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Reprogramming and development in vertebrate somatic cell nuclear transfer embryos and in interspecific systems

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Abstract

Nuclear transfer (NT) remains the most effective method to reprogram somatic cells to totipotency. Somatic cell nuclear transfer (SCNT) efficiency however remains low, but recurrent problems occurring in partially reprogrammed cloned embryos have recently been identified and some remedied. In particular, the trophectoderm has been identified as a lineage whose reprogramming success has a large influence on SCNT embryo development. Several interspecific hybrid and cybrid reprogramming systems have been developed as they offer various technical advantages and potential applications, and together with SCNT, they have led to the identification of a series of reprogramming events and responsible reprogramming factors. Interspecific incompatibilities hinder full exploitation of cross-species reprogramming systems, yet recent findings suggest that these may not constitute insurmountable obstacles.

Keywords

Nuclear reprogramming; Somatic cell nuclear transfer (SCNT); Interspecies SCNT (iSCNT); Hybrid; Nucleocytoplasmic hybrid (Cybrid); Nucleocytoplasmic incompatibility; Microcell-mediated chromosome transfer (MMCT); Interspecies intra-cytoplasmic sperm injection (iICSI); Cell fusion

The recent finding that non-enucleated human oocytes can efficiently reprogram transferred human somatic nuclei to pluripotency (21% triploid blastocyst formation and ~3% ES-like cell line derivation) [1] renews the interest in NT. SCNT using enucleated oocytes in other mammalian species seems to remain the most effective method to reprogram somatic cells to pluripotency [2,3]. In addition, since NT is the only available method to reprogram cells to totipotency, and chimera formation in primates is exclusively possible via aggregation of totipotent cells [4], the eventual generation of chimeric primates containing cells genetically-modified in culture would require NT. The mechanisms of reprogramming by NT are being investigated both directly in intraspecies SCNT embryos and in a variety of cross-species hybrid and cybrid systems. Interspecific hybrid cells or organisms comprise genomes, or parts of genomes, that originate from more than one species, while interspecific cybrids, also known as nucleocytoplasmic hybrids, originate from the combination of the nucleus or genome of one species with cytoplasm of another species.

The viability of cross-species (hybrid and cybrid) embryos tends to be inversely correlated with the genetic or evolutionary distance between the species that are combined [5,6]. A better understanding of the boundaries between species is not only fundamentally

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interesting, but it may also enable researchers, through intervention, to extend the actual range of viable hybrids and cybrids. This could be useful for various zoological, agricultural and biomedical purposes, including the identification of conserved reprogramming mechanisms. In addition to highlighting major advances in SCNT research, we review here the current hybrid and cybrid systems, the mechanistic insights they have recently provided into nuclear reprogramming, as well as the nature of interspecific incompatibilities.

Recent progress in somatic cell nuclear transfer

In mice, using cumulus cells as nuclear donors, up to ~65% of SCNT embryos develop to the morula/blastocyst stage, but only <3% of the transferred embryos develop to term [7]. With such a low implantation and post-implantation development efficiency, several investigators have asked whether recurrent defects could explain the lethality of SCNT embryos. Inhibition of histone deacetylases, such as with Trichostatin A (TSA) treatment, increases full-term development of cloned embryos [8] likely through improving several early events of nuclear reprogramming, including chromatin remodelling, histone modifications, DNA replication and transcriptional activity [9,10]. In pigs, DJ-1 is an ooplasm component that is, specifically in SCNT embryos, required for survival to the blastocyst stage through negatively regulating p53 signalling [11]. Inhibition of p53 also improves trophectoderm function of SCNT blastocysts in mice [12]. Since p53 normally acts to suppress cell division in response to DNA damage, these results suggest that DNA damage may often occur during the early cleavages in cloned embryos, activating p53, which then induces cell cycle arrest and/or apoptosis, thereby explaining some of the developmental delay and death of SCNT embryos. Perhaps in connection with this, errors in chromosome segregation often occur (>90% of the cases) during early cleavages in murine SCNT embryos, which appear not to affect survival until the blastocyst stage, but to impair only post-implantation development [13]. Interestingly, if the extra-embryonic lineage of a cloned blastocyst is replaced by one generated by *in vitro* fertilization (IVF) in a process called tetraploid complementation, the frequency of full-term development of cloned mice increases by about 6-fold [14]. In the converse experiment, where an IVF-derived inner cell mass is combined with cloned trophectoderm lineage, post-implantation development is reduced to a level near that of regular SCNT, suggesting that defects in the trophectoderm lineage may explain a large proportion of the post-transfer lethality observed in SCNT embryos [14]. Genome wide transcriptional analysis of cloned embryos revealed specific and recurrent decreased X-linked expression due to persistent Xist (a non-coding RNA that inactivates one of the two X chromosomes in females) expression from the active X chromosome (Xa) of transferred nuclei [15]. Inhibition of Xist function before the morula stage restored X-linked expression and increased implantation and post-implantation development of cloned embryos by more than 8-fold [15,16]. An overlapping set of genes (including many X-linked downregulated genes) is also often inappropriately reprogrammed in SCNT blastocysts across different nuclear donor cell types [15,17]. These results together suggest that multiple defects occur during early cleavage in SCNT embryos, including abnormal chromatin remodelling and chromosome segregation. When combined with persisting Xist expression from Xa and the resulting globally reduced X-linked expression, as well as recurrent failure to reprogram a relatively specific set of non X-linked genes, this may often impair trophectoderm function, and hence, implantation and post-implantation development of cloned blastocysts (Figure 1). This does not imply that cloned embryonic tissues are without flaws; SCNT-derived post-implantation stage epiblast stem cells and post-natal individuals often have aberrant transcriptional and/or epigenetic statuses [18,19], although whether these observed defects are cell/tissue-autonomous, or secondary to trophectoderm defects remains uncertain.

In mice, recent experiments [20] solidified the concept that essential reprogramming factors are located in the zygote nucleoplasm [21,22]. In order to leave these factors in the egg/zygote, enucleation must be performed during mitosis, when the nuclear envelope is broken down. In primates, SCNT is more problematic, and reproducible derivation of monkey SCNT ES cells required the development of a less invasive mitotic egg enucleation protocol [23,24]. In humans however, even with extreme care, enucleated mitotic eggs or zygotes were unable to support the development of somatic cell nuclei beyond the 6-10 cell stage [1,25]. Interestingly, the investigators asked what happens if the oocyte haploid genome was left intact, and development of the resulting NT embryos proceeded to the blastocyst stage, enabling the derivation of triploid human ES-like cells [1]. This suggests that in humans, as opposed to all the other species in which SCNT has been tried so far, essential reprogramming factors are tightly associated with, or even comprised within, the oocyte genome, such that even mitotic enucleation removes these factors. SCNT in humans, at least with current technology, is therefore unlikely to generate clinically relevant diploid ES cells.

Hybrid experimental systems and reprogramming insights

Three interspecies hybrid experimental systems currently exist: (i) hybrid cells generated by the fusion of cells or microcells from two species (we ignore here hybrids containing less than one chromosome from another species), (ii) interspecies NT (iNT) to *Xenopus* oocyte (at the first meiotic prophase), and (iii) hybrid embryos (Figure 2). When undifferentiated cells are fused with differentiated cells, differentiated cells are usually reprogrammed towards an undifferentiated state, even if the two cell types originate from different species [26,27]. Transcriptional analyses are greatly facilitated in interspecific hybrids due to genetic differences between species that make it possible to distinguish expression from each species' genome by RT-PCR [28]. Using interspecific cell fusion, it was shown that the Oct4 transcription factor, Polycomb repressive complex (PRC), and AID-dependent DNA demethylation activity are required for ES cells to successfully reactivate pluripotency gene transcription in somatic cells [28-30] (See review by A. Fisher in this issue). A cell fusion variant called microcell-mediated chromosome transfer interestingly enables the fusion of only one to a few chromosomes from one cell type/species to another cell [31]. Chromosome-wide analysis of murine cells that carried human chromosome 21 revealed that murine transcription factors were bound to and transcribed genes from human chromosome 21 in a human manner [32]. Thus, genetic sequence primarily directs transcriptional programs, even in an interspecific cellular environment. *Xenopus* oocytes are intensely active in transcription. It is possible to transplant up to hundreds of mammalian cell nuclei directly into the *Xenopus* oocyte nucleus, referred to as the germinal vesicle (GV), and this procedure induces the reactivation of transcriptionally silent mammalian loci [33]. Transcriptional reprogramming of mammalian somatic nuclei upon nuclear transfer to a *Xenopus* oocyte GV is inhibited by the presence of a histone variant called Macro H2A in somatic nuclei, and requires oocyte linker histone B4 and nuclear actin polymerization [34-36]. Interspecies intra-cytoplasmic sperm injection (iICSI) has been used mainly to study the early reprogramming effect of non-enucleated oocytes on interspecific sperm nuclei, including DNA demethylation, chromatin structure (HP1 levels), and several histone modifications (changes in H3 methylation, H3 and H4 acetylation) [37-39]. Interspecific cell fusion, iICSI and iNT to *Xenopus* oocyte experiments have thus significantly contributed to our current knowledge of reprogramming factors and mechanisms (reviewed in [40]), while they also suggest that these mechanisms are highly conserved.

Cybrid experimental systems and reprogramming insights

Current cybrid experimental systems can be subdivided into three categories: those that involve (i) nuclei incubated in interspecific cellular extract, (ii) cybrid cell lines generated

through cytoplast fusion, and (iii) cybrid embryos (Figure 3). *Xenopus laevis* egg and oocyte extracts can be easily prepared in large quantities and their ability to reprogram nuclei from other species has been investigated. Using this system, the chromatin remodelling ATPases ISWI and BRG1, as well as nucleoplasmin, were found as egg components required for reprogramming of somatic nuclei [41-43]. Extracts from a distantly related frog species, *Xenopus tropicalis*, whose embryonic cells and nuclei are smaller in size than those of *X. laevis*, have also been prepared, and the incubation of sperm nuclei from one species in egg extracts of another species and vice-versa, demonstrated that the magnitude of nuclear swelling, as well as spindle length, were dependent on egg cytoplasmic factors/species rather than on nuclear content/species. This was due to differences in the relative concentrations of two key nuclear transport factors in cells of the two species in the case of nuclear swelling, and to the gain of an inhibitory phosphorylation site in a microtubule-severing factor homolog in X. laevis in the case of spindle scaling [44-46]. Thus, there seem to exist quantitative and functional differences in relatively few key factors between species that may account for their divergent nuclear remodelling phenotypes.

Experiments in which cultured mtDNA-less human cells were fused with various cytoplasts isolated from other primate species showed that human nuclei are compatible with mtDNA of some of their closest non-human relatives, such as chimpanzees and gorillas, but not with mtDNA of species separated by 18 million years (MY) of evolution (orangutans) or more [47]. Interestingly, intraspecific fusion of human fibroblasts or hepatocytes with ES cell cytoplasts did not fully reprogram the somatic nuclei, suggesting that reprogramming to pluripotency by ES cell fusion requires the ES cell nucleus [48,49]. Many investigators have tested the ability of enucleated eggs to reprogram interspecific somatic nuclei and generate viable cybrid embryos/progeny, largely using interspecies SCNT (iSCNT) [5,6]. In most cases, a fraction of iSCNT embryos developed until the blastocyst stage, although reprogramming defects were observed in some instances, including a failure to properly activate pluripotency genes [50-52]. Several pluripotency genes were nonetheless properly activated in many cybrids [50,53,54], and ES-like cells have been isolated from iNT blastocysts in a few occasions, although the efficiency was low and the reproducibility of the results remains uncertain [55-57]. These results are nonetheless important as they suggest that homologous egg factors needed to reprogram cells to pluripotency may function across many mammalian species.

The nature of hybrid and cybrid incompatibilities

A long-standing question is whether the lethality and developmental failure of distantly related hybrid and cybrid embryos are due to cellular incompatibilities, reprogramming defects, and/or developmental failure. Hybrids generally survive better than cybrids of the same interspecific combinations, indicating that the ooplasm most often supports better the presence of an interspecific genome than the lack of an intraspecific genome [58,59]. Indeed, most cybrids made from divergent species could only develop until the blastocyst stage, and they were characterized by a range of defects, including developmental delay or failure, reduced cell numbers, nuclear/genome damage, structural disorganization, aberrant gene expression and energy levels, as well as defects in nucleologenesis [50-52,54,59-66]. Despite all these observed defects in cybrid embryos, very little is known about the nature of the underlying nucleocytoplasmic incompatibility. In iSCNT, treatment with TSA improves aspects of reprogramming in some cybrid embryos, as in same-species SCNT, but it does not improve survival [67-69]. This suggests that cybrid incompatibility may not be primarily due to reprogramming defects, at least of the types that are improved by TSA treatment [9,10]. Alternatively, cellular and developmental defects may underlie cybrid lethality. An interesting report in which ES cell extracts were transferred along with murine somatic nuclei into enucleated, mtDNA-depleted pig oocytes, showed that these changes (mtDNA

replacement and addition of ES cell extract components) improved cybrid development, although it is not known exactly how these changes were beneficial [70]. As discussed earlier, it was found that differences in the concentration or regulation of relatively few proteins between two *Xenopus* species could explain their different nuclear and spindle sizes but it remains unclear whether such differences affect the viability of interspecific cybrids [45,46]. Yet, a difference in the concentration of an embryonic transcription factor between the same two frog species may be responsible for some of the incapacity of cybrid embryos to undergo efficient gastrulation movements [59]. Specifically, the *Xenopus* cybrid had a relatively insufficient, cytoplasmic species-like, transcription factor concentration, which likely contributed to the inefficient induction response and subsequent gastrulation defects in embryos with nuclei that normally have a higher concentration of that same transcription factor. Viable cells have been isolated from cybrid embryos, but in most cases they could not be expanded normally *in vitro*, suggesting that their viability or ability to proliferate is reduced [56,57,71]. In an *in vivo* study, cybrid cell viability was improved if these cells were transplanted into embryos of the cytoplasmic species, suggesting that inter-cellular signalling with cytoplasmic species cells may rescue some of the cellular incompatibility of cybrids [72]. The cellular incompatibility of cybrids could result from nucleo-mitochondrial incompatibilities causing respiratory defects, as occurs in cybrids cells generated by cytoplast fusion in culture [47,73,74], or from other kinds of cellular nucleocytoplasmic incompatibilities, such as chromosomal loss/damage. More studies will be necessary to determine the full spectrum of possible nucleocytoplasmic incompatibilities between species, and whether it may be possible to correct them.

Conclusion

Analyses of nuclear reprogramming in SCNT and interspecific systems have led to the identification of several reprogramming factors and recurrent defects occurring in SCNT embryos, as well as some differentiation marks that restrict reprogramming in somatic nuclei. On the other hand, they have also led to the discovery of a nucleo-mitochondrial incompatibility between highly divergent species in culture *in vitro* [47,73,74], although the *in vivo* relevance of this phenomenon remains unclear [59,75]. Also, quantitative and functional differences in relatively few key factors between species may account for their divergent phenotypes, while these differences may underlie hybrid and cybrid lethality [45,46,59]. Comparative genome-wide analyses of chromatin and transcription in divergent species [76,77], as well as in hybrids and cybrids [54,78,79], are being carried out and may help to identify some of these disparities. A better understanding of the differences and incompatibilities between species may indeed help to develop a variety of widely relevant new tools and systems, including iNT-generated functional ES cells and interspecific chimeras [80,81].

An interesting point is that, at the moment, it appears that enucleated animal oocytes are better than their human counterpart at reprogramming intraspecies somatic cells [1,7,24,25]. For reasons of egg availability, and enucleated reprogramming capacity, we therefore wonder whether it would be most advantageous to investigate further the possibility of reprogramming human somatic nuclei using interspecific systems, including closely related non-human primate species with compatible mitochondrial genomes [47]. Obviously, the ethical implications of these experiments would need careful examination. Identifying and rescuing cross-species incompatibilities also becomes a priority.

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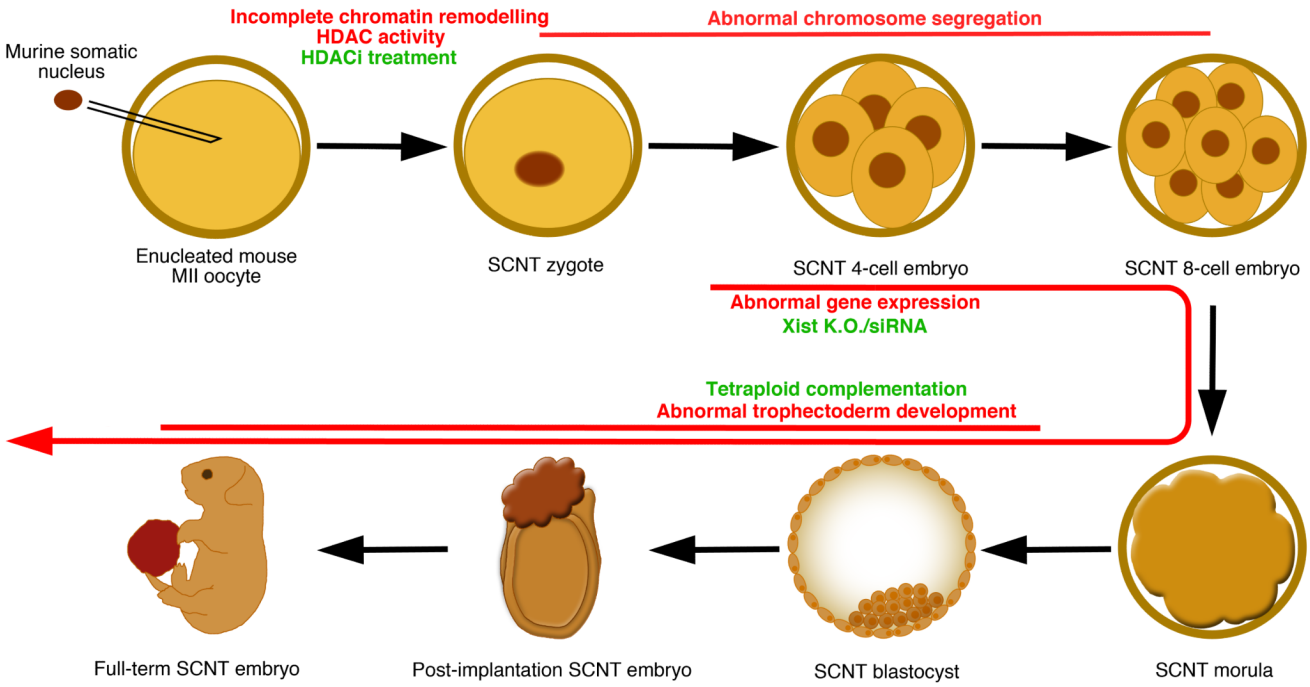

Figure 1. Recurrent reprogramming defects in SCNT embryos

In this example, a murine somatic cell nucleus is transferred into a mitotically-enucleated oocyte, containing cytoplasmic and nuclear reprogramming factors. After NT, the chromatin of the somatic nucleus is often not completely remodelled due to persistent histone deacetylase (HDAC) activity. This, and other reprogramming aspects, can be improved by HDAC inhibitor (HDACi) treatment. Abnormal chromosome segregation often occurs during the early cleavages and appears to be a major cause of developmental failure when it happens before the 8-cell stage. Following zygotic genome activation, abnormal gene expression, including Xist RNA from Xa and the subsequent under-expression of X-linked genes, further inhibits SCNT embryo development. This can be improved by removing Xist from Xa in donor nuclei, or injection of Xist siRNA in SCNT zygotes. Finally, incomplete reprogramming of the trophectoderm lineage, and the resulting defects in trophectoderm development are a major cause of the lethality of post-implantation stage SCNT embryos. This can be rescued by replacing the trophectoderm lineage with one generated from in vitro fertilized embryos through tetraploid complementation.

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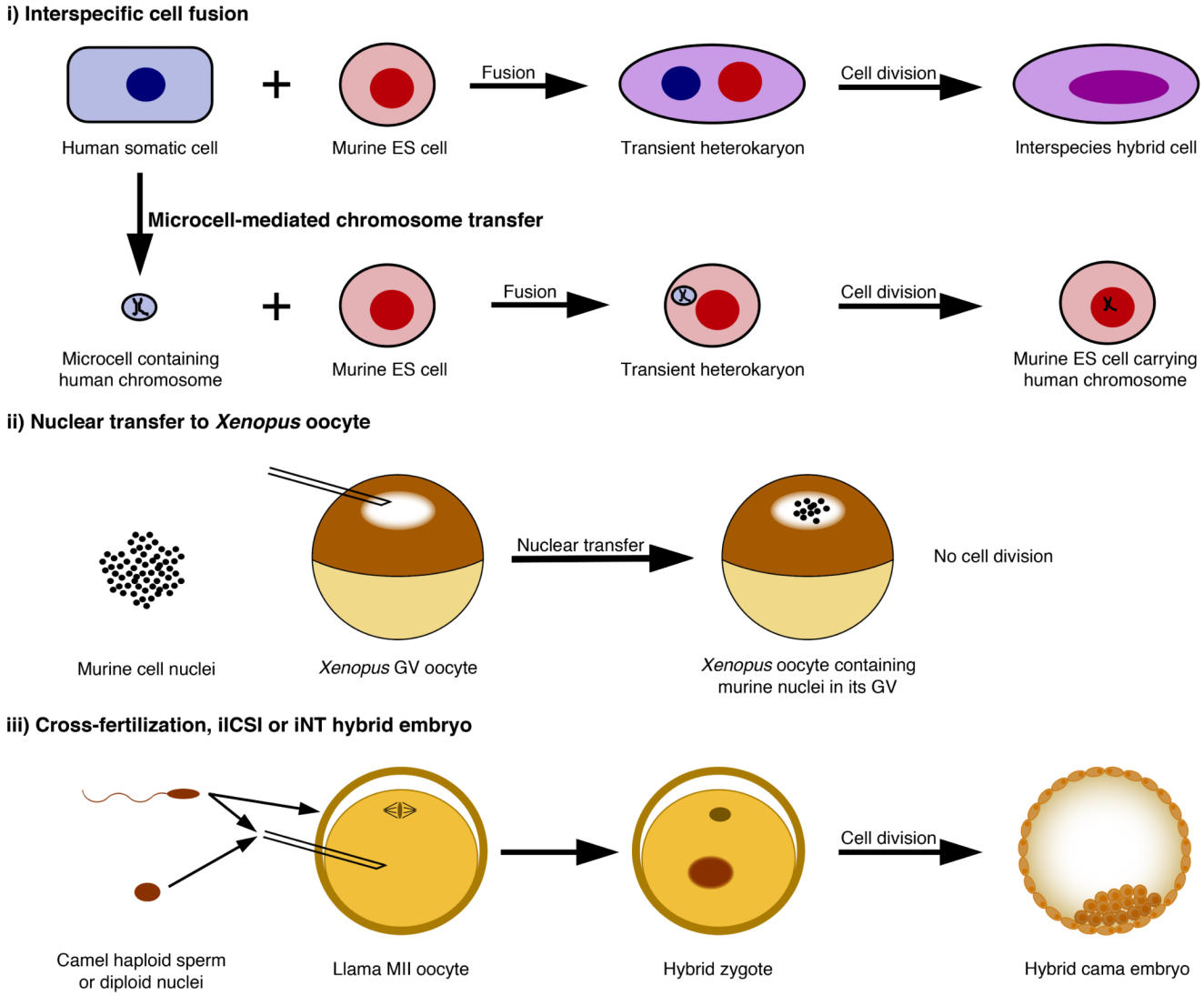

Figure 2. Interspecific hybrid reprogramming systems

(i) In interspecific cell fusion, cultured cells of one species are fused to cells of another species, resulting in the formation of transient heterokaryons in which one cell type is transcriptionally reprogrammed towards the other cell type. A heterokaryon may undergo mitosis, in which case the two nuclei will fuse to generate a proliferating hybrid cell. Interspecies microcell-mediated chromosome transfer is similar to cell fusion, except that the nuclei of the cells from one species are fragmented into microcells containing only one to a few chromosomes prior to fusing with interspecific cells. **(ii)** In NT to *Xenopus* oocyte GV, a few hundreds of mammalian nuclei are injected into the oocyte GV, which induces the transcriptional reactivation of previously silenced genes within a day or two in the absence of cell division. **(iii)** Hybrid embryos can be generated either by cross-fertilization, iICSI or iNT using non-enucleated interspecies oocytes.

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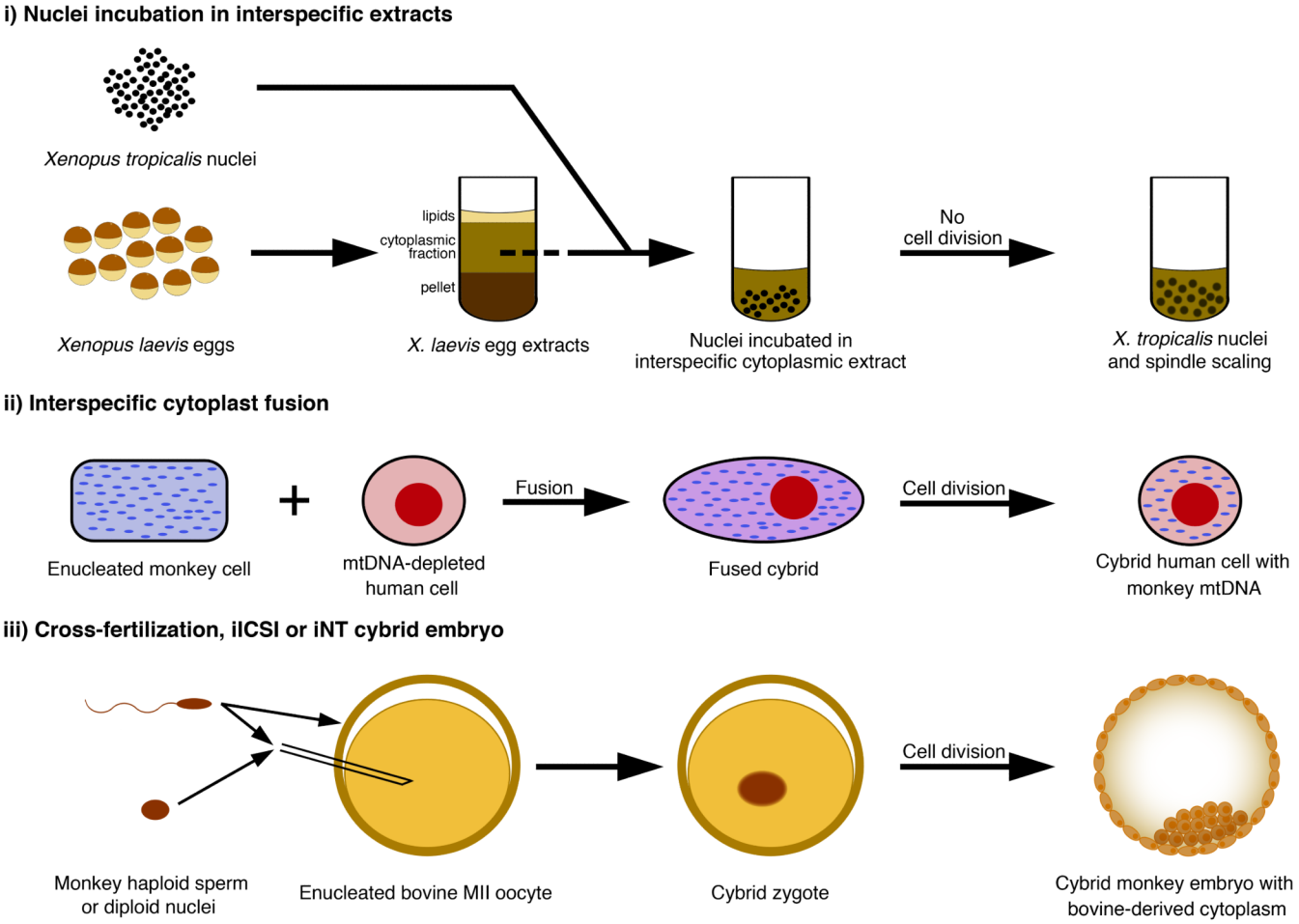

Figure 3. Interspecific cybrid reprogramming systems

(**i**) Nuclei from one species are incubated into M-phase egg extracts prepared from another species, resulting in nuclear and spindle scaling. (**ii**) In interspecific cytoplast fusion, cultured cells from one species are enucleated and fused with cells from another species. This can be combined with mtDNA-depletion of the other species cells to generate xenomitochondrial cybrid cells. (**iii**) Cybrid embryos can be generated either by cross-fertilization, iICSI or iNT using enucleated interspecies oocyte.