The embryonic stress response to *in vitro* culture: insight from genomic analysis

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Abstract

Recent genomic studies have shed light on the impact of *in vitro* culture (IVC) on embryonic homeostasis and the differential gene expression profiles associated with lower developmental competence. Consistently, the embryonic stress responses to IVC conditions correlate with transcriptomic changes in pathways related to energetic metabolism, extracellular matrix remodelling and inflammatory signalling. These changes appear to result from a developmental adaptation that enhances a Warburg-like effect known to occur naturally during blastulation. First discovered in cancer cells, the Warburg effect (increased glycolysis under aerobic conditions) is thought to result from mitochondrial dysfunction. In the case of IVC embryos, culture conditions may interfere with mitochondrial maturation and oxidative phosphorylation, forcing cells to rely on glycolysis in order to maintain energetic homeostasis. While beneficial in the short term, such adaptations may lead to epigenetic changes with potential long-term effects on implantation, foetal growth and post-natal health. We conclude that lessening the detrimental effects of IVC on mitochondrial activity would lead to significantly improved embryo quality.

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The purpose of studying embryogenomics in the assisted reproduction context

Embryo culture is an essential aspect of assisted reproductive technology since its purpose is to allow the zygote to grow, divide and reach a stage at which it can be transferred to the uterus. The first media formulated for *in vitro* culture (IVC) of mammalian embryos were quite unreliable, often causing early development to stall at the maternal–embryonic transition phase (Brinster 1963). Improvements in medium composition have largely overcome this problem (Brinster & Troike 1979, Schini & Bavister 1988) and commercially available culture media now support embryo development to the blastocyst stage in various species. The rise of assisted reproductive technologies (ART) is accountable for a growing portion of the livestock population. More than 5 million human beings are born due to ART since Louise Brown in 1978. This currently represents about 1–2% of births in developing countries.

Despite its widespread use, IVC continues to produce embryos of lower viability compared with natural conception (Nair 2008), making multiple/repetitive transfers necessary. Its impact on early development and hence on the quality of the transferred embryos remains considerable (Nelissen *et al.* 2012). On one hand, almost all ARTs use either superovulation or, in experimental procedures, abattoir-derived material, to obtain mature eggs for *in vitro* fertilisation. These eggs have usually

lower quality compared with naturally ovulating eggs, which is a major determinant of fertilisation and developmental success. On the other hand, sub-optimal culture conditions also reduce embryonic quality due to improper homeostasis regulation during early development (Summers & Biggers 2003). Moreover, the impact of IVC may be variable between embryos originating from *in vitro* or *in vivo* matured oocytes, inducing different stress responses that compromise the chances of successful implantation (Leese *et al.* 1998). Therefore, *in vitro* conditions could be optimised to reduce the impact of ART on embryo viability.

The first few cleavages after fertilisation take place under a regime of transcriptomic silence that persists until embryonic genome activation (EGA) is complete. During maturation, the gamete genomes therefore invested heavily in sustaining the first zygotic divisions and only the cytoplasmic compartment of the early embryo cells has any significant capacity to respond to stress. After EGA, the embryonic cells acquire a transcriptomic plasticity that provides certain responsiveness to external conditions (McKiernan & Bavister 1994, van Soom *et al.* 1997). Moreover, the acquisition of gap junctions during compaction provides a better co-ordination between embryonic cells in terms of metabolism, signal transmission and response to the external conditions. Because early embryos behave as autonomous cells until EGA, this contributes to their heightened sensitivity to IVC-related stress compared with the later stages

of preimplantation development (Brison *et al.* 2014). However, a considerable percentage of embryos still stall at the morula-to-blastocyst transition, and transcriptional state in blastocysts may remain compromised due to the adaptive response to the IVC environment (Summers & Biggers 2003).

Total transcript amplification allows screening for several thousands of cDNA probes using only a few cells (Rizos *et al.* 2002b, Zheng *et al.* 2007, Smith *et al.* 2009, Seli *et al.* 2010). This approach has been used to assess the degree of modulation of the expression level of developmentally important genes in cultured embryos of different species (Khosla *et al.* 2001b, Lucifero *et al.* 2004). Although gene expression levels do not necessarily equate to functional effects in terms of active proteins, transcriptomic analyses have been valuable in picturing the global genomic impact underlying the embryonic response to different *in vitro* environments. Notably, during pre-implantation development of cattle embryos (in which EGA is late, as in human embryos), large numbers of genes are expressed at different levels depending on whether the embryos are produced *in vivo* (under optimal conditions in the oviduct) or *in vitro* (Wrenzycki *et al.* 1996, Niemann & Wrenzycki 2000, Lazzari *et al.* 2002). The time-dependent sensitivity of early development to IVC is particularly apparent in cattle, in which transfer of embryos from the *in vitro* environment to the uterus is generally successful if timely (Gad *et al.* 2012). Using trophoblast biopsies before uterine transfer, a *posteriori* analysis of cases in which the recipient cow was not the oocyte donor has revealed IVC-specific differentially expressed genes (DEG) that are associated with gestational success or failure (El-Sayed *et al.* 2006, Ghanem *et al.* 2011).

Such breakthroughs have suggested that the measurement of transcriptomic perturbations could provide a diagnostic tool for assessing developmental competence (Lee *et al.* 2001, Jones *et al.* 2008). However, the importance of these perturbations for the quality of IVC embryos remains difficult to interpret in terms of direct links with the detrimental impact (stress) of the different culture components. In view of the considerable differences between *in vitro* and *in vivo* conditions, effects of IVC on embryonic gene expression patterns are to be expected. While there may be key DEGs that characterise inadequate stress responses indicating a detrimental impact on embryo quality, it cannot be ruled out that some and perhaps many DEGs represent developmental adaptations to IVC rather than damage to the embryo (Plourde *et al.* 2012).

The embryonic responses to the IVC stress conditions

Under IVC conditions, embryos are subjected to a variety of homeostatic pressures including

physicochemical (temperature, osmolality and pH), oxidative (pro-oxidant and anti-oxidant balance) and energetic (production, utilisation and storage) stresses, all of which can compromise further development (Summers & Biggers 2003).

Responses to physiochemical stress

Very few studies have examined the effects of osmolality and pH on early embryo transcriptomes. It is nevertheless known that these parameters vary during IVC and deviate from the corresponding *in vivo* conditions. Embryos are able to tolerate a range of osmolalities although first studies showed that a reduced osmolality during IVC (around 270 mosmol) may enhance developmental rate (Brinster & Troike 1979). However this is unlikely to be optimal, since *in vivo* data suggest osmolality of around 300 mosmol in the oviduct fluid. The hypertonic stress at physiological osmolality may be due to inappropriate concentrations of specific osmolytes in interaction with the salt level in the IVC medium (Richards *et al.* 2010, Baltz 2012). Osmolytes such as glycine and other amino acids become internalised through transporters and allow volume regulation during blastocyst expansion (Richards *et al.* 2010). More recent formulations of culture media therefore tend to include amino acids and organic osmolytes in order to increase osmolality to physiological levels, such as in oviduct fluid (Van Winkle & Dickinson 1995, Baltz 2012). Some studies have focused on the impact of hypertonic stress on responses mediated by mitogen-activated protein kinase pathways such as p38 activation (Fong *et al.* 2007, Xie *et al.* 2007) or aquaporin expression/localisation and apoptosis via MAPK14/11 and MAPK8 respectively (Bell *et al.* 2009). However, the extent to which the transcriptome responds to hypo-osmotic condition is still unclear.

The pH of a culture medium can increase quickly when the culture dish is handled outside of the incubator, but it can also change due to the metabolic activities of the embryo. Depending on the metabolic state of the cytoplasm (where glycolysis takes place) and the mitochondria, where oxidative phosphorylation (OXPHOS) takes place, ammonium ion can be liberated from amino acids, while lactate can be produced from pyruvate. Moreover, amino acids may spontaneously breakdown at 37°C, releasing ammonium. The bicarbonate buffer used in culture media is likely sufficient to counteract pH variations in regard to the slow metabolism of early embryos. However, stressed embryos may release excessive amount of ammonium and lactate into the culture medium, which could decrease the local pH and, in turn, compromise embryo development (Dagilgan *et al.* 2014). More importantly, ammonium build-up in interaction with the oxygen level negatively impacts on the metabolism and gene expression during IVC

of mouse embryos (Wale & Gardner 2013a). Fluidic culture systems have been developed to renew the medium continually, while closed chamber systems now make it unnecessary to handle embryos outside of the incubator (Krisher & Wheeler 2010). On this subject, detailed information on the effects of chemical and physical factors on mammalian embryo culture can be found in the review from Wales and Gardner (Wale & Gardner 2015).

Temperature shifts experienced by embryos handled outside of the incubator may cause heat shock (Sakatani *et al.* 2012). The early stages of bovine pre-EGA embryo development are particularly sensitive to heat shock; this is associated with major changes in the transcriptomic profile at the morula stage (Sakatani *et al.* 2013). Responses to heat shock include the production of homeostatic regulators involved in the unfolding response via the ubiquitination pathway and the glutathione scavenging response to oxidative stress (Arechiga *et al.* 1995). These regulators consist of not only chaperone proteins (Edwards & Hansen 1996) as well as apoptosis-related proteins such as Bax, Bid and Caspase-3 (Yadav *et al.* 2013) but also proteins associated with developmental competence genes, such as PLAC8 and CDX2 (Silva *et al.* 2013). A transcriptional dysregulation subject to *wnt* signalling in conjunction with heparin sulphate proteoglycans also may result from HS exposure (Sakatani *et al.* 2013).

In cattle, the impact of heat shock can be lessened by IGF1 stimulation through MAPKK and PI3K (Jousan & Hansen 2007), but this metabolic rescue is possible only when the shock occurs after EGA (Bonilla *et al.* 2011). Supplementation with free radical scavengers such as beta-mercaptoethanol has also been shown to decrease embryo mortality due to heat shock (Sakatani *et al.* 2008). In a mouse study, it was noted that expression of the X-linked gene for the metabolic enzyme G6PD was stronger in female embryos (Perez-Crespo *et al.* 2005), which would increase tolerance to heat-shock-induced free radicals during IVC. This indicates that the stress response during pre-implantation development results from the impact of heat shock on the metabolic state, which affects oxidative homeostasis and thus embryonic signalling.

Response to oxidative stress

Oxidative stress is the result of an imbalance between the production and elimination of reactive oxygen species (ROS). ROS of intracellular or extracellular origin include various free radicals such as superoxide anion, hydrogen peroxide, hydroxyl radical, hydroxyl ion and nitric oxide, which are formed during reactions that reduce oxygen (Agarwal *et al.* 2006). Oxidative stress can affect numerous physiological mechanisms and can be lethal to embryos (Yang *et al.* 1998, Betts & Madan 2008). At low concentrations, ROS can activate

cellular pathways that determine differentiation or proliferation, while higher concentrations appear to affect the integrity of cellular constituents such as lipids, proteins, amino acids and nucleic acids (Guerin *et al.* 2001). Early embryos produce ROS naturally even in the presence of low levels of oxygen, since they carry out OXPHOS as well as other redox reactions (Nasr-Esfahani & Johnson 1991). However, several analyses have shown that one of the ways in which culture conditions are detrimental to embryo developmental competence is by increasing oxidative stress (Nasr-Esfahani *et al.* 1990, Nasr-Esfahani & Johnson 1992).

Oxygen tension is an important regulator of oxidative metabolism (Wale & Gardner 2013b, Wale & Gardner 2015) and *in vivo* developing embryos are exposed to a lower oxygen concentration in the oviduct/uterus compared with the atmospheric condition (Khurana & Niemann 2000). An atmosphere containing 5% oxygen is recommended for human embryo culture (Gardner 2016), and numerous studies have shown the deleterious effects of oxygen at normal atmospheric concentrations (20–21%) on embryo development and quality (Fujitani *et al.* 1997, Orsi & Leese 2001, Balasubramanian *et al.* 2007). High oxygen tension increases H₂O₂ production, DNA fragmentation and apoptosis, and thereby decreases the percentage of IVC embryos reaching the blastocyst stage (Van Soom *et al.* 2002, Kitagawa *et al.* 2004). By affecting oxidative homeostasis, it might actually decrease energy production by the mitochondria (Harvey *et al.* 2004a). Uncouplers and inhibitors of OXPHOS appear to affect embryo development in a similar manner (Thompson *et al.* 2000, Machaty *et al.* 2001) as well as the expression of redox-sensitive genes such as *HIF1A* and *HIF2A* (Harvey *et al.* 2004b). Reduced oxygen tension increases the expression of metabolic genes involved in glycolysis such as LDHA (Harvey *et al.* 2007). Recent microarray analysis of human embryos has revealed the genome-wide impact of oxygen tension on gene expression related to metabolism, the cell cycle and OXPHOS (Mantikou *et al.* 2016).

Exogenous ROS will also stress the oxidative homeostasis of IVC embryos (Bedaiwy *et al.* 2004). Metal ions in the water, serum amine oxidase and visible light could contribute to increasing the concentration of ROS in a culture medium (Guerin *et al.* 2001, Wale & Gardner 2015). The pro-oxidant factor 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) is known to induce a self-perpetuating free radical formation that initiates cell membrane lipid peroxidation and generates ROS. Adding AAPH to the medium mimics the contribution of external ROS to developmental compromise during IVC and has been used to assess cellular responses (Feugang *et al.* 2003, 2005) and transcriptomic responses (Cagnone & Sirard 2013) to oxidative stress in bovine embryos.

Exogenous ROS (AAPH exposure) has a major impact on blastocyst integrity and activation of signalling pathways such as ERK1/2 as mechanisms of adaptation and survival. It also induces a rapid inflammatory response (TNF and IFN signalling), due possibly to lipid oxidation (Kim *et al.* 2010, Kopp *et al.* 2010). An inflammatory response during early development may further affect embryo competence (Van Sinderen *et al.* 2013). If this is true, cytokine release should be a useful marker of the oxidative stress experienced by IVC embryos before transfer (Johnson *et al.* 2003). Moreover, the use of anti-inflammatory factors (such as pre-implantation factor) might improve the embryonic signalling in response to stress (Stamatkin *et al.* 2011), although it would not likely prevent internal ROS damage. For this purpose, a redox-balanced environment still needs to be designed, based perhaps on a cocktail of oxidative regulators and anti-oxidant molecules (Takahashi *et al.* 1993). However, while the role of factors such as glutathione in maintaining the oxidative balance in the oviduct has been brought to light (Salmen *et al.* 2005), researchers are still troubleshooting the optimal metabolic conditions for embryonic oxidative homeostasis (Truong *et al.* 2016).

Both the maternal environment and the embryo itself provide defences against ROS (glutathione, vitamins and albumin), and different enzymes such as glutathione peroxidase (GPX) or superoxide dismutase (SOD) are produced to eliminate excess ROS (Lequarre *et al.* 2001). Reduced glutathione (GSH), synthesised primarily from cysteine, glutamate and glycine, plays a crucial role in scavenging hydrogen peroxide, an oxidative reaction catalysed by GPX. The enzyme GSH reductase ensures regeneration of GSH and thereby allows detoxification of the cell. This system is present in the oocyte and the embryo; GSH being stored during oocyte maturation is then utilised during pre-implantation development (Gardiner & Reed 1994). This dynamic pattern reflects the change in oxidative metabolism from a quiescent state before EGA to an active state after EGA. We note with interest that increased *GPX4* expression in response to oxidative stress has been observed in cattle blastocysts produced *in vitro* and that this transcriptomic response is correlated strongly with failure to bring about gestation (El-Sayed *et al.* 2006).

The sensitivity of cultured embryos to ROS may be due to impaired GSH turnover (Stover *et al.* 2000). Adding L-buthionine sulfoximine to the culture medium aggravates this deficiency by depleting the GSH reservoir in the embryo, thus causing a developmental blockage at the time of increased oxidative activity. Based on transcriptomic analysis of blastocysts, the resulting inflammatory response is clear (Cagnone & Sirard 2013), although genes involved in glycine metabolism (a component of GSH) are also overexpressed. Embryos that survive this treatment also show decreased expression of genes associated

with oxidative metabolism. Such a profile is typical in embryos produced *in vivo* and exhibiting a quiescent homeostasis, which might also be a marker of the best quality of blastocysts obtainable under conditions of extended culture. However, experimental validation is needed to ensure that conditions that select for this 'quiet' state do not do more harm than good.

Response to energetic stress

Accumulating evidence shows that IVC embryos bear the hallmarks of energetic stress in terms of nutrient utilisation (increased conversion of glucose to lactate), nutrient storage (lipid accumulation) and ATP synthesis (Spielmann *et al.* 1984, Thompson *et al.* 1991, Van Blerkom *et al.* 1995, Khurana & Niemann 2000, Swain *et al.* 2002, Romek *et al.* 2009). This metabolic state occurs because of inadequate support in the *in vitro* environment. Numerous attempts to replicate the conditions of the oviduct, including co-culturing embryos with feeder layers of somatic cells (Lee *et al.* 2001), have met with little success. As support for proper energy metabolism, *in vitro* conditions remain sub-optimal and require further improvement in order to lessen the energy stress response during IVC.

One of the first things understood about the impact of IVC on metabolism in embryos was that the glucose concentration used typically in culture media for somatic cells (around 5 mM) is deleterious for early embryos before MET, causing a Crabtree-like effect, that is, overstimulation of glycolysis, alteration of mitochondrial oxidative metabolism and increases in ROS production (Schini & Bavister 1988, Seshagiri & Bavister 1991, Leunda-Casi *et al.* 2002, Scott & Whittingham 2002, Diaz-Ruiz *et al.* 2008). While *in vivo* glucose concentration is low in the oviduct, glucose concentration increases in the uterus in order to support the proliferative homeostasis of embryonic cells during compaction, blastulation and implantation. These findings were crucial to reformulating IVC medium composition (Downs & Dow 1991, Fissore *et al.* 1992). Much lower glucose concentrations (around 0.2 mM) made it possible to overcome the developmental blockage at the MET in mouse embryos (Matsukawa *et al.* 2002). Afterwards, sequential media with increasing glucose concentrations were formulated in order to extend embryo culture until the blastocyst stage, thus selecting more competent embryos for transfer. However, the use of sequential media is arguably another stress since relocating suddenly the embryo from one media to another might have an impact on its homeostasis.

However, IVC embryos continue to suffer from energetic stress and around 50% of them go into developmental arrest before the blastocyst stage. To understand the molecular pathways associated with energetic stress, experimental models have been

developed in which embryos must cope with maternal hyperglycaemia or with increased glucose concentration during IVC. In murine embryos, hyperglycaemia can induce DNA fragmentation and apoptosis through the expression of cell death effector pathways (Moley *et al.* 1998). In one study of cultured cattle embryos, increasing the glucose concentration from 0.2 mM to 5 mM during early cleavage stage had a significant impact on blastocyst development, metabolism and the transcriptome profile (Cagnone *et al.* 2012). Early exposure to high glucose concentrations decreased the ATP content during blastocyst expansion and induced premature changes in the expression of two transcription factors (hepatic nuclear factor 4 alpha and pleomorphic adenoma-gene-like 1; Gae L M Cagnone, Isabelle Dufort, Melanie L Sutton-McDowall, Hannah Brown, Jeremy G. Thompson and Marc-André Sirard, unpublished data) at the morula stage.

Like the response of somatic cells under conditions of diabetic hyperglycaemia (Pampfer *et al.* 1997, Pavlinkova *et al.* 2009), the transcriptomic response to high glucose in bovine blastocysts includes polyol pathway activation (to clear excess glucose) and affects the oxidative homeostasis (Morrison *et al.* 2004). Excess glucose also induces an inflammatory reaction, possibly because of increased advanced glycation end products (Haucke *et al.* 2014) and activation of hexosamine pathways, in particular O-linked glycosylation (Pantaleon *et al.* 2010, Pantaleon 2015, Wong *et al.* 2015). Other pathways that are activated include protein kinase C signalling and extracellular matrix remodelling into the fibrotic-like phenotype. According to the long-standing consensus, the polyol, advanced glycation end product, PKC and hexosamine pathways are activated due to increased mitochondrial ROS production, which inhibits GAPDH activity (Nishikawa *et al.* 2000). Such an effect has been observed in embryos from diabetic mothers (Wentzel *et al.* 2001, Wentzel *et al.* 2003) or cultured under stress created by high glucose concentrations (Karja *et al.* 2006), suggesting that energetic stress in pre-implantation embryos is a result of compromised OXPHOS (Moley *et al.* 1996, Chi *et al.* 2002, Mitchell *et al.* 2009).

Besides having an energetic deficiency, IVC embryos also differ from their *in vivo* counterparts in terms of lipid metabolism (Romek *et al.* 2009). Lipids are an important source of energy and anabolic products required for embryo development. The lipid droplets in the cells of an embryo are composed primarily of triglycerides and cholesterol esters. It is noteworthy that adding serum to the culture medium increases lipid accumulation in embryos (Rizos *et al.* 2003), which may affect their ability to survive cryopreservation (Rizos *et al.* 2002a). The low cryotolerance (Sudano *et al.* 2014) and cell degeneration (Huang *et al.* 2010) observed in these embryos correlate with differential expression of genes involved in lipid biosynthesis and cholesterol metabolism (Van Hoeck *et al.* 2015). Such effect could

be due to any of the various factors present in serum (glucose, lipids, growth factors) (Leroy *et al.* 2010), and some studies have shown that the lipid fraction of serum increases the triglyceride and cholesterol contents of IVC embryos (Abe *et al.* 2002, Romek *et al.* 2009).

A recent analysis of bovine blastocysts produced in medium containing BSA plus the lipid fraction of serum revealed transcriptomic profiles exhibiting signs of lipid peroxidation and metabolic deregulation, in particular downregulation of *LDLR*, *HMGCS1* and *MSMO1*, suggesting a stress response to accumulated cholesterol and inhibition of SREBP and PPAR signalling. Pathway analysis showed activation of the metabolic repressor NR1P1, which is involved in triglyceride utilisation and balance between energy storage and expenditure (Cagnone & Sirard 2014). These findings are in agreement with those of Leroy *et al.* (2010) who showed the impact of IVC medium supplementation with high-fat and high-carbohydrate serum on early embryos (Leroy *et al.* 2010). This supplementation was associated with compromised blastocyst development and differential expression of genes involved in apoptosis, oxidative stress, metabolism and pluripotency. Finally, expression of *ACAT2*, a cytosolic acetoacetyl-CoA thiolase involved in mitochondrial utilisation of lipid and the synthesis of cholesterol in the placenta (Zolnierowicz *et al.* 1984), is regulated upwards in blastocysts that ultimately end up as miscarriages. This upregulation suggests impaired cholesterol metabolism, possibly in the mitochondria.

Supplementation of the medium with serum has been shown to accelerate development and increase the likelihood of blastocyst formation in cultured cattle embryos, but it also appears to increase the incidence of abnormal developments such as large offspring syndrome. In rodent studies, cultured blastocysts that developed faster had abnormal gene expression and imprinting patterns (Market Velker *et al.* 2012), while embryos developing in mothers fed a high-fat diet displayed compromised development (metabolic and inflammatory responses as well as mitochondrial stress) and long-term effects on offspring health (Shankar *et al.* 2011, Bermejo-Alvarez *et al.* 2012). These effects were enhanced when both parents were obese (Finger *et al.* 2015). The effect of the high-fat diet can be reversed by treating the mother with rosiglitazone (an agonist of PPAR gamma), which induces weight loss, improves oocyte quality and modifies the expression of PPAR target genes involved in cholesterol transport (Minge *et al.* 2008). Maternal exercise and diet management was also found to restore metabolic status in the embryos and the resulting fetuses (McPherson *et al.* 2013).

Common transcriptomic manifestations of IVC stress in low-quality embryos

The transcriptomes of pre-implantation IVC embryos tend to exhibit certain similarities that correlate with

decreased embryonic quality compared with their *in vivo* counterparts. For bovine blastocysts, we have compiled microarray data from the EmbryoGENE transcriptomic platform, which allows highlighting several stress response markers in association with developmental competency after embryo transfer, as summarised in [Supplementary Table 1](#), see section on [Supplementary data](#) at the end of the article. Based on differentially expressed genes between *in vivo* and *in vitro* produced blastocysts ([Cagnone & Sirard 2013](#)), we can appreciate the correspondence with another study comparing transcriptomic profiles from embryos culture *in vivo*, *in vitro* or sequentially before and after EGA ([Gad et al. 2012](#)). In addition, we have overlaid the transcriptomic profile of biopsies from *in vitro* ([El-Sayed et al. 2006](#)) and *in vivo* ([Ghanem et al. 2011](#)) produced blastocysts failing to result in calf delivery (i.e. compared with those resulting in calf delivery). Finally, we show how DEGs in IVC blastocysts reflect the response to different stress conditions of culture ([Cagnone et al. 2012](#), [Cagnone & Sirard 2013, 2014](#)). Data from mouse ([Heras et al. 2016](#)) and human ([Highet et al. 2015](#), [Kleijkers et al. 2015](#)) studies using different transcriptomic platforms are also provided. The transcriptomic biomarkers associated with compromised preimplantation development after IVC are involved in important biological functions such as metabolic homeostasis, extracellular matrix remodelling, inflammation and signal transduction.

Metabolic stress response

A large number of DEGs found in IVC embryos are associated with the metabolic stress response that involves the glucose (*TPI1* and *PGK1*), lipid (*LDLR* and *ACAT2*), oxidative (*GPX4*, *GPX8* and *OLR1*) and ion metabolism (*S100A10*, *S100A14*, *KCNIP4*, *FTH1* and *CLIC1*). Although the molecular consequences of their differential expression during pre-implantation development remain to be defined, impaired glucose and lipid metabolism are known to compromise development. Signs of free radical damage such as lipid peroxidation are clearly associated with embryonic degeneration. Finally, changes affecting the metabolism of calcium and other ions are also correlated with disruptions of cell function that further compromise embryo viability ([Im et al. 2007](#), [Wang et al. 2013a](#)). Culture conditions need to be optimised in order to lessen such detrimental metabolic responses, and here we indicate potential markers of the biological pathways involved in determining the impact of culture conditions on embryo quality.

Extracellular matrix synthesis and remodelling

Effects of IVC on genes involved in extracellular matrix synthesis and remodelling also appear in the transcriptome of blastocysts. This includes transcripts

coding for matrix structural proteins (*LUM*, *NDP*, *THBS1*, *CD9*, *JAM2*, *GJA1*, *COL3A1*, *LGALS3*, *LGALS3BP* and *EDN3*) and remodelling enzymes such as proteases *SERPINE1*, *SERPINA5*, *PLAT* and *PRSS23* and hydroxylase *PLOD2*. Extracellular matrix components are crucial for proper intercellular communication and adhesion during embryo development ([Aflalo et al. 2004](#)). In particular, morula compaction and formation of the blastocoel are dependent on tight cellular junctions, allowing polarised trophoblast cells to generate a cavity by inward movement of water. Remodelling is essential for trophoblast invasion into the endometrium and formation of the placenta ([Armant 2005](#)). In the murine endometrium, expression of the small leucine-rich proteoglycan called lumican (*LUM*, [Supplementary Table 1](#)) is regulated during decidualisation ([San Martin et al. 2003](#)), but the role of this molecule in embryo implantation remains unknown. Proteomic analysis of amniotic fluid has revealed that fetuses with Turner syndrome have a higher lumican content ([Mavrou et al. 2008](#)).

Impaired matrix remodelling capability in trophoblastic cells could interfere with the dynamics of endometrium invasion, with decisive consequences for embryo implantation ([Dimitriadis et al. 2010](#)). In the process of invasion by trophoblasts, the action of urokinase-type activator PLAU on plasminogen contributes to the activation of matrix metalloproteinase ([Martinez-Hernandez et al. 2011](#)). Expression of serine protease Prss23 has been detected in peri-implantation mouse embryos on day 7.5 but not in pre-implantation embryos on day 6.5 or earlier ([Diao et al. 2013](#)) and serine protease secretion is correlated positively with implantation potential ([Brosens et al. 2014](#)). Galectin 3 (*LGALS3*) promotes embryo implantation by regulating endometrial cell proliferation and adhesion through interaction with integrin $\beta 3$ ([Yang et al. 2011](#)), but oversecretion of Gal-3 is detrimental to trophoblast invasion. The receptivity of the human endometrium to blastocyst adhesion during the pre-implantation window depends in part on signal recognition via membrane domains rich in tetraspanin CD9, a receptor of pregnancy-specific glycoproteins ([Wynne et al. 2006](#), [Dominguez et al. 2010](#)). Overexpression of CD9 in blastocysts could inhibit implantation by reducing embryo invasiveness ([Liu et al. 2006](#)).

Embryo-maternal recognition

Maternal-embryo recognition is based on the regulated expression of inflammatory signals by the embryo and the inhibition of the immune response of the endometrium. The transcriptome of IVC blastocysts appears to contain features of excessive inflammatory response that might explain the failure of the maternal-embryo recognition. Stressed embryos have shown repeatedly differential expression of genes involved in immune signalling (*IFN- γ* ,

IL27RA, *OAS1*, *PSMD1* and *BOLA*) and inflammation (*TNFRSF1A*, *TNFAIP8L3*). The cytokine IFN- τ is well known in ungulate species to signal the presence of an embryo to the maternal immune system (Forde *et al.* 2015). However, it remains unclear how too much IFN- τ would affect endometrial receptivity. Overproduction of inflammatory factors by the embryo might be perceived by the maternal immune system as a danger signal, and the endometrium might block trophoblast invasion to prevent wastage of resources on the development of an embryo of poor quality. Placenta-specific protein 8 (PLAC8) is involved in placental development and might have a role in blastocyst hatching (Rekik *et al.* 2011) and embryo-maternal recognition (Klein *et al.* 2006, Ghanem *et al.* 2011). Platelet-derived growth factor is expressed normally by the embryo and the endometrium (Jaber & Kan 1998). PDGFC overexpression may interfere with implantation by inducing uterine fibrosis (Bonner 2004, Horne & Critchley 2007), which might otherwise result from excessive inflammation due to embryonic distress signals.

Mitochondrial signalling

Much evidence suggests that mitochondria play a central role in proper embryo development and metabolic adaptation. The lactate production/oxygen consumption ratio (essentially the glycolysis/OXPHOS ratio), lipid utilisation rate and amino acid turnover are under mitochondrial control and have been found to be good predictors of embryo viability (Ballantyne 2008). Aneuploidy has been correlated with altered metabolic status (Picton *et al.* 2010) and slower development and decreased viability (Lee *et al.* 2015) in human pre-implantation embryos. Moreover, increased mitochondrial DNA in the trophectoderm is associated with aneuploidy in humans and is a predictor of the post-implantation potential of embryos (Fragouli *et al.* 2015). The replication of mitochondrial DNA is under dynamic regulation during pre-implantation development (Cagnone *et al.* 2016) and stress responses could trigger the release of this DNA into the culture medium (due to cell death or clearance of damaged mitochondria). Recent studies have shown that an increased copy number of the mtDNA genome in embryonic cells and in spent media is a sign of a metabolic response to developmental stress (Stigliani *et al.* 2013, 2014).

Chromosomal abnormalities such as aneuploidy occur because of improper separation of chromosomes during meiosis in gametes or mitosis in the early embryos. Some PGS studies have shown the impact of IVC on the increase in the number of aneuploid cells among mosaic embryos (Bergh *et al.* 2004, Elaimi *et al.* 2012), but the underlying mechanisms are not completely understood (Labrecque & Sirard 2014, Taylor *et al.* 2014, Pfender *et al.* 2015). However, it appears likely that differential expression of genes controlling chromosomal segregation/

stability is involved (Treff *et al.* 2011, Vazquez-Diez *et al.* 2016). Moreover, communication between nuclear and mitochondrial DNA is crucial during meiosis, fertilisation and early pre-implantation development. Since the energetic metabolism is slowed-down until EGA, the early embryonic cleavages may not require important oxidative activity from the mitochondria. However, these early stages do require a synchronisation between the cytoplasmic accumulation of proteins, mRNA, mtDNA, etc. and the activation of the embryonic genome. In this context, intracellular bio-sensors such as the CHK2 kinase or the sirtuin deacetylases might intervene to communicate the state of cytoplasmic maturity to the nucleus, and regulate the proper segregation of chromosomes and the acquisition of developmental competence (Bolcun-Filas *et al.* 2014).

The response of embryos to IVC involves a variety of metabolic factors that act as signals (homeostatic sensors) of extracellular and intracellular conditions to which the cells adapt by modifying the developmental programme accordingly (Fig. 1). These sensors include notably the PI3K/AKT, mTORC and AMPK pathways, which are sensitive to the thermodynamic phosphate potential (Riley *et al.* 2005, Diaz-Ruiz *et al.* 2011, Guerke *et al.* 2016) and regulate energetic homeostasis (Li *et al.* 2013, Toyama *et al.* 2016). Other factors will also regulate mitochondrial metabolism, such as HIF1A in response to oxygen tension (Zhou *et al.* 2012), HNF4A in response to glucose concentration (Wang *et al.* 2000, Dankel *et al.* 2010), or PPAR γ and SREBP in response to lipid content. Others suggest that mitochondrial biogenesis is controlled in direct response to the energy demand via PGC1A or sirtuins and that these sensors should be targeted using suitable compounds to maintain mitochondrial homeostasis during IVC. For example, supplementing IVC medium with ADP and L-carnitine may be beneficial to OXPHOS, since they contribute to energy expenditure and lipid transport into the mitochondria. In addition, resveratrol (an activator of sirtuin deacetylases, which regulate proper mitochondrial function) could also enhance embryo quality through its action on mitochondrial maturation during oocyte growth and early embryonic development.

Changes in mitochondrial activity may also have an epigenetic impact on the developmental programming of energetic metabolism (metabolic memory) in the embryo. As biosensors of the surrounding milieu, mitochondria communicate with the nucleus to adjust cell metabolic homeostasis. In particular, mitochondrial production of acetyl-CoA and methyl groups, which are dependent on the availability of glucose, lipids (cholesterol) and amino acids (notably L-methionine, vitamin B12, folate and other substrates involved in one carbon metabolism), will regulate histone acetylation and the DNA methylation in the nucleus (Steegers-Theunissen *et al.* 2013, Xu & Sinclair 2015).

Figure 1 Proposed model for the IVC impact on the homeostasis of preimplantation development and the embryogenic response associated with compromised embryonic quality. In this model, the IVC conditions impact the mitochondrial maturation in early developing embryos, leading to OXPHOS impairment and ATP deficiency after embryonic genome activation (8–16 cells stage in bovine embryos). In order to survive and overcome the morula-to-blastocyst transition (MBT), embryos respond to IVC stress conditions by intensifying aerobic glycolysis (the Warburg effect), which creates a metabolic imbalance and ultimately compromises blastocyst quality. Such response induces a typical transcriptomic signature that should be considered when optimizing the IVC conditions to reduce mitochondrial stress in the embryos.

These so-called epigenetic marks regulate the expression of the genome to adjust the cellular homeostasis in response to the external energy supply

(Chason *et al.* 2011). Such regulation can be useful for short-term metabolic adaptation to overcome IVC-related stress. However, early changes in epigenetic programming, specifically epigenomic upheaval during pre-implantation development, can have long-term consequences on health and disease (Chavatte-Palmer *et al.* 2016). IVC and serum in particular have been shown to affect the establishment of epigenetic marks notably in gene regions such as *H19/IGF2* (Doherty *et al.* 2000, Khosla *et al.* 2001a), which are associated with developmental syndromes such as Beckwith–Wiedemann Syndrome or BWS (Lucifero *et al.* 2004, Fernandez-Gonzalez *et al.* 2007), thus providing compelling support for the hypothesis of the developmental origin of health and disease (Hart & Norman 2013, Chavatte-Palmer *et al.* 2016).

A unified mitochondrial hypothesis

The various interpretations of the repeatedly observed responses of the transcriptome to stress associated with IVC converge on a unified hypothesis according to which the central cause of compromised development of embryos is disturbed mitochondrial homeostasis. Molecular genetic analysis has shown that mitochondria originated from bacterial endosymbiosis, which established effective intracellular communication between prokaryotic and proto-nuclear DNA genomes, leading ultimately to the development of the first eukaryotic cells (Wallace 2009). As energy control units, mitochondria play a fundamental role in cellular homeostasis and in functions such as cell division, pluripotency, differentiation and apoptosis. Their metabolic functions include production of reduced dinucleotides NADH and FADH₂ via the tricarboxylic acid cycle and beta oxidation of fatty acids, oxidative phosphorylation of ADP to ATP (by the trans-membrane proton gradient generated by the electron transport chain), elimination of superoxide produced by the electron transport chain, production of ketone bodies such as hydroxybutyrate (as an alternative energy source), turnover of numerous amino acids via the TCA cycle, thiol metabolism, elimination of ammonia by the urea cycle, management of calcium pools, mitochondrial permeability transition, and the release of cytochrome C to induce apoptosis.

During oocyte maturation, mitochondria are in an immature state, containing few cristae and exhibiting a hooded shape (Scantland *et al.* 2014). This phenotype is maintained until embryonic compaction, when the demand for ATP increases. At this stage, mitochondria regain their regular shape, contain more cristae and produce most of the ATP made available to the cell by OXPHOS (Trimarchi *et al.* 2000). However, embryos produce ATP also by converting pyruvate into lactate, a process called aerobic glycolysis. Embryonic

metabolism is thus similar to cancer cell metabolism, first described by Dr Otto Warburg, Kaiser-Wilhelm-Institut (now Max-Planck-Institut) für Biologie, Berlin-Dahlem, Germany in the early 1920s and then called the Warburg effect (Vander Heiden *et al.* 2009). Numerous genes involved in the Warburg effect are expressed normally during blastocyst development (Krisher & Prather 2012, Redel *et al.* 2012), and this compensates for the limits on ATP synthesis via OXPHOS at low oxygen tension. Aerobic glycolysis also provides building blocks for anabolism in the developing embryos (e.g. production of nucleic acids from intermediates of the pentose phosphate pathway). In addition, the conversion of pyruvate to lactate limits the production of ROS that could otherwise arise from excessive OXPHOS activity.

Although post-EGA anaerobic glycolysis is normal and necessary (Krisher & Prather 2012, Redel *et al.* 2012), overexpression of the Warburg-like signature might be an energetic compromise in response to stress during IVC. Such compromise could result from altered mitochondrial function, albeit the direct causal mechanism is still hypothetical. During the early cleavages of the zygote, mitochondria and intracellular metabolism in general are quiescent. Compared with *in vivo*, this metabolic quiescence is disturbed *in vitro* due to the presence of nutrients in excessive amounts and the accumulation of metabolic intermediates that overstimulate the mitochondria (a Crabtree-like effect) and alter the OXPHOS coupling efficiency. Moreover, mitochondria also sense changes in homeostatic parameters such as the redox potential or the amino acid concentration. External conditions could thus induce damaging stress and affect mitochondrial maturation during early cleavage, forcing embryos to cope with decreased production of ATP by OXPHOS at the morula-to-blastocyst transition and to adapt in order to overcome an energy deficit. Although this model needs further investigation, it proposes an explanation for the overexpression of the Warburg-like metabolism observed in IVC embryos (Fig. 1).

The Warburg-like signature includes biomarkers such as TP11, a glycolic enzyme that is upregulated in cancer cells (Wang *et al.* 2013b, Poliakov *et al.* 2014). In addition, the extracellular matrix remodelling and inflammatory responses to IVC accompany transcriptomic changes are similar to those that accompany tumour progression and the micro-environmental modifications required for metastasis. Lumican, SERPINE1 and PLOD2 are involved in tumour invasion (Brezillon *et al.* 2013, Chen *et al.* 2015, Suh *et al.* 2015). Type I interferons have been shown to control the IFNR/STAT1-dependant expression of Warburg effect genes (Pitroda *et al.* 2009, Rautela *et al.* 2015) and to regulate tumour growth and chemoresistance (Sistigu *et al.* 2014, Rautela *et al.* 2015). Several other genes involved in tumorigenesis (*MLLT11*, *ZNF385B*, *TSC22D1*, *IFIH1* and *RPS12*) are also overexpressed in IVC embryos, although their functions

remain to be characterised. Moreover, alternative splicing of metabolic genes (which can be observed by RNA-seq or with the EmbryoGENE array) may control the Warburg-like effect in cancer cells (Redel *et al.* 2012, Yang & Lu 2013, Yang *et al.* 2013) and should be investigated in IVC embryos.

The differential gene expression associated with the Warburg-like signature may represent either a transient status regulated by transcription factors or the induction of epigenetic changes, or both (Salilew-Wondim *et al.* 2015). Based on our microarray results, several DEGs appear to be controlled by epigenetic modifications or parental imprinting (the epigenetic extinction of maternal or paternal alleles). For example, differential DNA methylation regulates *SERPINE1*, which appears to support long-term memory of metabolic disorders (Lopez-Legarrea *et al.* 2013, Takizawa *et al.* 2013). A histone-modifying enzyme under TGFβ1 stimulation targets the *PLOD2* promoter (Gjaltema *et al.* 2015). Overexpression of *IFN-τ* is associated with epigenetic reprogramming after somatic cell nuclear transfer in bovine elongated embryos (Rodriguez-Alvarez *et al.* 2010). In addition, *IFN-τ* and *IGF2* are regulated differently in slow-developing and fast-developing embryos (Gutierrez-Adan *et al.* 2004) and altered parental imprinting of *IGF2* in embryonic stem cells is associated with aberrant development (Dean *et al.* 1998). It should interest researchers that loss of imprinting on *IGF2*, which increases *IGF2* expression, is associated with mitochondrial OXPHOS deficiency, which causes cancer cells to shift toward glycolysis (Wallace & Fan 2010). These results suggest strongly that overexpression of the Warburg effect in response to mitochondrial stress in culture can have profound effects on the metabolic memory of the embryo and thus will likely influence its long-term homeostasis.

Conclusion

The focus of this review is the identification of stress response genes that might provide valuable biomarkers of blastocyst quality. These markers draw attention to specific pathways that are subject to transcriptome plasticity during early embryonic development and which would therefore become targets for media optimisation. These stress-induced pathways expressed in cultured embryos suggest a unified hypothesis of metabolic compromise in response to mitochondrial insult and excessive upregulation of a Warburg-like effect (aerobic glycolysis). After EGA, the embryo normally turns to aerobic glycolysis to complement oxidative energy production at low oxygen tension, an adaptation that limits ROS production by OXPHOS and provides anabolic substrates required for cell proliferation. Mitochondria are immature and vulnerable during early cleavage, and their maturation under IVC conditions may be incomplete, leading

to inadequate coupling efficiency during blastocyst development. In order to survive, embryos respond to IVC stress conditions by intensifying aerobic glycolysis (the Warburg effect), which creates a metabolic imbalance and ultimately reduces embryo quality. Moreover, the overexpression of a cancer-like developmental programme may compromise embryo-maternal recognition, since the endometrium might block the invasion of metabolically uncontrolled cells. The early energetic adaptation to IVC might also induce epigenetic modifications that affect foetal growth and induce developmental syndromes after birth (De Rycke *et al.* 2002, Gosden *et al.* 2003, Denomme & Mann 2012). In this equation, metabolic signalling is a critical factor for the establishment of a properly functioning embryonic epigenome. Culture media optimisation research therefore must take mitochondrial homeostasis into consideration in order to improve the quality of embryos and the health of offspring resulting from the use of assisted reproductive technologies.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-16-0391>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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