Mitochondrial Malate-Aspartate Shuttle Regulates Mouse Embryo Nutrient Consumption*

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Pyruvate has been considered the sole substrate that can support development of the mouse zygote to the two-cell stage, with lactate able to support development from the two-cell stage. This study has determined for the first time that mitochondrial reducing equivalent shuttles regulate metabolism in the early embryo. Activity of the malate-aspartate shuttle was found to be essential for the metabolism of lactate in the two-cell embryo. Furthermore, the inability of the mouse zygote to use lactate as an energy source was a result of a lack of malate-aspartate shuttle activity. The mRNA for the four enzymes for shuttle activity were detected at all stages of development. It was determined that aspartate was a rate-limiting factor in the activity of the malate-aspartate shuttle in mouse zygotes probably due to the high $K_m$ of the cytoplasmic aspartate aminotransferase. Addition of high concentrations of exogenous aspartate to the culture medium enabled mouse zygotes to utilize lactate in the absence of pyruvate and develop normally to the blastocyst stage as well as produce normal viable offspring. This study determined that the malate-aspartate shuttle is a key regulator of embryo metabolism and therefore viability and is the first report that mouse zygotes can develop normally to term in the absence of pyruvate.

Studies on the preimplantation mouse embryo have indicated that the zygote has an absolute requirement for pyruvate to complete the first cleavage division (1–3). Significantly, lactate is only able to support development from the two-cell stage (4), and glucose could support development from the eight-cell stage (5). Interestingly, mouse zygotes can metabolize both pyruvate and lactate (6, 7) even though cleavage only occurs in the presence of pyruvate. While this has been documented for more than 30 years, there is no explanation for this unusual pattern of metabolism at the zygote nor is it known what changes occur in the embryo that enable the two-cell embryo to use lactate to support development.

Energy metabolism in cells is compartmentalized to either the cytoplasm or the mitochondria. Pyruvate is transported into the mitochondria by specific transport proteins and is metabolized within the mitochondria. In contrast, lactate is converted to pyruvate within the cytosol with the concomitant conversion of NAD$^+$ to NADH. Cells must maintain a balance of metabolic intermediates between the cytosol and the mitochondria to enable lactate metabolism to continue. An inability to maintain this balance results in impairment of either mitochondrial and/or cytoplasmic metabolism. In addition to many enzymes existing in either the cytoplasm or mitochondria, the pyridine nucleotides (NAD$^+$, NADH, NADP$^+$, and NADPH) are also compartmentalized between the cytoplasm and the mitochondria. Intact mitochondria are impermeable to NADH (8). Therefore to transfer NADH across the mitochondrial membrane for oxidation and ATP production and for the regeneration of cytoplasmic NAD$^+$, there is an indirect pathway involving the movement of electrons from NADH, as opposed to the NADH itself, across the mitochondrial membranes. The most common mitochondrial shuttle present in cells is the malate-aspartate shuttle (MAS). This shuttle involves four enzymes mitochondrial aspartate aminotransferase (mAspAT), cytoplasmic aspartate aminotransferase (cAspAT), mitochondrial malate dehydrogenase (mMDH), and cytoplasmic malate dehydrogenase (cMDH) (Fig. 1). The MAS shuttle transfers NADH across the inner mitochondria for subsequent oxidation and also regenerates the NAD$^+$ in the cytoplasm required for further cytoplasmic conversion of lactate to pyruvate or for Embden-Meyerhof pathway activity. In the absence of these shuttles the NAD$^+$ pools within the cytoplasm quickly become exhausted and prevents the cell from using either lactate or glucose as an energy source. The only way cells can regenerate cytoplasmic NAD$^+$ pools in the absence of shuttle activity is by the energetically inefficient conversion of pyruvate to lactate. The presence or contribution of such shuttles to the control of embryo nutrient utilization, metabolism, and viability has not been determined previously.

We therefore hypothesized that control of carbohydrate preferences in the mouse embryo may be controlled by malate-aspartate shuttle activity. The aim of this study was therefore to examine the presence, kinetics, and contribution of the malate-aspartate shuttle to regulation of carbohydrate metabolism and therefore developmental progression in the preimplantation mouse embryo.

MATERIALS AND METHODS

Media—The base medium for embryo collection and cleavage stage embryo culture was a simplified version of G1.2 where the amino acids, EDTA, and pyruvate and glucose were omitted leaving a simple salt solution with lactate as the sole energy substrate (L-simple G1; Table I). For culture of embryos from the eight-cell to the blastocyst, medium G2.2 was used (Table I). All media were prepared monthly and stored at 4 °C. All salts were analar grade and

1 The abbreviations used are: MAS, malate-aspartate shuttle; AspAT, aspartate aminotransferase; mAspAT, mitochondrial AspAT; cAspAT, cytoplasmic AspAT; mMDH, mitochondrial malate dehydrogenase; cMDH, cytoplasmic malate dehydrogenase; MOPS, 4-morpholinepropanesulfonic acid; AOA, amino-oxoacetate; LDH, lactate dehydrogenase.

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purchased from BDH (Dorset, UK). Pyruvate, lactate, amino acids, alanyl-glutamine, MOPS, and amino-oxyacetate (AOA) were purchased from Sigma. Vitamin solutions were obtained from ICN (Aurora, OH). Human serum albumin was obtained from Vitrolife AB (Gothenburg, Sweden). All media components and disposables used for embryo culture were screened for ability to support embryo development with a mouse bioassay before use (9).

Embryo Collection—Mouse embryos for this study were collected from 4-week-old F1 female mice (C57Bl/6 × CBA). Multiple ovulations were induced by an intraperitoneal injection of 5 IU of pregnant mare’s serum gonadotrophin (Sigma) followed 48 h later by 5 IU of human chorionic gonadotrophin (Pregnyl, Organon Inc., West Orange, NJ). After the second injection females were placed with males of the same strain. Zygotes were collected at 21 h of human chorionic gonadotropin administration in simple-MOPS (Table I) and denuded by incubation with hyaluronidase (0.5 mg/ml, Sigma, bovine testes, type IV) for less than 1 min. Zygotes were washed twice in simple-MOPS and once in appropriate culture medium before analysis. Two-cell embryos were collected at 46 h post-human chorionic gonadotropin injection by flushing the oviduct with simple-MOPS.

Embryo Culture—Embryos were cultured in groups of 10 in 20-µl drops of culture medium at 37 °C in 6% CO2:5% O2:89% N2. Zygotes were cultured for 48 h and two-cell embryos for 24 h before transfer to medium G2.2 for a further 48 h to the blastocyst stage.

Analysis of Lactate Uptake—Lactate uptakes were assessed in individual embryos using quantitative microfluorescence (7) assays based on conventional methods of enzymatic analysis employing the pyridine nucleotide NADH in a coupled reaction. For lactate uptakes, individual embryos were placed in a 25-nl drop of medium simple-MOPS containing 0.5 mM lactate as the sole substrate. Embryos were incubated at 37 °C for up to 2 h. Serial 1-nl samples were taken at 15-min intervals and the lactate concentration determined. Linear rates of lactate uptake were determined for individual embryos and expressed as picomoles per embryo per hour.

Oxygen Consumption—Rates of oxygen consumption by embryos were assessed by an non-invasive pyrene method (10, 11). Embryos were incubated in 2 µl of media in a 5-µl polymerase chain reaction micropipette loaded with 1 µl of pyrene (Sigma) dissolved in paraffin oil (BDH) that had been pregressed overnight in a gas phase of 20% oxygen, and each end of the tube was then sealed (10). Oxygen consumption of embryos was determined by measuring the changes in fluorescence of the pyrene over a 5-h period using a fluorescence microscope with photometer attachments (Solamere Technologies, Logan, UT). For each experiment a control of 1 mg/ml yeast that had been incubated overnight in 60 mM glucose for zero oxygen and a 20% oxygen control where the PCR tube was loaded with medium and no embryos were included. The levels of oxygen consumption were then calculated by using a program that relates the linear change in pyrene fluorescence to changes in oxygen levels and models the diffusion of oxygen along the length of medium and pyrene (10, 11). Oxygen consumption was ex-

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**FIG. 1. Malate-aspartate reducing equivalent shuttle.** Schematic of reactions involved in the malate-aspartate shuttle, which transfers an electron across the inner mitochondrial membrane resulting in the net transfer of NADH from the cytoplasm into the mitochondria. The shuttle consists of reactions catalyzed by four enzymes: cAspAT, mAspAT, cMDH, and mMDH.

**TABLE I**

| Component          | Simple MOPS | L-simple G1 | G2.2 |
|--------------------|-------------|-------------|------|
| NaCl               | 90.08       | 90.08       | 90.08|
| KCl                | 5.5         | 5.5         | 5.5  |
| NaH2PO4            | 0.25        | 0.25        | 0.25 |
| MgSO4              | 1.0         | 1.0         | 1.0  |
| NaHCO3             | 5.0         | 25.0        | 25.0 |
| CaCl2              | 1.8         | 1.8         | 1.8  |
| Glucose            | 5.0         | 5.0         | 5.0  |
| Lactate            | 0.1         | 0.1         | 0.1  |
| Pyruvate           | 0.1         | 0.1         | 0.1  |
| Alanine            | 0.6         | 0.6         | 0.6  |
| Arginine           | 0.4         | 0.4         | 0.4  |
| Asparagine         | 0.1         | 0.1         | 0.1  |
| Aspartate          | 0.1         | 0.1         | 0.1  |
| Cystine            | 0.1         | 0.1         | 0.1  |
| Glutamate          | 0.1         | 0.1         | 0.1  |
| Glycine            | 0.1         | 0.1         | 0.1  |
| Histidine          | 0.2         | 0.2         | 0.2  |
| Isoleucine         | 0.4         | 0.4         | 0.4  |
| Leucine            | 0.4         | 0.4         | 0.4  |
| Lysine             | 0.4         | 0.4         | 0.4  |
| Methionine         | 0.1         | 0.1         | 0.1  |
| Phenylalanine      | 0.2         | 0.2         | 0.2  |
| Proline            | 0.1         | 0.1         | 0.1  |
| Serine             | 0.1         | 0.1         | 0.1  |
| Threonine          | 0.4         | 0.4         | 0.4  |
| Tryptophan         | 0.5         | 0.5         | 0.5  |
| Tyrosine           | 0.2         | 0.2         | 0.2  |
| Valine             | 0.2         | 0.2         | 0.2  |
| Calcium pantothenate | 0.0042  | 0.0042     | 0.0042|
| Choline chloride   | 0.0072      | 0.0072      | 0.0072|
| Folic acid         | 0.0023      | 0.0023      | 0.0023|
| Inositol           | 0.010       | 0.010       | 0.010|
| Nicinamidine       | 0.0082      | 0.0082      | 0.0082|
| Pyridoxal          | 0.0049      | 0.0049      | 0.0049|
| Riboflavin         | 0.0003      | 0.0003      | 0.0003|
| Thiamine           | 0.003       | 0.003       | 0.003|
| MOPS               | 20.0        | 20.0        | 20.0 |

a MOPS medium was adjusted to a pH of 7.35.
pressed as nmol of oxygen consumed per embryo per hour.

**Enzyme Extraction**—For whole embryo extraction, individual embryos were washed in saline and placed in 500 nl of enzyme extraction buffer containing 100 mM K2HPO4, 30 mM KF, 1 mM EDTA, 5 mM mercaptoethanol, 2 g/liter bovine serum albumin, and 0.5 g/liter phenylmethylsulfonyl fluoride at pH 7.5 (12). Extraction buffer containing embryos was taken up in a glass capillary tube, sealed in a plastic straw, and plunged into liquid nitrogen. Mitochondrial and cytoplasmic enzyme fractions were separated by a digitonin method as described in Fiskum et al. (13). Individual embryos were incubated in a 500-ml drop of enzyme extraction buffer (12), supplemented with 0.001% digitonin, for 2 min. This low concentration of digitonin permeabilizes the embryos and allows the cytoplasmic enzymes to leak out into the extraction buffer. After 2 min the embryos were removed from the drop and placed in a further 500-ml drop of enzyme extraction buffer to extract the mitochondrial enzymes. Extraction buffer containing the cytoplasmic enzymes and buffer containing the embryos (mitochondrial fractions) were taken up in a glass capillary tube, sealed in a plastic straw, and plunged into liquid nitrogen and stored at −80 °C.

Mitochondrial and cytoplasmic enzyme activities were determined by the digitonin permeabilization method. Mitochondrial enzyme activities were established on individual embryo extracts for cAspAT and mAspAT. For each analysis enzyme activity was determined at five substrate concentrations. Enzyme activities were determined using a Lineweaver-Burk plot (15).

**Analysis of Enzyme mRNA Expression**—The presence of mRNA for the mitochondrial shuttle enzymes was determined by reverse transcription-PCR. RNA was isolated from groups of 50–100 embryos (Qia-gen Inc., Valencia, CA) following the manufacturer’s protocol. RNA was reverse transcribed with oligo(dT) primers and superscript II reverse transcriptase (Invitrogen). For PCR amplification, 2–10 embryo equivalents were added to a PCR buffer of MgCl2, 0.2 mM dNTPs, 1 unit of Taq polymerase, and a 1 μM concentration of primer specific for the gene to be tested (Table II). PCR products were electrophoretically analyzed on a 1.6% agarose gel stained with ethidium bromide and visualized under a UV light. Bands were sequenced to ensure that the correct product was isolated.

**Analysis of AspAT Enzyme K_m and V_max**—Assessment of enzyme activity was determined by quantitative microfluorescence by determining the production or utilization of the pyridine nucleotides in the following reactions.

**REACTION 1**

\[ \text{AspAT} (EC2.6.1.1) \, + \, \alpha\text{-ketoglutarate} \rightarrow \text{oxaloacetate} + \text{glutamate} \]

**REACTION 2**

\[ \text{MDH} \, \text{oxaloacetate} + \text{NADH} \rightarrow \text{malate} + \text{NAD}^+ \]

The assay was performed in a 50 mM Tris buffer at pH 7.7 containing 40 mM aspartate, 2 mM α-ketoglutarate, 80 μM NADH, and 40 units of malate dehydrogenase (14). For each enzyme assay the enzyme extraction buffer was thawed and expelled under oil onto a siliconised glass slide and kept at 4 °C until analysis was completed (around 10 min). This extraction was used to assess enzyme activity (12). The K_m and V_max values were established using a Lineweaver-Burk plot (15).

**Assessment of Viability**—Four morphologically similar blastocysts were transferred to each uterine horn of pseudopregnant recipients on day 4 of pregnancy (day 1 of pregnancy is day of plug). Each treatment was transferred to a different female. Females were allowed to litter, and the number of pups born was assessed.

**Statistical Analysis**—Embryo development was assessed by general linear modeling using the log likelihood statistic. Replicate was fitted as a cofactor. Cell number and metabolic data were initially assessed for normality using the Kolmogorov and Smirnov test. For data sets that were found to be normally distributed between treatment differences, statistical analysis was performed using the Kruskal-Wallis test followed by a Dunn’s test.
RESULTS

Effect of Culturing Embryos with an Inhibitor of the Malate-Aspartate Shuttle—To determine whether the MAS was present in mouse embryos, the effect of culturing two-cell embryos in the presence of AOA, an inhibitor of the malate-aspartate shuttle (16, 17), was determined. Two-cell embryos were cultured in either L-simple G1 medium (containing lactate as the sole energy substrate), or L-simple G1 medium with 0.5 mM AOA (optimal concentration determined by a dose-response experiment; data not shown) with or without 0.32 mM pyruvate. Embryos cultured in the presence of lactate as the sole substrate or in the presence of lactate and pyruvate developed to the blastocyst stage at high rates (Table III). However, the majority of embryos cultured with lactate and AOA arrested development at the two- to four-cell stage. The addition of pyruvate to L-simple G1 medium containing AOA enabled embryos to develop to the blastocyst stage at similar rates to controls (Table III) demonstrating that the concentration of AOA used was not toxic to the embryos (Table III).

Lactate Uptakes and Oxygen Consumption by Mouse Embryos—The presence of a functional malate-aspartate shuttle is indicated by lactate uptake and oxygen consumption that is sensitive to AOA. Mouse zygotes and two-cell embryos were incubated in either L-simple G1 medium containing lactate or L-simple G1 medium containing lactate and AOA (0.5 mM) supplemented with pyruvate. Incubation of two-cell embryos with AOA significantly reduced oxygen consumption (p < 0.05) (Table IV). In contrast, lactate uptake by two-cell embryos was significantly reduced by the presence of AOA (p < 0.001; Fig. 2).

In contrast, lactate uptake by two-cell embryos was significantly reduced by the presence of AOA (p < 0.001; Fig. 2).

Similar to results for lactate uptakes, incubation of two-cell embryos with AOA significantly reduced oxygen consumption (p < 0.01; Table IV). In contrast, incubation of zygotes with AOA did not affect oxygen consumption compared with embryos incubated in the presence of lactate alone (Table IV).

Autofluorescence of Embryos Incubated with AOA—The measurement of autofluorescence of embryos was used as a method to assess changes in cytoplasmic redox state within the embryo. Zygote and two-cell embryos were incubated in a medium with lactate as the sole substrate with or without AOA for 30 min in the respective medium before measurement. Solid bars represent embryos incubated in medium containing lactate. Open bars represent embryos incubated in medium containing lactate and AOA (0.5 mM). Hatched bars represent embryos incubated in medium containing lactate and AOA (0.5 mM) supplemented with pyruvate. Gray bars represent in vivo developed embryos measured immediately after collection. n = a minimum of 20 embryos measured per treatment. *, significantly different from all treatments within stage of development (p < 0.05); **, significantly different from all treatments within stage of development (p < 0.01).

Lactate uptakes and oxygen consumption by mouse embryos—The presence of a functional malate-aspartate shuttle is indicated by lactate uptake and oxygen consumption that is sensitive to AOA.

Lactate uptake by zygotes incubated in the presence of lactate and aspartate and the inhibitor AOA. Lactate uptakes are mean ± S.E. n = 20 embryo extracts examined per stage of development. *, significantly different from embryos incubated in the presence of lactate (p < 0.05).

Oxygen uptakes by zygotes incubated in the presence of lactate and aspartate and the inhibitor AOA. Oxygen consumption is mean ± S.E. n = 5 replicate experiments. *, significantly different from embryos incubated in the presence of lactate (p < 0.05).
in both stages of development. Enzymes mAspAT, cAspAT, mMDH, and cMDH were present in embryos (data not shown) revealed that mRNAs for all four enzymes were measured in zygote and two-cell embryos. The activity and kinetics of the mAspAT and cAspAT were assessed (Fig. 5). The Km for aspartate of cAspAT was 1.59 mM. Similarly, the level of oxygen consumption by zygotes increased in lactate uptake was negated by the addition of 0.5 mM AOA. Similarly, the level of oxygen consumption by zygotes increased in lactate uptake was negated by the addition of 0.5 mM AOA. Similarly, the level of oxygen consumption by zygotes increased in lactate uptake was negated by the addition of 0.5 mM AOA. Similarly, the level of oxygen consumption by zygotes increased in lactate uptake was negated by the addition of 0.5 mM AOA. Similarly, the level of oxygen consumption by zygotes increased in lactate uptake was negated by the addition of 0.5 mM AOA.

Presence of mRNA for Enzymes of Malate-Aspartate Shuttle—Analysis of in vivo developed zygotes (Fig. 4) and two-cell embryos (data not shown) revealed that mRNAs for all four enzymes mAspAT, cAspAT, mMDH, and cMDH were present in both stages of development.

Assessment of AspAT Enzyme Activity and Kinetics—The activity and kinetics of the mAspAT and cAspAT were assessed in zygote and two-cell embryos. The Vmax activity of the cytoplasmic and mitochondrial enzyme was similar in zygote and two-cell embryos (Fig. 5). The Km for aspartate of cAspAT was measured to be 1.67 mM, and the Km for aspartate of mitochondrial AspAT is 2.15 mM in zygote stage embryos. In two-cell embryos the Km for aspartate for cAspAT was 0.78 mM and for mAspAT was 1.59 mM.

Effect of Aspartate Levels on Malate-Aspartate Shuttle Activity—In other cell types, the cytoplasmic levels of aspartate have been suggested to be a rate-limiting factor in the regulation and activity of the MAS (18). To assess whether the aspartate concentration may also be a rate-limiting factor in the activity of the shuttle in embryos, the effect on increasing the levels of aspartate in the culture media on the ability of zygotes to use lactate as the sole energy source was assessed.

Lactate uptake by zygotes was significantly increased by the presence of 10 mM aspartate in the medium (Fig. 6). This increase in lactate uptake was negated by the addition of 0.5 mM AOA. Similarly, the level of oxygen consumption by zygotes was significantly increased by the addition of aspartate to the medium. This increase in oxygen consumption was significantly reduced by adding 0.5 mM AOA to the medium (Fig. 7). In contrast, 78% of embryos cultured with 10 mM aspartate and lactate for the first 48 h developed to blastocyst stage. Culture in a medium containing lactate and aspartate with AOA for the first 48 h reduced the blastocyst development to 21% (of the four-cell or greater embryos) (Fig. 8). Ninety percent of embryos cultured in the control medium (lactate and pyruvate) developed to the blastocyst stage and the addition of AOA did not affect the rate of blastocyst development (Fig. 8).

Analysis of the resultant blastocysts determined that the embryos that were cultured in the presence of lactate and aspartate for the first 48 h had equivalent cell numbers and allocation to the inner cell mass and trophectoderm compared with those cultured in the presence of lactate and pyruvate (Table VI). The addition of AOA to the medium containing lactate and aspartate significantly reduced the cell numbers and trophectoderm cell numbers of the resultant blastocysts (Table VI).

Effect of Aspartate on Embryo Viability—To assess whether the blastocysts cultured in the presence of lactate and aspartate for the first 48 h are viable, blastocysts were transferred to pseudopregnant recipients. A total of 16 blastocysts cultured in the presence of lactate and aspartate for the first 72 h were transferred to two females. A total of 11 live and healthy pups were born to the two females. In comparison, 16 blastocysts cultured in the presence of lactate and pyruvate for the first 48 h resulted in the birth of 12 pups.

| Substrates in media   | One-cell | Two-cell | Four-cell | Eight-cell |
|-----------------------|----------|----------|-----------|------------|
| Lactate               | 51.4a    | 16.2b    | 25.7c     | 6.7d       |
| Aspartate             | 100d     |          |           |            |
| Lactate + aspartate   | 8.7c     | 11.6b    | 13.0c     | 75.4a      |
| Lactate + aspartate + AOA | 64.9a | 13.5b | 8.1d | 13.5b |
| Lactate + pyruvate    | 0c       | 4.2c     | 4.2c      | 91.6d      |
| Lactate + AOA + pyruvate | 0a    | 3.9b     | 6.9c      | 89.1d      |

a–d Different letters within a column are significantly different p < 0.05.
Mitochondrial Shuttles in Embryos

Cell numbers of mouse blastocysts cultured in the presence of different substrates (n = at least 70 embryos cultured per treatment)

| Substrates in medium | Total cell number | ICM cell number | TE cell number | % ICM cells/total cell number |
|----------------------|-------------------|----------------|---------------|-----------------------------|
| Lactate              | 11.5 ± 2.5*a       | 1.5 ± 0.5*a    | 10.0 ± 2.0*b  | 12.7 ± 1.6*a                |
| Lactate + pyruvate   | 78.3 ± 5.6*b      | 13.4 ± 1.2*b  | 64.9 ± 4.7*b  | 17.2 ± 0.9*b                |
| Lactate + aspartate  | 70.1 ± 3.9*b      | 11.8 ± 0.8*b  | 58.3 ± 3.2*b  | 16.9 ± 0.5*b                |
| Lactate + aspartate + AOA | 55.9 ± 6.1*c  | 10.5 ± 1.0*b  | 45.4 ± 5.1*c  | 18.9 ± 0.6*b                |
| Lactate + AOA + pyruvate | 75.5 ± 1.9*c  | 13.9 ± 0.4*c  | 60.5 ± 1.7*b  | 18.0 ± 0.6*b                |

*a,b,c Different letters within a column are significantly different p < 0.05.

DISCUSSION

To the best of our knowledge, this is the first study to report that the preimplantation mouse zygote does not have an absolute requirement for pyruvate in order for cleavage and development to proceed. Rather lactate, in the presence of a sufficient concentration of aspartate, can support the first cleavage division and result in the birth of normal offspring following embryo transfer. The previously reported inability to use lactate as an energy source appears to result from a lack of activity of mitochondrial shuttles, which maintain the balance of pyridine nucleotides between the mitochondria and cytoplasm and enable the continued metabolism of lactate. Without activity of these mitochondrial shuttles, the embryo can metabolize lactate only until the cytoplasmic pool of NAD⁺ within the cell is exhausted by the conversion into pyruvate and NADH. The ratio of NAD⁺:NADH in the embryo is low (1:10), and therefore it is likely that NAD⁺ is exhausted quickly before there is sufficient ATP produced to allow cleavage to occur. Therefore, lactate can only be used as an energy substrate for the mouse embryo when the mitochondrial shuttles are functional. In this study the addition of 10 mM aspartate to the medium enabled zygotes to metabolize lactate sufficiently to support development. Interestingly, at the two-cell stage shuttle activity is evident in all incubation conditions irrespective of the presence of aspartate in the medium thereby enabling lactate to be used as an energy substrate. These observations together suggest that shuttle activity in the mouse two-cell embryo is more robust and can maintain function in a wide variety of environments, while activity of the shuttle in the zygote requires more specialized conditions. This is the first study to determine that mitochondrial reducing shuttles contribute significantly to metabolic control and developmental competence in preimplantation embryos.

The malate-aspartate shuttle is found in the majority of cell types and is an important system for balancing cytoplasmic and mitochondrial metabolism (17, 18, 20–22). In this study, two-cell mouse embryos were found to have significant malate-aspartate shuttle activity as determined by the presence of AOA-sensitive lactate uptake and oxygen consumption. In contrast, mouse zygotes appear to lack AOA-sensitive lactate uptake and oxygen consumption. A lack of activity of the malate-aspartate shuttle in one-cell embryos correlates with previous studies that have determined that lactate cannot be used as an energy source until the two-cell stage (2, 4). In the absence of shuttle activity, a common adaptation by cells is to convert pyruvate to lactate to regenerate the NAD⁺ within the cytoplasm. However, the conversion of pyruvate to lactate is an energetically inefficient pathway (2 ATP) compared with pyruvate oxidation (12 ATP), and therefore there is a significant reduction in the net ATP production within the cell with this adaptation. This adaptation appears to occur in mouse zygotes incubated in the presence of high concentrations of lactate (20 mM). In these conditions the majority of pyruvate taken up by the zygote is converted to lactate presumably to regenerate the NAD⁺ in the cytoplasm and maintain the cellular redox balance (7). In contrast, later stage embryos that have significant shuttle activity oxidize the majority of pyruvate taken up by the embryo to maximize ATP production as the shuttle is able to maintain this metabolic balance (7).

The inability of the mouse zygote to use the malate aspartate shuttle to regulate the balance between cytoplasmic and mitochondrial metabolism was not a result of differences in the presence of the mRNA transcripts for the four enzymes of the shuttle. It has been demonstrated that the activity of another mitochondrial enzyme LDH that converts pyruvate to lactate was regulated by the differential availability of cofactors at different stages of embryo development (7). This therefore led us to the hypothesis that the malate-aspartate shuttle may be similarly controlled by levels of cofactors within the cells. In tumor cells, the activity of the malate-aspartate shuttle is partially dependent on the concentration of aspartate as the Km of aspartate aminotransferase can be as high as 5 mM (18, 23). Examination of the kinetics of AspAT in embryos in this study found that the Km for aspartate cAspAT in zygotes was 1.67 mM. If aspartate was a rate-limiting factor in the activity of the malate-aspartate shuttle then increasing the concentration of cytoplasmic aspartate levels should restore the activity of the shuttle. In this study, elevating the cytoplasmic aspartate concentration by adding 10 mM aspartate into the culture medium did restore MAS activity in zygotes and enabled zygotes to use lactate as the sole energy substrate resulting in normal development. Therefore, in the mouse zygote aspartate does appear to be the limiting factor in the activity of the MAS shuttle. Interestingly, there were no substantial differences in the activity and kinetics of the cytoplasmic AspAT between the zygote and the two-cell embryo implying that the differences in the activity of the shuttle appear to lie in the ability of the embryos to maintain sufficient levels of cofactors, one that appears to be the levels of cytoplasmic aspartate.

Glutamine metabolism can increase the activity of the malate-aspartate shuttle by increasing the concentration of aspartate within the cells (17). Addition of glutamine to culture media for the early embryo significantly improved early embryo development (24–26). Furthermore, most of the beneficial effects of glutamine on the early embryo could be substituted by the addition of non-essential amino acids that contain aspartate (27). Therefore, one of the beneficial effects of glutamine in early medium formulations that lacked aspartate may have been in supplying aspartate for shuttle activity. The role of glutamine in maintaining shuttle activity in the early mouse embryo is currently unknown; however, the observation that glutamine uptake is increased 6.4-fold from the zygote to the two-cell stage (28) suggests that this may be an important role for glutamine and may explain the differences in the ability of the shuttle to function between the zygote and two-cell stage. In support of this is the hypothesis that the addition of 0.4 mM glutamine to the culture medium significantly increases the concentration of intracellular aspartate of cultured mouse blastocysts (19).

2 M. Lane and D. K. Gardner, unpublished observations.
In other cells types, shuttle activity can be modulated by the levels of calcium on the external face of the inner mitochondrial membrane (29). The aspartate/glutamate carrier, which comprises one of the reactions of the MAS, is regulated by calcium levels in the cell. The differences in the activity of the shuttle at the different stages of development may therefore reflect differences in the ability to regulate calcium levels in the embryo. It has been demonstrated previously that hamster one-cell embryos collected from the oviduct soon after egg activation have reduced the ability to regulate intracellular calcium levels compared with later stage embryos (30). It is currently unknown whether calcium levels in embryos are correlated with the differences in malate-aspartate shuttle activity in the mouse.

Therefore, although this study has demonstrated that MAS activity can be detected in the mouse zygote, its activity depends on the environment. Activity can only be maintained when the cytoplasmic levels of aspartate are elevated, which in this study was achieved by the addition of extracellular aspartate. In contrast, from the two-cell stage MAS shuttle activity is detected in all conditions used in this study and is not reliant on extracellular aspartate for activity. What has yet to be elucidated is why the shuttle activity is regulated differently between the zygote and the later stage embryos as the enzymes appear able to be active at all stages of development. This study has demonstrated that the MAS is important in maintaining the balance between mitochondrial and cytoplasmic metabolism and that disruption of this metabolic balance resulted in loss in developmental competence.

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