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LETTER TO THE EDITOR

Mitochondrial metabolism and glutamine are essential for mesoderm differentiation of human pluripotent stem cells

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Dear Editor,

Human pluripotent stem cells (hPSCs) generate energy mainly by aerobic glycolysis, with glutamine oxidation in the tricarboxylic acid (TCA) cycle providing additional ATP required for survival.[1–3] During the exit from pluripotency and initial differentiation into multiple germ lineage precursors, energy production shifts from mainly aerobic glycolysis to mitochondrial oxidative phosphorylation (OXPHOS).[1] Until recently, consensus in the field was that as PSCs exit pluripotency, a metabolic switch from aerobic glycolysis to OXPHOS is required. However, a more detailed examination of nascent ectoderm (EC) metabolism showed unexpected maintenance of a high, MYC-dependent glycolytic flux, resembling sustained hPSC metabolism, in contrast to mesoderm (ME) and endoderm (EN),[4] generating questions for the role(s) of mitochondrial metabolism in early hPSC tri-lineage differentiation. To examine this issue, we differentiated hPSCs into early EN, ME, and EC lineages using a non-limiting, nutrient-balanced culture media that differed only by established lineage-driving cytokines,[5,6] so that intrinsic metabolic preferences were not derived from a variance in nutrient composition (Supplementary information, Data S1). Principal component analysis (PCA) of these early lineages using RNA-Seq was equivalent to a previous study using nutrient-balanced and chemically defined growth media.[4] Furthermore, transcriptomic and protein biomarker expression matched established profiles for hPSCs and hPSC-derived EN, ME, and EC (Supplementary information, Fig. S1a–c, Table S1),[7] confirming the validity of our model system.

To quantify the impact of mitochondrial OXPHOS on lineage potentiation, mitochondrial stress tests showed that ME has the highest basal and maximal oxygen consumption rate (OCR), greatest spare respiratory capacity, and largest approximate respiration-to-glycolysis ratio (OCR/ECAR) compared to hPSCs, EN, and EC (Fig. 1a, Supplementary information, Fig. S1d, e). To compare nutrient preferences, we used media footprint analysis, which showed decreased glucose (Glc) consumption, decreased lactate production, increased glutamine (Gln) consumption, and increased glutamate production in ME compared to EC (Fig. 1b). Live cell interferometry quantification of normalized biomass accumulation (growth) rate revealed that ME and EC cell clusters were statistically equivalent and higher than EN (Fig. 1c). Taken together, the data reveal a striking metabolic plasticity in that ME and EC differ dramatically in nutrient source and pathways for energy and metabolite production, yet yield the same growth rate during early lineage specification.

Prior studies show that cell biomass accumulation during proliferation depends mainly on the consumption of non-Gln amino acids.[8] Since our data reveal that ME and EC have equivalent growth rates, we examined biomass accumulation-independent roles for Glc and Gln, such as facilitating cell fate specifications in these two lineages. Initially, we determined whether Glc or Gln is most crucial for each lineage. Early EN, ME, and EC lineages did not robustly convert $^{13}C_6$-glucose into TCA cycle metabolites, suggesting that Glc is not a major carbon source for the germ lineages (Fig. 1d, Supplementary information, Fig. S2a). In contrast to Glc, Gln is a major fuel source for all three cell lineages, with >70% of $^{13}C_5$-glutamine metabolized to glutamate and subsequent TCA cycle metabolites (Fig. 1e, Supplementary information, Table S2). Specifically, the mass isotopologue distribution (MID) of $^{13}C_5$-glutamine shows increased $m+5$ αKG and $m+4$ succinate, fumarate, malate, and citrate levels in ME, indicating that ME preferentially uses Gln compared to EC (Fig. 1f). Increased $m+5$ citrate due to reductive carboxylation[9] was detected in EC compared to ME, suggesting that Gln may be preferentially shunted toward lipid biosynthesis and/or gluconeogenesis through citrate rather than into other TCA cycle intermediates in EC (Fig. 1f).[10] Overall, the data show that Gln is a major contributor to the TCA cycle in all three lineages, yet displays increased incorporation into first turn TCA cycle metabolites in ME compared to EC.

Since ME exhibits increased preference for Gln contribution into the TCA cycle, we next asked whether Gln deprivation would result in a bias in lineage potentiation in non-directed embryoid body (EB) differentiation, which has been previously demonstrated to recapitulate features of early embryogenesis.[11] As expected, Gln withdrawal from EB culture media at the start of non-directed differentiation resulted in significantly reduced levels of TCA cycle metabolites, with the exception of citrate at 48 h (Fig. 1g). Concurrent 1 mM dichloroacetate (DCA) treatment with Gln withdrawal to inhibit pyruvate dehydrogenase kinase and increase the flux of Glc supplied pyruvate into the TCA cycle[12] surprisingly did not replenish TCA cycle metabolite levels (Fig. 1g). This finding is consistent with a prior study reporting that hPSCs are highly dependent on both Glc and Gln oxidation, and that pyruvate addition cannot rescue Glc and Gln depleted conditions.[2] A mitochondrial stress test on EC and ME differentiated cells with Gln removed from the culture media revealed a decrease in maximal OCR and OCR/ECAR ratio in ME compared to EC, consistent with increased reliance on Gln in ME for respiration (Supplementary information, Fig. S2b, c). Additionally, differentiation under Gln deprivation did not affect EB formation, morphology, or viability (Supplementary information, Fig. S2d).[13] Interestingly, in EBs differentiated under Gln deprivation, immunoblot analysis revealed a decrease in the SLUG protein level, while qRT-PCR analysis showed significantly decreased *HAND1* expression, indicating that Gln is essential for ME production (Fig. 1h, Supplementary information, Fig. S2e). Additionally, phase contrast microscopy of ME lineage directed differentiation

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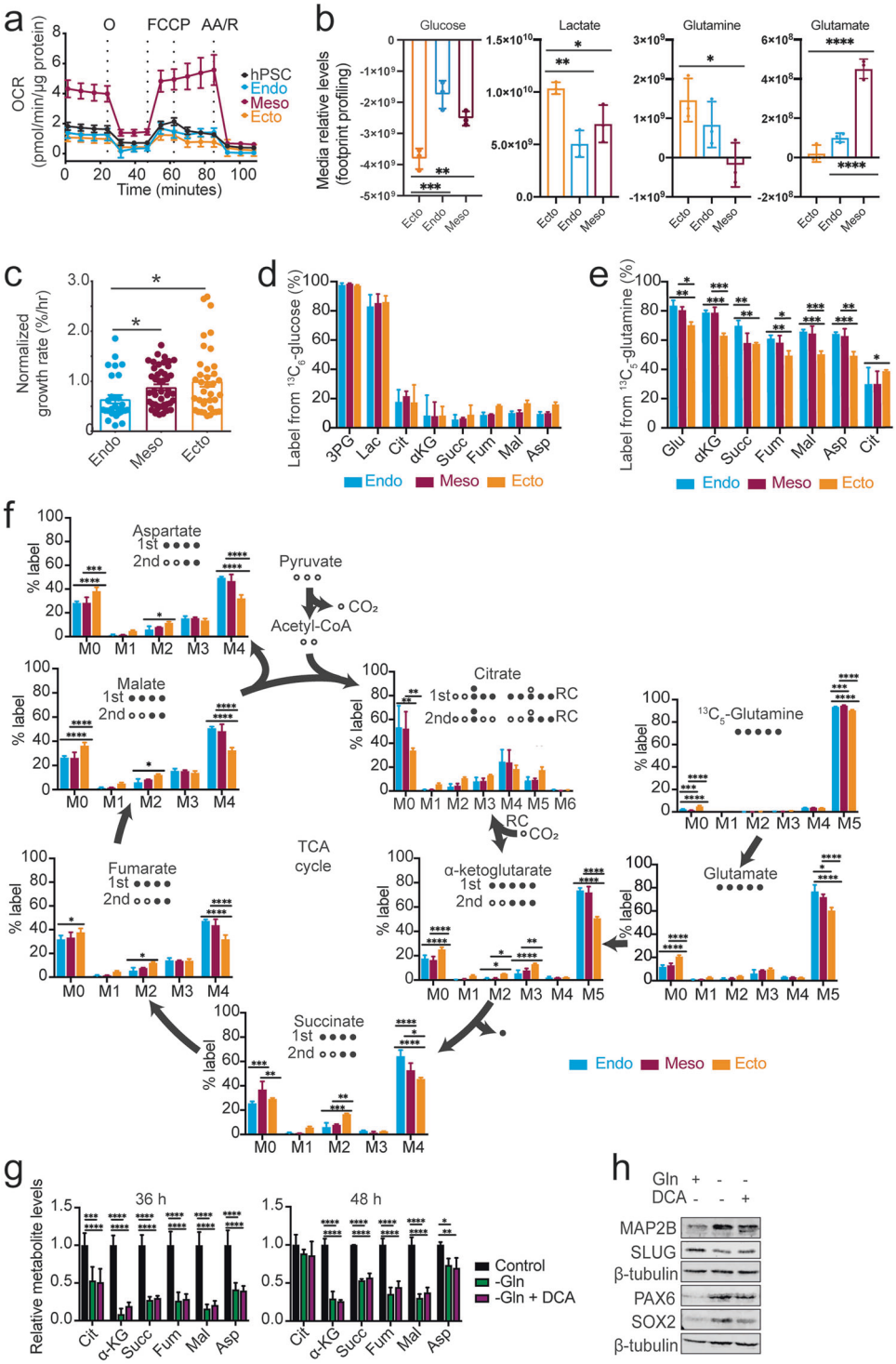

revealed widespread cell death under Gln deprivation, suggesting that Gln is indispensable for ME formation (Supplementary information, Fig. S2f). In contrast, Gln withdrawal led to an increase in EC specifying biomarker proteins PAX6 and MAP2B during 21 days of EB differentiation (Fig. 1h, Supplementary information, Fig. S2e). Increased EC production may be linked to a compensation for Gln deprivation by recovery of glycolytic metabolites (Fig. 1g, h, Supplementary information, Fig. S2g). Together, this EB differentiation profile suggests that Gln withdrawal skews potentiation of ME negatively and EC positively.

This report provides a deeper understanding of distinct metabolic shifts in early germ lineages by showing that mitochondrial respiration and Gln oxidation is essential for ME differentiation. Further studies are needed to elucidate the functional roles of metabolic reprogramming in early germ lineages, with possibilities including transcriptional program

Fig. 1 Human pluripotent stem cell-derived mesoderm differentiation requires glutaminolysis and distinct mitochondrial metabolism. **a** Oxygen consumption rate (OCR) measured by mitochondrial stress test of H9 hPSC and H9-derived endoderm (EN), mesoderm (ME), and ectoderm (EC) lineages at 5 days of directed differentiation. Data are normalized by microgram of protein content per well. Injections of 1 μM oligomycin, 0.33 μM FCCP, 0.5 μM FCCP, and 1 μM each Antimycin A/Rotenone were performed. Data represent $n = 6$ technical replicates of 1 biological experiment with additional $n \geq 2$ biological experiments provided in Supplementary information, Fig. S1e. **b** Media footprint analysis (relative metabolite consumption and production into media) of nutrient-balanced EN, ME, and EC samples, quantifying levels of (i) glucose consumption, (ii) lactate production, (iii) glutamine consumption, and (iv) glutamate production at 5 days of directed differentiation. Positive values indicate increased production of cellular metabolites into spent media, whereas negative values indicate cellular consumption of media metabolites. Data are normalized to initial unspent fresh media levels as measured by UHPLC–MS, and represent $n = 3$ independent experiments. Media of differentiated H9 hESCs were changed 24 h prior to spent media metabolite extraction. **c** Normalized single colony biomass accumulation and growth rates (%/hour) of nutrient-balanced H9-derived EN/ME/EC samples measured over 24 h. Data represent $n = 29$ EN, $n = 45$ ME, and $n = 37$ EC colonies. **d**, **e** Fractional contribution of $^{13}C_6$-labeled metabolites from [U-$^{13}C_6$] glucose (**d**) or $^{13}C_5$-labeled metabolites from [U-$^{13}C_5$] glutamine (**e**) after 18 h quantified by UHPLC-MS. **f** Mass isotopomer distribution (MID) of TCA cycle-associated metabolites from [U-$^{13}C_5$] glutamine. The carbon labeling of TCA cycle-associated metabolites from [U-$^{13}C_5$] glutamine is schematically illustrated in black for the first (1st) and second (2nd) turn or α-ketoglutarate (α-KG) reductive carboxylation (RC) into citrate. Media of differentiated H9 hESCs were changed 18 h prior to intracellular metabolite extraction. **g** Relative levels of TCA cycle metabolites in H9 hPSCs under 36 h and 48 h spontaneous differentiation in the presence or the absence of Gln in the culture media co-treated or untreated with 1 mM DCA, quantified by UHPLC-MS. **h** Immunoblot of ME (SLUG) and EC (PAX6, SOX2 and MAP2) markers in H9-derived EB at 21 days of differentiation in the presence or the absence of Gln in the culture media co-treated or untreated with 1 mM DCA. $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$. p values are determined using unpaired Student's t-test (**c**–**e**) or two-way ANOVA with multiple comparisons Bonferroni correction (**b**, **f**, **g**). Data represent mean ± SD (**a**, **b**, **d**–**g**) or mean ± SEM (**c**) of independent experiments indicated above

changes regulated by metabolite-sensitive epigenetic modifiers[14] or carbon source-specific dependencies.

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AUTHOR CONTRIBUTIONS

Conceptualization: P.D., V.L. M.A.T.; Methodology: P.D., V.L., M.A.T.; Software: F.M.A., D.H., D.B.; Formal Analysis: P.D., V.L., F.M.A., A.N.P., I.J.R., D.H., D.B., R.M.T.N.; Investigation: P.D., V.L., F.M.A., I.J.R., A.T.,Jr., R.M.T.N.; Resources: F.M.A., D.B., M.A.T.; Data Curation: V.L., F.M.A., A.N.P., I.J.R., D.B.; Writing-Original Draft: V.L., F.M.A., A.N.P., I.J.R., M.A.T.; Writing—Review & Editing: V.L., F.M.A., A.N.P., I.J.R., M.A.T.; Validation: V.L., F.M.A., A.N.P., I.J.R., M.A.T.; Visualization: V.L., F.M.A., A.N.P., I.J.R.; Supervision: P.D., M.A.T.; Project Administration: P.D., M.A.T.; Funding Acquisition: A.N.P., M.A.T.

ADDITIONAL INFORMATION

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