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Bcl-2-associated athanogene-1 (BAG-1): A transcriptional regulator mediating chondrocyte survival and differentiation during endochondral ossification

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Abstract

BAG-1, an anti-apoptotic protein, was identified by its ability to bind to BCL-2, HSP70-family molecular chaperones and nuclear hormone receptor family members. Two BAG-1 isoforms, BAG-1L (50 kDa) and BAG-1S (32 kDa) were identified in mouse cells and BAG-1 expression was reported in murine growth plate and articular chondrocytes. The present study aimed to elucidate the role of BAG-1 in the regulation of molecular mechanisms governing chondrocyte differentiation and turnover during endochondral ossification.

In long bones of skeletally immature mice, we observed expression of BAG-1 in the perichondrium, osteoblasts, osteocytes in the bone shaft, bone marrow, growth plate and articular chondrocytes. Monolayer cultures of murine chondrocytic ATDC5 cells, which exhibited robust expression of both BAG-1 isoforms and the *Bag-1* transcript, were utilized as an *in vitro* model to delineate the roles of BAG-1. Overexpression of BAG-1L in ATDC5 cells resulted in downregulation of *Col2a1* expression, a gene characteristically downregulated at the onset of hypertrophy, and an increase in transcription of *Runx-2* and *Alkaline phosphatase*, genes normally expressed at the onset of chondrocyte hypertrophy and cartilage mineralization in the process of endochondral ossification. We also demonstrated the anti-apoptotic role of BAG-1 in chondrocytes as overexpression of BAG-1 protected ATDC5 cells, which were subjected to heat-shock at 48 °C for 30 min, against heat-shock-induced apoptosis. Overexpression of the SOX-9 protein in ATDC5 cells resulted in increased *Bag-1* gene expression. To further investigate the regulation of *Bag-1* gene expression by SOX-9, CHO cells were co-transfected with the human *Bag-1* gene promoter–*Luciferase* reporter construct and the human pSox-9 expression vector. Activity of the *Bag-1* promoter was significantly enhanced by the SOX-9 protein.

In conclusion, a novel finding of this study is the role of BAG-1 as a transcriptional regulator of genes involved in chondrocyte hypertrophy and cartilage mineralization during the process of endochondral ossification. Additionally, we have demonstrated for the first time the regulation of *Bag-1* gene expression by SOX-9 and the anti-apoptotic role of BAG-1 in chondrocytic cells. Modulation of *Bag-1* expression can therefore mediate chondrocyte differentiation and turnover, and offer further insight into the molecular regulation of endochondral ossification.

Keywords: ATDC5; Chondrocyte differentiation; Endochondral ossification; *Runx-2*; *Alkaline phosphatase*

Introduction

Long bone formation occurs in the growth plate cartilage by the regulated process of endochondral ossification [1]. As a part of this process, growth plate chondrocytes undergo discrete differentiation stages whereby these cells sequentially proliferate, mature and undergo terminal differentiation or hypertrophy. Once fully differentiated, hypertrophic chondrocytes participate

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in mineralization of the cartilaginous matrix and undergo cell death. As early as 1973, dying hypertrophic chondrocytes have served to demonstrate a mechanism of cell death (non lysosomal degradation) morphologically distinct from the widely studied modes of physiological cell death namely, apoptosis and autophagic cell death [2]. In addition to hypertrophic chondrocytes, some chondrocytes of the proliferative and upper hypertrophic zones of the growth plate have been demonstrated to undergo cell death [3]. Previous studies have shown the coexistence of two morphologically distinct types of chondrocytes (light and dark) in the proliferative and hypertrophic zones of the growth plate [4,5]. Ultrastructural characterisation of the modes of cell death adopted by light and dark chondrocytes revealed two morphologically distinct mechanisms different from classical apoptosis [3,6]. The term 'chondroptosis' has been introduced in literature to describe the mechanism of cell death adopted by dark chondrocytes [7]. Another study has suggested that hypertrophic chondrocytes die through the induction of autophagy, which causes sensitisation of these terminally differentiated cells to apoptogens in the local microenvironment [8].

Cartilage formation is a complex process and intricate molecular mechanisms regulate differentiation of chondrocytes in the growth plate. Abundant *Sox-9* expression is observed during mouse embryonic development in mesenchymal condensations before overt chondrocyte differentiation, and expression of this transcription factor is found to persist during the subsequent stages of cartilage deposition [9]. Typically, chondrocytes express a set of genes encoding cartilage-specific extracellular matrix components such as Type II collagen (encoded by the *Col2a1* gene), Type IX collagen, Type XI collagen and aggrecan. SOX-9 binds directly to the *Col2a1* enhancer at a site which is essential for *Col2a1* expression in chondrogenic cells [10]. Induction of *Col2a1* expression occurs in the growth plate with the change in cellular phenotype from prechondrogenic cells to proliferating chondrocytes [11], while expression of *Col2a1* and *Sox-9* declines in hypertrophic chondrocytes [12,13]. The induction of *Col10a1* expression stimulates the change in cellular phenotype from proliferating/prehypertrophic chondrocytes to hypertrophic chondrocytes, and expression of Type X collagen is restricted to hypertrophic chondrocytes [14].

Bcl-2, an anti-apoptotic molecule, is expressed in the growth plate in late proliferative and prehypertrophic chondrocytes, while expression decreases in hypertrophic chondrocytes [15]. Apart from its role in preventing apoptosis, BCL-2 is involved in a number of pathways vital in the maintenance of a stable chondrocyte phenotype. Suppression of *Bcl-2* expression results in downregulation of *Sox-9*, *Col2a1* and *Aggrecan* expression [16,17]. Recently, a protein termed Bcl-2-associated athanogene-1 (BAG-1) has been demonstrated to be expressed in mouse growth plate and articular chondrocytes [18]. The study demonstrated changes in the pattern of BAG-1 expression by growth plate chondrocytes in mice aged 6 weeks, 6 months and 18 months, and expression of BAG-1 in chondrocytes decreased with age.

BAG-1 is a ubiquitously expressed protein, originally identified by its ability to bind to and enhance the anti-apoptotic activity of the BCL-2 protein [19], and interact with the members of the nuclear hormone receptor family [20]. Six genes in mammalian cells encode the BAG-family of proteins, namely, *Bag-1* (encoding its various isoforms including RAP46/HAP46, HAP50), *Bag-2*, *Bag-3* (CAIR-1, BIS), *Bag-4* (SODD), *Bag-5* and *Bag-6* (SCYTHE, BAT3) [21]. Proteins of the BAG-family are characterised by an evolutionarily conserved BAG domain that allows them to bind and modulate the activity of the HSP70-family molecular chaperones [22–25].

Multiple BAG-1 isoforms are expressed in human and murine cells, generated as a result of alternate translation initiation sites in a single mRNA transcript [26]. In addition to the cytosolic 32 kDa and 36 kDa BAG-1S isoforms identified in murine and human cells respectively, the existence of a larger 50 kDa BAG-1L isoform, containing the N-terminal nuclear localisation sequence (NLS), has been confirmed in the nucleus [26]. The BAG-1S isoform, however, has been reported to translocate from the cytoplasm to the nucleus in cells subjected to heat-shock [27]. The BAG-1L isoform is translated from a non canonical CUG codon, while the BAG-1S isoform is translated from a downstream AUG codon [26]. BAG-1S translation is also thought to be partially mediated via an internal ribosome entry segment [28]. In addition to the BAG-1L and BAG-1S isoforms, human cells express a 46 kDa BAG-1M isoform (RAP46/HAP46), which arises as a result of translation initiation at yet another site involving an AUG codon and this isoform partitions itself between the nucleus and cytoplasm [29].

Studies on the developing limb bud during mouse embryogenesis reveal that *Bag-1* is initially expressed throughout the mesenchyme of the developing limb bud between E10.5 and E11.5 [30]. At E14.5, when digit formation is nearly complete, *Bag-1* expression is not detected in the cartilaginous anlagen and is downregulated in the mesenchymal tissues programmed to undergo apoptosis i.e. in the interdigital spaces [30]. *Bag-1*

Table 1
qPCR primer sequences and their amplicon sizes

Gene	Primer pairs	Amplicon (bp)
β-Actin	F: 5′ TTG CTG ACA GGA TGC AGA AG 3′ R: 5′ GTA CTT GCG CTC AGG AGG AG 3′	85
Sox-9	F: 5′ GAG GCC ACG GAA CAG ACT CA 3′ R: 5′ CAG CGC CTT GAA GAT AGC ATT 3′	50
Col2a1	F: 5′ CGA GTG GAA GAG CGG AGA CT 3′ R: 5′ AAC TTT CAT GGC GTC CAA GGT 3′	66
Bcl-2	F: 5′ TCG CAG AGA TGT CCA GTC AG 3′ R: 5′ CCT GAA GAG TTC CTC CAC CA 3′	82
Col10a1	F: 5′ ACG GCA CGC CTA CGA TGT 3′ R: 5′ CCA TGA TTG CAC TCC CTG AA 3′	77
Bag-1	F: 5′ CAG ACG GAG GAA ATG GAA AC 3′ R: 5′ GCT GTG GGG TAA CAA GAA GG 3′	84
Runx-2	F: 5′ CCA CCA CTC ACT ACC ACA CG 3′ R: 5′ CAC TCT GGC TTT GGG AAG AG 3′	63
Alkaline phosphatase	F: 5′ CTG ACT GAC CCT TCG CTC TC 3′ R: 5′ CCA GCA AGA AGA AGC CTT TG 3′	82