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Cooperation of Rho family proteins Rac1 and Cdc42 in cartilage development and calcified tissue formation

Mikiko Ikehata [a, b], Atsushi Yamada [a, *], Koji Fujita [c], Yuko Yoshida [a, d], Tadashi Kato [a, e], Akiko Sakashita [e], Hiroaki Ogata [e], Takehiko Iijima [d], Masahiko Kuroda [c], Daichi Chikazu [b], Ryutaro Kamijo [a]

[a] Department of Biochemistry, School of Dentistry, Showa University, Tokyo, Japan
[b] Department of Oral and Maxillofacial Surgery, Tokyo Medical University, Tokyo, Japan
[c] Department of Molecular Pathology, Tokyo Medical University, Tokyo, Japan
[d] Department of Perioperative Medicine, Division of Anesthesiology, School of Dentistry, Showa University, Tokyo, Japan
[e] Department of Internal Medicine, Showa University Northern Yokohama Hospital, Yokohama, Japan

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ABSTRACT

Rac1 and Cdc42, Rho family low molecular weight G proteins, are intracellular signaling factors that transmit various information from outside to inside cells. Primarily, they are known to control various biological activities mediated by actin cytoskeleton reorganization, such as cell proliferation, differentiation, and apoptosis. In order to investigate the functions of Rac1 and Cdc42 in bone formation, we prepared cartilage-specific double conditional knockout mice, $Rac1^{fl/fl}$; $Cdc42^{fl/fl}$; Col2-Cre (Rac1: Cdc42 dcKO mice), which died just after birth, similar to $Cdc42^{fl/fl}$; Col2-Cre mice (Cdc42 cKO mice). Our findings showed that the long tubule bone in Rac1: Cdc42 dcKO mice was shorter than that in $Rac1^{fl/fl}$; Col2-Cre mice (Rac1 cKO mice) and Cdc42 cKO mice. Abnormal skeleton formation was also observed and disordered columnar formation in the growth plate of the Rac1: Cdc42 dcKO mice was more severe as compared to the Rac1 cKO and Cdc42 cKO mice. Together, these results suggest that Rac1 and Cdc42 have cooperating roles in regulation of bone development.

1. Introduction

Mammalian bone morphogenesis is divided into endochondral and intramembranous ossification. The skeleton is mostly formed through the process of endochondral ossification, in which replacement of cartilage in the growth plates of long bones, ribs, and vertebrae occurs in a series of distinct chondrocyte differentiations steps [1,2]. During endochondral ossification, proliferating growth plate chondrocytes undergo hypertrophy and die, and then are deposited as bone in the cartilage matrix, after which osteoblasts differentiate in the perichondrium and secrete cortical bone around the cartilage template [3,4].

The Rho family of GTPases, RhoA, Rac1, and Cdc42 regulate a variety of essential signaling pathways in eukaryotic cells. The major roles of Rho GTPases include regulation of actin cytoskeleton reorganization, gene transcription, and cellular polarity [5–7]. RhoA and Rac1 are known as inducers that assemble contractile actin and myosin filaments, as well as the actin-rich surface protrusion lamellipodia [8]. Cdc42 is known to be an inducer that promotes formation of actin-rich finger-like membrane extensions and is referred to as filopodia [8]. In particular, Rac1 and Cdc42 are known as essential proteins for development of various tissues, including bone and cartilage. However, they have not been sufficiently investigated, and the molecular details of their functions and mechanisms of regulation in chondrocytes are not fully understood. A recent study demonstrated that deletion of Rac1 in chondrocytes induced disorganization of growth plates and reduced the expression of chondrogenesis marker genes [9]. Similar growth plate abnormalities were detected in chondrocyte-specific Cdc42 conditional-knockout mice and, moreover, their neonates died within 1 day after birth [10]. Rac1 and Cdc42 have also been demonstrated to have both similarities and differences regarding their roles in hard tissue formation. In addition, previous studies have indicated that Dbl activates RhoA and Cdc42, and

* Corresponding author. 1-5-8 Hatanodai, Shinagawa, Tokyo, 142-8555, Japan.
E-mail address: yamadaa@dent.showa-u.ac.jp (A. Yamada).

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possibly Rac1 as well [11,12]. The Dbl proto-oncogene is a prototype guanine nucleotide exchange factor (GEF) that controls the functions of Rac1, Cdc42, and RhoA [13]. Using rheumatoid arthritis (RA) as an example of an osteochondral defect, that study reported that Dbl splicing variants were found to be with associated with RA in some affected patients and the variations showed weak GEF activity toward Cdc42. Additional research is necessary to better understand the functions of Rac1 and Cdc42 in regard to bone development, morphogenesis, and metabolism.

We speculated that Rac1 and Cdc42 have cooperating roles for regulation of bone development *in vivo*. Thus, for the present study we generated and analyzed chondrocyte-specific Rac1 and Cdc42 double-conditional knockout (dcKO) mice.

2. Materials and methods

2.1. Generation of mice

All animal experiments were conducted in accordance with the guidelines of Showa University. Rac1 flox mice and Cdc42 flox mice were gifted from Aiba A. Either or both Rac1 and Cdc42 were knocked out via Cre-loxP recombination by crossing Rac1 flox and Cdc42 flox mice with Col2-Cre transgenic (Col2-Cre) mice [14]. The conditional allele and Rac1/Cdc42 deleted alleles were identified using 50 mM NaOH/1 M Tris-HCl extracted tissue. The primers are used following: Rac1-A, 5′-TGGCTGGAATTCCTGGTCTG-3′, Rac1-B, 5′-GTGCTGGCATACCTGCTGCA-3′ and Rac1-C, 5′-GTGGTGCA-CATGTGTATAGA-3′, Cdc42-A, 5′- AGAGTGAGTTACAGAACAGC-3′, Cdc42-B, 5′-ATGCTTCATAACTTTCCAGA-3′, and Cdc42-C, 5′-CAGC-CACTCAAACCAGAACT-3', respectively.

2.2. Anatomical and histological analyses

For skeletal staining, mice were skinned, then eviscerated and dehydrated in 95% ethanol overnight. Next, the skeletons were stained overnight with 15% Alcian blue and 20% acetic acid in 95% ethanol, and soft tissues were dissolved overnight in 2% KOH, with the skeletons additionally stained overnight with 7.5% Alizarin red in 1% KOH. Finally, the skeletons were cleaned in 1% KOH and 20% glycerol for several days, and stored in glycerol/ethanol (1:1). For general morphological analyses, all samples were fixed in 4% paraformaldehyde and processed into serial paraffin sections using routine procedures. Deparaffinized sections were subjected to Safranin-O, Fast Green, and Villanueva staining. *In situ*

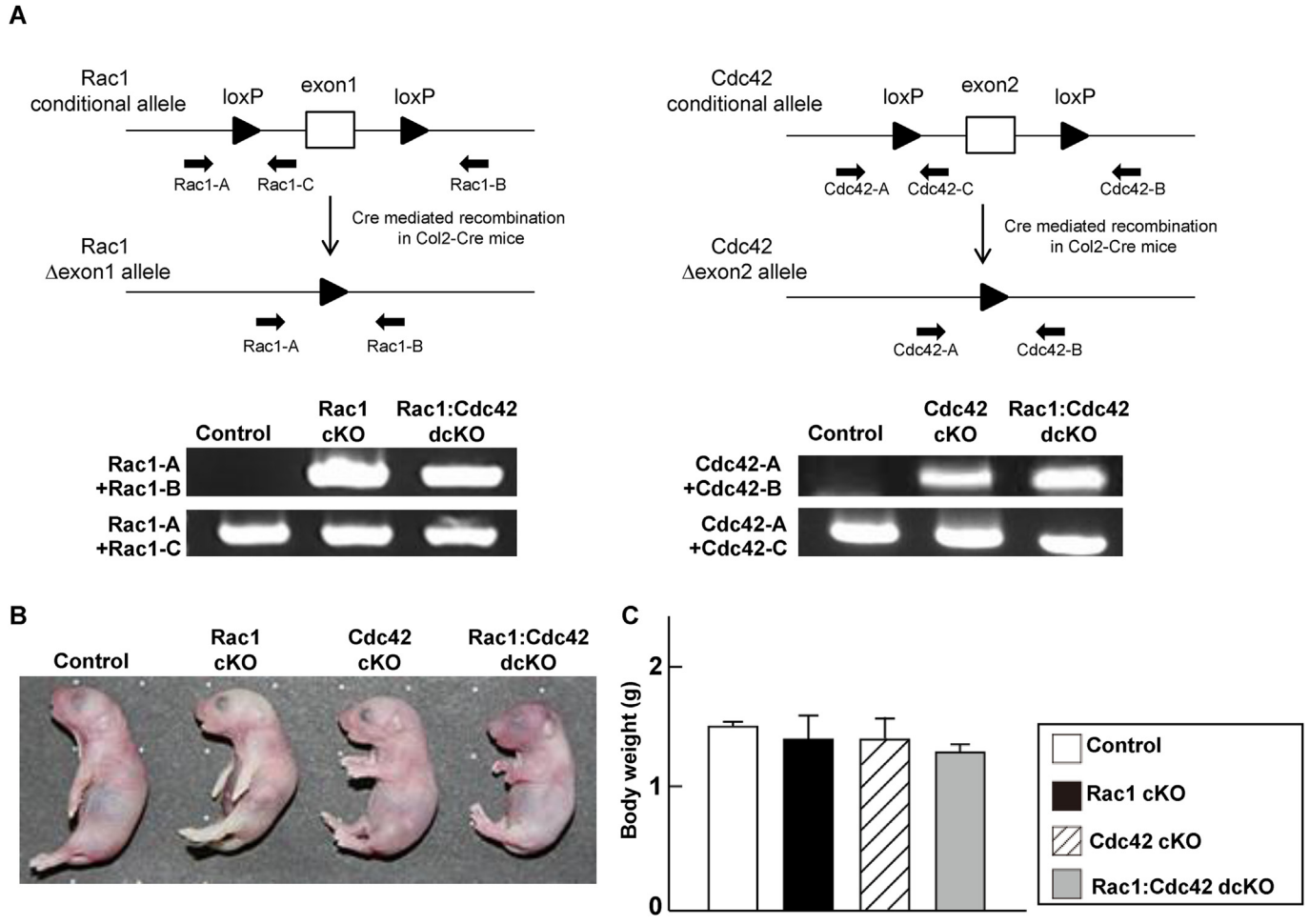

Fig. 1. Phenotypes of cartilage-specific Rac1 and Cdc42 conditional-knockout mice.
A) Schematic diagrams showing targeting strategy used for production of Rac1 and Cdc42 conditional-knockout mice. Primers used for PCR assessment of Rac1 exon1 (Δexon1) and Cdc42 exon2 deletion (Δexon2) were Rac1-A, -B, and -C, and Cdc42-A, -B, and -C, respectively. PCR analysis was performed using rib cartilage samples obtained from the 4 different types of mice on P0. Rac1 conditional allele-specific, Δexon1 allele-specific, Cdc42 conditional allele-specific, and Δexon2 allele-specific bands were detected. **B)** Whole-body morphology of mice on P0. As compared with control mice, the limbs and body were shortest in Rac1: Cdc42 dcKO mice, followed in order by Cdc42 cKO and Rac1 cKO mice. **C)** Comparison of birth weights. Rac1: Cdc42 dcKO mice had the lightest weight among the groups. Graphs show average and SD values obtained for the control (open column, n = 190), Rac1 cKO (closed column, n = 79), Cdc42 cKO (hatched column, n = 31), and Rac1: Cdc42 dcKO (shaded column, n = 6) mouse groups.