Metabolism of the Viable Human Embryo

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Regulation of energy production is fundamental to the survival and propagation of any cell type. What makes the preimplantation mammalian embryo so fascinating to study is the fact that the embryo undergoes major changes in its physiology and gene expression profiles during development. As the fertilised oocyte develops and differentiates into the blastocyst, embryonic genes are successively turned on (with the concomitant destruction of maternally derived mRNAs). Subsequently there is a growing energy demand as mitoses and biosynthesis increase post-embryonic-genome activation and as the blastocoel subsequently forms (through the activity of Na/K ATPase in the trophectoderm). Concomitantly, there are major changes in the regulation and relative activities of energy generating pathways. Of clinical interest is the fact that should an embryo at any stage of development have substantially altered energy production, i.e. if the flux of a specific nutrient through a metabolic pathway alters to a significant degree, even for a brief period, then this is associated with significantly impaired development in culture and reduction of viability post-transfer. Clearly it is in our interest to understand how the preimplantation embryo regulates its energy production, and to develop culture systems that best support an 'optimal' metabolism. Furthermore, it is evident that analysis of embryo metabolism is an appropriate means of assessing embryonic health and predicting subsequent viability. The task ahead is to determine the optimal range of metabolic functions that reflect viability at successive stages of embryo development.

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Changes in Metabolism with Development

The fertilised oocyte exhibits low utilisation of oxygen [1] and has a limited capacity to utilise glucose as an energy source [2]. In fact, glycolytic activity is restricted in the fertilised oocyte and early embryo by the allosteric regulation of phosphofructokinase (PFK) due to a high ATP:ADP ratio [3]. High ratio of these nucleotides is a consequence of the low energy demands at these stages, reflecting the dormant status of the oocyte from which it was derived. Consequently, in the absence of amino acids, the early embryo generates its energy from low levels of oxidation of pyruvate (fertilised oocyte) and lactate (from the 2-cell stage onwards), derived directly from oviduct fluid (and the cumulus cells in the case of the oocyte [4–6]) (Fig. 1). However, the early embryo can also utilise the malate-aspartate shuttle, and in the presence of specific amino acids, e.g. aspartate, the embryo can utilise lactate as an energy source through the conversion of lactate to pyruvate [7]. In the immediate post-fertilisation period of mouse zygotes, there is an absolute requirement

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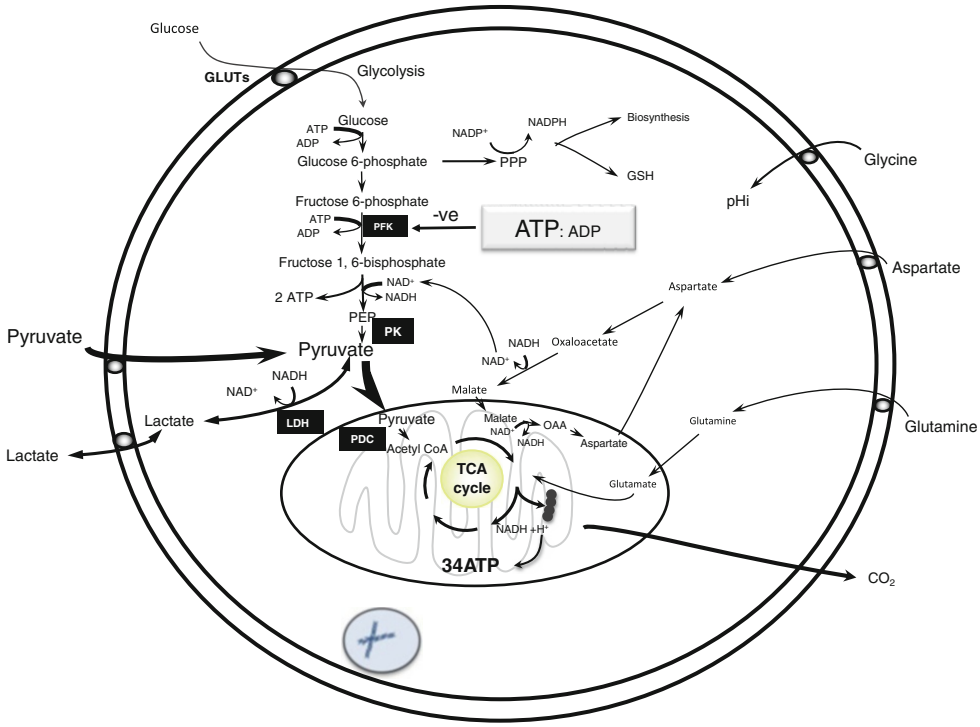

Fig. 20.1 Metabolism of the pronucleate oocyte and cleavage stage embryo. Prior to compaction the embryo has a metabolism based around low levels of oxidation of pyruvate, lactate and specific amino acids. The ovulated oocyte is surrounded by and is directly connected to cumulus cells, which actively produce pyruvate and lactate from glucose [4, 6]. This creates a high concentration of pyruvate and lactate and a low concentration of glucose around the fertilised oocyte. Upon dispersal of the cumulus cells, the human zygote and cleavage stage embryo still find themselves in a relatively high concentration of pyruvate (0.32 mM) and lactate (10.5 mM), and low levels of glucose (0.5 mM) [6]. Significantly, the relative abundance of nutrients affects the metabolism of the embryo. For example, the ratio of pyruvate:lactate in the surrounding environment directly affects the ratio of NADH:NAD^+ in the embryo, which in turn controls the redox state of the cells and hence the flux of nutrients through specific energy generating pathways [85]. Lactate dehydrogenase comprises ~5 % of the total protein of the mouse oocyte [86]. The oocyte, pronucleate oocyte and all stages of development to the blastocyst exhibit LDH isoform I [87], which then changes to predominantly isoform V at the late blastocyst stage upon outgrowth [88]. Isoform I favours the formation of pyruvate, and is typically found in heart tissue. Isoform V favours lactate formation, and is typically found in muscle tissues. This switch in isoforms is consistent with the changes in patterns of energy metabolism as the embryo develops, but cannot explain the significant production of lactate by the blastocyst (see Fig. 2). The early embryo is characterised by a high ATP:ADP level [3], which in turn allosterically inhibits PFK, thereby limiting the flux of glucose through the glycolytic pathway. Amino acids fill several niches in embryo physiology, such as the use of glycine as buffer of intracellular pH (pHi). Several amino acids are also utilised as energy sources by the early embryo such as glutamine and aspartate. Aspartate can be utilised through the malate-aspartate shuttle [7], the significance of which during embryo development we are only beginning to understand. The thickness of the lines represents the relative flux of metabolites through the pathway. *PFK* phosphofructokinase; *PK* pyruvate kinase; *LDH* lactate dehydrogenase; *PDC* pyruvate dehydrogenase complex; *PPP* pentose phosphate pathway; *OAA* oxaloacetate; *pHi* intracellular pH

for oxidative metabolism of pyruvate to support the completion of the second meiotic division and subsequent development [8].

With an increase in cell divisions and biosynthesis following the activation of the embryonic genome, the formation of a transporting epithelium when the morula is formed, and subsequent creation of the blastocyst, the need for cellular energy increases dramatically. Consequently there is a decline in the ATP:ADP ratio (and a dramatic increase in AMP levels) as ATP is readily consumed, thereby alleviating

inhibition on PFK and facilitating the flux of glucose through glycolysis. However, the overall regulation of carbon flux through glycolysis is also regulated at a number of sites (Fig. 2).

Interestingly, although the blastocyst exhibits a much higher oxygen consumption than the cleavage stage embryo [9–11], the late stage embryo does not oxidise all of the glucose consumed, but rather converts approximately 50 % (somewhat species-dependent) of glucose carbon to lactate, this even in the presence of adequate oxygen for complete oxidation of the glucose. This latter metabolic phenomenon is referred to as 'aerobic glycolysis' (not to be confused with anaerobic glycolysis found in tissues such as muscle when oxygen is limited). Aerobic glycolysis is a trait found in cancers and rapidly dividing cells, a phenomenon also known as the Warburg effect [12, 13] (Fig. 2).

What Is the Role of Aerobic Glycolysis/ Warburg Effect in the Blastocyst?

In the blastocyst of primates and rodents, high levels of aerobic glycolysis, have been interpreted as the embryos adaptation to its imminent invasion of the endometrium, which remains avascular for a period of up to 12 h, and will therefore be relatively anoxic [14, 15]. During the initial stages of blastocysts invasion, glycolysis will plausibly be the predominant means of generating energy. However, this may not be the sole explanation for the high levels of glycolysis observed in blastocysts of domestic animals, as they do not 'implant' for a further week. An alternative explanation for the rather idiosyncratic metabolism of the blastocyst was proposed by Gardner in 1998 [16]. A high level of aerobic glycolysis is a common characteristic of proliferating cells and tumours, with which the mammalian blastocyst shares several traits [17–19]. As well as being used to generate energy for increased mitoses and blastocoel expansion, high levels of glucose utilisation will be required for the synthesis of triacylglycerols and phospholipids and as a precursor for complex sugars of mucopolysaccharides and glycoproteins. Glucose metabolised by the pentose phosphate pathway (PPP) will generate ribose moieties required for nucleic acid synthesis and the NADPH and carbon skeletons required for the biosynthesis of lipids and other complex molecules [18, 20]. NADPH is also required for pathways of one carbon metabolism, which are vital for the methylation of various intracellular substrates, including DNA. It is now well appreciated that embryos undergo dramatic changes in the epigenetic modifications of their genomes including DNA methylation during preimplantation development [21]. NADPH is also required for the reduction of intracellular glutathione, an important antioxidant for the embryo [22]. Interestingly, although the absolute amount of glucose metabolised through the PPP increases with development [23], the percentage of the total glucose consumed which is metabolised through this pathway appears to be lowest at the blastocyst stage [24]. This again raises the question as to why such high levels of aerobic glycolysis are required? It is plausible that high levels of aerobic glycolysis, such as that observed in the mammalian blastocyst, will ensure that there is sufficient substrate and cytosolic ATP available for biosynthetic pathways, such as the synthesis of nucleic acids, proteins and new membranes, at the required times during cellular proliferation [25, 26]. This in turn suggests that there are times within the cell cycle when the PPP is more active than others (Fig. 2). Analysis of embryo energy metabolism with respect to cell cycle has not been thoroughly analysed, but it is speculated that at specific times during the cell cycle there will be increased energy demands, particularly during cytokinesis.

A further explanation for the high levels of aerobic glycolysis by the blastocyst could lie in the activity of mitochondrial shuttles in the oxidation of hydrogen equivalents formed through glycolysis. The malate-aspartate is one such shuttle that allows cytosolic NADH to be exchanged for NAD^+ that is derived from the mitochondrion. Once within the mitochondrion, the NADH can be more efficiently converted to ATP through oxidative phosphorylation. Interestingly, the malate-aspartate (NADH) shuttle of different tumours can contribute between 20 and 80 % of

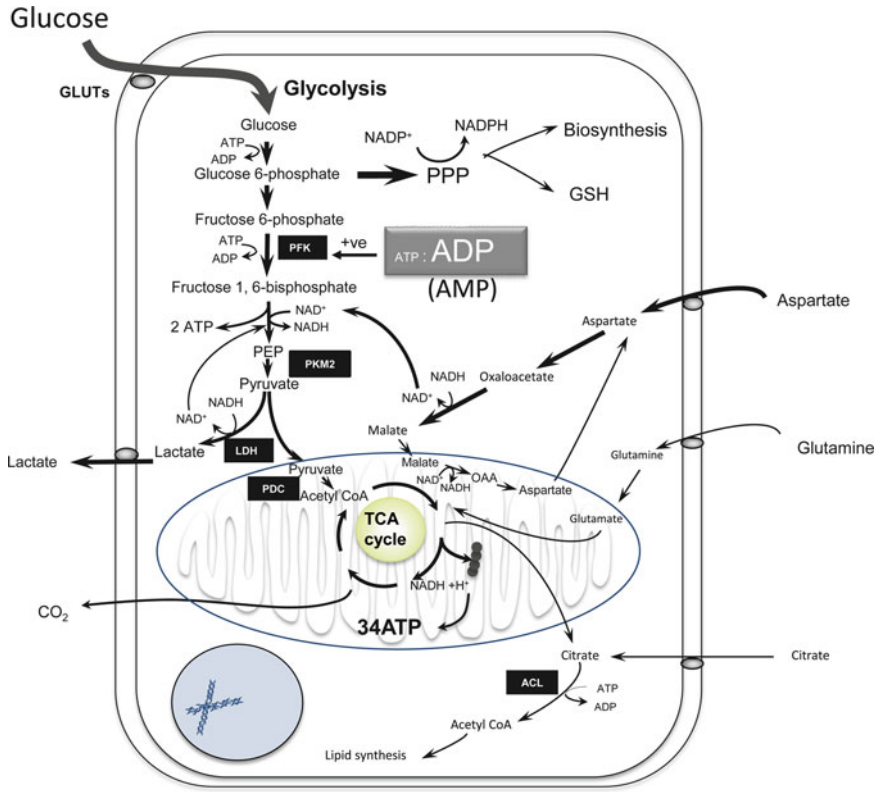

Fig. 20.2 Metabolism of the blastocyst. After compaction the embryo exhibits greatly increased oxygen consumption [1, 9, 10] and an increased capacity to use glucose as an energy source. The increase in oxygen consumption plausibly reflects the considerable energy required for the formation and maintenance of the blastocoel, while the increase in glucose utilisation reflects an increased demand for biosynthetic precursors [16]. Consequently, there is a reduction in the ATP:ADP ratio [3], and a concomitant increase in AMP, which will have a positive allosteric effect on PFK, thereby facilitating a higher flux of glucose through glycolysis. Rather than oxidise the glucose consumed, the blastocyst exhibits high levels of aerobic glycolysis [5]. Although this may appear energetically unfavourable, it does ensure that the biosynthetic arm of the pentose phosphate pathway has maximum substrate availability at all times. Activity of the pentose pathway will ensure that reducing equivalents are available for biosynthesis and ensure production of glutathione (reduced), a key intracellular antioxidant. In order for high levels of glycolysis to proceed the blastomeres need to regenerate cytosolic NAD^+. This can be achieved through the generation of lactate from pyruvate. A second means of generating cytosolic NAD^+ is through the activity of the malate-aspartate shuttle. Although it is evident that blastocysts do use aerobic glycolysis, it is proposed that the significant increase in oxygen utilisation at this stage of development could be largely attributed to the activity of the malate-aspartate shuttle and the resulting demand for oxygen to convert intramitochondrial NADH to ATP. Indeed many tumours that exhibit aerobic glycolysis also have high levels of the malate-aspartate shuttle [28]. Furthermore, inhibition of this shuttle has dire consequences for subsequent fetal development [21, 89]. A further key regulatory enzyme in glycolysis is pyruvate kinase (PK). In proliferating cells and in cancer cells, a specific isoform is present, PKM2 [90, 91]. This particular isoform of PK has been shown to promote aerobic glycolysis and anabolic metabolism [92], and recently has been identified in the mammalian blastocyst [93]. Further work is warranted on establishing the regulation of PKM2 in the embryo, with specific reference to its control by exogenous factors and signalling pathways involved. The pyruvate dehydrogenase complex catalyses the irreversible conversion or pyruvate to acetyl Co-A, and consequently functionally linking glycolysis to the activity of the oxidative TCA cycle. The activity of this enzyme complex is tightly regulated through three main mechanisms; (1) phosphorylation/dephosphorylation, (2) the redox state (NAD^+:NADH, ATP:ADP and the acetylCoA:CoA ratios) and (3) transcriptional regulation. However, relatively little is known about the regulation of this complex in mammalian embryos during the preimplantation period. Citrate, formed from either mitochondrial metabolism, or provided in the culture medium, could serve as a precursor in lipid synthesis, required for membrane generation associated with proliferation. As the embryo develops, it

the total respiratory rate [27, 28]. As the mammalian blastocyst has a high respiratory quotient [1] combined with high levels of lactate production [5], it is plausible that glycolysis is used to derive ATP oxidatively through the malate-aspartate shuttle. In support of this it has recently been demonstrated that in the mouse preimplantation embryo malate-aspartate shuttle activity is essential for the regulation of metabolism during the preimplantation period [7]. Furthermore, if the activity of the shuttle is inhibited in the blastocyst, then subsequent implantation and fetal development are significantly reduced [29]. Such data further highlight the significance of maintenance of embryonic metabolism to subsequent viability and normality.

Comparison of Metabolic Activities of Embryos Developed In Vitro Compared to In Vivo

One advantage of working with an animal model, such as the mouse, is the ability to access and analyse embryos developed in vivo, thereby being able to determine how similar, or otherwise, embryos are when maintained in vitro. If a mouse blastocyst is collected from the uterus and its metabolism quantitated immediately, it is observed that just less than 50 % of glucose consumed will be released into the surrounding medium as lactate [5]. It has been established that when developed in simple culture conditions (i.e. a culture medium lacking amino acids and vitamins, and with only glucose, pyruvate and lactate as energy sources, which is typical of the early human embryo culture media such as HTF [30]), resultant blastocysts exhibit a significant increase in lactate production [5, 31] and a concomitant reduction in carbohydrate oxidation [32], which has been documented to be associated with a significant loss of viability [33]. Lane and Gardner [34] demonstrated that placing a mouse blastocyst that had developed in vivo and collected directly from the uterus, into a simple culture medium induced aberrant metabolism within just 3 h of incubation. They went on to show that collecting mouse blastocysts in the presence of amino acids and vitamins greatly reduced this metabolic trauma. Furthermore, when in vivo developed blastocysts were incubated for just 6 h in a simple medium prior to transfer, they exhibited a significant reduction in implantation rate and subsequent fetal development. In contrast, blastocysts incubated in the presence of amino acids and vitamins for the 6 h period were not significantly compromised post-transfer.

Key points arising from the aforementioned study are: that culture-induced metabolic stress, i.e. incubation in a medium lacking amino acids and vitamins, resulted in a *rapid* loss of metabolic regulation, and that secondly, this metabolic perturbation not only reduced implantation but also impacted fetal development, culminating in lighter foetuses. So an incubation of just a few hours in a stressful environment can induce metabolic changes that have downstream effects leading to compromised fetal development. Hence, several studies have used this model to develop culture conditions which ensure that embryos

Fig. 20.2 (continued) exhibits a growing number of receptors for specific growth factors [94]. Given that growth factor signalling can reorganise metabolic fluxes independently of traditional allosteric means, it will be important to determine how exogenous factors affect key metabolic processes within the embryo. Analysis of blastocyst metabolism is a measurement of the relative activity of two cell types, the trophectoderm and inner cell mass (ICM). In the mouse blastocyst it has been determined that whereas the trophectoderm converts around half of the glucose consumed to lactate, the ICM is almost exclusively glycolytic [95]. It is not know what the relative activities of the two cell types are in the human blastocyst, but given that human embryonic stem cells are dependent upon a high glycolytic activity [96], it would be prudent to assume that the human ICM also exhibits low levels of glucose oxidation. Although the number of trophectoderm cells far outnumbers those of the ICM, it remains important to consider these cell-specific differences when analysing metabolic data. The thickness of the lines represents the relative flux of metabolites through the pathway. *PFK* phosphofructokinase; *PK* pyruvate kinase; *LDH* lactate dehydrogenase; *PDC* pyruvate dehydrogenase complex; *ACL* acetyl-citrate lyase; *OAA* oxaloacetate; *PPP* pentose phosphate pathway; *OAA* oxaloacetate; *GSH* reduced glutathione. From 97 with permission

in vitro exhibit a metabolic profile similar to those embryos developed in vivo [35]. These data firmly establish that quantifying embryo metabolism is an appropriate physiological parameter to relate to subsequent viability after transfer. Furthermore, the above study was performed on in vivo developed blastocysts, and it has been shown that embryos post-compaction are far more resilient to environmental stress than the cleavage stage embryo. Consequently, exposing cleavage stage embryos to stress, i.e. prior to compaction, may have even greater downstream effects [36, 37].

Relationship Between Carbohydrate Metabolism and Embryo Viability

As discussed, substantial perturbations in the relative activity of pathways involved in intermediary metabolism culminate in compromised embryo development in culture and a reduction of viability post-transfer. Data from such studies have been invaluable in the development of modern culture systems that support normal metabolic function in the developing embryo, thereby promoting the development of viable human embryos [38]. A relationship between carbohydrate utilisation by embryos and resultant viability has been established both in animal models [33, 39, 40] and the human [41, 42] (reviewed in detail in Chap. 23). Post-compaction glucose consumption is positively correlated with subsequent viability. Furthermore, should the metabolism of glucose by the blastocyst differ from that expected, i.e. excessive glycolytic activity, then it has been established that viability is significantly compromised in the mouse model. These data support the hypothesis that it is not only the rate at which a nutrient is consumed that reflects developmental potential but also the metabolic fate of the nutrient measured. Of further interest is the observation that female embryos are metabolically more active than males [41, 43]. This finding is consistent with the observed differences in gene expression profiles [44, 45], and subsequent proteome [46], between male and female embryos.

Beyond the Carbohydrate Triad: Analysis of Amino Acid Utilisation

Alternative energy sources for cells, other than those present in typical embryo culture media (i.e. glucose, pyruvate and lactate) include free fatty acids (FFA) and amino acids. Although FFA may have a role in the development embryos of certain species (typically those with a high endogenous lipid content, such as the cow and pig), and their uptake by human embryos has been quantitated [47], further work is required to understand how FFA impact the metabolism of the embryo. Consequently they will not be discussed further here. However, it would be prudent to watch developments in this area, especially with regard to the role of FFA metabolism in oocyte maturation [48]. The beneficial effects of amino acids on embryos in culture have been demonstrated for several years. Amino acids are abundant in oviduct and uterine fluids [49, 50] and are important regulators of embryonic function. As well as their documented effects as biosynthetic precursors [51], buffers of intracellular pH in the embryo [52], antioxidants [53] and in signalling and differentiation [54–56], amino acids also serve as energy sources [23]. Furthermore, our understanding of how embryo energy metabolism is regulated has changed recently with the determination that the malate-aspartate shuttle is involved in the control of carbohydrate metabolism [7]. A paradigm held for over 40 years was that the fertilised oocyte had an absolute requirement for pyruvate [2]. However, recent analysis of embryo physiology has revealed that lactate and aspartate can substitute for pyruvate at the 1-cell stage, the two nutrients presumably generating sufficient energy through the malate-aspartate shuttle to support development.

The relationship between amino acid utilisation and embryo development and viability has been analysed by Leese and colleagues [57–59], and reviewed in detail by Houghton (Chap. 24). Using HPLC to quantitate amino acids in embryo culture medium, it was observed that a different pattern of amino acid utilisation existed between embryos that went on to form a blastocyst compared to

those embryos that failed to develop in vitro. It was observed that leucine was taken up from the culture medium more by embryos which went on to develop [57]. The profiles of the amino acids alanine, arginine, asparagine, glutamine and methionine utilisation also correlated with blastocyst formation, although no relationship between amino acid utilisation and blastocyst quality or viability was established. Subsequently, Brison et al. [58] reported that changes in concentration of amino acids in the spent medium of human zygotes cultured for 24 h to the 2-cell stage in an embryo culture medium containing a mixture of amino acids, using HPLC. It was found that asparagine, glycine and leucine were all significantly associated with clinical pregnancy and live birth. Furthermore, and consistent with the data on glucose consumption, there appears to be gender differences with regard to amino acid utilisation [59, 60], consistent with differences in the proteomes of male and female embryos [43].

An important point to note is that all of the studies analysing the utilisation of amino acids by human embryos have been performed in the presence of 20 % oxygen, the significance of which is just now being realised and is discussed below.

Impact of the Culture Environment on Embryonic Metabolism

The relative concentrations of nutrients present in culture media directly affects embryo metabolism [61]. However, other factors of the culture system such as medium pH [62] and the concentration of oxygen used in the incubator can impact metabolic function. Historically, atmospheric oxygen (~20 %) has been in tissue culture, and human IVF laboratories subsequently adopted this level of oxygen for embryo culture. However, the physiological concentration of oxygen within the female reproductive tract has been reported to be below 10 % [63–65]. Consistent with such data, embryo development in all mammals studied to date is significantly improved by culture in an oxygen concentration of 5–7 %, compared to 20 % [66–69], with a high oxygen concentration having its most detrimental effects at the cleavage stages [70]. Similar data sets are now being documented for the human embryo [71–74].

A significant body of work has been amassed on the effects of oxygen concentration on mammalian embryo development. It transpires that atmospheric oxygen has a significant negative impact on blastocyst gene expression [36, 75], the embryonic proteome [76] and more recently it has been established that oxygen concentration has a significant impact on embryo metabolism, impacting the utilisation of both carbohydrates and amino acids [77] (Fig. 3). Of interest the effects of oxygen on metabolism were stage-specific. During the cleavage stages, 20 % oxygen was associated with an overall increase in amino acid turn over and pyruvate uptake by embryos. In contrast, post-compaction 20 % oxygen was associated with a decrease in amino acid turnover and glucose uptake. Given that it has been established that glucose uptake by the embryo post-compaction is positively correlated with viability, such findings are consistent with reports of lower pregnancy rates following culture in 20 % oxygen, i.e. the decrease in glucose utilisation in the presence of 20 % oxygen is related to the reduction in viability. Given the documented harm atmospheric oxygen imparts on the developing embryo and its physiology, its continued use in human IVF can no longer be condoned.

What Are the Accepted Patterns of Metabolic Activity? Quietness Revisited

A novel hypothesis proposed by Leese [78] suggested that those embryos with a less active, or a ‘quiet’ metabolism are those that go on to give rise to pregnancies. Data to support this hypothesis have been obtained from different species and reviewed recently [79]. In essence it was proposed that embryos with a low metabolic activity reflected a less stressed physiology, and consequently those embryos classified as viable would be those that had low nutrient uptake and turn

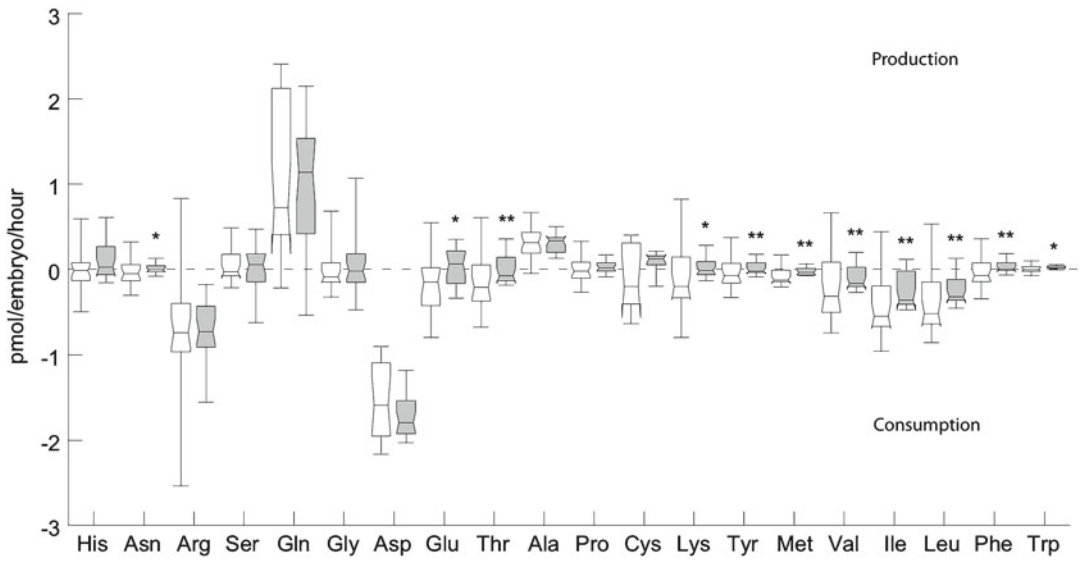

Fig. 20.3 Effect of oxygen concentration on amino acid utilisation by mouse post-compaction embryos from day 4 to 5. Five per cent oxygen, *open box* and 20 % oxygen, *grey box* (25 replicates per treatment, groups of 3, $n=75$ embryos). The line across the box is the median uptake or release. There were significant differences in the utilisation of asparagine, glutamate, tryptophan and lysine ($P<0.05$), with greater significance for utilisation of threonine, tyrosine, methionine, valine, isoleucine, leucine and phenylalanine ($P<0.01$), between the two oxygen concentrations. Consumption for each of these amino acids was greater at 5 % oxygen than atmospheric oxygen, and overall amino acid utilisation was higher at 5 % compared to 20 % ($P<0.05$). Notches represent the confidence interval of the median, and the depth of the box represents the interquartile range (50 % of the data), whiskers represent the 5 and 95 % quartiles. *Asterisks* indicate significant differences from embryos cultured in 5 % oxygen (*$P<0.05$, **$P<0.01$) (From 77 with permission)

over [79]. However, a growing number of recent studies have generated data that do not provide support for this hypothesis [33, 41, 80, 81], rather they indicate that viability is associated with increased metabolic activity. So how can this apparent paradox be resolved? An analysis of the studies upon which the 'quiet hypothesis' was built reveals that a common factor among them is the use of 20 % oxygen, either for embryo culture and analysis, or during the actual analysis of metabolism. Given the documented negative impact of 20 % oxygen on embryonic gene expression, proteome and metabolism described above, the significance of the 'quiet hypothesis' for embryos cultured under physiological oxygen conditions must be carefully reviewed and studies performed to determine what is the optimal nutrient utilisation profile under physiological conditions.

We know that when mammalian embryos are stressed, especially by 20 % oxygen, their metabolic profile changes significantly [77]. Data to date [79] indicate that those embryos cultured in 20 % oxygen which are able to develop do have a lower turnover of nutrients. One explanation for this finding may be related to the inability of unhealthy or developmentally compromised embryos to tolerate oxidative stress. Suboptimal embryos may have lower endowments of antioxidants or less robust systems for responding to oxidising agents and consequently may have to resort to other energetically consuming mechanisms to preserve their redox status. In this context, the quiet embryo hypothesis would pertain more to the embryo's ability to respond to oxidative stress than to its innate metabolic characteristics under physiologic conditions. Studies evaluating the metabolism of the same embryos

in both low and high oxygen conditions could test this hypothesis. Thus far, little attention has been devoted to the development and application of stress tests, i.e., exposing embryos to safe, well-controlled stresses and monitoring their responses, to evaluate embryonic health and developmental potential. There are certainly many clinical situations in which stress tests are used to diagnose disease or disease susceptibility such as exercise stress tests for cardiac disease and glucose tolerance tests for gestational diabetes. The challenge for the development of an embryonic stress test is to ensure that the stressor does not cause lasting harm. As mentioned previously, exposure to high oxygen tension is not an acceptable stressor based on our understanding of the short- and long-term effects of this exposure.

It is evident that when a 5 % oxygen concentration is used during culture, the metabolism of embryos on day 4 and 5 at the morula and blastocyst stages, is significantly higher in those embryos that give rise to pregnancies, compared to those embryos that fail to develop post-transfer. Such data, therefore, do not support the 'quiet hypothesis' for embryos beyond the 8-cell stage. In contrast, the data obtained for day 4 and 5 human embryos and their subsequent pregnancy outcome, were collected under low oxygen conditions. These data that reveal those embryos with the *more active* metabolism give rise to pregnancies. Interestingly, recent data on oxygen consumption by cleavage stage human embryos indicates that at the cleavage stage viability is associated with an increased oxygen consumption rate, reflecting an increase in respiration rate [81]. At present, we do not know if there is a value that is too high to be consistent with viability, although it is most plausible. There will likely be upper and lower values of metabolic normality, outside which embryo will show a decline in viability. Given that there is an overall increase in most metabolic activities as the preimplantation embryo proceeds through development, it would seem more likely for compromised embryos to have lower rather than higher metabolic activities relative to their healthy cohorts. Furthermore, as glucose uptake [41, 43] and amino acid utilisation [59] are related to the sex of embryo, such data need to be factored in to further hypotheses on embryo metabolism and optimal rates of metabolic activity for embryo selection.

Conclusions

Some 30 years after Renard et al. showed a relationship between glucose uptake by day 10 cow blastocysts and subsequent pregnancy outcome [39], data sets relating the patterns of nutrient utilisation by the human embryo with pregnancy outcome, are growing [41, 58, 59]. Further analysis of human embryo physiology will assist in establishing the metabolic algorithms for embryo selection. Growing data indicate that stage-specific and sex-specific differences in metabolism exist during the preimplantation period. Understanding the nature of these differences, the impact of culture conditions (such as medium composition and the concentration of oxygen used) has important implications for embryo selection. As development proceeds, there is an increase in asynchrony amongst embryos of a cohort and this will be manifest as an increased variation in nutrient utilisation (Fig. 4). Consequently it is predicted that differences in

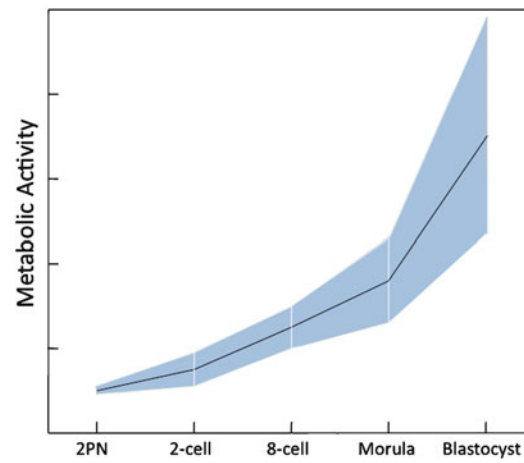

Fig. 20.4 Distribution of metabolic activity with respect to preimplantation embryo development. As development proceeds, asynchrony between embryos and metabolic activity increase. Consequently, it may be easier to identify a viable embryo within a cohort later in development

embryo metabolism will be more readily detected with increasing developmental stage.

It is also evident that changes in metabolic activity not only affect subsequent implantation potential, but have downstream effects through to fetal development and beyond. Given that perturbations in metabolic activity are linked to epigenetic regulation of cell function [82], assessment of a viable metabolism should facilitate the creation of conditions that support the genetic stability and epigenetic integrity of the embryo.

Whereas the technologies for such work currently resides in a handful of laboratories around the world, the rapid developments in laboratory-on-a-chip technologies may well ensure that the assays required will be able to be housed in microfluidic devices and subsequently such analyses will be readily available to the general IVF community [83, 84].

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References

1. Trimarchi JR, Liu L, Porterfield DM, Smith PJ, Keefe DL. Oxidative phosphorylation-dependent and -independent oxygen consumption by individual preimplantation mouse embryos. Biol Reprod. 2000;62:1866–74.
2. Biggers JD, Whittingham DG, Donahue RP. The pattern of energy metabolism in the mouse oocyte and zygote. Proc Natl Acad Sci USA. 1967;58:560–7.
3. Leese HJ, Biggers JD, Mroz EA, Lechene C. Nucleotides in a single mammalian ovum or preimplantation embryo. Anal Biochem. 1984;140:443–8.
4. Leese HJ, Barton AM. Production of pyruvate by isolated mouse cumulus cells. J Exp Zool. 1985;234:231–6.
5. Gardner DK, Leese HJ. Concentrations of nutrients in mouse oviduct fluid and their effects on embryo development and metabolism in vitro. J Reprod Fertil. 1990;88:361–8.
6. Gardner DK, Lane M, Calderon I, Leeton J. Environment of the preimplantation human embryo in vivo: metabolite analysis of oviduct and uterine fluids and metabolism of cumulus cells. Fertil Steril. 1996;65:349–53.
7. Lane M, Gardner DK. Mitochondrial malate-aspartate shuttle regulates mouse embryo nutrient consumption. J Biol Chem. 2005;280:18361–7.
8. Johnson MT, Freeman EA, Gardner DK, Hunt PA. Oxidative metabolism of pyruvate is required for meiotic maturation of murine oocytes in vivo. Biol Reprod. 2007;77:2–8.
9. Mills RM, Brinster RL. Oxygen consumption of preimplantation mouse embryos. Exp Cell Res. 1967;47:337–44.
10. Thompson JG, Partridge RJ, Houghton FD, Cox CI, Leese HJ. Oxygen uptake and carbohydrate metabolism by in vitro derived bovine embryos. J Reprod Fertil. 1996;106:299–306.
11. Houghton FD, Sheth B, Moran B, Leese HJ, Fleming TP. Expression and activity of hexokinase in the early mouse embryo. Mol Hum Reprod. 1996;2:793–8.
12. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science. 2009;324:1029–33.
13. Warburg O. On the origin of cancer cells. Science. 1956;123:309–14.
14. Rogers PAW, Murphy CR, Gannon BJ. Absence of capillaries in the endometrium surrounding the implanting rat blastocyst. Micron. 1982;13:373–4.
15. Rogers PA, Murphy CR, Rogers AW, Gannon BJ. Capillary patency and permeability in the endometrium surrounding the implanting rat blastocyst. Int J Microcirc Clin Exp. 1983;2:241–9.
16. Gardner DK. Embryo Development and culture techniques. In: Clark J, editor. Animal breeding: technology for the 21st century. London: Harwood Academic; 1998. p. 13–46.
17. Hume DA, Weidemann MJ. Role and regulation of glucose metabolism in proliferating cells. J Natl Cancer Inst. 1979;62:3–8.
18. Morgan MJ, Faik P. Carbohydrate metabolism in cultured animal cells. Biosci Rep. 1981;1:669–86.
19. Mandel LJ. Energy metabolism of cellular activation, growth, and transformation. Curr Top Membr Transp. 1986;27:261–91.
20. Reitzer LJ, Wice BM, Kennell D. The pentose cycle. Control and essential function in HeLa cell nucleic acid synthesis. J Biol Chem. 1980;255:5616–26.
21. Shi L, Wu J. Epigenetic regulation in mammalian preimplantation embryo development. Reprod Biol Endocrinol. 2009;7:59.
22. Rieger D. Relationships between energy metabolism and development of early mammalian embryos. Theriogenology. 1992;37:75–93.
23. Rieger D, Loskutoff NM, Betteridge KJ. Developmentally related changes in the uptake and metabolism of glucose, glutamine and pyruvate by cattle embryos produced in vitro. Reprod Fertil Dev. 1992;4:547–57.
24. Rieger D, Guay P. Measurement of the metabolism of energy substrates in individual bovine blastocysts. J Reprod Fertil. 1988;83:585–91.
25. Newsholme EA, Crabtree B, Ardawi MS. The role of high rates of glycolysis and glutamine utilization in rapidly dividing cells. Biosci Rep. 1985;5:393–400.
26. Newsholme EA. Application of metabolic-control logic to the requirements for cell division. Biochem Soc Trans. 1990;18:78–80.

27. Greenhouse WV, Lehninger AL. Occurrence of the malate-aspartate shuttle in various tumor types. Cancer Res. 1976;36:1392–6.
28. Greenhouse WV, Lehninger AL. Magnitude of malate-aspartate reduced nicotinamide adenine dinucleotide shuttle activity in intact respiring tumor cells. Cancer Res. 1977;37:4173–81.
29. Mitchell M, Cashman KS, Gardner DK, Thompson JG, Lane M. Disruption of mitochondrial malate-aspartate shuttle activity in mouse blastocysts impairs viability and fetal growth. Biol Reprod. 2009;80: 295–301.
30. Quinn P, Kerin JF, Warnes GM. Improved pregnancy rate in human in vitro fertilization with the use of a medium based on the composition of human tubal fluid. Fertil Steril. 1985;44:493–8.
31. Gardner DK, Sakkas D. Mouse embryo cleavage, metabolism and viability: role of medium composition. Hum Reprod. 1993;8:288–95.
32. Menke TM, McLaren A. Mouse blastocysts grown in vivo and in vitro: carbon dioxide production and trophoblast outgrowth. J Reprod Fertil. 1970;23: 117–27.
33. Lane M, Gardner DK. Selection of viable mouse blastocysts prior to transfer using a metabolic criterion. Hum Reprod. 1996;11:1975–8.
34. Lane M, Gardner DK. Amino acids and vitamins prevent culture-induced metabolic perturbations and associated loss of viability of mouse blastocysts. Hum Reprod. 1998;13:991–7.
35. Gardner D, Lane M. Towards a single embryo transfer. Reprod Biomed Online. 2003;6:470–81.
36. Gardner DK, Lane M. Ex vivo early embryo development and effects on gene expression and imprinting. Reprod Fertil Dev. 2005;17:361.
37. Lane M, Gardner DK. Understanding cellular disruptions during early embryo development that perturb viability and fetal development. Reprod Fertil Dev. 2005;17:371–8.
38. Gardner DK, Lane M. Culture and selection of viable blastocysts: a feasible proposition for human IVF? Hum Reprod Update. 1997;3:367–82.
39. Renard JP, Menezo Y, Heyman Y. Alternative tests to assess viability of bovine embryos. Theriogenology. 1982;17:106.
40. Gardner DK, Leese HJ. Assessment of embryo viability prior to transfer by the noninvasive measurement of glucose uptake. J Exp Zool. 1987;242:103–5.
41. Gardner DK, Wale PL, Collins R, Lane M. Glucose consumption of single post-compaction human embryos is predictive of embryo sex and live birth outcome. Hum Reprod. 2011;26:1981–6.
42. Conaghan J, Hardy K, Handyside AH, Winston RM, Leese HJ. Selection criteria for human embryo transfer: a comparison of pyruvate uptake and morphology. J Assist Reprod Genet. 1993;10:21–30.
43. Gardner DK, Larman MG, Thouas GA. Sex-related physiology of the preimplantation embryo. Mol Hum Reprod. 2010;16:539–47.
44. Kobayashi S, Isotani A, Mise N, Yamamoto M, Fujihara Y, Kaseda K, et al. Comparison of gene expression in male and female mouse blastocysts revealed imprinting of the X-linked gene, Rhox5/Pem, at preimplantation stages. Curr Biol. 2006;16: 166–72.
45. Bermejo-Alvarez P, Rizos D, Rath D, Lonergan P, Gutierrez-Adan A. Sex determines the expression level of one third of the actively expressed genes in bovine blastocysts. Proc Natl Acad Sci USA. 2010; 107:3394–9.
46. Epstein CJ, Smith S, Travis B, Tucker G. Both X chromosomes function before visible X-chromosome inactivation in female mouse embryos. Nature. 1978; 274:500–3.
47. Haggarty P, Wood M, Ferguson E, Hoad G, Srikantharajah A, Milne E, et al. Fatty acid metabolism in human preimplantation embryos. Hum Reprod. 2006;21:766–73.
48. Dunning KR, Cashman K, Russell DL, Thompson JG, Norman RJ, Robker RL. Beta-oxidation is essential for mouse oocyte developmental competence and early embryo development. Biol Reprod. 2010;83: 909–18.
49. Casslen BG. Free amino acids in human uterine fluid. Possible role of high taurine concentration. J Reprod Med. 1987;32:181–4.
50. Harris SE, Gopichandran N, Picton HM, Leese HJ, Orsi NM. Nutrient concentrations in murine follicular fluid and the female reproductive tract. Theriogenology. 2005;64:992–1006.
51. Crosby IM, Gandolfi F, Moor RM. Control of protein synthesis during early cleavage of sheep embryos. J Reprod Fertil. 1988;82:769–75.
52. Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the mouse preimplantation embryo: amino acids act as buffers of intracellular pH. Hum Reprod. 1998;13:3441–8.
53. Liu Z, Foote RH. Development of bovine embryos in KSOM with added superoxide dismutase and taurine and with five and twenty percent O2. Biol Reprod. 1995;53:786–90.
54. Martin PM, Sutherland AE. Exogenous amino acids regulate trophectoderm differentiation in the mouse blastocyst through an mTOR-dependent pathway. Dev Biol. 2001;240:182–93.
55. Martin PM, Sutherland AE, Van Winkle LJ. Amino acid transport regulates blastocyst implantation. Biol Reprod. 2003;69:1101–8.
56. Lane M, Gardner DK. Differential regulation of mouse embryo development and viability by amino acids. J Reprod Fertil. 1997;109:153–64.
57. Houghton FD, Hawkhead JA, Humpherson PG, Hogg JE, Balen AH, Rutherford AJ, et al. Non-invasive amino acid turnover predicts human embryo developmental capacity. Hum Reprod. 2002;17:999–1005.
58. Brison DR, Houghton FD, Falconer D, Roberts SA, Hawkhead J, Humpherson PG, et al. Identification of viable embryos in IVF by non-invasive measurement

of amino acid turnover. Hum Reprod. 2004;19: 2319–24.
59. Picton HM, Elder K, Houghton FD, Hawkhead JA, Rutherford AJ, Hogg JE, et al. Association between amino acid turnover and chromosome aneuploidy during human preimplantation embryo development in vitro. Mol Hum Reprod. 2010;16:557–69.
60. Sturmey RG, Bermejo-Alvarez P, Gutierrez-Adan A, Rizos D, Leese HJ, Lonergan P. Amino acid metabolism of bovine blastocysts: a biomarker of sex and viability. Mol Reprod Dev. 2010;77:285–96.
61. Gardner DK. Changes in requirements and utilization of nutrients during mammalian preimplantation embryo development and their significance in embryo culture. Theriogenology. 1998;49:83–102.
62. Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the preimplantation mouse embryo: effects of extracellular pH and weak acids. Mol Reprod Dev. 1998;50:434–42.
63. Mastroianni Jr L, Jones R. Oxygen tension within the rabbit fallopian tube. J Reprod Fertil. 1965;147: 99–102.
64. Ross RN, Graves CN. O_2 levels in female rabbit reproductive tract. J Anim Sci. 1974;39:994.
65. Fischer B, Bavister BD. Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. J Reprod Fertil. 1993;99:673–9.
66. Quinn P, Harlow GM. The effect of oxygen on the development of preimplantation mouse embryos in vitro. J Exp Zool. 1978;206:73–80.
67. Harlow GM, Quinn P. Foetal and placenta growth in the mouse after pre-implantation development in vitro under oxygen concentrations of 5 and 20%. Aust J Biol Sci. 1979;32:363–9.
68. Thompson JG, Simpson AC, Pugh PA, Donnelly PE, Tervit HR. Effect of oxygen concentration on in-vitro development of preimplantation sheep and cattle embryos. J Reprod Fertil. 1990;89:573–8.
69. Batt PA, Gardner DK, Cameron AW. Oxygen concentration and protein source affect the development of preimplantation goat embryos in vitro. Reprod Fertil Dev. 1991;3:601–7.
70. Wale PL, Gardner DK. Time-lapse analysis of mouse embryo development in oxygen gradients. Reprod Biomed Online. 2010;21:402–10.
71. Kovacic B, Vlaisavljevic V. Influence of atmospheric versus reduced oxygen concentration on development of human blastocysts in vitro: a prospective study on sibling oocytes. Reprod Biomed Online. 2008;17:229–36.
72. Kovacic B, Sajko MC, Vlaisavljevic V. A prospective, randomized trial on the effect of atmospheric versus reduced oxygen concentration on the outcome of intracytoplasmic sperm injection cycles. Fertil Steril. 2010;94:511–9.
73. Waldenstrom U, Engstrom AB, Hellberg D, Nilsson S. Low-oxygen compared with high-oxygen atmosphere in blastocyst culture, a prospective randomized study. Fertil Steril. 2009;91:2461–5.
74. Meintjes M, Chantilis SJ, Douglas JD, Rodriguez AJ, Guerami AR, Bookout DM, et al. A controlled randomized trial evaluating the effect of lowered incubator oxygen tension on live births in a predominantly blastocyst transfer program. Hum Reprod. 2009;24:300–7.
75. Rinaudo PF, Giritharan G, Talbi S, Dobson AT, Schultz RM. Effects of oxygen tension on gene expression in preimplantation mouse embryos. Fertil Steril. 2006; 86(4 Suppl):1252–65.
76. Katz-Jaffe MG, Linck DW, Schoolcraft WB, Gardner DK. A proteomic analysis of mammalian preimplantation embryonic development. Reproduction. 2005; 130:899–905.
77. Wale PL, Gardner DK. Oxygen regulates amino acid turnover and carbohydrate uptake during the preimplantation period of mouse embryo development. Biol Reprod. 2012;87:24, 1–8.
78. Leese HJ. Quiet please, do not disturb: a hypothesis of embryo metabolism and viability. Bioessays. 2002; 24:845–9.
79. Leese HJ, Baumann CG, Brison DR, McEvoy TG, Sturmey RG. Metabolism of the viable mammalian embryo: quietness revisited. Mol Hum Reprod. 2008; 14:667–72.
80. Ingerslev HJ, Hindkjaer J, Kirkegaard K. Effect of oxygen concentration on human embryo development evaluated by time-lapse monitoring. Hum Reprod. 2012;27:ii22–4.
81. Meseguer M, Tejera A, Herrero J, de los Santos M, Viloria T, Remohi J, et al. Oxygen consumption increases during cytokinesis in implanting human embryos; a time-lapse and microsensor combined analysis. Hum Reprod. 2012;27:ii22–4.
82. Donohoe DR, Bultman SJ. Metaboloepigenetics: interrelationships between energy metabolism and epigenetic control of gene expression. J Cell Physiol. 2012;227:3169–77.
83. Urbanski JP, Johnson MT, Craig DD, Potter DL, Gardner DK, Thorsen T. Noninvasive metabolic profiling using microfluidics for analysis of single preimplantation embryos. Anal Chem. 2008;80:6500–7.
84. Heo YS, Cabrera LM, Bormann CL, Smith GD, Takayama S. Real time culture and analysis of embryo metabolism using a microfluidic device with deformation based actuation. Lab Chip. 2012;12: 2240–6.
85. Lane M, Gardner DK. Lactate regulates pyruvate uptake and metabolism in the preimplantation mouse embryo. Biol Reprod. 2000;62:16–22.
86. Brinster RL. Lactate dehydrogenase activity in the preimplanted mouse embryo. Biochim Biophys Acta. 1965;110:439–41.
87. Auerbach S, Brinster RL. Lactate dehydrogenase isozymes in the early mouse embryo. Exp Cell Res. 1967;46:89–92.
88. Gibbons J, Hewitt E, Gardner DK. Effects of oxygen tension on the establishment and lactate dehydrogenase activity of murine embryonic stem cells. Cloning Stem Cells. 2006;8:117–22.
89. Wakefield SL, Lane M, Mitchell M. Impaired mitochondrial function in the preimplantation embryo perturbs fetal and placental development in the mouse. Biol Reprod. 2011;84:572–80.

90. Mazurek S. Pyruvate kinase type M2: a key regulator of the metabolic budget system in tumor cells. Int J Biochem Cell Biol. 2011;43:969–80.
91. Vander Heiden MG, Lunt SY, Dayton TL, Fiske BP, Israelsen WJ, Mattaini KR, et al. Metabolic pathway alterations that support cell proliferation. Cold Spring Harb Symp Quant Biol. 2011;76: 325–34.
92. Christofk HR, Vander Heiden MG, Harris MH, Ramanathan A, Gerszten RE, Wei R, et al. The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. Nature. 2008;452: 230–3.
93. Redel BK, Brown AN, Spate LD, Whitworth KM, Green JA, Prather RS. Glycolysis in preimplantation development is partially controlled by the Warburg effect. Mol Reprod Dev. 2012;79:262–71.
94. Hardy K, Spanos S. Growth factor expression and function in the human and mouse preimplantation embryo. J Endocrinol. 2002;172:221–36.
95. Hewitson LC, Leese HJ. Energy metabolism of the trophectoderm and inner cell mass of the mouse blastocyst. J Exp Zool. 1993;267:337–43.
96. Abu Dawud R, Schreiber K, Schomburg D, Adjaye J. Human embryonic stem cells and embryonal carcinoma cells have overlapping and distinct metabolic signatures. PLoS One. 2012;7:e39896.
97. Gardner DK, Wale PL. Analysis of metabolism to select viable human embryos for transfer. Fertil Steril. 2013;99:1062–72.