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Nutritional requirements of human induced pluripotent stem cells

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SUMMARY

The nutritional requirements for human induced pluripotent stem cell (hiPSC) growth have not been extensively studied. Here, building on our prior work that established the suitable non-basal medium components for hiPSC growth, we develop a simplified basal medium consisting of just 39 components, demonstrating that many ingredients of DMEM/F12 are either not essential or are at suboptimal concentrations. This new basal medium along with the supplement, which we call BMEM, enhances the growth rate of hiPSCs over DMEM/F12-based media, supports derivation of multiple hiPSC lines, and allows differentiation to multiple lineages. hiPSCs cultured in BMEM consistently have enhanced expression of undifferentiated cell markers such as *POU5F1* and *NANOG*, along with increased expression of markers of the primed state and reduced expression of markers of the naive state. This work describes titration of the nutritional requirements of human pluripotent cell culture and identifies that suitable nutrition enhances the pluripotent state.

INTRODUCTION

The first publications demonstrating the isolation and culture of human embryonic stem cells (hESCs) used the basal medium DMEM with 20% fetal bovine serum (FBS) (Thomson et al., 1998) in combination with a "feeder layer" of mouse embryonic fibroblasts. This was followed by replacement of DMEM with a low-osmolarity variant called knockout (KO)-DMEM (Amit et al., 2000) before settling on DMEM/F12 with 20% Knockout Serum Replacement (Schulz et al., 2004). Limited work has been completed on comparing suitable basal medium for chemically defined hESC/human induced pluripotent stem cell (hiPSC) culture, but it has been shown that growth in DMEM/F12 is similar to the comparatively simple MEMα (Chen et al., 2011), potentially suggesting that a complex basal medium might not be essential.

DMEM/F12 is one of the most complex of all common basal media and contains 52 components including 14 essential and 6 non-essential amino acids (NEAAs), 8 "B" vitamins plus myo-inositol and choline, 11 inorganic salts, the pH buffer HEPES, and other more unusual components such as hypoxanthine, linoleic acid, lipoic acid, putrescine, and thymidine. The formula of DMEM/F12 is derived from a 50:50 mix of two existing media which were developed in the 1950s and 1960s for mouse fibroblasts (DMEM) and Chinese hamster ovary cells (F12). DMEM is a derivative of the formulation basal medium Eagle's (BME) (Eagle, 1955b, 1955c; Eagle et al., 1956). BME was developed with a compromise approach still in use today: the addition of a small percentage of blood serum (either 2% dialyzed horse serum for mouse fibroblasts or 5% dialyzed human serum for HeLa cells) to provide certain undefined but necessary medium components. This work demonstrated that 13 amino acids were essential (arginine, cystine, glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, and valine) and that 7 amino acids (alanine, aspartic acid, glutamic acid, glycine, hydroxyproline, proline, and serine) were not essential. Later work went on to show that 8 vitamins (choline, folic acid, nicotinamide, pantothenic acid, pyridoxal, riboflavin, thiamine, and myo-inositol) are essential for human cells (Eagle, 1955a; Eagle et al., 1957) and biotin was found to be superfluous. Similar work on the inorganic salts demonstrated clear roles for Na^+, Cl^-, K^+, and $H_2PO_4^-$ in HeLa cells, along with a low concentration of Ca^{2+} and no (or very low) requirement for Mg^{2+} or HCO_3^- (Eagle, 1956). Work on cells in suspension suggested that doubling the concentrations of amino acids was appropriate for high-density cultures (McLimans et al., 1957), which was adopted and became the MEM formula (Lockart and Eagle, 1959). These MEM concentrations were doubled again and the vitamins quadrupled (without experimental evidence provided) in the DMEM formula (Dulbecco and Freeman, 1959). Of note, commercial versions of DMEM (e.g., Gibco or Corning) are often formulated with additional glycine, serine, iron nitrate, and high glucose (25 mM) not in the original formula. Further work suggested that the addition of NEAAs and glycine previously not included in the BME or MEM

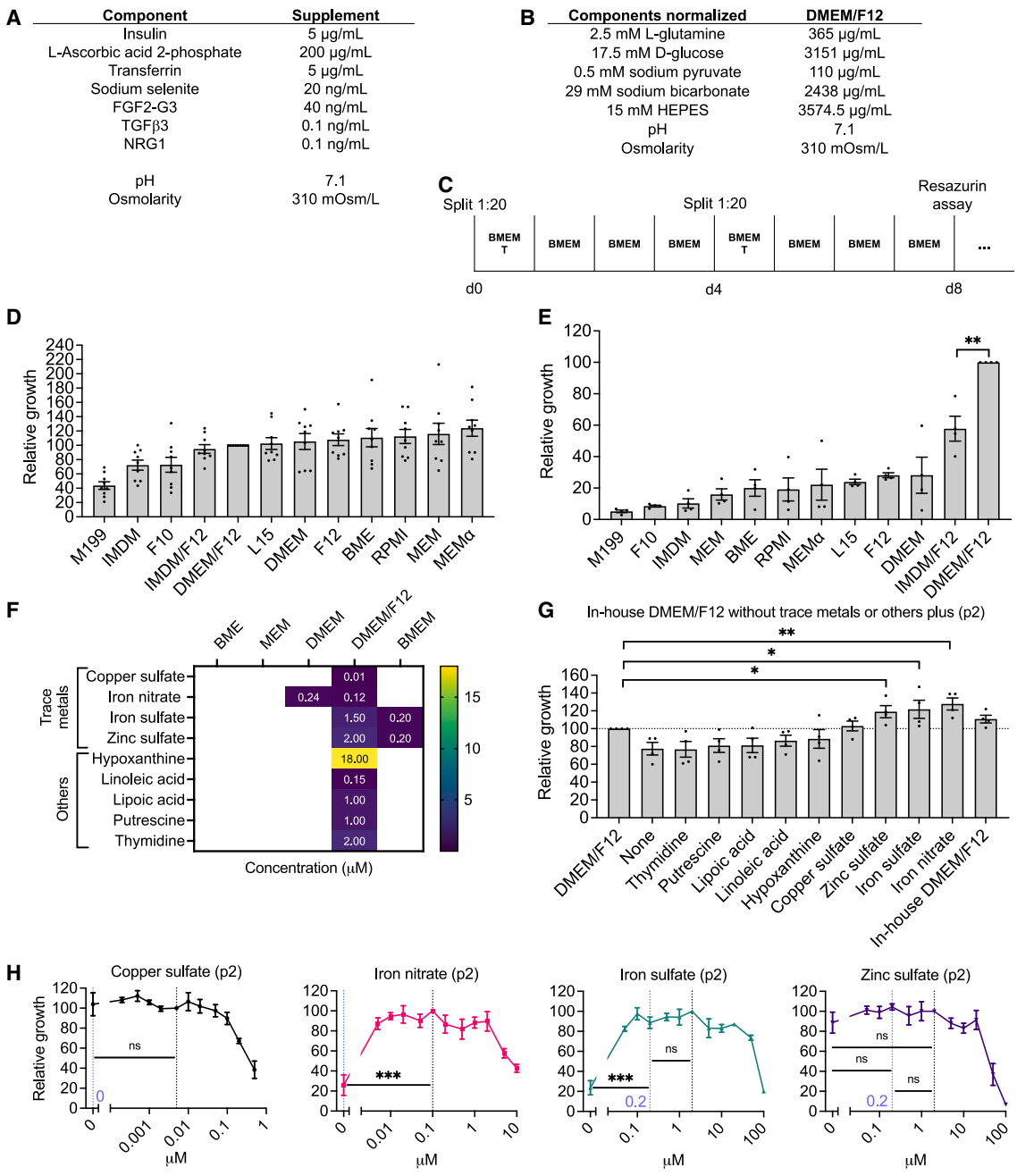

Figure 1. Comparison of existing basal media for hiPSC growth

(A) Concentrations of medium formula components previously optimized for B8 with modifications to reduce cost.

(B) Concentrations of the major components in DMEM/F12. For all commercial basal medium tested, the concentrations of these components were adjusted to match these DMEM/F12 concentrations.

(C) Schematic of daily medium exchange schedule.

(D) Comparison of hiPSC growth in B8 with various common commercial basal media, using a one-passage, daily medium change assay. Normalized to DMEM/F12. n = 9, two hiPSC lines (19c3 and 23c1). None were significantly better than DMEM/F12.

(E) Comparison of hiPSC growth in B8 with various common commercial basal media using a three-passage assay. Normalized to DMEM/F12. n = 4, two hiPSC lines (19c3 and 23c1). All were significantly worse than DMEM/F12.

(F) Heatmap-based comparison of media components found in DMEM/F12 but not in other common media formulations.

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formulations did enhance clonal growth of human cells, especially serine (Lockart and Eagle, 1959).

Contemporaneously, work on the "F"-series of media that resulted in F12 was completed, specifically studying single-cell growth. This started with numerous revisions of a "nutrient solution" supplemented with 30% serum (Cieciura et al., 1956; Puck et al., 1956; Sato et al., 1957) resulting in a serum-free but albumin- and fetuin-containing formula (Fisher et al., 1959). This was followed by studies in CHO cells producing iterations F7, in which hypoxanthine, thymidine, and iron were added (Ham, 1962), F10, in which zinc and copper were also added (Ham, 1963), and finally F12, which added putrescine, linoleic acid, and lipoic acid, and finally eliminated albumin and fetuin (Ham, 1965). In all cases during the development of the F-series media, the formulations contained multiple components that were known to not be required, and no data were provided to explain the rationale or concentration optimization.

As the predecessors of both DMEM and F12 were optimized in the presence of animal-derived serum or albumin and the rationale for the inclusion of many components or their concentrations was not provided, it is unlikely that DMEM/F12 is the optimal formulation for hiPSC growth, especially for chemically defined and weekend-free protocols. Here, it was our goal to develop a basal medium formula that promoted growth of hiPSCs to an identical or superior rate to that of DMEM/F12 and that the formula of this basal medium should use as few components as possible with titrated concentrations, without compromising robustness of growth, reprogramming, or subsequent differentiation. The simplification of this medium makes it more amenable for labs to generate in-house while providing insight into the nutritional and metabolic requirements for hiPSC, and the first knowledge of how the cellular nutrition can enhance the human pluripotent state.

RESULTS

Our prior work established "B8" medium consisting of DMEM/F12 and a supplement containing insulin, L-ascorbic acid 2-phosphate, transferrin, selenite, FGF2-G3, TGFβ3, and NRG1 as well as demonstrating that a pH of 7.1 and osmolarity of 310 mOsm/L was suitable for hiPSC growth (Kuo et al., 2020), and we have since further modified this supplement (Figure 1A). We began studying alternatives to the basal medium DMEM/F12 by assessing the suitability of 12 common commercial media formulations: M199, BME, MEM, MEMα, DMEM, IMDM, RPMI 1640, F10, F12, L15, IMDM/F12, and DMEM/F12. Each commercial medium was normalized to the DMEM/F12 levels of L-glutamine, D-glucose, sodium pyruvate, sodium bicarbonate, pH, and osmolarity (Figure 1B). To assess the effect of basal medium on hiPSC growth, we developed an assay consisting of plating cells in 12-well plates, and growing cells for 4 days with daily medium changes, before passaging at a 1:20 ratio. This was completed for 1–5 passages followed by a cost-effective resazurin viability assay (Figure 1C). The number of passages required for each titration assay was determined as the number required for the control "0 μM" concentration to have no surviving cells, up to a maximum of five passages. This was chosen to minimize the effect of variance caused by repeated manual passaging and replating. At least two hiPSC lines were used in parallel throughout the titration process. Identified adjusted concentrations of each group, such as amino acids, was incorporated prior to beginning the next group, such as vitamins, to bring subsequent titrations as close to final formulation as possible. Once the initial full cycle of titration of all components was complete, a second full cycle of was performed to confirm the results in the final formulation. We also completed identical experiments without daily medium change to identify components and concentrations that support this protocol (Figures 3, 5, and S1), and to allow for development of a simplified hiPSC culture strategy.

Using the common one-passage assay we found that MEM and MEMα were the most suitable formulations, in agreement with previous work (Chen et al., 2011) (Figure 1D). In contrast, when using a more representative three-passage assay, only DMEM/F12 was able to maintain a suitable grown rate (Figure 1E). This suggests that a more complex multi-passage assay is required to establish the basal medium requirements as some components take longer than 4 days to deplete, hence this method was used for subsequent experiments.

(G) Comparison of hiPSC growth in our in-house DMEM/F12 formula without trace metals or extras but with the addition individual components at concentrations used in DMEM/F12. Normalized to commercial DMEM/F12, two-passage assay, n = 4, two hiPSC lines (19c3 and 23c1).

(H) Titration of copper, iron, and zinc concentrations, with daily medium change, two-passage assay, n = 4, two hiPSC lines (19c3 and 23c1). Normalized to concentration in DMEM/F12, indicated by a dashed black line. Concentration selected for BMEM is indicated by a dashed blue line.

All error bars are SEM, and "n" indicates the number of independent replicates from separate experiments. ns, $p > 0.05$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

Low-molecular-weight nutrients

To establish the medium component requirements for hiPSC growth we first turned our attention to the more unique components of DMEM/F12 including the trace metals copper sulfate, iron nitrate, iron sulfate, and zinc sulfate; and the group we defined as "others" consisting of hypoxanthine, linoleic acid, lipoic acid, putrescine, and thymidine (Figure 1F). We developed an in-house formulation of DMEM/F12 without any of these components and tested their individual addition (Figure 1G). Using a two-passage assay, we completed a 4-log concentration titration to identify the suitable concentrations for each of these trace metals (Figure 1H) and identical experiments without medium change (Figure 3A). We found no advantage of the addition of copper to the medium and that it was toxic at higher concentrations (>0.1 μM). Iron is indispensable for respiration and metabolism and was shown to be essential, with either the nitrate or sulfate forms suitable, although both forms were toxic at high concentrations. Zinc, an essential element involved in insulin signaling, was not shown to be essential but did improve growth by approximately 10% and was therefore included. We conservatively selected concentrations of iron sulfate and zinc sulfate that were one dose above the lowest concentration that resulted in the highest growth for both the daily and no medium change data. All other components, hypoxanthine, linoleic acid, lipoic acid, putrescine, and thymidine, were found to be superfluous, suggesting that hiPSCs have no nutritional need for these components (Figure 1G).

Amino acids and metabolomics

We then proceeded to investigate the necessity and suitable concentrations of amino acids, again using a two-passage assay. They were separated into two groups based on their previously determined essentiality *in vitro*: essential amino acids (EAAs) glycine, arginine, cystine/cysteine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, valine, and glutamine; and NEAAs, alanine, asparagine, aspartic acid, glutamic acid, proline, and serine. An initial evaluation showed that cysteine alone can replace the cystine and cysteine combination found in DMEM/F12; therefore cystine, which has poor solubility, was eliminated. From concentration titration assessments, all EAAs were proven essential in the daily medium change experiments (Figure 2A). Interestingly, in the no medium change experiments, addition of glycine was superfluous, suggesting that hiPSCs can synthesize this glycine but at an inadequate rate (Figure 3C). Seven of the EAAs, including glutamine, were suitable at lower concentrations than those in DMEM/F12 (Figures 2B, 3D, and 3E), although none of the DMEM/F12 concentrations appear to have a negative effect on growth (Figure 2A). Conversely, the suitable concentration of tryptophan was found to be ~4-fold higher than that in DMEM/F12. Finally, DMEM/F12 concentrations of histidine, leucine, lysine, methionine, threonine, and tyrosine were shown to be approximately suitable. We also evaluated if the replacement of glutamine, a potential secondary source of energy *in vitro*, with the more stable L-alanyl-L-glutamine (GlutaMAX) was feasible. However, 2 mM L-alanyl-L-glutamine was required to maintain similar levels of proliferation to those of 1 mM glutamine, which would have a significant cost impact in the final formulation. Comparing these suitable concentrations, no difference in hiPSC growth rate was observed, even when using a no medium change strategy (Figures 3C–3E).

The necessity of NEAAs in medium formulae is well-known to be difficult to establish, and information on where the currently used concentrations of NEAAs were derived from is not described (Lockart and Eagle, 1959). To assess the necessity of NEAAs included in the DMEM/F12 formula (Figure 2C), we initially performed the same two-passage assay completed for the EAAs, but found no effect of the absence of any one individual NEAA (Figures S1A and S1B). This finding suggests that NEAAs may have some level of redundancy and/or require longer to deplete, and therefore led us to assess the effect of the addition of NEAAs individually, in pairs, triplets, quadruplets, or quintuplets, in a longer five-passage assay at the concentrations found in DMEM/F12. In this longer assay, the absence of all six NEAAs had no significant negative effect on growth and even the most successful asparagine/proline combination was not significantly better than no NEAAs (Figure 2D). Similar results were seen with the no medium change strategy, with no clear superior combination (Figure S1C). Upon further thorough testing the role of NEAAs with a daily medium change strategy, we found that BMEM versions without NEAA exacerbated the negative effect of normoxic conditions (21% O_2) on growth over our typical hypoxic (5% O_2) conditions (Figure S1D), and further validated the need of NEAAs with a daily medium change schedule. Therefore, although NEAAs are clearly not essential to the BMEM formula, we elected to keep the NEAAs for medium robustness under a variety of conditions, and only eliminate cystine.

Intracellular metabolite analysis found very little difference between cells cultured in BMEM vs. DMEM/F12 with either a daily or no change strategy (Figures S2A, S2B, S3A, and S3B). Phosphoribosylaminoimidazolesuccinocarboxamide (SAICAR), an intermediate in the formation of purines, was the sole metabolite equally upregulated in BMEM cells with and without daily medium change (Figures S2C–S2F).

We also formulated a version of BMEM without the six NEAAs and quantified their intracellular content to

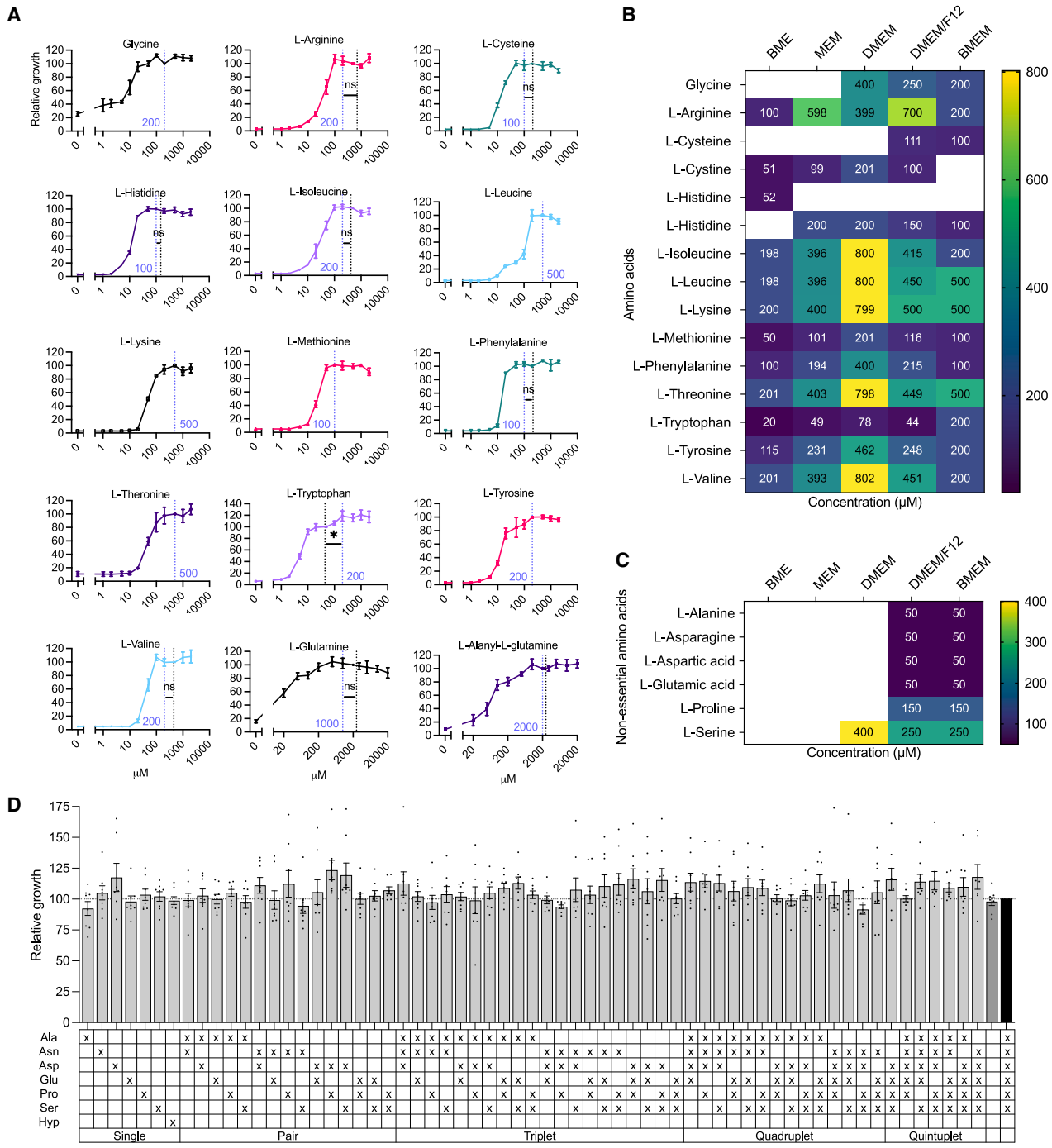

Figure 2. Titration of amino acid concentrations for hiPSC growth

(A) Titration of essential amino acid concentrations with daily medium change. Normalized to concentration in DMEM/F12, indicated by a dashed black line. Concentration selected for BMEM is indicated by a dashed blue line. Two-passage assay (except for L-glutamine and L-alanyl-L-glutamine, which was a three-passage assay), n = 4, two hiPSC lines (19c3 and 23c1).

(B) Heatmap-based comparison of essential amino acid concentrations found in common media formulations compared with BMEM.

(C) Heatmap-based comparison of non-essential amino acid concentrations found in common media formulations compared with BMEM.

(D) Addition of individual non-essential amino acid or combination in pairs, triples, quartets, or quintets, with daily medium change. Five-passage assay, n = 7–8, two hiPSC lines (19c3 and 23c1).

All error bars are SEM, and "n" indicates the number of independent replicates from separate experiments. ns, $p > 0.05$, *$p \leq 0.05$.

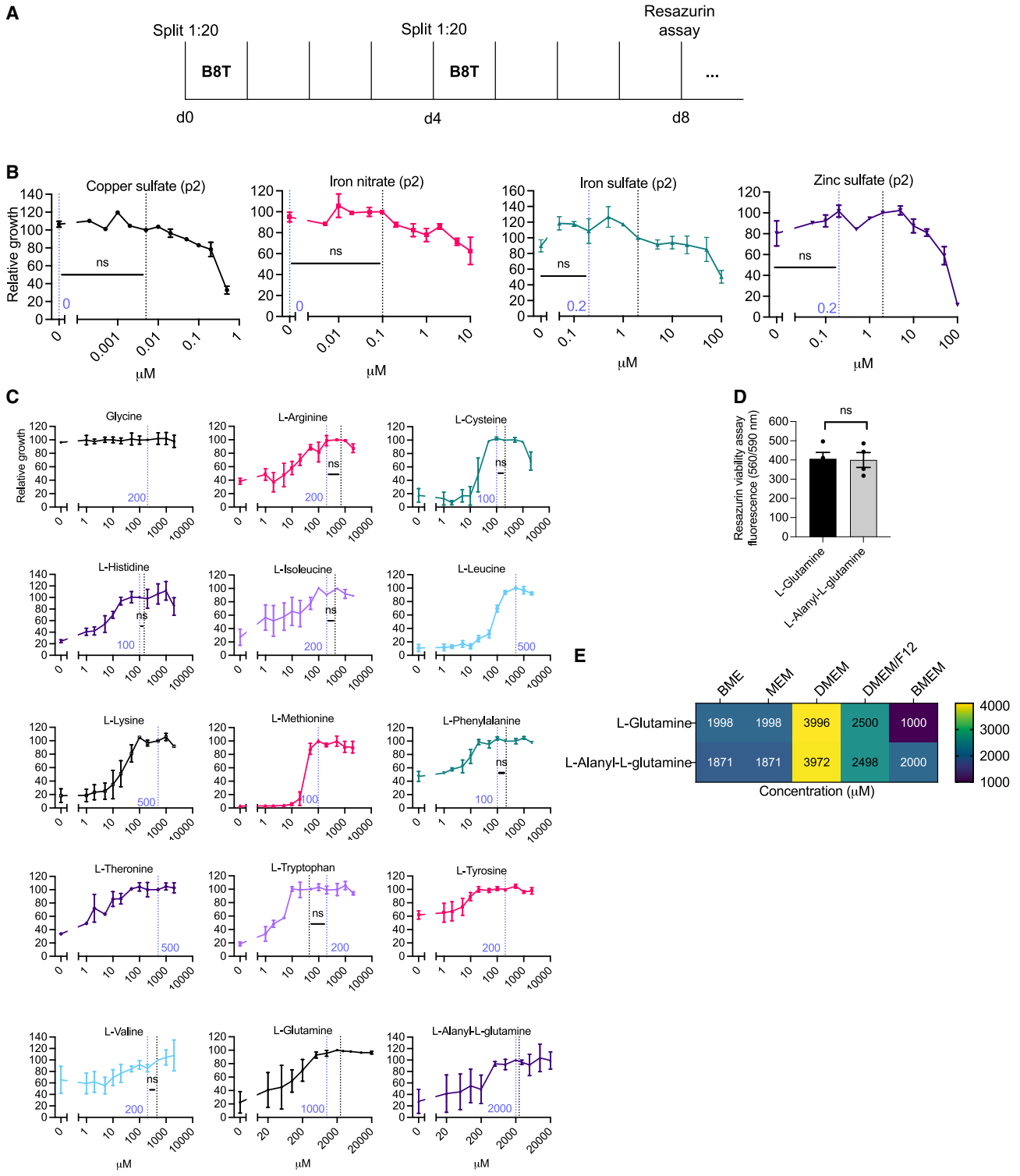

Figure 3. Titration of trace metal and essential amino acid concentrations for hiPSC growth using a "no medium change" strategy

(A) Schematic of no medium change schedule; using this method, thiazovivin is always in the medium.

(B) Titration of copper, iron, and zinc concentrations, without daily medium change, two-passage assay. n = 2, two hiPSC lines (19c3 and 23c1).

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investigate the hypothesis of their *de novo* synthesis by hiPSCs. Of the six NEAAs, only proline was not detected in hiPSCs, although 4-hydroxyproline was abundant. In addition, we found the intracellular concentrations of all NEAAs to be broadly similar (Figure S3C), suggesting that hiPSCs can synthesize these compounds as needed, after adaptation to culture conditions where they are absent.

Finally, our metabolomics analysis measured two "other" components, hypoxanthine and thymidine, that are present in DMEM/F12 but not BMEM. Hypoxanthine was at a higher concentration in BMEM cultured cells, suggesting that these cells can generate this by deamination of adenine. Thymidine was also measured but not found to be present in either DMEM/F12 or BMEM cultured cells. These results further validate the removal of both components in our formulation of BMEM.

Vitamins

To titrate vitamin concentrations, our first step was the replacement of choline chloride with choline bitartrate to avoid the hygroscopic clumping of choline chloride and improve the solubility. The following experiments demonstrated that biotin, folic acid, nicotinamide, pyridoxine, and riboflavin were all sufficient at concentrations lower than those in DMEM/F12, whereas higher concentrations of choline, D-pantothenic acid, and myo-inositol were beneficial (Figure 4A). Similar results were seen in the no medium change experiments (Figure 5A). The minimum number of passages required to identify the effect of deprivation of each vitamin was found to vary from two to five passages. We found that both thiamine and B_{12} are superfluous for hiPSC growth (Figure 4A), although thiamine was required under normoxic conditions (Figure 5B). Thiamine is a coenzyme involved in pyruvate metabolism, the pentose phosphate pathway, and the citric acid cycle, and is included in all common basal media formulae other than BMEM (Figure 4B). B_{12} is a coenzyme in fatty acid metabolism but is not included in the BME, MEM, or DMEM formulae.

Inorganic salts

We next studied the suitable concentrations of the inorganic salts of calcium, magnesium, and potassium. DMEM/F12 contains both $MgCl_2$ and $MgSO_4$, while other basal medium formulae typically use $MgSO_4$ alone, so we began by eliminating $MgCl_2$. We completed individual titration experiments and found that a two-passage assay was suitable and that, for the $CaCl_2$ and KCl ions, the existing concentrations in DMEM/F12 could be reduced (Figure 4C). Of note, rather than low viability, low calcium concentrations reduced the adhesion of hiPSCs to the plates, whereas high concentrations were not soluble. We also found that an increase in $MgSO_4$ concentration enhanced growth (Figure 4C). Similar results were seen in the no medium change experiments (Figure 5C).

Sodium phosphate, glucose, and sodium pyruvate

DMEM/F12 contains two types of sodium phosphate so we began by eliminating the dibasic version, which has poor solubility. We found that, when the medium was suitably pH adjusted, sodium phosphate monobasic was superfluous in the daily medium change assay (Figure 4D), whereas it was essential at around 2 mM in the no medium change assay (Figure 5D), likely due to its role in maintaining pH. The effect of glucose appeared relatively stable from 10 to 30 mM; therefore, 12.5 mM concentration was conservatively chosen (Figure 4D). Sodium pyruvate was shown to be essential and most efficacious at a slightly higher concentration than found in DMEM/F12. Of note, pyruvate, although predominantly known as a source of acetyl-CoA for the citric acid cycle, also has a role in cell culture as an antioxidant (Ramos-Ibeas et al., 2017).

Sodium bicarbonate, HEPES, and pH

All common basal media use a bicarbonate buffering system allowing for the balance of CO_2 in cell culture incubators (typically 5%) to maintain the pH of the cultures. Our previous work has shown that pH 7.1 is most suitable for hiPSC growth (Kuo et al., 2020). We found that, by normalizing the pH to 7.1 as we varied sodium bicarbonate concentrations, a lower level than was suggested previously (Chen et al., 2011) (22.5 mM) was most suitable for hiPSC growth (Figures 4E, 5E, and 5F). This concentration is even lower than is typically found in other basal media (Figure 4H). We also found that HEPES, a second buffering agent, is superfluous (Figure 3F), even in a no medium change method (Figure 5E). As HEPES is responsible for greater than half of the cost of BMEM, this is a useful

(C) Titration of essential amino acid concentrations without daily medium change. Two-passage assay (except for L-glutamine and L-alanyl-L-glutamine which was a three-passage assay), n = 2–4, two hiPSC lines (19c3 and 23c1).

(D) Comparison of growth using a no medium change strategy in media with either 1,000 μM of L-glutamine or 2,000 μM of L-alanyl-L-glutamine, three-passage assay, n = 4, two hiPSC lines (19c3 and 23c1).

(E) Heatmap of concentrations of L-glutamine or L-alanyl-L-glutamine (GlutaMAX) found in common medium compared with BMEM. Normalized to concentration in DMEM/F12, indicated by a dashed black line. Concentration selected for BMEM is indicated by a dashed blue line.

All error bars are SEM, and "n" indicates the number of independent replicates from separate experiments. ns, $p > 0.05$.

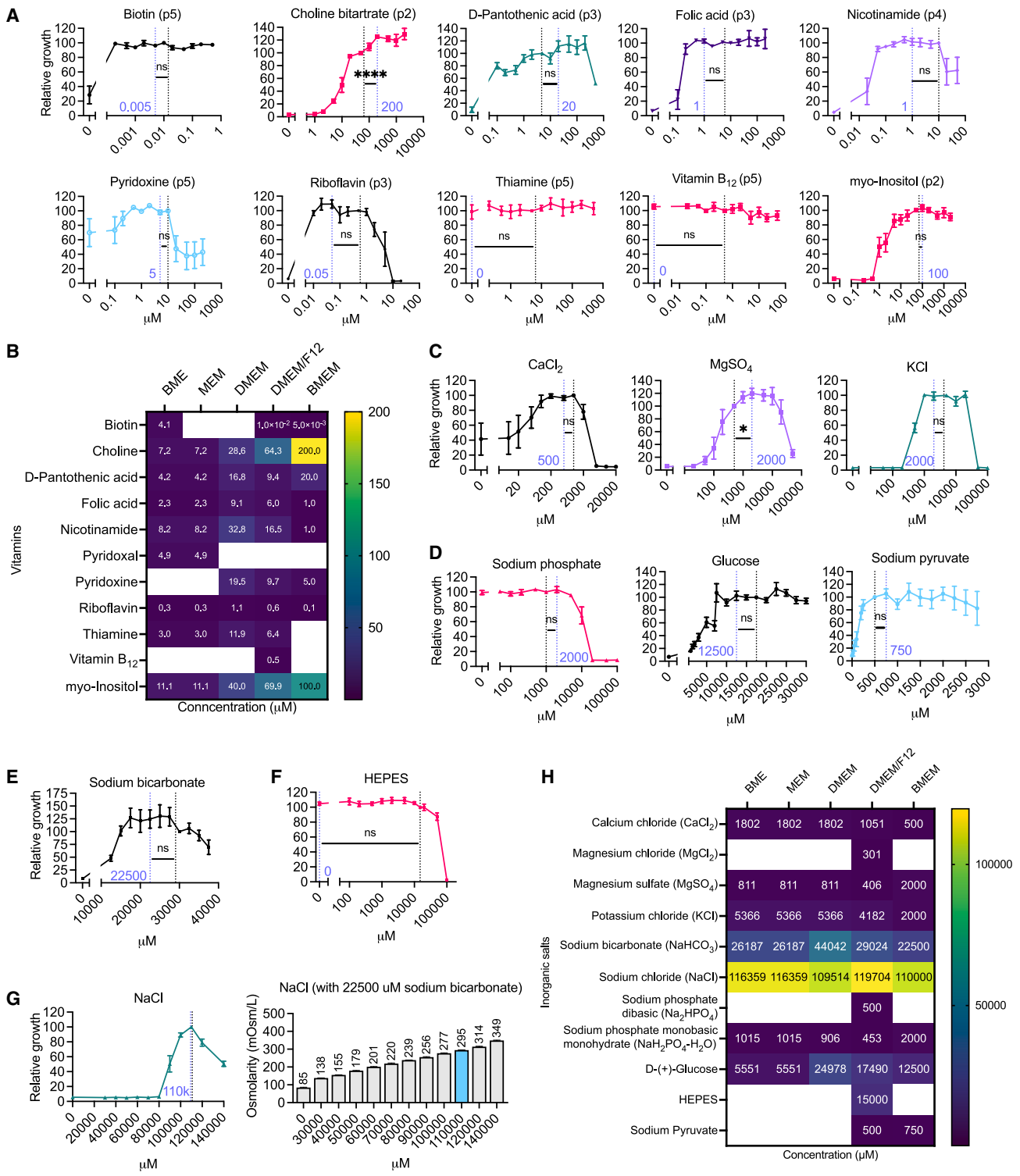

Figure 4. Titration of vitamin, salt, glucose, and pyruvate concentrations for hiPSC growth

Titration experiments with daily medium change, where relative growth is normalized to DMEM/F12 concentration indicated by a dashed black line. Selected BMEM concentrations are indicated by dashed purple lines.

(A) Titration of vitamin concentrations. n = 4–9, two hiPSC lines (19c3 and 23c1), number of passages required for absence of vitamin to result in complete cell death is indicated, up to five passages.

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component to be excluded. The elimination of HEPES from the basal medium allowed us to reduce our formula to 39 components. Of note, the primary role of HEPES is to maintain the pH of the medium when the plate is outside of CO_2 incubator. We found that during typical culture HEPES was not essential but if plates are going to be more than briefly observed under the microscope then the pH will quickly become basic and HEPES should be included.

NaCl and osmolarity

Maintenance of physiological osmolarity is essential for cell culture and NaCl makes up greater than 50% of the mass of typical powdered media. We previously found that an osmolarity of 310 mOsm/L is suitable (Kuo et al., 2020), which was broadly similar to 330 mOsm/L found by others (Ludwig et al., 2006). Here, we found that 110 mM of NaCl is suitable, which produced a slightly lower osmolarity of 295 mOsm/L (Figure 4G).

Effect of basal medium on gene expression

We completed RNA sequencing (RNA-seq) on 15 paired samples cultured either in BMEM or DMEM/F12. Hierarchical clustering of these samples based on the top 250 most variable genes demonstrated that all samples clustered based on the cell line rather than the medium in which they were cultured (Figure 6A). A principal-component analysis of the RNA-seq data further confirmed this observation (Figure 6B). We found that numerous undifferentiated cell genes were expressed significantly higher in hiPSCs cultured in BMEM compared with DMEM/F12, including *KDR*, *SPRY4*, *GDF3*, *NODAL*, *NANOG*, *POU5F1*, *TDGF1*, and *ZIC2* (Figures 6C and 6D). Conversely, *PODXL*, *DMNT3B*, *ZFP42*, and *SOX2*, all had lower expression in BMEM. Interestingly, we found markers of the primed state to be most upregulated in BMEM cultured cells such as *DUSP6*, *ZDHHC22*, *NEFM*, *THY1*, *FAT3*, *STC1*, *KLHL4*, *PLA2G3*, *PTPRZ1*, and *SOX11* (Messmer et al., 2019). Conversely, genes associated with the naive state such as *FST*, *CD24*, *ZIC3*, *DPPA3*, and *DPPA5* had lower relative expression in BMEM. Studying individual genes such as *POU5F1* and *NANOG* (Figures 6E and 6F) we see that the upregulation in BMEM is conserved across most of our samples. Using QIAGEN Ingenuity Pathway Analysis, the only pathway significantly upregulated in cells cultured in BMEM was "role of OCT4 in mammalian embryonic stem cell pluripotency" (Figure S4).

Knowing that many of the medium components that we have removed are involved in cellular respiration, we specifically studied genes associated with metabolism. We observed that in BMEM numerous genes associated with glycolysis are upregulated such as *ENO1*, *HK1*, *HK2*, *PGK1*, *GAPDH*, and *PFKFB3* (Figures S5A and S5C), and particularly glucose transporter proteins 1 and 2 (*SLC2A1* and *SLC2A3*) (Figure S5D). Conversely, mitochondrial metabolism-related genes were downregulated in BMEM-cultured cells, these include components of the pyruvate dehydrogenase complex, *DLAT*, *PDHB*, *SDHB*, *SDHC*, and *SDHD* (Figures S5B and S5E), components of the oxoglutarate dehydrogenase complex including *OGDH* (Figure S5F) and *DLST*, consistent with cases of thiamine deficiency (Bubber et al., 2004).

Characterization and differentiation of BMEM-cultured hiPSC lines

We next characterized hiPSC lines either cultured in BMEM for >30 passages or derived and cultured exclusively in BMEM, compared with those maintained in DMEM/F12-based medium. We evaluated all lines based on their undifferentiated cell marker expression and capacity to differentiate. Cells cultured long term in BMEM (minimum 25 passages) showed similar high-level expression of TRA-1-60 and SSEA4, markers of the undifferentiated state, assessed via flow cytometry (Figure 7A). BMEM-cultured hiPSCs also showed similar capacity to differentiate into cardiomyocytes, endothelial, vascular smooth muscle, neural, and hepatocyte-like cells (Figure 7B). BMEM-cultured cells showed higher, albeit non-statistically significant, growth rates compared with those cultured in DMEM/F12, when daily medium change was performed and only slightly slower growth when using a no medium change protocol (Figure 7C).

(B) Heatmap-based comparison of vitamin concentrations found in common media formulations compared with BMEM.
(C) Titration of inorganic salts concentrations. Two-passage assay (except for sodium phosphate which was a five-passage assay), n = 4–6, two hiPSC lines (19c3 and 23c1).
(D) Titration of sodium phosphate, glucose, and sodium pyruvate concentrations. Two-passage assay, n = 12–21, two hiPSC lines.
(E) Titration of sodium bicarbonate concentration with pH normalized to 7.1. Two-passage assay, n = 12, two hiPSC lines (19c3 and 23c1).
(F) Optimization of HEPES concentration pH normalized to 7.1. Two-passage assay, n = 7, two hiPSC lines (19c3 and 23c1).
(G) Titration of NaCl concentration in the presence of 22,500 μM sodium bicarbonate and its regulation of medium osmolarity. Two-passage assay, n = 4, two hiPSC lines (19c3 and 23c1).
(H) Heatmap-based comparison of inorganic salts and other component concentrations found in common media formulations compared with BMEM.
All error bars are SEM, and "n" indicates the number of independent replicates from separate experiments. ns, $p > 0.05$, *$p \leq 0.05$, ****$p \leq 0.0001$.

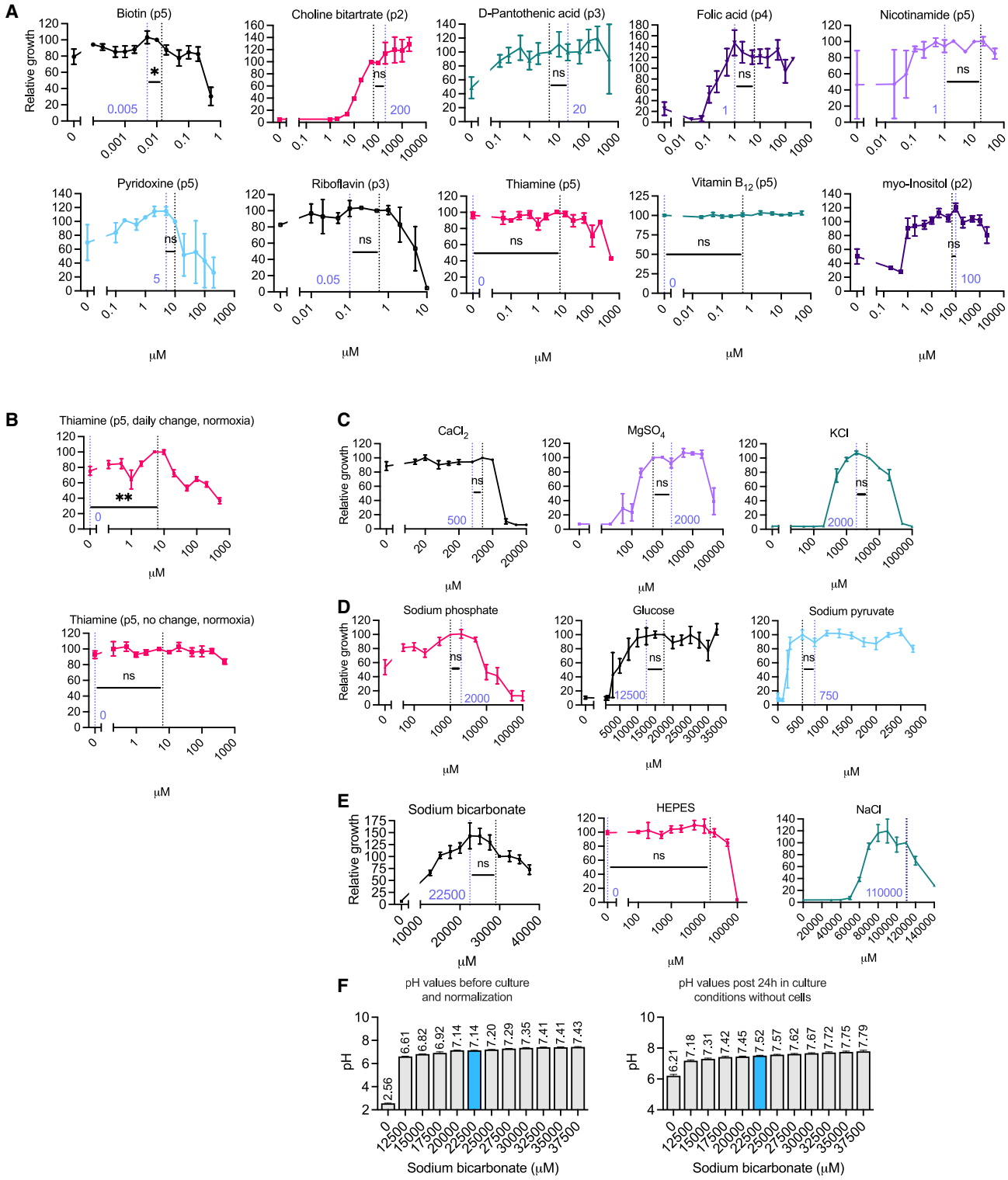

Figure 5. Titration of vitamin, salt, glucose, and pyruvate concentrations for hiPSC growth using a "no medium change" strategy

(A) Titration of vitamin concentrations, without medium change. n = 2–8, two hiPSC lines (19c3 and 23c1).

(B) Titration of thiamine concentrations, with and without medium change when culturing cells in a normoxic (21% O_2) rather than typical hypoxic (5% O_2) environment. n = 2–8, two hiPSC lines (19c3 and 23c1).

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Derivation of hiPSC lines in BMEM

We then investigated if BMEM was suitable for the derivation of hiPSC lines, deriving seven lines exclusively in BMEM. BMEM-derived lines (>p37) presented typical high levels of expression of the undifferentiated cell markers SSEA4, TRA-1-60, and TRA-1-81 (Figure 7D) and positive immunofluorescent staining for POU5F1, TRA-1-60, and SOX2 (Figure 7E). Both BMEM-cultured and BMEM-derived hiPSC lines presented similar morphology, with BMEM cells slightly elongated and skinnier compared with those maintained in DMEM/F12 (Figure 7F). This morphology remains after several passages, but cells still retain distinct hiPSC characteristics such as visible nucleoli, clearly identifiable nuclei, and white cell borders. As a final characterization step, we verified that both BMEM-cultured and BMEM-derived hiPSC lines, as those generated and maintained in DMEM/F12, presented normal karyotypes even after >30 passages, suggesting that both media maintain similar levels of genomic stability (Figure 7G).

Characterization of BMEM-cultured hiPSC lines on commercially available extracellular matrices

Finally, we investigated if BMEM was suitable for hiPSC culture using different commercially available extracellular matrices (ECMs) in addition to standard Matrigel-based culture. BMEM-cultured lines were seeded on Synthemax II-SC (a vitronectin peptide), recombinant human vitronectin (rh-VTN-N), recombinant human laminin 511, Cultrex, Geltrex, and Matrigel, and cultured for five passages. Irrespective of the ECM, BMEM-cultured hiPSC lines presented similar levels of growth (Figure S6A) and expression of undifferentiated markers (Figure S6B). As expected, the different ECMs lead to a small change in morphology (Figure S6C).

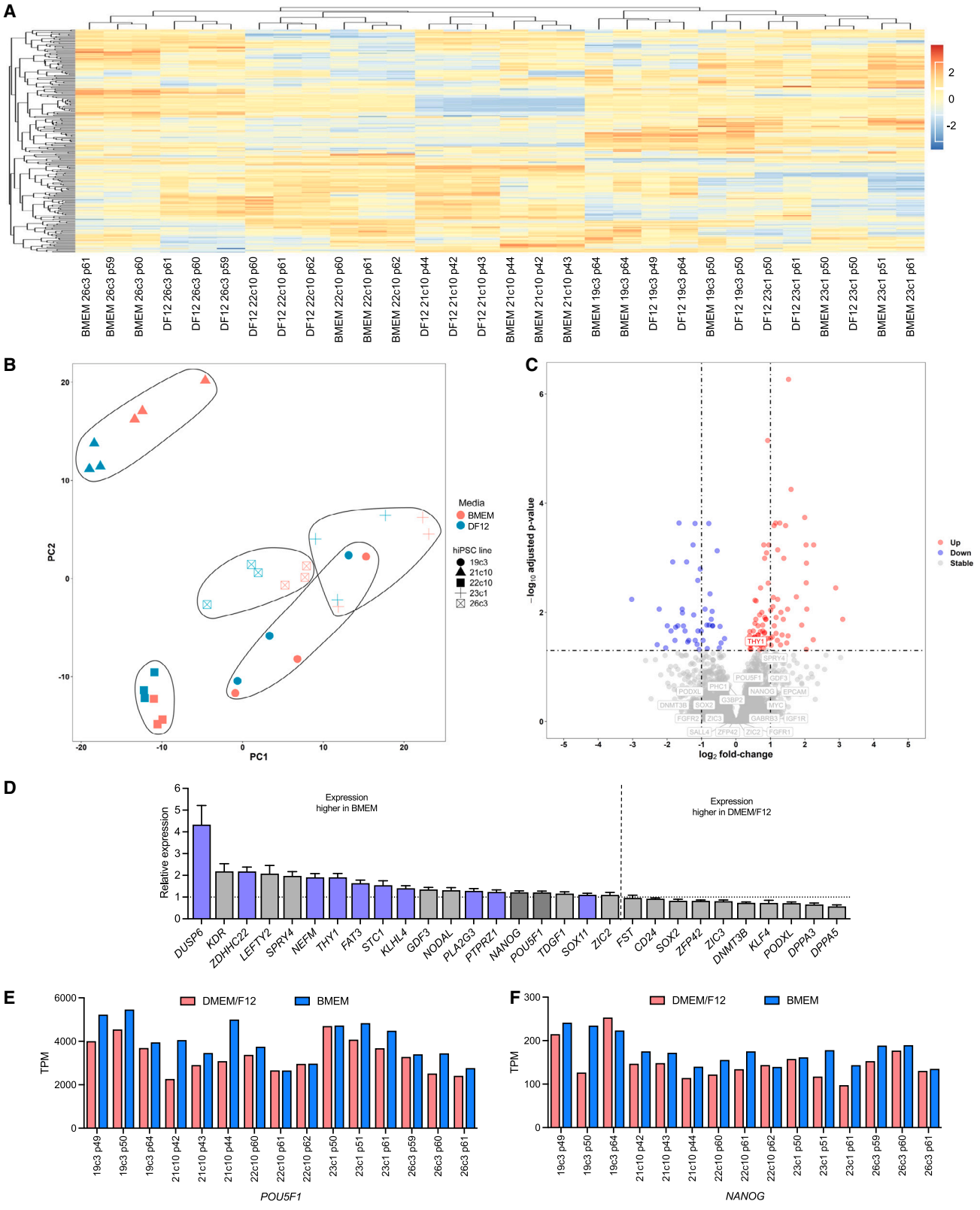
DISCUSSION

Stable and fast pluripotent cell growth is essential for all aspects of hiPSC use and especially integral to efficient differentiation (Burridge et al., 2014). Basal medium selection has an integral role in hiPSC growth in this process and it is therefore somewhat cryptic that the suitability of DMEM/F12 has not been questioned previously. In our prior B8 publication (Kuo et al., 2020), we developed simple methods for comparative analysis of manipulations to hiPSC growth conditions, allowing us here to study multiple components that influence growth that has not been feasible previously. Importantly, although there are many companies that supply DMEM/F12, the exact formula used varies slightly and the specifications for pH and osmolarity are typically wide (Figure S7). Because of this variability, some suppliers and even individual batches from suitable suppliers are suboptimal for hiPSC culture. Indeed, in our own lab we have traced prior issues with slow growth to specific batches of commercial basal media that are within specifications, but out of our defined appropriate range for hiPSC culture. When using BMEM, we specifically define the pH and osmolarity eliminating this source of variability.

From the outset, the development of BMEM was intended to mimic the development of Eagle's minimal essential medium, with the elimination of as many components as possible both to provide insight into the nutritional requirements for hiPSCs and to simplify the generation of this medium in-house. This contrasts with the trend toward creating vastly more complex media for the culture of cancer cell lines including the formulations of SMEM (Tardito et al., 2015), HPLM (Cantor et al., 2017), and Plasmax (Vande Voorde et al., 2019), with the rationale that historic media do not recapitulate the complex nutritional environment of the tumor (Ackermann and Tardito, 2019). A similar rationale has been applied to the culture of neurons with the "BrianPhys" formulation (Bardy et al., 2015). The formulations are theoretically based on the concentrations of metabolites in serum or tumor interstitial fluid, yet each still requires numerous additional components derived from other basal media formulations as well as supplementation with FBS (SMEM and HPLM at 10%, Plasmax at 2.5%), suggesting that merely recapitulating the *in vivo* nutritional environment is not appropriate for *in vitro* culture.

BMEM lacks several components that we would have originally suspected to be essential in a chemically

(C) Titration of inorganic salts concentrations. Two-passage assay, n = 2, two hiPSC lines (19c3 and 23c1).
(D) Titration of sodium phosphate, glucose, and sodium pyruvate concentrations. Two-passage assay (except for sodium phosphate which was a five-passage assay), n = 2–10, two hiPSC lines (19c3 and 23c1).
(E) Titration of sodium bicarbonate, HEPES, and NaCl, with no medium change. Two-passage assay, n = 2–3, two hiPSC lines (19c3 and 23c1).
(F) Assessment of pH value changes in culture conditions without cells. After initial pH measurement, values were normalized to 7.1 and medium was incubated at 37°C with 5% CO_2. All error bars are SEM. All values are normalized to the concentration in DMEM/F12, indicated by a dashed black line. Concentration selected for BMEM is indicated by a dashed blue line.
All error bars are SEM, and "n" indicates the number of independent replicates from separate experiments. ns, $p > 0.05$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.



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defined medium for hiPSC culture. Out of those, linoleic acid would seem to be the most irreplicable as it, and linolenic acid, are considered essential fatty acids and a precursor to many other fatty acids. This raises the question of how BMEM-cultured cells are generating their cell membranes without the lipid contribution to their phospholipid bilayers. We found fatty acid synthase (*FASN*) to be one of the most highly expressed genes in both BMEM- and DMEM/F12-cultured cells, expressed at similar levels to *POU5F1* and, along with the presence of abundant acetyl-CoA and acetyl-CoA carboxylase (*ACACA*), likely allows hiPSCs to synthesize fatty acids via *de novo* lipogenesis.

In typical culture we use three methodologies to reduce the risk of karyotype instability: clump rather than single-cell passaging, inclusion of a ROCK inhibitor (thiazovivin), and use of hypoxic (5% O_2) incubators (Thompson et al., 2020). We have demonstrated that three hiPSC lines derived or cultured in BMEM have no detectable karyotypic abnormalities by G-banded karyotype. These data are preliminary and further in-depth analysis will be required to compare genomic stability of hiPSCs in various media.

Finally, BMEM was developed with both daily medium change and an extreme no medium change feeding schedule. This was done to identify components that are limiting when skipping medium change days or weekends. Overall, the no medium change data did not identify any additional requirements except for the need for sodium phosphate. We conclude that a no change strategy, along a monolayer growth method, is compatible with hiPSC culture long term (44 passages), although it likely has negative effects on differentiation that we would expect are reversible upon resuming more frequent medium changes.

In summary, we have assessed the nutrient requirements for hiPSCs, providing a medium formulation that is simple and cost-effective to make, and that provides valuable insight into the nutritional requirements of human pluripotent cells that can be applied to the development of human pluripotent media. Importantly, BMEM pushes hiPSCs into a higher pluripotent state, potentially providing enhanced stability and differentiation reproducibility as well as providing a new nutrition-based avenue to study the human pluripotency.

EXPERIMENTAL PROCEDURES

Resource availability

Corresponding author

Further information and requests for resources and reagents should be directed to and will be fulfilled by the corresponding author, Paul W. Burridge (paul.burridge@northwestern.edu).

Materials availability

Full details required for the generation of BMEM are provided in the supplemental information.

Data and code availability

RNA-seq data have been deposited in the Gene Expression Omnibus repository at the NCBI (https://www.ncbi.nlm.nih.gov/geo/) under accession code GSE211100.

Human induced pluripotent cell culture

BMEM consists of BMEM basal medium and BMEM supplement. BMEM basal medium consists of a 25× salt solution, 200× vitamin solution (with 30 mL/L 1 N NaOH), and a 200× amino acid solution (with 80 mL/L 12 N HCl) (pH 7.1). These are added to ultrapure water and filter sterilized. BMEM supplement consists of 5 μg/mL *E. coli*-derived recombinant human insulin (Gibco), 200 μg/mL L-ascorbic acid 2-phosphate trisodium salt (AA2P, Wako), 5 μg/mL *Oryza sativa*-derived recombinant human transferrin (Optiferrin, InVitria), 20 ng/mL sodium selenite (Sigma), 40 ng/mL recombinant human FGF2-G3 (made in-house), 0.1 ng/mL recombinant human TGFβ3 (*E. coli*-derived, Qkine), 0.1 ng/mL recombinant human NRG1 (*E. coli*-derived, Shenodoah Biotechnology). Cells were routinely maintained in this medium on 1:800 diluted growth factor reduced Matrigel (Corning) diluted in a BMEM basal at 2 mL per well of a 6-well plate or equivalent. Matrigel-coated plates were kept in CO_2 incubators at 37°C for up to 1 month. BMEM was supplemented with 2 μM thiazovivin (LC Labs), hereafter referred to as BMEM-T, for the first 24 h after passage. For standard culture, cells were passaged at a ratio of 1:20 every 4 days using 0.5 mM EDTA (Invitrogen) diluted in $DPBS^{-/-}$ (without Ca^{2+} and Mg^{2+}, Corning), after achieving ~70%–80% confluence. Cell lines were used between passages 20 and 100. Throughout, pH was adjusted immediately after making the medium at room temperature and atmospheric oxygen and carbon dioxide levels but before filter sterilization. pH was adjusted with 12 N HCl or 1 N NaOH (both from Fisher) and measured using a SevenCompact pH meter (MettlerToledo). Osmolarity was measured using an osmometer (Advanced Instruments).

Basal media comparisons

Twelve basal media were purchased from Gibco: BME (21010046), MEM (11095080), DMEM (11965092), IMDM (12440053), MEMα

Figure 6. Comparison gene expression in hiPSCs after culture in BMEM or DMEM/F12

(A) Hierarchical clustering of 250 most variable genes.

(B) Principal-component analysis for the 30 samples, individual lines are encircled.

(C) Volcano plot with undifferentiated state-affiliated genes labeled; up, higher expression in BMEM.

(D) Comparative expression of undifferentiated state-affiliated genes in BMEM vs. DMEM/F12. Genes associated with the primed state are marked in blue and *POU5F1*, *NANOG*, and *SOX2* are marked in dark gray.

(E and F) Transcripts per million for *POU5F1* (OCT4) expression (E) and *NANOG* (F) showing consistent higher expression in BMEM. All error bars are SEM from 15 conditions/cell lines.

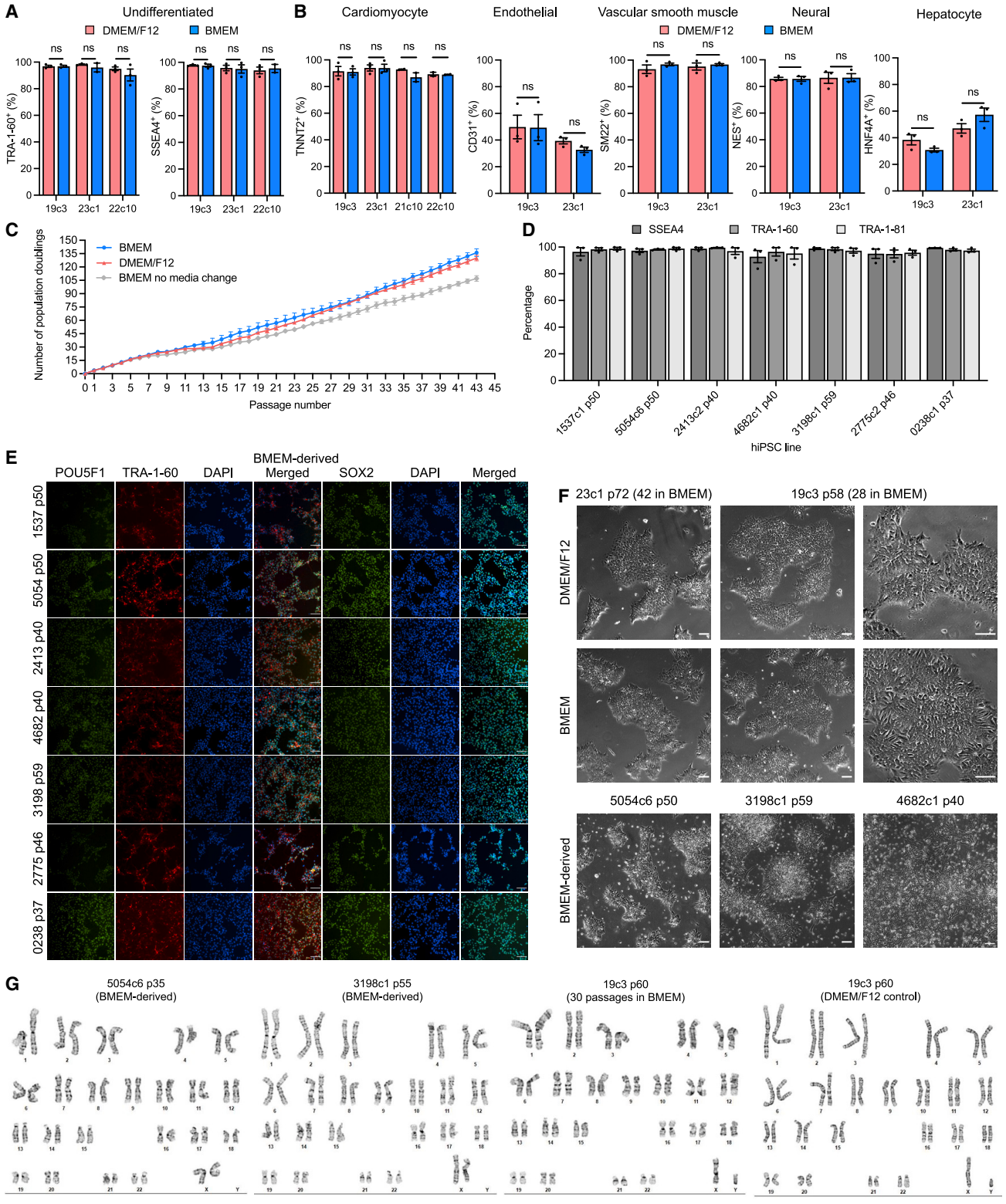

Figure 7. Comparison of hiPSCs cultured and/or derived in BMEM or DMEM/F12

(A) Undifferentiated cell markers for are similarly expressed in hiPSC cell lines 19c3, 23c1, and 22c10 cultured in DMEM/F12 or BMEM (>25 passages).

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(12560156), F12 (11765054), DMEM/F12 (11320032), RPMI 1640 (11875093), M199 (11150059), L15 (11415064), and F10 (11550043). Each was normalized to the DMEM/F12 levels of L-glutamine, D-glucose, sodium pyruvate, sodium bicarbonate, pH, and osmolarity.

Metabolite consumption

hiPSCs were cultured in BMEM, BMEM without NEAAs, or DMEM/F12 with and without daily medium change. Full details of the metabolite analysis protocol are provided in the supplemental experimental procedures.

hiPSC differentiation

hiPSCs were differentiated to cardiomyocytes, endothelial cells, neural progenitors, and hepatocytes using established protocols. Full details of the differentiation protocols are provided in the supplemental experimental procedures.

hiPSC derivation

These lines were used in this publication: 19c3, 21c10, 22c10, 23c1, 1537c1, 5054c6, 6955c3, 4682c1, 4138c2, 3198c1, 2775c2, and 0238c1. 19c3 and 23c1 were reported previously (Kuo et al., 2020). hiPSCs were derived as described previously (Kuo et al., 2020).

Immunofluorescent staining

hiPSCs were dissociated with 0.5 mM EDTA and passaged onto Matrigel-coated 96-well Clear Flat Bottom TC-treated Culture Microplates (Falcon) into BMEM supplemented with thiazovivin for 24 h. After 24 h, medium was changed to BMEM and changed every 24 h until day 4 and stained using typical protocols; further detailed in the supplemental experimental procedures.

Flow cytometry

For live cell staining, hiPSCs were dissociated using TrypLE Express (Gibco) for 3 min at 37°C and endothelial cells were dissociated using Accutase (Corning) for 5 min at 37°C. For fixed cell staining, cardiomyocytes were dissociated using Liberase TH and DPBS as described previously, smooth muscle cells were dissociated with Accutase, and neural and hepatocyte-like cells were dissociated with TrypLE. Cells were stained using typical protocols; further detailed in the supplemental experimental procedures. All cells were analyzed using a CytoFLEX (Beckman Coulter) with CytExpert 2.2 software and the acquired measurements were analyzed using the FlowJo v.10.8.2 software.

RNA-seq

Five hiPSC lines were cultured in BMEM or DMEM/F12 for a minimum of five passages before first RNA extraction. On the day of passage, a minimum of three wells for each condition were selected. Full details of the RNA-seq protocol are provided in the supplemental experimental procedures.

Statistical methods

Data were analyzed in Excel or R and graphed in GraphPad Prism 9.5. Detailed statistical information is included in the corresponding figure legends. Data are presented as mean ± SEM. Data were checked for normal distribution and comparisons were conducted via an unpaired two-tailed Student's t test with significant differences defined as *$p < 0.05$, **$p < 0.01$,***$p < 0.005$, and ****$p < 0.0001$. No statistical methods were used to predetermine sample size. The experiments were not randomized, and the investigators were not blinded to allocation during experiments and outcome assessment.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.stemcr.2023.05.004.

AUTHOR CONTRIBUTIONS

P.W.B. and D.M.L.-L. designed the research. D.M.L.-L., R.R.C., P.P.F., P.P., D.S., D.E.M., E.A.P., C.J.W., M.G., H.J., H.F., and P.W.B. performed the experiments. B.L. and Y.S. analyzed RNA-seq data. P.W.B. supervised the project. P.W.B. and D.M.L.-L. wrote the manuscript with input from all other authors.

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(B) BMEM-cultured hiPSCs (>25 passages) are equally capable of differentiating into cardiomyocytes, endothelial, vascular smooth muscle cells, neural, and hepatocyte-like cells.
(C) Comparison of growth when using BMEM with daily medium change (with thiazovivin for the first 24 h), BMEM without medium change (which has thiazovivin throughout) or DMEM/F12 with daily medium change (with thiazovivin for the first 24 h) over 44 passages, two hiPSC lines (19c3 and 23c1). 19c3 were kept in culture from passage 61 (after 31 passages in BMEM) to passage 105. 23c1 were kept in culture from passage 75 (after 45 passages in BMEM) to passage 119.
(D) Undifferentiated cell marker expression in nine hiPSC lines derived in BMEM at >p37.
(E) Immunocytochemistry analysis of seven hiPSC lines derived in BMEM at >p37. Scale bars, 100 μm.
(F) Phase contrast images of hiPSC lines cultured in DMEM/F12 or BMEM (19c3 [28 passages in BMEM] and 23c1 [40 passages in BMEM]), and hiPSC lines both derived and cultured in BMEM (5054, 3198, and 4682). Scale bars, 100 μm.
(G) Karyotyping for hiPSCs cultured in BMEM for >30 passages (19c3), and derived in BMEM (5054 and 3198) compared with standard culture conditions in DMEM/F12 (19c3). Each dot indicates an independent replicate from a separate experiment (n = 3–12).
All error bars are SEM.

CONFLICT OF INTERESTS

R.R.C., P.P.F., and D.E.M. are employees of Clever Carnivore, Inc. P.W.B is a co-founder of Clever Carnivore, Inc.

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