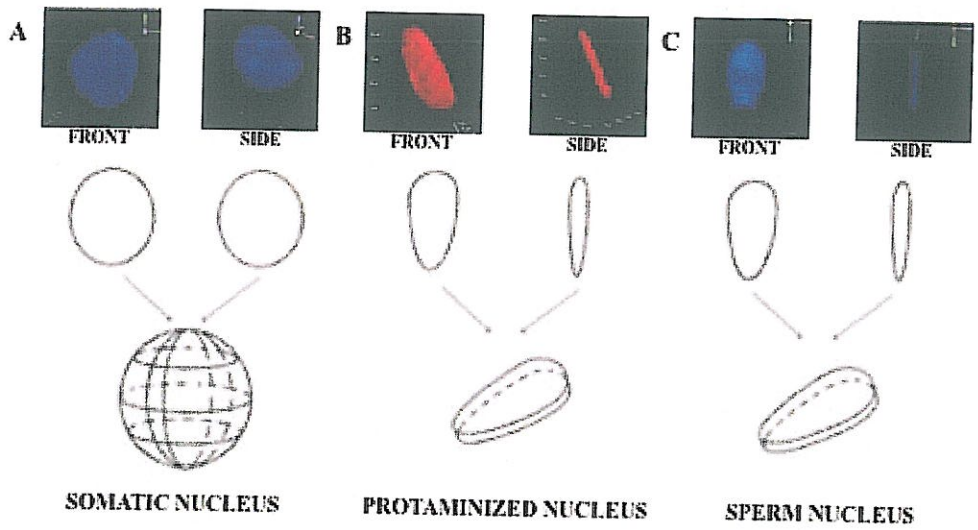


Figure 4. Photographic (top) and schematic (bottom) representation of spermatic and protaminized nuclei. *Scale bar, 5 µm (Czernik et al., 2016*; **work 3**).

Additionally, we have found that protamine sequence affected the shape of the remodelled nuclei. Human PRM1 compacted nuclei in a straight shape (Fig. 3A, C; Fig. 5D), while mouse PRM1 conferred nuclei a hooked conformation (Fig. 3B; Fig. 5F). It is worthy to point out here that mouse PRM1 has three more cysteins than human PRM1, therefore, we hypothesised that extra S-S intra/inter PRM1 bonds might be responsible for the hooked nuclear shape. To test this, we have removed cysteine residues by modifying the mouse *Prm1* gene, starting with cysteine 15 (Prm1-Mut 1) and then 29 (Prm1-Mut 2), and assessed the nuclear remodelling of transfected fibroblasts.

Interestingly, we have found that nuclear remodelling varied according to protamine sequence. In sheep fibroblasts expressing mouse *Prm1* (m*Prm1*) (Fig. 5F), 42% of nuclei compacted in a hooked shape (Fig. 5A, B, F), whereas a high proportion of cells transfected with human *Prm1* (h*Prm1*) (Fig. 5D) compacted in a straight shape (89%) (Fig. 5A, B), each reminiscent of the nuclear morphology of the corresponding mature male gametes and completely differ from somatic nucleus (Fig. 5C). Our data suggest that mouse PRM1 cysteine 15 and, more importantly, cysteine 29 (Fig. 5E), are responsible for the hooked shape of somatic cell nuclei expressing *Prm1*, an action likely modulated by intermolecular disulphide bridges and an extended protamine/DNA interaction. Those results do not make part of the habilitation application but strongly pertinent to this proposal. These findings suggested that protamine sequence might play a role in determining nuclear shape and have been submitted to a Nature Publishing Group Journal (*Czernik et al., (2019). Protamine sequence determines species-specific nuclear shape in a simplified in vitro model of nuclear*

*remodelling. **Nat. Communic.** submitted*). Moreover, part of the results has been performed by master student - Flavia Muccini, under my supervision, and took part of her master thesis "Does Protamine 1 (Prm1) sequence define the species-specific shape of spermatozoa nuclei. An *in vitro* study using mutated Prm1 vectors in somatic cells".

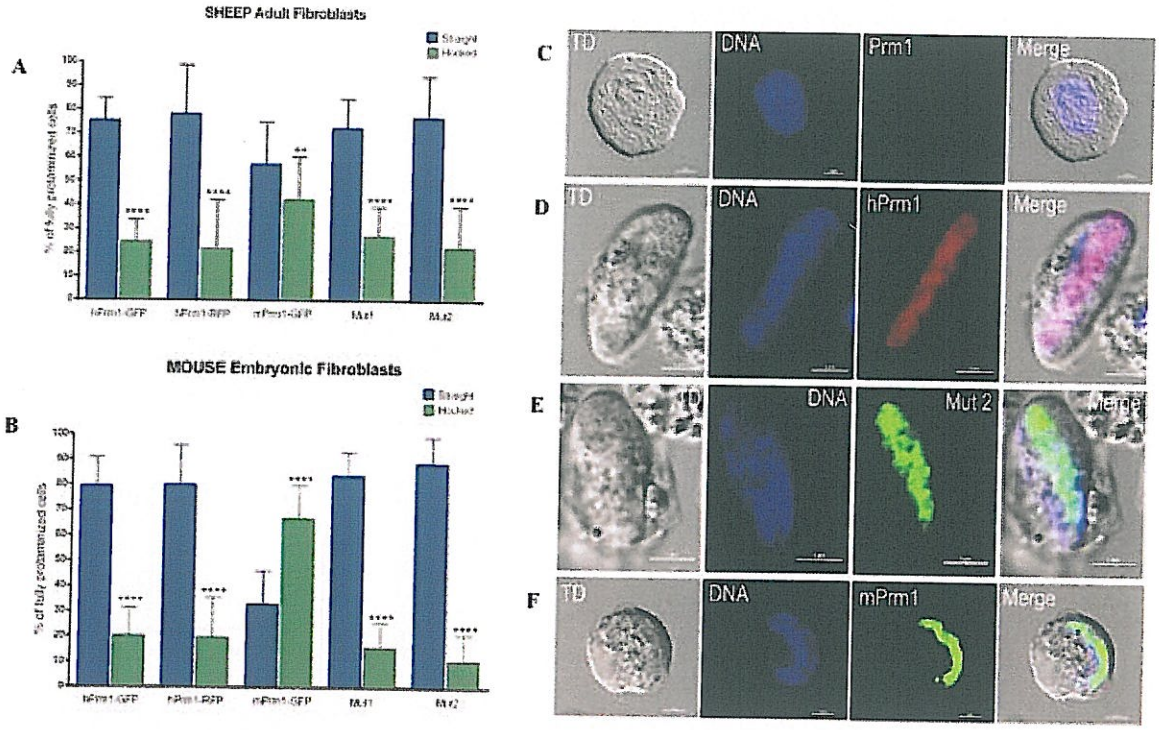


Figure 5. **Remodelling of somatic cell nucleus driven by protamine.** Graphs represent percentage of straight (blue column) and hooked (green column) nuclei in (**A**) sheep and (**B**) mouse fibroblasts expressing wild and mutated *Prm1* vectors (*** denotes $P<0.05$, **** denotes $P<0.0001$). (**C – F**), 2-dimensional structure of somatic cells, (C) non protaminized control, (D) straight somatic nucleus expressing *hPrm1* vector, (E) straight somatic nucleus expressing Mut2, (F) hooked somatic nucleus expressing *mPrm1*. (C - F): bright field (left pictures); blue – nucleus stained with Hoechst 33342, red – *hPrm1*; green – Mut2 on (e) or *mPrm*1 on (F).

Does Prm1 bind to the somatic DNA?

After morphological analysis, we wanted to make sure that Prm1 bind to DNA because Prm1 contains numerous cysteine residues that might form intra- Prm/inter-Prm disulfide cross-links (*Balhorn et al., 1992*). To exclude nuclear Prm1 polymerization, we sort Prm1 – positive cells and performed chromatin immunoprecipitation (ChIP)-seq assay. The ChIP-seq assay confirmed the effective binding of Prm1 to DNA at 42 DNA binding sites on 10 of 27 chromosomes, as early as 16-hr post-Prm1 transfection (*Iuso, Czernik et al., 201;* **work 5**).

Since our principal goal was improvement of somatic cell nuclear transfer we wanted to make sure that protamine efficiently remove /exchange histones from somatic nucleus. To test this, we transfected GFP-H2B fibroblasts with Prm1-RFP. A histone-to-Prm exchange was observed in GFP-H2B nuclei 48 hr after Prm1 transfection (*Iuso, Czernik et al., 2015;* **work 5**). Unequivocally, the red fluorescent signal of Prm replaced the green H2B, confirming the over competition of Prm1 on histones. Furthermore, we observed that the Prm1 replaced the histone H3 trimethylated on lysine 9 (H3K9me3), a critical epigenetic barrier of SCNT reprogramming (*Matoba et al., 2014)* in 30% of (36 of 120) of Prm1-positive cells. H3K9me3 was not longer detectable in fully condensed nuclei totally devoid of H3K9me3 (*Iuso, Czernik et al., 2015;* ***work 5***).

Does protaminization of somatic cells improved SCNT efficiency?

Those very important results, from SCNT point of view, make us more convince that protaminized cells may positively effects on SCNT efficiency. To verify that we decide to use somatic protaminized cells for nuclear transfer using sheep as an experimental model.

Many factors effect on SCNT efficiency. SCNT technique theoretically is a simple technique involve two major steps: the enucleation of matured (MII) oocytes, and subsequently replacing it with a donor somatic nuclei. Scientist, since first cloned animal born, try to improve - theoretically simple - but very invasive technique. The cytoplasm of large domestic animal's oocyte is dark, due to high lipid content, making it necessary to use fluorescence staining Hoechst 33342, ultraviolet (UV) exposure and cytochalasin B treatment during enucleation step. It has been shown that exposure to UV, however, has harmful effects on embryonic development (*Gil et al. 2012*). To avoid UV-related damages, I with my collaborators have been developed a new enucleation method in a large animal model, the sheep (*Iuso, Czernik et al. 2013*). We have showed significantly higher blastocyst rates when SCNT was performed without exposure of the oocytes to UV, as compared to the traditional method. That improved method have been used for SCNT using protaminized cells and have been in details described in Nature protocols paper signed as **work 3** in habilititon application (*Iuso, Czernik et al., 2013*; *Czernik et al., 2016*; **work 3**).

So far, several common errors observed in most cloned embryos were identified in epigenetic information such as DNA methylation, histone acetylation, methylation, and non-coding RNA transcripts. Regulating of these marks improves mammalian cloning efficiency, but mainly in mice model.

One of the well-investigated epigenetic events in embryo development is X chromosome inactivation (XCI), has an essential role for female mammalian cells. Since female cell has two X chromosomes, one copy is silenced for having the same proportion of gene products as that of males. Xist, a non-coding RNA that is transcribed from silenced X chromosome is one of the better indicators to investigate the condition of XCI. In fertilized pre-implantation embryos, Xist shows monoallelic expression from maternal X chromosomes however biallelic expression is observed in both inner cell mass and trophectoderm in cloned mice blastocysts (*Nolen et al., 2005*). Using Xist-deleted donor cells to prevent this ectopic Xist expression improves full-term development more than 7-8 times than that of normal procedures in mice (*Inoue et al., 2010*). Surprisingly, correction of Xist expression improves not only transcripts from X chromosome but also that from autosomes (*Inoue et al., 2010*). Moreover, injection of short interference RNA of Xist into reconstructed embryos represses Xist expression transiently and improves full-term development of male cloned embryos (*Matoba et al., 2011*). Ectopic expression of Xist is also observed in bovine (*Inoue et al., 2010*) and pig (*Yuan et al., 2014; Zeng et al., 2016*). Deletion of Xist from donor cells enhances of long-term development (*Ruan et al., 2018*) and RNAi-mediated knockdown of Xist increases full-term development in porcine cloned embryos (*Zeng et al., 2016*).

Actually, regulating Xist expression is less popular treatment because deletion process is time-consuming and RNAi is only effective in males but not in females (*Oikawa et al., 2013*). In biological aspect, expression pattern of Xist may be different between mouse and other mammals

Other barrier which somatic nucleus represents is methylation on histones which is generally associated with repression of gene transcription this prevents proper gene transcription at zygotic gene activation in cloned embryos. Removal of histone methylation has been achieved by injection a histone lysine demethylase into reconstructed embryos (*Matoba et al., 2014*). This treatment significantly improves full-term development of cloned mice in both sexes (*Matoba et al., 2014*) and macaque monkeys (*Liu et al., 2018*). In addition, injection of KDM4E improves pre-implantation development of bovine embryos. Also, treatment of donor cells with recombinant KDM4D protein also improves pre-implantation development of cloned ovine embryos (*Zhang et al., 2018*). So far, this application is widely used even in human SCNT (Chu*ng et al., 2015*).

Those promising results have been abolished by later publication where the same authors used a combination of *Xist* knockout donor cells and overexpression of *Kdm4*. They achieved more than 20% efficiency of mouse SCNT. However, post-implantation defects and

abnormal placentas were still observed, which can be indicating that additional epigenetic barriers impede SCNT cloning (*Matoba et al., 2018*).

All those methods talking about improvement of SCNT technique as well as reprogramming barriers, advantages and disadvantages are well described in publication presented **as work 1 and work 4** in habilitation application (*Loi et al., 2016; Czernik et al., 2019*; **work 4** and **work 1**).

Those **results may suggest that other improvements of SCNT techniques which are easier and less complicated are needed.**

We have previously shown that Prm1 convert somatic nucleus into spermatic like structure, bind to the DNA and that protamine sequence might play a role in determining nuclear shape in different species. Next, we use protaminized cells for SCNT as detailed described in (*Czernik et al., 2016*; **work 3**). Results shown that, starting from 6h post injection of protaminized nucleus into enucleated oocytes a large pronucleus-like structure was observed in the oocyte, and with time pronucleus were swelling in size (16.2 ±2.3 μm) by 8h. Meanwhile, protamine progressively disappeared in 77% of oocyte injected (28/36, Fig. 6A) and oocyte specific histones variant TH2B began to be assembled in the pronuclei (Fig. 6B). TH2B has recently described as the unique histone variant that plays a key role for nuclear reprogramming (*Shinagawa et al., 2014*), as it guides the protamine-histone chromatin transition post fertilization (*Montellier et al., 2015*).

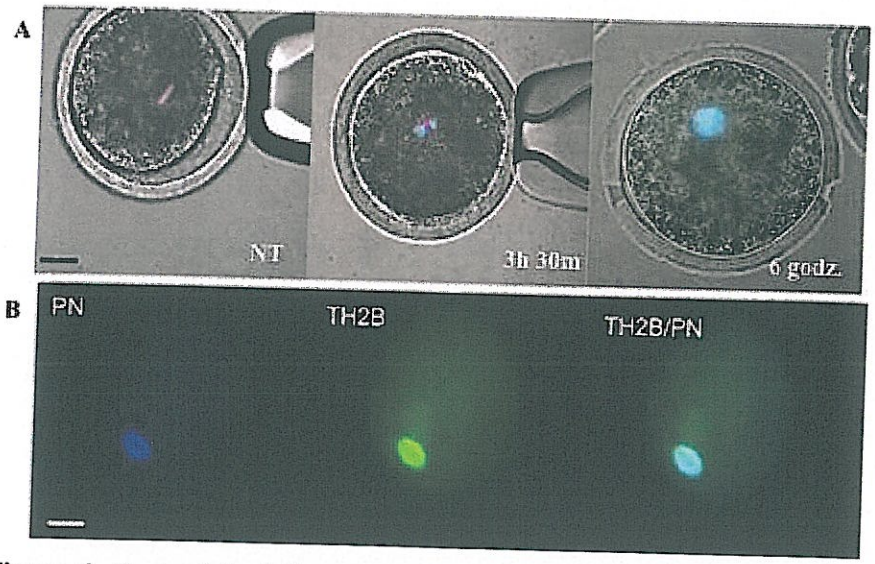
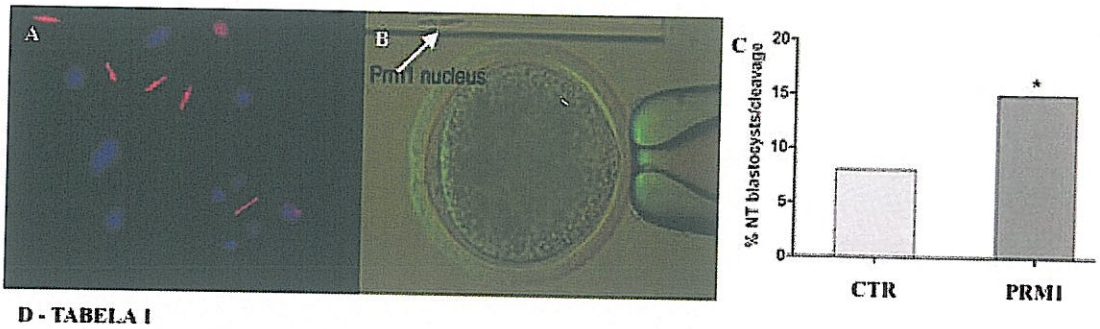


Figure 6. Protaminized Somatic Nuclei Re-acquire a Nucleosome Organization after Nuclear Transfer *(A) Displacement Prm1 during pronucleus formation after nuclear transfer (NT). Prm1 is red, and nucleus is blue. (B) Incorporation TH2B in pronucleus (PN) after NT of protaminized nuclei: pronucleus (PN, blue), TH2B (green), Merge (TH2B/PN). Scale bar represents 20 mm* (*Iuso, Czernik et al., 2015;* **work 5**).

Then, the enucleated oocytes reconstructed with protaminized somatic nuclei (Fig. 7A, B) were further cultured *in vitro*, indicating their full competence to direct early embryonic cleavages until the blastocyst stage. We were observed twice more cleaved embryos when protaminized cells were used (Fig. 7C; *Iuso, Czernik et al., 2015,* **work 5**). Moreover, the quality of the Prm1-embryos (total cell count and karyotype) was comparable to that of normal *in vitro* fertilized (IVF) embryos (Fig. 7D).



D - TABELA I

EXPERIMENTAL GROUPS	TOTAL CELL NUMBER	CELLULAR COMPOSITION (ICM/TE cell number)	KARYOTYPE COMPOSITION (cell with euploid chromosome number)
IVF	80.25±13.28	0.39±0.11	70% (28/40)
NT - Prm1 cells	82.00±15.64	0.40±0.07	72% (31/43)

Figure 7. Protaminised nuclei effect on SCNT efficiency. *(A) SCNT of fibroblast transfected with pPrm1-RFP. Picture represents Prm1 (red)-positive fibroblasts used as donors for SCNT. (B) Protaminized nucleus in injected capillary before nuclear transfer into enucleated MII sheep oocyte. (C) In vitro development of nuclear transfer embryos. (D) Table 1. Quality of blastocysts is as follows: IVF, in vitro fertilized; NT Prm1 cells. Total cell number is mean ± SD. Cellular composition is the number of inner cell mass/trophectoderm cell number. Karyotype composition is the cell with euploid chromosome number divided by total* cells (%) (*Iuso, Czernik et al., 2015*; **work 5**).

The work led to a major breakthrough, published in Cell Reports as a scientific report entitled "Exogenous Expression of Human Protamine 1 (hPrm1) Remodels Fibroblast Nuclei into Spermatid-like Structures", with my name as co- first author (*Iuso, Czernik et al., 2015,* **work 5**). Publication was widely reported in international media: http://www.ilcentro.it/teramo/clonazione-una-nuova-tecnica-scoperta-dall-ateneo-di-teramo-1.1353686; http://ilcentro.gelocal.it/teramo/cronaca/2015/11/27/news/clonazione-una-nuova-tecnica-scopertadall-ateneo-di-teramo-1.12521943?refresh_ce; https://motherboard.vice.com/it/article/pg34e9/cellule-spermatozoi-clonazione.

Moreover, "Exogenous Expression of Human Protamine 1 (hPrm1) Remodels Fibroblast Nuclei into Spermatid-like Structures" data have been presented during the

International Symposium on Future of Nuclear transfer and Nuclear Reprogramming in Yamanashi (Japan) on 10-11 March 2016 and won prize for best work awarded by **Sir John Gurdon**, the father of nuclear transfer (Nobel Prize in Physiology and Medicine 2012). Additionally, the results have been filed as an International patent (PCT/EP2015/058701, *Loi, Iuso, Czernik, et al., 2015,* **work 7**) on the protaminization method in which I am signed as co-inventor. Lastly, protaminized procedure is published in Nature Protocols journal, titled "Remodelling somatic nuclei through the exogenous expression of the protamine 1 to create spermatid-like structures for somatic nuclear transfer into sheep oocytes" (*Czernik et al., 2016;* **work 3**) and reported in two reviews (*Loi et al., 2016; Czernik et al., 2019*; **work 4 and work 1**). To broaden my knowledge of nuclear reprogramming, I applied for and was awarded a competitive postdoctoral fellowship from the Japan Society for the Promotion of Science (JSPS). The project awarded focuses on nuclear reprogramming and was being carried out at one of world's leading SCNT laboratories (that of Prof. A. Ogura, RIKEN Institute, Tsukuba, Japan).

Proposed protaminization of somatic cells nuclei is very optimistic and promising technique but the only think which do not fully unsatisfied us was efficiency of fully protaminized nuclei (30%).

Campbell and co-workers gave a significant breakthrough in research on mammal cloning (*Campbell et al., 1996*). They use a donor nuclei in G0 (state) G0. G0 stage is a stage of the resting state of the cell and exit from the cell cycle. It is characterized by a decrease in the level of transcription, degradation of most types of mRNA, changes in polyrybosomes and chromatin condensation. Campbell and co-workers assumed that this specific state of cells and the nuclear chromatin would enable proper reaction of all dormant genes (and possibly also nuclear structures) which help nuclear reprogramming. In addition, it should be noted that the sperm cell during fertilization is also at resting stage (G0). This observation suggests us first improvement of the protocol do transfect somatic cells with Prm1 in G0 stage.

Next, other big problem of cloned embryos is deacetylation on histones in transferred cell nucleus. Acetylation on histones is generally associated with activation of gene transcription. Zygotic gene activation, that is crucial event for beginning of transcription of zygotic genes, occurs during early embryo development. Therefore, histone acetylation on transferred cell chromatin is important for proper transcription and later embryo development. However, deacethylation is observed in cloned embryos. To repress these errors, several histone deacetylase inhibitors have applied. So far, one of the most effective and commonly

used inhibitors is Trichostatin A (TSA) (*Vanhaecke et al., 2004*). Treatment of cloned embryos with TSA represses deacethylation on histones in embryo chromatin, improves the transcriptional activities at 2-cell stage, and increases the efficiency of full-term development in mice (*Kishigami et al., 2007*). TSA treatment of porcine cloned embryos also improves both pre-implantation and fully development (*Li et al., 2008*). On the other hand, treatment of donor cells also improves preimplantation development of embryos reconstructed with treated cells in buffalo (*Luo et al., 2013*).

Base on that, we decided to refine the method of somatic cell protominization. Dr Luca Palazzese, who I was the auxiliary supervisor of the doctoral thesis, made the protaminization process even more similar to that occurring *in vivo* conditions by starving cells for 48 h in low % of serum (to synchronise all cells in G0 stage) and treat them with TSA to decrease deacetylation on histones in transferred cell nucleus (*Palazzese et al., 2018*; **work 2**). Results show that combination of cell culture conditions in 50 nM TSA, is more effective in terminating cell proliferation than starvation only and control groups (respectively 8%, 17.8% and 90.2% $p<0.0001$). Finally, starvation and 50 nM TSA jointly increased the number of fully protaminized nuclei (spermatid-like cell) (45%) (Fig. 8C; *Palazzese et al., 2018;* **work 2**). Currently there are ongoing experiments to check whether ameliorated protaminization technic affect on cloned embryo development.

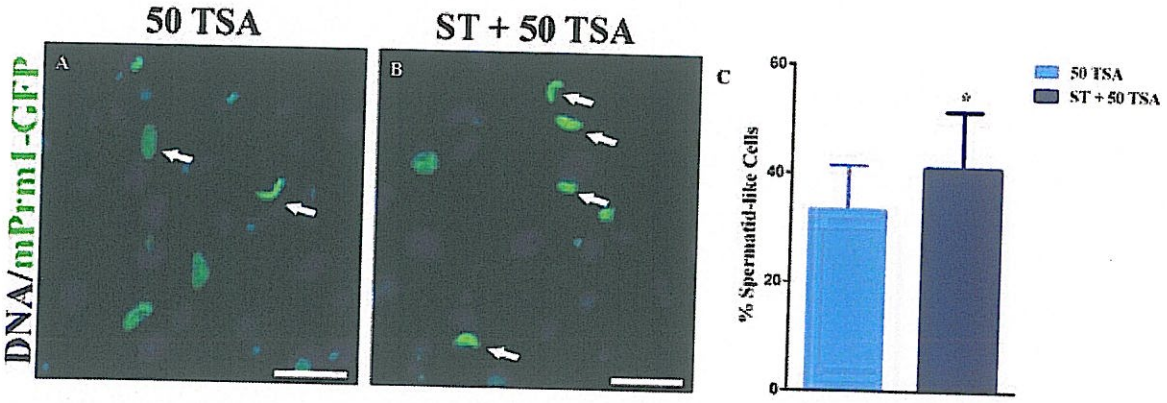


Figure 8. Number of spermatid-like cells at 48 h post-transfection. (A) Pictures of protaminized somatic nuclei *treated with 50 TSA (A) or ST + 50 TSA (B) at 48 h post-transfection. White arrows indicate spermatid-like cells. (C) Graphic representation of the number of spermatid-like cells at 48 h post-transfection. *mean value P = 0.0301. Scale bars represents: 20μm.*

The current efficiency of somatic cell nuclear transfer (SCNT) hampers its practical application. The strategies put forth to improve its efficiency have had a negligible effect in farm animals, and minimal advancements have been achieved only in the mouse. The

remodeling of an interphase nucleus of a somatic cell into a spermatid-like structure achieved by my group is the most recent, and promising approach published so far (*Iuso and Czernik et al., 2015; Czernik et al., 2016; Palazzese et al., 2018;* **work 2; work 5; work 3**).

Nuclear Reprogramming is a priority research topic worldwide for its implication in the advancement of Regenerative Medicine. SCNT remains still the best nuclear reprogramming tool, capable to erase the epigenome of a differentiated cell rapidly, within 24 hours, with high frequencies.

While the oocytes lack the molecular mechanisms to reprogram the complex nuclear structure of a somatic cell, formatting its nucleus like a spermatid one improved the extend of NR, and in turn SCNT efficiency. A reliable SCNT could be a unique tool to counteract the biodiversity decline, taking place on the planet. Far from suggesting SCNT as a routine reproductive tool, it might be applicable in critical situation, like the case of white Sumatra's rhinoceros for instance. The population has collapsed to two individual only, females, all sterile (*Saragusty et al., 2016*). SCNT might represent perhaps the only instrument to save this endangered species from the brink of extinction. SCNT might nicely complement the recent technique of genome manipulation like "genome" editing (*Laufer and Singh, 2015*). Genome editing empowers us to insert/abolish a gene conferring peculiar productivity characteristic, or the environmental adaptation genes to the global warming, in the cells, and use them for SCNT.

The main pioneering nature of the habilitation application is a major breakthrough for inducing a better nuclear reprogramming in somatic cells nuclear transfer (SCNT) by simply following Nature's nuclear transfer strategy: conferring somatic cell nuclei the same chromatin organization of a spermatozoon. My recent work has shown statistically higher percentages of embryonic development to blastocyst stage when "protaminized" cells were used for SCNT, compared to control, un-transfected fibroblasts (14% vs 7%) (Fig. 7C; *Iuso, Czernik et al., 2015;* **work 3** and **work 4**). Moreover, for pioneering nature of the discover votes the fact that, this is first nuclear reprogramming improvement proposed in large animal in the last 20 years, when original report was posted (*Wilmut et al., 1997*). Additionally, the main advantage of my habilitation application project in somatic cells protaminization technique is that is straightforward, fast (only 48h for full protaminization of nucleus), highly efficient and no advanced instruments, or particular molecular biology skills are required to carry out the protocol which make him universal.

5. *Additional scientific interests*

a) Deregulated expression of mitochondrial proteins affects development of cloned embryos: a rescue strategy (*in aim of the 2016/21/d/ NZ3/0210 SONATA, Narodowe Centrum Nauki (Principal Investigator*).

Investigations into mitochondria in SCNT have been limited to mitochondrial DNA (mtDNA) hetero/homoplasmy in the tissue of cloned offspring (*Bowles et al., 2007; Lee et al., 2010*), whereas no data are available on the eventual effect of mitochondrial dysfunction on the developmental failure of clones. Thus, the effect of mitochondrial dysfunction on SCNT pre-implantation embryos and offspring health remains unknown.

The mitochondrial genome transcribes only 13 proteins, while the remainder (1,500-2000) are encoded by the nucleus, where the crucial mitochondrial genes have been transferred to benefit from more accurate (error-free) DNA replication. There is a great deal of communication between the nuclear and mitochondrial genomes, and this communication strictly controls mitochondrial function (*Chappel, 2013*). It is important to remember that another reason for the low efficiency of SCNT embryos and abnormalities is incomplete NR of the somatic cell nucleus. It may be that incorrect NR does not activate the mitochondrial genome properly and this causes the malfunction of mitochondria, hence the abnormalities in cloned placentas and foetuses.

Mitochondria undergo numerous periods of fusion and division, which allows the mixing of damaged and high-quality mitochondria, and therefore an overall decrease in the pool of poorly performing mitochondria. There are three proteins involved in the fusion process: mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), and Opa1 (*Santel et al., 2001; Chen and Chan, 2010*). Mfn2 participates not only in the fusion process, but also bridges the mitochondria to the endoplasmic reticulum, and participates in calcium (Ca^{+2}) homeostasis and controlled cell death (*Kasahara and Scorrano, 2015*). Moreover, and of pertinence to this topic, Chen et al. (2003) found that mice deficient in Mfn2 died in mid-gestation mainly due to placental abnormalities, particularly disruption of the trophoblast binuclear cell layer. This observation has also been confirmed in humans: work by Pang et al., (2013) showed that deficient expression of Mfn2 in human trophoblastic cells might be responsible for unexplained early miscarriage.

Based on this evidence, I **hypothesize** that placental abnormalities and death of SCNT foetuses might result from deregulated expression of mitochondrial protein, a consequence of mitochondrial dysfunction. This hypothesis is supported by preliminary results which show

MCz

that damaged and malformed mitochondria, as well as swollen endoplasmic reticula in early placentas collected from cloned sheep are associated with deregulated expression of Mfn2 (*Czernik et al., 2017*).

SONATA project aims to gain a functional and molecular understanding of the intrinsic role of mitochondria in cloned embryo development, a topic never studied before. Project has the potential to improve SCNT efficiency by the injection of Mfn2, a mitochondrial protein, and hence partially or totally eliminate placental abnormalities. Since those placental abnormalities are a major cause of perinatal death in cloned embryos, our results may significantly improve cloned pre- and post-implantation embryo development. Moreover, preliminary results shown for the first-time fusion process in mouse embryos and confirm the role of nuclear reprogramming in placental/mitochondrial abnormalities.

b) Improvement of cloned blastocyst quality upon CHIR treatment of donor cells *(in aim of Japan Society for the Promotion of Science (JSPS) Fellowship, Japan, Tsukuba, RIKEN with collaboration with Teramo University, Teramo, Italy).*

The activation of WNT-pathway by inhibiting glycogen synthase kinase 3β (Gsk 3β) (CHIR99021) maintain the undifferentiated state of human and mouse embryonic stem cells, preserving the expression of main pluripotent genes (*Cook et al., 1996; Fagotto et al., 1997*). My JSPS fellowship aimed at verify whether CHIR-treated donor cells were better reprogrammable upon nuclear transfer. Mouse fibroblast were treated with CHIR for 24h and used as donors for SCNT. Then the subsequent *in vitro* quality and development of the reconstructed embryos were assessed. Results showed significant differences in the cleavage rate (Fig. 9A; 63% CTRL to 72% CHIR). There were not statistical differences observed in blastocyst formation (Fig. 9A; 24.2%vs 21.6%) but CHIR cloned blastocysts exhibited very robust, better-expanded morphologies than CTRL ones (Fig. 9B, C, D). Those results shown that donor cells, treated with Gsk 3β inhibitor, are easier reprogrammed upon nuclear transfer and help to improve quality of preimplantation cloned embryos. In ongoing experiments, we are trying to optimise the protocol to allow this approach to be effective on higher efficiency of live cloned offspring. Preliminary results are under review in **Theriogenology** (*Czernik et al., (2019). Improvement of cloned blastocyst quality upon CHIR treatment of donor cells, Theriogenology, submitted*).

			Number (%) of embryos that developed to		
A	**# Oocytes**	**Fusion rate**	**2-cells (%)**	**4- cells (%)**	**Blastocysts**
CTRL	231	**91/231 (39%)**	57/91 (63%)	26/57 (45.6%)	**22/91 (24.2%)**
CHIR	151	**97/151 (58%)**	70/97 (72%)	40/70 (57%)	**21/97 (21.6%)**
shame manipulation	32	-	28/32 (87.5%)	28/32 (87.5%)	26/28 (81%)

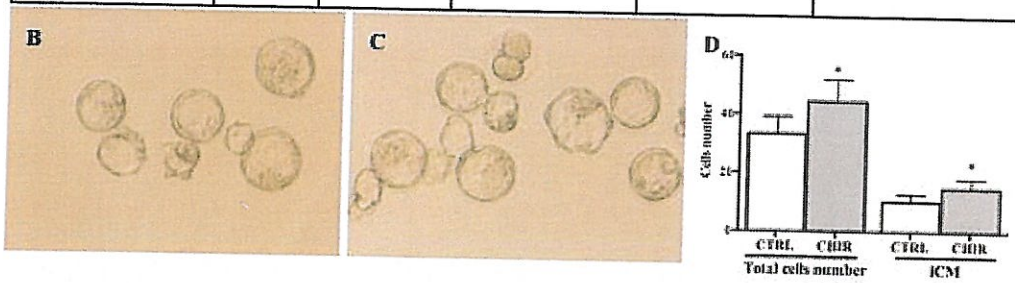


Figure 9. CHIR treated cells improved quality of the mouse cloned blastocysts. *(A) Nuclear transfer (NT) of tail tip fibroblasts (TTFs) (CTRL and CHIR) and subsequent in vitro embryo development in mouse models. P<0.05 (Fisher's exact probability test). Pictures of the cloned blastocyst produced by CTRL (B) and and CHIR (C) treated tail tip fibroblasts. Total and ICM cells number (B) of mouse blastocyst produced by NT using CTRL and CHIR treated TTFs as donors. Error bars indicate ±SEM, *p<0.05*

c) Late Embryogenesis Abundant (LEA) proteins confer water stress tolerance to mammalian somatic cells [*in aim of the H2020-MSCA-RISE-2016 project, titled: "Setting an interdisciplinary/sectorial/international research network to explore dry storage as an alternative strategy for cells/germplasm biobanking" - DRYNET, no. 734434 (Co-Principal Investigator)*].

As stated earlier, cloning efficiency does not allow yet its application, among the other things, to the salvage of animals threatened for extinction. Therefore, the establishment of genetic banks, in form of cell lines, from rare animals, is a wise endeavour. The number of cryopreserved biobanks of biological materials, not only from endangered species, but also for cell replacement therapies of toxicology studies, is increasing exponentially, representing an economic burden. In aim of the DRYNET project, where I am listened as co-principal investigator, we are trying to develop solutions for a dry storage of biobanks, as simpler, cheaper and environmental friendly alternative method to the current deep-frozen storage practices. Here, we show that natural xeroprotectants (Late Embryogenesis Abundant proteins) transiently expressed in somatic cells confer them desiccation tolerance.

Water is essential for life (*Hand et al., 2007*) yet many organisms are able to survive almost completely dehydrated (>99% of their body water is removed) (*Crowe et al., 1992*). In nature, this phenomenon is known as "anhydrobiosis", and is conserved across vegetal and

animal phyla (*Hincha and Thalhammer, 2012*). Anhydrobiosis allows seeds and small invertebrates to survive long time spans (decades/centuries) in the absence of water, thanks to the induced synthesis of sugars and various protein classes that can be collectively defined as "*xero-protectants*" (*Loi et al., 2013*). Among them, Late Embryogenesis Abundant proteins (LEAp) are the best characterized and perhaps the most interesting (Mar*unde et al., 2013*). LEA proteins were first discovered in cotton seeds more than 30 years ago (*Dure et al., 1981*) and were later also found in seeds and vegetative tissues of several other plants (*Shih et al., 2008*). A relatively recent survey, probably not updated, contains 769 LEAp entries from 196 organisms (*Hunault and Jaspard, 2009*). Late Embryogenesis Abundant (LEA) proteins are commonly found in plants and in other organisms capable of undergoing reversible dehydration, a phenomenon termed "anhydrobiosis". Here, we have produced tagged version of three LEA proteins: pTag-RAB17-GFP-N, *Zea mays* dehydrin-1dhn, expressed in the nucleo-cytoplasm; pTag-WCOR410-RFP, *Tricum aestivum* cold acclimation protein *WCOR410*, binding to cellular membranes, and pTag-LEA-BFP, *Artemia franciscana* LEA protein group 3 that targets the mitochondria. Sheep fibroblasts were transfected with individual vector or co-transfected with all three. The LEA proteins were detected in the expected subcellular localization (cytosol/nucleus, membranes and mitochondria, respectively). Then, fibroblasts transfected with single or all three LEA proteins were subjected to desiccation under controlled conditions, followed by rehydration, viability assessment and membrane/mitochondria functional tests. Control, un-transfected cells, were almost all not viable after four hours of desiccation (1% cell alive), while cells expressing LEA proteins showed high viability (more than 30%); the highest viability (58%) was observed in fibroblasts expressing all three LEA proteins. Growth rate was markedly compromised in control cells, while LEA-expressing cells proliferated at a rate comparable to non-desiccated cells.

Plasmalemma, cytoskeleton and mitochondria appeared unaffected in LEA-expressing cells, confirming their protective action during the entire desiccation and rehydration process. Our results confirm that LEA proteins protect somatic cells from desiccation injury and the combined effect between LEA proteins. Our findings advance the knowledge on the physiological response of mammalian cells protected from desiccation-related damages through the expression of LEA proteins, toward to ultimate goal of establishing biobanks of living cells at room temperature. Results are under review in Scientific Report Journal (*Czernik et al., (2019). Late Embryogenesis Abundant (LEA) proteins confer water stress tolerance to mammalian somatic cells,* ***Scientific Reports****, submitted*).

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