Early epigenetic reprogramming in fertilized, cloned and parthenote embryos

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Abstract:

Despite ongoing research in a number of species, the efficiency of embryo production by nuclear transfer remains low. Incomplete epigenetic reprogramming of the nucleus introduced in the recipient oocyte is one factor proposed to limit the success of this technique. Nonetheless, knowledge of reprogramming factors has increased -thanks to comparative studies on reprogramming of the paternal genome brought by sperm upon fertilization- and will be reviewed here. Another valuable model of reprogramming is the one obtained in the absence of sperm fertilization through artificial activation - the parthenote- and will also be introduced. Altogether the objective of this review is to have a better understanding on the mechanisms responsible for the resistance to reprogramming; not only because it could improve embryonic development but also as it could benefit therapeutic reprogramming research.

Keywords: Oocyte ; Nuclear transfer ; Embryonic genome activation ; Histones post-translational modifications ; DNA methylation

Introduction to Nuclear Reprogramming

The cells of an adult mammal show a striking variation in structure and function, conferred by the differential expression of tightly regulated and specific gene networks. With few exceptions, individual cell types have been shown to retain the entire genetic content of the totipotent embryo. Yet, specific gene expression patterns associated with differentiated cell states are highly stable and conserved after somatic cell division [1]. The process of restricting expression to lineage-appropriate subsets of genes is ongoing throughout development and is now understood to reflect an accumulation of "epigenetic" changes at specific gene loci [2] and [3]. The term epigenetics, coined by Conrad Waddington in the 1940s, is now used to refer to "the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail changes in DNA sequence" [4] and [5]. These changes include the large scale positioning of chromosomes and genes within the nucleus as well as local modifications to DNA and chromatin [6] and [7]. Epigenetic changes affect the accessibility of DNA to the transcription machinery, hence, gene expression [6] and [7]. Local modifications include histone posttranslational modifications (PTMs such as methylation, acetylation, phosphorylation, and so forth), DNA methylation, and remodeling of the chromatin [4] and [8]. Moreover, all these local modifications may specifically recruit factors, as in recruitment of bromodomain proteins to acetylated histones and of chromobox family proteins to methylated histones [9].

Each differentiated cell type has a specific profile of epigenetic modifications at key loci, resulting in expression of only type-appropriate genes. Deviations from this profile in vivo are frequently associated with disease [10]. It is also increasingly recognized that deviations from normally observed epigenetic patterning can contribute to the altered cell behavior found by cancer cells [11]. On the other hand, alteration of these epigenetic modifications with the aim of conferring a more developmentally plastic cell state is referred as nuclear reprogramming

and is attempted experimentally via a number of different techniques [12] and [13]. The first amphibian and mammalian cloned animals were achieved by inserting a donor nucleus into an enucleated recipient oocyte [14]. In this approach (cloning by nuclear transfer [NT]), the oocyte has to reprogram the injected nucleus, trying to mimic reprogramming of maternal and paternal DNA during natural fertilization (Fig. 1) [15]. Mammalian nuclei have also been reprogrammed by transfer to the germinal vesicle of Xenopus oocytes [13] and [16] or by the fusion of donor cells with an "embryonic dominant" cell type [12]. These techniques use the natural reprogramming abilities of oocytes, embryos, and embryonic cells, without requiring knowledge of the precise factors required for reprogramming. However, as knowledge of reprogramming factors has increased, alternative techniques involving exposure of cells to specific combinations of transcription factors have grown in popularity. Nowadays, somatic cells can be virally transfected, at least in mouse, with no more than four key transcription factors (Oct4, Sox2, Klf4, and Myc) to induce pluripotency (Fig. 1) [17]. The availability of induced pluripotent stem cells (iPS cells) from different species is also increasing rapidly [18], although the underlying molecular mechanisms remain to be investigated. Specific combinations of transcription factors have also been used to switch directly from one cell type to another, a process known as transdifferentiation [19].

The goals of this experimental nuclear reprogramming are twofold. First, to elucidate the roles of different epigenetic marks (and associated protein complexes) in nuclear reorganization at fertilization and during development and, second, to develop applications that benefit to human health. Such applications include the reprogramming of readily accessible cell types such as dermal fibroblasts to produce cell lines (iPS cells) to be used for drug screening or study of disease pathways [20] and [21]. These iPS cell lines could be used to select the most effective treatment for the individual patient or for the production of cells and organs for autologous

transplants without the ethical or immunological problems associated with allogeneic transplantation [20] and [21].

As a research tool, nuclear reprogramming continues to yield insights into the mechanisms and complexes involved in differential control of gene expression [13]. Despite this, and successful cloning experiments in a wide range of species, the efficiency of all techniques, as measured by proportion of nuclei leading to developmentally plastic cells or healthy adult animals, remains very low. Considering the possible therapeutic benefits of successful nuclear reprogramming, there is a great deal of interest in understanding the mechanisms responsible for this resistance to reprogramming.

Reprogramming at Fertilization

In mammalian species, the formation of the embryo begins with the fusion of two highly specialized haploid cells (sperm and oocyte) which gives place to a genetically new diploid organism: the zygote (or 1-cell stage embryo) with two haploid "pro" nuclei, the paternal and the maternal one (Fig. 1). The "early mammalian" or "preimplantation" embryo development compresses the time from fertilization until the implantation of the embryo in the mother's uterus. During this period of development, epigenetic reprogramming of the genome inherited from the gametes is crucial [22] and [23]. Indeed, during the formation of gametes, both oocyte and sperm cells are subjected to epigenetic changes that permit the expression of specific genes required for germ cell development. As gamete maturation is near to completion, a reorganization of the genome occurs. Paternal genome becomes highly methylated and compact as histones are replaced by protamines [24] and [25]. On the other hand, the oocyte undergoes a chromatin restructuring from a nonsurrounded nucleolus (open chromatin with few defined chromatin surrounding the nucleolus and transcriptionally active) to a surrounded nucleolus

conformation (highly condensed chromatin with clear presence of chromatin around the nucleolus and transcriptionally silent) (Fig. 2) [26] and [27].

From fertilization, both the incoming paternal DNA complement and that of the oocyte itself are reprogrammed in a number of steps, resetting chromatin to the embryonic form capable of undergoing further changes required during development [28] and [29]. The defined epigenetic status of the previous gametes' genome must now turn into a whole new epigenome proper of an early embryo with totipotent capacity [23], [30] and [31]. To do so, the paternal and maternal genome undergo global demethylation, and although many studies have led to contrasted results regarding the dynamics and the extend of this demethylation [32], it appears that the demethylation process continues after the first cell cycle in the preimplantation embryo up to the blastocyst stage in many mammals [33], [34], [35], [36] and [37]. At this point, the first cell lineage determination takes place (the formation of the inner cell mass (ICM) and of the trophectoderm (TE)) and new methylation patterns emerge together with cell differentiation and specialization until the whole organism is formed [35], [38] and [39].

In addition to this DNA demethylation occurring after fertilization, it has been shown in mouse that many of the histones replacing the protamines on the paternal genome are already acetylated such as lysines 8 and 12 of histone H4 [40]. Moreover, for a correct development, the paternal pronuclei has to be hyperacetylated with the further acetylation of lysines 5 and 16 of H4 and lysines 9, 14, 18, and 27 of histone H3 [31], [41] and [42]. On the other hand, some histone PTMs such as trimethylation of lysine 20 on histone H4 and trimethylation of lysine 9 on histone H3 (H3K9me3, Fig. 2) are inherited exclusively from the maternal pronucleus, creating an asymmetry between the two parental genomes in the embryo (it would not be possible to include in this work all known histone PTMs, their fluctuation and their roles; for a complete review of known histone PTMs see [30]). These asymmetries persist for varying lengths of time in the developing embryo. For an example, lysine 4 methylation on histone H3

is evenly distributed throughout DNA by the two-cell stage [42], whereas H3K9me3 remains asymmetrically distributed until the four-cell stage [30]. Other modifications are found to differ from the ICM and TE cells, such as H4/H2AS1P which is much frequent in the nucleosomes of TE than ICM cells [43] or the general methylation of H3K27 which is found only in the ICM, whereas in the TE it is only present in the inactivated X chromosome [44].

The function of this asymmetry just after fertilization has not yet been fully understood, although it is thought to be required for a proper development. Indeed, embryos are transcriptionally silent until the end of the one-cell stage, when a small number of embryonic genes are transcribed from the paternal genome [45] and [46]. This asymmetrical minor activation is followed by the major embryonic genome activation (EGA) later on, associated with a much more frequent rate of production of transcripts and the number of genes transcribed [46] and [47]. The reprogramming of histone modifications has been proposed to be significant for triggering transcription and EGA, correlating the accumulation of transcriptionally permissive marks on the paternal genome and minor activation and between more widespread reprogramming and EGA [48], [49] and [50]. Among the differences observed in preimplantation embryo between mammalian species, the timing of embryo genome activation is a major one. In mouse embryos, EGA occurs at two-cell stage, whereas in bovine and rabbit embryos it occurs at the eight-cell stage [51] and [52]. Remarkably though, it is believed that the fourth-fifth cell cycle in the bovine embryos is critical for chromatin remodeling and embryos that are unable to modify their chromatin structure for gene activation arrest at this stage. For example, distribution of H3K27me3 has been studied semiquantitatively in bovine embryos, where levels were found to decrease from oocytes to their minimum at eight-cell stage, corresponding with EGA [53] and [54]. The decline in H3K27me3 is independent of cell division, indicating an active removal mechanism, where histone demethylase KDM6B has been implicated as the enzyme catalyzing the removal [55]. Similarly, it appears that sheep

oocytes and embryos have a specific Dnmt1 transcript involved in DNA methylation maintenance whose levels decrease when the embryonic genome becomes active at the 8/16-cell stage. Interestingly, reducing Dnmt1(12b) by RNA interference prevents embryo compaction at the morula stage, showing the importance of DNA methylation for embryonic preimplantation development [56].

Therefore, it seems that although the dynamics of some epigenetic marks are not conserved between all mammalian species, they are always closely related with the formation of an "open" chromatin state allowing gene expression regulation during preimplantation development.

Reprogramming after cloning by Nuclear Transfer

Cloned embryos are the result of the enucleation of an oocyte and transfer of the diploid nucleus from another cell (Fig. 1). After such NT procedure, donor cell nuclei often get an incomplete reprogramming which is thought to lead to abnormal development in clones [15]. In particular, the donor chromatin needs to undergo epigenetic changes and modifications to get an embryonic-like chromatin structure as seen in sheep, mouse, bovine, and rabbit NT embryos [57], [58], [59] and [60]. The timing and manner to achieve this conformation will depend on the type of cell used as donor for NT. Embryonic stem (ES) cells proliferate fast and appear to have a more open chromatin conformation than cumulus cells, which may have a more compacted genomic structure. This property seems to make the chromatin of ES cells more accessible to the cytoplasm of the recipient oocyte and to efficient reprogramming [61]. Similarly, we observed that NT of murine iPS cells results in higher rates of blastocysts and live-born cloned mice than embryonic fibroblasts (46% blastocysts and 1.3% liveborn for iPS cells vs. 3.5% and 0% for fibroblasts, respectively) [62]. Altogether, it seems that chromatin of the donor cells often remains too compact.

Trimethylation of lysine 9 of histone H3 has been proposed to limit the success of nuclear reprogramming. H3K9me3 is indeed associated with the repression of transcription [63], and its localization has been shown to be strongly correlated with constitutive heterochromatin, where it recruits heterochromatin protein 1 (HP1β also called chromobox protein homolog 1) [64]. H3K9me3 distribution has also been revealed to significantly expand during the differentiation of human ES cells into fibroblasts, a process which involves spreading of heterochromatin [65]. Consistent with these observations, H3K9me3 has been shown to persist after bovine and mouse NT experiments (Fig. 2) [58], [66] and [67], and H3K9me3 levels in lymphocytes have been correlated with decreased potential for nuclear reprogramming [68].

A number of approaches have targeted H3K9me3 to improve nuclear reprogramming. In cell fusion experiments by Antony et al. [69], the transient induction of histone lysine demethylase KDM4D (also known as JMJD2B) in ES cells increased the proportion of cell reprogramming by 30% despite the rapid restoration of H3K9me3 levels thereafter. Similarly, the transient expression of KDM4D caused a twofold increase in the efficiency of reprogramming somatic cells into iPSCs [70]. Recently, it was shown that removal of H3K9me3 by overexpression of KDM4D can restore transcriptional reprogramming in mouse-cloned embryos [71]. Such transient overexpression of KDM4D in cloned embryos has also been proven to efficiently improve reprogramming both in mouse and human cloning experiments, giving much higher rates of blastocysts [71] and [72].

Histone acetylation is also very important for appropriate development in preimplantation embryos. Studies regarding histone acetylation patterns in rabbit embryos [73] and bovine embryos [74], produced either by in vitro fertilization or somatic cell NT, have shown significant differences. In vitro fertilized embryos always presented higher histone acetylation compared with their counterpart cloned embryos, underlying once again the compactness of chromatin after NT.

The use of histone deacetylase inhibitors (HDACi), as scriptaid (SA) or trichostatin A (TSA), to increase of acetylated histones and helping the chromatin opening in cloned embryos has been reported. The first successful group obtaining full-term developed embryos after NT from somatic cells was the group led by Kishigami et al. [75], although at almost the same time another study was reported demonstrating that TSA could improve clone development [76]. An increase of the blastocyst yield and improvement of embryo quality after TSA treatment has been obtained with various donor cells: fibroblasts, neural stem cells, spleen cells, and cumulus cells [77]. It has also been reported that this drug can help with gene expression regulation. For example, whereas cloned embryos reported a failure in the expression of Oct4—an important factor for pluripotency maintenance—TSA treatment favored Oct4 expression in the correct number of cells at the blastocyst stage [78] and [79].

Thereafter, SA was reported to be a novel HDACi with less toxicity than TSA because it had a high efficiency, not lethal even at high concentrations [80]. Moreover, SA treatment could support full-term development of inbred cloned embryos. In fact, it appears that inhibition of HDAC is an important factor of reprogramming [81]. Hence, the use of HDACi has resulted in significant improvements in cloning efficiency of many species including human [82].

Moreover, HDACi also favors global chromatin reprogramming and thereby gene expression in several species such as mouse or pig, by acting not only on acetylation of histones but also on H3K9me3 [83] or even DNA methylation [84] and [85]. HDACi improve genome-wide gene expression regulation bringing total gene expression profile of clones to resemble that of fertilized pups [86]. We also found that addition of HDACi during the first cell cycle in cloned mouse embryos could improve nuclear remodeling of pericentromeric heterochromatin that reorganized around nucleolar precursors such as in fertilized embryos [61]. Remarkably, the use of HDACi was also correlated with increased number of ICM cells and correct further development to term [61].

Research on somatic cell NT embryos has been very useful in portraying that these epigenetic modifications not only have the ability to alter the expression of genes but also strongly demonstrate how their misregulation can disturb preimplantation embryonic development. Developmental inefficiency of cloned embryos and aberrant chromatin state seem to be tightly linked. The use of HDACi and of histone demethylases transient expression can however promote the formation of an "open" chromatin structure after NT, improving the reorganization of early embryo nucleus and thereby reprogramming.

Reprogramming in Parthenotes

Research in early mammalian development is carried out mostly on fertilized embryos. However, there is another way to study embryo development. Parthenogenetic activation is another valuable model to produce embryos in the absence of sperm fertilization through the artificial activation of a metaphase II oocyte (Fig. 1) [87]. In some species (such as various fishes, ants, snakes, or amphibians) parthenogenesis is a common method of asexual reproduction in which an unfertilized oocyte is able to develop into a whole new individual. Nonetheless, in mammals, parthenogenesis does not occur naturally, and if it does, it is only a consequence of erroneous oocyte maturation and embryos never develop to term [87]. In mouse, developmental arrest of parthenotes occurs before Day 10 of gestation but this time varies among species [88].

In normal conditions, ovulated oocytes advance from metaphase I to metaphase II and they remain arrested at this stage until they are fertilized by sperm. For the first cell division to occur, a series of events triggered by the entrance of a spermatozoon, known as oocyte activation, must take place. Broadly, the main trigger factor is the phospholipase-Ç brought by the sperm into the oocyte's cytosol [89]. A number of signaling pathways are then activated, which result

in a calcium release inside the oocyte. This calcium increase is translated in the activation of Ca2+/calmodulin-dependent protein kinase II which in turn, will inactivate the "cycle blocking" proteins maturation promoting factor and cytostatic factor. The inhibition of these last two, releases the oocyte from its arrest, and activation can be confirmed by the exocytosis of cortical granules, resumption of meiosis, extrusion of the second polar body, and the formation of pronuclei. Without sperm, it is necessary to artificially induce oocyte activation if parthenogenetic embryos are to be obtained in the laboratory. There are different protocols capable of overcoming the arrested state of a metaphase II oocyte which may include temperature alterations, electrical pulses, and changes in osmolarity [87]. Contemporary protocols are mostly based on calcium mobilizing compounds (i.e., ethanol, strontium, or calcium ionophore) to foster the initial calcium release in the cytoplasm: protein kinase inhibitors or protein synthesis inhibitors (i.e., cycloheximide or 6-dimethylaminopurine) to inactivate the maturation promoting factor and/or the cytostatic factor and, finally, a microfilament inhibitor (i.e., cytochalasin B) to avoid the extrusion of the second polar body [90] and [91]. Indeed, avoiding the extrusion of the second polar body is necessary to maintain the diploidy in the future embryo (Fig. 1) [92]. Thus, diploid parthenotes only possess maternal genetic information and will be homozygous. In particular, diploid parthenotes will not present the two sets of maternal and paternal imprinted genes, reason why, mammalian parthenotes never develop completely unless genetically modified or by the production of chimeras with fertilized embryos [93], [94] and [95].

Therapeutically, because these embryos are not normally viable for full development, parthenotes are also being studied as a stem cell source as it would carry very few ethical issues [88]. Moreover, parthenotes are an effective tool to evaluate genetic effects on the process of maternal genomic imprinting [94] and [96]. They also offer a means to study the contribution of maternally derived factors, as well as the absence of paternal factors to early development.

In NT experiments, oocyte activation is performed after NT to induce the resumption of meiosis in the oocyte's cytoplasm. Comparing cloned embryos and parthenotes can, therefore, be particularly helpful when it comes to study the precise cytoplasmic factors required for reprogramming within the recipient oocyte. Chromatin reorganization has been compared between fertilized embryos, clones, and parthenotes in few studies. Parthenotes seem to have less problems than their counterpart cloned embryos in adopting the proper heterochromatin conformation at very early stages, at least in mouse and rabbit embryos (Fig. 2) [59] and [97]. On the other hand, some epigenetic modifications take place more rapidly in parthenotes. Acetylation of histone H4 after formation of the pronuclei has been observed earlier in bovine and mouse parthenotes, probably due to the absence of the paternal genome [41] and [98]. Remarkably, we observed in a preliminary study that supplementation of TSA during the first embryonic cycle as in NT experiments resulted in an even more open chromatin structure in term of histone acetylation and in extended survival of mouse parthenotes post implantation (unpublished data). All these observations make parthenotes an interesting model to study reprogramming by the oocyte's cytoplasmic factors, in the absence of any sperm supply.

Conclusion and Perspectives

Epigenetics is the area of molecular science which has been dusted off the shelves and gained a newfound interest. In order to have a better comprehension of the complex interrelationships between all the various components of the epigenome and the way that each individual part operates, it has been essential to decipher key elements of the nuclear reprogramming in early embryos. However, understanding the connection between chromatin structure, gene expression, genome organization, creation of the nuclear architecture, and how all these cellular processes come together during embryogenesis still needs further studies. What it also needs to be remembered is that epigenetic changes can arise from external agents such as environmental

cues, dietary, stress, and chemical contaminants to mention some examples, which in turn, cause a chain effect to the chromatin modifying agents and their respective genes or gene families affecting normal development and disease through their actions on the epigenome [10].

This is particularly important from a clinical point of view. Indeed, the main goal in a fertility clinic is to raise embryos under the best culture conditions after gamete retrieving and in vitro fertilization to afterward transfer the highest quality embryo to the mother's uterus and achieve a successful pregnancy [99]. This is nowadays an effective and common process thanks to all the research and advancements in assisted reproductive technologies which have been based on the knowledge obtained from studies mainly using mouse embryos because of their easy access and manipulation. Therefore, studies in early mammalian embryos (such as mouse or rabbit) and their reprogramming could possibly help to improve embryo culture conditions to promote development of better quality embryos with higher potential for further development, thus increasing the success rates of assisted reproductive technologies [30], [35] and [51].

Elucidation of the roles of epigenetic marks in nuclear reprogramming would also benefit human health, especially the reprogramming of iPS cells. In particular, some recent publications suggest that ES cells derived from cloned embryos may be closer to ES cells derived from in vitro derived embryos than iPS cells in terms of epigenome and transcriptome [82] and [100]. We hope better understanding of epigenetic remodeling mechanisms will shed some light on cell reprogramming and further application on stem cell therapies.

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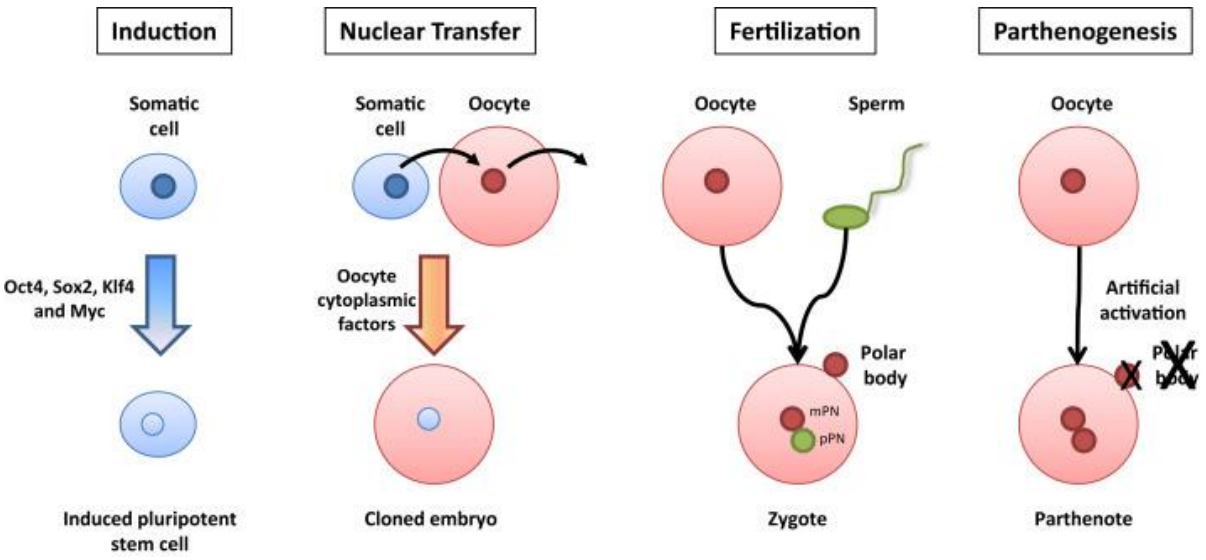
589 **Figure legends**

590 Figure 1: Strategies used to induce nuclear reprogramming include (from left to right):
591 induction by overexpression of embryonic pluripotent transcription factors, nuclear transfer of
592 somatic cell nuclei into enucleated recipient oocytes, fertilization through sperm penetration
593 and parthenogenesis by artificial activation.

594 Figure 2: Examples of H3K9me3 immuno-staining (green) with DNA counterstaining (red) on
595 nuclei from mouse oocytes in NSN (non-surrounded nucleolus) versus SN (surrounded
596 nucleolus) oocytes and in 1-cell stage embryos: either fertilized (zygotes), cloned (obtained by
597 nuclear transfer - NT) or parthenotes. Clear compaction of chromatin and accumulation of
598 H3K9me3 can be observed in SN oocytes. After fertilization, asymmetric distribution can then
599 be observed between the maternal and paternal pronuclei (mPN and pPN respectively) with
600 H3K9me3 accumulation around the nucleolus precursor; whereas cloned embryos present no
601 asymmetry with much more aggregates of H3K9me3, especially at the nuclear periphery. Bar=
602 10µm

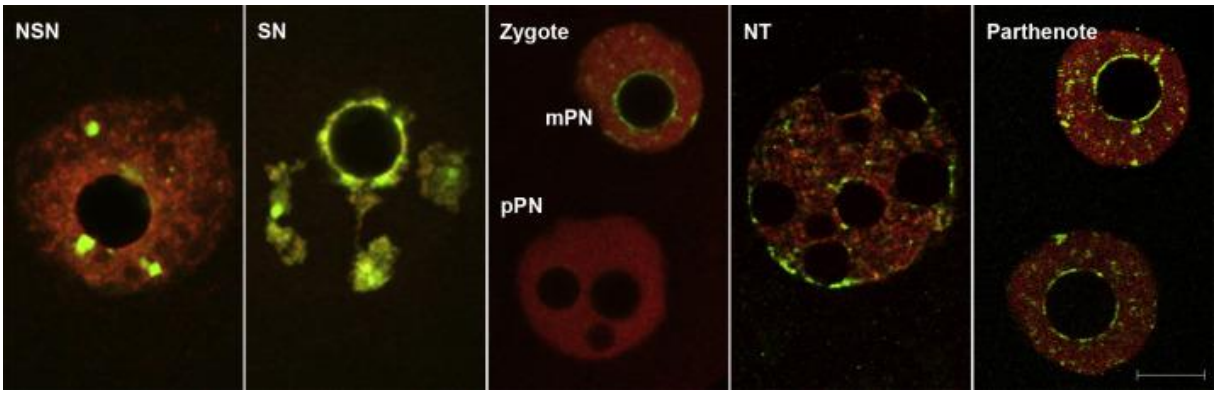
603

Figure 1


Induction
Somatic cell
Oct4, Sox2, Klf4 and Myc
Induced pluripotent stem cell
Nuclear Transfer
Somatic cell
Oocyte
Oocyte cytoplasmic factors
Cloned embryo
Fertilization
Oocyte
Sperm
Polar body
mPN
pPN
Zygote
Parthenogenesis
Oocyte
Artificial activation
Parthenote

607 **Figure 2**

608



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