Biomaterials 32 (2011) 6478–6486

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

The influence of Leucine-rich amelogenin peptide on MSC fate by inducing Wnt10b expression

Xin Wen [a], William P. Cawthorn [b], Ormond A. MacDougald [b,c], Samuel I. Stupp [d,e,f], Malcolm L. Snead [a], Yan Zhou [a,*]

[a] Center for Craniofacial Molecular Biology, Herman Ostrow School of Dentistry, University of Southern California, 2250 Alcazar St, Los Angeles, CA 90033, USA
[b] Department of Molecular and Integrative Physiology, University of Michigan Medical Center, Ann Arbor, MI, USA
[c] Division of Metabolism, Endocrinology and Diabetes, Department of Internal Medicine, University of Michigan Medical Center, Ann Arbor, MI, USA
[d] Department of Materials Science and Engineering and Institute for BioNanotechnology in Medicine, Northwestern University, Chicago, IL, USA
[e] Department of Medicine, Northwestern University, Chicago, IL, USA
[f] Department of Chemistry, Northwestern University, Evanston, IL, USA

ARTICLE INFO

Article history:
Received 2 May 2011
Accepted 15 May 2011
Available online 12 June 2011

Keywords:
ECM (extracellular matrix)
Bone marrow
Stem cell
Cell signaling
Osteogenesis
Adipose tissue engineering

ABSTRACT

Amelogenin is the most abundant protein of the enamel organic matrix and is a structural protein indispensable for enamel formation. One of the amelogenin splicing isoforms, Leucine-rich Amelogenin Peptide (LRAP) induces osteogenesis in various cell types. Previously, we demonstrated that LRAP activates the canonical Wnt signaling pathway to induce osteogenic differentiation of mouse ES cells through the concerted regulation of Wnt agonists and antagonists. There is a reciprocal relationship between osteogenic and adipogenic differentiation in bone marrow mesenchymal stem cells (BMMSCs). Wnt10b-mediated activation of canonical Wnt signaling has been shown to regulate mesenchymal stem cell fate. Using the bipotential bone marrow stromal cell line ST2, we have demonstrated that LRAP activates the canonical Wnt/β-catenin signaling pathway. A specific Wnt inhibitor sFRP-1 abolishes the effect of LRAP on the stimulation of osteogenesis and the inhibition of adipogenesis of ST2 cells. LRAP treatment elevates the Wnt10b expression level whereas Wnt10b knockdown by siRNA abrogates the effect of LRAP. We show here that LRAP promotes osteogenesis of mesenchymal stem cells at the expense of adipogenesis through upregulating Wnt10b expression to activate Wnt signaling.

1. Introduction

Amelogenin is the most abundant protein of the enamel organic matrix and is a structural protein indispensable for enamel formation [1–6]. A serendipitous finding showed that amelogenin also can be used to induce the regeneration of periodontal tissues in monkeys and humans [7–9]. Emdogain, a commercial product consisting largely of alternatively spliced and processed porcine amelogenins, can induce new bone, cementum and periodontal ligament formation in the jaws of dogs, monkeys and humans [7–11]. One naturally occurring amelogenin splicing isoform, Leucine-rich Amelogenin Peptide (LRAP), consisting of the N-terminal 33 and the C-terminal 26 residues of the full-length protein, has been shown to induce osteogenesis in various cell types [12–14]. We detected LRAP expression during osteogenesis of wild-type (WT) mouse embryonic stem (ES) cells and observed the absence of LRAP expression in amelogenin-null (KO) ES cells. LRAP treatment of WT and KO ES cells induces significant increases in mineral matrix formation, bone sialoprotein and osterix gene expression. In addition, the impaired osteogenesis of amelogenin-null ES cells is partially rescued by the addition of exogenous LRAP [15]. We also demonstrated that LRAP activates the canonical Wnt signaling pathway to induce osteogenic differentiation of mouse ES cells through the concerted regulation of Wnt agonists and antagonists [16].

Bone marrow mesenchymal stem cells (BMMSC) can differentiate into a number of cell types, including adipocytes and osteoblasts [17,18]. Compelling evidence from both *in vitro* and *in vivo* experiments indicate a reciprocal relationship between these two cell lineages [19–21]. For example, bone marrow stromal cells and immortalized clonal lines (e.g. ST2) are capable of undergoing both osteogenic and adipogenic differentiation, depending upon culture conditions. Moreover, single cell clones from bone marrow can differentiate *in vitro* into either adipocytes or osteoblasts [22].

* Corresponding author. Tel.: +323 442 2573; fax: +323 442 2981.
E-mail address: yzhou@usc.edu (Y. Zhou).

0142-9612/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2011.05.045

Activation of Wnt/β-catenin signaling inhibits adipogenesis and stimulates osteogenesis by a rapid suppression of the adipogenic transcription factors C/EBPα and PPARγ followed by an increase in osteoblastic transcription factors [23,24]. The endogenous Wnt signal may be initiated by Wnt10b, which is expressed in pre-adipocytes and stromal vascular cells but is rapidly suppressed upon induction of adipogenesis [25,26]. Although there is no evidence that Wnt10b deficiency in mice alters adipose tissue development, transgenic mice overexpressing Wnt10b in adipose tissues have ~50% less white adipose tissue and arrested development of brown fat [27,28]. Furthermore, these mice resist expansion of adipose tissue under conditions of diet-induced and genetic obesity [27,29]. Mice expressing the Wnt10b transgene also exhibit improved glucose homeostasis and increased insulin sensitivity [27,29]. Mice expressing the Wnt10b transgene in bone marrow have increased bone mass and strength and resist the loss of bone that occurs with aging or estrogen deficiency. In addition, Wnt10b-null mice have decreased trabecular bone mass and serum osteocalcin levels, indicating that Wnt10b is an endogenous regulator of bone mass [23].

Previously, we have shown that LRAP stimulates osteogenic differentiation of murine ES cells through activating the canonical Wnt/b-catenin signaling pathway [16]. Given that Wnt10b-mediated activation of Wnt/b-catenin signaling stimulates osteogenesis and inhibits adipogenesis of bone marrow mesenchymal stem cells [23–26], we hypothesized that LRAP might affect fate determination (osteogenesis *versus* adipogenesis) of mesenchymal stem cells through Wnt/b-catenin signaling. In this study, we used the bipotential bone marrow stromal cells ST2 to characterize the effect of LRAP on mesenchymal stem cells and to delineate the underlying mechanism.

2. Materials and methods

2.1. Reagents

LRAP was chemically synthesized and HPLC purified as described previously [16].

2.2. Cell culture

ST2 cells were maintained in α-minimal essential medium containing 10% fetal bovine serum and 100 units/ml penicillin/streptomycin. For adipogenesis, cells that had been confluent for a day were treated with 10% fetal bovine serum, 1 μM dexamethasone, 0.5 mM methylisobutylxanthine, 1 μg/ml insulin, and 5 μM troglitazone (day 0). Cells were fed with 1 μg/ml insulin in 10% fetal bovine serum media (day 2), and refed with 10% fetal bovine serum media every 2 days subsequently. To induce osteogenesis, over-confluent cells were switched to mineralization media containing 25 μg/ml ascorbic acid and 10 mM β-glycerophosphate.

2.3. Analysis of mineral deposition or lipid deposition

Two-week post-osteogenic-induction, cells were stained with Alizarin Red. Quantification of calcium concentration was measured by spectrophotometry at 612 nm (QuantiChrom Calcium Assay kit; BioAssay Systems). The total amount of protein in each sample was used as a standard with which to normalize calcium concentration. Eight-day post-adipogenic-induction, cells were stained with Oil Red O (0.5 g Oil Red O in 100 ml isopropanol) for analysis of triglyceride and lipid deposition. Oil Red O staining was extracted by isopropanol and measured by spectrophotometry at 490 nm.

2.4. RNA extraction and cDNA Synthesis

RNA was isolated using RNAqueous®-4PCR Kit (Ambion) following the manufacturer's instructions. Synthesis of cDNA was performed using RETROscript® Kit (Ambion). For cDNA template preparation, 1 μg of total RNA was used in a 20 μL reaction.

2.5. Western blot analysis

Cell lysate was prepared by washing the cells with PBS twice followed by the addition of M-PER mammalian extraction reagent (Pierce). An aliquot of the protein was added to 2X SDS loading buffer consisting of 4% v/v SDS, 200 mM dithiothreitol, 100 mM Tris pH 6.8, 20% v/v glycerol, 0.2% w/v bromphenol blue for polyacrylamide gel electrophoresis (PAGE). Protein concentration was measured using the Bio-Rad protein assay on the lysate samples, with known amounts of bovine serum albumin to establish a standard curve. Approximately 10 μg of protein from each experimental sample group was loaded to a 4–20% Tris-glycine SDS-polyacrylamide gel electrophoresis (PAGE) gel. The size-resolved proteins were transferred to Immobilon-P membranes (Millipore) for 1 h at 100 mA. The membrane was blocked with 5% non-fat milk in TBST (1 × TBS, 0.1% Tween-20) for 1 h at room temperature. Mouse anti-β-catenin antibody (1:2000; BD Bioscience) was added to the TBST and the membrane was incubated at 4 °C overnight. HRP-conjugated anti-mouse antibody (1:10000; Amersham Biosciences) was used as a secondary antibody and incubated with the membrane for 1 h. The antigen–antibody signal was detected by ECL detection system and normalized to the amount of β-actin from the same sample. Quantification of the signal was described previously [16].

2.6. Detection of Wnt reporter activity

MC3T3 cells grown in a 12-well culture dish were transiently transfected with the Wnt responsive TOPFLASH construct (1.6 μg/well), which contains 16 copies of a TCF/LEF site, using Lipofectamine 2000 (Invitrogen). For control, parallel dishes were transfected with the FOPFLASH construct, which contains 16 copies of a mutant TCF/LEF site. In each case, CMV-lacZ (0.16 μg/well) was co-transfected. Sixteen-hour post-transfection, the media was replaced with the conditioned media from LRAP-treated ST2 cells. Luciferase activity was detected using the Dual-Light reporter gene assay system (Applied Biosystems) 24 h later. Relative luciferase activity was calculated by normalizing the average luciferase activity to the β-galactosidase activity.

2.7. The siRNA knockdown assay

Knockdown of endogenous Wnt10b was accomplished using a commercially available siRNA (Ambion). The trypsinized ST2 cells were resuspended at 1×10^5 cells/mL in growth medium. Transfection agent siPORT NeoFX (3 μL) was diluted in OPTI-MEM I medium (47 μL). The siRNA was diluted in OPTI-MEM I medium by adding 1.5 μL of 20 μM siRNA to 48.5 μL of OPTI-MEM I medium, mixed with the transfection agent, and incubated at room temp for 10 min. The RNA/transfection agent complexes were dispensed into the empty wells of a 12-well culture plate and 900 μL of cells were transferred to each well of the culture plate containing the RNA/transfection agent complexes. The cells and RNA/transfection agent complexes were mixed gently and incubated at 37 °C for 48 h. An unrelated siRNA was included at the same concentration and conditions to serve as controls.

2.8. Quantitative RT-PCR

Quantitative PCR was performed according to the manufacturer's protocol. Briefly, a 25 μL reaction was prepared for each sample. Included in this reaction volume was 1 μL of the resulting cDNA, iQ SYBR green supermix (Bio-Rad) containing dNTP and iTaq DNA polymerase, and the appropriate primers. The resulting threshold cycle (C_T) value from each primer pair was normalized with the C_T value for *18S RNA*, which serves as an internal control. After amplification, melting curve analysis was performed as described in the manufacturer's protocol, and samples with aberrant melting curves were excluded. The corresponding primer sequences are 18S RNA (forward, 5′-CGATGCTCTTAGCTGAGTGT-3′; reverse, 5′-GGTCCAAGAATTTCACCTCT-3′), Runx2 (forward 5′-CCGTGGCCTTCAAGGTTGT-3′, reverse 5′-TTCATAACAGCGGAGGCATTT-3′), osterix (forward 5′-CCCTTCTCAAGCACCAATGG-3′, reverse 5′-AAGGGTGGGTAGTCATTTGCATA-3′), Dlx5 (forward 5′-GTCCCAAGCATCCGATCCG-3′, reverse 5′-GCGATTCCTGAGACGGGTG-3′), collagen I (forward 5′-GCTCCTCTTAGGGGCCACT-3′, reverse 5′-CCACGTCTCACCATTGGGG-3′), C/EBPα (forward, 5′-TGAACAAGAACAGCAACGAG-3′; reverse, 5′-TCACTGGTCACCTCCAGCAC-3′), PPARγ (forward, 5′-GGAAAGACAACGGACAAATCAC-3′; reverse, 5′-TACGGATCGAAACTGGCAC-3′), Wnt10b (forward 5′-TTCTCTCGGGATTTCTTGGATTC-3′, reverse 5′-TGCACTTCCGCTTCAGGTTTTC-3′).

2.9. Statistical analysis

All experiments were performed in triplicate unless stated otherwise. Final values were reported as means ± standard deviation (SD). Data were analyzed using Student's *t*-test and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of LRAP on osteogenic and adipogenic differentiation of ST2 cells

To decipher the mechanism underlying the signaling effect of LRAP on BMMSCs, we employed the bipotential stromal cell line