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Epigenetic and experimental modifications in early mammalian development: Part II

Culture of preimplantation embryos and its long-term effects on gene expression and phenotype

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A growing number of medical, scientific and biotechnological procedures rely on culture of mammalian preimplantation embryos. This review presents currently available data on aberrant offspring development that sometimes arises from commonly applied in-vitro procedures in humans, ruminant species and mice. Comparison between mammalian species reveals similarities in the phenotypic abnormalities that are observed at fetal and perinatal stages of development. In particular, aberrant effects on fetal growth have been observed in multiple studies in which serum complemented the preimplantation culture medium. Although it remains to be determined whether there is a common causal mechanism(s) involved, several hypotheses have been put forward to account for the variety of the observed developmental abnormalities. One of these postulates that culture can result in the epigenetic deregulation of developmentally important genes, and that such epigenetic alterations would affect in particular the expression of genes that are subject to genomic imprinting. Imprinted genes play key roles in the control of fetal growth, and altered imprinting can cause growth defects. Some recent in-vitro culture studies on mice and ruminant species now lend support to this hypothesis.

Key words: assisted reproduction/embryo culture/epigenetic/imprinting/large offspring syndrome

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Introduction

Many medical, scientific and commercial interventions today rely on in-vitro manipulation and culture of mammalian embryos at different time points during early development. Amongst others, these include generation of transgenic animals, gene targeting and assisted reproduction technologies in humans and domestic animals; cloning from embryonic and adult cells also involves manipulation and culture. In recent years, however, a growing number of reports have documented adverse effects of such procedures on post-implantation growth and development. From some of these studies it is evident that the in-vitro production of morphologically normal preimplantation embryos does not guarantee that their post-implantation development and post-natal life will be normal. With the current medical, commercial and scientific interest in cloning from somatic cells, and the likely increasing use of early embryos and cells for scientific and clinical purposes, it is appropriate to understand the precise nature of culture and manipulation-induced abnormalities and to investigate the causal mechanisms involved. In this review, the currently available data on procedures that involve culture of

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mammalian preimplantation embryos are summarized, and their consequences for growth and differentiation during subsequent stages of development are discussed. Phenotypic effects of different procedures are presented for commonly studied mammalian species that are representative of primates (humans), ruminants (sheep and cattle) and rodents (mouse). This comparison between mammalian groups reveals certain similarities in the phenotypic consequences that are observed, and this suggests that there may be common underlying mechanisms. Given the possibility of common mechanism(s), it is interesting to note that they all involve culture in synthetic media, in many instances, complemented with serum. In addition, it is the culture part of various procedures that has been shown to affect phenotype at post-implantation stages of development. This deregulation of developmentally important genes is likely to be epigenetic in nature. It has been postulated, by us and by others (Nagy *et al*., 1993; Moore and Reik, 1996; Dean *et al.*, 1998; Young *et al*., 2000; Khosla *et al*., 2001), that such epigenetic alterations would affect in particular the expression of genes that are subject to genomic imprinting. Several recent studies on mice and ruminant species lend support to this hypothesis.

The effects of preimplantation culture and manipulation

Phenotypic consequences of culture and manipulation of preimplantation embryos, and their frequencies of occurrence, are extremely diverse (Table I). Aberrant fetal growth is observed in all the different mammalian species studied and can arise as a consequence of different in-vitro interventions. Below, we list the phenotypic effects during fetal and perinatal development, of common in-vitro procedures on preimplantation human, ruminant and mouse conceptuses.

Humans

IVF and intracytoplasmic sperm injection (ICSI) procedures involve fertilization of mature oocytes with spermatozoa, followed by culture of fertilized embryos to usually the 2- to 4-cell stage (corresponding to day 2–3 embryos) (Tesarik and Mendoza, 1999). Embryos are then transferred to the uterus of a prospective mother. There have been several reports that consider the outcome of these assisted conception technologies, including articles on conventional in-vitro maturation (IVM) and IVF procedures, and on ICSI (van Steirteghem, 1998). Few studies, however, have systematically analysed the effects of these procedures in comparison with suitable control groups (Leese *et al.*, 1998), and interpretation of data is confounded by the fact that assisted reproduction technologies give rise to high rates of multiple pregnancies. Overall, it appears that, at least single births are not significantly affected by IVF procedures (Ménézo *et al*., 2000). However, in one large study (Tan *et al.*, 1992) it was reported that about 25% of babies produced by conventional IVF were born prematurely, and this was a significantly higher proportion than the value of 6% for non-assisted pregnancies in England and Wales (which were taken as the control group). An increase in the incidence of pre-term deliveries upon IVF was observed by several other groups as well (Australian In-Vitro Fertilization group, 1985; Doyle *et al.*, 1992; Wang *et al.*, 1994; Doyle, 1999; Tarlatzis and Grimbizis, 1999). Low birth weight and fetal growth retardation, as measured by ultrasonographic scanning revealing fetuses that were small for their gestational age, have also been reported in these latter studies. In addition, several groups described an increased risk of hypertension in the mother (requiring hospitalization) and vaginal bleeding at the time of delivery (due to placenta previa) upon IVF treatment (Doyle *et al.*, 1992; Tan *et al.*, 1992; Tanbo *et al.*, 1995; Doyle, 1999). However, in extensive French IVF studies (FIVNAT, 1995; Ménézo *et al*., 2000), these particular risks were found to be comparable with those in the general population. Similarly, a higher incidence of pre-term deliveries and low-birth weight for babies produced by ICSI have been reported in one study (Aytoz *et al.*, 1999), but this was not observed by others (Wennerholm *et al.*, 1996; Wisanto *et al.*, 1996; Bowen *et al*., 1998). The effect of cryopreservation of human embryos has also been investigated and again, results from different laboratories seem conflicting. One group (Sutcliffe *et al*., 1995) reported lower birth weight and a slightly shortened gestation period for frozen–thawed embryos relative to that for embryos in naturally conceived pregnancies, but this was not detected in another study (Wennerholm *et al*., 1997).

In recent years, assisted reproduction programmes in some countries have extended the period of in-vitro culture, sometimes to the blastocyst stage, in order to improve pregnancy rates after embryo transfer (Gardner and Lane, 1997). The effects of such extended periods of culture have not been documented. Given the sometimes dramatic consequences of in-vitro culture in ruminants and rodent species (see below), there may be a need to evaluate extended culture with great care (Gardner and Lane, 1997; Leese *et al.*, 1998; te Velde *et al.*, 1998).

Although in the studies which report negative consequences of in-vitro procedures there seems to be a down-regulation of growth, it should be recognized that early embryonic, non-genetic effects can also lead to enhanced growth in humans. For instance, Beckwith–Wiedemann syndrome (BWS) in humans is characterized by fetal and post-natal growth enhancement, organomegaly, macroglossia, hemihypertrophy, Wilms' tumour of the kidney and other complications. A number of studies implicate the imprinted insulin-like growth factor 2 (*IGF2*) gene and neighbouring imprinted genes on chromosome 11p15.5 in this fetal overgrowth syndrome. Since BWS is sporadic in a high proportion of cases, causal mutations are thought to be epigenetic in many of the patients, and such epigenetic alterations arise most likely during early development (Reik and Maher, 1997; Maher and Reik, 2000). Interestingly, several of the phenotypic abnormalities in BWS, including fetal overgrowth and disproportionate enlargement of internal organs, are not dissimilar to those observed in the culture-induced large offspring syndrome (LOS) in cattle and sheep (see below).

Cattle and sheep

There is extensive documentation on abnormalities that arise as a consequence of in-vitro culture of ruminant pre-elongation embryos (i.e. culture before elongation of the blastocyst; in ruminants, implantation occurs many days after the formation of the blastocyst only, at an early fetal stage). These aberrant phenotypes have been recently referred to as the LOS (Walker *et al*., 1996; Young *et al*., 1998; Sinclair *et al*., 2000). The main characteristic of LOS is increased fetal growth, and newborn animals are significantly heavier than control animals, frequently

Table I. Phenotypic consequences of in-vitro culture and manipulation procedures

Species	Procedure	Consequences	Key references
Humans	IVF	Reduced fetal growth and low birth weight Pre-term delivery Placental abnormalities	Australian In-Vitro Fertilization Group (1985); Tan *et al.*, (1992); Doyle *et al.* (1992); Wang *et al.* (1994); Aytoz *et al.* (1999)
	Cryopreservation	Low birth weight	Sutcliffe *et al.* (1995)
Sheep and cattle	Embryo culture	Increased birth weight Fetal and perinatal death Dystocia Increased gestation length Polyhydramnios Skeletal and organ abnormalities	Walker *et al.* (1992); Farin and Farin (1995); Thompson *et al.* (1995); Walker *et al.* (1996); Kruip and den Daas (1997); Sinclair *et al.* (1999)
	Cloning	Increased birth weight Fetal and perinatal death Dystocia	Keefer *et al.* (1994); Wilson *et al.* (1995); Kruip and den Daas (1997); Wilmut *et al.* (1997); Kato *et al.* (1998); Schnieke *et al.* (1997); Cibelli *et al.* (1998a,b); Kubota *et al.* (2000)
	IVF	Increased fetal growth and birth weight	Sinclair *et al.* (1995); Behboodi *et al.* (1995); Farin and Farin (1995)
Mouse	Embryo culture	Reduced fetal weight Increased fetal death	Bowman and McLaren (1970); Caro and Trounson (1984); Arny *et al.* (1987); van der Auwera *et al.* (1999); Khosla *et al.* (2001)
	Cloning	Increased placental weight Fetal and perinatal death	Wakayama *et al.* (1998, 1999); Wakayama and Yanagimachi (1999)
	ES cell derivation and culture	Increased fetal and neonatal weight Fetal and perinatal death Polyhydramnios	Nagy *et al.* (1990, 1993); Wang *et al.* (1997); Dean *et al.* (1998)
	Pronuclear transfer	Reduced fetal weight	Reik *et al.* (1993); Römer *et al.* (1997)
	Cryopreservation	Increased adult weight	Dulioust *et al.* (1995)

ES = embryonic stem.

with a 2-fold or more increase in weight. This extreme fetal growth has been observed both in sheep (Walker *et al.*, 1992, 1996; Thompson *et al.*, 1995) and in cattle (Behboodi *et al.*, 1995; Sinclair *et al.*, 1995; Kruip and den Daas, 1997; McEvoy *et al.*, 1998). Increased incidences of death during pregnancy and at around the time of birth are other commonly observed phenotypes associated with LOS. The perinatal death is due most frequently to complications during delivery, or to dysfunctional lungs, but in some cases specific congenital abnormalities have also been observed (Walker *et al.*, 1992, 1996; Behboodi *et al.*, 1995; Sinclair *et al*., 2000). Additional LOS phenotypes, in cattle and sheep, include extended gestational length (which only partially accounts for the increase in birthweight), polyhydramnios (Sinclair *et al*., 1999), dystocia (difficulty in giving birth), and poor development and reduced viability during the first few weeks after birth (Walker *et al.*, 1992; Behboodi *et al.*, 1995; Farin and Farin, 1995; Thompson *et al.*, 1995; Kruip and den Daas, 1997). Furthermore, LOS has been reported sometimes to involve also specific skeletal abnormalities, and can give rise to organomegaly of internal organs, in particular of the liver and heart (Farin and Farin, 1995; Walker *et al*., 1996; Kruip and den Daas, 1997; McEvoy *et al*., 1998; Sinclair *et al.*, 1999).

Cloning involves the transfer of nuclei from undifferentiated or differentiated somatic cells into enucleated oocytes. Reconstructed embryos are activated, cultured in synthetic medium for a defined period of time, and then transferred into the uterus of a synchronized recipient (Campbell *et al.*, 1996; Wells *et al.*, 1999). Recent reports on cloned sheep and cattle have described abnormalities during post-implantation development of reconstructed embryos that are similar to those associated with LOS. Unusually heavy offspring were obtained upon cloning in cattle (Wilson *et al.*, 1995; Wells *et al.*, 1999; Kubota *et al.*, 2000) and sheep (Schnieke *et al.*, 1997). Sudden perinatal death in up to half of the cloned animals has also been reported (Keefer *et al*., 1994; Campbell *et al.*, 1996; Schnieke *et al.*, 1997; Wells *et al*., 1997; Wilmut *et al.*, 1997; Cibelli *et al.*, 1998a; Kato *et al.*, 1998; Kubota *et al.*, 2000). In cattle, a proportion of these deaths is thought to be caused by hydrallantois (excess accumulation of allantoic fluid) (Stice *et al*., 1996; Wells *et al*., 1999). Like early embryo culture in cattle and sheep, cloning can also be associated with specific congenital abnormalities such as cleft palate and mandibular defects (i.e. undershot jaw; Schnieke *et al*., 1997; Wells *et al*., 1997).

Increased fetal growth and, as a consequence, higher than normal birth weights have been reported to occur as a consequence of IVM of oocytes and IVF (Behboodi *et al*., 1995; Farin and Farin, 1995; Sinclair *et al*., 1995; Table I). Again, these are procedures that involve culture.

Mice

Few studies on mice document post-implantation consequences of in-vitro culturing of preimplantation embryos. It has been reported (Bowman and McLaren, 1970) that fetuses derived from mouse embryos that had been cultured from the 8-cell to the blastocyst stage were lighter than control fetuses at day 17 of gestation. These authors also reported a reduced post-implantation viability of cultured embryos. Reduced viability as a consequence of in-vitro culture was also noted by several other groups (Caro and Trounson, 1984; Arny *et al*., 1987; Lane and Gardner, 1994; van der Auwera *et al.*, 1999; Khosla *et al.*, 2001). Indeed, a 20% reduction in weight for fetuses (on day 14 day of gestation) derived from blastocysts that had been cultured in a chemically defined medium containing serum has been described (Khosla *et al*., 2001).

Nuclear transfer experiments (cloning) in the mouse, that use either undifferentiated or differentiated somatic cells, were also reported to reduce post-implantation viability significantly (Wakayama *et al.*, 1998, 1999; Wakayama and Yanagimachi, 1999). Amongst other phenotypic consequences, increased incidences of perinatal death and abnormally large placentae were reported in these studies. Another procedure that involves culture of preimplantation embryos is the derivation of embryonic stem (ES) cells, and once derived, ES cell lines are frequently cultured for prolonged periods of time for their use in the generation of transgenic animals (Nagy *et al*., 1993). However, completely ES cell-derived mice obtained from high-passage cell lines were reported to be increased in weight at birth (Nagy *et al*., 1993; Wang *et al.*, 1997) along with high incidences of perinatal death. In agreement with the different studies on newborn ES-derived mice, increased weight of completely ES cell-derived fetuses was reported (Dean *et al*., 1998), and these authors also reported severe interstitial bleeding, polyhydramnios, and aberrant mandible development leading to an undershot jaw.

Pronuclear transfer experiments, where mouse pronuclei were exposed to the cytoplasmic environment of recipient fertilized oocytes, resulted in a growth retardation of about 15% in some of the experimental animals (Römer *et al.*, 1997). Cryopreservation of mouse embryos, though generally thought not to be a major cause of aberrances during post-implantation development, has been reported in one study to result in a small weight increase in adult animals between 39 and 67 weeks of age (Dulioust *et al*., 1995). Effects of cryopreservation, however, appear to depend on the sex of the animal and on the mouse strain used. For instance, in one particular genetic background, a slight influence was noted on the postnatal development of the mandible (Dulioust *et al*., 1995).

In-vitro culture experiments

Although the phenotypic abnormalities observed in different species are diverse, the various procedures applied seem all to lead to aberrant fetal growth in a proportion of the animals produced. It should be important to recognize that all the different procedures share incubation in culture medium. In studies that involve cloning from somatic nuclei (both in ruminant species and mice), for instance, donor cells are cultured prior to nuclear transfer and reconstructed embryos are cultured *in vitro* before transfer to uteri of recipient females (Campbell *et al.*, 1996; Schnieke *et al*., 1997; Wakayama and Yanagamachi, 1999). This is true also for in-vitro fertilized embryos (Tan *et al.*, 1992; Behboodi *et al.*, 1995) and for the derivation of ES cell-lines and their subsequent use for transgenic purposes. Several experiments have thus focused specifically on the consequences of in-vitro culturing of preimplantation embryos. The choice of medium for culture of preimplantation embryos is dependent on their metabolic and nutrient needs. Most culture media are based on the composition of the oviductal fluid, and differ mainly in the concentrations of the individual components (Lawitts and Biggers, 1993). In particular for ovine and bovine in-vitro culture, serum is added to culture media as a protein supplement to enhance the preimplantation development to the blastocyst stage (Bavister, 1995). However, some media do not include serum, or have bovine serum albumin (BSA) as a protein source. The virtuosity of a culture medium is usually judged by the ability of embryos to survive until a given developmental end-point, mostly the end of the preimplantation period (the pre-elongation period in ruminants) resulting in the production of blastocyst-stage embryos.

In sheep and cattle, many reports pertaining to the LOS relate to culture of pre-elongation embryos in culture medium (Walker *et al*., 1996; Young *et al*., 1998; Sinclair *et al*., 2000). Even though the culture systems with which LOS has been associated are varied, they frequently involve the addition of serum to enhance development to the blastocyst stage. Using different culture conditions [Synthetic Oviduct Fluid medium (SOF) + serum, or SOF + BSA], it was shown (Thompson *et al.*, 1995) that ovine embryos cultured in the presence of serum (SOF + serum) produced heavier lambs, even though the development of zygotes to the morula and blastocyst stages did not differ between different media. The effects of serum in the culture medium, and the consequences of co-culture with granulosa cells were also studied in detail (Sinclair *et al*., 1999). These studies on ovine embryos showed that both co-culture and presence of serum in the medium can affect fetal development, and thereby lead to LOS (Sinclair *et al*., 1999). Others (Behboodi *et al*., 1995; Farin and Farin, 1995) obtained similar results for bovine embryos.

With respect to the situation in rodents, it was shown that mouse fetuses derived from in-vitro cultured preimplantation embryos (from the 8-cell stage to blastocysts in Brinster's medium) were lighter than those derived from in-vivo control blastocysts (Bowman and McLaren, 1970). Another group (Lane and Gardner, 1994) were among the first to study the effects of specific components of the culture medium, and noted a beneficial effect of the inclusion of amino acids on post-implantation mouse development. In the same study, these authors demonstrated that (build-up of) ammonium in the culture medium retards fetal development and may give rise to exencephaly. In a recent study, the effects of serum in the medium (M16) were specifically addressed during culturing of preimplantation mouse embryos (Khosla *et al*., 2001). Serum was found to reduce the post-implantation viability of embryos, and the resulting fetuses were also significantly lighter than control fetuses. These findings agree with those of others (Caro and Trounson, 1984; Arny *et al*., 1987), who reported comparable effects of serum on the post-implantation viability of mouse embryos. In these studies on mouse embryos, the morphology of the blastocysts that had developed in

the presence of serum was normal. This emphasizes that an apparently normal in-vitro growth to blastocysts does not guarantee that post-implantation development will be unaffected. Taken together, the systematic studies on ruminants and mice clearly demonstrate that certain culture conditions can have negative consequences for growth and development of embryos after their transfer into recipient females, but these conditions do not necessarily affect the in-vitro development to the blastocyst stage.

Hypotheses

Could there be common, molecular, mechanisms underlying the growth and other abnormalities observed as a consequence of in-vitro culture? As summarized above, different interventions can lead to alterations in fetal growth. This involves mostly a reduction where effects were observed in mice and humans, whereas growth tends to be enhanced in sheep and cattle. It should be recognized, however, that in-vitro culture in mice can also give rise to enhanced fetal growth (Dean *et al.*, 1998), and in BWS in humans, epigenetic deregulation of the *IGF2* gene and of neighbouring imprinted genes is associated with fetal overgrowth (Reik and Maher, 1997; Maher and Reik, 2000). Directional differences between mammalian groups do not exclude the possibility of there being a common causal mechanism(s). The physiology of embryonic and in particular of extra-embryonic development is rather different in ruminant species as compared with that in humans and rodents. Furthermore, in contrast to ruminant species, mice have multiple offspring and the intrauterine growth of individual offspring is to a far greater extent regulated by maternal effects. However, it is clear that the interventions in different mammalian species have in common that early embryos are subjected to the external stress of in-vitro culture, and that this can lead to aberrations in pathways that control fetal growth and development. Such a directed 'acquisition' of a phenotypic character as a consequence of environmental stress (Leese *et al*., 1998) would agree with proposed theories on canalization and genetic assimilation (Waddington, 1942; McLaren, 1999). These theories are applicable in particular where the emergent character is transmitted to the next generation(s). It is unclear whether this happens with LOS in which some of the abnormalities persist to adulthood (McEvoy *et al*., 1998), and it is also too early to determine transgenerational effects related to assisted conception technologies in humans. However, heritability to the next generation has been demonstrated for pronuclear transfer-induced growth-retardation in mice (Römer *et al*., 1997). Considering stress-induced altered use of regulatory and signalling pathways, 'canalization' (Waddington, 1942), it might be pertinent that recent studies on mice and cattle provide initial evidence for the deregulation of the IGF and other signalling pathways as a consequence of in-vitro culture during preimplantation development (see below).

To explain the diversity of the abnormalities that occur as a consequence of culture, and to account for their stochastic nature of occurrence, specific hypotheses need to consider causal mechanisms that can have a wide spectrum of action. It had been observed that the cytoplasm of cells in bovine embryos fragments upon in-vitro culturing (Shamsuddin, 1994). Since interaction of various cytoplasmic factors with the nucleus is essential for normal development (Muggleton-Harris *et al.*, 1982; Evsikov *et al.*, 1990; Latham and Solter, 1991), this fragmentation of cytoplasm has been suggested to be a possible cause of the developmental deregulation (Walker *et al*., 1996). Another postulate is based on the fact that embryos developing inside the oviduct of the mother receive signals and cues that are required for their subsequent implantation and development. These cues are lacking during in-vitro culture, and this absence of signalling molecules could result in the deregulation of genes under their influence (Walker *et al.*, 1996).

It has been observed that in-vitro culture in the presence of serum may alter the kinetics of development to the blastocyst stage (Pinyopummintr and Bavister, 1991; Walker *et al*., 1992; van Langendonckt *et al*., 1997; Khosla *et al*., 2001). In ruminants, such alterations, in particular during the compaction phase, have been reported to influence the number of cells allocated to the inner cell mass (ICM) of the blastocyst (Thompson, 1997). It has been postulated, therefore, that culture-induced changes in the allocation of cells between the ICM and trophectoderm (TE) could contribute to the LOS, for instance by promoting an increased placental size (Walker *et al*., 1996; Leese *et al*., 1998). Further research is required to determine whether, indeed, an increased allocation of cells to the TE promotes the development of larger extra-embryonic membranes and thereby influences the size of the fetus (Sinclair *et al*., 2000). Whatever in-vitro culture does to the early embryo, it might introduce a discrepancy between the developmental age of the embryo and the receptive state of the uterine environment. Such discrepancies, though small, could result in enhanced fetal growth following transfer into recipient females. For instance, increases in fetal weight at days 21 and 37 of gestation was observed following the transfer of ovine blastocysts that had been temporarily exposed to a uterine environment that was three days advanced (Wilmut and Sales, 1981). Whether this corresponds to LOS, however, is unclear (Sinclair *et al*., 2000), and in one other report describing a similar treatment, no consistent effect on fetal growth was observed (Sinclair *et al*., 1998).

A molecular hypothesis put forward by us and others postulates that culture of preimplantation embryos and cells can give rise to aberrant epigenetic modifications in genes (Nagy *et al*., 1993; Moore and Reik, 1996; Dean *et al.*, 1998; Young *et al*., 2000; Khosla *et al*., 2001). After transfer of in-vitro cultured embryos, such alterations would be maintained somatically and might therefore affect gene expression at later, post-implantation, stages of development (Nagy *et al.*, 1993; Dean *et al*., 1998). A well-studied example of a heritable, epigenetic feature that regulates gene expression in mammals, is DNA methylation. During early embryonic development, extensive changes in genome-wide methylation take place (Monk *et al*., 1987; Howlett and Reik, 1991; Yoder *et al.*, 1997), and therefore any perturbation caused in the methylation process could potentially cause deregulation of development at later stages (Dean *et al*., 1998).

Aberrant growth and imprinted genes

A subset of genes within the mammalian genome is expressed from only one of the two alleles depending on whether the gene is inherited from mother or father. Genes that have such a parent-of-origin-specific manner of expression are known as 'imprinted' genes, and the corresponding epigenetic mechanism as 'genomic

imprinting' (Constância *et al*., 1998; Surani, 1998). Genetic studies have established that genomic imprinting is evolutionarily conserved among eutherian mammals, including humans, sheep and mice (Feil *et al*., 1998; Loi *et al*., 1998; Beechey *et al*., 1999; McLaren and Montgomery, 1999). Some 50 mammalian genes have been identified to be subject to imprinting, many of which play key roles in embryonic and extra-embryonic development (Beechey *et al*., 1999; Tilghman, 1999). Detailed studies on the role of DNA methylation in imprinting showed that at imprinted loci there are key regulatory sequences that are methylated on one of the two parental alleles only (Razin and Cedar, 1994). Significantly, at many of these 'differentially methylated regions' (DMRs) the allelic DNA methylation is established in either the male or the female germline, and is then somatically maintained throughout pre- and post-implantation development (Feil and Khosla, 1999). This contrasts with non-imprinted genes, which acquire their methylation patterns at later embryonic stages, upon or after gastrulation (Monk *et al*., 1987; Howlett and Reik, 1991; Yoder *et al*., 1997). This temporal difference in the ontogeny of methylation implies that imprinted genes might be particularly susceptible to methylation changes that occur as a consequence of in-vitro procedures during preimplantation development. It has been proposed, therefore, that fetal abnormalities observed as a consequence of culture during preimplantation development could be to a large part due to changes in the allelic methylation states of imprinted genes (Nagy *et al*., 1993; Moore and Reik, 1996; Dean *et al*., 1998). Furthermore, several imprinted genes are known to play key roles in fetal growth and development (Beechey *et al*., 1999), and alteration in expression of these imprinted genes can severely affect size and morphology of fetuses and newborn animals. This is possibly exemplified best by the studies on the mouse insulin-like growth factor-2 (*Igf2*) gene, which is located in an imprinted gene cluster on distal chromosome 7. Deregulation of the expression of *Igf2* has been shown to severely affect growth in mice (DeChiara *et al*., 1990; Eggenschwiler *et al*., 1997; Sun *et al*., 1997). In humans, epigenetic deregulation of the *IGF2* gene and flanking imprinted genes in the cluster has been implicated in the congenital overgrowth syndrome BWS (Reik and Maher, 1997; Maher and Reik, 2000). Some of the phenotypic abnormalities in BWS patients are not dissimilar to those observed in the LOS, and include fetal overgrowth and disproportionate enlargement of internal organs.

The postulated involvement of imprinted genes in culture-induced phenotypic abnormalities has gained initial support from several experimental studies. In our laboratory, we have used a mouse model system to determine the effects of in-vitro culture on the maintenance of genomic imprinting in early mammalian cells (Dean *et al*., 1998). Deregulation of imprinted genes was frequently observed upon derivation and prolonged culture of mouse ES cell lines (in medium containing serum). This aberrant imprinted gene expression (at the *Igf2*, *Igf2*-receptor, and other imprinted loci) was associated with changes in the allelic methylation status of their regulatory DMRs. Significantly, epigenetic alterations in the ES cells did not become corrected during post-implantation development, and were associated with aberrant gene expression and phenotypic abnormalities in completely ES cell-derived fetuses (Dean *et al*., 1998). Relative to culture-induced loss of imprinting in murine ES cells, it should be interesting to note that human and bovine stem cell-like cells have been derived recently (Cibelli *et al*., 1998b; Thomson *et al*., 1998). Dependent on how regulations will evolve during the coming years, it seems likely that human stem cell-like cells will be increasingly used for various differentiation studies. For the bovine ES-like cells, it has been shown that they can be used for the production of transgenic chimeric animals (Cibelli *et al*., 1998b). The chimeric animals in this study were reported to have LOS abnormalities that could have been caused by the derivation and culture of the stem cell-like cells, by the embryo culture and manipulation, or by a combination of these in-vitro procedures. Relative to the in-vitro use of differentiated cells, for instance for cloning purposes, it is interesting to note that relaxation of *Igf2* imprinting has been reported to occur upon culture of rat fibroblasts (Ungaro *et al*., 1997).

In addition to the studies on murine ES cells, we investigated whether deregulation of imprinted gene expression can also occur upon culture of preimplantation mouse embryos (Khosla *et al*., 2001). Decreased expression of the *Igf2* and the neighbouring, imprinted, *H19* gene was observed in many fetuses derived from blastocysts that had been cultured in the presence of fetal calf serum. On average, these 'serum group' fetuses were about 13% smaller than fetuses derived from blastocysts that had been grown in the absence of serum. In agreement with the altered gene expression, a differentially methylated region upstream of the *H19* gene that had been shown to be important for the imprinting of *H19* and *Igf2* (Thorvaldsen *et al*., 1998), was found to be hypermethylated (Khosla *et al*., 2001). It may be that culture effects on imprinting are dependent on the culture medium used. For instance, in a recent study (Doherty *et al*., 2000) it was reported that culture of 2-cell embryos to blastocysts in Whitten's medium (without serum) leads to loss of (paternal) DNA methylation and gain of expression at the *H19* gene. These recent studies complement an earlier report on disruption of imprinting of the *H19* gene in extra-embryonic tissues as a consequence of in-vitro culture of early mouse embryos (Sasaki *et al*., 1995). Also, the expression of *Grb10*, a growth-related imprinted gene that is thought to have a negative effect on cellular proliferation (He *et al*., 1998), was significantly increased in the fetuses derived from embryos that had been cultured in the presence of serum (Khosla *et al*., 2001). GRB10 belongs to a family of 'growth receptor binding' adapter proteins which function in a tissue-specific manner to link specific receptor tyrosine kinases and other tyrosine phosphorylated proteins to downstream effectors, though its exact role is at present unknown (Daly, 1998). Alterations in the levels of *IGF2* expression have also been documented in bovine fetuses that were derived from in-vitro-produced preimplantation embryos (Blondin *et al*., 2000). In this study, observed alterations seemed to be tissue-specific, with increased expression in the liver.

Few imprinting studies have been performed on the effects of IVF and ICSI procedures. Using a mouse model system, it was found that, at a low frequency, *H19* expression was deregulated in embryos derived following spermatid or sperm injection (Shamanski *et al*., 1999). In particular, these authors observed that in extra-embryonic tissues the normally repressed paternal *H19* allele had become de-repressed in a proportion of the cells, most likely as a consequence of the fertilization procedure. In a recent human IVF study, children born after the application of ICSI were analysed for their allelic methylation status at a key

regulatory region in the imprinted Prader–Willi syndrome (PWS) and Angelman syndrome (AS) regions on chromosome 15q11-q13 (Manning *et al*., 2000). No irregular DNA patterns were detected in 92 children analysed, and none showed clinical symptoms of the neurodevelopmental imprinting disorders of PWS or AS.

Outlook

In this review we have discussed in-vitro procedures that can lead to aberrant fetal growth and development in different mammalian species. Although the protocols used are rather diverse, they all involve culture or incubation in media during varying lengths of time. Based on a large number of studies, it is thought that such in-vitro culture can be a possible cause of the phenotypic abnormalities observed, in particular where serum is present in the medium. Several hypotheses have been put forward to explain the phenotypic consequences of preimplantation in-vitro culture. Although, at present, data seem insufficient to draw firm conclusions, we favour the proposal that epigenetic deregulation of genes—in particular of imprinted genes—may be causally involved (Nagy *et al*., 1993; Moore and Reik, 1996; Dean *et al*., 1998; Young *et al*., 2000; Khosla *et al*., 2001). This molecular hypothesis proposes a memory mechanism that is heritable from the time of intervention to the later stages at which phenotypic abnormalities become apparent. Recent studies in the mouse provide evidence for epigenetic deregulation of imprinted genes as a consequence of in-vitro culture during preimplantation development (Dean *et al*., 1998; Khosla *et al*., 2001). As expected, the methylation changes observed were maintained throughout development and were associated with aberrant gene expression.

How culture can lead to epigenetic alterations in genes is unclear at present, and requires further study. One possibility would be that culture of early embryos and cells deregulates the kinetics of their growth and that this, in turn, interferes with the complex maintenance of methylation imprints (Feil and Khosla, 1999; Khosla *et al*., 2001). Indeed, it has been observed in different species, that embryos cultured in the presence of serum undergo some of the cleavage divisions at different rates as compared with those cultured without serum (Pinyopummintr and Bavister, 1991; Walker *et al*., 1992; van Langendonckt *et al*., 1997; Khosla *et al*., 2001). Culture, in particular in the presence of serum, could thus interfere with components of the cell cycle in undifferentiated cells, and this would result in the improper maintenance of epigenetic marks. Interestingly, several of the known methyltransferase enzymes (Robertson *et al*., 2000) and chromatin regulatory proteins (LeGouy *et al*., 1998; Voncken *et al*., 1999) are cell cycle-regulated and show major alterations in their expression during preimplantation development. Relative to a possible cell cycle deregulation of such enzymatic activities, it is interesting to note that imprinted genes also undergo epigenetic alterations in many types of tumours (where cells have lost control over their cell cycle), and frequently show expression from both parental chromosomes (Squire and Weksberg, 1996). This raises the question as to whether mechanism(s) involved in the deregulation of genes in cancer cells could be similar to the ones implicated in the deregulation of genes upon in-vitro culture. Future research should elucidate the molecular mechanisms and chromatin components that are involved in the normal cell cycle maintenance of epigenetic marks, to then be able to address the question as to how deregulation of the cell cycle can lead to heritable epigenetic alterations and altered gene expression.

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Note added in proof

A recent study by Young and co-workers (Young *et al*., 2001) demonstrates that in sheep, culture-induced LOS can be associated with *IGF2R* expression and methylation.

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