Rewiring of embryonic glucose metabolism via suppression of PFK-1 and aldolase during mouse chorioallantoic branching

Hidenobu Miyazawa1, Yoshifumi Yamaguchi1,2,*, Yuki Sugiuira2,3, Kurara Honda3, Koki Kondo1, Fumio Matsuda4, Takehiro Yamamoto3, Makoto Suematsu3 and Masayuki Miura1,5,*

ABSTRACT
Adapting the energy metabolism state to changing bioenergetic demands is essential for mammalian development accompanying massive cell proliferation and cell differentiation. However, it remains unclear how developing embryos meet the changing bioenergetic demands during the chorioallantoic branching (CB) stage, when the maternal-fetal exchange of gases and nutrients is promoted. In this study, using metabolome analysis with mass-labeled glucose, we found that developing embryos redirected glucose carbon flow into the pentose phosphate pathway via suppression of the key glycolytic enzymes PFK-1 and aldolase during CB. Concomitantly, embryos exhibited an increase in lactate pool size and in the fractional contribution of glyceraldehyde 3-phosphate pathway via suppression of the key glycolytic enzyme PFK-1 and aldolase during mouse chorioallantoic branching (CB). Perturbation of glucose metabolism rewiring by suppressing Lin28a downregulation resulted in perinatal lethality. Thus, our work demonstrates that developing embryos rewire glucose metabolism following CB for normal development.

KEY WORDS: Energy metabolism, Chorioallantoic branching, Lin28a, Phosphofructokinase-1, Mouse, Imaging mass spectrometry

INTRODUCTION
Understanding how cellular metabolism is coordinated with various biological processes at the tissue or organismal level has been a challenge for modern biology (Boroughs and DeBerardinis, 2015; Pavlova and Thompson, 2016; Vander Heiden et al., 2009). Recent metabolic research in the cancer and stem cell fields has highlighted the importance of cellular metabolism in dictating cell proliferation and cell differentiation; actively dividing cells favor glycolysis for efficient biomass production, whereas terminally differentiated cells mainly rely on oxidative phosphorylation (OXPHOS) for efficient energy production (Agathocleous and Harris, 2013; Christofk et al., 2008; Folmes et al., 2011; Vander Heiden et al., 2009).

This also seems to be the case during development, when cell proliferation and cell differentiation occur concurrently (Agathocleous et al., 2012; Tennessen et al., 2011). In flies, for example, aerobic glycolysis is activated during larval development for efficient biomass production, thereby achieving a dramatic increase in body mass (Tennessen et al., 2011). In mammals, it is proposed that the center of embryonic energy metabolism shifts from glycolysis to OXPHOS during the transition from gastrulation to the neurulation stage [embryonic day (E)6-9 in mice] (Clough, 1985; Clough and Whittingham, 1983). These stages following gastrulation are accompanied by alterations in the intrauterine environment of embryos, namely, the establishment of vitelline circulation and, subsequently, chorioallantoic branching (CB) (Arora and Papaioannou, 2012; McGrath et al., 2003; Rossant and Cross, 2001; Zohn and Sarkar, 2010). CB facilitates the uptake/circulation of gases and nutrients between the mother and the fetus and impacts the growth and development of the whole embryo (Luo et al., 1997). These changes require precise coordination between embryonic energy metabolism and the maternal environment. Embryos harboring mutations in key regulators of energy metabolic pathways exhibit defects around these stages of development (Bamforth et al., 2001; Davis et al., 1993; Iyer et al., 1998; Larsson et al., 1998).

Although studies in the late 20th century proposed that glycolysis decreases and OXPHOS increases following mammalian CB, this widely accepted notion should be reconsidered owing to the technical limitations that existed during that era for studying cellular energy metabolism within developing embryos. Those studies measured only lactate and CO2, which were secreted from cultured embryos (Clough and Whittingham, 1983; Tanimura and Shepard, 1970), and therefore might not capture the complete picture of metabolic change in vivo. Furthermore, a simple shift from glycolysis to OXPHOS does not explain how mammalian embryos adapt their energy metabolism state to increasing metabolic demands for biomass production and for energy production in order to support cell proliferation, cell differentiation, and morphogenesis during the neurulation stage.

In this study, we conducted a global metabolome analysis of intracellular metabolites by taking advantage of recent technical advances in mass spectrometry, including imaging mass spectrometry. These state of the art techniques revealed a novel perspective on the rewiring of embryonic glucose metabolism during mouse CB. Furthermore, we demonstrated that downregulation of the heterochronic gene Lin28a could be a component of the developmental programs mediating metabolic rewiring during this stage of development.
RESULTS

Transition of embryonic metabolome profiles during CB

To reveal how the embryonic metabolome profiles change during CB (E8.5 to E10.5 in mice) (Rossant and Cross, 2001), we performed a metabolome analysis of embryos at E8.5 [somite stage (ss) 10-12], E9.5 (ss 24-26) and E10.5 (ss 35-37) using capillary electrophoresis-mass spectrometry (CE-MS) (Soga et al., 2006). This allowed us to construct the first comprehensive metabolome of CB stage embryos.

We detected 219 intracellular metabolites, and discovered that the embryonic metabolome profiles clearly changed from E8.5 to E10.5 (Fig. S1A, Table S1). A supervised statistical analysis by partial least-square discriminant analysis (PLS-DA) revealed a statistically significant difference between the developmental stages (Fig. S1A). Whereas PLS2 discriminated the difference between E8.5/E10.5 and E9.5 embryos, PLS1 clearly discriminated the difference between E8.5, E9.5 and E10.5 embryos. Metabolites involved in glycolysis and the tricarboxylic acid (TCA) cycle ranked highly among the metabolites that contributed the most to the separation within the PLS1 analysis and whose amounts changed significantly between these stages (Fig. S1B).

Increased pool size of lactate and TCA cycle metabolites following CB

Although glucose metabolism is known to be crucial for embryonic development during CB (Hunter and Tugman, 1995; Yang et al., 2013), it remains to be fully understood how embryonic glucose metabolism changes at this stage. Our metabolome data showed that intracellular metabolites in the TCA cycle increased significantly from E8.5 to E10.5 (Fig. 1). The increase in TCA cycle metabolite abundance was accompanied by increased expression of mitochondrial electron transport chain (ETC) genes from E8.5 to E9.5 (Fig. 2A). This increased gene expression was not due to the increased number of mitochondria (Fig. 2B). Consistent with the results of previous studies, these results imply that developing embryos prime the ETC for efficient ATP production through OXPHOS during the CB stage (Mackler et al., 1971; Shepard et al., 1998). Interestingly, although previous studies suggested that glycolysis is decreased at this stage (Clough and Whittingham, 1983), the amount of intracellular lactate, which is the end product of glycolysis, also increased significantly from E8.5 to E10.5 (Fig. 1).

Fig. 1. Metabolome analysis during chorioallantoic branching. Intracellular metabolite levels were quantified by CE-MS analysis and normalized to the total amount of protein extracted from the embryos. Relative amount refers to the amount of intracellular metabolites in the TCA cycle and glycolysis relative to E8.5 embryos. Data are represented as mean±s.d. n=3 independent sample replicates. *P<0.05, **P<0.001, versus E8.5 embryos (two-tailed Welch’s t-test). E8.5, somite stage (ss) 10-12; E9.5, ss 24-26; E10.5, ss 35-37. DHAP, dihydroxyacetone phosphate; GA-3-P, glyceraldehyde 3-phosphate; PGI, phosphoglucose isomerase; PFK-1, phosphofructokinase-1; TPI, triose phosphate isomerase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGAM, phosphoglycerate mutase; PK, pyruvate kinase; LDH, lactate dehydrogenase.
Tissue distribution of glycolytic and TCA cycle intermediates within embryos revealed by imaging mass spectrometry

We next explored which embryonic structures contribute to the increased lactate level during CB. We investigated the distribution of lactate within E9.5 embryos using matrix-assisted laser desorption/ionization-imaging mass spectrometry (MALDI-IMS), a useful method for visualizing the spatial distribution of individual biomolecules at a spatial resolution of ∼30 µm pitch on intact tissues (Bailey et al., 2015; Hamilton et al., 2015; Sugiuira et al., 2014). Fresh frozen embryos were sectioned and analyzed by MALDI-IMS. Whereas hexose phosphate (glucose 1-phosphate, glucose 6-phosphate and fructose 6-phosphate) and glutamate (an abundant amino acid in the uterus) tended to be distributed evenly, higher levels of lactate and citrate were observed within specific tissues, including the dorsal or posterior neural tube, somites and head mesenchyme, as compared with such as the extraembryonic tissues (Fig. 3). Such decreases may increase the intracellular pool of PPP metabolites following CB.

Increased contribution of glycolysis to lactate production during CB

Although the results of the above analyses represent snapshots of metabolome profiles, the observed changes in metabolite abundance do not necessarily reflect the flow of each metabolic pathway. To understand changes in the carbon flow of glycolysis and the TCA cycle during development, we traced and compared the metabolic fate of glucose using a fully labeled form (13C6-glucose) in CB stage embryos in vivo. 13C6-glucose was administered to pregnant mice along the rostral-to-caudal axis of the embryo.

Increased pool size of the pentose phosphate pathway (PPP) and the biosynthetic pathway during CB

We next aimed to assess how suppression of PFK-1/aldolase affects the pool sizes of other metabolic pathways branching from the glycolytic pathway. Suppression of PFK-1/aldolase reactions can result in glucose being directed into the PPP, which is involved in biomass production and maintenance of the cellular redox balance (Patra and Hay, 2014; Yamanoto et al., 2014). Since the pool size of PPP metabolites is small compared with those of glycolysis and the TCA cycle, we utilized ion chromatography connected to a Fourier transform orbitrap mass analyzer (IC-MS) (Hu et al., 2015). The amount of PPP metabolites, including 6-phosphogluconate (6-PG), ribose 5-phosphate (R-5-P) and sedoheptulose 7-phosphate (S-7-P), increased significantly from E8.5 to E9.5 (Fig. 6A). These results suggest that suppression of PFK-1/aldolase may increase the intracellular pool of PPP metabolites following CB.
We also found that the abundance of glycerol 3-phosphate (G-3-P) and glycine increased significantly from E8.5 to E10.5 (Fig. 6B,C). G-3-P and glycine are synthesized from the glycolytic metabolites dihydroxyacetone phosphate (DHAP) and 3-phosphoglycerate (3-PG), respectively. G-3-P is the starting material for glycerolipids, including the mitochondria-specific phospholipid cardiolipin (Houtkooper and Vaz, 2008). Therefore, the reduced amount of glycolytic intermediates (DHAP and 3-PG; Fig. 1) might be a result of redirection from the glycolytic pathway to the biosynthetic pathway, producing G-3-P and glycine.

**Oxygen conditions play a key role in glucose-derived carbon entry into the TCA cycle**

CB is thought to promote maternal oxygen supply to embryos. To examine whether oxygen conditions affect the metabolic rewiring observed above, we performed labeling experiments with $^{13}$C$_6$-glucose in whole-embryo culture (WEC). In WEC, E8.5 embryos require low oxygen conditions (5% O$_2$ is optimal) for normal development, whereas at E9.5 or later the embryos require high oxygen conditions (60% O$_2$ or higher is optimal) (Cockroft, 1990).

Accordingly, we cultivated embryos under optimal oxygen conditions as well as under higher (20% for E8.5) or lower (5% for E9.5 and E10.5) oxygen conditions. FC(lactate) and the lactate pool size remained relatively constant between different oxygen conditions at all of the stages of development examined (Fig. 7, Fig. S4A,C). This suggests that glucose carbon flow into lactate is independent of oxygen concentrations. However, FC(citrate) and the citrate pool size were increased under higher oxygen conditions (E8.5, 20% O$_2$; E9.5/E10.5, 60% O$_2$; Fig. 7; Fig. S4B,D). These results suggest that CB stage embryos have the capacity to promote the entry of glucose-derived carbon into the TCA cycle depending on the oxygen supply.

**Lin28a as a possible intrinsic timing determinant of glucose metabolism rewiring**

The gradual decrease of Aldoa expression along the body axis raises the possibility that embryonic glucose metabolism rewiring is intrinsically regulated by the developmental program. We therefore focused on the heterochronic gene Lin28a, which regulates various cellular processes including embryonic stem cell self-renewal and
glucose metabolism (Shyh-Chang and Daley, 2013; Shyh-Chang et al., 2013; Zhang et al., 2016). Lin28a mRNA is reported to be expressed throughout the embryo at E8.5, but gradually becomes restricted to the posterior part of embryos as development proceeds (Balzer et al., 2010), resembling the dynamic expression pattern of Aldoa during CB (Fig. S3). We confirmed that Lin28a mRNA expression dramatically decreases across the whole embryo from E8.5 to E10.5, whereas the expression of mature let-7 microRNAs (miRNAs), which are negatively regulated by Lin28a, dramatically increased (Fig. 8A,B).

To examine whether the developmentally regulated expression of Lin28a is involved in the rewiring of embryonic glucose metabolism during CB, we used a conditional transgenic mouse line (tetO-Lin28a:rtTA) that overexpresses Lin28a in a doxycycline (Dox)-dependent manner (Fig. S5) (Zhu et al., 2010). Lin28a downregulation was prevented by overexpressing Lin28a via Dox treatment at E8.5 and E9.5. This resulted in a significant increase in some glycolytic intermediates downstream of the PFK-1 reaction (3-PG, phosphoenolpyruvate and pyruvate) in E10.5 embryos compared with non-treated embryos (Fig. 8C). The amount of PPP metabolites did not differ between tetO-Lin28a:rtTA E10.5 embryos with or without Dox treatment (Fig. S6). These observations suggest that the rewiring of embryonic glucose metabolism during CB is partly regulated by Lin28a. Furthermore, we found that perturbation of glucose metabolism rewiring by Lin28a overexpression during CB led to perinatal lethality at postnatal day (P)0 without obvious morphological defects (Fig. 8D).

Taken together, these results suggest that Lin28a is involved in the regulation of the timing of glucose metabolism rewiring following CB, which is essential for postnatal survival.

DISCUSSION

Our global analysis of intracellular metabolites using metabolomics with mass-labeled glucose revealed that embryos have increased pools of lactate and citrate during CB, accompanying the increased fractional contribution of glycolysis to lactate biosynthesis. Furthermore, our data suggest that glucose carbon flow is redirected into the PPP via suppression of PFK-1 and aldolase during this stage of development (Fig. 9). These notions advance the classical view that embryos simply suppress glycolysis while accelerating the TCA cycle following CB. The observed increase in FC(lactate) can be explained by: (1) increased glycolysis, which is expected to result in increased lactate secretion; or (2) decreased lactate production from carbon sources other than glucose. Previous studies proposing decreased glycolysis relied on evidence that cultured embryos had reduced lactate secretion as development proceeded, a result that we reproduced (Fig. S7). However, measurement of lactate secretion alone is not indicative of glycolytic activity within embryos. The amount of secreted lactate can be strongly influenced by the surface area to volume ratio of the embryo, which decreases as the embryo enlarges substantially during CB. Additionally, the rate of lactate secretion and/or uptake may differ between distinct cell types. Consistent with this idea, our IMS analysis revealed an uneven distribution of lactate within embryos. Thus, future studies will address how glycolytic activity is coordinated with developmental progression at the tissue or cellular level.

Interestingly, the reduction of glycolytic intermediates precedes the changes in PFK-1/aldolase expression at E9.5. The enzymatic activity of aldolase first decreases from E8.5 to E9.5, but that of PFK-1 is suppressed only from E9.5 to E10.5. Suppression of both enzymatic activities at E10.5 could be attributed to the decreased expression of their genes, but the mechanisms of glycolytic flow suppression at E9.5 remain to be elucidated. PFK-1 activity is regulated by many allosteric inhibitors and activators, and thus allosteric regulations of PFK-1 could be involved in the glucose
metabolism rewiring at E9.5. Citrate levels, a potent allosteric
inhibitor of PFK-1, increased ∼2-fold from E8.5 to E9.5, suggesting
an involvement in glucose metabolism rewiring. It is interesting to
note that the expression of Pfkm mRNA, which is more sensitive to
allosteric inhibition by citrate than Pfkl (Staal et al., 1987), increased
at this stage (Fig. 5B). Although the enzymatic activity assay using
cell lysates reflects not only the amount of enzymes but also, to
some extent, the effects of endogenous metabolites (Yamamoto
et al., 2014), such allosteric regulation of PFK-1 is presumably
difficult to detect in the presence of excessive amounts of ATP,
which is a substrate as well as an allosteric inhibitor of PFK-1. In
addition, it should be noted that the enzymatic assay disrupts
cellular structure and therefore does not detect the regulation of
enzymatic activity according to cellular localization. This is
important because some glycolytic enzymes, including Aldo, are
localized to specific cellular compartments in addition to the
cytoplasm (Hu et al., 2016; Mamczur et al., 2013), and therefore
regulation of the cellular localization of glycolytic enzymes could
be one of the mechanisms that regulate glucose metabolism rewiring
during CB.

Fig. 6. The increased pool size of the pentose phosphate pathway (PPP) during CB. (A) PPP metabolites were quantified by IC-MS. Area values were
normalized to the amount of protein extracted from the embryos, and the relative ratios to E8.5 embryos are shown. n=4 independent sample replicates. *P<0.05,
**P<0.01 versus E8.5 (two-tailed Welch’s t-test). E8.5, ss 8-11; E9.5, ss 24-25; E10.5, ss 34-35. G-6-P, glucose 6-phosphate; F-6-P, fructose 6-phosphate; F-1,6-
P2, fructose 1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; GA-3-P, glyceraldehyde 3-phosphate; 6-PG, 6-phosphogluconate; Ru-5-P, ribulose
5-phosphate; R-5-P, ribose 5-phosphate; S-7-P, sedoheptulose 7-phosphate; E-4-P, erythrose 4-phosphate. (B,C) Amounts of glycerol 3-phosphate and glycine determined by CE-MS were normalized to the total amount of protein extracted from the embryos, and the relative ratios to E8.5 embryos are shown. n=3 independent sample replicates. *P<0.05 versus E8.5 embryos (two-tailed Welch’s t-test). E8.5, ss 10-12; E9.5, ss 24-26; E10.5, ss 35-37. Data are represented as mean±s.d.

Fig. 7. Oxygen concentration affects glucose-derived carbon entry into the TCA cycle. Fractional contribution of 13C
from 13C6-glucose to lactate [FC(lactate)] or citrate [FC(citrate)] in ex vivo culture. After
pre-culturing for 60-90 min under 5% (L), 20% (M) or 60% (H) O2 conditions, embryos
were cultured with 13C6-glucose for 60 min. Each bar represents an independent
sample. E8.5, ss 8-12; E9.5, ss 24-28; E10.5, ss 35-39.
Accompanying the onset of mammalian gastrulation and neurulation, the intrauterine environment of embryos changes following vitelline circulation development and CB, which enhances the maternal-fetal interaction for the exchange of gases and nutrients (Arora and Papaioannou, 2012; McGrath et al., 2003; Zohn and Sarkar, 2010). Given the close association between the timing of metabolic changes and CB, previous studies have suggested that the metabolic change was the result of a presumptive elevated oxygen supply to the embryos (Clough, 1985; Clough and Whittingham, 1983). Our 13C labeling experiment using WEC also showed that glucose-derived carbon entry into the TCA cycle is enhanced by high oxygen concentrations (Fig. 7), suggesting that embryos at the CB stage have the capacity to modulate the entry of glucose-derived carbon into the TCA cycle depending on the concentration of oxygen. However, our results also demonstrate that embryos at this stage sustain glucose carbon flow into lactate irrespective of the concentration of oxygen (Fig. 7). This implies that intrinsic developmental programs may be regulating glucose carbon flow through the glycolytic pathway irrespective of the presumptive increases in oxygen supply during CB. It should be noted that WEC might not recapitulate the metabolic state in vivo owing to differences in the environmental conditions surrounding the embryo, as suggested by inconsistencies between FC(citrate) and FC(lactate) in vivo and ex vivo. Nevertheless, our study suggests that WEC can be used to investigate the responsiveness of embryos to environmental challenges.

It is interesting to note that downregulation of Aldoa mRNA proceeds along the body axis during CB, which enhances the maternal-fetal interaction for the exchange of gases and nutrients (Arora and Papaioannou, 2012; McGrath et al., 2003; Zohn and Sarkar, 2010). Given the close association between the timing of metabolic changes and CB, previous studies have suggested that the metabolic change was the result of a presumptive elevated oxygen supply to the embryos (Clough, 1985; Clough and Whittingham, 1983). Our 13C labeling experiment using WEC also showed that glucose-derived carbon entry into the TCA cycle is enhanced by high oxygen concentrations (Fig. 7), suggesting that embryos at the CB stage have the capacity to modulate the entry of glucose-derived carbon into the TCA cycle depending on the concentration of oxygen. However, our results also demonstrate that embryos at this stage sustain glucose carbon flow into lactate irrespective of the concentration of oxygen (Fig. 7). This implies that intrinsic developmental programs may be regulating glucose carbon flow through the glycolytic pathway irrespective of the presumptive increases in oxygen supply during CB. It should be noted that WEC might not recapitulate the metabolic state in vivo owing to differences in the environmental conditions surrounding the embryo, as suggested by inconsistencies between FC(citrate) and FC(lactate) in vivo and ex vivo. Nevertheless, our study suggests that WEC can be used to investigate the responsiveness of embryos to environmental challenges.

It is interesting to note that downregulation of Aldoa mRNA proceeds along the body axis during CB, implying regulation of glucose metabolism rewiring by a developmental program that also proceeds in a rostral-to-caudal direction (Stern et al., 2006). This view of glycolysis regulation by intrinsic factors, but not intrauterine environmental factors, is consistent with the idea that rapid embryonic growth during CB determines metabolic changes (New, 1978). In this regard, it is interesting to note that Lin28a expression is dramatically suppressed along the anterior-posterior axis from E8.5 to E10.5, similar to changes in the expression of Aldoa.

**Fig. 8. Lin28a as a possible determinant of glucose metabolism rewiring timing.** (A) qPCR analysis of Lin28ab mRNA. Actb was used as an internal control. n=4 independent sample replicates. **P<0.01, ***P<0.001 versus E8.5 (two-tailed Welch’s t-test). E8.5, ss 10; E9.5, ss 23-26; E10.5, ss 34-36. (B) qPCR analysis of let-7 miRNAs. U6 snRNA was used as an internal control. n=4 independent sample replicates. *P<0.05, **P<0.01, ***P<0.001 versus E8.5 (two-tailed Welch’s t-test). E8.5, ss 9-11; E9.5, ss 24-26; E10.5, ss 34-36. (C) Measurement of glycolytic metabolites within tetO-Lin28a:rtTA E10.5 embryos by IC-MS. Dox was administered intraperitoneally at E8 and E9. Size-matched E10.5 embryos (ss 33-35) were used for analysis, and the relative peak area per embryo is shown. Control, rtTA; Tg, tetO-Lin28a:rtTA; (−), Dox non-treated; (+), Dox treated. n=4 independent sample replicates. *P<0.05, **P<0.01 (two-tailed Welch’s t-test). (D) Lethality of tetO-Lin28a:rtTA P0 neonates treated with Dox at E8 and E9. Data are represented as mean±s.d.
Interestingly, Lin28a knockout E10.5 mouse embryos show aberrant glucose metabolism represented by decreased levels of glycolytic intermediates downstream of the PFK-1 reaction (Shinoda et al., 2013). These metabolic changes are complementary to those induced by sustained expression of Lin28a in E10.5 embryos, suggesting that downregulation of Lin28a from E8.5 to E10.5 is involved in the regulation of glucose metabolism rewiring. Because the expression of PFK-1 and aldolase mRNAs was not affected by exogenous Lin28a overexpression at E10.5 (data not shown), how Lin28a downregulation controls embryonic glucose metabolism remains an open question.

Perturbation of glucose metabolism rewiring by suppression of Lin28a downregulation at the CB stage resulted in perinatal lethality, suggesting that metabolic rewiring is crucial for normal development. How these metabolic changes affect development during and after the CB stage remain to be elucidated and will be explored in future studies. Suppression of PFK-1 can divert glucose carbon flow into PPP in cancer cells and endothelial cells (De Bock et al., 2013; Yamamoto et al., 2014). This is also the case during embryonic development from E8.5 to E10.5, as the suppression of PFK-1 is accompanied by an increase in PPP metabolites such as S-7-P. Since PPP is important for NADPH production and de novo nucleotide synthesis, such rewiring of glucose metabolism might enable embryos to enhance biomass production while generating energy mainly through OXPHOS, thereby meeting increasing metabolic demands for the dramatic expansion in body mass, cell number and cell types from E9.5 onwards. In addition, the rewiring of glucose metabolism from E8.5 to E10.5 results in an increase in G-3-P and glycine, which can be further converted to glycerolipids and nucleotides, respectively. In addition to biomass production, redirecting glucose carbon flow into the PPP might potentiate embryonic antioxidant capacity by producing NADPH to counteract the oxidative stress associated with enhanced OXPHOS capacity at these stages, and also to avoid excessive cell death (L’Honoré et al., 2014). Consistent with these ideas, embryos during CB have been shown to be especially susceptible to PPP inhibition (Chamberlain and Nelson, 1963; McCandless and Scott, 1981; Turbow and Chamberlain, 1968).

Metabolic changes are known to precede cellular fate changes during the reprogramming of differentiated cells into induced pluripotent stem cells (Folmes et al., 2011), raising the possibility that metabolic rewiring constitutes a crucial part of the reprogramming/cellular differentiation processes. In addition, several lines of evidence suggest that enzymes and metabolites that are involved in glycolysis also function as signaling molecules (Chang et al., 2013; Ho et al., 2015; Lee et al., 2015; Luo et al., 2011; Yang et al., 2011). For instance, lactate promotes angiogenesis by activating the Raf-ERK pathway under hypoxic conditions in cancer cells (Lee et al., 2015). The Lin28 genes act as gatekeepers between stemness and differentiation via regulation of both early proliferative cell fates and later differentiating cell fates (Faas et al., 2013; Polesskaya et al., 2007; Tsialikas and Romer-Seibert, 2015; Vadla et al., 2012; Yu et al., 2007). Therefore, downregulation of Lin28a might constitute a part of the developmental program that coordinates cell fate transitions with energy metabolic rewiring as a means to fulfill the changing metabolic demands of embryos for cell proliferation, cell differentiation, and morphogenesis during organogenesis.

The glucose metabolism rewiring observed in our study reflects changes in the whole embryo over time. Whether reorganization simultaneously occurs throughout the embryo, or whether there is a specific population of cells or a cell lineage that exhibits drastic metabolic rearrangement currently remains unclear. However, as discussed above, hurdles remain in studying metabolism within...
heterogeneous multicellular tissues. In this study, we demonstrated the potential of MALDI-IMS for metabolic studies during development as revealed by the unexpected distribution of lactate-rich tissues (i.e. the posterior/dorsal neural tube, somites and head mesenchyme in E9.5 embryos). Further technical advances, such as quantitative IMS analysis and genetically encoded metabolic probes to monitor metabolic pathway activity at the single-cell level in vivo, will aid in understanding how cellular metabolism contributes to development.

MATERIALS AND METHODS

Mice

Pregnant Jcl:ICR mice were purchased from CLEA Japan. Collalt-1etO-Lin2ka (Zhu et al., 2010) and ROSA26-rTA*M2 (Hochedlinger et al., 2005) transgenic mice were obtained from the Jackson Laboratory. 9-tert-butyl doxycycline (Echelon) dissolved in water was administered to pregnant mice intraperitoneally at 0.25 mg/mouse. All experiments were performed with approval from the Animal Experiment Ethics Committee of the University of Tokyo, and in accordance with the University of Tokyo guidelines for the care and use of laboratory animals.

Metabolic profiling of developing embryos by CE-MS

Embryos were dissected from the uterus and removed from yolk sac, amnion and allantois in cold PBS, and washed twice with 5% mannitol solution. Dissected embryos were immediately frozen with liquid nitrogen and stored at −80°C until use. Metabolite extraction was performed from the embryo with methanol containing an internal standard solution (Human Metabolome Technologies) as described (Soga et al., 2003). Metabolite quantities were determined by CE-MS (Agilent Technologies) from 11 (E8.5), 5 (E9.5) or 3 (E10.5) embryos/sample; n=3 independent sample replicates (Soga et al., 2006). PLS-DA was performed based on an algorithm described previously (Barker and Rayens, 2003). Multiple testing correction via false discovery rate (FDR) estimation was performed using R software (Storey, 2002).

Tracing 13C6-glucose metabolic pathways in developing embryos by CE-MS

For whole-embryo culture (WEC) experiments, embryos were carefully isolated from the uterus with the yolk sac intact in prewarmed (37°C) dissection medium. Embryos were precultured for 60-90 min in DMEM containing 50% immediately centrifuged (IC) rat serum in a culture bottle placed in a rotational drum culture system (Ikemoto Rika, Tokyo, Japan). Then, 13C6-glucose (2 mg/ml final concentration) was added to the culture medium and the embryos were cultured for another 60 min. For intravenous injection, 13C6-glucose (24 mg/mouse) was administered to pregnant mice, and embryos were retrieved 60 min later. The amount of intracelular 13C-labeled metabolites in the embryos was determined by CE-MS as described previously (Sugiura et al., 2014). For details, see the supplementary Materials and Methods.

FC(citrate) and FC(lactate) were determined from the 13C labeling patterns of citrate and lactate acquired by CE-MS. The effects of naturally occurring isoforms were removed from the raw mass spectrometry data to obtain the corrected mass distribution vectors (MDVs) of the carbons in the metabolites (Kajihata et al., 2014; van Winden et al., 2002). Fractional contributions were calculated as described (Buescher et al., 2015). Although the small sample number prevents statistical testing, our data show a clear tendency for metabolic changes and embryonic response to environmental conditions.

MALDI-IMS

Embryos were dissected from the uterus with the Reichert’s membrane and yolk sac intact in cold PBS. Dissected embryos were washed with 5% mannitol solution, embedded in Super Cryoembedding Medium (SCEM)-L1 (SECTION LAB, Hiroshima, Japan) and stored at −80°C until use. Frozen SCEM blocks in which embryos were embedded were sectioned at −16°C using a cryostat (Leica CM 3050) to a thickness of 8 µm. Kawamoto cryofilm was used to support fragile embryo tissues during cutting (Kawamoto, 2003). Sections were attached onto indium-tin oxide (ITO)-coated glass slides (Bruker Daltonics, Billerica, MA, USA) with electrically conducting double-adhesive tape (Shimadzu Corporation, Kyoto, Japan). Prepared tissues were coated with 9-aminoacridine as the matrix (10 mg/ml dissolved in 80% ethanol) (Benabedolah et al., 2009) by manually spraying with an artistic brush (Procon Boy FWA Platinum, Mr. Hobby, Tokyo, Japan). The matrix was simultaneously applied to multiple tissue sections in order to maintain consistent analyte extraction and co-crystallization conditions (Setou, 2010; Sugiura et al., 2009). MALDI imaging was performed using an Ultraflextreme MALDI-TOF mass spectrometer (Bruker Daltonics) and 7T FT-ICR-MS (Solarix Bruker Daltonics, Bremen, Germany) equipped with an Nd:YAG laser. Data were acquired in the negative reflectron mode with raster scanning by a pitch distance of 30 µm. Each spectrum was the result of 300 laser shots at each data point. In this analysis, signals between m/z 50 and 1000 were collected. In FT-ICR-MS (Fourier transform ion cyclotron resonance mass spectrometry) imaging, signals between m/z 100 and 400 were collected using the ‘continuous accumulation of selected ions’ mode. Image reconstruction was performed using FlexImage 4.1 software (Bruker Daltonics). The high mass accuracy provided by FT-ICR-MS allowed the selective ion signals of the metabolites to be obtained within a mass window of 5 ppm, enabling identification of the specific elemental composition of the compounds by comparing the highly accurate masses in databases (Marshall et al., 1998).

Measurement of PPP metabolites by IC-MS

Trace amounts of PPP metabolites in developing embryos (n=4 independent sample replicates) were measured using an orbitrap-type mass spectrometer (Q-Exactive Focus, Thermo Fisher Scientific) connected to a high-performance ion chromatography (HPIC) system (ICS-5000+, Thermo Fisher Scientific) that enables highly selective and sensitive metabolite quantification owing to the ion chromatography separation and Fourier transform mass spectrometry principle (Hu et al., 2015). The sample preparation method was the same as for CE-MS analysis. For details, see the supplementary Materials and Methods.

Lactate assay

Embryos were retrieved from the uterus in prewarmed (37°C) dissection medium. Embryos without the yolk sac, amnion and allantois were cultured under ambient oxygen conditions in DMEM containing 100 U ml−1 penicillin and 100 µg ml−1 streptomycin at 37°C in a 5% CO2 incubator (E8.5 and E9.5 embryos, 2 h; E10.5 embryos, 2.5 h; n=3 independent sample replicates). The amount of lactate released into the culture medium was determined using the Lactate Assay Kit (Biovision) following the manufacturer’s protocol.

Enzymatic activity assay

Activities of PFK-1 and aldolase were measured using a published protocol with modifications (Tian et al., 1998). Embryos [18-22 (E8.5), 4 (E9.5) or 1 (E10.5) embryo/sample; n=7 independent sample replicates] were sonicated in 0.1 ml lysis buffer containing 50 mM Tris acetate (pH 8.0), 10 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA and Protease Inhibitor Mix (Roche), and then centrifuged at 20,000 g for 30 min at 4°C. The supernatant was used in the assays. The activities of PFK-1 and aldolase were determined by coupled enzyme assays, in which fructose 6-phosphate (a substrate of PFK-1) or fructose 1,6-bisphosphate (a substrate of aldolase) is added to the embryo lysates, and the resulting dihydroxyacetone phosphate from the substrate is evaluated by the rate of conversion of NADH to NAD+ by glyceral-3-phosphate dehydrogenase (GPDH), which is proportional to the activity of PFK-1 or aldolase; the conversion of NADH to NAD+ was measured by the decrease in absorbance at 340 nm by a spectrophotometer.

aldolose activity assay: 2.5 mM D-fructose 1,6-bisphosphate, 0.15 mM NADH (all these substrates were from Sigma).

**Microarray and qPCR analysis**

Total RNA was extracted from embryos using the RNeasy Plus Micro Kit (Qiagen) and RNA quality was checked using a Bioanalyzer (Agilent). Total RNA with RNA integrity number (RIN) values ≥7 was utilized for microarray analysis. One hundred nanograms of total RNA was used for analysis with the mouse GE 4 × 44K V2 microarray (Agilent) according to manufacturer’s instructions. Data were analyzed using GeneSpring software (Agilent).

For qPCR, cDNA was synthesized from 200 ng total RNA using a PrimeScript RT Reagent Kit with gDNA Eraser (Takara). qPCR was performed with Takara SYBR Premix Ex Taq II (Tli RNaseH Plus) using a LightCycler 480 (Roche). Primers for qPCR are listed in Table S2. All primers gave a similar PCR amplification efficiency (∼2.0), allowing comparison of estimated gene expression levels among different genes using crossing point (Cp) values. For quantification of mature let-7 microRNAs, cDNAs were synthesized using the TaqMan MicroRNA RT Kit (Applied Biosystems) from total RNA. RNA was extracted from embryos using Trizol (Invitrogen) and the mirVana miRNA Isolation Kit (Ambion). qPCR was performed with TaqMan Fast Advanced Master Mix (Applied Biosystems) using the LightCycler 480 system. We reproduced the result twice, and a representative result is shown in Figs 5 and 8.

**Mitochondrial DNA quantification**

Embryos were lysed with lysis buffer containing proteinase K, and mitochondrial (mtDNA) and nuclear (nDNA) DNAs were purified by phenol-chloroform extraction ([n=7 (E8.5) or n=4 (E9.5)] independent sample replicates). The amount of mtDNA and nDNA was quantified by qPCR using the following primers (5′-3′, forward and reverse): mtDNA, CCTATCACCCTTGCCATCAT and GAGGCTGTTGCTTGTGTGAC; nDNA (Pecam1), ATGGAAAGCCTGCCATCATG and TCCTTGTTGTTGCTGAC; nDNA (Pecam1), ATGGAAAGCCTGCCATCATG and TCCTTGTTGTTGCTGAC. We reproduced the result at least three times and a representative result is shown in Fig. 2.

**Western blotting**

Western blotting to detect Lin28a and β-tubulin in E10.5 embryo extracts was performed as outlined in the supplementary Materials and Methods.

**Whole-mount in situ hybridization (WISH)**

Detection of Aldoa by WISH at E8.5, E9.5 and E10.5 was performed as described in the supplementary Materials and Methods.

**Acknowledgements**

We thank M. Yamamoto and all members of the M.M. laboratory for helpful discussions and comments; and M. Hamachi, N. Saito, Y. Matsumoto, Y. Chayama, and M. Hamachi, N. Saito, Y. Matsumoto, Y. Chayama for experimental assistance. We thank M. Yamamoto and all members of the M.M. laboratory for helpful discussions and comments; and M. Hamachi, N. Saito, Y. Matsumoto, Y. Chayama, and M. Hamachi, N. Saito, Y. Matsumoto, Y. Chayama for experimental assistance. We thank M. Yamamoto and all members of the M.M. laboratory for helpful discussions and comments; and M. Hamachi, N. Saito, Y. Matsumoto, Y. Chayama, and M. Hamachi, N. Saito, Y. Matsumoto, Y. Chayama for experimental assistance.

**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

H.M., Y.Y. and M.M. designed the study. H.M. performed most of the experiments and data analysis. Y.S. and K.H. performed the labeling experiments, IC-MS analysis and data analysis. Y.S. and K.H. performed MALDI-IMS. K.K. performed the WISH analysis. F.M. analyzed the data from the labeling experiments. T.Y. and M.M. provided essential advice. H.M., Y.Y. and M.M. prepared the manuscript.

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**Data availability**

Microarray expression data are available from the Dryad Digital Repository at http://dx.doi.org/10.5061/dryad.fdqg (Miyazawa et al., 2016).


Figure S1. Alterations in the embryonic metabolome profile during chorioallantoic branching. (A) PLS-DA of the intracellular metabolite profiles of E8.5, E9.5, and E10.5 embryos (n = 3 independent sample replicates). E8.5, E9.5 and E10.5 embryos were clearly separated by PLS1. (B) List of the differentially expressed metabolites that contributed the most to separation within the PLS1 [*q < 0.05, **q < 0.01; Welch’s t-test with multiple testing correction via false discovery rate (FDR) estimation]. This included metabolites involved in glycolysis (blue) and the TCA cycle (orange).
Figure S2. $^{13}$C-enrichment of lactate and citrate revealed by labeling experiment with $^{13}$C$_6$-glucose in vivo. (A, B) Isotopomer abundance of lactate (A) and citrate (B) within embryos 60 min after $^{13}$C$_6$-glucose administration. $^{13}$C$_6$-glucose was administered to pregnant mice by intravenous injection. (C, D) MDV of lactate (C) and citrate (D) within embryos. The values were collected for naturally occurring isotopes by using OpenMebius (Kajihata et al., 2014). Each bar represents an independent sample (E8.5, ss 8–12; E9.5, ss 22–26; E10.5, ss 32–36).
Figure S3. Aldoa expression becomes localized to the posterior neural tube and somites as development proceeds. Whole mount in situ hybridization for Aldoa mRNA. Aldoa expression becomes localized to the posterior neural tube and mesodermal tissues as development proceeds from E8.5 to E10.5. Scale bar, 500 µm.
Figure S4. $^{13}$C-enrichment of lactate and citrate as revealed by labeling experiment with $^{13}$C$_6$-glucose in whole embryo culture. (A, B) Isotopomer abundance of lactate (A) and citrate (B) within embryos 60 min after $^{13}$C$_6$-glucose labeling in ex vivo culture. Embryos were cultured under 5% (L), 20% (M) or 60% (H) O$_2$ conditions with $^{13}$C$_6$-glucose. (C, D) MDV of lactate (C) and citrate (D) within embryos. The values were collected for naturally occurring isotopes by using OpenMebius. Each bar represents an independent sample (E8.5, ss 8–12; E9.5, ss 24–28; E10.5, ss 35–39).
Figure S5. Transient overexpression of Lin28a in tetO-Lin28a:rtTA E10.5 embryos. (A) Induction of Lin28a protein was confirmed by western blotting with anti-LIN28A antibody. Arrows indicate exogenous Lin28a. Dox was administered intraperitoneally at E8 and E9. (B) qPCR analysis of let-7 miRNAs in tetO-Lin28a:rtTA E10.5 embryos (ss 33–35). U6-snRNA was used as an internal control (n = 3 independent sample replicates; *p < 0.05, **p < 0.01, ***p < 0.001 vs. wild-type; two-tailed Welch’s t-test).
Figure S6. Measurement of PPP metabolites in tetO-Lin28a:rtTA E10.5 embryos by IC-MS.

Relative peak area of PPP metabolites. Dox was administered intraperitoneally at E8 and E9. Size-matched E10.5 embryos (ss 33-35) were used for analysis, and relative peak area per embryo is shown. Control, rtTA; Tg, tetO-Lin28a:rtTA; (-), Dox non-treated; (+), Dox treated (n = 4 independent sample replicates; *p < 0.05, **p < 0.01; two-tailed Welch’s t-test).
**Figure S7. Lactate secretion from embryos is decreased from E8.5 to E9.5.** Lactate assay using spent medium. Amount of lactate released into the culture medium was measured. Embryos were cultured under ambient oxygen conditions. Data are represented as mean ± SD (n = 3 independent sample replicates; **p < 0.01, ***p < 0.001 vs. E8.5; two-tailed Welch’s t-test). Amount of lactate released from the embryos decreased significantly from E8.5 to E9.5.
Table S1.
Click here to Download Table S1

Table S2. Sequence of primers for qPCR analysis

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<th>Gene name</th>
<th>Primer Type</th>
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<td></td>
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Supplementary Materials and Methods

Whole-mount in situ Hybridization (WISH)

WISH was performed as previously described (Nonomura et al., 2013). Aldoa cDNA template (FANTOM clone, 8530401H18) was digested using Eco47III, and antisense and sense probes were synthesized using T3 and T7 RNA polymerases, respectively. We reproduced the result at least three times, and representative images are shown in the figure.

Western Blotting of Embryos

Western blotting was performed as described previously (Yamaguchi et al., 2011) with slight modifications. Briefly, embryos dissected from the uterus at E10.5 were removed from the yolk sac, immediately frozen in liquid N2 and stored at –80°C until use. Frozen embryos were lysed with RIPA buffer containing protease inhibitor cocktail (Roche), and 2–4 µg of total protein was loaded in each lane. Lin28a and β-tubulin were detected by using anti-LIN28A antibody (1:1000; #3978; Cell Signaling Technology) (Triboulet et al., 2015) and anti-β-tubulin antibody (1:1000; MAB3408; Millipore), respectively.

Tracing 13C6-Glucose-Metabolic Pathways in Developing Embryos by CE-MS

Metabolites were extracted from the tissues by homogenizing frozen embryos together with the internal control compounds (see below) in ice-cold methanol (500 µL) using a manual homogenizer (Finger Masher (AM79330); Sarstedt, Tokyo, Japan) (WEC experiments: E8.5, 4–6 embryos/sample; E9.5, 2–4 embryos/sample; E10.5, 1 embryo/sample; and i.v. experiments: E8.5, 7–16 embryos/sample; E9.5, 4–5 embryos/sample; E10.5, 2 embryos/sample). Then, an equal volume of chloroform and 0.4 times the volume of ultrapure water (LC/MS grade; Wako) were added to the homogenates. The suspension was centrifuged at 15,000 × g for 15 min at 4°C. After centrifugation, the aqueous phase was ultra-filtered using an ultrafiltration tube (Ultrafree-MC, UFC3 LCC NB; Human Metabolome Technologies, Tsuruoka, Japan). The filtrate was concentrated with a vacuum concentrator (SpeedVac; Thermo, Yokohama, Japan); this condensation process helped to quantitate trace levels of metabolites. The
concentrated filtrate was dissolved into 50 µL ultrapure water and used for CE-MS.

CE-ESI-MS experiments were performed using an Agilent CE System equipped with an air pressure pump, an Agilent 6520 Accurate Q-Tof mass spectrometer, an Agilent 1200 series isocratic high-performance LC pump, 7100 CE-system, a G1603A Agilent CE-MS adapter kit, and a G1607A Agilent CE-MS sprayer kit (Agilent Technologies).

*Internal* (added to tissue before extraction) and *external* (added to sample after extraction) standard compounds were used. To normalize the shifts in migration (retention) times among datasets, we used two internal standard compounds set to be eluted in the beginning and at the end, to measure the time difference between two standard peaks as a calibrant. We then corrected the relative migration times of all peaks by normalizing those of the internal standards. For anionic metabolites, 2-morpholinoethanesulfonic acid (MES) and 1,3,5-benzene-tricarboxylic acid (trimesate) were used, while L-methionine sulfone and 3-aminopyrrolidine dihydrochloride were used for cationic metabolites. These compounds are not present in the tissues and therefore serve as ideal standards. Loss of metabolites during sample preparation was also corrected for. Furthermore, before sample measurement, the mixture of authentic compounds of target metabolites was measured at three different concentrations to generate calibration curves. Quantification (amount of metabolites; pmol/mg protein) was performed by comparing the normalized peak areas to the calibration curves.

**Measurement of PPP metabolites by IC-MS**

The IC was equipped with an anion electrolytic suppressor (Thermo Scientific Dionex AERS 500) to convert the potassium hydroxide gradient into pure water before the sample enters the mass spectrometer. The separation was performed using a Thermo Scientific Dionex IonPac AS11-HC, 4-µm particle size column. IC flow rate was 0.25 mL/min supplemented post-column with 0.18 mL/min makeup flow of MeOH. The potassium hydroxide gradient conditions for IC separation are as follows: from 1 mM to 100 mM (0–40 min), 100 mM (40–50 min), and 1 mM (50.1–60 min), at a column temperature of 30°C.

The Q Exactive focus mass spectrometer was operated under an ESI negative mode for all detections. Full mass scan (*m/z* 70–900) was used at a resolution of 70,000. The automatic gain control
(AGC) target was set at $3 \times 10^6$ ions, and maximum ion injection time (IT) was 100 ms. Source ionization parameters were optimized with the spray voltage at 3 kV and other parameters were as follows: transfer temperature at 320°C, S-Lens level at 50, heater temperature at 300°C, Sheath gas at 36, and Aux gas at 10.

**Supplementary References**

