1-Proline induces differentiation of ES cells: a novel role for an amino acid in the regulation of pluripotent cells in culture

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Washington JM, Rathjen J, Felquer F, Lonic A, Bettes MD, Hamra N, Semendric L, Tan BS, Lake JA, Keough RA, Morris MB, Rathjen PD. 1-Proline induces differentiation of ES cells: a novel role for an amino acid in the regulation of pluripotent cells in culture. Am J Physiol Cell Physiol 298: C982–C992, 2010. First published February 17, 2010; doi:10.1152/ajpcell.00498.2009.—The development of cell therapeutics from embryonic stem (ES) cells will require technologies that direct cell differentiation to specific somatic cell lineages in response to defined factors. The initial step in formation of the somatic lineages from ES cells, differentiation to an intermediate, pluripotent primitive ectoderm-like cell, can be achieved in vitro by formation of early primitive ectoderm-like (EPL) cells in response to a biological activity contained within the conditioned medium MEDII. Fractionation of MEDII has identified two activities required for EPL cell formation, an activity with a molecular mass of <3 kDa and a second, much larger species. Here, we have identified the low-molecular-weight activity as l-proline. An inhibitor of l-proline uptake, glycine, prevented the differentiation of ES cells in response to MEDII. Supplementation of the culture medium of ES cells with >100 M l-proline and some l-proline-containing peptides resulted in changes in colony morphology, cell proliferation, gene expression, and differentiation kinetics consistent with differentiation toward a primitive ectoderm-like cell. This activity appeared to be associated with l-proline since other amino acids and analogs of proline did not exhibit an equivalent activity. Activation of the mammalian target of rapamycin (mTOR) signaling pathway was found to be necessary but not sufficient for l-proline activity; addition of another activators of the mTOR signaling pathway failed to alter the ES cell phenotype. This is the first report describing a role for amino acids in the regulation of pluripotency and cell differentiation and identifies a novel role for the amino acid l-proline.

PROTOCOLS THAT ACHIEVE THE formation of highly enriched or homogenous populations of functionally normal cells from pluripotent cells in culture will have applications in a number of developing areas. They will provide a more sophisticated understanding of the mechanisms regulating cellular differentiation in vitro, augment understanding of differentiation events in the early embryo, and may have implications in understanding the behavior of cells undergoing rapid growth and change in normal and disease states. Clinically, they have potential utility in the formation of therapeutically relevant cells, capable of replacing damaged, dysfunctional, and/or deficient cell populations within the body. The identification of regulatory molecules, including bioactive small molecules, is critical to the successful development of differentiation protocols. They will provide probes for identifying and manipulating signaling pathways and scalable technologies capable of delivering enriched cell populations for scientific and clinical use.

Differentiation of pluripotent cells within the mammalian embryo initiates with the formation of the epiblast, or primitive ectoderm, from the inner cell mass (ICM). In mouse, primitive ectoderm formation initiates around 4.75 days postcoitum (dpc) in response to signals from the adjacent extraembryonic endoderm (1, 64) and is characterized by the loss of the ICM-specific markers Nanog, Rex1, Spp1, Gbx2, and Crtr1 and upregulation of the epiblast bud marker PRCE (5, 9, 48). This is accompanied by an increase in cell number and projection of the pluripotent cell mass into the blastocoel cavity. Between 5.0 and 5.25 dpc, expression of Fgf5 is upregulated and the pluripotent cells rearrange into an epithelial sheet, the primitive ectoderm (15, 31). The maintenance of pluripotency within the primitive ectoderm is demonstrated by continued expression of the pluripotent markers, Oct4, Sox2, and alkaline phosphatase (29, 67, 69), and by the ability of the cell population to differentiate into all cell types of the embryo and adult (2, 3, 40). The molecular mechanisms required for formation of primitive ectoderm from ICM are not well understood.

Mouse embryonic stem (ES) cells are derived from pluripotent cells of the ICM (24, 43) and maintain many of the properties of this population, including the ability to differentiate to form cell populations representative of the three primary germ layers, extraembryonic endoderm, and the germ lineage (37, 53). ES cells are able to contribute to all lineages of the embryo and adult after injection into the blastocyst (6), suggesting that these cells can respond appropriately to the inductive signals present during embryonic development, including those regulating the formation of primitive ectoderm. Culturing ES cells in a conditioned medium, MEDII, derived from the human hepatocarcinoma cell line HepG2, results in the formation of early primitive ectoderm-like (EPL) cells (39, 50, 51). Expression of Oct4, Sox2, and alkaline phosphatase (34, 51) and a differentiation potential in culture that includes the formation of populations of the mesoderm, endoderm, and ectoderm lineages (39, 50, 51) (Rathjen J, unpublished observations) identifies EPL cells as pluripotent. The alteration in colony morphology (51), downregulation of ICM- and ES cell-specific markers Rex1, CRTR-1, Psc, Gbx2, Spp1, and Nanog, upregulation of the primitive ectoderm and EPL cell...
markers Fgf5, Otx2, Dnmt3b, and PRCE (34, 39, 48, 51), and a restricted ability to form cell populations characteristic of the primitive endoderm lineage (39), however, are consistent with the formation of primitive ectoderm. The ability to recapitulate the formation of primitive ectoderm from ICM with the formation of EPL cells from ES cells in culture provides a manipulatable system to understand the signals regulating this process.

It has become increasingly clear that amino acids can function as signaling molecules in the regulation of many cellular processes. Within the nervous system the excitatory amino acids, l-glutamate, l-aspartate, and d-aspartate, act as neurotransmitters (59). Similarly, glycine, through the glycine receptor chloride channel receptor, has roles in neuronal excitation and inhibition in the developing and mature central nervous system and has been implicated in inflammatory pain sensitization (4). The aromatic amino acids have been shown to be effective activators of the extracellular calcium-sensing receptor and intracellular calcium mobilization; amino acid activation of this receptor has been implicated in gastric acid and pancreatic hormone regulation in the gut and water excretion in the kidney (13). Potential roles for amino acid regulation of ES cell function have not been explored despite the demonstrated involvement of amino acids in regulating early embryonic development (27).

Amino acids and amino acid receptors have been shown to activate the mammalian target of rapamycin (mTOR) signaling pathway. mTOR is an evolutionarily conserved serine/threonine kinase, belonging to the phosphoinositide kinase-related kinase (PIKK) family (16, 32, 57, 68). mTOR activity integrates signals from nutrients, growth factors, energy status, and stress and regulates cellular processes including cell cycle progression, protein synthesis, autophagy, ribosome biogenesis, glucose homeostasis, and metabolism (32, 57, 68). mTOR<sup>−/−</sup> mouse embryos die around the time of primitive ectoderm formation from a failure of ICM development and trophodermctoderm proliferation (26, 46). In vitro, mTOR signaling pathway is required for ES cell proliferation (46); involvement in cell differentiation has not been determined.

Fractionation of MEDII has identified two bioactivities required for EPL cell formation, a low-molecular-mass activity (<3 kDa) and larger species (>30 kDa) (51). In this article, we identify the low-molecular-mass activity as l-proline. Addition of an inhibitor of l-proline uptake to MEDII prevented differentiation, suggesting that uptake of free proline is required for the formation of EPL cells. Addition of l-proline to ES cells at concentrations >100 μM, in the presence of 1,000 U/ml leukemia inhibitory factor (LIF), was accompanied by changes in colony morphology, gene expression, and differentiation kinetics consistent with differentiation toward a primitive ectoderm-like cell. The activity associated with l-proline appeared restricted to this molecule since other amino acids tested and analogs of proline did not exhibit the bioactivity. Many, but not all, l-proline-containing peptides were active. An inhibitor of mTOR signaling, rapamycin, prevented l-proline activity, suggesting a requirement for mTOR signaling. The inability of l-leucine or glycine to act like l-proline, despite activating mTOR in ES cells, suggests that the kinase activity is required but not sufficient for l-proline activity. These data suggest a novel role for the imino acid l-proline in the regulation of pluripotency and identify a small molecule regulator of pluripotent cell differentiation.

**EXPERIMENTAL PROCEDURES**

**Chromatography.** HepG2 cells (38) were maintained as previously described (51), and the conditioned medium from these cells, MEDII, was prepared as described by Rathjen and Rathjen (52). Serum-free MEDII (sMEDII) was produced from HepG2 cells as previously described (51).

sMEDII (250 ml) was separated into eluted (E) and retained (R) fractions using a Diaflo YM3 membrane (Amicon). The E fraction was further purified by sequential chromatography over a Sephacryl G10 column (11 cm × 11.3 cm ID; Pharmacia) and a 10-μm normal-phase silica HPLC column (Waters Radial Pak LC 10 μm column; 8 mm ID) as previously described (51). Active fractions from HPLC were pooled, lyophilized, and resuspended in 50 μl water. A 10-μl aliquot was applied at a flow rate of 25 μl/min to a Superdex PE 3.2/30 gel filtration column connected to a SMART micropurification system (Pharmacia) equilibrated in water at room temperature. Twenty-five microliter fractions were collected. Equivalent fractions from the five runs (125 μl total) were pooled, and 50 μl of the pool was tested for its ability to induce an alteration in ES cell colony morphology.

**Amino acid analysis.** Active fractions from the Superdex chromatography were analyzed for amino-acid content. Fractions were lyophilized, derivatized with 9-fluorenylmethylchloroformate (FMOC) and o-phthalaldehyde (OPA), and amino-acid analysis was conducted with and without hydrolysis using a Hewlett-Packard Amino-Quant II analyzer.

**ES cell culture.** ES cells were cultured as described by Rathjen and Rathjen (52) in ES cell medium [DMEM with high glucose and no HEPES (Life Technologies no. 11995-065) supplemented with 10% fetal calf serum (FCS), 40 μg/ml gentamycin (Pharmacia and Upjohn), 0.1 mM β-mercaptoethanol (β-ME; Sigma Aldrich), and ~1,000 units mouse LIF (produced as described in Ref. 51)]. A clonally derived ES cell line, EPLBR, isolated from <i>day 4 </i>D3 embryoid bodies (EBs) (52) was used in experiments shown in Fig. 1A and Table 1; this line was used in assays developed for factor purification because it displayed a robustly stable morphology when cultured as ES cells but responded well to MEDII. In all other experiments, feeder-independent D3 cells were used (20). MEDII was used at 50% concentration and was supplemented with LIF where denoted in the text.

To determine the ability of MEDII, medium fractions, and test compounds to alter ES cell colony morphology, ES cells were seeded at a density of 2.8 × 10<sup>5</sup> cells/cm<sup>2</sup> in a 2-ml well containing 1 ml of ES cell medium supplemented with MEDII (50%), chromatographic fractions of sMEDII, including R (50 μg/ml protein) and E (50%) and peptides, amino acids, and proline analogs (Sigma) prepared as aqueous stocks and diluted in ES cell medium. Before addition, R was desalted over a Sephadex PD10 column (according to the manufacturer’s instructions; Pharmacia). All medium was adjusted to contain a final concentration of 10% FCS, 0.1 mM β-ME, and 1,000 μg/ml LIF.

In active samples, an EPL cell-like colony morphology was observed after 2–4 days. On <i>day 5</i>, all wells were stained for alkaline phosphatase (Sigma; used as described in Ref. 51) and scored for colony morphology. Activity was considered to be present when >95% of the alkaline phosphatase-positive colonies in a well had EPL cell-like colony morphology.

For RNA analysis and formation of EBs, ES cells were seeded at 1.3 × 10<sup>4</sup> cells/cm<sup>2</sup> on tissue culture plasticware (Falcon) that had been pretreated with gelatin in ES cell medium supplemented as stated in the text. Cells were passaged every 2 days and replated at 1.3 × 10<sup>4</sup> cells/cm<sup>2</sup>. EBs were formed and maintained as described by Lake et al. (39) and Rathjen and Rathjen (52) except that cells were maintained in culture for 4 days before body formation. The mTOR
inhibitor rapamycin (Sigma) was used at a concentration of 10 nM and was added to culture medium 30 min before the addition of l-proline.

Northern blot analysis. RNA was isolated from cells using the method of Edwards et al. (22). Northern blot analysis was performed as described by Thomas et al. (65). 32P-labeled DNA probes were synthesized using a Gigaprime (Geneworks) or Megaprime (Amersham) kit. The probes used were as previously described (39, 51). Northern blot filters were exposed to phosphor screens (Molecular Dynamics) for 24–48 h and imaged and analyzed using ImageQuant software on a PhosphorImager (Molecular Dynamics).

Real-time PCR. RNA was isolated from cells using RNAwiz RNA isolation reagent (Ambion). Synthesis of cDNA was performed using Omniscript RT kit (Qiagen). Real-time PCR was performed using Platinum SYBR Green qPCR Supermix UDG (Invitrogen) and a Chromo4 real-time machine (MJ Research) using primers listed in Table SI (supplemental data for this article can be found online at the American Journal of Physiology-Cell Physiology website). Data were analyzed using Q-Gene software (63).

In situ hybridization analysis. Whole mount in situ hybridization analysis of cells was performed using the method of Rosen and Beddington (56) with modifications (39, 51). Anti-sense and sense probes were synthesized using a DIG RNA Labeling Kit (Roche). Details for the generation of an Oct4 anti-sense probe were as described previously (51).

Western blot analysis. Cells were lysed in 100 μl RIPA buffer supplemented with phosphatase and protease inhibitors at 4°C, and cell debris was removed by centrifugation. Proteins were separated on a 10% SDS-polyacrylamide gel and analyzed by immunoblot using standard conditions; signals were developed using enhanced chemiluminescence [SuperSignal West Pico substrate (Pierce)]. Anti-p44/42 MAP kinase antibody, anti-phospho-S6 ribosomal protein (Ser235/236), and anti-phospho-4E-BP1 (Thr37/46) antibodies were used at a 1:1,000 dilution and were purchased from Cell Signaling Technology.

Cell growth assays. Cell growth was quantified using the WST-1 colorimetric assay (Roche) following the manufacturer’s instructions and measured at 450 nm with a reference wavelength of 650 nm using an Emax Precision Microplate Reader.

Statistical analysis. Experiments were analyzed using an unpaired two-tailed Student’s t-test, and significance was achieved at P ≤ 0.05, unless otherwise stated in the text.

Immunofluorescence. EBs were seeded onto gelatin-coated glass coverslips on day 9 of differentiation and allowed to differentiate for a further 3 days. Adherent EBs were fixed with 4% paraformaldehyde, permeabilized with PBS containing 0.25% Triton X-100, and blocked with 10% FCS. Primary antibodies were applied at 1:250 (mouse anti-neuronal nuclei; Chemicon) or 1:1,000 (mouse anti β-tubulin III; Sigma); secondary antibodies were used at 1:1,000 (Invitrogen). All images were taken on an Olympus BX50 microscope with an Olympus FVII digital camera.

RESULTS

Physicochemical properties of the low-molecular-weight bioactivity within MEDII. The addition of fractions of sfMEDII [eluate (E) and retentate (R)], with LIF, to ES cells in culture induces an alteration in colony morphology, whereas addition of R/LIF does not (51). No loss in activity of E was seen with any of the following treatments: 20° or 4°C for several days, or 20° or 4°C for several days. Fig. 1. L-proline is the active component within MEDII. A: serum-free MEDII was subjected to sequential fractionation over Sephadex G10 (not shown), normal-phase HPLC (not shown), and Superdex peptide column. The trace represents the absorbance (A) recorded at 215 nm. The arrow represents the position of the 25-μl fraction containing bioactivity, as measured by morphology assays. B–E: embryonic stem (ES) cells were cultured in ES cell medium [with leukemia inhibiting factor (+LIF)] (B), MEDII + LIF (C), and MEDII + LIF supplemented with 10 mM glycine (D) or 10 mM L-leucine (E). F: cells were collected on day 4 and the RNA was analyzed for Oct4, Dmnt3b, Otx2, and Fgf5 expression by real-time PCR. Expression was normalized to β-actin and is expressed relative to ES cells. Error bars represent SE; n = 3 (Oct4, Dmnt3b, Otx2); n = 5 (Fgf5). *P < 0.05; **P < 0.01 using a one-tailed Student’s t-test.
showed that the majority of L-alanine and L-proline was present in abundance (150 μM) compared with equivalent material prepared from unconditioned medium. A comparison of hydrolyzed and unhydrolyzed samples with molecular weight standards.

Assessment of the ability of other amino acids, L-proline analogs, and short bioactive or L-proline-containing peptides to alter the colony morphology, gene expression, and differentiation potential of embryonic stem cells is shown in Table 1.

The active fractions from Superdex column chromatography were fractionated sequentially over a Sephadex G10 gel filtration column, a normal-phase silica HPLC column (51), and a Superdex gel filtration column. Fractions were analyzed for the ability to induce an alteration in colony morphology, in amino acid uptake, including L-proline uptake, can be inhibited by the addition of other amino acids to excess (23), identifying a number of molecules that could be used to antagonize L-proline uptake in culture. ES cells were cultured in MEDII + LIF supplemented with 10 mM glycine, an amino acid shown to allow L-proline cellular uptake. ES cells were cultured in MEDII + LIF, however, resulted in a change in morphology and the formation of EPL cell-like colonies (data not shown).

The addition of other amino acids can inhibit the formation of EPL cells in response to MEDII. L-proline transport into cells is mediated by several amino acid transporters, including SNAT1, SNAT2, and SNAT3, PAT1 and PAT2, and IMINO (35). The ability of many of these transporters to mediate amino acid uptake, including L-proline uptake, can be inhibited by the addition of other amino acids to excess (23), identifying a number of molecules that could be used to antagonize L-proline uptake in culture. ES cells were cultured in MEDII + LIF supplemented with 10 mM glycine, an amino acid shown to antagonize the cellular uptake of L-proline, or 10 mM L-leucine, an amino acid shown to allow L-proline cellular uptake. Cells were analyzed on day 4 for morphological changes and the expression of the pluripotent cell marker Oct4 and primitive ectoderm markers Dnmt3b, Fgf5, and Otx2 (Fig. 1, B–F). Addition of MEDII + LIF induced a change in colony morphology consistent with the formation of EPL cells (Fig. 1, B and C), maintenance of Oct4 expression, and increased expression of the primitive ectoderm markers (Fig. 1F). Addition of 10 mM L-leucine did not affect the activity of MEDII + LIF (Fig. 1, E and F). In contrast, addition of 10 mM glycine resulted in maintenance of an ES cell-like colony morphology.

Table 1. Assessment of the ability of other amino acids, L-proline analogs, and short bioactive or L-proline-containing peptides to alter the colony morphology, gene expression, and differentiation potential of embryonic stem cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration Range, μM</th>
<th>Morphology, μM</th>
<th>Gene Expression/Differentiation Potential, μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Proline</td>
<td>20–1,000</td>
<td>40</td>
<td>&gt;100</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>10–1,000</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>L-Alanine</td>
<td>30–3,475</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>L-Proline</td>
<td>390–3,900</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>L-Lysine</td>
<td>55–5,500</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Proline analog</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Prolinamide</td>
<td>1–1000</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>N-acetyl-L-proline</td>
<td>64–650</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>N-L-BOC-L-proline</td>
<td>230–4,650</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Pyrrolidine</td>
<td>10–1,000</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>cis-4-Hydroxy-L-proline</td>
<td>80–460</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>trans-4-Hydroxy-L-proline</td>
<td>270–550</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>3,4-Dehydro-L-proline</td>
<td>1–500</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>L-Azetidine-2-carboxylic acid (AZET)</td>
<td>10–1,000</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>L-Proline</td>
<td>390–15,500</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Peptide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro-Ala</td>
<td>20–1,000</td>
<td>50</td>
<td>–</td>
</tr>
<tr>
<td>Ala-Pro</td>
<td>20–1,000</td>
<td>80</td>
<td>400</td>
</tr>
<tr>
<td>Ala-Pro-Gly</td>
<td>40–1,000</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Pro-OH-Pro</td>
<td>20–1,000</td>
<td>40–80</td>
<td></td>
</tr>
<tr>
<td>Pro-Gly</td>
<td>20–1,250</td>
<td>50</td>
<td>–</td>
</tr>
<tr>
<td>Gly-Pro</td>
<td>20–1,000</td>
<td>40</td>
<td>200</td>
</tr>
<tr>
<td>Gly-Pro-Ala</td>
<td>20–1,000</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Gly-Pro-OH-Pro</td>
<td>40–5,850</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>Gly-Pro-Arg-Pro (inhibitor of fibrin polymerization)</td>
<td>40–1,000</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Gly-Pro-Gly-Gly (inhibitor of dipeptidyl peptide IV)</td>
<td>1–1,200</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Substance P (RPKPQFFGLM-NH₂)</td>
<td>0.005–500</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Substance P (free acid (RPKPQFFGLM-COOH)</td>
<td>7–200</td>
<td>40</td>
<td>200</td>
</tr>
<tr>
<td>Substance P₁₋₄ (RPKP)</td>
<td>40–1,000</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Substance P₁₋₇</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Substance P₃₋₁₁ (QQFFGLM-NH₂)</td>
<td>25</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Bradykinin (RPQGFSPFR)</td>
<td>10–100</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Neurokinin A (HKDSVFGLM-NH₂)</td>
<td>10–100</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Senktide (succinyl-DFMFGLM-NH₂)</td>
<td>10–100</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

*Concentration at which early primitive ectoderm-like cell-like colonies were first observed. A dash (–) indicates no activity over the concentration range tested. Concentration at which downregulation of Rex1 and expression of the mesoderm marker brachyury in embryoid bodies were found to be similar to that observed in cells cultured in MEDII + leukemia inhibitory factor. No gene expression change or change in differentiation potential at 250 μM. Tested at one concentration only. No change in differentiation potential at 1.25 mM. Determined for gene expression change only.
1-Proline induces alterations in colony morphology, gene expression, proliferation, and the differentiation behavior of ES cells. ES cells and EPL cells were compared to identify changes in morphology, proliferation, gene expression, and differentiation kinetics resulting from the addition of 1-proline. Cells cultured with 200 μM 1-proline showed a change in colony morphology, similar to that seen when cells are cultured in MEDII (Fig. 2, A–C). Cell counts showed a 55% increase in cell number with addition of 100 μM 1-proline (Fig. 2D). Analysis of gene expression by real-time PCR or whole mount in situ hybridization showed no significant change in the level or distribution of Oct4 expression (Fig. 2, E and H), suggesting that addition of 1-proline did not lead to a loss of pluripotency. 1-Proline addition resulted in decreased expression of the ES cell markers Rex1 and Spp1 (Fig. 2E). The formation of EPL cells is accompanied by increased transcription of Otx2 (34) and the DNA methyltransferase gene Dnmt3b (Fig. S1); these genes were also increased in cells cultured in the presence of 1-proline (Fig. 2E). These alterations are consistent with differentiation to a primitive ectoderm-like cell. The increase in Fgf5 expression in response to 1-proline was modest and not comparable with the increased expression of this gene seen in cells cultured in MEDII or MEDII + LIF (Fig. 2F; data not shown) suggesting that 1-proline alone could affect some, but not all, of the changes in gene expression seen on the addition of MEDII to ES cells. The addition of 5 mM glycine with 1-proline resulted in decreased expression of the ES cell markers (Fig. 2H). The kinetics of differentiation resulting from the addition of 1-proline was diminished in cells cultured for 8 days.

These data suggest that at concentrations of 100 μM or greater, 1-proline induces changes in the morphology, gene expression, proliferation, and the rate of differentiation in ES cells.

Specificity of the inductive activity. Proline analogs and peptides were tested for the ability to induce changes in ES cells in a manner analogous to 1-proline (Table 1). Analogs of proline (proline, cis-4-hydroxy-l-proline (cis-OH-pro), L-azetidine-2-carboxylic acid (AZET), and 3,4-dehydro-l-proline), including those with alterations to the carboxyl region (γ-prolinamide), amino region (N-acetyl-l-proline and N-BOC-l-proline), and ring (including pyrrolidine, trans-4-hydroxy-l-proline (trans-OH-pro)), and pipocelic acid, were unable to substitute for l-proline and alter the morphology of ES cell colonies at any concentration tested (Table 1).

Several proline-containing peptides, including peptides containing two or more proline residues (e.g., Gly-Pro-Arg-Pro, bradykinin) or hydroxylated proline (Pro-OH-Pro and Gly-Pro-OH-Pro), were tested and shown to induce an EPL cell-like colony morphology at concentrations similar to l-proline (Table 1). Gly-Pro (200 μM) and Ala-Pro (400 μM) exhibited l-proline-like activity, inducing changes in morphology, gene expression, and differentiation kinetics (Table 1). In contrast, Pro-Gly and Pro-Ala did not change the gene expression or differentiation kinetics of ES cells, despite inducing changes in colony morphology (Table 1). Substance P (SP)-COOH (200 μM) and SP1–7 also induced morphology and gene expression changes similar to those observed with 200 μM l-proline, whereas wild-type SP, which is COOH-terminally amidated, failed to induce a morphology change at concentrations ≤500 μM (Table 1). Peptides lacking proline, including several with known bioactivity such as RGD, failed to induce a morphology change.

Collectively, these data suggest that the activity of l-proline and active proline-containing peptides is structurally restricted and cannot be substituted by γ-proline or proline analogs. Some, but not all, proline-containing peptides were active; the position of l-proline within these peptides appears to affect activity.

Activation of the mTOR signaling pathway is required for l-proline activity. The mTOR signaling pathway plays a role in mediating cellular response to amino acid availability (30). The involvement of this pathway in l-proline activity was tested using the specific pharmacological inhibitor of mTOR, rapamycin. ES cells were cultured in ES cell medium supplemented with 200 μM l-proline with or without 10 nM rapamycin and were analyzed for morphology, gene expression, and kinetics of differentiation on day 4. The addition of rapamycin with l-proline prevented the l-proline-associated changes in morphology (data not shown). Consistent with this, cells cultured
in L-proline and rapamycin expressed significantly higher levels of Rex1 and significantly lower levels of Dnmt3b when compared with ES cells cultured in L-proline; the expression of these genes was not significantly different from expression in ES cells (Fig. 4A). Similarly, the timing of brachyury upregulation during the differentiation of cells cultured in L-proline and rapamycin was equivalent to that seen during ES cell differentiation (Fig. 4B). These data suggest that rapamycin inhibited L-proline activity. Consistent with this, Rex1 and Dnmt3b expression levels in ES cells cultured in L-proline and rapamycin were not significantly different from those in ES cells. It should be noted that rapamycin did exert influence on ES cells, shown by the elevated expression of Oct4.

The increased ES cell numbers seen with the addition of L-proline (Fig. 2D) was estimated by a colormetric assay based on cleavage of the tetrazolium salt WST-1 (Fig. 4C). The addition of rapamycin to ES cells growing in ES cell medium or ES cell medium with L-proline reduced the cell number to ~60% of untreated ES cells (Fig. 4C). When analyzed with a bromodeoxyuridine incorporation assay, cells cultured in rapamycin showed an analogous decrease (~50%) in cell proliferation (data not shown).

The addition of amino acids to cells in culture has previously been shown to induce mTOR activity and result in the phosphorylation of 4Ebp1 and rpS6 (7, 8, 11, 28, 61). The phosphorylation state of 4Ebp1 and rpS6 was determined by Western blot using phospho-4Ebp1 and phospho-rpS6 antibodies. L-Proline induced phosphorylation of both 4Ebp1 and rpS6 in ES cells after 30 min, suggesting that mTOR activation occurred in response to L-proline (Fig. 4D). Phosphorylation of mTOR targets was reduced when L-proline was added with rapamycin.

L-Leucine and glycine activate the mTOR pathway but do not induce ES cell differentiation. Other amino acids have been shown to activate mTOR signaling (32), raising the possibility that they may be capable of inducing mTOR activity and cell differentiation in ES cells in a manner analogous to L-proline. ES cells were cultured in ES cell medium supplemented with 200 μM L-proline and compared with cells cultured in 1 mM L-leucine or 1 mM glycine. The addition of L-leucine or glycine to ES cells induced the phosphorylation of 4Ebp1 and rpS6 (Fig. 5A); phosphorylation was reduced by rapamycin. Unlike ES cells cultured in L-proline, however, cells cultured in L-leucine or glycine did not show increased expression of Dnmt3b on day 4 and showed differentiation kinetics consistent with the maintenance of an ES cell phenotype (Fig. 5, B and C). The expression of Rex1 in the presence of L-leucine or glycine was variable. These data indicate...
that amino acids other than l-proline can activate the mTOR signaling pathway in ES cells but are unable to induce changes in ES cell gene expression or differentiation kinetics, suggesting that activation of this pathway is necessary, but not sufficient, for l-proline activity.

**DISCUSSION**

l-Proline is a bioactive component within MEDII. In culture, the formation of a primitive ectoderm-like cell occurs as ES cells differentiate in EBs (54) and can be recapitulated by the formation of EPL cells from ES cells in response to MEDII. Here, we identify l-proline as a bioactivity within MEDII. ES cells cultured in l-proline and LIF maintained pluripotency, as indicated by the maintenance of Oct4 expression and alkaline phosphatase activity (data not shown), but adopted an altered colony morphology and showed reduced expression of ICM-specific markers (Rex1, Spp1) and increased expression of the EPL cell markers Otx2 and Dnmt3b. The changes were...
abrogated by the addition of an excess of amino acids predicted to inhibit proline uptake, suggesting that they were dependent on L-proline transport into the cell. Regulation of L-proline entry into the cell, through increasing transcriptional activity of the Slc38a2 gene, has been shown to be one mechanism whereby transforming growth factor-β1 promotes growth of smooth muscle cells and collagen synthesis (23). Similarly, L-proline influx may be required to trigger ES cell differentiation.

The gene expression changes that occurred in cells cultured in L-proline were not equivalent to those seen in response to MEDII, illustrated by the negligible expression of Fgf5 expression in cells cultured in L-proline when compared with cells cultured in MEDII + LIF, despite the considerable variability seen in the latter (Fig. 2F). Similarly, cells cultured in L-proline showed intermediate differentiation kinetics when compared with ES and EPL cells. These data suggest that although the addition of L-proline to ES cell medium is able to effect alterations in ES cells consistent with differentiation, EPL cell formation from ES cells requires the presentation of L-proline within the context of MEDII. This may reflect the requirement to maintain LIF in cultures containing L-proline. LIF has been shown to delay the formation of EPL cells in response to MEDII (51), and the primitive ectoderm in EBs and the mouse embryo (47, 62).

The ability of L-proline to induce changes in ES cells in culture in the presence of LIF appears, prima facie, to be inconsistent with the presence of L-proline within many medium formulations and supplements commonly used for the propagation of these cells, such as Ham’s F-12 medium and nonessential amino acids (33, 55, 58, 66). The ability of glycine and other amino acids to modulate amino acid uptake, including the uptake of L-proline, however, suggests that the balance of amino acids present may have important and unexpected effects within the culture medium. The potential that the interplay between amino acids and pluripotent cells could regulate cell fate suggests that medium constituents used for the maintenance of ES cells need to be carefully appraised and any associated bioactivities understood.
Specificity and action of L-proline. The ability of amino acids to function as biological regulators in cell growth, differentiation, and communication has precedent in the diverse roles of amino acids that have been documented to date (4, 13, 27, 42, 44, 59). Attribution of regulatory activities to L-proline in mammals is novel. The activity of L-proline observed here was associated with free L-proline and some proline-containing peptides. Alterations in ES cell colony morphology could not be induced by any of the L-proline analogs tested, including D-proline, indicating strict structural constraints on the activity. The interaction between L-proline and the transcription factor Put3b in yeast, which induces a conformational change and activation of transcription from a number of proline-specific genes (19, 60), is also subject to structural constraints, with some stereospecificity suggested by a preference for the L-form of the amino acid. The strict structural requirement for L-proline observed here was associated with free L-proline and some proline-containing peptides. Alterations in ES cell colony morphology could not be induced by any of the L-proline analogs tested, including D-proline, indicating strict structural constraints on the activity. The interaction between L-proline and the transcription factor Put3b in yeast, which induces a conformational change and activation of transcription from a number of proline-specific genes (19, 60), is also subject to structural constraints, with some stereospecificity suggested by a preference for the L-form of the amino acid. The strict structural requirement for L-proline observed here was associated with free L-proline and some proline-containing peptides. Alterations in ES cell colony morphology could not be induced by any of the L-proline analogs tested, including D-proline, indicating strict structural constraints on the activity. The interaction between L-proline and the transcription factor Put3b in yeast, which induces a conformational change and activation of transcription from a number of proline-specific genes (19, 60), is also subject to structural constraints, with some stereospecificity suggested by a preference for the L-form of the amino acid. 

Fig. 5. L-Leucine and glycine activate the mTOR signaling pathway but are unable to induce changes in gene expression or differentiation kinetics in ES cells. A: ES cells, untreated or stimulated with 200 μM L-proline, 1 mM L-leucine, or 1 mM glycine for 30 and 360 min with and without rapamycin, were analyzed by Western blot using anti-phospho- rpS6 and anti-phospho-4Ebp1 antibodies. Total ERK1/2 protein levels were used to show equal protein loading. B: ES cells were cultured in ES cell medium (+LIF) or ES cell medium supplemented with 200 μM L-proline, 1 mM glycine, or 1 mM L-leucine, as indicated, for 4 days. Cells were analyzed for the expression of Oct4, Rex1, and Dnmt3b and were normalized to β-actin by real-time PCR. Expression was normalized to β-actin and is expressed relative to the positive control (ES cells formed from ES cells on day 4 of culture). Notation on the x-axis describes culture conditions of the starting cell population. Error bars represent SE; n = 3.

Supports this (Lonic A and Washington JM, unpublished observations). The failure of peptides Pro-Ala and Pro-Gly, and the amidated form of substance P, to induce the transition may reflect differences in the efficiency of peptide bond cleavage and the structural constraints presented by proline in this conformation (17, 18, 36).

The inhibitor of mTOR, rapamycin, effectively inhibited the ability of L-proline, suggesting a requirement for mTOR activation downstream of L-proline uptake. As might be expected from the inability of L-alanine and L-lysine to induce changes in morphology at high concentrations, neither L-leucine nor glycine was able to induce changes in gene expression or differentiation kinetics in ES cells at 1 or 5 mM. This is despite activating downstream targets of mTOR signaling, 4Ebp1 and rpS6, in a manner analogous to 200 μM L-proline. These data suggest multiple pathways downstream of L-proline in ES cells and implicate at least one of these pathways as specific to L-proline. The identities of other pathways are yet to be elucidated.

It is tempting to speculate that L-proline may function as a general mediator of primitive ectoderm formation in embryos and embryoid bodies. In EBs and the embryo the signals regulating the formation of primitive ectoderm originate from the extraembryonic endoderm (1, 10, 12, 14, 15, 21, 64). Within EBs a small, diffusible signal from the extraembryonic endoderm has been proposed to be involved in the transition of

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ES cells to primitive ectoderm (14, 15). Conditioned media from extraembryonic-like cell lines, END2 and PYS2, appear to have similar activities to MEDII (Washington JM, Lake JA, and Bettess MD, unpublished observations), and L-proline has been detected in medium conditioned by END2 cells at a concentration similar to MEDII (Washington JM, unpublished observations). Lastly, ablation of mTOR resulted in embryonic lethality around the time of primitive ectoderm formation (26, 46), consistent with a role for an amino acid at this time in development.

Few low-molecular-weight regulators of ES cell differentiation have been identified, yet the availability of such activities will provide valuable tools for modulating ES cell differentiation and cell fate choice. We have shown that the L-proline induces formation of a distinct pluripotent cell population with characteristics of the primitive ectoderm in culture and have identified, for the first time, a potential role for amino acid regulation in the growth and differentiation of the pluripotent lineage. The inductive abilities of L-proline provide a first step in the development of technologies for the production of specified cell lineages from pluripotent cells via the addition of small, nontoxic organic molecules.

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DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES

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