A method for cold storage and transport of viable embryonic kidney rudiments

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Organ culture of mouse embryonic kidneys is a powerful system for studying normal renal development and for researching the developmental effects of experimental perturbations (drugs, antibodies, interfering RNA, and so on). In standard protocols, embryonic kidneys are isolated by delicate micro-dissection and placed in organ culture as soon as possible after the death of the donor mouse, before there is time to genotype them or to transport them elsewhere. This report demonstrates that fully viable embryonic kidneys can be isolated and cultured from crudely dissected caudal portions of embryos that have been stored on ice or at 4°C for several days. This very simple technique can save considerable resources in laboratories that culture kidneys of transgenic mice: (i) cold storage allows embryos to be genotyped before their kidneys are cultured, and (ii) cold transport allows kidney research laboratories to study kidneys of transgenic mice raised elsewhere without the need for expensive importing of the mouse line itself. It will therefore substantially augment the ability of kidney research labs to perform pilot experiments on large numbers of different transgenic animals and to collaborate in new ways.

Organ culture of embryonic kidneys is a widely used method for studying the molecular cell biology of renal development. Kidney rudiments, isolated from embryos shortly after formation of the ureteric bud, develop organotypically when supported on filters and show many features of normal renal development. These include branching morphogenesis, mesenchymal condensation, epithelial and stromal differentiation, boundary formation, proliferation, and apoptosis.[1–4] Organ culture has the advantage that developing kidneys are highly accessible to imaging,[5] and also to exogenous growth factors, drugs, and interfering RNAs.[6–10] Organ culture also allows the combination of tissues of one genotype with tissues of another genotype, or with tissues pre-treated with an experimental reagent. This allows researchers to test the cell autonomy of a genetic defect or to test the fate choices available to different cells.[11–14]

The use of organ culture to study mutant kidneys suffers from problems not experienced with wild-type kidneys. The first is that importing a colony of mutant mice is an expensive and time-consuming business, particularly for what might only be a pilot experiment. The second is that, for homozygous mutant embryos produced from the crossing of two heterozygotes, cultures have to be set up before the genotype of the donor embryos is known. This procedure results in only pause' the growth of cell lines (Chinese hamster ovary and human embryonic kidney 293), without significant detrimental effect, by storing them at 4–6°C for several days.[15] This raises the possibility that metanephric rudiments may be similarly 'paused' by low temperature. I have therefore performed a simple study of the ability of kidney rudiments isolated from E11.5 mouse embryos, or left inside the caudal part of their embryos for storage and subsequently isolated, to remain viable while being stored on ice or at 4°C.

The results show that ability to develop organotypically in organ culture can be maintained over several days of storage – long enough to permit extra-renal genotyping and long enough to permit the rudiments to be transported almost anywhere in the world. Surprisingly, the method works best when crudely dissected caudal part-embryos are stored with the kidney rudiments still inside them, and kidneys are isolated from these only after storage. This has the useful consequence that genetic laboratories sending transgenic kidneys to collaborating kidney research labs do not require any expertise in fine micro-dissection.

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RESULTS

Storage of isolated kidneys at 0–4°C

Kidneys that were used for organ culture immediately on isolation developed in an organotypic manner over the 72 h culture period, as has been described before.[1] Their ureteric buds, initially T-shaped, branched to produce a mean of 27 ($\sigma = 7.6$) tips, whereas the mesenchyme produced a mean of 9.2 ($\sigma = 5.9$) epithelialized nephrons. Kidneys that were isolated and then stored at either 0 or 4°C before being used for 72 h-organ culture, however, showed a pronounced decrease in their ability to develop normally. The ability of the ureteric bud to branch, for example, declined exponentially with time of storage at 0°C (Figure 1a); according to the equation $tips = 10^{(1.4702 - 0.0241t)}$, where t is time in hours, with correlation coefficient $r^2 = 0.998$ (Figure 1b). Cold storage of already-isolated kidneys is therefore not a useful technique.

Storage of part-embryos at 0–4°C

The conventional method for isolation of kidneys from mouse embryos begins with a transverse cut in the upper lumbar region of the embryo, which divides the embryo into an anterior part (discarded) and a posterior part (Figure 2) that contains the metanephric rudiments. This operation is simple, requiring only a few seconds and no specialized training (unlike subsequent steps); it would therefore be useful if it were possible to store embryos at this stage of the dissection whereas the rest of the embryo was genotyped.

Caudal part-embryos that were stored at 0 or 4°C yielded kidney rudiments that had retained their viability much better than those that were isolated before storage.

Kidneys that had been stored within their part-embryos for 24, 48, and even 72 h showed no significant deterioration of either ureteric bud branching or nephron formation (Figure 3a–e). Morphologically, there were no obvious differences between kidneys that had been isolated and cultured at once and those that have been isolated only after storage for 24, 48, and 72 h. Quantitative analysis of both ureteric bud branching and nephron formation (Figure 3g and h) showed no significant differences between these stored samples and the controls cultured at once. By 96 h of storage, however, the ability of kidneys to develop normally was beginning to be lost, and whereas some degree of ureteric bud branching and nephron formation still took place, its extent was diminished and the shape of the kidneys was somewhat abnormal (Figure 3f). By 120 h of storage, viability was essentially lost (at least in terms of the kidneys' ability to undergo organotypic development). The results were similar whether the storage temperature was for 0 or 4°C.

The cold storage method also worked for embryos containing kidneys at earlier stages. Kidneys with short, unbranched ureteric buds produced a mean of 10.5 ± 4.9 branch tips when cultured for 72 h following their immediate isolation (this is lower than the 25–30 tips produced when T-bud kidneys are cultured for 72 h, partly because the younger rudiments contain less mesenchyme and because the T-bud stage have a 'head start', in terms of number of generations of
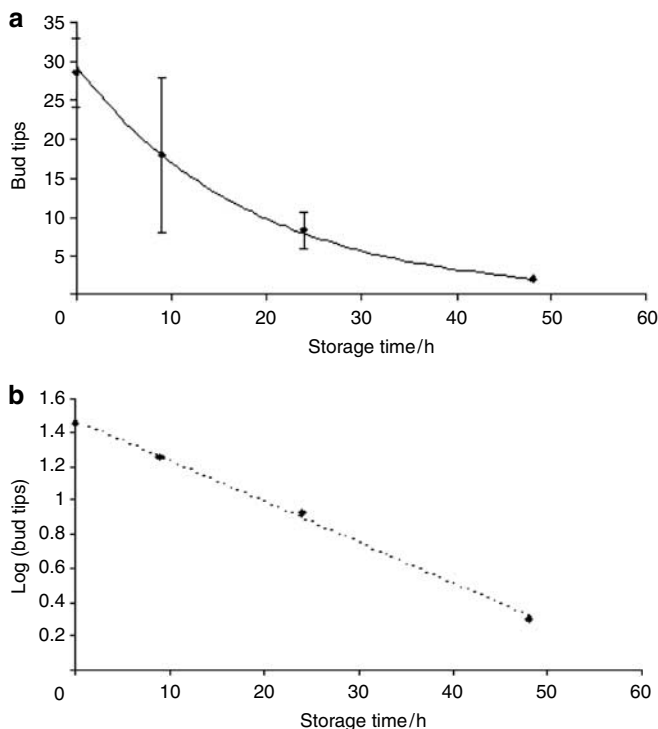
Figure 1 | Ability of isolated kidneys, stored at 0°C, to develop organotypically and to generate a branched ureteric bud tree during 72 h of organ culture after storage. (**a**) Viability of isolated kidney rudiments declines rapidly during storage. (**b**) A log-linear plot of the data presented in (**a**) demonstrates that the ability of stored kidneys to generate new ureteric tips declines exponentially ($r^2 = 0.998$). At the time of isolation, the ureteric bud had two tips, so a *y* axis value of 2 in (**a**), and of 0.3 ($= \log 2$) in (**b**), represents the baseline of no further development.

Figure 2 | An example of a 'caudal part-embryo', produced by cutting the embryo transversely through the lumbar region. Bar = 500 μm.
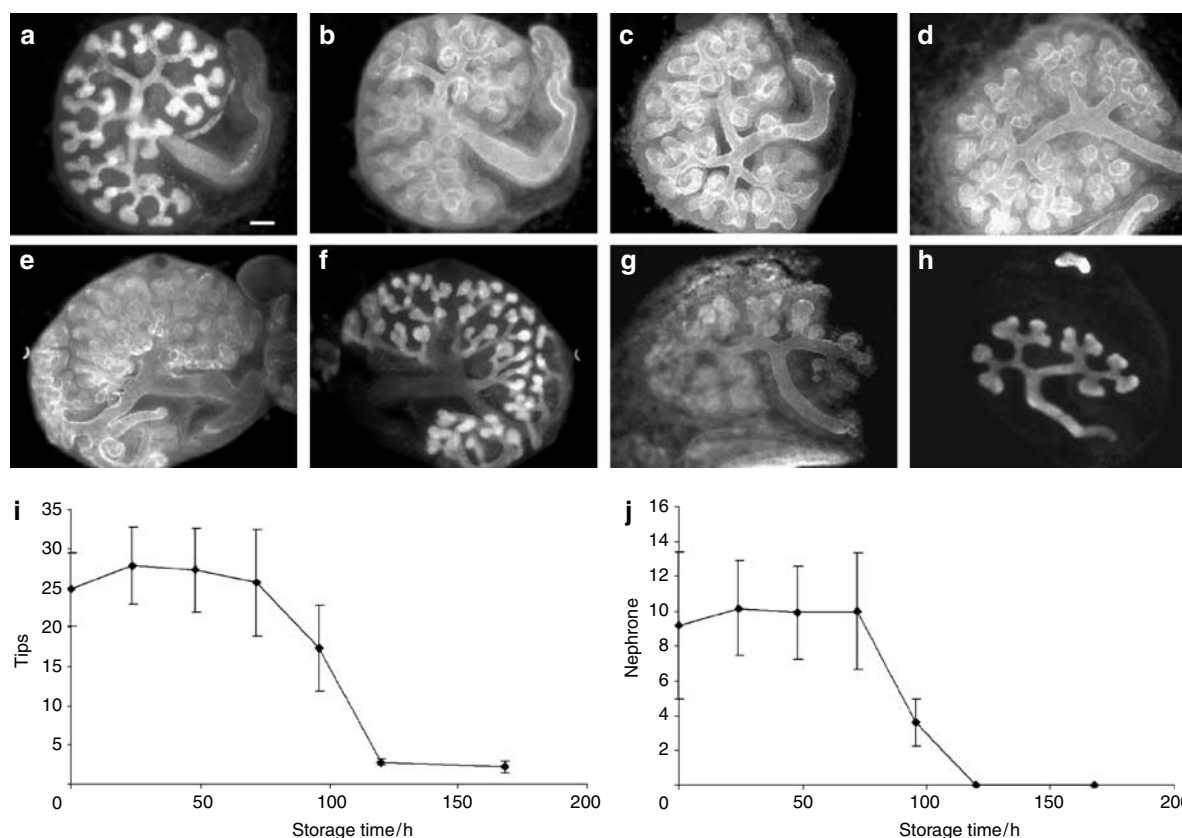
Figure 3 | Ability of kidneys stored in part-embryos to develop organotypically and to generate a branched ureteric bud tree and nephrons. (**a** and **b**) Kidneys that were isolated immediately and cultured for 72 h; they developed normally: (**a**) Anti-calbindin immunostaining, which identifies the ureteric bud/collecting duct system; (**b**) Anti-laminin immunostaining, which binds to both the ureteric bud and nephrons, and allows nephrons (a few of which are marked by arrow heads) to be counted. (**c–e**) Kidneys isolated from part-embryos that had been stored for 24, 48, and 72 h, respectively, are shown; these too develop organotypically. (**f**) A similar 72 h kidney stained for calbindin-D-28k is shown. (**g** and **h**) Kidneys isolated from a part-embryo that had been stored for 96 h, stained for laminin and calbindin-D-28k, respectively; development is now not as good. (**i** and **j**) How the abilities of kidneys isolated from stored part-embryos to form ureteric bud branches and to make nephrons are maintained for up to 72 h of storage, but then decline (two tips represents the starting condition). Bar = 100 μm; error bars represent s.e.m.

branching, compared to ones with unbranched buds). They produced a mean of 12 ± 1.4 tips, not significantly different from the immediately cultured controls, when stored in their part-embryos for 72 h at 4°C before culture. In embryos harvested at approximately E10.25, the future ureteric bud was visible only as a slight bulge in the nephric duct. Culture of the caudal nephric duct and its associated mesenchyme (the future metanephros) resulted in the formation of a small ureteric bud tree with 4.8 ± 0.77 tips (the very small size of the mesenchyme, which did grow well in this medium, restricted the development of renal rudiments isolated at this very early stage). Part-embryos of this age, stored at 4°C, yielded kidneys whose development (4.7 ± 0.37 tips) again showed no significant difference from those isolated at once.

DISCUSSION

The results above demonstrate that, although isolated kidneys quickly lose their ability to develop normally when stored at 0 or 4°C, kidneys can be stored in the context of the caudal part-embryo for up to 72 h and still develop normally

in subsequent organ culture. They can therefore be 'paused' as cell lines can,[15] but not for as long. This storage time is easily long enough for embryos to be genotyped (e.g., by using DNA from their heads). It is also long enough for caudal part-embryos to be shipped from one laboratory to another, even from one continent to another. The fact that crude bisections of embryos, of which any laboratory would be capable, offer the best method of storage is a particular advantage because it allows a laboratory with an interesting mouse line but no renal expertise to collaborate with a specialist renal group.

The finding that kidney rudiments retain their developmental potential less well when isolated than when they are in part-embryos was unexpected. Their demise cannot be due to low temperature alone, as rudiments still in part embryos experienced the same low temperature as those that were isolated. It is possible that the continued health of kidneys depends on factors synthesized by tissues that surround them, but this seems unlikely given that the rate of synthesis of these factors at 0 or 4°C would be expected to be very low.

It is also possible, however, that tissues that surround kidney rudiments in caudal part-embryos function to impede the loss of critical diffusible molecules from the rudiments themselves. The measured temperature coefficient (Q_{10}, the factor by which reaction rate rises for a $10°C$ rise in temperature) for vertebrate protein biosynthesis is typically $\geqslant 2.4$[16] (meaning that rates of synthesis at $4°C$ are $\approx 5\%$ those at $37°C$), whereas measured Q_{10} values for diffusion are typically < 2.0 and often ≈ 1.0 (i.e., almost temperature-independent).[17] If the continuing potential of a kidney rudiment to grow organotypically were to depend on diffusible molecules synthesized within that rudiment (e.g., survival and growth factors already identified[18]), low temperature would mean that synthesis could not keep up with loss by diffusion to bulk medium, unless the rudiment were to be shielded from that bulk medium by other tissues.

Whatever the explanation turns out to be, the fact that kidney rudiments can be 'paused' by storage in caudal part-embryos for up to 3 days is a very useful one and is likely to find applications in many labs devoted to mechanisms of renal development.

MATERIALS AND METHODS

E11.5 CD1 mouse embryos were cut transversely about one hind-limb width cranial to the anterior margin of the hind limb, to produce what is referred to in the rest of this report as the 'caudal part-embryo' (Figure 1a). E10.5 embryos were treated in a similar way. Kidney rudiments were isolated from the part-embryo, either immediately or after cold storage of that region, by micro-dissection in Earle's modified minimum essential medium (Sigma M5650, Poole, England). At this stage, the rudiments had reached the 'T'-bud stage (two ureteric tips).

For cold storage, part-embryos or isolated kidneys were transferred to a 5 ml 'bijou' tube that was filled completely with dissecting medium, which had been pre-equilibrated with a 5% CO_2 atmosphere. The tube lid was tightened fully, sealed with parafilm, and the tube was then transferred either to a bucket of ice or to the $4°C$ compartment of a refrigerator. After cold storage for various lengths of time, kidney rudiments were isolated from the part-embryo (if not already isolated), and were pipetted on to Millipore track-etched polycarbonate filters held by a Trowell screen at the gas/medium interface, the culture medium being Sigma M5650 with 10% newborn calf serum, penicillin, and streptomycin. They were cultured for 72 h at $37°C$.

Cultured kidney rudiments were fixed in cold methanol, washed in phosphate-buffered saline, then stained overnight with rabbit anti-laminin (Sigma L9393; marks basement membranes of all epithelia[19]) and mouse anti-calbindin D-28 K (AbCam, Cambridge, England; marks only ureteric bud/collecting duct in organ culture[20]). After another wash and an overnight incubation in secondary antibody, they were sealed between coverslips using nail varnish (Lizzie cosmetics: 'pretty in pink') and viewed on a Leitz epifluorescence microscope.

The exponential decay equation quoted in the first paragraph of results was obtained by producing a log-linear plot of bud tips (b) against time, and using the linear regression function built into Microsoft Excel to determine the intercept, gradient, and correlation coefficient r^2. The decay equation was derived from the intercept (i) and gradient (g) according to $y = 10^{(i + gt)}$

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