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The Oncogene LRF Stimulates Proliferation of Mesenchymal Stem Cells and Inhibits Their Chondrogenic Differentiation

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Abstract

Objective. The oncogene leukemia/lymphoma-related factor (LRF) enhances chondrosarcoma proliferation and malignancy. This study aimed to investigate the roles of LRF in chondrogenic differentiation of primary human bone marrow–derived mesenchymal stem cells (BMSCs). Design. LRF was overexpressed in BMSC by lentiviral transduction. Chondrogenic differentiation of BMSC was induced by high-density pellet culture. Western blotting and real-time polymerase chain reaction were used to investigate changes in protein and mRNA levels, respectively, during chondrogenesis. Safranin-O staining, immunohistochemistry, and glycosaminoglycan contents were used to assess cartilage matrix deposition. BMSC proliferation was determined by mitochondrial dehydrogenase activity and cell counting. Cell cycle profiling was performed by flow cytometry. Results. LRF overexpression effectively inhibited protein and mRNA expression of chondrocyte markers and cartilage matrix deposition during chondrogenesis of BMSC. Endogenous LRF expression was constitutively high in undifferentiated BMSC but remained low in primary articular chondrocytes. Endogenous LRF protein was downregulated in a time-dependent manner during chondrogenesis. BMSCs overexpressing LRF had higher proliferation rates and cell population in the S phase. LRF suppressed p53 expression during chondrogenesis and this might prevent differentiating chondrocytes from entering a quiescent state. Conclusion. Our data showed that LRF is important for stimulating stem cell proliferation and cell cycle progression. It is known that LRF is highly expressed in the mouse limb buds prior to overt chondrogenesis; thus, LRF might function to prevent premature chondrogenic differentiation of stem cells.

Keywords
chondrogenesis, cells, mesenchymal stem cells, proliferation, chondrocytes, LRF

Introduction

The leukemia/lymphoma-related factor (LRF) is a transcription factor containing an N-terminal POZ (poxvirus and zinc finger) domain and a C-terminal zinc finger domain. LRF belongs to the POK (POZ and Krüppel) protein families that are important for cellular differentiation and tumorigenesis.1,2 LRF directly represses the transcription of the tumor suppressor p19ARF, which in turn is required for p53 stability and function.3 Hence, aberrant overexpression of LRF is associated with some human cancers, such as lymphoma, glioma, breast, and non–small cell lung carcinomas.3,4 Ectopic overexpression of LRF in B and T lymphoid cells also causes aggressive lymphoma in mice.5 Apart from its oncogenic activity when overexpressed, normal expression of LRF is required to maintain cellular proliferation and prevent the induction of senescence in mouse embryonic fibroblast.1,5

A high level of LRF is detected in chondroprogenitor cells at the developing mouse limb buds at 10.5 dpc prior to overt chondrogenesis,5 suggesting the involvement of LRF in skeletal development. In addition, we have shown that aberrant expression of LRF contributes to the development of chondrosarcoma and tumor malignancy.7 Depletion of LRF in chondrosarcoma cell lines dramatically reduces proliferation and enhances their sensitivity to the anticancer drug doxorubicin. Furthermore, other evidences suggest a
Sox9 on COMP gene expression. However, LRF also suppresses expression of other cartilage matrix genes, such as collagen type II, type X, and aggrecan, during chondrogenesis. This suggests that LRF may also function through a distinct mechanism to suppress the expression of other cartilage matrix genes to inhibit chondrogenesis.

Our objective is to investigate the functions of LRF in chondrogenesis of primary human mesenchymal stem cells. Here, we determined the effects of ectopic overexpression of LRF on chondrogenesis of human bone marrow–derived mesenchymal stem cells (BMSCs). We also examined the steady state and temporal expression of endogenous LRF during chondrogenic differentiation of primary BMSCs. We found that LRF inhibited chondrogenesis by stimulating proliferation of BMSCs and suppressing p53 expression, and thus preventing the maturing chondrocytes from entering a quiescent state.

**Methods**

**Cell Culture**

Human bone marrow aspirates and cartilage from patients undergoing total knee arthroplasty were obtained with institutional review board approval. Primary BMSCs were isolated according to a previously described protocol. BMSCs grown in monolayer media (PT-3001 from Lonza, Basel Switzerland), supplemented with 1 ng/mL basic fibroblastic growth factor (Roche, Basel, Switzerland), supplemented with 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 mM HEPES, 1 U/mL penicillin, 100 mg/mL streptomycin, 29 mg/mL l-glutamine, 10% (v/v) fetal bovine serum (Lonza), and 5 ng/mL FGF-2 (R&D Systems, Minneapolis, MN). For chondrogenic differentiation in vitro, 4 × 10⁴ cells were cultured in micromass pellet cultures in polypropylene screw-cap tubes, with media changes every 3 days. Chondrogenic medium was Dulbecco’s modified Eagle medium (high glucose) with 1% ITS+ (BD Biosciences, San Jose, CA), 100 nM dexamethasone (MP Biomedicals, Santa Ana, CA), 50 µg/mL ascorbic acid 2-phosphate (Sigma, St. Louis, MO), 40 µg/mL Proline, 15mM HEPES, 10 ng/mL TGF-β1 (PeproTech, Rocky Hill, NJ), and 200 ng/mL rhBMP-2 (Medtronic, Minneapolis, MN).

**Generation of Lentivirus**

Human LRF cDNA (clone ID 8322563) was purchased (Open Biosystems, Huntsville, AL) and subcloned into a pCCL-based lentiviral vector with the addition of an N-terminal FLAG tag. A pCCL-based construct expressing green fluorescent protein (GFP) was also generated as control. Replication incompetent lentiviruses were packaged in 293T cells as described. BMSCs grown in monolayer were infected with lentivirus at 10 multiplicity of infection with 1 µg/mL polybrene and the media was replaced 16 hours later. Seventy-two hours after infection, BMSCs were used for in vitro chondrogenesis in pellet culture.

**In Vitro Chondrogenesis**

Primary human mesenchymal stem cells were isolated from discarded tissues obtained during total knee arthroplasty operations, with institutional review board approval and patient consent. Bone marrow was diluted 10 times in Hanks’s balanced salt solution, and nucleated cells stained with crystal violet and counted. Nucleated cells were plated onto tissue-culture treated plastic at a density of 10,000 cells/cm², in α-MEM (GIBCO, Carlsbad, CA) supplemented with 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 mM HEPES, 1 U/mL penicillin, 100 mg/mL streptomycin, 29 mg/mL l-glutamine, 10% (v/v) fetal bovine serum (Lonza), and 5 ng/mL FGF-2 (R&D Systems, Minneapolis, MN). For chondrogenic differentiation in vitro, 4 × 10⁴ cells were cultured in micromass pellet cultures in polypropylene screw-cap tubes, with media changes every 3 days. Chondrogenic medium was Dulbecco’s modified Eagle medium (high glucose) with 1% ITS+ (BD Biosciences, San Jose, CA), 100 nM dexamethasone (MP Biomedicals, Santa Ana, CA), 50 µg/mL ascorbic acid 2-phosphate (Sigma, St. Louis, MO), 40 µg/mL Proline, 15mM HEPES, 10 ng/mL TGF-β1 (PeproTech, Rocky Hill, NJ), and 200 ng/mL rhBMP-2 (Medtronic, Minneapolis, MN).

**Real-Time Reverse Transcriptase-Polymerase Chain Reaction**

Total RNA from cells grown as pellet culture for various times were extracted using an RNasy Mini Kit with DNase I digestion (both from Qiagen, Germantown, MD). The quantities and qualities of total RNA were assessed by a nanodrop instrument (Thermo Scientific, Waltham, MA), and 50 to 100 ng of RNA were reverse transcribed with random hexamers using a Superscript first-strand kit (Invitrogen). Quantitative real-time polymerase chain reaction was performed as triplicate measurements with the 7900HT qPCR instrument according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA). The TaqMan Gene Expression Assays used are as follows: COL2A1 Hs01060345_m1, COMP Hs00164359_m1, COL10A1 Hs00166657_m1. The level of each target gene was normalized to 18s rRNA and expressed as fold-change relative to the levels at time 0 (Ct method; Applied Biosystems).
Western Blot Analysis

BMSCs in monolayer or pellet cultures, and primary articular chondrocytes (passage 1) were harvested and lysed with sample loading buffer (50 mM Tris–HCl, pH 6.8; 100 mM dithiothreitol; 4% 2-mercaptoethanol; 2% sodium dodecyl sulfate [SDS]; 10% glycerol). Lysates were resolved by 8% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (BioRad, Hercules, CA). The membranes were blocked with 2% bovine serum albumin in TBST (25 mM Tris–HCl, pH 7.5; 125 mM NaCl; 0.1% Tween 20), followed by incubation with rabbit polyclonal antiserum specific for human COMP,8 rabbit anti-LRF,7 mouse anti-p53 (Santa Cruz Biotechnology, Santa Cruz, CA), and mouse anti-GAPDH (Ambion, Carlsbad, CA) at 4 °C overnight. Blots were then probed with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology), and reactive protein bands were visualized with Western Lightning Plus-ECL (PerkinElmer, Waltham, MA).

Safranin-O Staining

Twenty-one-day-old BMSC pellet cultures were fixed in 10% formalin overnight and embedded in paraffin. Three-micrometer sections mounted on glass slides were stained with Safranin-O and counterstained with hematoxylin as described.13

Immunohistochemistry

Sections of paraffin-embedded BMSC pellets were processed for immunohistochemistry using mouse anti-human collagen type II (MP Biomedical clone II-4C11), and then visualized with ImmPRESS anti-mouse Ig reagent and ImmPACT NovaRED peroxidase substrate (Vector Laboratories, Burlingame, CA). Negative controls included an irrelevant primary antibody and omission of the secondary antibody.

DMMB Assays

Proteoglycan content of the cell pellets was determined using DMMB (dimethylmethylene blue). Briefly, pellets were digested overnight in proteinase K, DMMB dye (Sigma) was bound, and absorbance at 525 nm was measured and compared with that of chondroitin sulfate standards.

Cell Proliferation Assay

BMSCs were infected with GFP- or LRF-expressing lentivirus as described above and used at 4-day post-infection. For proliferation assays, 1 x 10^5 BMSCs were seeded in 96-well plates with 100 µL media. At various days, the activities of mitochondrial dehydrogenase from live cells were determined by adding 1 µL of water-soluble tetrazolium substrate (Quick Cell Proliferation Kit, BioVision, Milpitas, CA) and the optical density was measured at 440 nm with a spectrophotometer after 2 hours. An empty 96-well with 100 µL media was used as blank. For the determination of cell number, 10 x 10^3 BMSCs were seeded in 12-well plates (as day 0), and the numbers of cells were counted in a hemocytometer at days 2, 4, and 6.

Cell Cycle Profiling

Approximately 1 x 10^6 uninfected, GFP- or LRF-transduced BMSCs were harvested by trypsinization at 9 day post-transduction. After centrifugation (500 x g, 5 minutes), the cell pellets were washed with phosphate-buffered saline and fixed with cold 70% ethanol for 30 minutes on ice. Cells were washed and resuspended in phosphate-buffered saline containing RNase (200 µg/mL) and incubated at 37 °C for 30 minutes. Propidium iodide was added to a final concentration of 25 µg/mL and cells were incubated on ice for 30 minutes before acquiring by flow cytometry (FACS Fortessa LSR, Becton Dickinson, Franklin Lakes, NJ). The data were analyzed by FlowJo software.

Statistical Analysis

JMP software (version 8.0; SAS Institute, Cary, NC) was used for data analyses. The mRNA expression, growth rates, and cell cycle distribution profiles of LRF-infected BMSCs were compared to that of the uninfected and GFP-infected BMSCs (unpaired Student’s t test, P < 0.05 is considered significant).

Results

LRF Inhibits Expression of Chondrocyte Markers during Chondrogenesis of BMSC

Our previous studies showed that LRF depletion reduces proliferation in chondrosarcoma cell lines, whereas ectopic overexpression of LRF in the mouse mesenchymal stem cell line C3H10T1/2 inhibited in vitro chondrogenesis. To gain more insight into the mechanism that regulates LRF’s function during chondrogenesis, we examined the effects of LRF overexpression on chondrogenesis of primary human BMSC in pellet culture. Lentivirus harboring human LRF, or GFP as a control, was used to transduce BMSC and chondrogenesis was induced by pellet culture. The mRNA levels of 2 chondrocyte markers, COMP and Col2a1, and the hypertrophic marker Col10, were monitored by quantitative polymerase chain reaction in a 21-day time course. The data showed that uninfected or GFP-infected BMSCs produced increasing amounts of COMP, Col2a1, and Col10 in a time-dependent manner during chondrogenesis as expected (Fig. 1). In contrast, LRF overexpression...
significantly reduced Col2a1 mRNA expression during chondrogenesis. COMP and Col10 expression were also lowered in LRF-expressing BMSC, compared to control and GFP-infected BMSCs, although not achieving statistically significant (Fig. 1). Similar results were also obtained in pellet cultures of primary chondrocytes overexpressing LRF (data not shown), demonstrating a negative role of LRF in cartilage matrix gene expression.

**LRF Inhibits Chondrogenesis of Primary Human BMSC**

Next, the pellet cultures from uninfected and LRF-infected BMSCs at day 21 were stained with Safranin-O to assess proteoglycan deposition. The results showed that proteoglycan staining of the pellet culture overexpressing LRF is markedly reduced when compared with uninfected control (Fig. 2A, top panels). In addition, consistent with the mRNA results, Col2a1 protein was markedly reduced in LRF-infected pellets (Fig. 2A, bottom panels). Western analysis showed that the production of COMP protein was greatly reduced in LRF-infected pellet culture when compared to control (Fig. 2B). Thus, the protein expression profiles of COMP correlated well with the mRNA levels of COMP (Fig. 1) during chondrogenesis of BMSC. Similar results were obtained using BMSC from 2 other donors (not shown). Western analysis also demonstrated that the lentiviral expression system used in this study is effective in maintaining the long-term expression of LRF protein at least through day 14 of pellet culture.

We next examined the effects of LRF overexpression on the size and glycoaminoglycan (GAG) content of the pellet cultures. As shown in Figure 3A, the physical dimension of the pellets expressing LRF at various days was markedly reduced, compared with control and GFP-expressing pellets.
Furthermore, the total GAG contents in LRF-expressing pellets were also reduced (Fig. 3B) at days 14 and 21. Taken together, the above results indicate that overexpression of LRF effectively blocks chondrogenesis of primary human BMSC, thus confirming previous findings in C3H10T1/2 mouse cell line.8

Endogenous LRF Is Constitutively High in Proliferating Stem Cells but Remains Low in Adult Chondrocytes

To examine the regulation of LRF expression during chondrogenesis, we next determined the endogenous levels of LRF in undifferentiated stem cells and adult chondrocytes. Western analysis revealed that the endogenous LRF protein was readily detectable in primary human BMSC isolated from 2 different donors (Fig. 4A, lanes 1 and 2), and in the mouse mesenchymal stem cell line C3H10T1/2 (lane 3). In contrast, the levels of endogenous LRF were barely detectable in adult articular chondrocytes isolated from 2 donors (Fig. 4A, lanes 4 and 5), and in the immortalized human chondrocytic cell line C20/A4 (lane 6). These results indicate that endogenous LRF expression is low in chondrocytes but high in BMSC, and in turn suggest that LRF expression might be suppressed during differentiation of BMSCs into chondrocytes. Indeed, when the levels of endogenous LRF were monitored during chondrogenesis of primary BMSCs in pellet culture, there was a time-dependent decrease of LRF protein (Fig. 4B). The decrease in LRF protein coincided with a gradual increase in the expression of the chondrocyte-specific COMP protein, and hence correlated well with the progression of chondrogenesis. Collectively, these data are consistent with the model that LRF is an inhibitor of chondrogenesis, and therefore, LRF expression is suppressed during chondrogenesis.

LRF Enhances Proliferation of BMSC

We next examined the effects of LRF expression on BMSC proliferation to gain insight into the mechanism of which LRF inhibits chondrogenesis. Since LRF is an oncogene that is required for maintaining normal cellular proliferation, we tested the effects of LRF overexpression on the growth rate of BMSC. LRF was overexpressed in BMSC through lentiviral transduction to ~5-fold the level of
endogenous LRF (Fig. 5A). The proliferation rates of uninfected BMSCs, or BMSCs infected with GFP- or LRF-expressing lentivirus were measured by mitochondrial dehydrogenase activity (Fig. 5B) and by counting the cell numbers (Fig. 5C) over a 6-day period. The results demonstrated a similar growth rate increased in both uninfected and GFP-infected BMSCs. In contrast, LRF-infected BMSC showed significantly higher dehydrogenase activity and cell numbers that were apparent at day 4 and 6 (Fig. 5B and C). These results indicate that LRF enhances proliferation of BMSCs.

**LRF Enhances Cell Cycle Progression in BMSC**

It has been shown that LRF suppresses p19ARF transcription, thus enhancing degradation of p53 that is important for regulating cell cycle arrest. This prompted us to investigate the effects of LRF overexpression on the cell cycle profile of BMSCs. Flow cytometry analysis revealed that uninfected and GFP-infected BMSCs demonstrated similar cell cycle profiles (representative cell cycle profiles from a single donor are shown in Fig. 6A-C). Data determined from three BMSC donors showed that on average, there were ~80% cells in G1 phase and ~10% cells in each of S and G2/M phase in uninfected and GFP-expressing cells (Fig. 6). However, compared with control cells, LRF-infected BMSCs showed a significant decrease in G1 population (~63%), and this is accompanied by a corresponding increase in the S phase (~27%; Fig. 6D). On the other hand, the population of cells in the G2/M phase did not change significantly for all samples measured. Taken together, these data indicate that LRF overexpression increases the S1 population that are primed for mitosis, and correlates with the above results that LRF enhances cellular proliferation.

**LRF Overexpression Reduces p53 Levels in BMSC Pellet Cultures**

Apart from functioning as a tumor suppressor to induce cellular senescence, p53 is necessary for terminally
differentiating cells to establish a quiescent state.\textsuperscript{14,15} Chondrocytes are enclosed within their own pericellular matrix with limited space for dividing, so entering a quiescent state is critical for maintaining the chondrocytic phenotype. We therefore examined the changes in the protein levels of p53 during chondrogenesis of BMSC in pellet cultures. The results showed that p53 level increased significantly during chondrogenesis of normal BMSC (\textbf{Fig. 7}, compared lanes 1 and 2). In contrast, there was a significant decrease in p53 level during chondrogenesis of BMSC overexpressing LRF (\textbf{Fig. 7}, compared lanes 3 and 4). These data indicate that high levels of LRF suppress p53 expression in differentiating chondrocytes.

\textbf{Discussion}

Expression of LRF is detected in a variety of different cell types.\textsuperscript{6} Numerous studies have indicated that LRF may have diverse physiological functions during embryonic development and also in the regulation of tissue homeostasis and senescence.\textsuperscript{3,16-19} To our knowledge, this is the first study to investigate the role of LRF in chondrogenesis of
Our findings reveal a difference between the expression of LRF in undifferentiated BMSC (high LRF) and in articular chondrocytes (low LRF). Moreover, expression of endogenous LRF is suppressed during differentiation of BMSC into chondrocytes, suggesting that LRF is a natural inhibitor of chondrogenesis that is downregulated during this process. This is supported by the results that chondrogenesis is inhibited when the expression of LRF in BMSC (Figs. 1 and 2) and chondrocytes (not shown) is artificially maintained at high levels.

Our current study demonstrates that LRF enhances the proliferation of primary human BMSCs by facilitating cell cycle progression. This is in agreement with our previous finding that LRF depletion severely suppresses the proliferation of chondrosarcoma cell lines. High LRF expression is known to enhance p53 degradation and prevent p53-mediated cell cycle arrest. As a result, excessive LRF leads to uncontrolled cellular proliferation and this may contribute to the malignancy of chondrosarcoma and other human cancers. On the other hand, mouse embryonic fibroblasts deficient in LRF have reduced growth and increased senescence rates, indicating that normal level of LRF is required to maintain continuous cellular proliferation. We postulate that this aspect of LRF function is especially important in maintaining the self-renewal capacity of stem cells and preventing their premature senescence.

Our previous data in a mouse mesenchymal stem cell line and our present study in primary human BMSCs both confirm that ectopic overexpression of LRF effectively inhibits chondrogenesis. However, the precise mechanism remains unknown. Since a high level of LRF is detected in the developing mouse limb bud prior to overt chondrogenesis, we postulate that LRF may function to promote proliferation of chondro-progenitor cells, while preventing their premature differentiation into chondrocytes. Adult chondrocytes in the cartilage rarely divide and they last for the entire life span of an organism. As chondrocytes are surrounded by pericellular and extracellular matrix in the cartilage, there is very limited space for proliferation. As a result, high levels of LRF that promote cell division would seem detrimental to the chondrocyte phenotype.

We therefore propose a model to describe the function of LRF in chondrogenesis (Fig. 8). In this model, high level of LRF in BMSCs mainly functions to prevent senescence by promoting their self-renewal and proliferation capacity. While a decrease in LRF expression during chondrogenesis allows the accumulation of p53 and facilitates the transition of maturing chondrocytes into a quiescent state as they are enclosed within the cartilage matrix. This hypothesis is supported by the findings that the tumor suppressor p53 is important for establishing cellular quiescence in terminally differentiated cells, and therefore, low level of LRF in chondrocytes may in turn enhance p53’s function in this respect, as evident by our data in Figure 6.

In summary, we demonstrated in this study the function of LRF in stimulating BMSC proliferation and in inhibiting chondrogenesis. Our data suggest that by modulating the activity of LRF, one might be able to increase the

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**Figure 7.** Leukemia/lymphoma-related factor (LRF) overexpression suppresses p53 levels during human bone marrow–derived mesenchymal stem cells (BMSCs) chondrogenesis. (A) p53 levels during chondrogenesis of BMSC. The protein levels of cartilage oligomeric matrix protein (COMP), LRF (endogenous and FLAG-tagged), p53, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) in day 0 and day 14 pellet cultures of uninfected BMSC, or BMSC overexpressing LRF, are determined by Western blot. (B) The expression of p53 detected by Western blots were quantified by densitometry and normalized to GAPDH levels. The levels of p53 from day 0 pellet cultures were set to 100%. Results with standard deviation were the average of experiments using BMSC from 2 different donors.
proliferation and self-renewal capacity of stem cell and affects their chondrogenic differentiation.

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Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval
This study was approved by our institutional review board.

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