Intermediary Metabolism and Energetics During Murine Early Embryogenesis*

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1The abbreviations used are: TCA, tricarboxylic acid cycle; HK, hexokinase; PFK-1, phosphofructokinase-1; LDH, lactate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; GPI, glucose-6-phosphate isomerase; PDC, pyruvate dehydrogenase complex; E1α, pyruvate dehydrogenase α subunit; dpc, days postcoitum; and E3, dihydrolipoamide dehydrogenase.

Running title: Intermediary Metabolism during Early Embryogenesis
All living cells rely on intermediary metabolism to maintain an adequate state of energetics. Considerable progress has been made over the last century in defining many of the pathways and regulatory mechanisms of intermediary metabolism. Following from this mechanistic understanding, much insight has been gained into how organisms modulate the metabolism of their various cell types to achieve energy homeostasis during different physiologic states and developmental stages. In mammals, the prenatal period is less well characterized in terms of energy metabolism, principally due to technical difficulties associated with the small size of and limited accessibility to samples. The initial stages of prenatal development, when there is rapid growth and striking morphologic changes, stand out as a particularly important period for investigating energy metabolism. Furthermore, the increasing utilization of in vitro fertilization techniques for human and domestic animal reproduction mandates a better understanding of early embryonic metabolism to improve culture conditions. This review will provide an overview of the current understanding of intermediary metabolism associated with energy production in the early murine embryo, the most fully characterized of mammalian embryos. Several approaches that have been used to investigate embryonic metabolism will be considered, with a special emphasis being given to recently introduced mutations affecting pathways implicated in energy homeostasis of the early embryo.

**Substrate Utilization During Early Embryogenesis**

Since the first reports of successful culture of rabbit embryos almost a century ago, much effort has been devoted to defining the substrates required for mammalian embryo viability and then characterizing how these substrates are utilized. Before considering studies investigating the metabolism of substrates in vitro, it is important to appreciate what is known about the
substrates that are available to the embryo in vivo. The embryo begins life with an endowment of maternally provided substrates that are carried over from the oocyte. Unlike a number of vertebrate and invertebrate species, oocytes from many mammalian species, including the mouse, contain relatively limited amounts of glycogen or lipids (1). Mammalian oocytes do, however, have substantial stores of amino acids and protein. It has been estimated that the endogenous protein store within the one-cell mouse embryo could meet the all of its energetic needs for the first several days of development (2). The observation that the murine embryo loses approximately 25% of its total protein content during the first 2-3 days of development indicates that endogenous protein is catabolized during early embryogenesis (3). Beyond the first several days of development, little is known about the relative amounts of endogenous substrates present in the embryo, and no studies have determined how these endogenous substrates are utilized.

Studies of the female reproductive tract fluid in the mouse have identified a number of substrates that could potentially serve as exogenous sources of energy. Miniaturized assays have documented considerable concentrations of glucose, lactate, pyruvate and glutamine (0.65-3.4, 0.37-0.98, 0.07-4.8 and 0.2 mM, respectively), with concentrations varying in different anatomic regions (4). The concentrations of amino acids have not been measured in the murine tract, but studies in the rabbit indicate that there are 1-4 mM concentrations of glutamine, taurine, glycine, threonine and serine present in uterine fluid (5). Concentrations other potential substrates such as lipids and fatty acids have not been determined. Studies in other rodent systems have shown that oxygen tensions within the reproductive tract lumen (30-60 mm Hg) are similar to those seen in a number of tissues (6).
The body of literature investigating substrate utilization by the early embryo in vitro is quite extensive. A brief summary of this work is presented to highlight general trends in the relative utilization of these pathways during early embryogenesis. For more comprehensive treatment of the subject, the reader is referred to previously published reviews by Leese (2) and Gardner (7). To date, studies have principally focused on a relatively small number of substrates, namely glucose, lactate and pyruvate, and their metabolism through the central pathways of energy metabolism (Fig. 1A). The period of development covered in this review extends from conception until gastrulation, which encompasses the first week of development in the mouse and corresponds to the first 2 weeks of human development. During this period of development, the embryo goes through 4 morphologically distinct stages (Fig. 1B). Up to the late blastocyst stage, the embryo exists as a free-living organism that migrates through the female genital tract before implanting into the uterine wall; this entire early period of development is often referred to as the preimplantation period.

At the very beginning of development, the one-cell embryo appears predominantly to utilize oxidative pathways of metabolism as supported by the preferential metabolism of pyruvate over glucose (8). The reliance on aerobic metabolism is felt to represent a continuation of the oocyte’s metabolic pattern, which is also characterized by oxidative metabolism (9). The first two days of development are considered to be associated with a relatively low metabolic rate since there is no increase in size, and there is an actual decrease in biomass of the embryo (Fig. 1B). During this period, glucose uptake increases gradually and then abruptly at the 8-cell stage, when the embryo differentiates to form the tightly compacted structure referred to as the morula (Fig. 2A). The relative rates of glucose and pyruvate uptake, when measured separately, are roughly equivalent at the morula stage, with a significant portion of glucose being
anaerobically metabolized to lactate (10). In concert with this increase in glucose uptake at the morula stage, the embryo, which has been expressing the low-affinity glucose transporter-1 since conception, begins to express the higher affinity glucose transporter-3 (11). One day later, when the embryo begins the process of developing into a blastocyst with the generation of a fluid-filled cavity, glucose uptake has dramatically outpaced pyruvate or lactate uptake, with most of the imported glucose being accounted for by lactate production (10). It has been posited that the switch from aerobic to anaerobic metabolism during the preimplantation period occurs in anticipation of the oxygen-poor environment that the embryo will be exposed to during implantation into the uterine wall (12).

The period of development beyond the blastocyst stage presents considerable challenges to studies of embryonic metabolism in vitro because embryos are no longer free-living and retrieval of embryos is very disruptive to the integrity of the embryo and closely approximated maternal tissues. By day 6, when it is first possible to retrieve postimplantation embryos, the pattern of metabolism shows an even greater rate of glucose uptake, with virtually all exogenously provided glucose being accounted for by lactate production. In addition to the increased glycolytic flux, there appears to be an induction in the potential for oxidative metabolism as demonstrated by an increased ability to metabolize pyruvate (Figs. 1B, 2A). Over the next few days of postimplantation development, the embryo shows a declining ability to convert glucose to lactate. Approximately 77-89% of glucose is converted to lactate by day 9 of development (13).

The in vitro studies of substrate utilization have revealed a series of dramatic changes that occur in intermediary metabolism during early embryogenesis. The embryo appears to switch from oxidative metabolism to glycolytic metabolism during the preimplantation period and then
begins to reinduce oxidative metabolism following implantation. As with all in vitro studies, the conclusions from these studies must be tempered by concerns as to how accurately these results reflect the metabolic processes in vivo. The use of physiologically relevant concentrations of substrates more recently and agreement of results using a number of different culture systems suggest that these studies probably do reflect the general trends of metabolism in vivo (2,7).

Clearly, additional work is needed to address the utilization of endogenous substrates and other potential exogenous substrates. There are substantial data indicating that mammalian embryo viability increases with the addition of amino acids to culture media, but only one study to date has investigated amino acid metabolism, and it was restricted to glutamine (14). In terms of lipid metabolism, there is also need for further investigation since only one study has assessed the metabolism of one fatty acid, palmitate (15). With the development of extremely sensitive techniques employing mass spectrometry or NMR spectroscopy, it should soon be possible to assess more quantitatively the metabolic flux of a large number of substrates in mammalian embryos (16,17).

Regulation of Pathways of Intermediary Metabolism

Studies investigating the regulation of intermediary metabolism during early embryogenesis have thus far mainly focused on determining how the activities of key regulatory enzymes change. Since most in vitro assays are designed to measure the maximal velocities of the enzymes, these studies are useful for assessing regulation that involves enzyme abundance, but are unable to evaluate other mechanisms that may modulate enzymatic activity in vivo, such as allosteric regulation or feedback inhibition. Studies of the oocyte have demonstrated that the embryo begins life with a large number of enzymes, including many involved in the glycolytic,
pentose-phosphate and fatty acid oxidation pathways as well as the tricarboxylic acid (TCA\(^1\)) cycle (18). For a few enzymes, total enzymatic activity levels have been followed throughout the preimplantation period (Fig. 2B). Of the surveyed enzymes, hexokinase (HK) shows the most dramatic change in activity over the preimplantation period. The increase in HK activity coincides temporally with an increase in glycolysis, suggesting that HK may play a role in upregulating glycolysis at the morula stage (Fig. 2A and B). At present, the mechanism(s) responsible for HK upregulation has not been determined. The lack of upregulation of phosphofructokinase-1 (PFK-1) (Fig. 2B) and several other glycolytic enzymes suggests that synthesis of glycolytic enzymes is not coordinately regulated during this stage of development (19).

For a number of enzymes, there appears to be general decline in activity during early embryogenesis. The decrease in the total activity of glycogen synthase may in part explain the general decline in embryonic glycogen content that occurs in the latter stages of preimplantation development. The significance of the 85% and 89% decreases in activity of glucose-6-phosphate dehydrogenase (G6PDH) and lactate dehydrogenase (LDH), respectively, is uncertain since the initial activities of these enzymes are in excess of activities required for the estimated flux through their associated pathways. From the analysis of this small group of enzymes, it appears that intermediary metabolism is not generally regulated during early embryogenesis by affecting abundance of key regulatory enzymes.

In addition to studies of enzymatic activity, several other approaches have provided insight into the regulatory mechanisms of intermediary metabolism in the early murine embryo. Using a NAD\(^+\)-linked enzymatic cycling technique, Oliver Lowry and colleagues assessed a number of intracellular metabolites within preimplantation embryos following starvation and
refeeding with glucose and other substrates (18). The accumulation of several substrates in the glycolytic pathway upon refeeding with glucose suggested that early cleavage stage embryos may have a block at PFK-1, the key regulatory glycolytic enzyme, in addition to an impairment in HK (20). The quantitation of nucleotides within the preimplantation embryo by several groups has revealed another potential regulatory mechanism. Throughout the preimplantation period, it has been shown that the total amount of ATP and the ATP/ADP ratio decrease whereas the ratio of NADH/NAD⁺ remains relatively constant (10,21). Considering that ATP is a potent allosteric inhibitor of PFK-1, the decrease in ATP concentration may play a role in releasing the inhibition of this enzyme during the preimplantation period.

Mutations in Enzymes of Intermediary Metabolism and Their Effects on Early Embryogenesis

At present, a relatively small number of mutations have been identified that affect the previously considered pathways of intermediary metabolism (Table I). The few existing mutations can be explained, in large part, because most mutations introduced into the murine genome have been designed to produce postnatal phenotypes, with the intention of either serving as models for human disorders or allowing classical investigations of metabolism. Considering the many technical barriers to investigating early embryonic metabolism in vitro, recently developed genetic techniques hold tremendous promise for assessing pathways of intermediary metabolism during early embryogenesis in vivo. As with the other previously considered approaches, genetic studies also have their limitations. Caution must always be exercised when phenotypes are interpreted since an introduced mutation may have effects on other unrelated physiologic or metabolic processes. For example, introduced mutations may have secondary
effects arising from accumulation of toxic substrates or deficiencies of essential biosynthetic precursors.

Among several mutations affecting enzymes of the glycolytic pathway, the mutation ablating glucose-6-phosphate isomerase (\textit{Gpi}) is the most extensively studied (Fig. 1A) (22). GPI null embryos die in the early postimplantation period at around 7.5 days post coitum (dpc). Chimeric animals have been created by fusing GPI-null and wild-type embryos. Analysis of chimeric progeny indicates that GPI-null cells are at a selective disadvantage in most tissues during development, but are nonetheless capable of surviving in adult tissues (23). A recent report analyzing progeny from chimeric females has shown that liveborn animals can be produced from GPI-null oocytes (24). The identification of progeny arising from GPI-null oocytes indicates that GPI is not essential for production of a normally functioning oocyte nor is it required for early embryogenesis prior to activation of the genome, which occurs at the 2-cell stage. The survival of GPI-null oocytes and early embryos most likely can be explained by a combination of two factors. Within the ovary, oocytes are connected by gap junctions to surrounding follicle cells which may provide necessary metabolites to the oocyte. Secondly, a block in glycolysis may not be detrimental due to the predominant utilization of oxidative metabolism during these developmental stages. The importance of glycolysis beginning in the postimplantation period is corroborated by the identification of three chemically-induced mutations that affect other glycolytic enzymes (Table I). All of these mutations result in postimplantation lethality, although the interpretation of the \textit{Ldh1} mutation is complicated by the presence of other isozymes in the embryo at this stage (25,26,40). Recently, a report has described introduction of a targeted mutation into the gene encoding G6PDH, the enzyme that catalyzes the first step of
the pentose-phosphate pathway. G6PDH null embryos become delayed in development approximately one day later than that described for the GPI null phenotype (27).

Two mutations have been introduced into nuclear-encoded enzymes that affect mitochondrial oxidative metabolic pathways. Both mutations inactivate pyruvate dehydrogenase complex (PDC), the mitochondrial multienzyme complex that catalyzes the first irreversible step in the commitment of glucose to oxidative metabolism (Fig. 1A). One mutation is PDC-specific, whereas the other additionally impairs three other enzyme complexes (28,29). The PDC-specific mutation allows for conditional inactivation of $Pdha1$, the X-linked gene that codes for the pyruvate dehydrogenase $\alpha$ (E1$\alpha$) subunit of PDC, using the Cre-loxP recombination system. Embryos that predominantly carry the $Pdha1$ null mutation become delayed at 9.5 dpc, which corresponds to the period when gastrulation has been completed and the neural tube is beginning to be formed. The other targeted mutation inactivates the dihydrolipoamide dehydrogenase (E3) component, which is shared among four multienzyme complexes: PDC, $\alpha$-ketoglutarate dehydrogenase complex, branched-chain $\alpha$-ketoacid dehydrogenase complex (Fig.1A) and the glycine cleavage system. Embryos deficient in E3 become developmentally delayed at 7.5 dpc and die within the subsequent 48 hours. One potential explanation for the 2-day difference in survival between E1$\alpha$- and E3-deficient embryos is that the conditional nature of the E1$\alpha$ mutation may not inactivate PDC in all cells of the embryo, thereby allowing these mosaic embryos to survive longer due to residual enzymatic activity. Alternatively or additionally, the E3 mutation may have a more severe phenotype as a result of the impairment of the TCA cycle. The requirement for mitochondrial oxidative metabolism during early embryogenesis has been further supported by several mutations that impair mitochondrial biosynthesis or function as presented in Table I.
Insight into embryonic metabolism has also come from the study of mutations that have no apparent effect on prenatal development. Mice carrying a null mutation in short- or long-chain acyl-CoA dehydrogenase, which are both involved in mitochondrial β-oxidation of particular groups of fatty acids, develop normally throughout the prenatal period (30,31). Several enzymes in the pathway of glycogen degradation have been inactivated and have been shown to have no apparent untoward effects during prenatal development. The absence of effects from mutations in the fatty acid oxidation and glycogenolytic pathways (Table I) can most likely be explained by the lack of a dependence on endogenous stores during prenatal development due to the constant supply of substrates provided by the maternal system.

One of the more interesting observations arising from the group of mutations thus far described is that virtually all mutations do not affect viability during the preimplantation period. There are at least two possible explanations for preimplantation survival: the embryo is able to compensate for deficiencies by using alternative metabolic pathways and/or the mutations have no effect on the earliest stages of development, presumably due to the presence of maternally-encoded proteins. Since the developing oocyte contains both alleles for each autosomal gene until completion of the first meiotic division at the time of ovulation, wild-type enzyme will be present in each oocyte at the time of fertilization regardless of its genotype. At present, there is little direct evidence addressing either possible explanation for preimplantation survival since no studies investigating the metabolism of mutant embryos outlined in Table I have been performed. Some insight into the kinetics of the elimination of maternally-encoded proteins during embryogenesis has come form studies of GPI. Using breeding schemes to distinguish maternally- and embryonically-encoded isozymes, it has been shown that maternal GPI protein (originating from the maternal allele not present in the oocyte) persists to at least 6.5 dpc (32).
Considering the long half-lives of mitochondrial proteins, which are often in the range of several days, the maternal enzyme theory seems even more plausible for mutations affecting oxidative metabolism. The battery of mutations listed in Table I provide a means to assess the kinetics of elimination of a number of maternally encoded enzymes during early embryogenesis.

The mutations affecting glycolysis and the TCA cycle have provided some of the first insight into the metabolic requirements of the postimplantation embryo in vivo. The importance of glycolysis in the postimplantation period was expected in light of the high rates of glycolysis that occur during this stage (Fig. 1B). It has been previously hypothesized that the postimplantation period relies on glycolysis due to the limited availability of oxygen during this period. Histologic analysis has shown that the peri-implantation site is poorly vascularized (33). Recent analysis using pimonidazole, a marker of hypoxia, has demonstrated that many regions of the embryo at 8.5 dpc are hypoxic (34). The phenotype of the G6PDH null embryo is more difficult to interpret because the pentose-phosphate pathway is also utilized for maintenance of the cellular redox state and biosynthetic processes such as nucleotide synthesis.

The phenotypes arising from mutations impairing enzymes involved in mitochondrial metabolism were, on the other hand, less anticipated. The collection of mutations impairing mitochondrial oxidative metabolism provides convincing evidence that oxidative metabolism is required during the early postimplantation period, which has been generally viewed as being heavily, if not solely, reliant on anaerobic metabolism. In retrospect, the importance of oxidative metabolism may not be such a surprise when one considers the rapid growth of the embryo during this stage of development. In certain regions of the early postimplantation embryo, cell cycle times have been shown to be as short as 5 hours (35). Oxidative metabolism, with its roughly 19-fold greater ATP yield from glucose metabolism, may be essential for meeting the
tremendous biosynthetic energy requirements during this developmental period. Furthermore, the TCA cycle may be important for generating biosynthetically important substrates such as oxaloacetate and succinate for pyrimidine synthesis.

Concluding remarks

It has become clear that the embryo undergoes dramatic shifts in intermediary metabolism during its early stages of development as it shifts from oxidative metabolism, to glycolysis and then to utilize both pathways. The early mammalian embryo is an attractive system for determining how central pathways of intermediary metabolism are regulated. It is anticipated that an improved understanding of embryonic metabolism will also improve embryo culture conditions and thereby benefit many of the assisted reproduction technologies. Additionally, a greater understanding of embryonic metabolism may provide new approaches to assessing embryonic viability and screening for inborn errors of metabolism. For example, a recent study reported that higher rates of lactate production in murine embryos cultured in vitro were associated with reduced viability following uterine transfer (36). The size and accessibility of the early mammalian embryo no longer represent such significant impediments to investigating early embryonic metabolism. Highly sensitive biochemical analyses and novel techniques in genetics now provide the means to investigate this technically challenging and incredibly fascinating period of development.
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References


Figure Legends:

**FIG. 1.** Overview of pathways of energy metabolism and their relationship to early embryogenesis. A. Cartoon of pathways of intermediary metabolism. Glycolysis is depicted in pink with enzymes noted in yellow boxes: HK-hexokinase, GPI-glucose 6-phosphate isomerase, PFK-1-phosphofructokinase-1, PGK-phosphoglycerate kinase-1 and LDH-lactate dehydrogenase. The pentose phosphate pathway (PPP) is denoted by the blue arrow and includes the enzyme glucose 6-phosphate dehydrogenase (G6PDH). The mitochondrial metabolic pathways are depicted below the double line that represents the mitochondrial membranes. The orange pathway depicts the TCA cycle. Enzymes shown are PDC-pyruvate dehydrogenase complex, BCKADC- branched-chain α-keto acid dehydrogenase complex, α-KGDC-α-ketoglutarate dehydrogenase complex, and E3-dihydrolipoamide dehydrogenase. The blue box depicts the electron transport chain (ETC). The interconnections with amino acid (AA) and fatty acid (FA) metabolic pathways are also indicated. B. Utilization of metabolic pathways at various stages of development. The colors of the lines represent the pathways shown above. The thickness of lines and percentages represent relative utilization of substrates (adapted from ref. 37). Embryonic age presented as days post coitum (dpc), cell number and depictions of embryonic morphology are presented beneath the pathways.

**FIG. 2.** Substrate utilization and enzyme activity during early embryogenesis. A. Relative utilization of substrates normalized to the one-cell stage values (shown in the parentheses). Conversion of [U-14C]-glucose to 14C-lactate (0.3 pmoles/embryo/hr, blue diamond; oxidation of [U-14C]-glucose to 14CO2 (0.8 pmoles/embryo/hr), pink square; oxidation of [U-14C] pyruvate to
$^{14}$CO$_2$ (6.95 pmoles/embryo/hr), red triangle; and consumption of oxygen (0.157 nl/embryo/hr), green diamond, (38). B. Relative activities of several enzymes normalized to the one-cell embryo values (shown in the parentheses as nmoles/embryo/hr). HK(0.00176); PFK-1(0.0324); LDH (46.2); G6PDH (1.36); glycogen synthase (0.030); and glutamate dehydrogenase(0.043). The values are from ref. (1).
B  One cell  Morula  Blastocyst  Egg cylinder

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Morphology

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