Original Research

Mouse embryonic stem cells require multiple amino acids

Boyang Zhang and Jian Feng

Department of Physiology and Biophysics, The State University of New York at Buffalo, Buffalo, NY 14203, USA
Corresponding author: Jian Feng. Email: jianfeng@buffalo.edu

Impact Statement

We examined which amino acids were needed for the survival and growth of mouse embryonic stem cells (mESCs). In contrast to an influential study on the unique requirement of mESCs for threonine, we found that mESCs required many essential and some non-essential amino acids to survive and proliferate in a commonly used medium containing serum or in a chemically defined medium without serum. The study aims to set the record straight on the amino acid requirements of mESCs.

Abstract

Previous studies suggest that mouse embryonic stem cells (mESCs) have a unique requirement for threonine when cultured in serum and leukemia inhibitory factor (LIF). Here, we replicated the experiments and found that the growth of mESCs (E14 and AB2.2) in serum/LIF was significantly attenuated by the individual absence of multiple amino acids. When mESCs were maintained in naïve pluripotency by the MEK inhibitor, GSK3 inhibitor (2i), and LIF, their growth was significantly affected by the lack of any one of the nine essential amino acids or some non-essential amino acids. There was no unique requirement for threonine in both culture conditions. This study shows that, like many other cells, mESCs do not have any special requirements for amino acids.

Keywords: Stem cells, cell metabolism, amino acid, serum, LIF, MEK inhibitor, GSK3 inhibitor

Experimental Biology and Medicine **2022; X: 1–9. DOI: 10.1177/15353702221096059**

Introduction

Mouse embryonic stem cells (mESCs) can be maintained in an undifferentiated state indefinitely in medium containing fetal bovine serum (FBS) and leukemia inhibitory factor (LIF), a condition known as serum/LIF.[1–3] LIF promotes mESC self-renewal primarily by activating the JAK/Stat3 pathway,[4] which is critical for maintaining the expression of pluripotency factors, such as Oct4 and Nanog.[5] LIF alone is insufficient to maintain the pluripotency of mESCs; bone morphogenic proteins (BMP) in serum are required as well.[6] BMP-induced signaling causes phosphorylation of Smad proteins that induce expression of Id genes.[6] This inhibits the expression of genes involved in the differentiation of mESCs. However, mESCs cultured in serum/LIF exhibit fluctuating levels of pluripotency markers and express some lineage-specific genes.[7,8] Batch-to-batch variations in the concentrations of serum components, many of which are undefined, may introduce unspecified heterogeneity in mESCs. Because of these issues, the serum/LIF condition can be used to maintain pluripotency only in a few lines of mESCs derived from special strains of mice.[9]

Alternatively, indefinite self-renewal of mESCs can be maintained in a defined, serum-free medium termed 2i/LIF (2iL), which contains LIF and two small molecule inhibitors (2i) of MEK (PD0325901) and GSK3 (CHIR99021).[10] The two inhibitors block signaling pathways critical for the differentiation of mESCs and obviate the need for BMP (which is in serum) to sustain pluripotency in mESCs.[10] In contrast to the situation in the serum/LIF condition, mESCs cultured in 2iL display a homogeneous expression of pluripotency markers[8] and capture the "ground state" of pluripotency analogous to the inner cell mass of pre-implantation blastocysts.[11–13] As such, 2iL can be used to derive and maintain pluripotent stem cells from virtually any mouse stains and many mammalian species.[9]

The rapid proliferation of mESCs demands a continuous supply of biomolecules, including amino acids (AAs). Nine AAs, namely histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine, are essential amino acids (EAA) because they cannot be synthesized by mice.[14,15] It is reported that only the lack of threonine significantly compromises the growth of mESCs in serum/LIF.[16] This claim leads to the finding that one-carbon metabolism downstream of threonine is critical in mESCs.[16,17] The two papers have stimulated considerable interests in understanding the reportedly unique AA requirement of mESCs for threonine and its implications in other types of cells capable of indefinite self-renewal.[18–20] In our effort to understand the metabolic requirements of naïve pluripotency, we were unable to observe the unique requirement of mESCs for threonine under essentially the same condition. Instead,

we found that the growth and proliferation of mESCs in either serum/LIF or 2i/LIF required many essential and non-EAAs.

Materials and methods

Materials

All mESCs were purchased from ATCC and passaged 5–20 times in the study. DMEM, DMEM/F12, Neurobasal, N2, B27, TrypLE, penicillin-streptomycin, β-mercaptoethanol, sodium pyruvate, non-EAAs, and Trypan Blue were purchased from Thermo Fisher Scientific. Bovine serum albumin (BSA), mitomycin C, mouse LIF, and all cell-culture grade AAs were purchased from MilliporeSigma. HyClone standard FBS was purchased from Fisher Scientific. CHIR99021 and PD0325901 were purchased from Tocris.

Culture of mESCs

AB2.2 and E14Tg2A were cultured on mitomycin C-treated CF1 feeder cells at 37°C with 5% CO_2 in either serum-containing media (DMEM, 10% FBS, 0.1 mM β-mercaptoethanol, mouse LIF [1000 U/mL], 1 × non-EAAs, 1 × penicillin-streptomycin, 1 mM sodium pyruvate) or 2iL media[6] (1:1 DMEM/F12 and Neurobasal, N2, B27, 0.1 mM β-mercaptoethanol, mouse LIF [1000 U/mL], 1 × non-EAAs, 1 × penicillin-streptomycin, BSA [5 mg/mL], 1 μM PD0325901, and 3 μM CHIR99021). mESCs were passaged every 2 days using TrypLE. For AA drop-off assay, media was created using either AA-free DMEM (C4150, Genaxxon Bioscience) or an AA-free 2iL base media (i.e. DMEM/F12 and Neurobasal) that was custom-made by UCSF Media Production Core. Appropriate concentrations of AAs, dissolved in water or HCl, were then added along with the remaining media components (e.g. mouse LIF, BSA, etc.) to make complete media. Similarly, AA drop-off media was made by omitting the selected AA. The final pH of the media was adjusted using either HCl or NaOH.

AA drop-off assay

mESCs were trypsinized into single cells and seeded onto feeders (12-well plate format) in normal serum/LIF or 2iL media. After 6 h, each well was washed twice with C4150 or AA-free 2iL base media to remove residual AAs. Cells were then cultured in normal media, complete media, or AA drop-off media for 36 h, during which media was changed once. Alkaline Phosphatase (AP) staining (ab242286, Abcam) or OCT4 immunostaining was later performed. AP staining was performed according to vendor's protocol. Immunostaining was conducted by fixing the cells in 4% paraformaldehyde for 15 min at room temperature, followed by 0.1% Triton X-100 for 30 min, and then incubating with primary antibody (MAB4419; MilliporeSigma; 1:1000) overnight at 4°C and secondary antibody (A21121; Thermo Fisher; 1:2000) for 1 h at room temperature.

The same assay conditions (6 h in normal media and 36 h in normal/complete/AA drop-off media) were performed for MEF (Mouse Embryonic Fibroblasts) cells. Afterward, MEF cells were trypsinized into single cells, stained with Trypan Blue, and counted using a hemocytometer.

Image and statistical analyses

Bright-field images of AP staining and fluorescence images of OCT4 immunostaining were taken on a Leica AF6000 inverted microscope. Leica Application Suite X (LASX) was used to analyze images. For monochromatic bright-field images, the intensities were first inverted to convert the light background into dark and the dark-colored AP^+ colonies into light-colored. Using the Analysis module, the threshold was manually adjusted to coincide with the original image as close as possible. In the measurements tab, the accepted object count and integrated intensity were selected to obtain the colony count and colony intensity, respectively. For a colony to be counted as an accepted object, the pixel cut-off was set to 1000. The integrated intensity of a colony can be described as the total number of pixels inside the colony multiplied by the average colony intensity. The sum of all integrated intensities in an image is defined as total intensity. Phase-contrast images were taken on an EVOS FL Cell Imaging System. All statistical analyses were performed with GraphPad Prism version 7.0. Results were deemed statistically significant at $p < 0.05$ using one sample *t*-test (two-tailed).

Results

Growth and proliferation of mESCs in the serum/LIF condition require multiple AAs

In our effort to understand the metabolic requirements of naïve pluripotency, we performed AA drop-off experiments as reported previously.[16] We chose Dulbecco's Modified Eagle Medium (DMEM) instead of Glasgow's Minimum Essential Medium (GMEM) because an AA-free version of DMEM (C4150) is commercially available. The components of GMEM are identical to DMEM, either at the same or lower concentrations (Table S1). To maintain consistency, we cultured the same E14 mESCs[16] in the serum/LIF condition using normal DMEM, C4150 with all 20 AAs added back (complete media), and C4150 with all but one AA added back (AA drop-off media). The experiments were performed as described previously:[16] 6 h in normal media after seeding mESCs and 36 h in AA drop-off media, followed by AP staining. Gray-scale images of AP staining were used for accurate counting and quantification. The numbers of AP^+ colonies had no statistical difference when E14 mESCs were cultured in DMEM, C4150 DMEM with all 20 AAs (complete media), or C4150 missing 1 of the following 11 AAs: Ala, Asn, Gln, Glu, Gly, Leu, Phe, Pro, Ser, Trp, and Tyr (Figure 1[a] to [w]). The absence of His or Ile produced moderate but statistically significant reductions in AP^+ colony numbers, while the lack of Arg, Cystine (dimeric Cysteine, dCys), or Lys resulted in less than half the AP^+ colony number compared to complete media. The most severe reduction in AP^+ colony number was observed in the absence of certain EAAs: Met (Figure 1[o]), Thr (Figure 1[s]), or Val (Figure 1[v]), as quantified in Figure 1(w) and Table S2. Phase-contrast images of the mESCs in each condition prior to AP staining showed the corresponding changes in the number and size of mESC colonies (Figure S1).

Under the above culture conditions, mESCs had varying sizes and AP intensity, with smaller colonies tended to

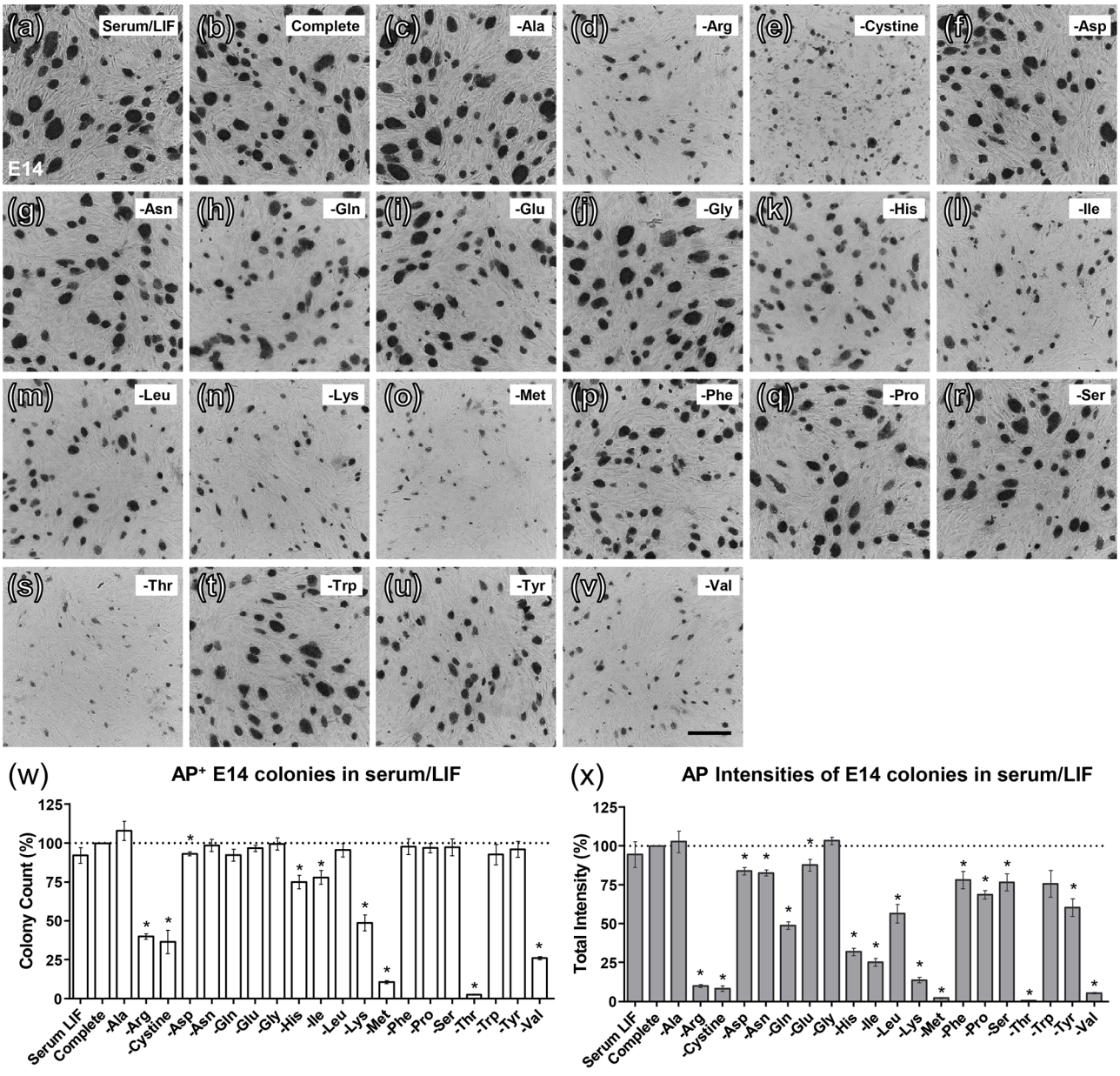

Figure 1. AA dependence of E14 mESCs cultured in serum/LIF. (a to v) AP staining of E14 mESCs cultured with serum and LIF in DMEM (a), complete C4150 DMEM (b), or complete C4150 DMEM without an indicated AA (c to v). The image for each condition is 1 of 36 images in a 6 × 6 tilescan (10x magnification); it is at the center of the well of a 12-well plate. Scale bar, 200 μm. (w) The number of AP$^+$ colonies in the tilescan was counted by software for each condition. (x) The integrated intensity of AP staining of all colonies in the tilescan was measured by software. Error bar, standard error of measurement, $n = 4$ independent experiments, *, $p < 0.05$ versus complete C4150 DMEM, one sample *t*-test (two-tailed).

have weaker AP staining. Images of colonies in a well of a 12-well plate were acquired by tilescan. We measured the integrated intensity of each colony by multiplying average colony intensity by the number of pixels in that colony. The sum of integrated intensities of all colonies in tilescan is designated as total intensity, which accurately reflects the representative images in Figure 1(a) to (v). This more accurate quantification produced results (Figure 1[x]) that generally paralleled colony counts (Figure 1[w]) and confirmed that the lack of non-EAAs Arg or dCys, or the lack of EAAs Lys, Met, Thr, or Val severely inhibited the growth of E14 mESCs (Figure 1[a] to [v], [x]). Compared to colony count, we observed more pronounced changes in total intensity for almost every AA drop-off condition (Figure 1(x), Table S2). To confirm these results, we used another well-characterized mESC line, AB2.2. When deprived of a single AA, AB2.2 cells exhibited the same profiles as E14 in terms of colony count and total intensity in the serum/LIF condition (Figure 2 for AP staining and Figure S2 for phase-contrast images). The observed changes in total AP intensity in AB2.2 cells were recapitulated using OCT4 immunostaining (Figure S3). Consistent with the previous observation,[16] we found that the survival of MEF cells was impaired only by the absence of dCys in serum/LIF (Figure 3). Thus, the

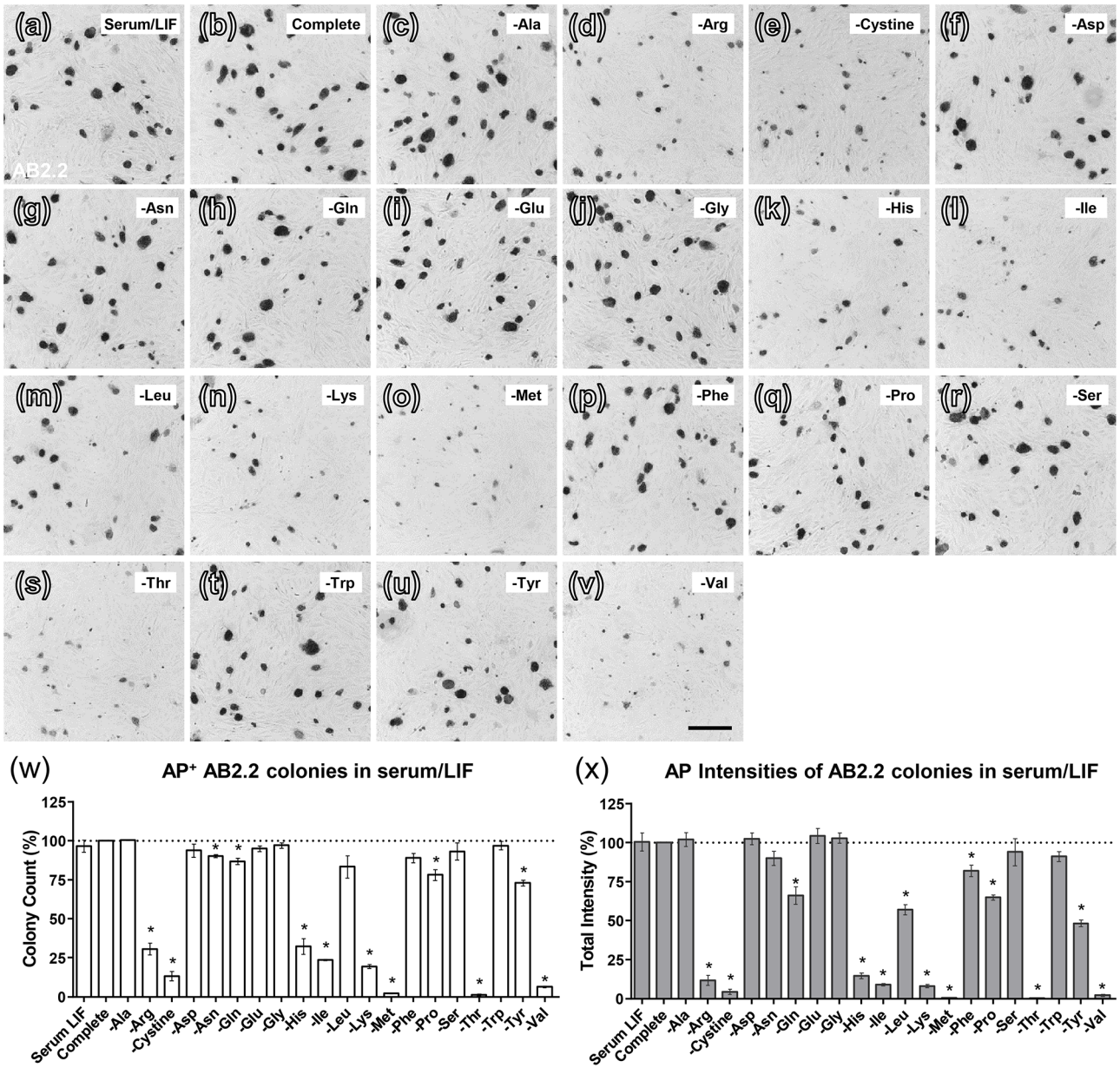

Figure 2. AA dependence of AB2.2 mESCs cultured in serum/LIF. (a to v) AP staining of E14 mESCs cultured with serum and LIF in DMEM (a), complete C4150 DMEM (b), or complete C4150 DMEM without an indicated AA (c to v). The image for each condition is 1 of 36 images of a 6 × 6 tilescan (10x magnification); it is at the center of the well of a 12-well plate. Scale bar, 200 μm. (w) The number of AP + colonies in the tilescan was counted by software for each condition. (x) The integrated intensity of AP staining of all colonies in the tilescan was measured by software. Error bar, standard error of measurement, $n = 3$ independent experiments, *, $p < 0.05$ versus complete C4150 DMEM, one sample t-test (two-tailed).

requirement of dCys by E14 (Figure 1[e], [w] to [x]) and AB2.2 (Figure 2[e], [w] to [x]) may be affected by the loss of MEF cells in the absence of dCys.

Growth and proliferation of mESCs in the 2i/LIF condition require multiple AAs

To remove the potentially confounding influence of serum, which has AAs in variable concentrations, we examined the growth of E14 in 2iL medium[6] and a custom-made AA-free 2iL medium where all AAs were added back (complete media) or complete media with an individual AA missing. In the 2iL condition, the absence of any one of the nine EAAs led to a severe decrease in colony count and total intensity for E14 (Figure 4 for AP staining, Figure S4 for phase-contrast images, and Table S4 for quantification). The same were true for AB2.2 cultured in 2iL (Figure 5 for AP staining, Figure S5 for phase-contrast images, and Table S5 for quantification). Without the EAA Leu, Phe, or Trp, total intensities became 10% or less relative to complete media. Removing the EAA His, Ile, Lys, Met, Thr, or Val, total intensities fell below 2% relative to complete media. There were also large decreases in total intensity on removing the non-essential AA Arg, Gln, or Tyr, which were (relative to complete media): 7.3%, 17%, and 4.4% for E14, and 14%, 20%, and 5.1% for AB2.2, respectively. E14 and AB2.2 grown in conditions lacking Ala, Cys,

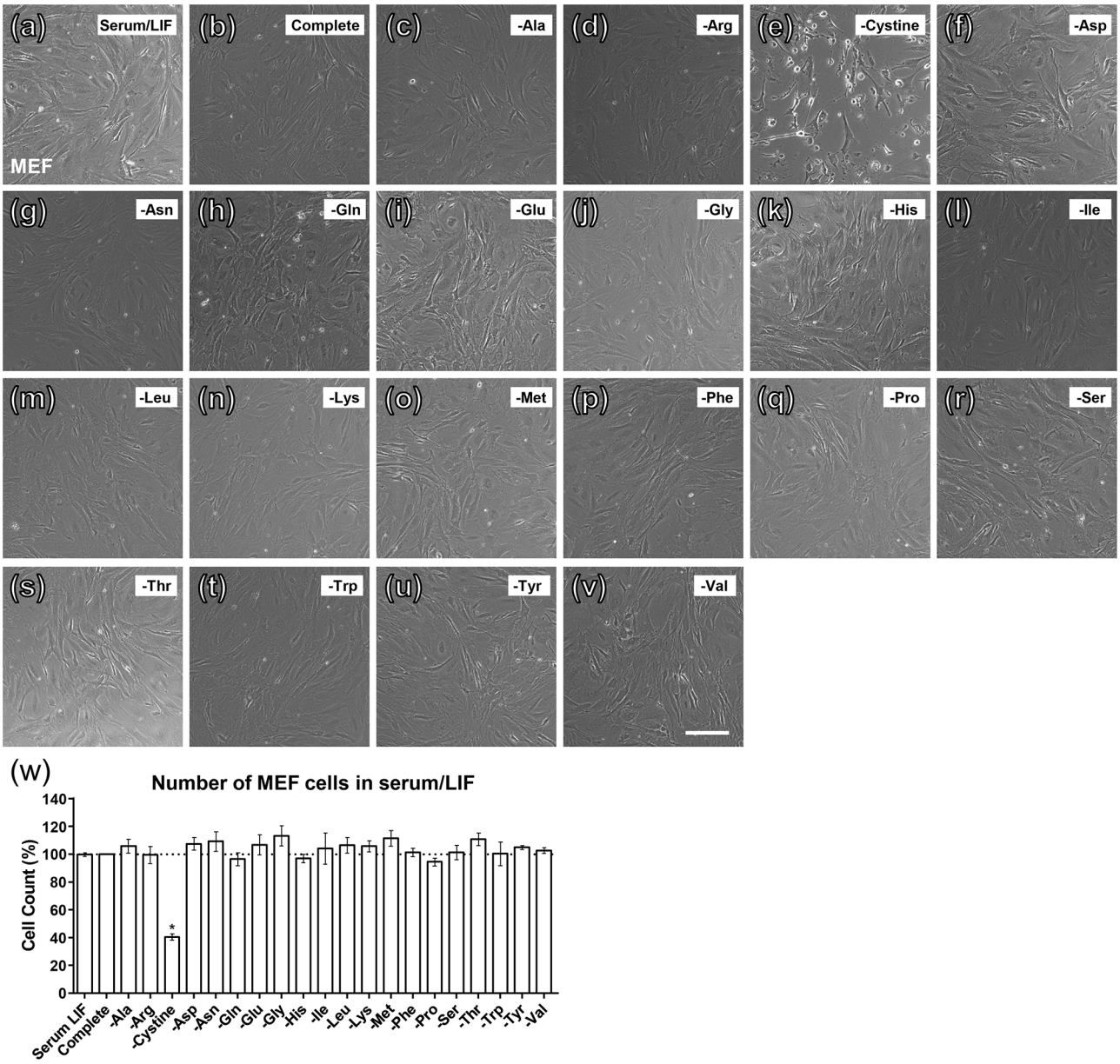

Figure 3. MEF survival is affected by cystine deprivation in the serum/LIF condition. (a to v) Phase-contrast images show live MEF cells cultured with serum and LIF in DMEM (a), complete C4150 DMEM (b), or complete C4150 DMEM without an indicated AA (c to v). Scale bar, 200 μm. (w) Analysis of MEF cells by their live cell count. Error bar, standard error of measurement, $n = 3$ independent experiments, *, $p < 0.05$ versus complete C4150 DMEM, one sample *t*-test (two-tailed).

dCys, Asp, Asn, Glu, or Gly exhibited similar colony number and total intensity as those grown in conventional 2iL or complete media. This trend in total intensities from AP staining in AB2.2 was also witnessed using OCT4 immunostaining (Figure S6). It is important to note that DMEM does not contain Cys (but has dCys), while the 2iL base media contains both Cys and dCys. Therefore, in AA drop-off experiments in 2iL, we also tested a condition where both Cys and dCys were omitted to eliminate all thiol-containing AAs and to parallel the removal of dCys in DMEM serum/LIF. Total intensities were diminished in media devoid of both Cys and dCys, and Pro or Ser, but these changes were not as drastic as removing an individual EAA (Figures 4 and 5; Figures S4 to S5; Tables S4 to S5). In 2iL, we found that removing any single AA or removing both Cys and dCys did not impact the number of live MEF cells compared to those grown in 2iL or complete media (Figure 6). It is unclear why MEF cells require dCys when cultured in the serum/LIF condition, but not in the 2iL condition.

Discussion

A previous study shows that the growth and proliferation of mESCs in the serum/LIF condition is significantly affected by the lack of threonine, but not other AAs.[16] However, Figure 4(a) of that paper clearly shows substantial decreases in mESCs colonies when Cys, His, Met, Pro, or Val is individually removed; yet, there is no statistical analysis of the data.[16] The most severe effect caused by the lack of threonine and the high expression of threonine dehydrogenase

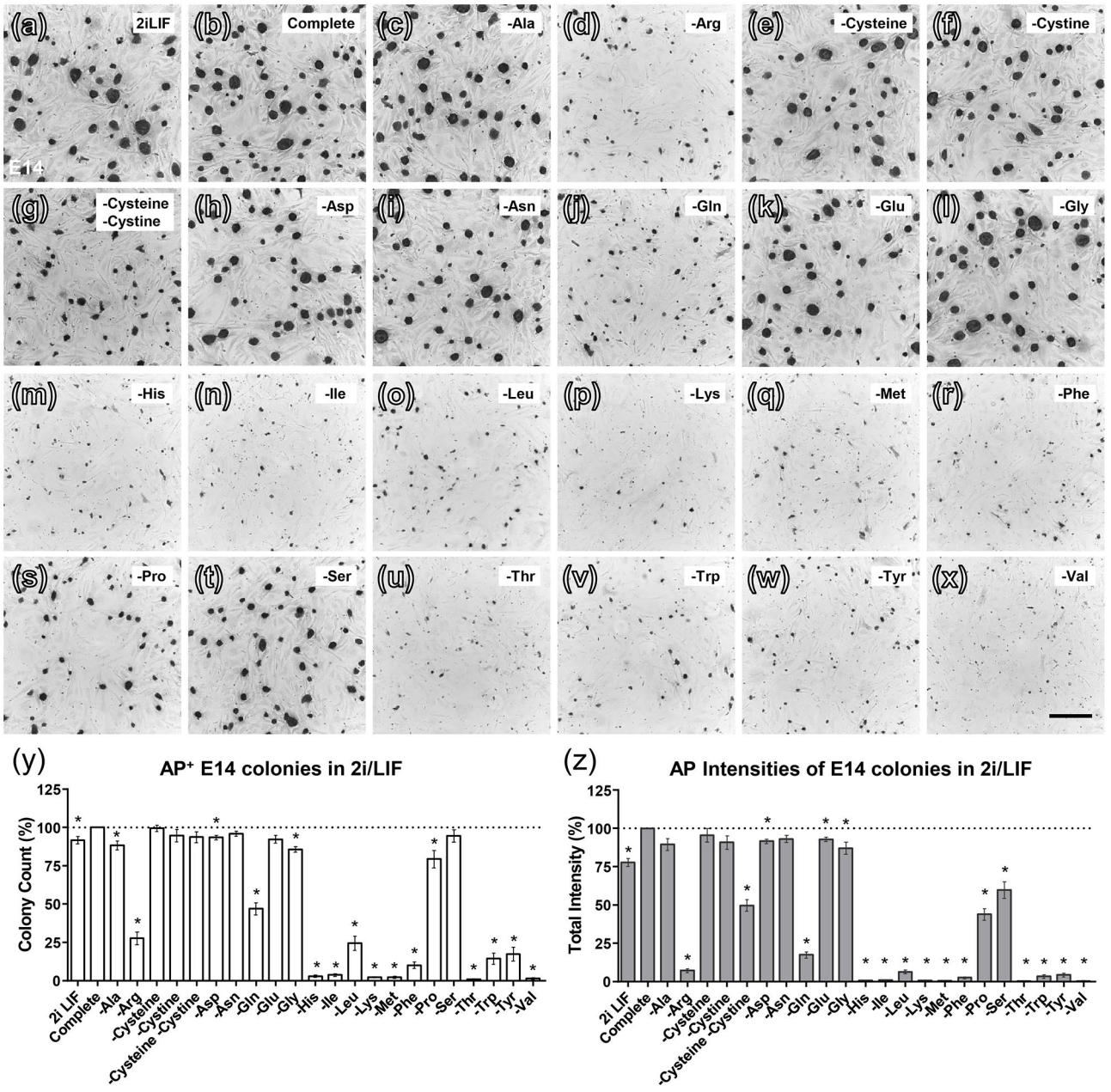

Figure 4. AA dependence of E14 mESCs cultured in 2i/LIF. (a to x) AP staining of E14 mESCs cultured with 2iL (a), complete 2iL-based media (b), or 2iL-based AA drop-off media (c to x). The image for each condition is 1 of 36 images of a 6 × 6 tilescan (10x magnification); it is at the center of the well of a 12-well plate. Scale bar, 200 μm. (y) The number of AP$^+$ colonies in the tilescan was counted by software for each condition. (z) The integrated intensity of AP staining of all colonies in the tilescan was measured by software. Error bar, standard error of measurement, $n = 4$ independent experiments, *, $p < 0.05$ versus complete 2iL-based media, one sample *t*-test (two-tailed).

(TDH) in mESCs lead to the suggestion that TDH-mediated catabolism of threonine to glycine and acetyl-coenzyme A is uniquely critical for the survival of mESCs.[16] The claim of threonine as the only amino acid critically required for the self-renewal of mESCs leads to the finding that threonine generates a substantial portion of glycine and acetyl-coenzyme A for the synthesis of S-adenosyl-methionine, which influences the epigenetic state of the cell by affecting the methylation of histone H3 lysine-4.[17] These two influential papers have stimulated many subsequent investigations on the metabolism of mESCs and other types of cells capable of indefinite self-renewal, for example, cancer cells.[18–20]

Intrigued by these findings, we studied the metabolism of naïve pluripotency and found that not only Thr but also Arg, Lys, Met, and Val were critically important in sustaining the growth of E14 and AB2.2 in serum/LIF, similar to the results of Wang *et al.*,[16] which does not mention these changes. Whereas Wang *et al.* describe little to no change in colony size or colony number except when Thr is removed, we observed substantial reductions in colony size in media lacking Arg, dCys, His, Ile, Leu, Lys, Met, Thr, or Val in the serum/LIF condition (Figures 1 and 2). Smaller colonies also tended to have weaker AP staining. To account for differences in colony sizes and AP levels, we measured the intensity of AP

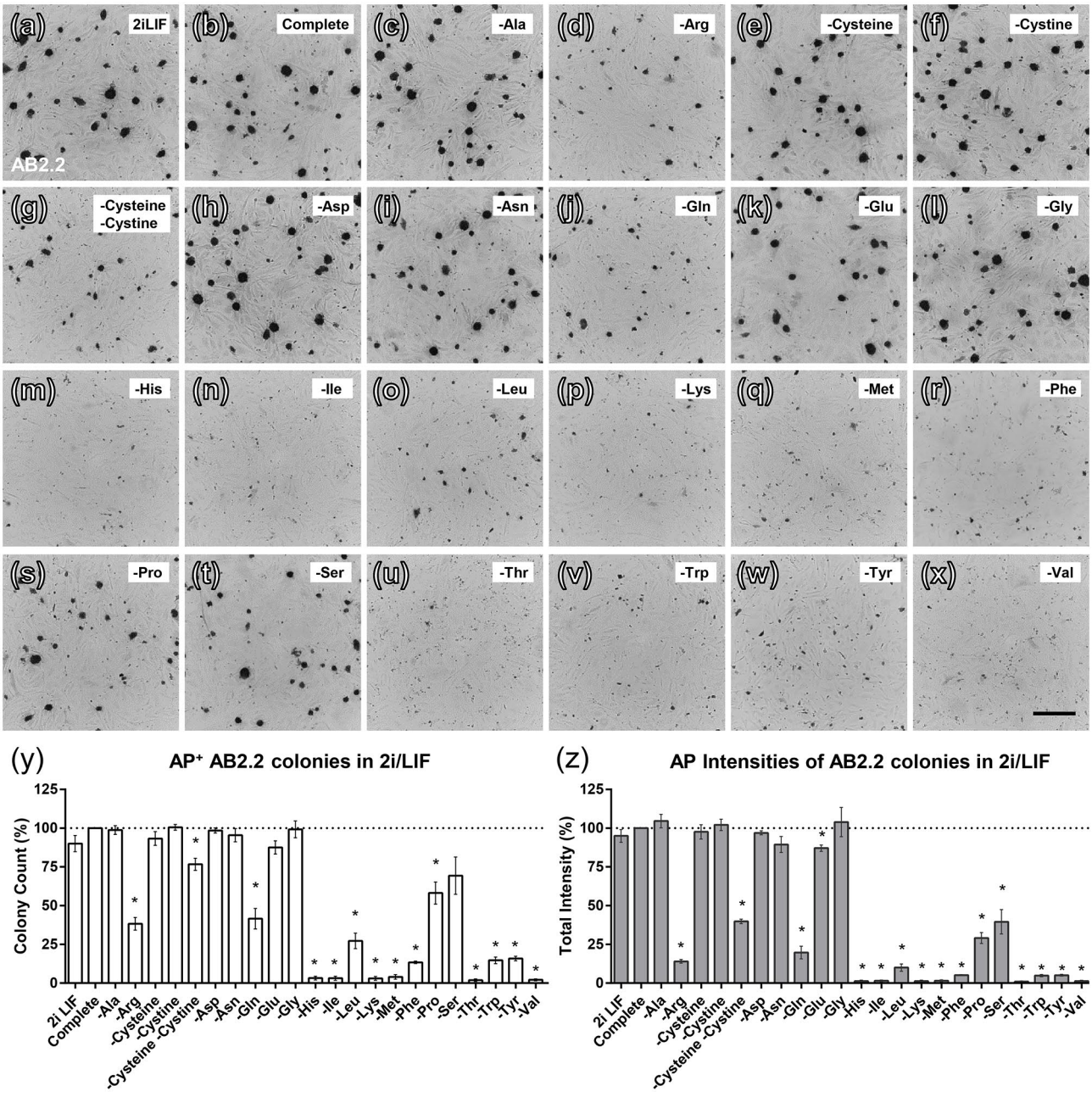

Figure 5. AA dependence of AB2.2 mESCs cultured in 2i/LIF. (a to x) AP staining of AB2.2 mESCs cultured with 2iL (a), complete 2iL-based media (b), or 2iL-based AA drop-off media (c to x). The image for each condition is 1 of 36 images of a 6 × 6 tilescan (10x magnification); it is at the center of the well of a 12-well plate. Scale bar, 200 μm. (y) The number of AP + colonies in the tilescan was counted by software for each condition. (z) The integrated intensity of AP staining of all colonies in the tilescan was measured by software. Error bar, standard error of measurement, $n = 3$ independent experiments, *, $p < 0.05$ versus complete 2iL-based media, one sample *t*-test (two-tailed).

staining to more accurately assess the impact of removing individual AAs. Under the 2iL condition, withdrawing any one of the EAAs resulted in almost complete abolishment of mESC colonies (Figures 4 and 5).

Signs of differentiation, such as flattened or dispersed cells, were not observed. Colony morphology and cell morphology remained unchanged, even in conditions lacking a critical AA, which reduced the number and size of colonies (Figure S1 to S2 and S4 to S5). Both AP (Figures 1, 2, 4 and 5) and OCT4 (Figure S3 and S6) staining showed that the mESCs were not differentiated. The reduced AP or OCT4 signal paralleled the decrease in colony size and colony number, which were caused by attenuated cell proliferation and/or increased cell death in the absence of a critical AA. It is possible that many activators of cell death pathways, including Caspase 3, may be engaged when the lack of a critical AA blocked protein synthesis. It takes separate studies to dissect the underlying mechanism, as the lack of different AAs may engage cell death pathways in different manners.

By culturing mESCs in the serum-free 2iL condition, we uncovered a confounding factor in using serum, which may inflate the survival of mESCs in AA drop-off experiments.

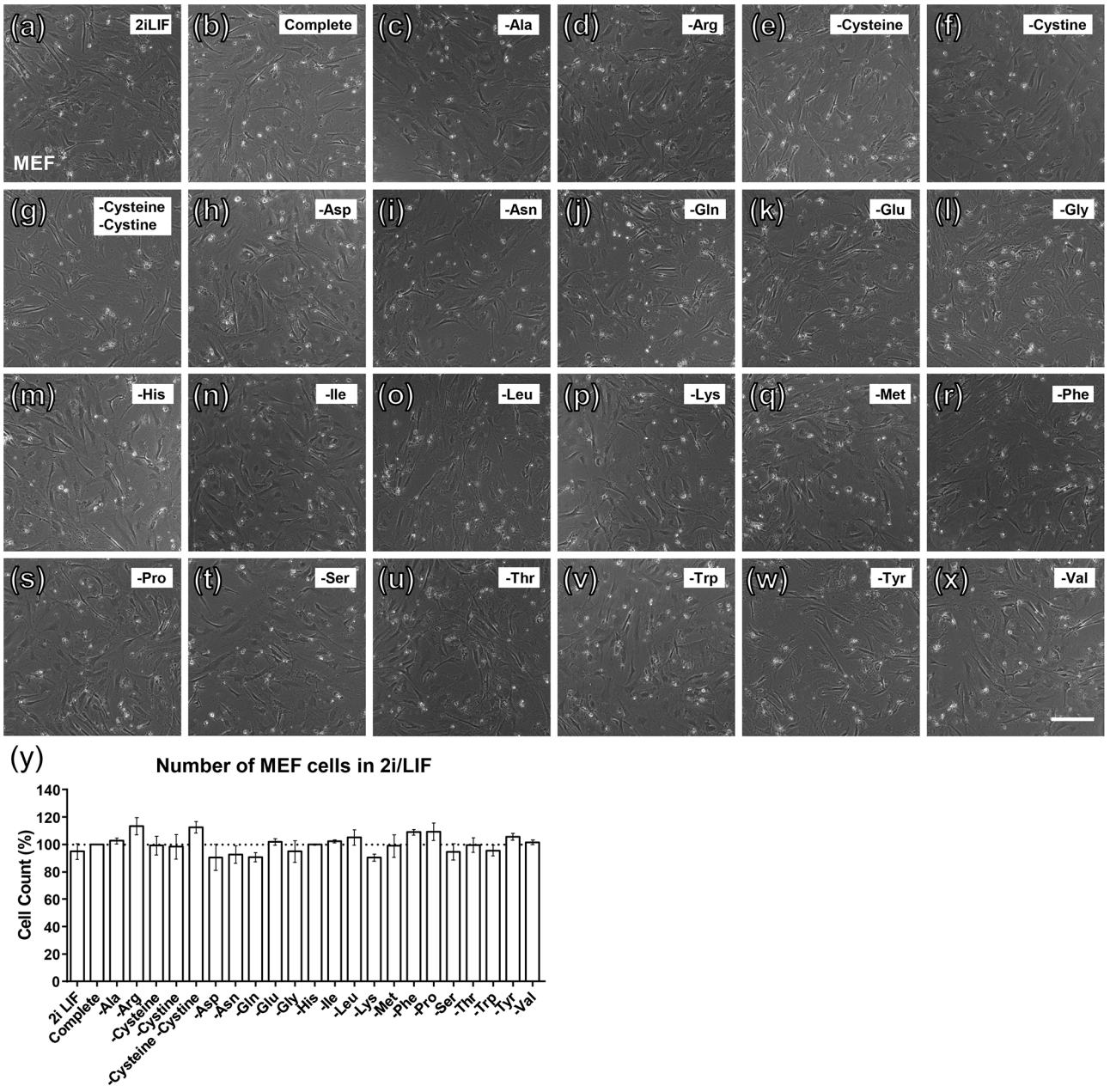

Figure 6. MEF survival is not affected by AA deprivation in the 2i/LIF condition. (a to x) Phase-contrast images show live MEF cells cultured with 2iL (a), complete 2iL-based media (b), or 2iL-based AA drop-off media (c to x). Scale bar, 200 μm. (y) Analysis of MEF cells by their live cell count. Error bar, standard error of measurement, $n = 3$ independent experiments, *, $p < 0.05$ versus complete 2iL-based media, one sample *t*-test (two-tailed).

Using E14 as an example, we observed the greatest total intensity disparities among the two culture conditions (serum/LIF versus 2iL, relative to complete media) on the removal of Phe (78% versus 2.5%), Trp (76% versus 3.4%), Tyr (60% versus 4.4%), or Leu (56% versus 6.4%) (Figures 1 and 4 and Tables S2, S4). It is possible for mESCs to use AAs in the serum or hydrolyze serum proteins into free AAs. The undefined compositions of serum and fluctuating concentrations in different batches make it impossible to pinpoint the sources of these discrepancies. Among the non-EAAs, removing Arg, Gln, or Tyr also greatly diminished the survival of mESCs in 2iL. The rapid proliferation of mESCs requires robust anabolic activities, which may place higher demands for these non-EAAs. While FBS is widely used to culture cells, including mESCs, it is estimated to have over 1000 components, including enzymes, lipids, vitamins, nucleosides, AAs, and carbohydrates.[21,22] It may introduce confounding variables that are impossible to control. For example, it is well-known that serum contains significant amounts of endogenous exosomes that can affect cell behavior.[23] The use of the serum-free, defined 2iL medium obviates these issues and maintains mESCs in the ground state of pluripotency.[24] Under such chemically defined condition, it becomes even clearer that mESCs require many AAs to proliferate, similar to many other types of cells. There is no unique requirement for threonine.

AUTHORS' CONTRIBUTIONS

B.Z. performed all experiments and data analyses. B.Z. and J.F. conceived the project and wrote the article. The authors thank Meredith A. Juncker for reading the article. Correspondence and requests for materials should be addressed to J.F. (jianfeng@buffalo.edu).

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: The work was supported in part by the National Institutes of Health grants NS102148 (J.F) and Buffalo Blue Sky Initiative (J.F.).

ORCID ID

Jian Feng https://orcid.org/0000-0001-7630-8800

SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

REFERENCES

1. Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. *Nature* 1981;**292**:154–6
2. Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci USA* 1981;**78**:7634–8
3. Smith AG, Heath JK, Donaldson DD, Wong GG, Moreau J, Stahl M, Rogers D. Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* 1988;**336**:688–90
4. Niwa H, Burdon T, Chambers I, Smith A. Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. *Genes Dev* 1998;**12**:2048–60
5. Do DV, Ueda J, Messerschmidt DM, Lorthongpanich C, Zhou Y, Feng B, Guo G, Lin PJ, Hossain MZ, Zhang W, Moh A, Wu Q, Robson P, Ng HH, Poellinger L, Knowles BB, Solter D, Fu XY. A genetic and developmental pathway from STAT3 to the OCT4-NANOG circuit is essential for maintenance of ICM lineages in vivo. *Genes Dev* 2013;**27**:1378–90
6. Ying QL, Nichols J, Chambers I, Smith A. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell* 2003;**115**:281–92
7. Cahan P, Daley GQ. Origins and implications of pluripotent stem cell variability and heterogeneity. *Nat Rev Mol Cell Biol* 2013;**14**:357–68
8. Marks H, Kalkan T, Menafra R, Denissov S, Jones K, Hofemeister H, Nichols J, Kranz A, Stewart AF, Smith A, Stunnenberg HG. The transcriptional and epigenomic foundations of ground state pluripotency. *Cell* 2012;**149**:590–604
9. Martello G, Smith A. The nature of embryonic stem cells. *Annu Rev Cell Dev Biol* 2014;**30**:647–75
10. Ying QL, Wray J, Nichols J, Batlle-Morera L, Doble B, Woodgett J, Cohen P, Smith A. The ground state of embryonic stem cell self-renewal. *Nature* 2008;**453**:519–23
11. Boroviak T, Loos R, Bertone P, Smith A, Nichols J. The ability of inner-cell-mass cells to self-renew as embryonic stem cells is acquired following epiblast specification. *Nat Cell Biol* 2014;**16**:516–28
12. Habibi E, Brinkman AB, Arand J, Kroeze LI, Kerstens HH, Matarese F, Lepikhov K, Gut M, Brun-Heath I, Hubner NC, Benedetti R, Altucci L, Jansen JH, Walter J, Gut IG, Marks H, Stunnenberg HG. Whole-genome bisulfite sequencing of two distinct interconvertible DNA methylomes of mouse embryonic stem cells. *Cell Stem Cell* 2013;**13**:360–9
13. Ficz G, Hore TA, Santos F, Lee HJ, Dean W, Arand J, Krueger F, Oxley D, Paul YL, Walter J, Cook SJ, Andrews S, Branco MR, Reik W. FGF signaling inhibition in ESCs drives rapid genome-wide demethylation to the epigenetic ground state of pluripotency. *Cell Stem Cell* 2013;**13**:351–9
14. John AM, Bell JM. Amino acid requirements of the growing mouse. *J Nutr* 1976;**106**:1361–7
15. Leib DE, Knight ZA. Re-examination of dietary amino acid sensing reveals a GCN2-independent mechanism. *Cell Rep* 2015;**13**:1081–9
16. Wang J, Alexander P, Wu L, Hammer R, Cleaver O, McKnight SL. Dependence of mouse embryonic stem cells on threonine catabolism. *Science* 2009;**325**:435–9
17. Shyh-Chang N, Locasale JW, Lyssiotis CA, Zheng Y, Teo RY, Ratanasirintrawoot S, Zhang J, Onder T, Unternaehrer JJ, Zhu H, Asara JM, Daley GQ, Cantley LC. Influence of threonine metabolism on S-adenosylmethionine and histone methylation. *Science* 2013;**339**:222–6
18. Kaelin WG Jr, McKnight SL. Influence of metabolism on epigenetics and disease. *Cell* 2013;**153**:56–69
19. Folmes CD, Dzeja PP, Nelson TJ, Terzic A. Metabolic plasticity in stem cell homeostasis and differentiation. *Cell Stem Cell* 2012;**11**:596–606
20. Pavlova NN, Thompson CB. The emerging hallmarks of cancer metabolism. *Cell Metab* 2016;**23**:27–47
21. Lawson MA, Purslow PP. Differentiation of myoblasts in serum-free media: effects of modified media are cell line-specific. *Cells Tissues Organs* 2000;**167**:130–7
22. Freshney RI. Culture of animal cells: a manual of basic technique and specialized applications. 6th ed. Hoboken, NJ: Wiley-Blackwell, 2010
23. Shelke GV, Lässer C, Gho YS, Lötvall J. Importance of exosome depletion protocols to eliminate functional and RNA-containing extracellular vesicles from fetal bovine serum. *J Extracell Vesicles* 2014;**3**:24783
24. Mulas C, Kalkan T, von Meyenn F, Leitch HG, Nichols J, Smith A. Defined conditions for propagation and manipulation of mouse embryonic stem cells. *Development* 2019;**146**:dev173146

(Received January 17, 2022, Accepted April 5, 2022)