REDOX regulation of early embryo development

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Preimplantation embryonic development is associated with a change in preference in energy metabolism pathways. Although oxidative phosphorylation is obligatory in most species throughout preimplantation development, an increasing role for energy derived from glycolysis is associated with compaction and blastulation. Such a shift in metabolic pathway preference is desirable as the embryo faces an increasingly hypoxic environment *in utero*. We hypothesize that this shift in metabolic preference is associated with a change in the reduction–oxidation (REDOX) state within the embryo, affecting not only the energy production required for development, but also the activity of REDOX-sensitive transcription factors, which may alter gene expression patterns. Shifts in intracellular REDOX state may also contribute to spatial differences in cell activity, especially after compaction, and perhaps even major embryonic events such as fertilization, genome activation and cellular differentiation.

Early embryo development requires energy (that is, the formation of adenosine triphosphate, ATP), which is produced through two possible mechanisms: glycolysis, using glucose as a substrate, and oxidative phosphorylation, using pyruvate or oxaloacetate as a substrate. The pioneering work of Brinster, Biggers, Whitten, Whittingham and Wales from the late 1950s to the early 1970s revealed that for mouse embryo development to the blastocyst stage, pyruvate or oxaloacetate are essential for early cleavage, but that glucose is an effective substrate from the eight-cell stage (Bavister, 1995; Wales, 1975). These studies were the first to reveal that a change in metabolic state is involved in the control of early development and led many other workers to examine the concentrations of exogenous energy substrates required for optimal *ex vivo* embryo development (Bavister, 1995).

Another key energy substrate (although usually not considered as such) is oxygen (Fig. 1). Oxygen is essential for the conversion of ADP to ATP in oxidative phosphorylation through its role as an electron acceptor in the electron transport chain. However, the use of oxygen as an energy substrate also results in the production of reactive oxygen species (ROS), particularly the superoxide anion ($O_2^{\bullet-}$) and the hydroxyl radical ($OH^{\bullet}$). ROS are highly active electron acceptors, able to strip electrons from other molecules that, in turn, become free radicals. Hydrogen peroxide (H_2O_2) is not a radical *per se*, but is a product of $O_2^{\bullet-}$ and metal ion catalysis. However, both H_2O_2 and $O_2^{\bullet-}$ can form the extremely reactive $OH^{\bullet}$. The over-generation of intracellular ROS during culture of mammalian embryos *in vitro* is generally thought to be detrimental to embryo development (reviewed by Johnson and Nasr-Esfahani, 1994; Guerin

et al., 2001). The consensus view is that ‘over-production’ of ROS is unfavourable for embryo development, coincident with perturbed metabolic activity. We believe this to be an overly simplistic view and prefer to think in terms of altered reduction–oxidation (REDOX) states, in which a prolonged oxidized state within the embryo, especially after early cleavage, is not favourable for embryo development. Other *in vitro* culture conditions may also shift the REDOX state unfavourably. Furthermore, there are some specific events in development that appear to be associated with a change in the REDOX state, indicating that REDOX state has a causative role. These events include sperm-mediated oocyte activation, embryonic genome activation and embryonic hatching from the zona pellucida.

Intracellular reduction–oxidation states

The intracellular REDOX state describes a complex interaction of the relative concentrations of reduced and oxidized forms of a variety of molecules, including the nicotinamide adenine nucleotides (NAD(P)⁺/NAD(P)H), flavins (FAD⁺/FADH), ubiquinones, peroxides and thiols–disulphides (for example, glutathione (GSH/GSSG)), and others (Fig. 1). Estimation of the REDOX state is difficult to measure with accuracy, and is usually achieved by direct or indirect measurement of REDOX molecules, such as intracellular glutathione or peroxides, [NAD⁺]/[NADH] or [pyruvate]/[lactate].

REDOX state is also significantly influenced by factors that stimulate the generation or neutralization of intracellular ROS. ROS are produced primarily through electron transport at the inner mitochondrial membrane, but also by cytoplasmic or membrane-bound NADPH-oxidase, cytochrome p450 enzymes and the xanthine–xanthine oxidase systems (Gilbert and Colton, 1999). Mammalian cells have

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Fig. 1. REDOX regulation of early embryo development incorporating oxygen as a key modulator of ATP production via metabolic pathways (oxidative phosphorylation (OXPHOS) and glycolysis) and REDOX state. A consequence of OXPHOS is production of O₂•⁻, the amount of which significantly affects the REDOX state by the interaction with REDOX-cycling molecules such as the nicotinamide adenine nucleotides (NAD(P)⁺/NAD(P)H) and glutathione (GSH/GSSG). Glycolytic pathway activity is affected by both oxygen availability (through the stabilization of hypoxia-inducible factor 1 (HIF-1), which is regulated by the oxygen sensor and subsequent gene expression) and indirectly through the REDOX state (via the influence of availability of NAD(P)⁺/NAD(P)H). Other cellular functions, such as apoptosis and cell cycle control, are also significantly influenced by oxygen availability and REDOX state, via transcription factors such as nuclear factor kappaB (NFκB). ROS: reactive oxygen species.

several mechanisms to protect against the generation of ROS. Several enzymes catalyse their destruction, including Cu–Zn and Mn-superoxide dismutases, catalase and glutathione peroxidase, some of which are known to be transcribed in preimplantation mammalian embryos (Harvey *et al.*, 1995). Many compounds have antioxidant activity and act as electron donors for oxidant species and either yield inactive molecules themselves (such as thiols forming disulphides) or are sufficiently stable as radicals to be virtually non-reactive (for example, α -tocopherol). However, ROS present a paradox in that they may act as second messengers in mammalian cells (Schreck and Baeuerle, 1991; Wenger, 2000). Several genes are activated in response to alterations in ROS concentration including those for protein kinases (Burdon, 1996), tyrosine kinases and growth factors (Nose, 2000). The interaction of cytokines and growth factors with cell receptors has been shown to elicit increases in ROS production (Nose, 2000). However, it is most likely that the balance between ROS production and elimination (that is, the REDOX state), rather than ROS themselves, determines these responses.

Therefore, REDOX state regulation has important functions for optimal growth responses (Burdon, 1996).

Environment of the reproductive tract

After ovulation, fertilization, and early embryo development in the oviduct, embryos migrate to the uterus, where implantation occurs. The timing of implantation relative to the development of the embryo differs among species. Despite these differences, the timing of the transition from oviduct to uterus for most species occurs around the time of compaction and blastulation (Boyd and Hamilton, 1952), indicating that the oviduct is excluded physiologically as a site for significant post-hatching development.

Oviductal O₂ concentration is about 40% or less than atmospheric concentration (Leese, 1995) and it appears that the uterine environment has an even lower O₂ concentration than that of the oviduct (Fischer and Bavister, 1993). Thus embryos encounter a decreasing O₂ concentration gradient as they progress down the reproductive tract. Furthermore, during early implantation, hypoxic and even anoxic

conditions confront the invading trophectoderm (reviewed by Leese, 1995). In addition, fluid compositional studies have established that oviductal fluid generally has relatively low glucose concentrations ($< 1 \text{ mmol l}^{-1}$) compared with those in uterine fluid, indicating an environment more favourable for glycolytic activity in the uterus.

Metabolic evidence for REDOX regulation of embryo development

Energy (ATP) production increases at the onset of compaction and blastocyst development to support increased protein synthesis and activity of ion-transport systems, notably the Na^+/K^+ -dependent ATPase (Wales, 1975; Leese, 1995). An increase in ATP production is inferred from the increases in uptake of oxygen and energy substrates, such as pyruvate and glucose (Houghton *et al.*, 1996; Thompson *et al.*, 1996). Furthermore, measurements of ATP:ADP ratios imply that demand for ATP increases after major activation of the embryonic genome (Rozell *et al.*, 1992). The increase in glucose uptake and metabolism from compaction appears to be a common phenomenon for embryos across a range of species. This increase has been attributed to increased glycolytic enzyme activity (Houghton *et al.*, 1996) and increased capacity for glucose uptake by facilitated transporters (Pantaleon and Kaye, 1998). During development to the blastocyst stage, there is a significant shift from a dependence on ATP generation by oxidative phosphorylation to a dependence on ATP generation by glycolysis. In particular, glycolytic activity leading to lactate production accounts for all glucose uptake in post-compaction stage embryos of several species including rats (Brison and Leese, 1991), sheep (Gardner *et al.*, 1993), cows (Thompson *et al.*, 1996) and humans (Gott *et al.*, 1990). Collectively, these data can be interpreted as signifying that post-compaction development is associated with a significant shift in the REDOX state to a more reduced state, favoured by post-compaction embryos to accommodate an increasingly hypoxic environment. This is not to say that oxidative phosphorylation is not important during post-compaction development. Indeed, mouse blastocysts that have a reduced capacity for oxidizing substrates via the Krebs cycle (that is, they convert 100% glucose to lactate) are associated with poor post-transfer development (Lane and Gardner, 1998).

Manipulation of substrates and metabolic pathways during development *in vitro*

Studies using inhibitors of oxidative phosphorylation have revealed significant differences in developmental ability, dependent on whether treatment is applied before or after compaction. Rat (Brison and Leese, 1994), cattle (Thompson *et al.*, 2000) and pig (Macháty *et al.*, 2001) embryos continue development after compaction when oxidative phosphorylation is either partly or completely inhibited. In contrast, application of inhibitors during pre-compaction development is highly inhibitory to development

(Thomson, 1967; Brison and Leese, 1994; Thompson *et al.*, 2000).

However, compounds that either suppress glycolysis, such as ethylene diamine tetra-acetic acid (Gardner and Lane, 1996), or stimulate the tricarboxylic acid (TCA) cycle, such as 2-dichloroacetic acid (Penzias *et al.*, 1993), during pre-compaction development, have been found to improve development.

Decreasing the O_2 concentration within the incubation atmosphere from atmospheric to more physiological concentrations during culture *in vitro* is beneficial for mammalian embryo development (for examples, see Thompson *et al.*, 1990; Li and Foote, 1993; Dumoulin *et al.*, 1999). Both the number of cells and the proportion of embryos developing to the blastocyst stage increase when oxygen concentrations during incubation are in the 5–7% range. Furthermore, metabolic activity of embryos cultured under decreased O_2 tension correlates more closely with that of *in vivo* recovered embryos (Hooper *et al.*, 2001). These results have largely been interpreted in terms of reduced O_2 tension decreasing the production of ROS within the embryo. However, this conclusion is based on little direct evidence. Fluorescence of the intracellular peroxide sensor, dichlorodihydrofluorescein diacetate (DCHFDA), was not correlated with O_2 concentrations during incubation in one study (Nasr-Esfahani *et al.*, 1992) but was in another (Goto *et al.*, 1993). In addition, reducing O_2 tension is more effective in promoting embryo development *in vitro* than is treatment with detoxifying enzymes (superoxide dismutase (SOD) and catalase) (Orsi and Leese, 2001). Furthermore, restricting O_2 availability after compaction by further reducing the O_2 tension from 7 to 2% is beneficial for bovine embryo development after compaction (Thompson *et al.*, 2000). More convincingly, incubation in 5% O_2 compared with 20% O_2 increases oxygen uptake and pyruvate oxidation (Hooper *et al.*, 2001). This finding is significant as the effect of a reduction in O_2 tension from atmospheric levels to 5% within embryos has also been examined using a mathematical model (Byatt-Smith *et al.*, 1991). Byatt-Smith *et al.* (1991) showed that for small mammalian embryos, such as mouse embryos, O_2 from a 5% atmosphere would be able to sustain oxidative phosphorylation throughout development, albeit under hypoxic conditions. However, larger embryos, such as human or cattle embryos, would establish a significant gradient leading to anoxia in the centre of the embryo, which may explain some of the metabolic differences among embryos of different species. Such a gradient, especially during compaction, may also provide spatial information to cells within the embryo (Fig. 2). This prediction is supported by the observation that the metabolic profile of inner cell mass cells differs from that of trophectoderm (Hewitson and Leese, 1993).

Intracellular NAD^+/NADH during embryo development

Mouse embryos contain concentrations of NAD^+ and NADH of 0.25 and 0.85 mmol kg^{-1} , respectively, similar

Fig. 2. Hypothetical model of O_2 gradient in early cleavage and post-compaction stage embryos during development *in vivo*. Early cleavage stage embryos encounter a physiological O_2 concentration during transport through the oviduct, where a minimal gradient is encountered by individual blastomeres. However, post-compaction stage embryos encounter a significantly lower (hypoxic) O_2 environment *in utero*, potentially creating a more marked gradient within the embryo and resulting in the establishment of spatial relationships between blastomeres.

concentrations to those in adult tissues (Wales, 1975). The intracellular ratio of $[NAD^+]:[NADH]$ does not vary greatly with embryonic stage; however, this finding does not account for possible variations within mitochondrial and cytoplasmic pools (Wales, 1975), which would be influenced by concentrations of $[pyruvate]:[lactate]$ in the cytosol. However, considerable variation occurs among individual embryos at either the one- or two-cell stage (Wales, 1975). Blastocyst development from the two-cell stage is significantly affected by the relative concentration of pyruvate and lactate in the medium, which is postulated to affect intracellular $[pyruvate]:[lactate]$, with an optimum ratio of approximately 1:10 of pyruvate:D,L-lactate.

Intracellular glutathione and $NADP^+/NADPH$ during embryo development

Glutathione, a tripeptide of glycine, cysteine and glutamine, is an important regulator of REDOX status (Munday and Winterbourn, 1989; Fig. 3) and has been detected in reproductive tract fluid (Gardiner *et al.*, 1998). Glutathione plays a major role in regulating ROS concentrations within the cytoplasm, both directly as a free-radical scavenger, and

as a substrate with NADPH for detoxifying ROS using GSH peroxidase (Guerin *et al.*, 2001). Glutathione concentrations measured throughout preimplantation embryo development decrease as early cleavage proceeds (Gardiner and Reed, 1995). Preimplantation stage embryos have little capacity for GSH synthesis (Gardiner and Reed, 1995). However, increasing intracellular concentrations of reduced glutathione, especially during oocyte maturation, are associated with both improved fertilization and subsequent embryo development *in vitro* (Abeydeera *et al.*, 1999; de Matos and Furnus, 2000). Appropriate GSH status is also usually achieved by the inclusion of its constituent amino acids, cysteine, glycine and glutamine, within the incubation medium.

Pronuclear formation after fertilization in mice is also accompanied by increased glycolysis and glucose oxidation through the pentose phosphate cycle, with no change in pyruvate or glutamine entry into the TCA cycle (Pantaleon *et al.*, 2001). This is a sperm-mediated effect, as parthenogenesis does not affect metabolism whereas the degree of polyspermy influences metabolic rate (Pantaleon *et al.*, 2001). As the pentose cycle regulates concentrations of NADPH (Fig. 3), such activity may alter $[NADP^+]:[NADPH]$. Similarly, a cell cycle-linked REDOX mechanism (experimentally mediated through addition of thioredoxin) has been implicated in the cessation of sperm-mediated Ca^{2+} spiking after fertilization (Day *et al.*, 2000).

Periods of ROS production or extinction during embryo development

The effect of ROS on embryo development is paradoxical. Most studies have shown that prolonged, experimentally induced ROS production severely inhibits embryo development (Johnson and Nasr-Esfahani, 1994; Guerin *et al.*, 2001). Controversially, the inclusion of antioxidants or radical-detoxifying enzymes to embryo culture medium produces a varied response (Noda *et al.*, 1991; Payne *et al.*, 1992; Orsi and Leese, 2001). Hydrogen peroxide concentrations increase during the two–four-cell transition period, at about the time of embryonic genome activation, in mouse embryos cultured *in vitro*, but not in those developed *in vivo* (Nasr-Esfahani *et al.*, 1990). This finding indicates that an increase in ROS may be associated with the arrest of development at the two-cell stage, observed under some *in vitro* culture conditions. However, increases in H_2O_2 concentrations *in vitro* were also observed in embryos from a strain of mice that develop successfully *in vitro*. A very brief pulse of H_2O_2 delivered to bovine embryos at the eight-cell stage, the developmental stage at which major activation of the embryonic genome occurs in cattle, had a positive (although not significant) influence on their capacity to develop to the blastocyst stage (Morales *et al.*, 1999). Treatments administered at other stages of development inhibited development. Similarly, mouse embryo hatching, in embryos derived both *in vivo* and *in vitro*, has also been associated with a specific burst of ROS production (Thomas

Fig. 3. Relationship between the pentose phosphate pathway (PPP), reactive oxygen species (ROS) and glutathione (GSH) REDOX status.

Fig. 4. Hypoxic regulation of hypoxia inducible factor (HIF-1) protein stabilization and DNA binding. Changes in oxygen are detected through a putative oxygen sensor. Under normoxic conditions, HIF-1 α is ubiquitinated and undergoes proteasomal degradation. Conversely, under hypoxic conditions, HIF-1 α is stabilized enabling translocation to the nucleus where it dimerizes with the β subunit (aryl hydrocarbon receptor nuclear translocator (ARNT)) to form the active DNA binding complex, which binds to the hypoxia response element (HRE) in target genes. In the preimplantation embryo, induction of HIF activity is a potential mechanism through which responses to changes in oxygen concentration, including alterations in metabolism and gene expression, may be mediated.

et al., 1997). The production of ROS may also be an important regulatory system for programmed cell death (apoptosis) in the blastocyst (Pierce *et al.*, 1991).

Transcription factors mediated by the REDOX state

Can a change in metabolic activity during early embryo development elicit a molecular response? As mentioned

earlier, a number of genes are known to be responsive to a change in REDOX state. Such transcriptional control is mediated largely by transcription factors sensitive to the intracellular REDOX state and several such factors may be involved in embryo development. Members of the hypoxia inducible factor family of transcription factors are influenced directly by the intracellular oxygen concentration, and may be important for embryonic development within

the hypoxic reproductive tract, whereas nuclear factor kappa β may be involved in stress-related and maternal recognition (cytokine) responses.

Hypoxia inducible factor family

Hypoxia inducible factors (HIFs) are basic helix–loop–helix Per-ARNT-Sim (bHLH-PAS) transcription factors that mediate oxygen-dependent expression of a range of genes involved in cellular and systemic processes (Fig. 4), including angiogenesis, erythropoiesis, glucose transport and glycolysis (reviewed in Semenza, 1999, 2000; Wenger and Gassman, 1999; Wenger, 2000). HIF-1, the first HIF to be identified, is a heterodimer composed of HIF-1 α and HIF-1 β subunits. HIF-1 α was identified as a novel protein, whereas the HIF-1 β subunit is identical to the aryl hydrocarbon receptor nuclear translocator (ARNT). HIF-1 α protein concentrations are regulated precisely according to cellular oxygen concentration. More recently, other similar hypoxia responsive bHLH-PAS proteins, HIF-2 α and HIF-3 α , have also been identified.

Regulation of HIF-1 activity can occur at a number of levels, including mRNA expression, nuclear localization and transcriptional activation, and changes in the protein stability of HIF-1 α appear to be the principal control mechanism (reviewed in Semenza, 1999, 2000; Wenger and Gassman, 1999; Fig. 4). Under normoxic conditions, HIF-1 α protein is degraded rapidly by the ubiquitin–proteasome system. This process is mediated through binding of the von Hippel-Lindau (pVHL) tumour suppressor protein to HIF-1 α . Multiple mechanisms have been proposed to account for the nature of the oxygen sensor that detects changes in oxygen concentration (Semenza, 1999; Wenger and Gassman, 1999). A study by Jaakkola *et al.* (2001) indicates that the binding of pVHL to HIF-1 α is promoted through oxygen-regulated hydroxylation of a proline residue in HIF-1 α . Under hypoxic conditions, the HIF-1 α protein is not targeted for degradation and can translocate to the nucleus to dimerize with the β subunit, thus forming the active DNA-binding complex. HIF-1 can activate the expression of a number of genes involved in the adaptation to low oxygen, including erythropoietin, vascular endothelial growth factor, inducible nitric oxide synthase, haem oxygenase 1, glucose transporter 1 and several glycolytic enzymes (Wenger and Gassman, 1999; Semenza, 1999, 2000). Many of these genes are known to play important roles during late preimplantation development and early placental development.

The involvement of HIF in embryonic development has been demonstrated by the production of gene knockout mice. Homozygote *hif-1 α ^{-/-}* mice die around embryonic day 10.5–11.0 and exhibit neural tube defects, cardiovascular malformations, cell death within the cephalic mesenchyme and impaired vascularization (Iyer *et al.*, 1998; Kotch *et al.*, 1999). Similarly, *hif-1 β* knockout mice die at midgestation, with primary defects including defective yolk sac angiogenesis and compromised fetal capillary develop-

ment (Maltepe *et al.*, 1997) or failure of the embryonic part of the placenta to vascularize and form the labyrinthine spongiotrophoblast (Kozak *et al.*, 1997). Mice lacking HIF-2 α also die between embryonic day 12.5 and embryonic day 16.5, primarily as a result of defects in catecholamine synthesis (Tian *et al.*, 1998).

Preliminary data indicate a differential pattern of expression of HIF-1 α in mouse embryos derived *in vivo* and bovine embryos derived *in vitro* (Harvey *et al.*, 2001, 2002). HIF-2 α mRNA has also been observed in cultured bovine blastocysts (Harvey *et al.*, 2001). HIF-1 β (ARNT) mRNA has been detected in rabbit morulae and blastocysts derived *in vivo* (Tscheudschilsuren *et al.*, 1999). Nevertheless, further studies are required to determine whether HIF-1 α or HIF-2 α protein nuclear translocation and DNA binding are responsive to hypoxic conditions in the preimplantation embryo.

Nuclear factor kappaB

The transcription factor nuclear factor kappaB (NF κ B) has key roles in the regulation of cell proliferation and programmed cell death, as well as in immune, inflammatory and stress responses, and controls numerous genes, including those involved in cell adhesion and the cell cycle (Janssen-Heininger *et al.*, 2000). NF κ B consists of two subunits and is normally sequestered in the cytoplasm through its association with the inhibitory molecule I κ B (Mercurio and Manning, 1999). NF κ B can be activated by a variety of stimuli associated with stress, and potentially that encountered during culture *in vitro*. Potent inducers include the cytokines tumour necrosis factor α (TNF- α) and interleukin-1 β (IL-1 β). ROS have been shown to be involved in NF κ B activation through REDOX mechanisms in many, but not all, cell lines (Meyer *et al.*, 1994; Brennan and O'Neill, 1995). NF κ B has also been shown to be induced by hypoxia (Koong *et al.*, 1994). In addition to investigations showing knockout embryo lethality, recent evidence documents the expression and distribution of NF κ B during preimplantation embryo development in mice (Parrott and Gay, 1998; Nishikimi *et al.*, 1999). The expression of NF κ B subunits during embryo development may be required for responses to environmental stimuli such as stress (Parrot and Gay, 1998). It has been suggested that activation of NF κ B at the early one-cell stage is required for the development of mouse embryos beyond the two-cell stage (Parrot and Gay, 1998).

Other REDOX-sensitive transcription factors

Activator protein-1 (AP-1) and p53 are other transcription factors that are REDOX-regulated (Yao *et al.*, 1994; Giaccia and Kastan, 1998). AP-1 is a heterodimer of c-fos and c-jun proteins and tends to elicit opposing effects to those elicited by NF κ B. It is unclear whether AP-1 protein is present in the mammalian embryo; indeed, c-fos and c-jun proteins were not detectable in the early cleaving mouse embryo (Ahmad and Naz, 1993). The tumour-suppressor

protein p53, which is known to regulate apoptosis, interacts with HIF-1 α , providing an alternative protein stabilization pathway (Carmeliet *et al.*, 1998). Moley and Mueckler (2000) have suggested that hyperglycaemic-induced apoptosis in mouse embryos is mediated through HIF-1 α stabilization with p53.

Concluding remarks

Many studies have examined the effect of medium composition, in particular energy substrate concentrations, on embryo development up to the blastocyst stage as the end-point. Few of these studies examine the mechanism by which substrate concentrations and culture conditions affect development. We propose here that one mechanism that should receive close attention is alteration of the intracellular REDOX state. A perturbed REDOX state, as a consequence of sub-optimal culture conditions, may not only alter ATP production, but also gene expression patterns during development, leading to altered placental and fetal growth patterns.

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