A medium-chain fatty acid as an alternative energy source in mouse preimplantation development

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To further optimize the culturing of preimplantation embryos, we undertook metabolomic analysis of relevant culture media using capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS). We detected 28 metabolites: 23 embryo-excreted metabolites including 16 amino acids and 5 media-derived metabolites (e.g., octanoate, a medium-chain fatty acid (MCFA)). Due to the lack of information on MCFAs in mammalian preimplantation development, this study examined octanoate as a potential alternative energy source for preimplantation embryo cultures. No embryos survived in culture media lacking FAs, pyruvate, and glucose, but supplementation of octanoate rescued the embryonic development. Immunoblotting showed significant expression of acyl-CoA dehydrogenase and hydroxyacyl-CoA dehydrogenase, important enzymes for β-oxidation of MCFAs, in preimplantation embryo. Furthermore, CE-TOFMS traced [1-13C8] octanoate added to the culture media into intermediate metabolites of the TCA cycle via β-oxidation in mitochondria. These results are the first demonstration that octanoate could provide an efficient alternative energy source throughout preimplantation development.

Improvements in culture media formulations have enhanced our ability to maintain healthy mammalian embryos in culture throughout the preimplantation stages. A recently developed sequential media for human embryos is based on the characteristic switch in energy source preference from pyruvate during the early cleavage stages to glucose during compaction and blastocoel formation1–3. The presence of certain amino acids in the culture media also improves preimplantation development of mammalian embryos4–7, such as glutamine and glycine being important for osmoregulation. Amino acids also provide precursors for protein and nucleotide synthesis, and contribute to metabolic regulation and paracrine signaling in preimplantation embryos8–10. However, the precise combination of amino acids that should be added to the culture media for embryos is still unclear. Furthermore, very few published studies have analyzed the requirements for low-molecular-weight metabolites other than amino acids (e.g., fatty acids) in culture media for preimplantation embryos. On the other hand, recent reports emphasize the renewed focus in the field on in vitro environmental effects on preimplantation-embryo development (e.g., an increased incidence of both monozygotic twins and Beckwith-Wiedemann syndrome)11–13. Therefore, optimization of culture media for preimplantation embryos is an urgent issue. In this context, we sought to analyze the effects of low-molecular-weight metabolites in culture media on the growth of mouse preimplantation embryos, to better understand the metabolic demands of such embryos and inform both the further optimization of culture condition and efforts to identify a potential biomarker for embryo quality.

Omnics technologies, including transcriptomics, proteomics, and metabolomics, have been successfully applied to screen for biomarkers in biological fluids for cancers and autoimmune diseases14. Oligo DNA microarrays with RNA amplification was first employed as an omics technology to demonstrate the dynamics of global gene expression changes in mammalian preimplantation embryos15–17. However, transcriptomic analyses often fail to predict protein abundance or function. Advances in proteomic technologies including mass spectrometry (MS) have enabled groups of proteins within limited amounts of complex biological fluids and tissues to the identified18,19. Specifically, surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) MS was used to
generate protein profiles in culture media at all stages of mammalian embryonic development, demonstrating remarkable profile differences between early and expanded blastocysts and between developing blastocysts and degenerate embryos. These studies also suggested that each preimplantation stage during the fertilized egg to blastocyst development has a distinctive secretome signature.

Metabolomics profiling has the potential to simultaneously determine hundreds of small-molecule metabolites in cells and biological fluids such as culture media, serum, and urine. Nuclear magnetic resonance spectroscopy, gas chromatography–mass spectrometry, and capillary electrophoresis–mass spectrometry (CE-MS) have been often used in metabolomic studies. Each of these technologies detects only a subset of all the metabolites present. Among them, CE-MS is a powerful tool to quantitatively determine small amounts of most ionic intermediate metabolites in the primary metabolic pathways (e.g., carbohydrates, amino acids, nucleotides, and molecules involved in energy metabolism), and thus is a suitable profiling technique to study preimplantation embryos in the context of both hormone and metabolite analysis. Furthermore, CE-MS has successfully identified metabolite biomarkers for several diseases, including cancer and fulminant hepatitis. Therefore, we applied CE-MS to the exploration of metabolic systems in preimplantation embryos in this study, focusing first on a candidate medium-chain fatty acid as an alternative energy source for preimplantation embryo culture.

**Results**

We analyzed 50-μl drops of either culture media from in vitro cultures of 100 mouse embryos during early and late preimplantation stages (the embryo group) or media just incubated without any embryos (the control group) (Fig. 1), and 28 metabolites were detected by CE-TOFMS.

**Embryo-derived metabolites.** Twenty-three metabolites including 15 amino acids were significantly detected in the embryo group, but not in the negative control group (Fig. 2A). These are considered as embryo-derived metabolites generated via excretion from embryos. Comparison between the early- and late-stage embryos revealed that 17 metabolites were differentially excreted. Of these, 2 were excreted more at the early stages: glycine, an osmolarity regulator, and hexanoate (Fig. 2A & Table 1). On the contrary, 15 metabolites (alanine, cis-aconitate, histidine, leucine, malate, methionine, phenylalanine, proline, putrescine, threonin, tryptophan, tyrosine, valine, 2-hydroxy-4-methylpentanoate, and 2-hydroxypentanoate) were excreted in larger amounts in the later stages (Fig. 2A & Table 1).

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**Figure 1** | Strategy for microdrop culture of synchronizing preimplantation embryos and the timing of culture media collection. Embryos were collected from mated superovulated mice at 18 hours post-hCG, and embryos with two pronuclei were discarded. Other embryos were incubated in KSOM and then those with 2PN were selected to synchronize in vitro embryo development (at 24 hours post-hCG). A hundred of zygotes were cultured in 50-μl drops of KSOM under oil at 37°C in an atmosphere of 95% air/5% CO2. The embryos were then transferred into fresh KSOM at 55 hours post-hCG and further cultured until the blastocyst stage. Embryo-free control drops were incubated alongside the embryo-containing drops. Each of the spent media was analyzed by CE-TOFMS.
Culture media-derived metabolites including octanoate, a medium-chain fatty acid. Five metabolites were significantly detected in both the negative control and embryo groups (Fig. 2B & Table 1). These metabolites are considered as culture media-derived metabolites, although only three of these metabolites (glutamine, lactate, and pyruvate) are listed as media components in the manufacturer’s information.
Compared to the negative control group, the embryo group media showed significantly lower concentrations of pyruvate especially at the early stages, suggesting it as an important energy substitute at the cleavage stages. The two metabolites not found in the manufacturer’s media formulation list were 5-oxoproline and octanoate. Octanoate is a medium-chain fatty acid that is thought to be carried into culture media by bovine serum albumin (BSA). Compared to the control group, the embryo group media showed significantly lower concentrations of octanoate throughout the preimplantation stages, and based on this we hypothesized that preimplantation embryos consume octanoate as an alternative energy substitute.

BSA is commonly used as the macromolecular component in media for culturing mammalian embryos, and several analyses of the fatty acid content of BSA\(^{28,29}\) have implicated BSA-binding fatty acids in both mouse and rat embryo development\(^{30}\). However, all these studies analyzed only long-chain fatty acids, with neither medium-chain nor short-chain fatty acids studied. In this study, we therefore focused on the kinetics of medium-chain fatty acids in embryonic development and metabolism.

**Effect of octanoate on preimplantation development.** To study the effect of octanoate on preimplantation development, embryos were cultured in vitro until the blastocyst stage in Potassium Simplex Optimized Medium (KSOM), fatty acid-deficient culture media, or octanoate-supplemented culture media, and counted at 2.5, 4.5, and 5.5 days post-coitum (d.p.c.). Whereas only 42.0 ± 12.6% of the embryos cultured in fatty acid-deficient media reached the 8-cell or morula (8C/Mo) stage at 2.5 d.p.c., 65.6 ± 14.9% of embryos cultured in octanoate-supplemented media reached the 8C/Mo stage (P < 0.05) (Fig. 3A). Similarly, while 54.6 ± 10.3% of embryos cultured in fatty acid-deficient media had reached the blastocyst stage at 3.5 d.p.c., 76.9 ± 10.3% of embryos cultured in octanoate-supplemented culture media were classified as blastocysts at the same period post-coitum (P < 0.01) (Fig. 3B). In contrast, there was no significant difference in the hatching rate between embryos cultured in fatty acid-deficient and octanoate-supplemented culture media (35.6 ± 9.9% and 38.2 ± 5.2%, respectively) (Fig. 3C). These results revealed the important role of fatty acids in vivo culture of preimplantation embryos, and that octanoate alone could almost completely fulfill that role.

To further investigate the effect of octanoate on preimplantation development under energy-depleted conditions, embryos were cultured in vitro until the blastocyst stage in media lacking glucose, pyruvate, and fatty acids (energy-depleted media) or the media supplemented with octanoate during preimplantation development. Few of the starting embryos (9.8 ± 2.9%) cultured in energy-depleted media reached the 8C/Mo stage, whereas 50.4 ± 2.9% of those embryos cultured with octanoate-supplemented media reached the 8C/Mo stage (P < 0.01) (Fig. 3D). Furthermore, only 8.3 ± 4.9% of starting embryos cultured in energy-depleted media reached the blastocyst stage, compared to 60.4 ± 7.4% incubated in octanoate-supplemented media (P < 0.01) (Fig. 3E). No embryos incubated in energy-depleted culture media showed blastocyst hatching, whereas octanoate supplementation allowed a successful rate of blastocyst hatching up to 21.3 ± 4.7% (P < 0.05) (Fig. 3F).

**Expression changes in genes related to β-oxidation during preimplantation development.** Medium-chain fatty acids enter the mitochondrial matrix directly without any help from the carnitine transport system that is necessary for long-chain fatty acids, and are first used as a substrate for medium-chain acyl-CoA synthetases (ACSM) in the matrix β-oxidation system. Such activation of fatty acids by conversion into acyl-CoA thioesters allows their participation in catabolic pathways\(^{31}\). The β-oxidation of fatty acids constitutes a repeated sequence of four reactions catalyzed by acyl-CoA dehydrogenases (ACADVL, ACADL, ACADM, ACADS), 2-enoyl-CoA hydratases (EHs), 3-hydroxy acyl-CoA dehydrogenases\(^{32}\), and 3-keto acyl-CoA thiolases (KATs), each of which comprise 2–4 distinct enzymes with different, but overlapping, chain-length substrate specificities (Fig. 4A). The substrate specificity for ACADVL, ACADL, ACADM, and ACADS ranges from C20 to C12, from C20 to C8, from C12 to C4, and from C6 to C4, respectively\(^{33}\). Hydroxyacyl-CoA, a product of the fatty acid β-oxidation cycle, is catalyzed by the mitochondrial trifunctional protein, which is a heterooctamer multienzyme complex consisting of four α-subunits (hydroxyacyl-CoA dehydrogenase alpha, HADHA) and the β-subunit (hydroxyacyl-CoA dehydrogenase beta, HADHB)\(^{34-37}\). Western blotting analysis using oocytes, 8-cell embryos, and blastocysts showed significant expression of ACADM and HADHA throughout the preimplantation stages including the unfertilized egg stage, whereas ACADL was expressed only at the blastocyst stage, but not at the earlier stages (Fig. 4B). It therefore seems that all stages of preimplantation embryos are well equipped for mitochondrial β-oxidation systems to metabolize octanoate.

### Table 1: Uptake and release of metabolites in culture media during preimplantation development

| Stage     | Metabolites produced by embryos                                                                 | Metabolites consumed by embryos | Metabolites that were not consumed by embryos |
|-----------|-------------------------------------------------------------------------------------------------|---------------------------------|---------------------------------------------|
| Early Stage | Hexanoate*, Isoleusine, Glutamate, Glycine, Citramolate, Tryptophan***, cis-Aconitate***, Thrreonine***, Proline***, Putrescine***, Malate***, Leucine***, Succinate, Phenyllalanine***, Valine***, Ornithine, Methionine*, Tyrosine*, Lysine, Histidine***, 2-Hydroxy-4-methylpentanoate***, 2-Hydroxy nepentanoate**, Alinine*** | Glutamine, Pyruvate*, Octanoate*** | 5-Oxoproline                                          |

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Octanoate and the tricarboxylic acid (TCA) cycle. We hypothesized that octanoate supports preimplantation development by providing ATPs via β-oxidation and the TCA cycle. To test this idea in the present study, we used stable isotope \(^{13}\)C-labeled octanoate as a tracer to follow the TCA cycle activity and establish whether octanoate is truly used as an energy substitute in preimplantation embryos. Embryos were incubated with octanoate (control group) or \([1-^{13}\text{C}]\) octanoate (experimental group) from the 1-cell stage to the blastocyst stage. \(^{13}\)C was detected in intermediate metabolites of the TCA cycle and we compared the isotope ion peaks (M’ + 1) – molecular ion peaks (1/M”) (M’ + 1/M”) ratios between the groups. Interestingly, there were significant differences in M’ + 1/M” ratio between the groups in the metabolite levels of the initial part of the TCA cycle, including citrate, cis-aconitate, fumarate, and malate, which were markedly higher in embryos incubated with \([1-^{13}\text{C}]\) octanoate (Fig. 4C), while three TCA metabolites, iso-citrate, 2-oxoketogluarate and succinyl CoA, were not detected. These findings indicated that octanoate was incorporated into the TCA cycle via β-oxidation and thereby used as an alternative energy source in the mitochondria of preimplantation embryos.
In this study, we used CE-TOFMS to quantitatively determine small amounts of ionic intermediate metabolites in embryo culture media, including 16 amino acids, 2 amino-acid derivatives (putrescine and 5-Oxoproline), and other 10 molecules involved in primary energy pathways after in vitro culture of preimplantation embryos (Table 1).

These 28 metabolites included 2 metabolites differentially excreted at the early stages of development (glycine and hexanoate) and 15 metabolites differentially excreted at the late stages (Fig. 2 and Table 1), indicating that late-stage preimplantation embryos excreted more metabolites into the culture media than early-stage embryos. This could reflect that embryonic metabolism is activated.

**Discussion**

In this study, we used CE-TOFMS to quantitatively determine small amounts of ionic intermediate metabolites in embryo culture media, including 16 amino acids, 2 amino-acid derivatives (putrescine and 5-Oxoproline), and other 10 molecules involved in primary energy pathways after in vitro culture of preimplantation embryos (Table 1).
Figure 4 | The metabolic process of fatty acids and incorporation of label into the TCA cycle from [1-13C8] octanoate. (A) Schematic diagram of fatty-acid metabolic process. Long-chain fatty acids are catalyzed by ACSL1, 3–6 (acy-CoA synthetase long-chain 1, 3–6) and CPT1 (carnitine O-palmitoyltransferase 1). Then, long-chain fatty acids are transferred from the cytosol to the mitochondria by CACT (carnitine acylcarnitine translocase) for subsequent β-oxidation. On the other hand, medium-chain fatty acids enter directly into the mitochondrial matrix, and subsequently acyl-CoAs are synthesized. Acyl-CoA fatty acid products are dehydrogenased by ACADM and ACADL, and the products are subjected to the β-oxidation spiral. (B) Western blotting analysis of ACADM, ACADL, and HADHA in oocytes, 8-cell embryos, and blastocysts (U: unfertilized oocyte, 8: 8-cell embryo, and B: blastocyst). Actin was used as a loading control. An amount of extracted protein corresponding to 100 oocytes or embryos was loaded per lane. The representative result is shown from three independent experiments. (C) Incorporation of 13C label into TCA-cycle metabolites in mitochondria from [1-13C8] octanoate-containing culture media. Box plot showing the amount of metabolites derived from octanoate (shaded boxes) and [1-13C8] octanoate (open boxes) cultures. Error bars represent SD. N.D. means that the metabolite concentration was below the detection limit of the analysis. P-value was calculated by Student’s t-test (*P < 0.05 and **P < 0.01).
by a switch in energy source preference, from pyruvate during the early cleavage stages, to glucose during compaction and blastocoele formation (Baltz & Tartia, 2009; Biggers & Summers, 2008; Lane & Gardner, 2007). The current study also showed that pyruvate was dramatically consumed in culture media at early stages rather than at late stages. Interestingly, Houghton et al. reported a sharp rise in metabolic activity at the morula stage, measured in terms of oxygen consumption in all species studied.

Amino acids provide precursors for protein and nucleotide synthesis and contribute to osmoregulation, metabolic regulation, and paracrine signaling in preimplantation embryos. Glutamine is the amino acid most commonly added to culture media. It is metabolized to glutamate and then to 2-Oxoketoglutarate, which is further oxidized through the TCA cycle to generate ATP, and then excreted as alanine. On the other hand, 16 amino acids were excreted from preimplantation embryos in this study (Fig. 2A). Of these, glycine is an important component of culture media for embryos due to its role in osmoregulation (Baltz & Tartia, 2009), while histidine, an amino acid excreted from porcine and human blastocyst, is known to play a role in signaling to the uterine endometrium where it is decarboxylated into the signaling agent histamine by uterine histidine decarboxylase. However, this latter putative role is hypothetical, and might be species specific, since histidine production has not been detected in cattle embryos. Methionine is also likely to play an important role in metabolic regulation and nucleotide synthesis, particularly in the methylation cycle involving folate and vitamin B12, which leads to subsequent DNA methylation. An amino acid profiling study of human embryos showed that developing embryos produced significantly more methionine than arresting embryos.

In the present study, 10 molecules involved in primary energy pathways were detected in the media after in vitro culture of preimplantation embryos. Out of them, we focused on octanoate, a medium-chain fatty acid. Although the kinetics of medium-chain fatty acids in preimplantation embryos has never been reported, long-chain fatty acids are known to contribute to early embryonic development, and the late stages of preimplantation mouse embryo development are known to involve fatty acid uptake and metabolism in short-term culture experiments. However, long-chain fatty acids need a carnitine transport system to reach the mitochondrial matrix and generate ATP via β-oxidation. Indeed, the inhibition of β-oxidation during zygote cleavage by etomoxir, an inhibitor of carnitine palmitoyltransferase CptIβ, impaired subsequent blastocyst development in mice. Mouse blastocysts incubated with methyl palmitate, another inhibitor of carnitine palmitoyltransferase, significantly reduces lactate production and cell numbers in the trophectoderm and inner cell mass. Using radiolabeled palmitic acid added directly to the media, a highly significant increase in fatty acid oxidation was identified during the morula and blastocyst stages, with CO₂ production rising to 200–300% of the levels observed in earlier stages. All these findings clearly show an important role for long-chain fatty acids as an energy source at the late stages of preimplantation embryonic development. Furthermore, long-chain fatty-acid acyl-CoA dehydrogenases (ACADL)-deficient mice often show spontaneous death and gestational loss, and ACADL-deficient preimplantation embryos show a lower survival ratio to the blastocyst stage than the wild-type controls. The ACADL-null phenotype thus points to the overall importance of mitochondrial oxidation of long-chain fatty acids in embryonic development.

On the other hand, ACADM is responsible for catalyzing the dehydrogenation of medium-chain length (C4–C12) fatty acid thioesters. ACADM-deficient mice show significant neonatal mortality, distinctive from ACADL-deficient mice, with approximately 60% of the ACADM-deficient pups dying prior to weaning at 3 weeks of age. It is likely that neonatal ACADM-deficient pups are manifesting sensitivity to fasting with decompensation in a short period of time if maternal milk is not ingested. Although ACADM-deficient embryos may have little problem in vivo, they may show suppressed preimplantation development during in vitro culture, which may not provide such an ideal condition as in vivo and fail to provide adequate and balanced energy substitutes. We demonstrated herein that preimplantation embryos consumed octanoate throughout the preimplantation stages and traced [1,13C₆] octanoate to intermediate metabolites of the TCA cycle via β-oxidation in mitochondria. Furthermore, western blotting analysis indicated the protein expression of ACADM and HADHA throughout the preimplantation stages from oocyte to blastocyst (Fig. 4B), whereas ACADL, which catalyzes long-chain fatty acids, was not expressed in oocytes or 8-cell embryos. These findings together suggested that medium-chain fatty acids are useful for all the stages of preimplantation embryos as an energy substitute for β-oxidation in mitochondria.

Interestingly, Berger et al. investigated if preimplantation development of ACADL-deficient mice could be rescued in culture by supplying excess medium-chain, long-chain, or very-long-chain fatty acids. However, they failed to demonstrate any rescue by supplementing the culture media with fatty acids of a wide-range of chain-lengths. This result is not consistent with our results. Their failure in the rescue of ACADL-deficient preimplantation embryos by octanoate may be caused by the unfavorable culture conditions used, which grows only 40–60% of wild-type embryos (as positive controls with fatty-acid-binding BSA) to the blastocyst stage. On the contrary, 97.6% of embryos (with fatty-acid-binding BSA) developed to the blastocyst stage in our study; 54.6% of embryos reached the blastocyst stage with all the fatty acids eliminated from culture; and supplementation of octanoate significantly recovered the developmental rate to the blastocyst stage up to 76.9%. This suggests that preimplantation embryos can consume octanoate as an alternative energy substitute.

In conclusion, all these results support our hypothesis that octanoate is an efficient alternative energy substitute throughout preimplantation embryo development, by directly entering the mitochondrial matrix without a chaperone transport system. Although follicular fluid, the physiological environment in which oocytes mature in vivo, contains both lipoproteins and free fatty acids, current commercial defined media formulations used for oocyte and embryo culture do not intentionally include any fatty acid substrates. Because the rate of human embryos reaching the blastocyst stage in vitro is not yet satisfactory compared to that of other animals such as mouse and bovine, in vitro culture conditions for human preimplantation embryos need further optimization. The epigenetic adverse effect on preimplantation-embryo development (e.g., an increase in monozygotic twinning and Beckwith-Wiedemann syndrome) are the subject of recent studies and are likely to be attributed to the in vitro environment. Therefore, optimization of culture media for preimplantation embryos is an urgent issue. Because fasting or shortness of the existing energy substitutes may occur to in-vitro-cultured embryos under such inadequately optimized condition of human preimplantation embryos without fatty acids, the inclusion of medium-chain fatty acids in culture media formulations may be effective for the optimization of chemically defined culture media and for improvements in the quality and safety of human preimplantation embryos.

**Methods**

Collection and manipulation of embryos and culture media. Six- to eight-week-old B6D2F1 mice were superovulated by injecting 5 IU of pregnant-mare serum gonadotropin (PMSG; Calbiochem, La Jolla, CA) followed by 5 IU of human chorionic gonadotropin (hCG; Calbiochem) 48 h later according to a standard published protocol. After hCG injection the females were placed with fertile males of the same strain, and checked the following morning for copulation plugs. Embryos were collected from mated superovulated mice at 18 hours post-hCG injection, and embryos with two pronuclei were selected to synchronize the collection and manipulation of embryos and culture media. Methods not intentionally include any fatty acid substrates. Because the rate of human embryos reaching the blastocyst stage in vitro is not yet satisfactory compared to that of other animals such as mouse and bovine, in vitro culture conditions for human preimplantation embryos need further optimization. The epigenetic adverse effect on preimplantation-embryo development (e.g., an increase in monozygotic twinning and Beckwith-Wiedemann syndrome) are the subject of recent studies and are likely to be attributed to the in vitro environment. Therefore, optimization of culture media for preimplantation embryos is an urgent issue. Because fasting or shortness of the existing energy substitutes may occur to in-vitro-cultured embryos under such inadequately optimized condition of human preimplantation embryos without fatty acids, the inclusion of medium-chain fatty acids in culture media formulations may be effective for the optimization of chemically defined culture media and for improvements in the quality and safety of human preimplantation embryos.
(1) Culture media and reagents. Prior to being used for research, 100 yzotes were cultured in 50-μl drops of KSOM under oil. The embryos were then transferred into fresh culture media at 55 hours post-hCG and cultured until the blastocyst stage at 120 hours post-hCG (Fig. 1). Embryo-free control drops were incubated alongside the embryo-containing drops to allow for any non-specific amino acid degradation/appearance. Culture media were immediately frozen and stored at −80°C until metabolite extraction.

(2) To investigate the effect of octanoate during preimplantation development, embryos were cultured in KSOM, fatty acid-deficient culture media, octanoate-supplemented fatty acid-deficient culture media, energy-depleted culture media, or octanoate-supplemented energy-depleted culture media until 5.5 days postcoitum (d.p.c.). Embryonic morphology was assessed daily and the embryos were cultured differently as follows: on 2.5 d.p.c., embryos at the 8-cell to morula stage, on 4.5 d.p.c., blastocyst stage, and on 5.5 d.p.c., hatching blastocyst. The rate of development was assessed daily as the percentage of embryos meeting the development criteria from the starting number of embryos.

All experiments were conducted in accordance with the Laboratory Animal Care and Use Committee of Keio University School of Medicine, and the protocol was formally approved (Permit number #10240-1838).

14C Tracer Studies in preimplantation embryos. Collection of embryos cultured in octanoate- or [1-13C8] octanoate-supplemented and fatty acid-deficient culture media. Fertilized eggs were cultured in octanoate- or [1-13C8] octanoate (Iwai Chemicals Corporation, Tokyo, Japan)-supplemented fatty acid-deficient culture media at 37°C in an atmosphere of 95% air/5% CO2. The embryos were cultured until the blastocyst stage at 120 hours post-hCG. Embryos were thoroughly washed, and then immediately frozen and stored at −80°C until metabolite extraction.

Metabolite extraction of embryos for CE-TOFMS. To extract metabolites, preweighed deep-frozen samples from 100–300 embryos were left at room temperature to thaw. A deep-frozen sample from 100–300 embryos was left at room temperature to thaw. A small volume of culture media was added to the sample to bring the volume to 2 ml. The sample was then frozen at −80°C until ready for analysis.

CE-TOFMS conditions for cationic metabolite analyses. Cationic metabolites were separated in a commercially available COSMO(+) capillary (50 μm i.d. × 100 cm total length, Nacalai Tesque, Kyoto, Japan), which was chemically coated with a cationic polymeric, and filled with 50 mmol/l ammonium acetate solution (pH 8.5) as the reference electrolyte. Sample solution was injected at 5 kPa for 30 s (approximately 30 nl) and a negative voltage of 30 kV was applied. Ammonium acetate (boiled for 30 min) in 50% methanol/water mixture (v/v) containing 1 μmol/l reserpine was delivered as the sheath liquid at 10 μl/min. ESI-TOFMS was conducted in negative ion mode and the capillary voltage was set at 3,500 V. The fragmentor, skimmer, and Oct RF voltages in TOFMS were set at 100, 50, and 200 V, respectively. An automatic recalibration function was performed using the following masses of two reference standards: [13C isotopic ion of the deprotonated acetic acid dimer (2CH3COOH-H)+], m/z 120.03834; and [deprotonated reserpine (M-H)-], m/z 607.26611. Mass spectra were acquired at a rate of 1.5 cycles per second from m/z 50 to 1,000. All other conditions were the same as described above for cationic metabolite analysis.

CE-TOFMS data processing. The raw data were processed using our proprietary software (MasterHands)12. The overall data processing flow consisted of noise filtering, baseline correction, peak detection, and integration of the peak areas from the starting number of embryos.

Immunoblot analysis. Protein samples from embryos were solubilized in Sample Buffer Solution without 2-ME (Nacalai Tesque, Kyoto, Japan), resolved by NuPAGE Novex on Tris-acetate mini gels (Invitrogen), and transferred to Immobilon-P transfer membrane (Millipore). The membrane was soaked in protein blocking solution (Blocking One solution, Nacalai) for 30 min at room temperature before an overnight incubation at 4°C with primary antibody, and then washed three times with TBST. The signal was detected by enhanced chemiluminescence (SuperSignal West Dura Extended Duration Substrate, Thermoscientific, Rockford, IL, USA) following the manufacturer’s recommendations.

Statistical analysis. The data was analyzed by Student’s t-test; differences with a P value < 0.05 were considered significant.

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