Developmental consequences of embryo and cell manipulation in mice and farm animals*

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Advances in biotechnology in recent decades have revolutionized our understanding of early mammalian development and promise to provide ever more finely tuned and precisely targeted techniques for genetic enhancement of domestic animal species. In demonstrating what is both technically and biologically possible, not only in mice but also in larger animal species, research has provided hope that previously intractable diseases and genetic defects can be successfully combated. Crucial to this research is the ability to culture oocytes, embryos and somatic cells *in vitro* and to sustain their development without inducing adverse short- or long-term consequences. There is a need to refine current culture strategies in farm animal species to avoid jeopardizing their dependent technologies. A key to resolving current limitations of culture strategies is to identify, acknowledge and then address those features of *in vitro* culture that compromise early regulation of mammalian development. The aim of this review is to appraise critically *in vitro* embryo and somatic cell production strategies in the context of their impact on developmental competence and normality at embryonic, fetal and later stages. In addition, effects of physically manipulating embryos and cells, most notably via nuclear and gene transfer technologies, are considered with a view to identifying how detrimental consequences can be avoided.

Recent advances in gamete and embryo manipulation are transforming mammalian reproduction and biotechnology. Some advances have rescinded long-held 'certainties' and also promise to improve the odds in the battle against cancers, hereditary diseases and other illnesses. Scientists today are privileged to witness and contribute to this unfolding story. Privilege, however, brings responsibilities, primarily to ensure that normal development is safeguarded. It is important to monitor the consequences, positive or adverse, of our actions in manipulating gametes (both oocytes and spermatozoa), embryos and cells, whether such manipulation be via exposure to artificial environments, microinjection, nuclear transfer, gene-targeting or other means.

The large offspring syndrome (LOS), first reported in cloned calves (Willadsen *et al.*, 1991) and then after *in vitro* culture of intact embryos (for review, see Young *et al.*, 1998), demonstrates that manipulation of the environment of an embryo can alter subsequent fetal, placental and peri- and post-natal development profoundly in farm animals (McEvoy *et al.*, 1998; Sinclair *et al.*, 1999; Peterson *et al.*,

2000; van Wagten-donk-de Leeuw *et al.*, 2000). This essentially biochemical manipulation of early-stage embryos reflects two features of *in vitro* culture. Firstly, the egg is removed from the oviduct and uterus, which normally provide developmental cues and trophic signals orchestrated to safeguard development of the new entity. Secondly, *in vitro* environments, being thus far based more on empiricism than on precise knowledge of embryo needs, inevitably provide a discordant repertoire of biochemical signals that confound the genetic blueprint of at least some embryos. When such disruption coincides with the commencement of embryonic genome activation (from the two-cell stage onwards, depending on species) errors may increase even though, in contrast to some physical manipulations, genetic codes are conserved. Ironically, the subacute nature of at least some of the aberrant changes induced by *in vitro* production of embryos allows the changes to remain undetected in the short term. Blastocyst production, a hallmark for the efficiency of *in vitro* embryo culture systems, can often be achieved despite detrimental environmental effects. Indeed, Walker *et al.* (1992) reported that more blastocysts were produced from ovine zygotes *in vitro* than from equivalent zygotes *in vivo*. This finding should cause us to question the normality of blastocysts produced in artificial environments where subnormal embryos are perhaps less stringently de-selected than in dynamic conditions *in vivo*.

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This article reviews some of the short- and long-term changes that can be wrought in mammalian oocytes and embryos up to the blastocyst stage (Table 1), and also in somatic and germline cells, by culture or physical manipulation.

Impact of *in vitro* environments

A major requirement for normal long-term development of domestic animal embryos, whether generated entirely *in vivo* or produced *in vitro*, is that sufficient numbers of their constituent somatic cells are diploid. Although mixoploidy is a common occurrence in morphologically normal domestic animal embryos, ploidy may be compromised further as a consequence of conventional *in vitro* production. For example, Viuff *et al.* (1999) reported that the incidence of mixoploidy was significantly higher among bovine blastocysts produced *in vitro* than among *in vivo* embryos from superovulated donors (78 versus 25%); furthermore, 17% of *in vitro* blastocysts but none of those produced *in vivo* had a > 10% incidence of polyploidy. As noted by Viuff *et al.* (1999), advances in detection techniques, for example the availability of chromosome-specific probes for fluorescence *in situ* hybridization (FISH) analysis, are beginning to provide more informative data for individual embryos than were previously available. Thus, it should soon be possible to determine the extent to which mixoploidy is aggravated by *in vitro* culture and whether, as a consequence, subsequent development is distorted. In one study in mice, James *et al.* (1995) reported that diploid-tetraploid chimaeras exhibited retarded development at day 7.5 of gestation and placental overgrowth at day 12.5. This placental overgrowth may have been a consequence of the fact that tetraploid cells were preferentially allocated to extra-embryonic membranes, but it is not yet known whether the same phenomenon occurs in domestic animal species.

Gene expression

Early post-fertilization embryonic genome activation occurs gradually and includes ribosomal RNA (rRNA) gene activation, a prerequisite for the synthesis of proteins with housekeeping and other roles (Hyttel *et al.*, 2000a). Ribosomal RNA gene activation in pig embryos can be adversely modified in culture (Hyttel *et al.*, 2000b), with the result that localization of essential nucleolar proteins is lacking in *in vitro* derived embryos compared with embryos produced *in vivo* (Hyttel *et al.*, 2000a). Laurincik *et al.* (2000) described some key features of rRNA gene activation in bovine embryos *in vitro*, but equivalent data for *in vivo* embryos have not been reported.

In addition to rRNA gene expression, scrutiny of other transcripts has been used to elucidate effects of culture in a number of animal species. Included in such studies are stress-sensitive genes that fulfil various protective roles in preimplantation embryos. In response to elaborate developmental checkpoint criteria aimed at co-ordinating the

multiplicity of events during cell proliferation, these and other genes overcome set-backs during development by slowing or arresting growth to facilitate repair of damage or, where this is excessive, by recourse to selective induction of cell death (King and Cidlowski, 1998). Accordingly, significant increases in the abundance of transcripts from such specialized genes are indicative of the reaction of the embryo to adverse artificial conditions. At Aberdeen, W. D. Rees and colleagues have investigated the expression of growth arrest genes such as *gas5*, *gas6* and *CHOP-10* (*gadd153*) in mouse and cattle embryos and in bovine granulosa cells cultured *in vitro* (Fleming *et al.*, 1997; Fontanier-Razzaq *et al.*, 1999, 2001). These studies have helped to determine the impact of culture constituents, notably serum, on embryonic well-being *in vitro* and may lead to the identification of culture systems that avoid imposing negative developmental stresses on the embryo. When embryonic damage cannot be repaired, cell death may be an inevitable and sometimes self-imposed consequence. This 'self-pruning' strategy mimics normally programmed cell death or 'apoptosis', which is a natural and necessary mechanism used, even in optimal conditions, to sculpt and manage mammalian development, from earliest embryonic cell lineages through to adult limbs (Allan *et al.*, 2001). However, growth arrest and apoptosis may be invoked during culture to an extent that is severely detrimental or even lethal. Moreover, once they have been induced, these processes might not subsequently be sufficiently or appropriately deregulated in artificial environments, for example when such conditions prevent proper regulation of the balance between these and the antagonistic processes of cell proliferation (for a review, see King and Cidlowski, 1998).

The precise threshold beyond which apoptotic activity becomes detrimental to the early embryo is not known, although conditions wherein the number of cells is significantly compromised are generally considered to be suboptimal. An example is the study by Byrne *et al.* (1999) in which the presence of fetal calf serum was associated with increased apoptotic activity and a reduction in the number of blastocyst cells. In this report, apoptosis was detected predominantly in the inner cell mass (ICM), unlike the situation in mice or humans in which the occurrence of apoptosis in blastocysts produced *in vitro* is more random and includes trophectoderm (TE) cells (Jurisicova *et al.*, 1998; Hardy, 1999). Taking into account the likely impact of apoptosis on both ICM cell populations and ICM:TE ratios, Byrne *et al.* (1999) concluded that any alteration in the extent of cell death could influence subsequent fetal development. The implications of alterations in the extent of cell death would depend on whether healthy and vital cells were killed or, in circumstances of depressed apoptotic activity, whether abnormal cells survived. There is not yet any definitive link between the incidence of apoptosis in *in vitro* derived mammalian embryos and the normality or otherwise of subsequent fetal development.

In addition to the rRNA and stress-sensitive gene

Table 1. Impact of mammalian egg culture on some key developmental events and processes up to the blastocyst stage

Event / process	Impact	Developmental consequence	Reference
Karyotype	Aggravation of chromosomal complement	Increased incidence of mixoploidy and polyploidy	Viuff <i>et al.</i> , 1999
Nucleolar protein status	Aberrant rRNA gene activation	Lack of proper localization of key nucleolar proteins	Hyttel <i>et al.</i> , 2000a,b
Apoptosis	Upregulation in adverse conditions	Reduced cell proliferation and altered inner cell mass:trophoblast ratios	King and Cidlowski, 1998; Byrne <i>et al.</i> , 1999
Stress-sensitive gene expression	(a) <i>gadd153</i> upregulation in adverse environments; (b) possible downregulation in some cultures using serum	(a) Developmental retardation; (b) persistence of unwanted or degenerate cells or lineages	Fontanier-Razzaq <i>et al.</i> , 1999, 2001
Growth-regulating ligand/receptor gene expression	Altered expression of imprinted genes (for example <i>Grb7</i> , <i>H19</i> , <i>IGF2r</i>)	Modification or disruption of short- and long-term development	Ho <i>et al.</i> , 1995; Doherty <i>et al.</i> , 2000; Khosla <i>et al.</i> , 2001; Young <i>et al.</i> , 2001
Cell compaction and blastocyst formation	Inadequate or deviant gene expression, including that of the Cx43 gap junction protein gene	Poor cell–cell compaction and other limitations, leading ultimately to hatching failure	Krisher <i>et al.</i> , 1999; Watson <i>et al.</i> , 1999; Wrenzycki <i>et al.</i> , 1999
Protein and amino acid metabolism	Altered uptake in defined media	Reduced protein content and possible depletion of intracellular amino acid pools	Eckert <i>et al.</i> , 1998; Thompson <i>et al.</i> , 1998
Ultrastructural characteristics	Accumulation of lipids and alteration of long-chain polyunsaturated fatty acid complement	Increased risk of oxidative damage; compromised mitochondrial function; structural deformities (for example poorly developed apical microvilli in trophectoderm)	Abe <i>et al.</i> , 1999a,b; Ferguson and Leese, 1999; Crostier <i>et al.</i> , 2000; Reis and McEvoy, unpublished; see Fig. 1, this review
Mitochondrial activity	Increased vulnerability to free radical damage and other stressors	Altered oxidative phosphorylation; possible failure to utilize lipid reserves; risk of degenerative changes	Dorland <i>et al.</i> , 1994; Barnett and Bavister, 1996; Leese <i>et al.</i> , 1998; Walker <i>et al.</i> , 1998; Smith <i>et al.</i> , 2000
Embryonic signalling	Altered interferon τ production	Compromised capacity to establish pregnancy	Wrenzycki <i>et al.</i> , 1999; Kubisch <i>et al.</i> , 2001

expression studies, several reports have cited differences between embryos produced *in vivo* and *in vitro* in respect of transcripts required for other developmental processes (for a review, see Niemann and Wrenzycki, 2000). Likewise, a number of reports contrast gene expression patterns among embryos generated in different culture conditions *in vitro*. Ho *et al.* (1995) observed that culture of mouse embryos in potassium simplex optimized medium (KSOM) supplemented with amino acids induced gene expression patterns that closely resembled those of *in vivo* embryos and which, in respect of *insulin-like growth factor receptor I (IGF-I)*, *IGF-II* and *IGF-II receptor* genes, for example, exceeded the amounts of transcript detected after culture in Whitten's medium. Wrenzycki *et al.* (1999) reported that connexin 43 mRNA transcripts were detectable throughout development in bovine embryos cultured in medium supplemented with polyvinyl alcohol (PVA) but were virtually absent between the 8–16-cell and hatching stages in embryos cultured in the presence of serum. The same authors observed that the abundance of mRNA transcripts for interferon τ , a glycoprotein required for maternal recognition of pregnancy, was significantly increased at the hatched blastocyst stage only in embryos cultured in the presence of PVA. They concluded that gene expression in bovine embryos exposed to exogenous protein *in vitro* was affected in a stage-specific manner. However, Kubisch *et al.* (2001) suggested that increased interferon τ production by bovine embryos cultured in the presence of PVA may be a stress-responsive reaction by the embryos to suboptimal culture conditions.

Embryo composition and constituent integrity

Many reports have demonstrated that 'blastocyst stage domestic animal embryos' produced *in vitro* differ from those produced *in vivo* (Thompson, 1997; Holm and Callesen, 1998; Abe *et al.*, 1999a), and the extent of these differences is dictated by the *in vitro* production system used (Thompson *et al.*, 1995, 1998; Abe *et al.*, 1999b; Krisher *et al.*, 1999). In general, extended exposure of embryos to serum-supplemented media results in more marked changes than exposure to supplements such as albumin or other macromolecules (Thompson *et al.*, 1995; Wrenzycki *et al.*, 1999; Crosier *et al.*, 2000). Ferguson and Leese (1999) reported nearly twofold increases in the triglyceride content of bovine embryos cultured to the blastocyst stage in the presence of serum, and a number of authors have observed that lipid inclusions are a feature of morulae and blastocysts generated in the presence of serum (see McEvoy *et al.*, 2000a) (Fig. 1). Such exogenously derived lipids tend to be enriched with polyunsaturated fatty acids (PUFAs) and consequently constitute a threat to cattle, sheep and pig embryos, which have been shown preferentially to avoid long-chain PUFAs in their endogenous reserves (McEvoy *et al.*, 2000b). The most significant threat, aside from complications posed during conventional cryopreservation (Pollard and Leibo, 1994), is that of oxidative damage to which the preimplantation

embryo is susceptible. Therefore, it is not surprising that Pangestu *et al.* (1996) were able to demonstrate that injection of α -tocopherol, a potent antioxidant, improved cryo-survival of bovine embryos produced *in vitro*. Nasr-Esfahani *et al.* (1990) noted that mouse embryos produced *in vitro* generated more hydrogen peroxide than did embryos produced *in vivo*, predisposing embryos produced *in vitro* to a greater risk from reactive oxygen species. Cattle embryos seem less able than mouse embryos to express antioxidant genes during early development (Harvey *et al.*, 1995) and expression of these genes is also stage-dependent: 9–16-cell eggs are better able to combat oxidative stress than are either zygotes or blastocysts (Van Langendonck *et al.*, 1998). One popular strategy aimed at minimizing oxidative stress *in vitro* is to culture embryos in 'low oxygen atmospheres' (see Bavister, 1995; Barnett and Bavister, 1996; Thompson, 1996).

In contrast to mere compositional changes, which can perhaps be offset by recourse to antioxidants, the occurrence of degenerative anomalies in mammalian embryos poses a sinister threat to the viability and continued normal function of blastocysts produced *in vitro*. For example, Abe *et al.* (1999a) reported that apical microvilli and cell–cell junctional complexes were not as well developed in the trophectoderm of blastocysts produced in the presence of serum as in *in vivo*-derived blastocysts. The presence of protein during embryo culture has been associated with degenerative changes in mitochondria. Crosier *et al.* (2000) reported that compact morulae produced *in vitro* had fewer mitochondria but more lipid than did those produced *in vivo*, and that morulae produced in the presence of serum throughout culture were the most deviant. Although there is not yet any definitive evidence linking altered mitochondrial localization or appearance with either concurrent or later developmental repercussions (Krisher and Bavister, 1998), it is difficult to imagine that severe degeneration could occur with impunity. Leese *et al.* (1998) speculated that increased glycolytic activity of embryos cultured in the presence of serum reflects mitochondrial degeneration of the type reported by Dorland *et al.* (1994). Likewise, Barnett and Bavister (1996) noted that free radical-induced mitochondrial damage could be the reason for altered embryo metabolism *in vitro*. It has been suggested that, in circumstances of mitochondrial degeneration, genomes of surviving mitochondria mutate more frequently than normal (Walker *et al.*, 1998).

Large offspring

It has been stated that changes induced by the *in vitro* environment *per se* are achieved without altering the fundamental genetic code of an individual. However, access to that code and the means whereby it is deciphered, phenotypically expressed, or perhaps eventually transmitted, can be altered *in vitro*. This scenario extends beyond upregulated expression of genes with apoptotic or growth arrest roles because, albeit probably at amounts and times at odds with normal expression *in vivo*, these stress-

Fig. 1. Choice of culture medium influences embryo composition. Expanding blastocysts stained with Sudan black B to highlight the relative abundance of lipid droplets after culture for 5 days of sheep zygotes in synthetic oviduct fluid (SOF) supplemented with either 0.4% (w/v) serum albumin (a) or 10% (v/v) serum (b). Although lipid inclusion rates are variable and lack uniformity of distribution even within individual embryos (A. Reis and T. G. McEvoy, unpublished), exposure to serum during culture tends to increase lipid abundance and droplet size. Scale bars represent 10 μ m.

responsive genes are merely fulfilling their intended roles. Instead, at issue here is the induction of changes in other genes, including those that are imprinted during gametogenesis and early embryogenesis. Imprinted genes express only the paternal or the maternal allele and a number of these genes influence fetal size and viability and their epigenetic modification during culture may underlie the phenomenon referred to as large offspring syndrome, which occurs in ruminant (cattle, sheep) species (see Young and Fairburn, 2000; Young *et al.*, 2001). Apparently analogous consequences also have been detected after more invasive manipulations of oocytes or embryos, notably nuclear transfer, but the extent to which the physical intervention and the inevitable *in vitro* culture component of such procedures are at fault is not yet known. Nevertheless, large offspring syndrome (for reviews, see Kruip and den Daas, 1997; Young *et al.*, 1998; Sinclair *et al.*, 2000) provides emphatic evidence for long-term developmental consequences of domestic animal embryo culture. Epigenetic alterations mediated by non-invasive culture also have been demonstrated in mice after culture of embryos and embryonic stem (ES) cells. For example, Doherty *et al.* (2000) showed that choice of culture medium for mouse embryos significantly and selectively influences *H19* gene expression and DNA methylation in the putative imprinting control domain on the paternal allele.

Consequences of domestic animal embryo culture include aberrations in fetal or placental development *in utero* (Maxfield *et al.*, 1998; Sinclair *et al.*, 1999; Farin *et al.*, 2000; Peterson *et al.*, 2000) and peri- and post-natal differences (Brown and Radziewicz, 1998; McEvoy *et al.*, 1998; van Wagendonk-de Leeuw *et al.*, 2000). A study of post-natal development of oversized (> 60 kg at birth) calves derived *in vitro* demonstrated that, when killed at 13 months of age, these calves did not outweigh calves with normal birth weights derived *in vivo* or *in vitro* but the hearts of oversized calves were abnormally large (McEvoy

et al., 1998; Fig. 2). This finding indicates that *in vitro* effects on developmental programming persisted for more than 1 year after birth and, as with sudden unexplained deaths among lambs derived *in vitro* (Walker *et al.*, 1996), questions whether offspring conceived *in vitro* can match the life expectancy and health of those gestated entirely *in vivo*. In contrast, Sangild *et al.* (2000) suggested that calves derived from embryos produced *in vitro* were more mature than control animals with respect to certain perinatal health parameters.

Physical manipulation of embryos and cells

Physical interventions aimed at revising or copying the genome of a mammalian embryo have been transformed from science fiction into fact. Much of the recent progress has occurred in the UK, with production of gene-targeted sheep via nuclear transfer among the latest advances (McCreath *et al.*, 2000). In response to the remarkable achievements that have demonstrated what is technically possible, additional research is aimed at improving and refining new technologies and, for example, in sperm-mediated gene transfer, establishing the true feasibility of strategies that have yet to be universally accepted for application to domestic animal species.

Undoubtedly, a series of nuclear transfer studies involving sheep has been responsible for most of the scientific headline news in recent years. These advances have placed the prospect of gene-targeted animal production within reach and, in so doing, offer genuine hope for novel therapies and treatments to combat diseases and genetic defects that have thus far thwarted medical efforts. In view of this valuable prize, it is important that the technologies do not fail because of limitations that, if addressed, can be overcome or at least kept to an acceptably low incidence. These technologies are invasive and therefore have repercussions in terms of subsequent

Fig. 2. Postnatal development of cattle from embryos produced *in vitro*: points represent heart:carcass masses of individual bulls categorized as normal or with large offspring syndrome (LOS). Postnatal legacies of embryo production *in vitro* include grossly enlarged hearts among 13-month-old Simmental bulls reared from oversize calves (> 60 kg at birth) derived from embryos produced *in vitro*. In contrast, the hearts of yearling bulls reared from calves weighing 46–59 kg at birth were not enlarged, regardless of whether the embryos the dams had received had been produced *in vivo* or *in vitro*. Neither size at birth nor embryo origin influenced the carcass masses of these animals, all 18 of which were reared together on *ad libitum* feedlot diets and killed at the same live weight (redrawn from McEvoy *et al.*, 1998).

survival and development. Much of the remainder of this review will highlight some of the detrimental developmental consequences associated with nuclear and gene transfer technologies and will consider those features of current strategies that are relevant to the problems encountered. However, before considering how physically invasive procedures such as nuclear transfer and gene or sperm injection can have detrimental as well as beneficial consequences, it is important to note that, as in the non-invasive scenarios already considered, *in vitro* culture *per se* may contribute to some of the problems that have been encountered. It is perhaps impossible to distinguish the effects of culture from those of physical manipulations undertaken *in vitro*, but it would be erroneous to presume that conditions *in vitro* cease to influence cells or embryos that undergo physical manipulations. One question is whether reliance on serum in cloning strategies, for example, to avoid or induce cell quiescence or to support blastocyst production thereafter (Yang *et al.*, 2000), is a safe procedure. It seems incongruous that state-of-the-art nuclear transfer and gene-targeting technologies (for example, see McCreath *et al.*, 2000) rely on a culture constituent that is an ill-defined, inconsistent and pathological fluid (Bavister,

1995). Perhaps some of the adverse consequences of cloning, several of which are analogous to those identified with LOS after serum-supplemented culture of intact embryos (Kruip and den Daas, 1997), are due to the culture of karyoplasts, cytoplasts and reconstituted embryos in the presence of serum. Eggs undergoing the trauma of nuclear transfer might be expected to be more vulnerable to culture-mediated developmental aberrations than intact eggs. The same arguments apply, of course, to other manipulations including ES cell-mediated genetic transformations and, albeit to a lesser extent, sperm-mediated gene transfer and intracytoplasmic sperm injection (ICSI). Therefore, it is important to appraise critically those culture procedures used currently to generate karyoplast donor cells, to facilitate physical manipulation and to support subsequent development of reconstituted or otherwise manipulated ova *in vitro*.

Nuclear transfer – lessons and limitations

The procedure whereby a cell nucleus (karyoplast) can be introduced into an enucleated oocyte (cytoplast) to generate a new individual whose genetic make-up is determined by the transferred nucleus is remarkable (Fig. 3). In many respects, the wonder is that it ever succeeds. However, knowing that it can occur even when the karyoplast is from adult tissue (Wilmot *et al.*, 1997), the quest is to improve its efficiency and to eliminate incidental abnormalities. Nuclear transfer is not a robust technology in either murine or domestic animal studies and most reconstituted eggs never generate viable offspring. Initial enucleation of the oocyte, via a skilled yet crude excision process, and subsequent introduction of a donor nucleus is the equivalent of major transplant surgery and undoubtedly traumatizes both the cytoplasm and nucleus. Consequently, it may be unreasonable to expect a high survival rate, but it remains to be seen whether the efficiency of generating viable blastocysts, fetuses and offspring can be improved significantly.

As progression from the time of nuclear transfer to the birth of resultant offspring is a multi-stage process, it is difficult to predict how efficiently mouse, cattle or sheep clones can be produced. For example, sometimes only a selected cohort of surviving embryos is transferred, as in the study by Wells *et al.* (1999), with the possibility of the outcome thereby exaggerating the overall efficiency relative to studies in which all surviving embryos are transferred. Of course, selection of cloned embryos at the blastocyst stage is a valid strategy which, in terms of surrogate recipient requirements, facilitates efficient generation of offspring. Selection stringency at egg transfer undoubtedly differs across species and laboratories, but the survival of those eggs that are placed into a receptive uterine environment nevertheless provides insights into the developmental competence and normality of embryos generated by nuclear transfer. Evidence from most nuclear transfer studies indicates that losses during pregnancy and at or soon after birth are considerable, with a range of

Fig. 3. Production of clones and genomic copies in calves. As well as the natural occurrence of monozygotic twin or multiple offspring, simple bisection or further subdivision of domestic animal embryos can be used to produce limited numbers of true, genetically identical clones (a). Alternative procedures, using nuclear transfer technologies (b) generate individuals that share identical or near-identical nuclear DNA but these differ in respect of their cytoplasmic mitochondrial DNA (mtDNA) inheritance. Hence, as noted by Campbell (1999), these animals are more properly described as 'genomic copies' than as definitive clones.

abnormalities contributing to this wastage (cattle: Kruij and den Daas, 1997; Cibelli *et al.*, 1998; Wells *et al.*, 1999; Hill *et al.*, 2000; sheep: Wells *et al.*, 1997; De Sousa *et al.*, 2000; and mice: Wakayama *et al.*, 1998). Kato *et al.* (1998) cited the loss of four of eight calves cloned from somatic cells but these losses were attributed to perinatal complications rather than to any developmental abnormalities. The authors indicated that signs of labour and mammary development were subdued in most dams in this study, a feature also noted in some calvings after transfer of intact embryos produced *in vitro* (see Sinclair *et al.*, 2000). Mortality has also been reported some time after calving, notably in a cloned calf derived from tissue taken from a donor that was itself a clone (Renard *et al.*, 1999). In this study, post-natal echocardiography indicated the presence of an enlarged right ventricle, but death at day 51 was due to severe anaemia, and postmortem examination revealed lymphoid hypoplasia and thymic atrophy. The authors concluded that the defect was attributable to epigenetic events during reprogramming of the reconstructed embryo and expressed confidence in their *in vitro* system, which

had already facilitated the generation of > 100 healthy calves via nuclear transfer. In the targeted gene transfer study of McCreath *et al.* (2000), of 14 lambs born alive, seven died within 2 days and four more died within 3 months. Abnormalities included a high incidence of kidney defects and brain and liver pathologies and were similar to those reported after nuclear transfer involving the same cells (Schnieke *et al.*, 1997), leading the authors to conclude that they were probably due to some feature of cell treatment or nuclear transfer rather than to gene targeting *per se*.

One factor that influences the efficiency of nuclear transfer and normality of resultant offspring is the choice of cells for provision of donor nuclei. Renard (1998) noted that late abortions and neonatal deaths in cattle were more frequent after nuclear transfer of somatic nuclei than after nuclear transfer of embryonic nuclei, indicating that karyoplast origin may influence the extent of deviant development in clones. In choosing cells for cloning of domestic animals or mice, decisions may be constrained by end-use considerations as much as by the resilience or age of potential donor cells. In particular, genetically

transformed cells may be most valuable in domestic species with gene knockouts perhaps a priority in mice. Kato *et al.* (1998) noted that it may be preferable to use cells from a proven adult, for example, in respect of milk yield, than cells from a genetically elite but untried embryonic or fetal source. Among adult-derived tissues that have been used, cumulus or granulosa cells have generated promising results. Wells *et al.* (1999) found that nuclear transfer with cultured granulosa cells generated ten calves, all of which survived, although development of re-cloned fetuses did not extend beyond 100 days of gestation. In contrast, mice have been cloned sequentially by the transfer of adult cumulus cell nuclei without any adverse effects (Wakayama *et al.*, 1998). In a novel two-stage procedure, pigs free of developmental and health defects were generated from granulosa cell-derived karyoplasts (Polejaeva *et al.*, 2000). In reporting that nuclear transfer using cumulus cells as donor nuclei generated a 49% blastocyst yield compared with a 23% efficiency when oviductal cells were used, Kato *et al.* (1998) proposed some possible advantages to be gained from use of cumulus cells as donor nuclei. In addition to citing the potential of cumulus cells to interact compatibly with oocyte cytoplasm *in vivo*, the authors also noted the possibility that cumulus cell populations are protected from ageing. This latter argument is based on evidence of telomerase activity in human cumulus cells (Dorland *et al.*, 1997), and raises a significant issue concerning the consequences of generating offspring from nuclear transfer procedures that use adult donor nuclei.

In somatic cells, ageing is accompanied by progressive telomere shortening owing to incomplete replication of terminal sequences of double-stranded DNA; when telomeres become critically shortened, replicative senescence may result through the action of cell cycle checkpoints (Stampfer *et al.*, 1997). In contrast, in situations in which cell ageing is to be avoided, a facility to restore telomere length is achieved by telomerase activity. Therefore, it might be argued that cells with such capability are more suitable sources of karyoplasts for nuclear transfer than ageing cell populations if telomere shortening cannot be reversed during nuclear transfer. In this context, ewe 6LL3 (Dolly) took the lead as a candidate for scrutiny of ageing effects. Analysis of terminal restriction fragment (TRF) length, a proxy for telomere size, in 6LL3 and in other cloned sheep was carried out and compared with TRF length data from relevant cell populations (Shiels *et al.*, 1999a). The results demonstrated that TRF lengths in cells from Dolly were consistent with her being derived from transfer of a nucleus from a cell of a 6-year-old adult, with TRF lengths of other animals reflecting their derivation from younger nuclear material. Shiels *et al.* (1999a) noted that, despite the evidence of apparent telomere shortening, Dolly had not demonstrated any adverse health effects or evidence of premature physiological ageing. Dolly has given birth three times and it is reassuring that the TRF length of her first offspring was consistent with age-matched lambs rather than reflecting the limitations of its dam (Shiels

et al., 1999b). Elsewhere, Lanza *et al.* (2000) reported that transfer of senescent donor nuclei resulted in the birth of six healthy cloned calves and, moreover, that telomere length in nucleated blood cells was restored and even extended, relative to newborn and age-matched controls. Gene expression data for dermal fibroblast cells from these animals were consistent with this finding. The same report also provided evidence of a 50% greater proliferative lifespan among fetal clone-derived fibroblast cells than equivalent control cells *in vitro*, echoing similar findings from another cattle study by Cibelli *et al.* (1998). In contrast, Betts *et al.* (2001) did not detect any evidence of extended telomere lengths in tissues of their cloned calves and suggested that an activated immune response played a role in the telomere-lengthening scenario reported by Lanza *et al.* (2000).

The effectiveness and efficiency of nuclear transfer depends on a range of other factors beyond the scope of this review. Among the topics that need to be taken into account are quiescence of donor nuclei, strategies for cytoplasm production, and oocyte activation (Wakayama *et al.*, 1998; Campbell, 1999; Dominko *et al.*, 1999; Kishikawa *et al.*, 1999; Polejaeva and Campbell, 2000). Differentiation status of successful karyoplasts should also be considered. In this context, Dorland *et al.* (1997) suggested that stem cells may be present in human cumulus cell populations, a possibility that also applies to other tissues (Wilmot, 1999). Ultimately, ongoing refinement of the overall process, including avoidance of hazards of culture *in vitro* referred to previously, should help to eliminate many of the inefficiencies and aberrant developmental features associated with production of cloned offspring. In support of this contention, Polejaeva *et al.* (2000) reported the successful cloning of pigs using a strategy of minimal *in vitro* and, in most steps, serum-free culture. In this study, none of the five offspring born was abnormal or unhealthy although, because the nuclear transfer process also differed from earlier procedures in other respects, the extent to which better piglet vitality was due to more appropriate culture is not known.

In a study indicative of the long-term impact of cloning on animal production, Tamashiro *et al.* (2000) reported that nuclear transfer of adult somatic cell nuclei to produce cloned mice may delay some developmental milestones but does not adversely compromise postnatal behaviour. A curious feature of the cloned mice in this study was that their body weights were increased significantly after birth. Wakayama *et al.* (1998) reported that clones and cloned clones were produced with comparable efficiency, leading them to conclude that successive generations do not undergo changes that influence the outcome of the cloning process.

Gene transfer technologies

In a review of transgenic domestic livestock production, Ward and Brown (1998) highlighted the achievements and limitations of preceding years and concluded that nuclear

Fig. 4. Gene transfer technologies. Production of transgenic animals via pronuclear injection of mouse (a) or cattle (b) zygotes has low efficiency and is likely to be superseded by nuclear transfer of genetically transformed karyoplasts, a process that facilitates the production of gene-targeted animals. Alternative strategies remain elusive, but some researchers believe that individual spermatozoa (c) may yet prove useful as carriers of large constructs or artificial chromosomes.

transfer was required to bolster the capabilities for genetic transformation of farm animal species. In particular, they argued, nuclear transfer would facilitate testing of novel gene constructs, largely because of the greatly increased likelihood of generating transgenic offspring. Nuclear transfer should also help overcome the inefficiency of microinjection, which is a major obstacle to effective application of gene transfer technology in domestic animal species, especially in the context of agricultural production (Murray, 1999; Brink *et al.*, 2000).

Before the landmark study of McCreath *et al.* (2000), the production of transgenic but non-targeted animals via nuclear transfer in sheep (Schnieke *et al.*, 1997), cattle (Cibelli *et al.*, 1998) and goats (Baguisi *et al.*, 1999) was reported. Therefore, it is reasonable to expect that much future research relating to gene transfer in domestic livestock will be hand-in-glove with cloning technology. Consequently, many of the hazards and limitations will be those already described for nuclear transfer and culture *in vitro*. However, some additional steps merit careful consideration, among them the *in vitro* screening process required to identify suitably transformed cells before selection of karyoplasts. Gene constructs are designed to ensure that the gene of interest is co-integrated with a 'marker' gene, frequently one that confers antibiotic resistance, so that only appropriately transformed cells should survive an antibiotic challenge. However, it is important to ensure that, firstly, the antibiotic challenge does not induce undesirable alterations among surviving cells intended for nuclear transfer and, secondly, that the antibiotic resistance component of the inserted gene construct does not compromise the health of resultant offspring. Similar considerations also apply to alternative marker genes, including those for firefly luciferase, green fluorescent protein (GFP) and enhanced GFP (EGFP). Expression of such genes is readily detectable, making them valuable research tools that promise to enhance efficiency of gene transfer (Menck *et al.*, 1998; Gutierrez-Adan and Pintado, 2000; Hadjantonakis and Nagy, 2000), although the risk of mutagenic or toxic effects, and of exposure to ultraviolet excitation light, must be appreciated (Kato *et al.*,

1999; Perry *et al.*, 1999). In addition, there is a genuine risk that, however useful fluorescent animals might be, their profoundly unnatural appearance may generate adverse public reaction. In addition to such eye-catching features, more apparently mundane aspects of transgenic technology also can influence embryo survival after gene microinjection. For example, Wall *et al.* (2000) demonstrated that choice of either of two DNA purification methods influenced embryo survival and pregnancy rate in both mice and sheep, without altering transgene integration rate. Likewise, improvements in gene constructs have meant that some of the extreme consequences associated with the early gene transfer studies in farm animals are now avoidable (see Murray, 1999). Murray (1999) noted that insufficient attention has been paid to anticipating problems that might arise in respect of transgene introgression into populations with different genetic backgrounds.

Although genetic transformation via nuclear transfer promises to supersede or at least complement conventional gene injection procedures, some other strategies, including sperm-mediated gene transfer and ICSI, also merit consideration (Fig. 4). Sperm-mediated gene transfer has had a chequered history and, as noted by Gandolfi (2000), opinion remains divided over claims that genuinely transgenic offspring have been born. Among the many reports, which refer to honey bees, fish species and chickens as well as mice and domestic animals, one report at least has demonstrated transgene transmission to the F₁ generation. In that study, sperm-mediated gene transfer was achieved via an ICSI procedure that involved co-injection of 'killed' spermatozoa with an EGFP gene construct into the cytoplasm of mouse oocytes (Perry *et al.*, 1999). The success of transgene integration was probably due to the fact that sperm membranes were sufficiently disrupted to bind exogenous DNA that subsequently, and thus far inexplicably, survived degradation within the oocyte to become incorporated into the genome of the resultant zygote. Thereafter, the EGFP transgene was transmitted to F₁ offspring, which expressed it in a manner consistent with Mendelian inheritance of a single gene locus. Perry *et al.* (1999) observed that among the advantages of ICSI and

sperm-mediated gene transfer is a capacity to cope with large constructs or artificial chromosomes and perhaps to ensure additional sperm-associated protection of constructs. These authors also advocated ICSI as a more 'user-friendly option' than pronuclear microinjection in the opaque lipid-dense eggs of cattle and pigs. The first report of ICSI in cattle involved the use of 'dead' spermatozoa (Goto *et al.*, 1990) and more recent investigations have demonstrated not only that bovine male pronuclear formation rates differ from those obtained via *in vitro* fertilization but also that, unlike in mouse ICSI, there is a requirement for chemical pretreatment of the spermatozoa (Wei and Fukui, 1999).

Conclusions

In recent years, embryo manipulation technologies *in vitro* have developed to a stage where targeting of precise genetic modifications, already a valuable research tool in mice, is a realistic prospect in farm animal species. Although the technology is still in its infancy (Piedrahita, 2000), successful gene-targeted sheep production represents not only a major advance beyond alternative strategies for gene transfer, notably pronuclear gene-injection, but also a fulfilment of the promise offered by nuclear transfer technology, which was itself made possible by earlier but less spectacular advances in embryo and cell culture. However, it is possible that we will only capitalize fully on the ever more sophisticated nuclear transfer and gene targeting technologies when unspectacular refinements to *in vitro* culture systems finally establish an environment that is hazard-free and that avoids perturbing the early regulation of mammalian development. Having already demonstrated what is possible, scientists must now realize that application of recent and remarkable research advances for the benefit of animal and human health will hereafter depend on minimizing, and preferably eliminating, adverse consequences arising from *in vitro* manipulation of animal cells and embryos. Future research must focus on strategies that help to achieve this, thereby safeguarding offspring and their progeny as well as ensuring that benefits accrue at acceptable costs. Otherwise, promising biotechnologies will fail to fulfil their potential.

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