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Adenovirus-mediated transfer of siRNA against Runx2/Cbfa1 inhibits the formation of heterotopic ossification in animal model

Lin Lin [a], Lianxu Chen [a], Haijun Wang [a], Xuelei Wei [a], Xin Fu [a], Jiying Zhang [a], Kangtao Ma [b], Chunyan Zhou [b,*], Changlong Yu [a,*]

[a] *Institute of Sports Medicine, Peking University Third Hospital, No.49, North Garden Road, Haidian District, Beijing 100083, PR China*
[b] *The Department of Biochemistry and Molecular Biology, Peking University School of Basic Medical Science, Beijing 100083, PR China*

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Abstract

The heterotopic ossification of muscles, tendons, and ligaments is a common problem faced by orthopaedic surgeons. Runx2/Cbfa1 plays an essential role during the osteoblast differentiation and is considered as a molecular switch in osteoblast biology. RNA interference technology is a powerful tool for silencing endogenous or exogenous genes in mammalian cells. In this study, we investigated the effect of Runx2/Cbfa1-specific siRNA on osteoblast differentiation and mineralization in osteoblastic cells, and then constructed adenovirus containing siRNA against Runx2/Cbfa1 (Ad-Runx2-siRNA) to inhibit the formation of heterotopic ossification induced by BMP4, demineralized bone matrix, and trauma in animal model. Our results showed that the Runx2/Cbfa1-specific siRNA could inhibit the expression of Runx2/Cbfa1 at the level of mRNA and protein. Analysis of the expression of osteoblast maturation genes including type I collagen, osteopontin, bone sialoprotein, and osteocalcin, alkaline phosphatase activity, and matrix mineralization (von kossa) revealed that osteoblast differentiation was inhibited in cultured primary mouse osteoblasts transduced with Ad-Runx2-siRNA. Furthermore, adenovirus-mediated transfer of siRNA against Runx2/Cbfa1 could inhibit the formation of heterotopic ossification induced by BMP4, demineralized bone matrix, and trauma in animal model. It is likely that the inhibition of Runx2/Cbfa1 by RNAi could be developed as a powerful approach to prevent or treat heterotopic ossification.

Keywords: siRNA; Runx2/Cbfa1; Osteoblast; Gene therapy; Heterotopic ossification; Adenovirus

Heterotopic ossification can be defined as the formation of bone in tissues which normally exhibit no properties of ossification. The heterotopic ossification of muscles, tendons, and ligaments most frequently is seen with musculoskeletal trauma, neurologic injury, severe burns, and elective surgery [1–10]. In addition, there is the rare hereditary form known as fibrodysplasia ossificans progressive [11]. Reports of radiographic heterotopic ossification following total hip arthroplasty have ranged from 15% to 90% of patients, with 1% to 27% of patients reporting clinically important pain and loss of motion [12]. The prevalence of heterotopic ossification associated with all burn injuries has ranged between 1% and 3% but can be as high as 35% for larger, more severe burns [9,13,14]. The development of heterotopic ossification after spinal cord injury has been reported in 16–53% of patients, one-third of whom had clinically important manifestations [15,16]. Heterotopic ossification has also been reported in nonskeletal tissues such as the gastrointestinal tract [17].

While the exact etiology of heterotopic ossification remains unclear, the ectopic bone is thought to originate from mesenchymal stem cells which lie dormant in soft tissues and differentiate into osteogenic cells under appropriate stimuli [18]. However, mesenchymal stem cells alone cannot produce heterotopic ossification without stimulating agents

* Corresponding authors. Fax: +86 10 62021305 (C. Yu), +86 10 82802417 (C. Zhou).
E-mail addresses: chunyanzhou@bjmu.edu.cn (C. Zhou), YCL123@vip.sina.com (C. Yu).

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existed. It is possible that the overexpression of certain bone morphogenetic proteins (BMPs) which belong to TGF-β superfamily, such as BMP2 and BMP4, plays a role in heterotopic ossification formation. It has been demonstrated that the addition of BMPs to normal skeletal muscle can induce heterotopic ossification [19,20]. BMP4, in particular, seems to be up-regulated in diseases such as fibrodysplasia ossificans progressiva, a rare autosomal dominant disease characterized by the progressive ossification of skeletal muscle, ligaments, and tendons [11,21,22].The addition of other stimulating factors such as interleukin-1β to bone morphogenetic proteins has been shown to further enhance bone formation [23]. Growth hormone, prolactin, insulin-like growth factor type I and basic fibroblast growth factor have been implicated in the formation of heterotopic ossification after head injury [24,25]. It would appear that a complex and as yet poorly understood interaction of a wide variety of stimulating factors is involved.

During the osteoblast differentiation process, a set of bone-specific genes (e.g., alkaline phosphatase and osteocalcin genes) are activated. Runt-related protein 2/core binding factor 1 (Runx2/Cbfa1), hereafter called Runx2, is a key regulator of osteocalcin and alkaline phosphatase gene promoters and cooperates with BMP-specific R-Smads [26]. Runx2 is a transcription factor recently shown to be a master regulator of osteoblast differentiation and is considered as a molecular switch in osteoblast biology [27–30]. Furthermore, Ducy et al. [31] and Banerjee et al. [32] demonstrated that disruption of Runx2 by antisense oligonucleotides in osteoblast cultures inhibited expression of osteoblast differentiation markers and formation of mineralized nodules.

In the present study, we designed recombinant adenoviral vectors encoding siRNA against Runx2, and investigated their effects on Runx2 expression and osteoblast differentiation in cultured osteoblastic cells. And then the vectors were tested against three different models of heterotopic ossification (BMP4, demineralized bone matrix, and trauma), all of which previously have been shown to induce heterotopic ossification [19,33,34].

Materials and methods

Cell culture

Primary osteoblasts were isolated from the calvariae of 21-day fetal mice as previously described [35]. In brief, cells were obtained from the calvariae (the dura and periosteum removed) by five sequential digestions of 10 min at 37 °C in phosphate-buffered saline (PBS) containing 0.05% collagenase. Cells from the fourth and fifth digests were used in the present study. MC3T3-E1 was obtained from Chinese Academy of Medical Sciences (Peking, China). Cells were suspended in Dulbecco's modified Eagle's medium (DMEM; Gibco, Laboratories, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT, USA), penicillin (100 units/ml), and streptomycin (100 units/ml) at 37 °C in a humidified atmosphere of 5% carbon dioxide and 95% air.

siRNA synthesis and transfection

Five target sequences (Table 1) for mouse Runx2 mRNA were chosen according to Ambion's siRNA design online tool. The specificity of all sequences was confirmed by BLAST search. All siRNAs were synthesized with the silencer siRNA construction kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol. The negative control (scrambled siRNA) siRNA was purchased from Ambion. Transfection of siRNA was performed by the siPORT™ Lipid (Ambion) according to the manufacturer's protocol.

Construction of adenovirus siRNA vector

The adenovirus siRNA vector was constructed with the pSilencer™ adeno 1.0-CMV System (Ambion, Austin, TX, USA) according to the manufacturer's protocol. Briefly, the siRNA templates were designed and synthesized according to Ambion's siRNA design online tool, and then inserted into the shuttle vector 1.0 CMV at the *Xho*I and *Spe*I restriction enzyme sites. The resultant DNA and the adenoviral LacZ backbone were linearized with *Pac*I and transfected in 293 cells using a calcium phosphate method for adenovirus packaging. The control recombinant adenovirus carrying the scrambled siRNA was constructed with a similar method. The adenovirus siRNA vector was purified by cesium chloride ultracentrifugation. Purified viruses were dialyzed in phosphate-buffered saline (PBS) with 10% glycerol and stored at −70 °C until use. Adenoviral infection was performed essentially as described previously [34]. All infection experiments were performed at an MOI (multiplicity of infection: pfu/cell) of 200, a condition that enabled about 80% transduction of transgenes into osteoblastic cells used in this study (data not shown).

Table 1
siRNA template sequences

Sequence range (GC%)	siRNA oligonucleotide template
siRNA$^{96–116}$ (47.6)	
Antisense	5′-AATCTCTGCAGGTCACTACCACCTGTCTC-3′
Sense	5′-AATGGTAGTGACCTGCAGAGACCTGTCTC-3′
siRNA$^{758–778}$ (47.6)	
Antisense	5′-AAGTAGCCAGGTTCAACGATCCCTGTCTC-3′
Sense	5′-AAGATCGTTGAACCTGGCTACCCTGTCTC-3′
siRNA$^{922–942}$ (38.1)	
Antisense	5′-AAGCTTGATGACTCTAAACCTCCTGTCTC-3′
Sense	5′-AAAGGTTTAGAGTCATCAAGCCCTGTCTC-3′
siRNA$^{1064–1084}$ (42.9)	
Antisense	5′-AAGGACAGAGTCAGATTACAGCCTGTCTC-3′'
Sense	5′-AACTGTAATCTGACTCTGTCCCCTGTCTC-3′
siRNA$^{1657–1677}$(38.1)	
Antisense	5′-AATGGCAGCACGCTATTAAATCCTGTCTC-3′
Sense	5′-AAATTTAATAGCGTGCTGCCACCTGTCTC-3′