Mitochondrial transformation: time for concerted action

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The mitochondrial genome is essential for the expression of several crucial proteins. Defects in mitochondrial DNA (mtDNA) are known to cause a wide variety of clinical disorders, and mutations have been implicated in the ageing process. Our detailed understanding of the mechanisms that govern mammalian mitochondrial gene expression, however, is still surprisingly sketchy, particularly in comparison with our successes in unravelling gene expression in prokaryotes and in the nucleus or cytosol of many eukaryotes. Given the relatively small size of the mitochondrial genome, why is this the case? The answer, in large part, is due to our inability to manipulate the mitochondrial genome or to interrogate the role *in vivo* of various *cis*-acting sequences in mtDNA replication, transcription and messenger RNA expression.

Mitochondrial transfection is complicated for several reasons. First, the mitochondrion is a dynamic organelle surrounded by two membranes, the innermost of which is impermeable to large hydrophilic polyanions such as DNA or RNA. Thus, any successful nucleic-acid-mediated transformation of mitochondria requires that the molecule crosses the plasma membrane, targets the mitochondrion and is imported across two membranes into the matrix, in which it can be expressed. Second, assuming the molecule is able to access the matrix, DNA would need to be stably recombined into an endogenous copy of mtDNA, or maintained independently. This is complicated because the level of *in organellar* recombination in some species, including mammals, is believed to be low in most tissues. This is compounded by our limited understanding of the crucial elements for DNA replication and faithful

transmission. This means that the entire genome needs to be used as a potential vector for replication, and even then we are unsure whether an incoming genome would associate with the soluble and membrane-bound factors necessary to promote mtDNA transmission. Finally, any transfecting molecule would need to carry either a selectable marker or express a foreign element that could be identified, such as a mitochondrially translated fluorescent protein. As many hundreds or thousands of copies of mtDNA are present in any mammalian cell, selection would have to be highly efficient, as it is unlikely that methods of transfection would introduce many copies of foreign DNA.

There have, nevertheless, been many attempts to transfect the mitochondrion, with varying degrees of success and reproducibility. The field has been punctuated by many claims of success, yet no method has been accepted or repeated by independent research groups. Thus, with the exception of biolistic-mediated transformation of yeast mitochondria, we are still frustratingly devoid of robust methods in nearly all species.

Khan & Bennett (2004) suggested an intriguing approach to the transfection of mammalian mitochondria, termed 'protfection'. This methodology has been continually updated and is notable for a history of impressive claims (for example, Keeney *et al*, 2009), including *in vivo* mitochondrial transfection in live rats in 2004 (<http://www.sens.org/files/conferences/sens2/talks/Smigrodzki.mp3>). Although the details of the methods were hard to come by, several more recent papers have detailed the agent vector used for mtDNA protfection. The inventors have tagged the well-characterized mtDNA-binding protein TFAM with a virally based amino-terminal

protein transduction domain immediately upstream from a mitochondrial-targeting sequence. Theoretically, this allows the pre-packaged mtDNA–fusion protein complex to cross the plasma and mitochondrial membranes. Constructs with an N-terminal viral-transduction domain upstream from a mitochondrial presequence have also been used to import extracellular fusion proteins into mitochondria (for example, Rapoport *et al*, 2011). However, the way in which a construct relying on an ionic interaction between DNA and binding protein is able to facilitate mitochondrial import of DNA through three membranes remains unclear. Although the data are intriguing, the methodology has only been applied by one research group so far. Until experiments using protfection can be reliably reproduced by other researchers, it must remain a promising yet unfulfilled method, particularly given its long gestation period.

Another approach has been to use nanocarriers, including DQAsomes and MITO-Porter. DQAsomes—derived from the compound dequalinium—are cationic, self-assembling vesicles that target the mitochondrion. These molecules can condense with DNA and have been shown to localize to mitochondria, in which their DNA cargo is released (Weissig *et al*, 2001). The problem of how DNA could be delivered across the mitochondrial membranes has been addressed by the production of another liposome-based carrier, MITO-Porter, which enters cells by macropinocytosis and mediates mitochondrial membrane fusion (for example, Yasuzaki *et al*, 2010). Import into the mitochondrial matrix requires DNA transfer through both the inner and outer membranes. Although MITO-Porter has yet to be shown to deliver large DNA molecules, it seems to be a promising

approach to mitochondrial transfection. However, no researchers outside the laboratories of the inventors have successfully reported the use of MITO-Porter-mediated mitochondrial transformation.

Evidence for RNA-based transfection processes has also been presented. For several years, Adhya and colleagues have claimed that a specific multi-subunit complex of approximately 500 kDa, present in the mitochondrion of one strain of *Leishmania* (*Leishmania tropica*), can act to import transfer RNA. These authors have reported that this isolated complex can be added to cultured human cells, in which it travels to the mitochondrion and facilitates the import of human transfer RNA from the cytosol (Mahata *et al*, 2006). Furthermore, this complex can be pre-programmed with RNA and has been used to promote RNA-interference-mediated depletion of human mitochondrial messenger RNA (Mukherjee *et al*, 2008). These are remarkable observations, but even after several years there is no evidence that anyone unconnected to the Adhya laboratory has used this complex to promote mitochondrial transfection. Moreover, an expression of concern over data presented in a 2006 publication from the Adhya laboratory has recently been published by the journal *Proceedings of the National Academy of Sciences USA* (Schekman, 2010), and a second correction to a paper published by Dr Adhya in *EMBO reports* is included in this issue of the journal.

Finally, several reports of the introduction of DNA or RNA into isolated mitochondria have been published. There have been claims of successful import by using the protein-import pathway (Seibel *et al*, 1995; Vestweber & Schatz, 1989), electroporation (Collombet *et al*, 1997) or natural competence (for example, Koulintchenko *et al*, 2006). Bacterial conjugation with isolated organelles shows particular promise and has been reported (Yoon & Koob, 2005). None of these methods, however, is commonly used or generally accepted by research scientists for mitochondrial transformation, although natural competence has reportedly been used by several groups. Nevertheless, assuming that one method becomes accepted, the question then becomes how these organelles can be returned to a host for propagation. In this context too, a standard method remains to be found, although there have been sporadic reports that mammalian cells can take up isolated organelles in culture (Clark & Shay, 1982; Spees *et al*, 2006).

The time to verify methods in independent laboratories is overdue. The point of this letter is not to directly question the highlighted methodologies. Indeed, the author is also associated with a simple method of DNA import into isolated mitochondria that defies all efforts of explanation at the molecular level (see Koulintchenko *et al*, 2006). The mitochondrial research community needs to try and validate any methodology, old or new, that purports to promote nucleic-acid-based mitochondrial transformation in intact mammalian cells. If a method can be established as robust and uniformly accepted by the community, it is inevitable that our understanding of basic mitochondrial gene expression and its relation to disease will be accelerated dramatically.

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Complexity: The ultimate frontier?

Simon Conway Morris

Murmur the word ‘complexity’ and it is a bit like tasting a fine wine: ‘forward oaty nose, thrilling undertones of blackberry, whiff of giraffe urine ...’. Everybody knows what you are talking about, but it is all so very elusive. Oats? Giraffe pee? The history of life shouts ‘Look! Once there was bacteria, now there is New York’: thermogenic plants, Bombardier beetles, ballistic fungal spores. The biological world is not only fascinating, but dazzlingly complex, but how do we capture it? Take two pinches of Shannon, a dash of self-organization, sprinkle Kolmogorov liberally, stir with a fractal spoon, and serve immediately. In the computer such a recipe might work, but in the forests and oceans, concepts of complexity slip through our fingers.

Perhaps we need to take a step back; if we can define some of the boundaries, then maybe our mathematical colleagues can step into the cage and pin the beast with their equations. Let’s begin by applying Conway Morris’ Fourth Law of Biology: whenever the word ‘surprising’ is used, be prepared to smell a rat. Such terminology

is employed when we look at ancestral forms. Far from being slobberingly simple, such ancestors are ‘surprisingly’ complex. A striking example involves the earliest eukaryotes. In terms of gene complements crucial for subsequent multicellularity and bodyplan construction, such as SNAREs (Kloepper *et al*, 2007) and homeodomain TALE/non-TALEs (Derelle *et al*, 2007), the archaic eukaryote must have been, well, unexpectedly complex. Much the same can be inferred from other molecular machines, such as the kinesins (Wickstead *et al*, 2010). That such may be the norm, even the rule, is apparent from the nature of the first vertebrates (Heimberg *et al*, 2010). As Alysha Heimberg and co-workers remark, the first worm-like vertebrate “was a more complex organism than conventionally accepted”. To be sure, there are further elaborations, not least by the engine of gene duplication. But ancestral complexity is a non-trivial problem. In part, the solution must lie in co-options (and maybe horizontal gene transfer), but the suspicion remains that self-organization has a crucial role as each biological threshold is breached.