Activation of the amino acid response modulates lineage specification during differentiation of murine embryonic stem cells

Jixiu Shan, Takashi Hamazaki, Tiffany A. Tang, Naohiro Terada, and Michael S. Kilberg

1Department of Biochemistry and Molecular Biology, McKnight Brain Institute, Shands Cancer Center, and Center for Nutritional Sciences, University of Florida College of Medicine, Gainesville, Florida; and 2Department of Pathology, McKnight Brain Institute, Shands Cancer Center, and Center for Nutritional Sciences, University of Florida College of Medicine, Gainesville, Florida

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IN SOMATIC CELLS, dietary protein or amino acid (AA) deprivation have on ESC properties, as well as on specific lineage formation during in vitro differentiation.

Self-renewal and pluripotency are characteristics of ESC that distinguish them from other cell types (20). The pluripotent nature is demonstrated by their capacity to be differentiated in vitro into all three primitive germ layers, endoderm, ectoderm, and mesoderm (20, 48). Several signaling pathways have been identified as key ESC regulators, and, although many of the mechanistic details remain obscure, the transcription factors octamer-binding transcription factor 4 (Oct4), Nanog, and sex-determining region Y-box 2 (Sox2) play key roles (50). One aspect of ESC self-renewal and pluripotency regulation that has not been extensively explored is the possible impact of nutrient limitation. In vivo, ESC may encounter a microenvironment in which nutrients are limited before vascularization or because of dietary deprivation. Such nutrient limitation may have long-term, detrimental effects on the survival, function, and differentiation of ESC. A significant number of U.S. babies are born small for gestational age as the result of intrauterine growth retardation (IUGR) (reviewed in Ref. 10). IUGR can result from either decreased placental function or poor maternal nutrition during the pregnancy; in either case the net outcome is a reduction of maternal-to-fetal transfer of AA (2). Placentas from human IUGR pregnancies have elevated eIF2 phosphorylation (52), revealing the activation of the Gcn2-eIF2-Atf4 AAR pathway. Beyond the immediate effects of AA limitation on organ development in the fetus, it is now recognized that long-term consequences are also a factor (1). Maternal dietary protein limitation during pregnancy causes genome-wide changes in DNA methylation of the fetus and, subsequently, altered gene expression during adulthood of the resulting offspring (24). These epigenetic effects are thought to contribute to the collection of diseases placed under the umbrella term “metabolic syndrome.”

Links between AA availability and stem cell biology have been reported recently. For example, it was discovered that mouse ESC are uniquely sensitive to threonine deprivation because the conversion of threonine to glycine (for one-carbon metabolism) and to acetyl-CoA (for energy production) is essential for stem cell survival (43). Interestingly, threonine deprivation of ESC selectively suppresses di- and trimethylation of lysine-4 in histone-3 (32), which could lead to epigenetic-related changes during differentiation. In separate studies, Washington et al. showed that elevated proline in the culture medium promotes differentiation of ESC into primitive ectoderm, even in the presence of the differentiation suppressor leukemia inhibitory factor (LIF) (44). Casalino and colleagues subsequently extended those studies and confirmed this unique
influence of proline (4). The data suggest that, at the relatively low proline concentration in normal culture medium, the presence of other AA likely compete with proline for uptake and thereby keep its intracellular levels moderated. In fact, this concept was illustrated by the observation that the addition of glycine to high-proline culture medium results in competition for proline transport (37), and, consequently, glycine blocks the elevated proline induction of differentiation to primitive ectoderm (44). Tan et al. have suggested two important consequences of these observations (37). 1) The recognized heterogeneity within ESC cultures might result from activation of extrinsic differentiation programs through culture medium components, such as proline. 2) On the other hand, suppression of ESC differentiation by reducing culture medium proline levels might be useful as an experimental tool to minimize the heterogeneity within ESC cultures. Collectively, these investigations begin to provide a window into the fundamental impact that AA availability has on ESC function.

The present data demonstrate that ESC and differentiating embryoid bodies (EB) exhibit a functional AAR. Neither AAR activation nor Atf4 deficiency caused significant detrimental effects on ESC self-renewal and pluripotency. However, triggering even a low level of the AAR during differentiation caused significant changes in lineage specification, favoring endodermal lineages. Surprisingly, knockdown of one of the principal AAR mediators, Atf4, further enhanced this endodermal formation, indicating that alternative pathways are responsible for the shift toward endoderm. These results contribute to the emerging evidence that AA availability is a critical consideration during culture and differentiation of stem cells in vitro.

METHODS

Cell culture and differentiation. The R1 murine embryonic stem cell line was maintained on gelatin-coated dishes in a maintenance medium made with Knock-out Dulbecco’s modified Eagle medium (KDMEM) (GIBCO-BRL, Grand Island, NY) supplemented with 10% knockout serum replacement (GIBCO-BRL), 1% FBS (Atlanta Biologicals, Norcross, GA), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 25 mM HEPEs (GIBCO-BRL), 300 µM monothioglycolic acid (Sigma, St. Louis, MO), and 1,000 U/ml recombinant mouse LIF (Chemicon, Temecula, CA). Cells were maintained at 37°C in a 5% CO2-95% air incubator. ESC were plated at a density of 2 × 10⁴ cells/60 mm dish, the medium was replaced every other day, and the cells were passaged every 3rd day. To ensure the cells were in the basal (“fed”) state, cell cultures were replenished with fresh medium at 12 h before initiating experimental treatment. To activate the AAR pathway, cells were incubated in ES-LIF medium containing 2 mM histidinol (HisOH) (Sigma). HisOH blocks charging of histidine onto the corresponding tRNA and thus activates AAR signaling in a manner analogous to histidine deprivation (14). We have documented that HisOH treatment mirrors histidine-deficient medium with regard to induction of the AAR and the associated increase in transcription for AA-responsive downstream genes (18, 39).

To induce differentiation, ESC were resuspended in Iscove’s Modified Dulbecco’s Medium (IMDM) lacking LIF as a differentiation medium (no. 12440–053; Life Technologies) containing 20% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 300 µM monothioglycolic acid (Sigma) at a cell density of 60–70 cells/µl. To generate EB, about 70 “hanging drops,” 2,000 cells in 30 µl, were placed on the inside surface of a polystyrene petri dish top (Fisher Scientific), and then the lid was carefully inverted and put back on the bottom of the dish in which there is 5–10 ml of sterilized water to maintain humidity within the dish. After 2 days, petri dish lid, with the hanging drops still attached, was removed, and the EB from about 70 hanging drops were collected and pooled, transferred to the bottom of a new polystyrene petri dish with fresh differentiation medium, and then cultured for two more days. On day 4, the EB were transferred to 60-mm tissue culture dishes (Sarstedt) coated with 0.1% gelatin (Millipore, Phillipsburg, NJ). For HisOH treatment during differentiation, EB were cultured in IMDM differentiation medium containing 0.5 mM HisOH. As described in results, this HisOH concentration was chosen based on preliminary studies.

Cell counting and flow cytometry. For cell counting, ESC were treated with 0.125% trypsin (GIBCO-BRL), and resuspended in KDMEM, as described above. The ESC suspension was diluted appropriately, and cell numbers were analyzed with a Coulter counter (Coulter ZM). For cell cycle analysis, about 1 × 10⁵ cells were fixed with ice-cold 100% ethanol and stained for 30 min at room temperature in a solution containing 2 ml 1% propidium iodide, 0.1% Triton X-100, and 0.2 mg/ml DNase-free RNase A. The data were acquired on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA), using a 15-mW argon ion laser at 488 nm to excite propidium iodide, and fluorescence emission was collected at 585 nm. Cell Quest Version 3.3 software was used to collect and store the data. ModFit software Version 3.1 (Verity Software House, Topsham, ME) was used to perform the cell cycle analysis. For each time point, three independent samples were analyzed, and the studies were performed with at least two different batches of cells. For expression analysis of stage-specific embryonic antigen (SSEA1), 2 × 10⁴ cells were resuspended in PBS solution containing 0.1% FBS and a 1:500 dilution of either anti-SSEA1 (no. sc-21702) or a control mouse IgM (no. sc-2870), each labeled with phycoerythrin (Santa Cruz Biotechnology), and incubated on ice for 30 min. Stained cells were washed once with PBS containing 0.1% sodium azide and then resuspended in 500 µl of this solution containing 0.5% paraformaldehyde. Data collection was performed with a FACS Calibur flow cytometer and Cell Quest Version 3.3 software, as described above. The fluorescence was plotted as a histogram with markers set to calculate the percent-positive cells, using the isotype-matched control sample to adjust for background fluorescence.

RNA isolation and real-time quantitative RT-qPCR analysis. Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. A 2-µg aliquot of total RNA was used to synthesize first-strand cDNA with the SuperScript III First-Strand Synthesis Kit (Invitrogen). For real-time quantitative PCR (RT-qPCR), synthesized cDNA was diluted 10× with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), and then 2 µl were mixed with 10 µl of SYBR Green master mixture (Applied Biosystems, Warrington, UK) and 5 pmol of forward and reverse primers in a total volume of 20 µl. The qPCR was performed with a DNA Engine Opticon 3 system (Bio-Rad), and the mixture was subjected to 40 cycles at 95°C for 30 s and 60°C for 60 s. The primers used are listed in Table 1. After qPCR, melting curves were acquired by a stepwise increase of the temperature from 55 to 95°C to ensure that a single product was amplified in the reaction. Either glyceraldehyde-3-phosphate dehydrogenase or ribosomal protein large ribosome subunit protein 7a (L7a) mRNA content was used as an internal control. All calculations were based on the difference of C(T) (the threshold cycle) of the analyzed gene relative to the internal control mRNA content in the same sample. Transcription activity of the Asna gene was monitored by RT-qPCR as described previously (6), using a pair of primers (Table 1) that amplify the junction of intron 4 and exon 5 so that the relative amount of short-lived, unspliced heteronuclear RNA is measured.

Measurement of protein synthesis. Protein synthesis rate was measured by culturing ESC or differentiating EB in 2 mM HisOH as described above. During the last 1 h of incubation, 0.6 µCi/ml [3H]leucine was added to the culture medium. After the cells were washed with ice-cold PBS, the cells were incubated for 30 min in
ice-cold 5% trichloroacetic acid. The precipitates were washed one time with fresh ice-cold 5% trichloroacetic acid and two times with water and then solubilized with 0.1 M NaOH and 0.5% SDS. The counts per minute of [3H]leucine incorporated were measured by liquid scintillation counting and the data normalized to the total precipitated protein for each sample. The data are presented as counts per minute [3H]leucine incorporated per microgram protein, and the value for the DMEM control was set to 100%.

Knockdown of Atf4. The expression of Atf4 was suppressed by vector-based short-hairpin RNA (shRNA) using the following sequence: forward: 5'-TCGAGACCGTATCTGTCATCTACATGAAATC-3', and reverse: 5'-GATCCTTCCAAAAAAGTATCTGAAAGACCTGATATTCA-3'. This target sequence was selected from three separate sequences within the Atf4 mRNA that were chosen with the aid of computer analysis (51) and then tested in a preliminary series of studies. A scrambled DNA sequence was selected from three separate sequences within the Atf4 sequence was chosen with the aid of computer analysis (51) and then subjected to immunoblotting as described previously (31). The anti-Atf4 polyclonal (1:5,000) was a custom antibody; the rabbit anti-Atf3 polyclonal (1:200, no. sc-150); mouse anti-Oct4 monoclonal (1:500, no. sc-17320) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit anti-Nanog polyclonal (1:1,000, no. SC1000; Calbiochem, Darmstadt, Germany); rabbit anti-Ser51 phospho-eIF2α and anti-total eIF2α polyclonal (nos. 9721 and 9722, Cell Signaling Technology, Danvers, MA). The effect of R1 ESC respond to the AAR in a similar manner, cells were treated with HisOH, which mimics histidine deprivation by blocking His-trNA synthesis (14), and thus activates Gcn2. A concentration of 2 mM HisOH was chosen for the ESC experiments because this was within the middle of the range that enhanced Atf4-driven asparagine synthetase (Asns) transcription (data not shown) but resulted in only a 20% decline in protein synthesis (Fig. 1A). The effect of

### RESULTS

**A functional AAR exists in murine R1 embryonic stem cells.** In somatic cells, triggering the AAR through AA limitation activates the Gcn2 kinase, which leads to a cascade of increased phosphorylation of eIF2α, synthesis of the transcriptional activator Atf4, and, subsequently, the Atf4 feedback suppressors Atf3, C/ebp homology protein (Chop), and C/ebpβ (reviewed in Ref. 21). To determine if mouse R1 ESC respond to the AAR in a similar manner, cells were treated with HisOH, which mimics histidine deprivation by blocking His-trNA synthesis (14), and thus activates Gcn2. A concentration of 2 mM HisOH was chosen for the ESC experiments because preliminary experiments revealed that this amount was within the middle of the range that enhanced Atf4-driven asparagine synthetase (Asns) transcription (data not shown) but resulted in only a 20% decline in protein synthesis (Fig. 1A). The effect of

### Table 1. Primers used for real-time quantitative PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>Asns</td>
<td>5-CTTGTGGTGGCGGTTCCATTTAC-3</td>
<td>5-ACGCCAGATTGTCTTTCAGGTCCTC-3</td>
</tr>
<tr>
<td>Asns</td>
<td>5-GGCCTGCTCTCAAAAGGTCT-3</td>
<td>5-AAGGAAGAGCTCAGATTTT-3</td>
</tr>
<tr>
<td>Atf4</td>
<td>5-ACCTCATGCTCTGCAAGCAGA-3</td>
<td>5-GCCCAAGCATCACCTATAGGC-3</td>
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<td>Oct4</td>
<td>5-CCAGAGAGTCTCAGGAAGAC-3</td>
<td>5-ACGCCAAGCTGCTGCTCTTG-3</td>
</tr>
<tr>
<td>Nanog</td>
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<td>5-GCGATTGCTGGAAGATG-3</td>
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<tr>
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<td>5-GCGTTCTCAGAAAAATTTCTTCAA-3</td>
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<td>5-GATTCTGCTGAGAAATGGACCC-3</td>
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<td>5-GAACAGAGAACGAGGCGG-3</td>
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<td>5-GAACAGAGAACGAGGCGG-3</td>
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<td>ActC</td>
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<td>5-GGAGGACTGACAGCCTACTGTA-3</td>
</tr>
<tr>
<td>Gapdh</td>
<td>5-GGAGTGCTGGAGGCGGACTCA-3</td>
<td>5-GCCCAAGCTCAGGTCCTCAG-3</td>
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<tr>
<td>L7a</td>
<td>5-AATGCCGGTCAAGTTAGGCAC-3</td>
<td>5-GAGGAGACTGACAGCCTACTGTA-3</td>
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Asns, asparagine synthetase; Atf4, activating transcription factor 4; Oct4, octamer-binding transcription factor 4; Sox2, sex-determining region Y-box 2; Fgf5, fibroblast growth factor 5; Sdc4, syndecan 4; Dab2, disabled homolog 2; Hnf3β, hepatocyte nuclear factor 3β; Krt8, keratin 8; Nfn, neurofilament medium protein; ActC, actin c; Mhc-α, myosin heavy chain α; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; L7a, large ribosome subunit protein 7a.
a 24-h HisOH treatment on cell growth showed a statistically significant 30% decline in total cell number after 24 h of growth (Fig. 1B), and the percentage of cells in the G1 phase of the cell cycle rose from 20 to 28% (Fig. 1C). For somatic cells, AA deprivation is known to cause accumulation of cells in G1 (26). The observable morphology of individual cells and colonies was not changed to a visible degree, and trypan blue staining revealed little or no cell death in the control or HisOH-treated population (data not shown). All of these observations are consistent with those reported for somatic cells after AAR activation.

No published reports have documented the AAR in ESC. The AAR is a collection of signaling pathways that lead to transcriptional programs mediated by c-Jun, Atf2, and Atf4 (21). NF-κB is also activated by AA limitation (19), but no specific downstream AAR-associated gene targets have been identified. Thus far, studies show that Atf2 action is dependent on either c-Jun (12) or Atf4 (5), and ESC are known to lack c-Jun expression, an observation that we have confirmed for the murine R1 ESC used in the present studies. Consequently, we focused on the function of the Gcn2-eIF2-Atf4 pathway by monitoring the levels of phosphor (p)-eIF2α, Atf4, Atf3, and C/ebpβ. All three isoforms of C/ebpβ were induced (LAP* and LAP, two forms of "liver-activating protein" and LIP, "liver-inhibitory protein"). The blot was also reprobed for tubulin as the loading control. The immunoblot shown is from a single experiment, but the study was repeated with an independent batch of cells to confirm qualitative reproducibility of the changes observed. E: ESC were incubated in KDMEM ± 2 mM HisOH, and, at the times indicated, transcription activity for the AAR marker gene asparagine synthetase (Asns), relative to the glyceraldehyde-3-phosphatase (Gapdh) internal standard, was measured by real-time quantitative PCR (RT-qPCR). The results represent the averages ± SD from three biological replicates, and the experiment was repeated to determine reproducibility. Where not shown, the SD bars are within the symbol, and an asterisk designates a P value of ≤0.05 relative to the KDMEM control at the same time point.
C/ebpβ after 2 mM HisOH treatment (Fig. 1D). The results show that within 2 h p-eIF2α was increased and remained elevated throughout the 24-h period investigated. Likewise, Atf4 protein content was increased by 2 h, peaked at 8 h, and then declined. Expression of Atf3 and C/ebpβ was also observed, but, consistent with their proposed function as feedback suppressors of AAR-activated genes (6, 27, 39), their accumulation lagged behind that for Atf4 (Fig. 1D). As the result of translational control mechanisms (8), C/ebpβ is expressed as three isoforms, and all three C/ebpβ isoforms were elevated in the ESC during HisOH treatment (Fig. 1D). It is interesting to note that after a 24-h incubation in complete KDMEM, detectable induction of p-eIF2 and Atf4 also occurred. This result suggests that, during routine cell culture, even at low density, ESC may deplete the medium of nutrients such that cell stress pathways are activated. The most likely candidates to be responsible for this increase are the eIF2 kinases Gcn2 (amino acid limitation) or RNA-dependent protein kinase-like ER kinase (glucose deprivation) (45).

Transcription activity from the Asns gene was monitored during a 24-h time course as a marker for Atf4-driven gene expression during the AAR (33). Compared with cells cultured in control medium (KDMEM), those incubated in HisOH exhibited an increase in Asns transcription activity that was detectable at 2 h, reached a peak of about sixfold at 12 h, and then declined to about two- to threefold by 24 h (Fig. 1E). The decline in Asns transcription between 12 and 24 h (Fig. 1E), despite maintenance of elevated p-eIF2α and Atf4 (Fig. 1D), is consistent with results obtained in somatic cells in which Atf4-dependent induction of Atf3, C/ebpβ, and Chop serves as feedback repressor of the Atf4-driven Asns induction, even with continued AAR activation (6, 35). Collectively, the data demonstrate that mouse ESC exhibit a functional AAR that exhibits properties similar to those observed in fully differentiated cells.

Characterization of the ESC response to AAR activation. The self-renewal and pluripotency properties of ESC are controlled by a gene regulatory network in which Oct4, Nanog, and Sox2 play central roles (50). To determine if activation of the AAR affects the expression of these ESC-specific genes, mRNA and protein for each was measured. As shown in Fig. 2A, during a 24-h time course of HisOH treatment, no significant difference was detected in the mRNA content of Oct4 and Sox2, but a slight decline occurred for Nanog. Compared with extracts from ESC incubated in KDMEM control medium, the protein content of Sox2 was unaffected, but...
Oct4 and Nanog protein content was decreased in the HisOH-treated cells (Fig. 2B). The SSEA1, a membrane surface marker for mouse ESC (3, 16), was analyzed by flow cytometry in ESC incubated for 12 or 24 h in HisOH (Fig. 2C). The data revealed no major change in the number of SSEA1-expressing cells. The results demonstrate that ESC-specific marker genes are not strongly activated or repressed by the AAR pathway, but, consistent with a moderate decline in global protein synthesis, the protein content for selected markers was suppressed.

**Activation of the AAR differentially modulates lineage specification.** The “hanging drop” method was used to induce differentiation of ESC, and, after the indicated time of EB development, example gene markers for specific lineages were monitored by RT-qPCR (Fig. 3A). Over the 12-day period investigated, distinct cell morphologies were clearly visible within each colony. For example, beginning at day 11, islands of beating cardiomyocytes were readily apparent (data not shown). Analysis of specific lineages illustrated that, as ESC abundance declined, as illustrated by Oct4, markers reflective of primitive ectoderm [fibroblast growth factor 5 (Fgf5)] and mesendoderm (brachyury) were transiently detectable between days 2 and 8 (Fig. 3B). The gene markers for the three primary germ layers ectoderm [neurofilament medium protein (Nfm)], mesoderm [myosin heavy chain α (Mhc-α)], and endoderm [keratin 8 (Krt8)] were readily detected by day 12.

To assess the influence of a low level of AA deprivation on lineage formation, AA limitation was mimicked by activation of the AAR through HisOH treatment of the EB beginning at day 2 of culture and continuing through day 12. A preliminary series of experiments determined that 0.5 mM HisOH was the minimal concentration that caused a significant induction of the AAR marker genes Asns and Atf4 (Fig. 4A) while still permitting continued cell growth and differentiation. Assessment of protein synthesis rates during the HisOH treatment revealed that on day 7 there was no significant effect, whereas on day 11 protein synthesis was decreased by about 35% (data not shown), a level of protein synthesis suppression similar to that observed in somatic cells. Note that activation of the AAR through the use of the HisOH, rather than histidine deprivation, does not reduce the cytoplasmic concentration of histidine for metabolism. For each of the lineages monitored, the mRNA expression of markers was analyzed by RT-qPCR, and the results were normalized to the mRNA abundance for ribosomal protein L7a, which is not affected by AA limitation or HisOH treatment. By day 8, 6 days after initiating HisOH treatment, the ESC markers Oct4 and Nanog were significantly reduced in the control cells, whereas activation of the AAR caused a suppression of ESC loss (Fig. 4B). When Fgf5, a marker for primitive ectoderm, was measured, the day 2 and day 4 control cultures exhibited the typical transient existence of this lineage, but the Fgf5 expression was largely inhibited by HisOH treatment. This result suggests that the AAR minimized the accumulation of this stage of differentiation within the cultures (Fig. 4C), either because the differentiation process is blocked at an earlier step or because the transition through this stage is enhanced. As discussed below, formation of primary germ layers within the cultures, despite the presence of HisOH (see Fig. 4F), indicates that transition through the primitive ectoderm stage must occur.

**Primitive endoderm** (Fig. 4D: Gata4, Gata6) and visceral endoderm [Dab2 and syndecan 4 (Sdc4); Fig. 4E] are cell lineages that, in part, lead to extraembryonic tissues (28, 36). Markers for both were increased within 48 h of HisOH addition (i.e., day 4) and remained elevated relative to control values through day 12. Using Nestin and neurofilament medium protein (Nfm) as two independent measures for cell lineages that are ectodermal, it was observed that the abundance of Nestin was significantly suppressed from day 4 to day 12, whereas the Nfm marker was largely unchanged (Fig. 4F). These data illustrate that, within the spectrum of lineages arising from a primary germ layer, there are differences in the response to nutrient availability. The increase in abundance for the two markers for the mesoderm lineage, Mhc-α and ActC, was strongly suppressed when the AAR was activated (Fig. 4G). Consistent with these mesodermal marker studies, in the control differentiating cultures incubated for 14 days, each EB colony exhibited beating cardiomyocytes, whereas those cultures maintained in HisOH from day 2 to day 14 did not

Fig. 3. Differentiation of murine R1 ESC into embryoid bodies (EB) prepared by the hanging-drop method. A: the genes that were used as markers that reflect differentiation stages and tissue lineages, Fgf5, fibroblast growth factor 5; Dab2, disabled homolog 2; Sdc4, syndecan 4; Nfm, neurofilament medium protein; ActC, actin c; Mhc-α, myosin heavy chain α; Krt8, keratin 8; Hnf3β, hepatocyte nuclear factor 3β. B: as described in METHODS, ESC were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM) differentiation medium to produce EB, which eventually contain numerous cell lineages. On the indicated days (d), total RNA was isolated, and the mRNA levels for genes representing specific cell lineages, as listed in A, were analyzed with RT-qPCR (B). The data are normalized to the mRNA level for ribosomal protein L7a, which is not affected by AA limitation or HisOH treatment. By 10.220.33.6 on June 20, 2017 http://ajpendo.physiology.org/ Downloaded from
contain any beating cells (data not shown). Interestingly, if the day 2 EB were transferred to HisOH for 4 days and then switched back to HisOH-free control medium for an additional 14 days, the abundance of beating cardiomyocytes that developed was equal to that in control cultures incubated in the absence of HisOH for the entire 18-day period. These latter studies indicate that the effect of AAR activation simply delays mesoderm formation rather than irreversibly blocking their development.

Fig. 4. Effect of AAR activation on lineage specification during in vitro ESC differentiation. Murine R1 ESC were cultured in IMDM differentiation medium to produce EB, which by day 12 contain the cell lineages outlined in Fig. 3A. To test the effect of the AAR, beginning on day 2 the EB were incubated in either control IMDM medium (open bars) or IMDM containing 0.5 mM HisOH (filled bars) and on the days indicated; total RNA was collected and analyzed for lineage-specific markers by qRT-PCR. The marker gene products analyzed were: positive controls for the AAR (Asns and Atf4, A), nondifferentiated ESC (Oct4 and Nanog, B), primitive ectoderm (Fgf5, C), primitive endoderm (Gata4 and Gata6, D), visceral endoderm (Dab2 and Sdc4, E), and the primary germ layers, ectoderm (Nestin and Nfm, F), mesoderm (Mhc-α and ActC, G), endoderm (Krt8 and Hnf3β, H). The data were normalized to the mRNA level for ribosomal protein L7a, which was assayed as an internal control and plotted as the fold change relative to the value of ESC (d0). For each experiment, the RT-qPCR was performed using three biological replicates, and the data represent the averages ± SD. An asterisk indicates a P value of ≤0.05 vs. the IMDM control value at each time point. I: to illustrate that the changes in mRNA were also observed at the level of protein expression, immunoblotting for representative lineage markers was done using protein extracts from EB on days 10 and 12 of differentiation. For all of the panels, the results presented are from an individual experiment, but qualitatively similar results were obtained in replicate experiments.
differentiation. For two independent markers of the endoderm, Krt8 and hepatocyte nuclear factor 3β (Hnf3β), the effect of HisOH treatment slightly decreased Krt8 abundance, but the level of Hnf3β was increased at day 8 (Fig. 4H). However, by day 12, both markers were significantly increased in response to the AAR. To establish that the changes observed in lineage formation could be detected at the level of protein expression, example markers for ESC and lineages that were increased or decreased by HisOH were monitored by immunoblotting (Fig. 4I). The level of p-eIF2α was measured to illustrate the AAR activation. A decrease in the mesodermal marker ActC as well as increases in Gata4 (primitive endoderm) and Dab2 (visceral endoderm) showed that protein expression correlated with the AAR-induced changes in differentiation. Collectively, these results illustrate that activation of the AAR pathways leads to significant changes in cell specification during differentiation of mouse EB.

An alternative explanation for the observed induction of the primitive and visceral endoderm is that the change in mRNA could result from AA-dependent upregulation of the marker gene rather than a change in cell abundance within the culture. To address this issue, EB were differentiated until day 12, exposed to HisOH for 8 h to activate the AAR, and then Gata4, Gata6, Sdc4, and Dab2 were analyzed. Although the positive control for the AAR (Asns) showed an increase in expression, none of the marker genes was significantly increased (data not shown). These results indicate that the large and significant changes observed in the experiments depicted in Fig. 4, D and E, were the result of differences in cell number rather than a change in gene expression within a constant population of cells.

Role of ATF4 in the AAR-induced changes in cell specification.

To investigate the possible role of Atf4 in EB differentiation, ESC were transfected with a control shRNA (shCtr) or an shRNA specific for mouse Atf4 (shAtf4) and selected for stable expression. After induction by HisOH treatment, the shAtf4-ESC exhibited significantly reduced Atf4 mRNA and protein expression relative to the shCtr-ESC population (Fig. 5A). To illustrate the functional consequences of the Atf4 knockdown, it was established that the AAR induction of the Atf4 target genes Atf3 and Chop was blocked in the shAtf4-ESC (Fig. 5B). Conversely, relative to the shCtr-ESC, the Atf4-deficient ESC showed no significant changes in the Oct4, Nanog, and Sox2 stem cell markers (Fig. 5C).

With the use of the shCtr- and shAtf4-ESC lines, differentiation of EB was performed in the presence or absence of 0.5 mM HisOH for 12 days, and then the abundance of markers for specific lineages was analyzed to assess the consequences of Atf4 deficiency (Fig. 6). As shown in Fig. 4 for wild-type day 12 EB, the AAR-induced enhancement of visceral endoderm (Dab2, Sdc4; Fig. 6A) and of the endodermal marker Hnf3β (Fig. 6B) was still evident in the shCtr cells. Although the original hypothesis was that Atf4 was the likely mediator of this enhancement and that loss of Atf4 would blunt the AAR enhancement of lineage specification, surprisingly, the knockdown of Atf4 actually resulted in a further increase in abundance for each of the endodermal lineages in the HisOH-treated EB (Fig. 6). Although the overall abundance of ectoderm (Nestin) was increased in the shAtf4 cultures incubated in control medium, consistent with the data shown in Fig. 4, HisOH caused a partial suppression in the shCtr cells, and this effect was also observed in the Atf4-deficient cultures (Fig. 6B). The results in the wild-type day 12 EB revealed a strong inhibition of the mesodermal lineage (Mhc-α), and that result was also observed for both the shCtr and the shAtf4 cultures (Fig. 6B). Collectively, the results indicate that the enhancement of the endodermal lineages by the AAR was increased even further in Atf4-deficient cells.
caused no detectable change in ESC self-renewal or pluripotent differentiation toward primitive endoderm, visceral endoderm, and certain mesoderm, and ectoderm. In contrast, differentiation and inhibited the increase in the formation of primitive ectoderm, even in the presence of the extraembryonic tissue, such as the yolk sac (28, 36).

Collectively, the results in this report indicate that mouse ESC and EB respond to environmental nutritional queues with regard to AA availability. We show that mimicking AA deprivation by activation of the AAR alters the differentiation program such that endosomal lineages are favored, whereas others are suppressed. These results are consistent with interesting observations showing enrichment of specific lineages after manipulation of nutrients by other laboratories. Based on gene expression and metabolic flux studies, Tohyama et al. reasoned that a medium lacking glucose but containing lactate might favor cardiomyocyte survival (41). Culture of differentiating EB from either human or mouse ESC in the high-lactate medium resulted in an enrichment of cardiomyocytes up to 99% purity. Similarly, Tomizawa et al. also used tissue-specific differences in metabolic pathways to propose that a medium deficient in arginine, tyrosine, glucose, and pyruvate but enriched in ornithine, phenylalanine, galactose, and glyceral would enrich the population for hepatocytes (42). After 14 days of growth in their “hepatocyte-selection medium” compared with normal differentiating medium, the number of cells was reduced by 60-fold, but those cells remaining exhibited morphology and a gene expression pattern indicative of hepatoblast-like cells. In a third example of metabolic modulation of lineage selection, two independent groups have documented that high levels of culture medium proline promotes differentiation/survival of primitive ectoderm, even in the presence of the differentiation suppressor LIF (4, 44). Thus, there is growing evidence that supports the concept that changes in cell culture nutrients can modulate lineage specification.

In somatic cells, activation of the AAR leads to a transient suppression of global protein synthesis as the result of a decrease in translation initiation (22). Our results show that mouse ESC respond in a similar manner, exhibiting about a 20% decrease in protein synthesis after an 8-h HisOH treatment. Consistent with this decline, after a 24-h HisOH treatment, Oct4 and Nanog protein levels were suppressed, but neither Sox2 nor SSEA1 protein levels were affected. Such differences likely reflect the half-lives of these ESC marker proteins. Although a decline in Oct4 and Nanog protein content might lead one to speculate that differentiation may be triggered by the AAR, based on visual morphology and differentiation markers, this did not appear to be the case. Guo et al. also observed that downregulation of ESC Oct4 did not induce differentiation (13). Given that a much greater proportion of ESC are within the cell cycle S phase compared with somatic cells, the effect of the AAR on the cell cycle was not predictable. However, ESC behaved in a manner similar to somatic cells in that a reduction at the G1/S transition was observed. In HepG2 hepatoma cells, it has been documented that the AAR pathway results in increased expression of the cyclin kinase inhibitors p21 and p27, which likely contributes to G1 cell cycle arrest (23). It has been reported that p21 and p27 are undetectable in mouse ESC (47), so the AAR-induced mech-}

**Fig. 6.** Influence of Atf4 knockdown on cell lineage specification in differentiating EB. ESC with (shAtf4) and without (shCtr) stable Atf4 knockdown were used to generate EB by the protocol described in METHODS. After incubation in control IMDM differentiation medium (IMDM) or IMDM containing 0.5 mM HisOH (HisOH) from days 2 to 12, total RNA was isolated, and the mRNA levels for genes representing specific cell lineages were analyzed by RT-qPCR. The selected markers were as follows: visceral endoderm (Dab2 and Sdc4) (A), and ectoderm (Nestin), mesoderm (Mhc-α), and endoderm (Hnf3β) (B). The data are normalized to the mRNA level for ribosomal protein L7a and plotted as the fold change relative to the value in ESC. The RT-qPCR was performed on three independent samples of ESC for each condition within an experiment, and the data are shown as the averages ± SD. The results presented are representative of multiple experiments, and the asterisk indicates a P value of ≤0.05 vs. the corresponding shCtr condition.

**DISCUSSION**

This manuscript provides new evidence that the AAR is functional in mouse ESC and influences the in vitro differentiation outcome in EB. Investigation of nutrient sensing and regulatory mechanisms in ESC and EB is limited, and this report represents the first characterization of the AAR. The data support the following observations. 1) The Gcn2-εIF2-Atf4 signal transduction pathway is functional in ESC and was activated by the AA-deprivation mimic HisOH. 2) The mRNA and protein content of the ESC markers Sox2 and SSEA1 were largely unaffected by AAR activation for 24 h, whereas Nanog and Oct4 protein expression was decreased. 3) After a 24-h HisOH treatment, there was no observable change in ESC morphology, but the rate of protein synthesis, cell proliferation, and cell cycle were moderately suppressed in a manner similar to the response in somatic cells. 4) Maintenance of differentiating EB for 12 days in an amount of HisOH that activated a consistent but low level of the AAR suppressed the loss of ESC and inhibited the increase in the formation of primitive ectoderm, mesoderm, and ectoderm. In contrast, differentiation toward primitive endoderm, visceral endoderm, and certain endoderm-derived lineages was enhanced by AAR activation. 5) Stable knockdown of Atf4, a primary mediator of the AAR, caused no detectable change in ESC self-renewal or pluripo-
anism that causes ESC to accumulate at the G1/S transition will require further study.

Beyond AA control of Gcn2 activity, increased Atf4 synthesis can be triggered by additional eIF2 kinases that are activated by cellular stresses such as arsenite, endoplasmic reticulum stress, ultraviolet irradiation, hypoxia, and viral infection (reviewed in Ref. 45). Although not definitive proof, the existence of the AA-responsive Atf4 pathway suggests that the ESC may also respond to these other stimuli through Atf4-dependent mechanisms. Consistent with its primary role as a stress-inducible factor, stable knockdown of Atf4 in the ESC did not appear to significantly alter the basic functions of self-renewal and pluripotency. However, even in the absence of exogenous stress, Atf4 has critical roles during mouse development. Atf4 knockout mice exhibit deficiencies in formation of bone (49), lens of the eye (17, 38), and hematopoietic cells (25). Given that in vitro differentiation of EB represents a model system for the earliest events of development, the effect of Atf4 deficiency was tested in the present studies. In the absence of stress (culture in IMDM), Atf4 deficiency caused an increase in ectoderm (Nestin) but no effect on formation of mesoderm or endoderm. Surprisingly, when the Atf4-deficient EB were subjected to AAR activation by HisOH treatment, rather than suppressing the AAR-triggered increase in the visceral endosome and endosome lineages observed in control cells, the reduction of Atf4 actually resulted in a further enhancement of those lineages. These results suggest that an AAR-associated pathway other than the Gcn2-eIF2-Atf4 cascade mediates the observed changes in selective lineage formation in the presence of the AAR. Given that Atf4 knockdown enhanced this differentiation process, it is possible that Atf4 may actually counterbalance or feedback inhibit the pathway to endosomal lineages by some unknown mechanism.

There are multiple candidates that may participate in this Atf4-independent signaling, including NF-κB (19), MAPK (40), and c-Jun (11). It may be possible to correlate their expression or activity with the AAR-induced promotion of the endodermal lineage. However, unlike Atf4, each of these proteins has many fundamental cellular functions independent of AA-regulated gene expression, and, consequently, even partial knockdown is likely to produce detrimental effects unrelated to the AAR over the required 12-day period of differentiation. Alternative approaches will be necessary to investigate the possible role of these factors.

In addition to the obvious immediate impact of decreased AA supply on fetal tissue development and function, it has become clear that long-term consequences of poor fetal nutrition occur through epigenetic mechanisms. Such epigenetic effects are the basis for the “fetal origins of adult disease” hypothesis (1). For example, maternal protein restriction during development leads to genomewide changes in fetal DNA methylation, which in turn leads to altered gene regulation during adulthood of the offspring (24). A recent expression microarray analysis after activation of the AAR by HisOH treatment revealed 19 enzymes associated with histone methylation/demethylation that were increased by more than twofold (30). As an example, the impact of the AAR on one of these, Junomji domain-containing 3, has been more extensively investigated to document its transcriptional control (29). For many of these AA-responsive histone-modifying enzymes, critical contributions to the regulation of ESC differentiation have been well documented (7). Thus, AAR-induced changes in both DNA and histone methylation may influence early development of the primary germ layers, long-term organ development, and organ function during adulthood. Consequently, in the context of proposing therapeutic use of ESC, induced pluripotent stem cells, or in vitro differentiated tissue, investigators should consider nutrient supply as a critical factor during maintenance in cell culture and throughout differentiation protocols.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

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