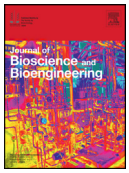

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Major amino acids in collagen hydrolysate regulate the differentiation of mouse embryoid bodies

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To take advantage of the therapeutic potential of embryonic stem cells (ESCs), it is necessary to regulate their differentiation in response to defined factors. In this study, in order to explore novel molecules that regulate the differentiation of ESCs, we investigated whether collagen hydrolysate, collagen-characteristic amino acids, glycine (Gly), L-proline and *trans*-4-hydroxy-L-proline (L-Hyp); or dipeptides, proline-hydroxyproline and hydroxyproline-glycine regulate the differentiation of mouse embryoid bodies (EBs). We identified that treatment with collagen hydrolysate or Gly repressed the expression of the mesendodermal markers, Brachyury and Foxa2 in EBs and maintained the undifferentiated state of mESCs in a feeder-free monolayer culture. In contrast, L-Hyp promoted the expression of Brachyury, Mixl1, Gsc and Foxa2 in EBs. And the treatment with L-Hyp promoted cardiac differentiation within EBs, which was proven by the spontaneous contraction of cardiomyocytes and the expression of the cardiac markers, α-MHC, MLC-2v and Nkx2.5. Results suggest that L-Hyp is a promising new inducer for reproducible and efficient differentiation of mesendoderm lineages.

[**Key words:** Collagen hydrolysate; Glycine; *trans*-4-Hydroxy-L-proline; Embryonic stem cells; Embryoid bodies; Mesendoderm; Cardiomyocyte]

Embryonic stem cells (ESCs) are capable of self-renewal and differentiation to a broad spectrum of lineages *in vitro*, providing a putative source of replacement cells for regenerative therapies. The development of cell therapeutics based on ESCs requires technologies that direct cell differentiation to specific somatic cell lineages in response to defined factors. Recently, considerable efforts have been made to identify novel small molecules that can modulate the pluripotent state of ESCs (1,2) or direct differentiation of ESCs into specific cell lineages such as cardiomyocytes (3) and neural progenitors (4,5).

In this study, we focused on collagen hydrolysate and its constituents such as amino acids and dipeptides. Some animal experiments and preclinical human trials have also suggested that oral ingestion of collagen hydrolysate may have beneficial effects on skin (6), bone metabolism (7), and articulation (8). When ingested collagen hydrolysate is digested and absorbed, it appears in blood in the form of amino acids or peptides. On the basis of an *in vitro* study using a cell culture system, it has been revealed that some collagen-derived peptides have some bioactivities. In particular, proline-hydroxyproline (Pro-Hyp) and hydroxyproline-glycine (Hyp-Gly), which were detected as major collagen hydrolysate digests (9), activate the growth of mouse primary fibroblasts (10) and Pro-Hyp enhances chondrocyte differentiation under pathological conditions (11). Also, it has become increasingly clear that amino acids can function as signaling molecules in the regulation of many physiological functions (12). As an example of a collagen-characteristic amino acid, glycine (Gly) itself is reported to have bioactivities such as the prevention of tumor growth by inhibiting angiogenesis (13).

Currently, it is widely accepted that amino acids such as L-threonine (14) and L-ornithine (15) function as signal molecules regulating ESC metastability.

To explore novel molecules that regulate the differentiation of ESCs, we applied collagen hydrolysate or its characteristic constituents [Gly, L-proline (L-Pro), *trans*-4-hydroxy-L-proline (L-Hyp), Pro-Hyp and Hyp-Gly] to embryoid bodies (EBs) formed with mouse ESCs. We then observed that collagen hydrolysate and Gly suppressed mesendoderm differentiation in EBs and maintained the undifferentiated state of mESCs. Meanwhile, L-Hyp activated mesendodermal differentiation in EBs and enhanced cardiac differentiation.

MATERIALS AND METHODS

Reagents Collagen hydrolysate was obtained by ultrafiltration and gel filtration fractions of the enzymatic hydrolysate derived from fish skin collagen (Ihara & Co., Ltd., Hokkaido, Japan) with an average molecular weight less than 290 Da. Gly, L-Pro and L-Hyp were purchased from Wako Chemical Industries (Osaka, Japan). Pro-Hyp and Hyp-Gly were purchased from Bachem (Bubendort, Germany).

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mESC culture and formation of EBs Bruce-4 ESCs (derived from C57/BL6 mice) were cultured on a feeder layer of mouse embryonic fibroblasts inactivated with mitomycin C, and maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM: Nacalai Tesque, Kyoto, Japan) supplemented with 15% fetal bovine serum (FBS: Nichirei Biosciences Inc., Tokyo, Japan), 2 mM L-glutamine, 1% EmbryoMax ES Cell Qualified 2-mercaptoethanol, 1% EmbryoMax ES Cell Qualified non essential amino acids, 1% EmbryoMax ES Cell Qualified Nucleosides (Millipore, Billerica, MA, USA), and 1000 U/ml LIF (ESGRO: Chemicon, Temecula, CA, USA). For formation of EBs, Bruce-4 ESCs were dissociated with 0.25% trypsin-EDTA for 3 min at 37°C. 500 cells of the ESCs were placed into each well of a 96-well low attachment round-bottomed plate (Lipidure-coat: NOF corporation, Tokyo, Japan), and incubated with ES medium without LIF. Medium was changed every other day. On the sixth day, individual EBs were transferred to gelatin-coated 48-well culture plates. 48-well plates were monitored daily to detect the appearance of spontaneously contracting cardiomyocytes, and the percentage of the EBs that exhibited spontaneous contraction was calculated as cardiac differentiation efficiency.

Quantitative real-time PCR Total RNA was extracted from ESCs or EBs using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was synthesized from total RNA using SuperScript III Platinum Two-Step qRT-PCR kit (Invitrogen). Quantitative real-time PCR was performed by using Applied Biosystems StepOnePlus Real-Time PCR System. Relative levels of gene expression were normalized to the GAPDH gene by using the comparative CT method according to the manufacturer's instructions. The primer sequences were as follows: GAPDH, 5′-TGCACCACCAACTGCTTAGC-3′ sense and 5′-TCTTCTGGGTGGCAGTGATG-3′ antisense; Nanog, 5′-ATGCCTGCAGTTTTTCATCC-3′ sense and 5′-GAGGCAGGTCTTCAGAGGAA-3′ antisense; Otx2, 5′-GAAAATCAACTTGCCAGAATCCA-3′ sense and 5′-GCGGCACTTAGCTCTTCGAT-3′ antisense; Sox1, 5′-GCCGAGTGGAAGGTCATGT-3′ sense and 5′-TGTAATCCGGGTGTTCCTTCAT-3′ antisense; Brachyury, 5′-CAGCCCACCTACTGGCTCTA-3′ sense and 5′-GAGCCTCGAAAGAACTGAGC-3′ antisense; Mixl1, 5′-CGCCAGAGTGGGAAGTCA-3′ sense and 5′-CAGGGCAATGGAGGAAAAC-3′ antisense; Gsc, 5′-GCACCGCACCATCTTCA-3′ sense and 5′-AAACCAGACCTCCACCTTC-3′ antisense; Foxa2, 5′-GGCCCAGTCACGAACAAAGC-3′ sense and 5′-CCCAAAGTCTCCACTCAGCCTC-3′ antisense; Flk1, 5′-CTGTGGCGTTTCCTACTCCT-3′ sense and 5′-AGGAGCAAGCTGCATCATTT-3′ antisense; Sox17, 5′-CAGAACCCAGATCTGCACAA-3′ sense and 5′-GCTTCTCTGCCAAGGTCAAC-3′ antisense; Rex1, 5′-TGTGCTGCCTCCAAGTGTTG-3′ sense and 5′-ACTGATCCGCAAACACCTGC-3′ antisense; Fgf5, 5′-TGTACTGCAGAGTGGGCATC-3′ sense and 5′-ACAATCCCCTGAGACACAGC-3′ antisense; α-MHC, 5′-TAAAGGCAAAGGAGGCAAGAAG-3′ sense and 5′-ACAAAGTGAGGGTGGGTGGT-3′ antisense; MLC-2v, 5′-CGACAAGAATGACCTAAGGGACA-3′ sense and 5′-CCCAAACATCGTGAGGAACA-3′ antisense.

PicoGreen dsDNA assay for measuring cell viability The effects of exogenous factors on EBs viability were compared quantitatively by Quant-iT PicoGreen dsDNA assay kit (Invitrogen). PicoGreen provides a stable measurement parameter by quantifying double stranded DNA (dsDNA) that was present in live cells. The measurement is based on fluorescence enhancement of the dye upon binding to dsDNA. Individual EBs on day 6 were lysed with proteinase K (50 μg/ml; Wako) at 56°C for 4 h and then incubated at 98°C for 10 min to inactivate proteinase K. DNA samples were incubated with PicoGreen reagent for 5 min at room temperature, protected from light. Fluorescence of the sample mixtures was measured at excitation and emission wavelengths of 485 and 520 nm, respectively. Each analysis was performed in triplicate. A standard curve was made using lambda DNA standard in the PicoGreen Kits.

Alkaline phosphatase staining Alkaline phosphatase staining of ESCs on day 4 was assessed using Alkaline Phosphatase Substrate Kit I (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions.

Immunostaining Immunohistochemistry for detection of mesendodermal Brachyury was carried out as described previously (16). For cryosections, EBs on day 6 were fixed in 2% paraformaldehyde for 1 h on ice, followed by incubation in 30% sucrose in phosphate-buffered saline overnight at 4°C, and were frozen in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan). Cryosections (10 μm) were incubated with the primary antibody against Brachyury (diluted 1:100; Santa Cruz Biotechnology, CA, USA) for 2 h at room temperature. Alexa 594 labeled Donkey anti-goat IgG antibody (diluted 1:1000; Molecular Probes, Eugene, OR, USA) was used as the secondary antibody. Cryosections were incubated with 4′, 6-diamidino-2-pheylindole (DAPI: Wako) for nucleic acid staining.

On the twelfth day of culture, EBs plated at day 6 were fixed in 4% paraformaldehyde at room temperature for 30 min on ice, and then permeabilized with 0.1% Triton X-100 in PBS. After blocking with 3% BSA in PBS, cells were incubated with the goat antibody against Nkx2.5 (diluted 1:100; Santa Cruz) for 1 h at room temperature. Alexa 594 labeled Donkey anti-goat IgG antibody (diluted 1:1000) was used as the secondary antibody. Cells were incubated with DAPI for nucleic acid staining.

RESULTS

Sequential expression of differentiation stage-related genes in EBs As it has been revealed that EBs formed under various culture conditions have the heterogeneity in differentiation status (17), to explore when differentiation programs of the three germ layers are initiated within EBs that were formed in a round-bottomed low-adherence 96-well plate (Fig. 1A), mRNA expression of marker genes was analyzed by real-time PCR (Fig. 1B). During EB formation, gradual downregulation of the pluripotency marker, Nanog was observed. The primitive ectodermal marker, Otx2 began to be expressed on day 4. The neuroectodermal marker, Sox1 began to be expressed on day 6. The pan-mesendodermal markers, Brachyury and Mixl1; and anterior mesendodermal marker, Gsc were strongly expressed on day 6, indicating a wave of mesendodermal induction. The another anterior mesendode rmal marker, Foxa2; mesodermal marker, Flk1; and endo dermal marker, Sox17 were expressed, respectively, from day 6. Collectively, these data indicate that three germ layer differentiation programs in EBs start on day 6.

Effects of collagen hydrolysate, collagen-characteristic amino acids, or dipeptides on the differentiation and growth of EBs To investigate whether collagen hydrolysate, collagen-characteristic amino acids (Gly, L-Pro, L-Hyp), or dipeptides (Pro-Hyp, Hyp-Gly) regulate the differentiation of EBs, we analyzed the expressions of the mesendodermal markers and neuroectodermal marker in EBs treated with each compound for 6 days (Fig. 2A). Treatment with collagen hydrolysate (0.5 and 1 mg/ml) or Gly (5 and 10 mM) repressed the expression of Brachyury and Foxa2 in EBs. In contrast, treatment with L-Hyp (5 and 10 mM) promoted the expression of Brachyury and Foxa2, and repressed the expression of Sox1 in EBs. These results suggest that collagen hydrolysate and Gly repress, and L-Hyp promotes the differentiation of the mesendoderm in EBs.

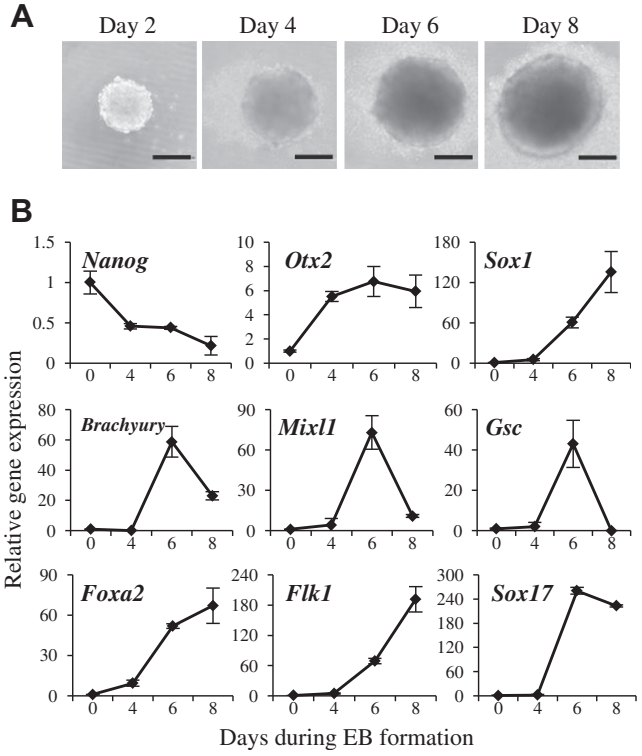

FIG. 1. Growth and differentiation of EBs. (A) EB on days 2, 4, 6 and 8 of differentiation, derived from 500 mouse ES cells placed in a well of a round-bottomed low-adherence 96-well plate. Scale bar: 200 μm. (B) Sequential expression of differentiation stage-related genes in EBs. The gradual loss at the pluripotency marker and sequential acquisition of mRNA indicative of specific stages of embryonic development were obtained. Sequential expression analyzed by real-time PCR were expressed relative to ESCs on day 0. GAPDH was used as an internal control. Data are expressed as means ± SD ($n = 3$).