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Genome cryopreservation: a valuable contribution to mammalian genetic research

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Summary

Mouse embryo banking has become an important asset to geneticists. Individual laboratories can now maintain a far greater diversity of stocks than by conventional breeding alone. Also, many mutations that in the past would have been discarded due to lack of space, can now be preserved for future use. Recent advances in cryopreservation techniques have simplified procedures and, in certain cases, resulted in increased rates of survival.

1. Introduction

The ability to preserve mouse embryos at low temperature was first demonstrated 18 years ago (Whittingham *et al.* 1972; Wilmut, 1972). Since that time, the technique has been used effectively for the conservation of at least 15 different mammalian species, including the human. The potential benefits of embryo banking to mouse genetics were anticipated immediately; i.e. to expand resources by preserving all special genetic combinations for future use, as well as protecting against hazards such as fire or disease (Whittingham, 1974). Mouse geneticists were initially somewhat reluctant to consign their valuable stocks to the liquid nitrogen refrigerator, but at this early stage Dr Mary Lyon had the foresight to initiate an investigation, with David Whittingham, into the feasibility of long-term cryopreservation of the unique stocks held by the Genetics Division of the M.R.C. Radiobiology Unit, Harwell.

As a tribute to Dr Mary Lyon, in recognition of her contribution to the establishment of embryo banks in genetic research, we present an overview of the current status of genome cryopreservation.

2. Long term storage

Initially, the possible risk of genetic damage during long-term storage, induced by a cumulative dose of background radiation or by the process of freezing and thawing *per se*, was of major concern. Prolonged storage at normal background levels of radiation (~ 0·1 cGy/year) was simulated by exposing more than 5000 frozen 8-cell embryos to various doses of gamma radiation over periods of up to five years. The effects were assessed using the following parameters: survival after freezing and thawing, continued development *in vitro* and *in vivo*, breeding performance of the liveborn, and the occurrence of mutations amongst their offspring. Apart from the initial embryonic loss inherent in the freezing procedure, which was similar in all groups, no significant difference was found in any of these parameters, up to radiation doses equivalent to approximately 2000 years storage at a normal level of background radiation (Lyon *et al.* 1977; Whittingham *et al.* 1977*a*; Lyon *et al.* 1981; Glenister *et al.* 1984; Glenister & Lyon, 1986). A recent study (Mobraaten & Bailey, 1987) found that freezing and thawing had no effect on the spontaneous mutation rate of genes in the MHC region, as measured by skin-graft rejection.

In the earlier studies on the effect of background radiation, embryo viability was found to be unaffected by storage for up to five years (Lyon *et al.* 1977; Whittingham *et al.* 1977*a*). In addition, feasibility studies were carried out by freezing and re-establishing genetically diverse stocks that were thought to be typical of those that researchers might wish to cryopreserve in the future (Whittingham *et al.* 1977*b*). The stocks were: HT and PT, mutation testing stocks homozygous for *a,bp,fz,ln,pa* and *pe* and *a,b,c,*^{ch}*,d,se,p* and *s*, respectively; Mo^{dp}, an X-linked gene lethal in the male and thus only a third of the surviving offspring carry the dappled gene, Mo^{dp}*; XO*, where

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again only a small proportion of the embryos would give rise to *XO* females (*OY* also being lethal); CBA/CaH and CBA/H-T6, examples of inbred strains, the latter being homozygous for the reciprocal translocation T(14;15)6Ca. Embryos of each type were frozen and stored for between 6 and 21 months before samples were thawed and the surviving embryos transferred to pseudopregnant foster mothers. Breeding lines were successfully re-established from each genotype although only one *XO* female was recovered from the frozen *XO* stock.

Subsequently we have shown that there is no change in the viability of embryos stored for as long as 14·5 years (Table 1). At Harwell, the overall survival of the PT and HT stocks frozen in 1974 and thawed 11 and 14·5 years later was similar to that reported after 15–17 months (Table 1). A randombred stock, CFLP, frozen in 1972 showed similar survival (Table 1) when thawed after 3 months in Cambridge (Department of Physiology), after five years in London (MRC Mammalian Development Unit) and after 13 years in Carshalton (MRC Experimental Embryology and Teratology Unit).

Although the survival of each genotype did not change during storage, there appeared to be differences between the randombred and inbred stocks (Table 1). These studies were not contemporary comparisons but others have reported, in more controlled studies, that genotype has a significant effect on survival (Schmidt *et al.* 1985, 1987). Although the reasons for the differences in overall survival are unknown, interactions between genetic background, cryoprotectants, cooling & warming procedures, choice of sample container, e.g. ampoule or straw, and general sensitivity to manipulation *in vitro* may be contributory factors.

3. Embryo banks

In 1974, Mary Lyon estimated that 32% of the cage space available at Harwell was occupied by rarely used stocks and a further 37% by others used only occasionally (Lyon, 1976). She also noted that even though mutations were arising continuously during experimental studies, it was impossible to keep more than one or two alleles at any particular locus.

> However, despite the value of these mutants, we can in fact keep only a proportion of those we find. For example, we obtain many new alleles at already known loci. If all these could be kept, they could be compared and tested against each other. From this, considerable knowledge might be gained concerning the structure of the loci and the types of mutational damage that are being produced ... Our present resources do not enable us to make such studies.

Once the feasibility of long term embryo storage was established, the first embryo bank for conserving mutant genes and chromosome anomalies was started at Harwell in 1976. Since then, the introduction of powerful mutagens such as ethylnitrosourea and sophisticated methods of manipulating the mouse genome to produce transgenic animals have resulted in a rapid proliferation of new mutations and experimentally altered genomes. In 1974, the gene list published in *Mouse News Letter* contained less than 500 entries. Today, that figure has escalated to more than 2400. Together with the many hundreds of chromosome anomalies, recombinant, congenic and inbred strains, this represents a very substantial increase in the genetic pool available for study. Cryoconservation enables any one laboratory to maintain a wider range of stocks than is possible by conventional breeding alone. Before the embryo bank was established, the Genetics Division at Harwell maintained approximately 120 different stocks; today over 350 are available and more than 200 of these are stored in liquid nitrogen.

The embryo banking policy at Harwell is to cryopreserve all genotypes not needed for current research programmes. Generally, new mutations, whether unique events or new alleles at known loci, are genetically mapped and characterized before

Table 1. *Survival of 8-cell mouse embryos after long-term storage*

Length of storage (years)	Strain	No. thawed	No. transferred	No. foetuses	No. liveborn	Overall survival (%)*
1–1·5	HT[1]	321	128	17	25	13
14·5	HT[2]	40	17	—	7	18
1–1·5	PT[1]	95	40	6	7	14
11	PT[3]	40	18	—	5	13
< 1	CFLP[4]	80	56	—	34	43
5	CFLP[4]	80	62	—	32	42
13	CFLP[4]	80	60	—	37	46

[1] Whittingham *et al.* 1977*b*; [2] Glenister, unpublished data; [3] Glenister & Lyon, 1986; [4] Whittingham, unpublished data.
* Calculated as the percentage of foetuses and liveborn obtained out of number of embryos thawed.

freezing. Since 1976, embryo banks have been set up in many other laboratories world wide storing a variety of inbred, congenic and mutant stocks. These banks play an important role in maintaining the genetic integrity of inbred strains by avoiding the consequence of genetic drift and providing a source for the replacement of genetically contaminated or diseased stocks.

An example of genetic contamination was discovered at Harwell in 1988 when the results of biochemical analysis showed that the 101/H inbred strain carried *Gpi-1s*^{b} and *Pgm-1*^{b}, in addition to the *Gpi-1s*^{a} and *Pgm-1*^{a} alleles characteristic of the strain. It was assumed that the contamination had originated from the C3H/HeH strain since it is homozygous for the alternative alleles and was housed in the same room. Fortunately, it was possible to re-establish the strain from frozen embryos held in the bank at Carshalton since 1980, when breeding of a colony originally obtained from Harwell was discontinued.

Horizontal transmission of disease via the transfer of frozen embryos has not been reported. However, there is no evidence to suggest that the common infectious viral diseases of the mouse are transmitted by embryo transfer (Carthew *et al.* 1983, 1985). Embryo transfer is already used routinely to eliminate viral and bacterial pathogens in mice and rats (Wood, 1981; Glenister *et al.* 1989; P. H. Glenister, D. G. Whittingham & M. J. Wood, unpublished data).

Information about the genetic material contained in embryo banks throughout the world is mainly disseminated by publication in *Mouse Genome* (formerly *Mouse News Letter*). Harwell receives many requests for stocks to be re-established; between 1985 and 1989 thirty-five different stocks were recovered from the bank. For example, in 1988 four of sixteen pink-eye alleles stored since 1979 were thawed for scientists at the Jackson Laboratory, USA. They had cloned a DNA sequence from the pink-eye locus and these stocks provided a unique opportunity for them to analyse *p* alleles at the molecular level. Similarly, five alleles at the albino locus were re-established from the bank for molecular analysis by Dr J. Peters at Harwell. Since 1981, twelve stocks were recovered from the bank at Carshalton e.g. 101/H (see above).

4. Application of recent advances in embryo freezing to banking procedures

The initial success of embryo freezing depended upon slow controlled rates of cooling and warming in the presence of dimethylsulphoxide (DMSO) (Whittingham *et al.* 1972; Wilmut, 1972). As embryo banks were established in laboratories worldwide, efforts were made to simplify and streamline the procedures. Today, all the pre-implantation stages of the mouse, including the oocyte, may be preserved in a variety of containers (plastic insemination straws, glass or plastic ampoules), using one or more cryoprotectants (e.g. DMSO, propylene glycol (PROH), glycerol), at rates of cooling and warming ranging from < 1 to > 2000 °C/min. Also, the non-permeating property of sucrose has been used effectively to achieve some dehydration before freezing and to assist in the

Table 2. *Studies reporting the survival of mouse embryos to foetuses or liveborn after storage in various permeable cryoprotectants*

		Developmental stage			
Cryoprotectant	Cooling	1-cell	2-Cell	8-Cell	Morula/ blastocyst
DMSO	Slow	1	1	1	1
	Plunge LN_2	2	2, 3 and 4	2 and 5	NT*
Glycerol	Slow	NT	6	7 and 8	9
	Plunge LN_2	NT	NT	10	11 and 12
PROH	Slow	13	13	13, 14 and 15	13
	Plunge LN_2	No survival, 2	No survival, 2	2	NT
Methanol	Slow	NT	NT	NT	16
Mono, di, tri & poly, ethylene, glycol, erythritol	Slow	NT	NT	8, 15 and 17	NT

[1] Whittingham *et al.* (1972); [2] Wilson & Quinn (1989); [3] Trounson *et al.* (1987); [4] Shaw & Trounson (1989); [5] Trounson *et al.* (1988); [6] Bernard & Fuller (1983); [7] Wood & Rall (1987); [8] Miyamoto & Ishibashi (1983); [9] Massip *et al.* (1984); [10] Szell & Shelton (1987); [11] Williams & Johnson (1986); [12] Reichenbach & Rodrigues (1988); [13] M. J. Wood & D. G. Whittingham (unpublished data); [14] Renard & Babinet (1984); [15] Miyamoto & Ishibashi (1978); [16] Rall *et al.* (1984); [17] Miyamoto & Ishibashi (1977).

* NT, Not tested.

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Table 3. *Summary of freezing protocols for storing embryos in straws*

Stage	Cryoprotectant (M)	Cooling	Warming	Diluent (M)	Estimated overall survival
Morula and blastocyst[1]	Glycerol (2·0) + sucrose (0·5)	Plunge LN_2 vapour	Rapid 37 °C bath	Glycerol (0·25)	33 %
8-cell[2]	Glycerol (4·0) + sucrose (0·5)	Plunge LN_2 vapour	Rapid 35 °C bath	Sucrose (1·0)	25%
8-cell[3]	Glycerol (1·5)	Slow to −30 °C Plunge LN_2	Rapid room temperature	Sucrose (1·0)	77 %
8-cell[4]	PROH (1·5)	Slow to −30 °C Plunge LN_2	Rapid 30 °C bath	Sucrose (1·0)	66 %
8-cell[5]	DMSO (3·5) + sucrose (0·25)	Plunge LN_2	Rapid 37 °C bath	Sucrose (0·25)	26 %
8-cell[6]	DMSO (3·5) + sucrose (0·25)	Plunge LN_2	Rapid 36 °C bath	Sucrose (0·25) stepwise	60 %
2-cell[6]	DMSO (3·5) + sucrose (0·25)	Plunge LN_2	Rapid 36 °C bath	Sucrose (0·25) stepwise	57 %
2-cell[7]	DMSO (3·0) + sucrose (0·25)	Plunge LN_2	Rapid 35 °C bath	Sucrose (0·25)	68 %
2-cell[7]	DMSO (1·5)	Slow to −80 °C Plunge LN_2	slow	No sucrose stepwise	62 %

[1] Williams & Johnson (1986); [2] Szell & Shelton (1987); [3] Wood & Rall (1987); [4] Renard & Babinet (1984); [5] Trounson *et al.* (1988); [6] Wilson & Quinn (1989); [7] Shaw & Trounson (1989).

Table 4. *Examples of protocols used for vitrification of mouse embryos*

Stage	Cryoprotectant	Cooling	Warming	Diluent	Estimated overall survival
8-cell[1]	DMSO (2·62 M) Acetamide (2·62 M) PROH (1·3 M) PEG (6·0 M)	Plunge LN_2	Rapid 0 °C bath	No sucrose	18 %
8-cell[2]	Glycerol (6·5 M) BSA (6 %)	Plunge LN_2 vapour	Rapid 20 °C bath	Sucrose (1 M)	68 %
Morula and blastocyst[3]	Glycerol (25 %) PROH (25 %)	Plunge LN_2	Rapid 20 °C bath	Sucrose (1 M)	31 %

[1] Rall *et al.* (1987); [2] Wood & Rall (1987); [3] Scheffen *et al.* (1986).

removal of the higher concentrations of cryoprotectants necessary for ultra-rapid freezing and vitrification.

Late stage foetuses and live-born have been obtained from mouse embryos preserved with a wide range of penetrating cryoprotectants (Table 2). Of these DMSO, PROH and glycerol protect most preimplantation stages during slow cooling and all three compounds protect at least some embryonic stages during ultra-rapid cooling. To the best of our knowledge, nobody has reported the successful preservation of 1-cell embryos using glycerol. Practically, there is little to choose between these three widely used compounds. DMSO and PROH permeate the cells more rapidly than glycerol allowing exposure of the embryos in a single-step before cooling (with the exception of blastocysts in DMSO) and rapid removal to isotonic media after thawing. Although glycerol can also be added in a single step, survival depends upon its gradual removal after thawing. Excessive swelling can be avoided by the addition of sucrose to the diluent.

The practical advantages of storing embryos in plastic insemination straws, namely: visual observation of embryos during loading, easy retrieval and reduction in loss of embryos, and economy of storage space, outweigh those of other containers. A summary of the freezing protocols that have been used for

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storing embryos in straws and where overall viability is assessed by development of thawed embryos to late foetuses and liveborn is given in Table 3.

In our experience, the most suitable and reproducible method for storage in straws is that originally described by Renard & Babinet (1984). Essentially, this method involves equilibration with 1·5 M-PROH at 20 °C, ice nucleation at −6 °C, slow cooling to −30 °C at 0·3 °C/min, plunge into liquid nitrogen for storage, rapid warming in a 20 °C waterbath and dilution of the cryoprotectant with medium containing 1 M-sucrose. The technique is suitable for all preimplantation stages. This is of great value for embryos obtained after natural mating where different developmental stages from a given group of females can be collected and frozen on the same day. At Harwell the adoption of this method for storage in straws has resulted in a dramatic increase in survival to liveborn (40% straws versus 14% ampoules). However, this difference was not observed at the MRC Experimental Embryology and Teratology Unit in London (50% straws versus 54% ampoules), but the comparisons were not contemporary and the data was obtained from a variety of strains and mutant stocks.

The introduction of straws, with their large surface area to volume ratio, has enabled survival of embryos to be studied after cooling and warming at very high velocities (Tables 3 and 4). The so-called ultra-rapid methods use higher concentrations of cryoprotectants and sucrose to achieve a certain amount of dehydration before freezing (Table 3). When even higher levels of cryoprotectants are employed, ice formation in the surrounding medium is completely avoided and the whole sample vitrifies (Table 4). With these methods, high rates of survival can be achieved but there is considerable variability in survival reported in the different studies. In our opinion, the current methods require very precise attention to detail, leave little margin for error and require further evaluation before they can be recommended for routine use in embryo banking. Nevertheless, the perfection of such techniques should provide much simpler and cheaper procedures for storage in the future.

5. Conclusion

Embryo banking has become an integral part of mouse genetic research. Already, there are many examples of the rederivation of strains and mutants to check authenticity and for specific research projects. Procedures for the exchange of information have been established. The introduction of straws has improved methods for storage and in the future the cost effectiveness of banking may be further enhanced by the adoption of ultra-rapid cooling and thawing techniques. With the rapid increase in production of transgenic mice, greater demands for both short and long-term embryo banking are foreseen.

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