Journal of Oral Biosciences 55 (2013) 116–121

Contents lists available at ScienceDirect

Journal of Oral Biosciences

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

Review

The essential roles of the small GTPase Rac1 in limb development

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ARTICLE INFO

Article history:
Received 7 March 2013
Received in revised form
4 April 2013
Accepted 23 April 2013
Available online 21 June 2013

Keywords:
Conditional knockout mice
Limb development
Rac1

ABSTRACT

Vertebrate limbs develop through complicated interactions between various types of cells of the ectoderm and mesenchyme, which facilitate the proper steps of growth and formation by cell proliferation, differentiation, and apoptosis. These processes are regulated by several signaling proteins such as Fgf, Bmp, and Wnt. Recently, several types of *Rac1* conditional knockout mice were created, and Rac1 was observed to play essential roles in each of the developing limb tissues. Mice with genetic deletion of *Rac1* in the chondrocytes, the limb bud ectoderm, or the limb bud mesenchyme exhibit dwarfism with short limbs, severe truncations of limbs, or syndactyly with short limbs, respectively. Analyses of these mice demonstrated that Rac1 regulates limb development by controlling many kinds of gene expression and various cellular functions.

Contents

1. Introduction

1.1. Limb development

The vertebrate limb is composed of various cells and tissues, including bones, muscles, blood vessels, and skin. Limb development in mice during the embryonic stages originates from extensions of the limb primordium (limb bud), which is composed of mesenchymal cells derived from the lateral plate mesoderm and ectoderm covering the mesenchyme. The limb bud has specialized regions such as the apical ectodermal ridge (AER) and the zone of polarizing activity (ZPA). The AER, which is located at the tip of the limb buds, maintains outgrowth of the limb bud by expressing fibroblast growth factor 8 and 4 (Fgf8, Fgf4), which in turn keeps the underlying mesenchymal cells in the progress zone (PZ) in an undifferentiated state [1–4]. A group of cells located in the posterior mesenchyme of the limb bud, the ZPA, acts as the organizer of the anterior–posterior (AP) polarity of the limb bud; the polarizing activity of the ZPA is mediated by sonic hedgehog (Shh) [5]. The analysis of various gene functions has revealed the existence of complex interactions between signaling pathways operated by secreted factors of the HH (hedgehog), TGF-β/BMP, WNT, and FGF superfamilies, which interact with many other genetic networks to control limb positioning, outgrowth, and patterning [6].

After maturation of the limb buds, mesenchymal cells are recruited via migration and condensed at the position of each of the bones during the patterning stage, and then the chondrocyte progenitors are differentiated from condensing mesenchyme to proliferate and differentiate in the fixed regions during the next step [7]. Cells undergoing chondrogenesis acquire a distinct

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http://dx.doi.org/10.1016/j.job.2013.05.002

spherical cell morphology and initiate expression of the transcription factors Sox9 [8], Sox5, and Sox6, which regulate the genes encoding the extracellular matrix (ECM) molecules collagen II and aggrecan [9–11]. Chondrocytes differentiate by hypertrophy, downregulate the expression of collagen II, and initiate the expression of collagen X [12], the matrix molecule bone sialoprotein (Bsp) [13], and secreted factors such as matrix metalloproteases 9 and 13 (Mmp9, Mmp13). The metalloproteases degrade the ECM during the remodeling process for hypertrophic enlargement, proper vascularization, and ossification [14,15]. Hypertrophic chondrocytes mineralize their surrounding matrix and undergo apoptosis [16]. The vascular tissue is stimulated to invade into this region, allowing the entrance of osteoclast and osteoblast precursors, which remodel the remaining hypertrophic matrix and lay down the bone tissue [17].

2. Rac1

The proteins in the mammalian family of Rho proteins were initially isolated as Ras-like small GTP-binding proteins and they were found to be composed of the Rho, Rac, and Cdc42 subfamilies [18]. These proteins act as molecular switches; they are inactive when bound to GDP, but upon exchange of GDP for GTP, they bind to and activate a number of downstream effectors that regulate multiple cellular processes such as actin dynamics, gene expression, and cell cycle progression [19,20]. The switching of Rho GTPases between these two states is regulated by three sets of proteins: guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide-dissociation inhibitors (GDIs).

Rac proteins form a subfamily within the Rho GTPases. They occur in one of three isoforms, stimulate lamellipodium and membrane ruffle formation, and induce membrane extension during phagocytosis [21]. The three Rac isoforms have different expression patterns. *Rac1* is the best-studied member of this family and is ubiquitously expressed, whereas *Rac2* expression is mostly restricted to cells of hematopoietic origin, and *Rac3* mRNA is most abundant in the brain [22–24].

Recently, analysis of the functions of genes *in vivo* by using genetically modified mice has become a routine procedure. In the case of *Rac1* global knockout mice, the mice displayed the characteristic of embryonic lethality between embryonic days 8.5 and 9.5 because of an abnormal formation of the three germ layers during gastrulation [25]. Therefore, we had to create conditional knockout mice by using the Cre-loxP system, which have a deletion of *Rac1* in specific tissues, for more detailed analyses of the functions of Rac1 in specific cells and organs during development [26,27]. This review focuses on the current knowledge of the roles of Rac1 during limb development and the factors that are involved in this process, according to the results of analyses using *Rac1* conditional knockout mice.

2.1. The roles of Rac1 in chondrocytes

Some *in vitro* data have shown important roles of Rac1 in the control of mesenchymal condensation, proliferation, and differentiation of chondrocytes, as well as apoptosis. Pharmacological inhibition of Rac1 expression by NSC23766 in micromass culture resulted in reduction of the essential chondrogenic transcription factors *Sox9*, *Sox5*, and *Sox6*, decrease in the chondrogenic markers *Col2a1* (collagen II) and *Acan* (aggrecan), and a decrease in the accumulation of glycosaminoglycans [28]. In contrast, overexpression of Rac1 in the chondrogenic ATDC5 cell line increased *Sox9*, *Sox5*, *Sox6*, *Col2a1*, and *Acan* expression, reduced cell numbers, and markedly accelerated hypertrophic differentiation and apoptosis [28,29]. Rac1 overexpression results in activation of the p38 MAP kinase (MAPK) pathway in ATDC5 cells. Inhibition of p38 MAPK signaling blocks the upregulation of collagen X promoter activity and activation of caspase-1 and -3 by Rac1 overexpression [29]. These results suggest that Rac1 signaling accelerates the progression of chondrocyte hypertrophy and apoptosis through a p38 MAPK-dependent mechanism (Fig. 1A).

In addition, activation of Rac1 increased with maturation compared with immature primary chondrocytes. Activated Rac1 overexpression induced chondrocyte enlargement and increased matrix expression of *Mmp9* and *Mmp13*, which are characteristic of mature chondrocytes. Conversely, Rac1 inactivation by expression of dominant negative forms of Rac1 diminished adhesion, decreased alkaline phosphatase activity, and stimulated functions typical of immature chondrocytes [30]. These data provide evidence that Rac1 coordinates changes in chondrocyte phenotype and function and stimulates the maturation process (Fig. 1A).

Genetic deletion of *Rac1* in proliferating collagen II-expressing chondrocytes using *Col2-Cre* transgenic mice resulted in skeletal deformities, severe kyphosis, and dwarfism (Fig. 1B) [31,32]. These *Rac*1-deficient mice (*Rac1*$^{fl/fl}$*; Col2-Cre*) have disorganized growth plates, reduced proliferation, hypertrophic zones with chondrocytes of abnormal shape and size, reduced proliferation by expression of the cell cycle genes cyclin D1 and p57, and increased apoptosis. Moreover, phosphorylation of p38 MAPK is greatly reduced and *Col10a1* (collagen X), *Ibsp* (bsp), and *Ihh* (Indian hedgehog), key regulators of cartilage development, are increased in *Rac1*$^{fl/fl}$*; Col2-Cre* mice. In addition, a recent study showed the molecular pathways of reduced chondrocyte proliferation in *Rac1*$^{fl/fl}$*; Col2-Cre* mice [33]. The *Rac1*$^{fl/fl}$*; Col2-Cre* mouse growth plates have reduced inducible nitric oxide synthase (iNOS) and nitric oxide (NO) expression and increased activation of the transcription factor 3 (Atf3), a known suppressor of cyclin D1 expression in chondrocytes [34]. The growth plate of *iNOS* knockout mice showed reduced chondrocyte proliferation and expression of cyclin D1, resembling the phenotype of *Rac1*$^{fl/fl}$*; Col2-Cre* growth plates, including increased Atf3. These data suggest that Rac1 is required for iNOS expression and NO production in chondrocytes. NO suppresses the expression of Atf3, which acts as a transcriptional repressor of cyclin D1, which in turn slows chondrocyte proliferation and induces premature exit of the cell cycle.

There are some different results between *in vitro* and *in vivo* studies in this field. The most likely potential explanation for this discrepancy is that a standard two-dimensional cell culture system as used *in vitro* cannot recapitulate an *in vivo* model; *e.g.*, the three-dimensional growth plate organization and tightly controlled cell-ECM interactions [32]. According to *in vitro* data, Rac1 regulates *Sox9* expression [28,29]; however, *Rac1*$^{fl/fl}$*; Prx1-Cre* mice, which have Rac1 deleted in the limb mesenchyme before the initiation of *Sox9* expression (more detailed information is indicated in the third main paragraph), did not show changes in the *Sox9* expression pattern during mesenchymal condensation [35]. Although Rac1 overexpression in ATDC5 cells suppressed cell proliferation, *Rac1*$^{fl/fl}$*; Col2-Cre* mice demonstrated reduced expressions of cyclin D1 and p57, BrdU-positive cells in the growth plates, and a shorter proliferative zone. *Rac1*$^{fl/fl}$*; Col2-Cre* mice also had reduced phosphorylation of p38 MAPK and increased expression of the hypertrophic chondrocyte marker genes *Col10a1* and *Ibsp. Col2a1-MKK6EE* mice, transgenic mice with expression of a constitutively active mutant of MKK6 (a MAPK kinase that specifically activates p38 MAPK) in chondrocytes, showed a reduced zone of hypertrophic chondrocytes and reduced expression of *Ihh* and *Col10a1* [36]. These *in vivo* data imply that Rac1 inhibits chondrocyte hypertrophy by activation of p38 signaling even though *in vitro* data showed that Rac1 induced hypertrophy and apoptosis in chondrocytes through p38 MAPK (Fig. 1A).