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On the cellular and developmental lethality of a Xenopus nucleocytoplasmic hybrid

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Nucleocytoplasmic hybrid (cybrid) embryos result from the combination of the nucleus of one species, and the egg cytoplasm of another species. Cybrid embryos can be obtained either in the haploid state by the cross-fertilization or intra-cytoplasmic injection of an enucleated egg with sperm from another species, or in the diploid state by the technique of interspecies somatic cell nuclear transfer (iSCNT). Cybrids that originate from the combination of the nucleus and the cytoplasm of distantly related species commonly expire during early embryonic development, and the cause of this arrest is currently under investigation. Here we show that cells isolated from a Xenopus cybrid (*Xenopus (Silurana) tropicalis* haploid nucleus combined with *Xenopus laevis* egg cytoplasm) embryo are unable to proliferate and expand normally in vitro. We also provide evidence that the lack of nuclear donor species maternal poly(A)$^+$ RNA-dependent factors in the recipient species egg may contribute to the developmental dead-end of distantly-related cybrid embryos. Overall, the data are consistent with the view that the development promoted by one species' nucleus is dependent on the presence of maternally-derived, mRNA encoded, species-specific factors. These results also show that cybrid development can be improved without nuclear species mitochondria supplementation or replacement.

Introduction

The generation of hybrids or chimeras sharing characters of distant species is a fascinating possibility. Perhaps for the same reasons, ancient mythologies and legends were sprinkled with various human-animal chimeras, such as the minotaur of the Greeks and many Egyptian gods. Equally fascinating is the possibility to preserve endangered species, or even revive extinct species such as the woolly mammoth, by nuclear transfer from preserved cells or by DNA injection into the eggs of a more available species. Not surprisingly, the potential resolution of the roadblocks that currently prevent such realizations has inspired one of the most famous science-fiction novel of these times: "Jurassic Park."[1] From a medical perspective, nuclear transfer arguably remains the most efficient method to generate pluripotent embryonic stem (ES) cells,[2-5] and the only realistic route for mitochondrial gene replacement therapies.[6,7] Thus, if the barriers that are currently associated with iSCNT are better understood, it may be possible to overcome these incompatibilities, such that iSCNT would become a viable approach to generate human ES cells from human nuclei and animal oocytes, and these could be used for research or stem cell-based therapies. In addition, iSCNT studies may reveal novel nucleocytoplasmic interactions that occur during early embryonic development.

Over the past 50 years several investigators have performed iSCNT in a variety of fish, amphibian and mammalian combinations to reach the general conclusion that development of cybrids to adulthood is only successful if the two parental species are very closely related. If they are too evolutionarily distant, cybrid embryos usually arrest development at an early stage due to a variety of potential nucleocytoplasmic incompatibilities.[8-11] These may include defects in embryonic genome activation (EGA) and/or nucleo-mitochondrial incompatibilities,[12-18] but these hypotheses still remain poorly tested. Our recent work involving a distant Xenopus lethal cybrid formed by the combination of a *Xenopus (Silurana) tropicalis* haploid nucleus and a *Xenopus laevis* egg cytoplasm (these two species being separated by 50–65 million years of evolution),[19,20] provided compelling evidence to suggest that differences in the concentrations of key proteins between species could lead to inefficient induction signaling and contribute to cybrid developmental defects.[21,22] In this specific case, embryos of the recipient species typically have a lower concentration of Xbra protein, a key transcription factor that is necessary to induce efficient convergence-extension movements during gastrulation, than the embryos of the nuclear donor species do. Interestingly, in the cybrid, the Xbra concentration is similar to that of the cytoplasmic species, and thereby lower than it is normally in the nuclear species, and this seems to explain, at least in part, why these cybrid embryos have reduced convergence-extension movements.[21] Here we present two experiments that complement this study and further define the nature of the embryonic lethality in this Xenopus cybrid combination. In the first instance, we evaluate the in vitro culture potential of cybrid cells isolated from cybrid embryos.

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Table 1. Failure of in vitro expansion of cybrid embryonic cells

Embryo (N, n)	Cells attached*	Cells reached confluence*	Maximum number of passages§
lxl (2, 28)	10/10	10/10	> 30
[*l*]x*l* (1, 16)	8/8	8/8	> 6
txt (1, 16)	5/5	3/5	> 9
[*t*]x*t* (1, 12)	4/4	2/4	> 7
lxt (2, 28)	14/14	14/14	> 4
[*l*]x*t* (5, 42)	13/18	0/18	0

N, Number of experimental repeats (different male/female combinations); n, Total number of dissociated embryos; *, Number of dishes where the condition was true/total number of dishes; §, Confluent cultures passaged 1/2 to 1/3.

After finding that cybrid embryonic cells have a reduced potential for in vitro culture, we asked whether some of the defects of cybrid cells and embryos may originate from the lack of nuclear species maternal factors.

Results

Limited in vitro viability and expansion of cybrid embryonic cells. We will use a previously defined nomenclature to refer to the diverse kinds of embryos used in this study. Briefly, a first italicized letter represents the egg species, followed by an "x" which stands for "fertilized," or "cross-fertilized" with, and a second italicized letter indicates the sperm species. Square brackets indicate that a component's nucleus has been inactivated using UV irradiation.[21] Our previous work has indicated that [*l*]x*t* cybrid (enucleated *X. laevis* eggs cross-fertilized with *X. tropicalis* sperm) embryos, much like their iSCNT diploid counterparts, form normal late blastulae, but fail to respond properly to induction signals, do not fully close their blastopore during gastrulation due to inefficient convergence-extension, and eventually die as poorly developed, abnormal postneurulae.[21,23] Cybrid embryonic lethality may result from developmental nucleocytoplasmic incompatibilities, but also from "cellular" nucleocytoplasmic incompatibilities if the resulting cybrid cells themselves have a reduced viability. Here we asked whether [*l*]x*t* cybrid embryonic cells are viable and can proliferate normally in vitro, as with the embryonic cells of both Xenopus species.[24,25] Despite multiple trials, we were unable to derive viable cell lines from [*l*]x*t* cybrid embryos, while we could easily derive multiple lines from *lxl* and *txt* diploid, *lxt* hybrid, or [*l*]x*l* and [*t*]x*t* haploid control embryos (**Table 1**). Following their dissociation and exposure to standard in vitro culture conditions, [*l*]x*t* cells attached normally to the dishes and appeared viable for several days, but consistently failed to expand normally or reach confluence (**Fig. 1**, **Table 1**). In one occasion, we passaged a sub-confluent 8-d [*l*]x*t* culture to another dish and the cells attached, indicating that some of the cells were still viable but again, the population did not expand (**Fig. 1**). One possible explanation for this is if the mitochondrial DNA (mtDNA) from the egg species is incompatible with the nuclear DNA of the other species, which could lead to defects in oxidative phosphorylation in cybrid cells.[26,27] In vitro culture and expansion of mtDNA-less human cells required the addition of pyruvate and uridine to the culture medium,[28] but adding uridine (50 μg/ml) to our culture medium (which already contains pyruvate) did not improve the in

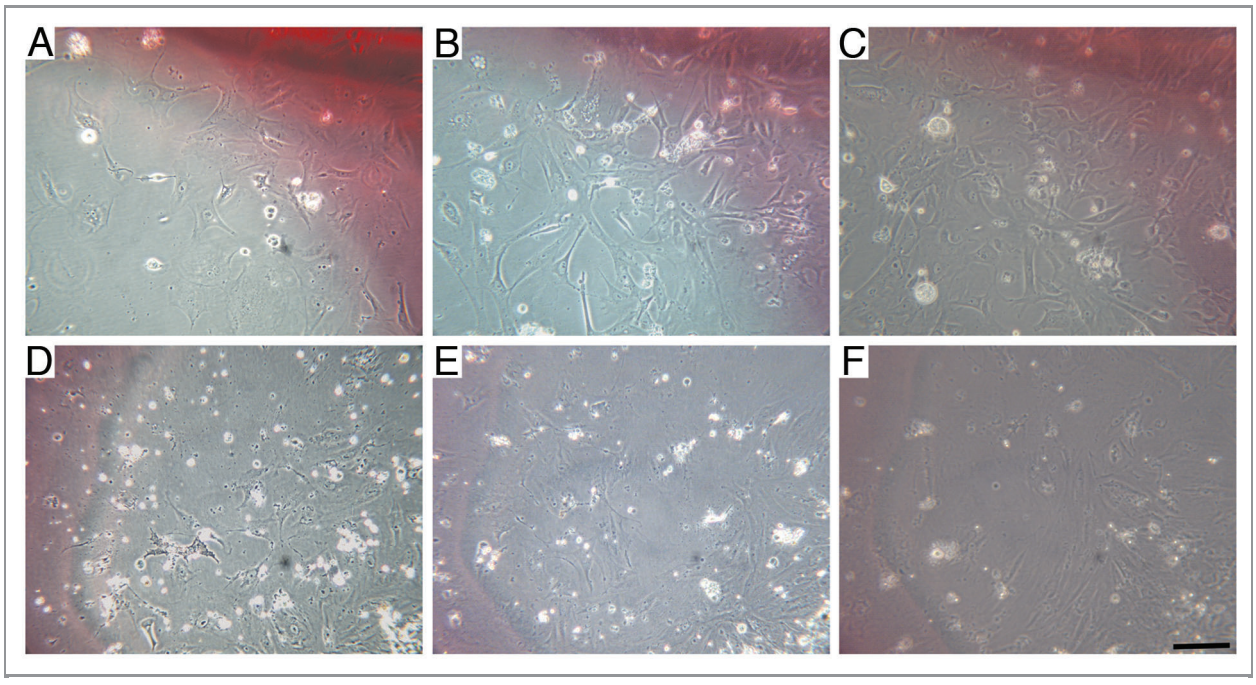

Figure 1. Defective in vitro expansion of cybrids cells. The concentration of control haploid [*l*]x*l* embryonic cells (A-C) in a given area of a culture dish visibly increased over time, while that of [*l*]x*t* cybrid cells (D-F) did not. Pictures in (B, E) and (C, F) were taken 5 and 13 d, respectively, after those in (A, D). Scale bar: 0.1 mm.

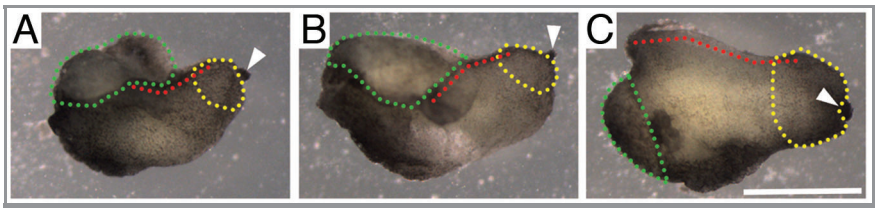

Figure 2. Improved cybrid embryo development by nuclear species maternal poly(A)$^+$ RNA injection. The most developed [*l*]x*t* cybrid embryo that was obtained following injection at the 1-cell stage with (A) $_dH_20$, (B) *X. laevis* oocyte poly(A)$^+$ RNA, or (C) *X. tropicalis* oocyte poly (A)$^+$ RNA from one experiment are shown as an example. All three embryos had a rudimentary sucker (arrowhead), yet noticeable improvements in the development of the animal shown in (C) include a better blastopore closure (green dotted line), axis formation and elongation (red dotted line), muscular response, and an increased head size (yellow dotted line). Embryos are shown (anterior to the right; dorsal up) at 48 h post-fertilization, about 24 h before they were finally scored for inclusion in **Table 2**. Scale bar: 1 mm.

vitro expansion potential of [*l*]x*t* cybrid cells (unpublished data). This suggests that the inviability of cybrid cells may not, or not only, result from oxidative respiration incompatibilities. This cellular nucleocytoplasmic incompatibility of [*l*]x*t* cells may contribute to the developmental failure and lethality of [*l*]x*t* cybrid embryos.

Improvement of cybrid development by injection of nuclear species maternal mRNA. The substances present in the *X. laevis* egg cytoplasm cannot sustain the in vitro viability and development promoted by a *X. tropicalis* nucleus (**Fig. 1**, **Table 1**).[21] The cytoplasm of [*l*]x*t* cybrids thus lacks factors that are normally present in the *X. tropicalis* cytoplasm, and which are required for in vitro viability and expansion of cells with a *X. tropicalis* nucleus. To partly test this idea, we isolated poly(A)$^+$ RNA from either *X. laevis* or *X. tropicalis* oocytes and injected it into [*l*]x*t* cybrid embryos at the one-cell stage, so as to supplement them with nuclear maternal species-specific poly(A)$^+$ RNA and their encoded factors, to test whether this would improve their development. Injection of 15 ng of *X. tropicalis* poly(A)$^+$ RNA in [*l*]x*t* cybrid zygotes indeed significantly improved some aspects of their development, although not to a dramatic extent (**Fig. 2**, **Table 2**). None of the poly(A)$^+$ RNA injected cybrid embryos formed swimming tadpoles. Yet, among the embryos that reached a postneurula stage, a significantly higher proportion of the *X. tropicalis* poly(A)$^+$ RNA-injected population had pigmented rudimentary eyes and/or demonstrated muscular activity (**Table 2**). The data therefore suggest that the incompatibilities in [*l*]x*t* cybrid cells and embryos arise, at least in part, from the absence in *X. laevis* eggs (or presence in different concentrations), of substance(s) that exist in *X. tropicalis* oocytes. These substance (s) likely include either poly(A)$^+$ RNA or proteins synthesized from these maternal molecules. The lack of these substance(s) may partly explain the reduced potential for in vitro culture of cells isolated from cybrid embryos, as well as the developmental failure of cybrid embryos.

Discussion

Cybrid embryonic lethality has been observed in many distant nucleocytoplasmic combinations.[8-11] We have shown here that the cells of [*l*]x*t* cybrid embryos themselves have a reduced in vitro viability and expansion capacity. Thus, distant cybrid embryos may not suffer only from various developmental incompatibilities,[8,9,21] but also because the cells from which they are made may not possess the ability to participate in many normal developmental processes, such as those that involve cell proliferation. Consistent with this, others have had difficulties to maintain or expand ES cells isolated from mammalian iNT embryos in vitro,[29,30] and observed reduced implantation capacities of cybrid cells in in vivo transplantation experiments in fish.[31] This could be due to nucleo-mitochondrial incompatibilities, and/or to any other form of nucleocytoplasmic cellular incompatibility. It was previously recognized that mitochondrial cybrid cell lines, produced by the fusion of mtDNA-less cells of one species with the cytoplasts of another divergent species, can have defects in oxidative phosphorylation, the severity of which is related to the evolutionary distance between the two species.[26,32,33] Yet, even though mitochondrial dysfunction was observed in cell culture in one such distant murine cybrid cell line, mice formed with the same cybrid cells were perfectly normal, suggesting that the functions impaired in this cell line are not necessary for normal development, or that compensation occurred.[34] Cybrid cellular incompatibilities may thus further complicate the analysis and interpretation of some experiments that involve cybrid

Table 2. Embryonic development of poly(A)$^+$ RNA-injected [*l*]x*t* cybrid embryos

Injection[‡]	Normal four-cell[†] (n)	Regular late blastulae (%)	Died during gastrulation (%)	Died during neurulation (%)	Died as an abnormal postneurulae with			
					no distinct features (%)	protruding sucker (%)	muscular response (%)	pigmented eye(s) (%)
$_dH_2O$	84 (5)	77 (91.7)	6 (7.1)	16 (19.0)	40 (47.6)	21 (25.0)	3 (3.6)	9 (10.7)
X. laevis RNA[a]	74 (4)	66 (89.2)	13 (17.6)	17 (23.0)	28 (37.8)	15 (20.3)	4 (5.4)	5 (6.8)
X. tropicalis RNA[b]	73 (5)	64 (87.7)	5 (6.8)	11 (15.1)	26 (35.6)	30 (41.1)	14 (19.2)[c]	26 (35.6)[d]

[‡]Embryos were injected at the one-cell stage with either $_dH_2O$ or 15 ng of oocyte poly(A)$^+$ RNA isolated from the indicated species, in a volume of 9.2 nl. A relationship exists between the injection treatment and development ($p < 0.001$; Chi-square analysis). n, Number of different male-female combinations used to generate the embryos. [†]Embryos that showed abnormal early cleavages were excluded from this analysis. [a]This row does not differ significantly ($p = 0.53$) in pairwise Chi-square analysis vs. $_dH_2O$. [b]This row differs significantly ($p = 0.002$; $p < 0.001$) in pairwise Chi-square analysis vs. $_dH_2O$ and *X. laevis* RNA, respectively. [c,d]Value differs significantly (c: $P_1 = 0.005$; $P_2 = 0.02$, d: $P_1 = 0.003$; $P_2 < 0.001$) in pairwise Chi-square analysis vs. $_dH_2O$ (P_1) and *X. laevis* RNA (P_2).

embryos as any of their developmental defects may be modified/amplified by cellular defect(s) or corresponding compensatory mechanisms. It is therefore imperative that the cellular incompatibilities of distantly related cybrids are further investigated in a cell culture model system.

We have further provided evidence that nuclear species-specific maternal mRNAs, or derived proteins, can help to support the development that is promoted by that species' nucleus, within the context of another species' cytoplasm. The improvement was however quite subtle, yet this could be due to the many technical limitations of the experimental design, as only a certain number of full-length protein copies may be synthesized from any mRNA molecules in the embryo before it reaches a stage where the function of that protein is required. If the concentration of any key protein has not reached a functional threshold in time, then this technique cannot be expected to fully rescue cybrid incompatibilities. Also, it could be that the supplied factors may not localize properly in the recipient cytoplasm. An experiment resembling this one has been recently tried in a murine-to-porcine iSCNT system, whereby mouse ES cell extracts were injected along with a mouse nucleus into mtDNA-depleted recipient porcine oocytes.[17] This significantly improved cybrid development to the blastocyst stage, yet because there was more than one difference between the control and treated iSCNT embryos, it is difficult to conclude whether the improvement came from the mtDNA exchange, and/or from anything else contained in the extracts. Our results are therefore key in that they suggest that improvement of cybrid development can be achieved in the absence of nuclear species mitochondrial supplementation or replacement.

Materials and Methods

Xenopus eggs and embryos. *Xenopus laevis* and *Xenopus (Silurana) tropicalis* adults were purchased from Nasco. They were maintained and induced to lay eggs as previously described, and the eggs were UV-irradiated and cross-fertilized as previously described.[21]

Cell culture. Neurula stage embryos were dissociated in Ca^{2+}/Mg^{2+}-free MBS containing 0.5mM EDTA. Dissociated cells were transferred to gelatin-coated tissue culture dishes (2–3 embryos per well of a 24-well plate) in modified L15 medium (Sigma) [diluted 2/3 with $_dH_2O$, containing 10% FCS, penicillin (100 U/ml), streptomycin (0.1mg/ml), Gentamycin (50 ug/ml) and GlutaMAX I (Invitrogen)]. Cells were then incubated at 23°C and periodically observed until a large number of cells were obviously attached and/or the culture had reached confluence. Cultures were then gradually expanded, when possible, by subplating to dishes of increasing sizes.

RNA injection. Oocytes were collected from *X. laevis* or *X. tropicalis* mature females and defolliculated with Liberase (Roche) as described elsewhere.[35] Total RNA was extracted from the oocytes using a standard Trizol (Invitrogen) based method, followed by poly(A)$^+$ RNA extraction using the Dynabeads® Oligo $(dT)_{25}$ system (Invitrogen), according to the manufacturer's recommendations. Fertilized enucleated eggs were de-jellied using a 2% L-Cysteine (Sigma) (pH 8) solution, placed in a 6% Ficoll (type 400, Sigma), 0.4x MMR solution, and injected at the one-cell stage using a Drummond micro-injector. One *Xenopus laevis* egg contains about 80 ng of poly(A)$^+$ RNA[36] and thus to introduce a significant proportion (~16%) of exogenous poly(A)$^+$ RNA, while also staying within a non-toxic range, we chose to inject 15 ng per embryo as a starting point. The one-cell stage was chosen for injection in order to allow cybrid embryos to translate a maximum amount of *X. tropicalis* proteins before they begin to gastrulate. Embryos were subsequently transferred to solutions with progressively reduced Ficoll and MMR concentrations as previously described.[21]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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