REVIEW

Biological time machines: a realistic approach for cloning an extinct mammal

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ABSTRACT: A recent paper entitled 'Resurrection of DNA function *in vivo* from an extinct genome' (Pask et al. 2008; PLoS ONE 3:e2240) suggests that the return to life of extinct animals could be just around the corner. Indeed, every time a mammoth is unearthed, hot debates on the possibility of 'resuscitating' it take place worldwide. However, most of this on-going discussion is purely speculative, and is not backed by rigorous scientific criteria. In the present review we describe a step-by-step approach to test the functionality of nuclei collected from a non-living mammal through nuclear transplantation into enucleated oocytes.

KEY WORDS: Extinct animal · Nuclear integrity · Cloning · Embryo transfer

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INTRODUCTION

Bringing back to life extinct animals that inhabited the planet in geological times has always been a fascinating issue. The first concrete step taken in this respect was to look at the state of preservation of genetic material in ancient biological remains. This was first done in 1985, when a report on the successful amplification of DNA sequences from a 2300 year old Egyptian mummy was published (Pääbo 1985). Another surprising finding related to such ancient samples is that individual nuclei were still recognizable in the mummy's skin through Hoechst 33342 staining and ultraviolet irradiation, indicating good preservation of tissue structures (Pääbo 2008). Since then, the new field of ancient DNA (aDNA) has progressed at an impressive

pace, and fragments of mitochondrial DNA (mtDNA) have been successfully amplified from the remains of mammoths, ancient wolves and bears (Hofreiter et al. 2001). The most impressive of these early achievements is a landmark paper reporting, for the first time, a short sequence of Neanderthal mtDNA (Krings et al. 1997). However, extracting and processing DNA from archaeological remains is a complex process, with many pitfalls related to its degradation, as well as to bacterial, fungal and modern human DNA contamination (Briggs et al. 2007). These technical hurdles can be overcome only by using highly stringent conditions, thus confining the study of aDNA to a handful of world-leading laboratories.

The advent of the last generation of high-throughput sequencing machines, which allow

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sequencing of up to 10 billion base pairs in a single shot (Nowrousian 2010), has provided a powerful platform for the study of aDNA, culminating in the publication of the Neanderthal mtDNA sequence (Green et al. 2008), followed shortly thereafter by the almost complete sequence (80%) of the extinct mammoth *Mammuthus primigenius* genome (Miller et al. 2008). Moreover, the same team announced in early 2009 that a project to sequence the genome of the extinct Tasmanian tiger (or thylacine) *Thylacinus cynocephalus* was about to begin (www.the-scientist.com/news/display/55333/), while its mtDNA genome has just been sequenced (Miller et al. 2009). These studies are of particular relevance, for they tell us how much of the genome from these extinct animals and hominids is still viable in their extant relatives, providing at the same time a better understanding of the genetic basis of evolution. Such an example was given recently when, after sequencing the entire genome of the Neanderthal, its similarity to modern non-African humans was demonstrated (Green et al. 2010). However, soon after the human genome was sequenced, it became clear that simple reading of the sequence does not provide much information; the crucial aspect would be to understand its function. Deciphering the function of single genes or large chromosomal domains is currently feasible in living organisms (Mikkelsen et al. 2007), but it becomes an almost impossible task when it comes to dealing with the genome of an extinct animal, even if the best error-free sequence is available.

The technological advances in DNA sequencing have further nourished the discussion on the possibility of bringing an extinct animal back to life. A commentary accompanying the publication of the mammoth genome described all the theoretical steps required to make a mammoth from scratch (Nicholls 2008). That article provided in detail the required steps, starting from synthesis of the entire genome, allocating the sequence into individual chromosomes, packaging these into a newly formed nucleus ready to be transplanted into an enucleated elephant oocyte. The resulting cloned embryo could then be transferred into a surrogate elephant foster mother, which would deliver the cloned baby mammoth. Nevertheless, the take-home message from Nicholls' commentary is unambiguous: such a project is still fantasy.

The question then, is: What can effectively be achieved, using the available experimental and embryological know-how?

THEORETICAL APPROACH TO CLONING A NON-LIVING ANIMAL USING SOMATIC CELL NUCLEAR TRANSFER

The animal to be brought back to life should ideally have an extant relative with well-characterized genetics and reproductive physiology. Scientists are very familiar with the genetics and reproductive biology of mice, so a frozen, or otherwise well-preserved, small rodent would be the perfect animal to start with. Unfortunately, frozen small ancient rodents have not been found yet, or at least no such findings have been publicized. The mammoth is therefore the first on the resuscitation list. Indeed, the mammoth has an extant relative, the elephant, particularly the Asian elephant, which is phylogenetically closer to the mammoth than the African species (Miller et al. 2008).

We should first focus on the selection of cells with the most stable genome. In the fortunate case that a well-preserved male mammoth is available, we should try to retrieve spermatozoa from the testes. The spermatozoa genome is a long string of transcriptionally inactive DNA, tightly packed around a protamine core and some testis-specific H2A-like histone variants, resulting in a very stable chromatin structure. Sperm DNA stability over time has been assessed particularly in mice. Spermatozoa collected from mice kept frozen for up to 15 yr were able to fertilize oocytes following intra-cytoplasmic sperm injection (ICSI), and the resultant embryos produced viable normal pups following embryo transfer into surrogate females (Ogonuki et al. 2006). Spermatozoa collected from the frozen bodies were not viable because of the extensive membrane damage inflicted by chilling and freezing injuries, but their nuclei maintained full development potential. These findings suggest that, at least in principle, spermatozoa from a male mammoth preserved in permafrost might turn out to have a functional nucleus as well.

Unfortunately, another set of chromosomes, of maternal origin, in addition to spermatozoa, is required to make an animal, and it is certainly impossible to rely on mammoth oocytes for this purpose. Therefore, the use of diploid somatic cells appears to be the only option available. Differentiated somatic cells injected into enucleated oocytes (somatic cell nuclear transfer [SCNT], also known as cloning) are reprogrammed to a condition of totipotency and are potentially capable of producing offspring after transferring the cloned embryo into a suitable foster mother (Wilmut et al. 1997). To date, almost all farm, pet and laboratory animals have been cloned, and SCNT has also been car-

ried out between species (interspecific somatic cell nuclear transfer, ISCNT; Loi et al. 2001, Tao et al. 2009; for a recent review see Loi et al. 2011).

A STEP-BY-STEP APPROACH TO PRODUCING A MAMMOTH USING SCNT

The preconditions for SCNT of a mammoth are: (1) availability of individual mammoth cells or isolated nuclei and (2) availability of a suitable recipient oocyte.

Availability of individual mammoth cells or isolated nuclei

The only scientific data published to date on mammoth tissue histology were produced by a Japanese team (Kato et al. 2009). In that study, samples of skin, muscle, bone and bone marrow were collected from a leg of a 14 000 to 15 000 yr old mammoth and transported frozen to the laboratory. The samples were processed according to standard histological procedures, mounted on glass slides and stained with haematoxylin and eosin. Nuclei were clearly identifiable in all tissues except skin. Obviously, none of the cells present in a mammoth carcass are viable after 15 000 yr in permafrost, but this is not an insurmountable problem.

Cloning pioneer Marie di Berardino stated that a set of chromosomes and a centriole are all you need for successful cloning (di Berardino 1999). Recent data support her statement. Nuclei isolated from frozen carcasses of mice stored in conventional freezers (without cryoprotectant) for 16 yr have been used to generate embryos by injection into enucleated oocytes (Wakayama et al. 2008). Embryonic stem cells have been derived from such embryos and used for a second round of nuclear transfer (NT) that gave rise to normal, viable offspring. Non-viable cells have successfully been used for NT before (e.g. Loi et al. 2008, Li & Mombaerts 2008); however, what strikes one as interesting in Wakayama et al. (2008) is the long storage time of the frozen carcasses. These findings have been confirmed by similar studies on other species (Hoshino et al. 2008). The non-equivalence between cell viability and nuclear viability have further been highlighted by several studies demonstrating that freeze-dried cells can direct embryonic development following NT, giving rise to embryonic stem cells (Loi et al. 2008, Ono et al. 2008), even after 5 yr of storage at ambient temperature (Loi et al. 2008).

Hence, some cells in a mammoth carcass might have an intact genome, and, thus, it might be worth attempting NT assays. In cases of mild DNA damage, we could still rely on the oocyte's DNA-repairing mechanisms. This capacity in oocytes, first documented in frog *Xenopus laevis* oocytes, is truly amazing. In an elegant experiment, scientists injected previously damaged DNA into *X. laevis* oocytes and recovered it a couple of hours later completely repaired (Lehman et al. 1993). This proves that *X. laevis* eggs can catalyze homologous ligation and illegitimate recombination, suggesting that multiple pathways are available for repairing double-strand breaks. Such a remarkable capacity might turn out to be a powerful ally in the envisioned mammoth project.

Availability of a suitable recipient oocyte

Undoubtedly, the genome of a large number of mammoth cells will have to be tested and, keeping in mind the currently relatively low efficiency of SCNT, 1000s of oocytes will be required for the NT experiments. Ideally, elephant cows would be used as oocyte donors. Three alternatives can be envisioned for this purpose:

(1) Live elephant cows as a source: the elephant ovarian cycle has been studied extensively (for a review see Hildebrandt et al. 2011). Ovulation occurs once every 13 to 18 wk. Under natural conditions, however, ovulation would occur only once every 4 to 5 yr, as cows are anestrus during gestation (20 to 22 mo) and parts of the nursing period (3 to 5 yr). Normally, only 1 oocyte develops to maturation in 99 % of cases (twins occurs at a rate of approximately 1 % in elephants). Alternatively, ovarian stimulation to induce the development of a large number of oocytes could be attempted. This procedure, however, has not yet been reported in elephants and would need to be developed. Such an invasive procedure as *in vivo* oocyte collection would require anesthesia, putting the animals at risk. All these constraints limit the number of oocytes that can be collected from each cow. Three different collection techniques can be considered:

(a) Collection by endoscopy: artificial insemination in elephants is routinely done using an extra-long video-chip flexible endoscope (Brown et al. 2004). Given their long reproductive tract, ovum pick-up procedure in elephants, however, would require the development of an even longer endoscope than that currently available (2.5 m long) for use in artificial insemination.

(b) Collection by laparoscopy: oocyte collection by conventional laparoscopy is out of the question as, in the absence of a pleural cavity in elephants (Brown et al. 1997), inflation of the abdominal cavity might cause collapse of the animal's lungs, leading to its death. A possible alternative to overcome this risk would be to conduct laparoscopy without inflating the abdominal cavity, a technique that is already available (Paolucci et al. 1995). This technique, however, has never been tested on elephants, and, given the enormous size and weight involved, it would have to be modified for the equipment to be able to lift the abdominal wall.

(c) Transrectal collection: in another large terrestrial mammal, the rhinoceros, a technique for transrectal ovum pick-up has been developed (Hermes et al. 2009). This technique might be adapted for elephants, although the much larger size and the location of the ovaries would make such adaptation very challenging at best.

(2) Collection from culled elephants: culling has been used for many years as a management technique for population control in some south African nations, and much of the knowledge on elephant reproduction and anatomy has been generated through studies on culled elephants. The number of culled animals these days is not high, so one could rely on a steady supply of ovaries. Culling is limited to the African savannah elephant *Loxodonta africana*, as the numbers of the more relevant Asian elephant *Elephas maximus*, which is listed on the IUCN Red List of Endangered Species, are far too low.

(3) Elephant ovarian tissue transplantation: ovarian cortical tissue contains a large number of immature oocytes, which, after transplantation, can mature to some extent *in vivo* before being harvested. Live birth following ovarian tissue transplantation was reported >50 yr ago (e.g. Parrott 1960) and has been successfully tested in various species, including humans, using auto-, allo-, or xenotransplantation. In the only report available to date on elephants, African elephant ovarian tissue xenografted into the ovarian bursa of nude mice showed development of antral follicles (Gunaseena et al. 1998). While highly vascular tissue is most suitable for transplantation, transplants can also be performed under the skin for easy access. Ovarian tissue can be harvested from deceased or culled cows, allotransplanted under the skin of captive elephants and, later, at the right stage of the ovarian cycle, retrieved for oocyte collection. While this can be a good source for a large number of elephant oocytes, many such grafts would have to be transplanted because graft loss rate and follicular

death due to ischemia are high (Candy et al. 1997), and even the surviving grafts have short lifespans (Liu et al. 2002, Kim et al. 2005). The recipient elephants would have to be treated against graft rejection, putting them at risk of various infections.

Regardless of the collection technique to be used, most harvested oocytes would have to be matured *in vitro*. While oocyte *in vitro* maturation has been demonstrated in a large number of species, it has not yet been performed on elephants. Although gaps still exist in our understanding of the endocrinology of the elephant estrous cycle, and although at least some hormones work differently in elephants as compared to other mammals (Hildebrandt et al. 2011), we see no reason to believe that, with some intensive experimental work, elephant oocyte *in vitro* maturation cannot be achieved. The availability of an effective *in vitro* oocyte maturation system for elephants would enormously simplify the project.

The efficiency of cloning with living somatic cells is very low, with only 1 to 5 % of cloned embryos developing into offspring (Loi et al. 2007). Using current protocols, the number of oocytes reconstructed with somatic cells that reach the advanced stage suitable for embryo transfer (blastocyst) is around 30 to 40 %. This means that ~300 embryos would have to be produced in order to produce about 100 cloned embryos, which is the minimum number needed to ensure reasonable probability of producing a living cloned offspring following the transfer of such embryos into elephant surrogate mothers. These numbers are not impossible, and can be handled by many experienced embryo biotechnology laboratories. Naturally, the embryo transfer procedure would have to be developed, as it has never been attempted in elephants. In the case of the mammoth, however, it is very likely that the majority of nuclei found in tissues would have extensive DNA damage; therefore, the number of NTs into enucleated oocytes that would have to be carried out with the hope of finding partially damaged and/or 'reparable' DNA is likely to be on the order of 1000s.

In this case, a valuable option could be the use of oocytes from widely available sources, such as mice or the ovaries of slaughtered cattle. Ovulated mouse oocytes or *in vitro* matured cattle oocytes are two equally suitable sources for developmentally competent oocytes. The production of cloned embryos by injecting mouse or cattle enucleated oocytes with mammoth nuclei is technically easy; however, the phylogenetic distance between *Mammuthus* and *Bos* and/or *Mus* genera could give rise to the following problems:

(1) Mitochondrial (mt)/genomic DNA incompatibility: mtDNA encodes some of the subunits of the electron transfer chain responsible for ATP production. Nuclear DNA encodes factors required for mtDNA replication, transcription and translation. Therefore, coordinated mt/genomic DNA cross-talk is essential for normal development (St. John et al. 2004, Bowles et al. 2007). Interspecies NT has been successfully carried out between sheep and mouflon (Loi et al. 2001) and between cattle and gaur (Lanza et al. 2000), closely related species in both cases. In the case of major mt/genomic DNA incompatibility due to high phylogenetic distance, abnormal embryonic growth with alterations in its metabolic profile can be expected. Mammoth/cattle or mammoth/mouse embryos, derived from the injection of isolated mammoth nuclei devoid of any mitochondria, would probably be homoplasmic for cattle or mouse mtDNA, certainly the most extreme mt/genomic DNA hybrid attempted so far.

(2) Zygotic genome activation (ZGA) failure of the mammoth genome in cattle or mouse oocytes: new genome, established at fertilization, becomes transcriptionally active at different stages in pre-implantation embryos, depending on the species. Maternally expressed transcription factors, accumulated and stored in the oocyte cytoplasm, trigger ZGA (Liang et al. 2008). Preliminary inter-generic NT experiments (cow/pig and sheep/pig) showed that, in all cases, the transplanted genome failed to actively transcribe in the host cytoplasm, invariably leading to embryonic arrest (Fulka et al. 2008, Lagutina et al. 2011). Indeed, success of hybrid NT embryonic development is a function of the evolutionary distance between donor nuclei and recipient cytoplasm. An encouraging note comes from the positive, albeit preliminary, results obtained in inter-generic, bovine/primate NT experiments (Wang et al. 2009).

There are, however, possible exit strategies. Inter-order *Mammuthus*–*Bos* or *Mus* embryos could first be produced by NT so as to assess the viability of the mammoth genome. In the fortunate case that hybrid embryos start cleaving, a second round of NT could be carried out, this time by transferring early blastomeres from the cloned hybrid embryo (before ZGA) into enucleated elephant oocytes. In theory, hybrid mammoth/elephant embryos would have a better chance of developing normally, given their phylogenetic relatedness and the genetic similarity between them (99.41%) (Miller et al. 2008). The elephant cytoplasmic factors inducing ZGA would most probably recognize the relative mammoth target gene(s). Although the collection of large numbers of

elephant oocytes might not be easy to accomplish, we believe it can be done using one or more of the above techniques.

Finally, the hybrid mammoth/elephant embryos would have to be transferred into elephant surrogate mothers for development to term. Of course, for the transfer of elephant/mammoth-cloned embryos, a dedicated facility housing the elephant females would be required. Alternatively, the cloned embryos could be frozen and transported to several centres where elephant females would be available. Such a procedure, however, would reduce efficiency even further.

Immunological incompatibility between the genetic mammoth embryo and the elephant uterus is not expected, although we cannot absolutely rule this out. In the unfortunate case that such incompatibility did arise, the last, extreme rescue strategy could be the insertion of inner cell mass (ICM) cells of the hybrid mammoth embryo into elephant trophoblast vesicles isolated from normally fertilized elephant embryos by micromanipulation, as has already been demonstrated (Loi et al. 2007). In this case, the elephant trophoblastic cells would shelter the ICM of the mammoth, allowing the pregnancy to develop to term.

CONCLUSIONS

Cloning an extinct mammal might be seen as purely speculative at the moment. Nevertheless, some of the steps presented in this article have already been accomplished. Kato et al. 2009 successfully isolated mouse nuclei from frozen-thawed bone marrow and injected them into enucleated mouse oocytes. Almost 46% of these nuclei formed pronuclear-like structures. We do not know the level of functionality of the mammoth pronuclei within the mouse oocyte cytoplasm; the simple assessment of DNA duplication activity could have been very informative. However, the fact that a 15 000 yr old chromatin was still able to be recognized by—and respond to—the remodelling molecular machinery of the oocyte is indeed remarkable.

The possibility of restoring an extinct mammal through cloning still remains remote, but not impossible. We must also remember that we are just at the dawn of the artificial life era, or synthetic biology (Gibson et al. 2010), and many of the breakthroughs being achieved might complement the existing embryological procedures, thus enhancing our chances of accomplishing such a pro-

ject. The question then will be what to do with a revived mammoth. A single individual would probably be regarded as a curiosity and would be little more than a circus exhibit, becoming very likely a target for animal rights activists. In the lucky case that a few individuals could be produced, preferably from both sexes and from different nuclei sources, such a group of mammoths would represent a golden mine for evolutionary biologists and animal behaviourists.

Acknowledgements. The research leading to these results received funding from the European Research Council under the European Community Seventh Framework Programme (FP7/2007-2013)/ERC Grant Agreement No. 210103. P.L. acknowledges the support of the EU FP7-KBBE-2009-3 Programme, Project No. 244356, NextGene. J.F.'s laboratory is supported by MZe 0002701401.

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Editorial responsibility: Mike Bruford,
Cardiff, UK

Submitted: November 11, 2010; Accepted: July 8, 2011
Proofs received from author(s): August 23, 2011