mTORC1 signaling controls mammalian skeletal growth through stimulation of protein synthesis

Jianquan Chen1 and Fanxin Long1,2,3,*

ABSTRACT

Much of the mammalian skeleton is derived from a cartilage template that undergoes rapid growth during embryogenesis, but the molecular mechanism of growth regulation is not well understood. Signaling by mammalian target of rapamycin complex 1 (mTORC1) is an evolutionarily conserved mechanism that controls cellular growth. Here we report that mTORC1 signaling is activated during limb cartilage development in the mouse embryo. Disruption of mTORC1 signaling through deletion of either mTOR or the associated protein Raptor greatly diminishes embryonic skeletal growth associated with severe delays in chondrocyte hypertrophy and bone formation. The growth reduction of cartilage is not due to changes in chondrocyte proliferation or survival, but is caused by a reduction in cell size and in the amount of cartilage matrix. Metabolic labeling reveals a notable deficit in the rate of protein synthesis in Raptor-deficient chondrocytes. Thus, mTORC1 signaling controls limb skeletal growth through stimulation of protein synthesis in chondrocytes.

KEY WORDS: mTOR, mTORC1, Raptor, Rptor, Cartilage, Chondrocyte, Mouse

INTRODUCTION

The limb skeleton of mammals is derived from cartilage templates through endochondral ossification (Kronenberg, 2003; Long and Ornitz, 2013). The process begins with condensation of mesenchymal cells within the embryonic limb bud. Subsequently, cells at the core of mesenchymal condensation differentiate into chondrocytes, whereas those at the periphery give rise to the perichondrium. Following the initial proliferation that produces an elongated cartilage template, chondrocytes become increasingly organized into morphologically distinct domains. At either end of the template the proliferating chondrocytes exhibit a rounded morphology (round chondrocytes), but become flattened and stacked in columns (columnar chondrocytes) towards the middle of the cartilage rod. The columnar chondrocytes produce a large amount of extracellular matrix and also proliferate at a higher rate than the round cells (Long et al., 2001). Further towards the center of the template, the columnar chondrocytes eventually stop proliferating and enter the hypertrophic stage. A recent study indicates that chondrocyte hypertrophy can be further divided into three progressive stages of volume enlargement based on changes in the density of cellular dry mass (Cooper et al., 2013). During the first phase, cells increase both dry mass and fluid volume, thus maintaining the same density of dry mass as the prehypertrophic cells; this is then followed by a swelling phase that reduces the dry mass density, and the final phase when both dry mass and fluid volume increase again without changing the dry mass density. Concurrent with chondrocyte hypertrophy, cells within the perichondrium surrounding the hypertrophic zone differentiate into osteoblasts that produce a nascent bone collar. The hypertrophic chondrocytes are generally believed to undergo apoptosis, followed by invasion of blood vessels from the perichondrium. The invading vasculature not only triggers resorption of the hypertrophic cartilage matrix and formation of the bone marrow cavity, but also brings in osteoblast precursors that eventually produce cancellous bone within the marrow cavity (Maes et al., 2010). Overall, proper regulation of chondrocyte progression through proliferation and hypertrophy is crucial for skeletal growth, but relatively little is known about the intracellular signaling mechanisms responsible for these transitions.

Mammalian (or mechanistic) target of rapamycin (mTOR) is an evolutionarily conserved serine/threonine kinase that integrates various inputs from growth factors and nutrients to regulate cell growth, proliferation and survival (Sengupta et al., 2010b). mTOR functions as the catalytic subunit in two functionally distinct signaling complexes: mTOR complex 1 (mTORC1) and complex 2 (mTORC2) (Sengupta et al., 2010b; Thoreen et al., 2009). The complexes are distinct by virtue of specific components, such as Raptor (also known as Rptor) for mTORC1 and Rictor for mTORC2, and by their different downstream effectors (Jacinto et al., 2004; Sarbassov et al., 2004). mTORC1 is best known for phosphorylating p70 S6 kinase (p70S6K; also known as RPS6KB1) and eukaryotic translation initiation factor 4E binding protein 1 (4EBP1; also known as EIF4EBP1) to regulate protein synthesis (Thoreen et al., 2012). Global deletion of mTOR or Raptor in the mouse leads to early postimplantation lethality (Gangloff et al., 2004; Guertin et al., 2006; Murakami et al., 2004). Subsequent tissue-specific knockout studies have identified crucial roles for mTORC1 in several tissues, but its function in skeletal development has not been examined genetically (Bentzinger et al., 2008; Polak et al., 2008; Yilmaz et al., 2012).

Here, through deletion of either mTOR or Raptor, we demonstrate that mTORC1 signaling is required for optimal protein production in chondrocytes, thus controlling cell size, the amount of cartilage matrix and, ultimately, skeletal size. This study therefore identifies mTORC1 as a crucial regulator of skeletal growth during embryogenesis.

RESULTS

mTORC1 signaling during endochondral bone development

To gain insight into mTOR signaling in the developing long bone, we performed immunofluorescence staining for phosphorylation of ribosomal protein S6 (P-S6) by p70S6K at residues S240 and S244, an established readout for mTORC1 signaling, on sections of mouse embryonic limbs (Sengupta et al., 2010a). We first examined the humerus at embryonic day (E) 15, before the marrow cavity was formed. Here, P-S6 was detected at a relatively low level in a ‘salt and pepper’ pattern among the round chondrocytes (Fig. 1A,B, yellow...
boxes). The staining was notably increased in both intensity and uniformity within the columnar region, with the prehypertrophic and early hypertrophic chondrocytes exhibiting the most prominent, nearly homogeneous signal (Fig. 1A,B, green boxes). The P-S6 signal declined rapidly beyond the early hypertrophic stage, resulting in little staining within much of the hypertrophic region (Fig. 1A,B). P-S6 was somewhat reactivated in cells at the final stage of hypertrophy (Fig. 1A,B, blue boxes). At E16.5, the staining pattern of P-S6 within the different zones of cartilage was identical to that at E15 (Fig. 1C,D). However, at this stage, the central hypertrophic region was replaced by a nascent marrow cavity, and the terminal hypertrophic chondrocytes were found near the chondro-osseous junction. These cells, like those at E15, also exhibited some reactivation of P-S6 (Fig. 1C, asterisk). Besides chondrocytes, the osteoblast precursors within either the perichondrium or the primary spongiosa also exhibited a robust P-S6 signal (Fig. 1C, arrows, PS in blue box). Because deletion of Raptor abolished the P-S6 signal in chondrocytes and osteoblast precursors, we conclude that P-S6 faithfully reflects mTORC1 signaling in the developing skeleton (supplementary material Fig. S2B). Overall, the dynamic pattern of mTORC1 signaling indicates that the pathway is likely to play a role in normal skeletal development.

mTORC1 is crucial for embryonic skeletal growth

To examine directly the role of mTOR in skeletal development, we deleted the gene with Prx1-Cre, which targets mainly the limb, the cranial and the interlimb flank mesenchyme (Logan et al., 2002). Briefly, Prx1-Cre; Mtor<sup>fl/fl</sup> male mice were mated with Mtor<sup>fl/fl</sup> females to produce Prx1-Cre; Mtor<sup>fl/fl</sup> embryos (hereafter mTORCKO). The mutant mice were born alive but died shortly after birth; their limbs were severely diminished, and ~50% also exhibited exencephaly (supplementary material Fig. S1A,B). Whole-mount skeletal staining at E18.5 revealed a clear deficiency in ossification of the skull and the sternum, in addition to the marked shortening of appendicular bones (supplementary material Fig. S1C-H). The limb skeleton was correctly patterned but each element was greatly reduced in size (Fig. 2A-D). Direct measurements of the humerus indicated that the total length and the relative bone-collar length (normalized to total length) were decreased to 34.2% and 70.6% of normal values, respectively (Fig. 2E). Histological analyses of the ulna revealed that, in contrast to the well-established marrow cavity that is normally present at E18.5, the mutant element maintained a cartilaginous core (supplementary material Fig. S1L,J). Similar defects were observed with the other limb bones of mTORCKO mice. Thus, loss of mTOR severely impairs skeletal growth.

Because mTOR can function through either mTORC1 or mTORC2, we next assessed the specific contribution of mTORC1. For this, we deleted the gene encoding the mTORC1-specific Raptor with Prx1-Cre in the same way as for mTOR removal. Western blot analyses of limb bud protein extracts confirmed that Raptor and P-S6 were markedly reduced in Prx1-Cre; Raptor<sup>fl/fl</sup> embryos (hereafter RapCKO) at E12.5 (supplementary material Fig. S2A). The residual signal of Raptor and P-S6 in RapCKO could be due to the ectoderm that Prx1-Cre did not target, or to incomplete deletion in the mesenchyme at this early stage. Regardless, when examined by immunostaining at E16.5, the P-S6 signal was undetectable from the cartilage and the perichondrium (supplementary material Fig. S2B). Importantly, the RapCKO embryos exhibited a perinatal phenotype strikingly similar to that of mTORCKO, including very short limbs,
the periosteum and the chondro-osseous junctions (Fig. 3G,I). In the control at E16.5 and E18.5, apoptosis was detected at both E15.5 (Fig. 3C,E). After the formation of a bone marrow cavity in E14.5, and then among the terminal hypertrophic chondrocytes at RapCKO embryos at any stage. In the control, apoptotic cells first appeared within the perichondrium flanking the hypertrophic region at E14.5. We therefore examined the status of hypertrophy in the RapCKO embryo. Histological sections revealed a much shorter hypertrophic zone in the humerus of RapCKO embryos at both E14.5 and E15.5, and this shortening was disproportionate to the reduction in total length (Fig. 4A,B). As shown above, at E18.5 the central hypertrophic cartilage persisted in the mutant humerus, instead of being replaced by a marrow cavity (Fig. 4D). In situ hybridization showed that the central hypertrophic chondrocytes in the mutant sample no longer expressed Col10a1 but activated Mmp13, indicating their status of terminal hypertrophy (Fig. 4D). The terminal status of the cells was also supported by the observation above that some were undergoing apoptosis (Fig. 3I). However, a closer examination revealed that these cells were considerably smaller than their normal counterparts (Fig. 4E,F). The reduced size of RapCKO hypertrophic chondrocytes was unlikely to be due to the absence of a primary ossification center in the mutant bones because, even when compared with E15 wild-type embryos that had not yet developed the ossification center, the E18.5 RapCKO mice still had significantly smaller hypertrophic chondrocytes (Fig. 4F). Thus, in the absence of mTORC1 signaling, hypertrophic chondrocytes failed to attain a normal size. Taken together, the results so far demonstrate that mTORC1 controls not only the initiation of hypertrophy, but also the ultimate size and the eventual removal of the hypertrophic chondrocytes.

Besides hypertrophy, the size of other chondrocytes and the amount of cartilage matrix also contribute to the size of skeletal elements. Therefore, we examined the round and columnar regions by histomorphometry. Indeed, both areas exhibited a higher cell density in the humerus of E18.5 RapCKO than in the littermate control (Fig. 5A-C). Further quantification of the columnar region identified a clear decrease in both cell size and matrix area per cell (Fig. 5D,E). Analyses of the humerus at E13.5 provided similar results (supplementary material Fig. S4). Thus, loss of mTORC1 reduces both the size of chondrocytes and the amount of extracellular matrix that they produce.

These findings prompted us to determine whether mTORC1 normally stimulates protein translation in chondrocytes. We performed metabolic labeling experiments with primary cultures of chondrocytes with Raptor either intact or deleted in vitro with an adenovirus expressing Cre. Western blot analyses confirmed...
effective Cre-mediated deletion of Raptor and the expected decrease in the phosphorylation of 4EBP1 and S6 (Fig. 6A). By contrast, phosphorylation of AKT (at S473), a known target of mTORC2, was not impaired but rather increased upon mTORC1 disruption, a phenomenon previously reported in other systems (Fig. 6A) (Laplante and Sabatini, 2012). Importantly, the Raptor-deficient chondrocytes exhibited a marked deficiency in protein synthesis compared with the control cells (Fig. 6B).

Overall, this study establishes mTORC1 as a crucial determinant of chondrocyte size and matrix production during endochondral skeletal development through its stimulation of protein translation.

DISCUSSION

We have identified mTORC1 as a crucial regulator of embryonic skeletal growth in mice. Our data indicate that physiological mTORC1 signaling increases the cell size and the amount of extracellular matrix proteins produced by chondrocytes at all stages of maturation. In addition, normal mTORC1 activity is necessary for the timely transition of chondrocytes to hypertrophy, as well as for the final removal of hypertrophic chondrocytes. The direct impact of mTORC1 on the overall rate of protein translation in chondrocytes is likely to be central to the function of this protein complex in skeletal growth.

Chondrocyte hypertrophy is a principal driving force in skeletal growth. Recent studies have identified three distinct phases of volume increase during hypertrophy. The first and third phases involve increases in dry mass production, whereas the second phase of expansion is due to fluid accumulation in the cell (Cooper et al., 2013). Interestingly, our data show that mTORC1 signaling is robustly activated in the prehypertrophic/early hypertrophic stage, but then becomes undetectable until being reactivated in the final phase of hypertrophy. Therefore, mTORC1 signaling might drive the increase in dry cell mass in the first and third phases of hypertrophy, therefore contributing to the final size of hypertrophic chondrocytes. The final stage of chondrocyte hypertrophy is normally followed by apoptosis and replacement by a marrow cavity formed through blood vessel invasion. Previous work in bat and mouse limbs has indicated that the entire hypertrophic zone in a growth plate is normally turned over within ~24 h (Cooper et al., 2013; Farnum et al., 2008). Here we show that, without mTORC1, the hypertrophic chondrocytes failed to turn over even though they transitioned to the final stages of expressing MMP13 and undergoing apoptosis. This observation raises the intriguing possibility that mTORC1 activity in the terminal hypertrophic chondrocytes might be intrinsically necessary for blood vessel invasion and for the removal of hypertrophic cartilage, but we cannot exclude the possibility that the defect might be secondary to the loss of mTORC1 in other cell types in the limb.

It should be noted that because mTOR or Raptor was deleted by Prx1-Cre at the mesenchymal progenitor stage in our study, potential defects in the progenitors might have contributed to the dramatic size reduction in the limb skeleton. Indeed, we have observed smaller cartilage primordia in the limbs of E11.75 RapCKO embryos when compared with littermate controls. Thus, mTORC1 signaling appears to stimulate embryonic skeletal growth by regulating both the initial formation and the subsequent growth of the cartilage template. Future experiments with more stage-specific approaches (e.g. Col2-Cre) will be necessary to distinguish the relative contribution of each stage to overall skeletal growth.

It is worth noting that mTORC1 activity markedly decreases following the onset of hypertrophy. A recent study showed that hyperactivation of mTORC1 via the deletion of Lkb1 caused overgrowth of the columnar region, highlighting the importance of mTORC1 suppression for the transition of the cells to hypertrophy (Lai et al., 2013). It is not yet clear at present whether Lkb1
activation represents a normal regulatory step during the progression of chondrocyte hypertrophy.

Similarly, it is unclear which extracellular signals are responsible for the dynamic regulation of mTORC1 activity at the various stages of chondrocyte maturation. Insulin-like growth factor (IGF) signaling is likely to play a role because it is known to activate mTORC1 in a variety of tissues, and IGF2 together with the signaling receptor IGF1R are expressed in the growth plate chondrocytes (Oldham and Hafen, 2003; Wang et al., 1995). Disruption of Igf1, IGF2 or IGF1R reduces overall skeletal growth (Baker et al., 1993; Liu et al., 1993; Long et al., 2006). In particular, deletion of Igf1 resulted in a reduced final size of hypertrophic chondrocytes, apparently due to failure in the third-phase expansion (Cooper et al., 2013; Lupu et al., 2001; Wang et al., 1999). Thus, IGF signaling is likely to contribute to mTORC1 activity in chondrocytes. However, because the skeletal defect caused by mTORC1 deletion is more severe than that in the Igf1r knockout embryo (Liu et al., 2013), other signals must also contribute to mTORC1 activation to ensure proper skeletal growth.

MATERIALS AND METHODS

Mouse strains

Prx1-Cre, Mtorf/f and Raptorf/f mouse lines are as previously described and were purchased from the Jackson Laboratory (Logan et al., 2002; Risson et al., 2009; Sengupta et al., 2010a). The Animal Studies Committee at Washington University approved all mouse experimental procedures.

Analyses of mouse embryos

Whole-mount embryonic skeleton was prepared and stained with Alizarin Red/Alcian Blue essentially as described previously (McLeod, 1980). For analyses on sections, embryonic limbs were dissected out in PBS, fixed in 10% formalin overnight at room temperature, and then processed for paraffin embedding prior to sectioning (6 µm). For detection of mineralization, sections were stained with 1% silver nitrate (von Kossa method) and counterstained with Nuclear Fast Red. For other histology-based analyses on E16.5 or older embryos, limbs were decalcified in 14% EDTA for 24 h after fixation and prior to processing. Hematoxylin and Eosin (H&E) staining and Alcian Blue/Picrosirius Red staining were performed on paraffin sections following standard protocols. In situ hybridization was performed with 35S-labeled riboprobes as previously described (Hu et al., 2005; Joeng and Long, 2009; Long et al., 2004; Long et al., 2001).

BrdU and TUNEL staining

Pregnant females were injected with BrdU at 0.1 mg/g body weight 2 h before harvest. Embryonic limbs were collected, decalcified, processed and sectioned in paraffin. BrdU detection was performed with a BrdU staining kit (Zymed Laboratories). For quantification of BrdU labeling, sections from at least three animals of each genotype were scored for the percentage of BrdU-positive cells. TUNEL assay was performed with the In Situ Cell Death Detection Kit TMR Red (Roche).

Western blot and immunofluorescence

For western blot analyses, total proteins were isolated from mouse forelimb buds using RIPA buffer [20 mM Tris (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate]. Protein samples (30 µg) were separated on 10% SDS-polyacrylamide gels and subjected to a standard western procedure. Antibodies for S6 (Cell Signaling, catalog number 2215), P-S6 (2240/244; Cell Signaling, catalog number 2217), AKT (Cell Signaling, catalog number 9272), P-AKT (S473; Cell Signaling, catalog number 9271), 4EBP1 (Cell Signaling, catalog number 9452), P-4EBP1 (S65; Cell Signaling, catalog number 9451), Raptor (Cell Signaling, catalog
Metabolic labeling of protein synthesis

To isolate chondrocytes, the cartilage portion of the rib cage and sternum was dissected from newborn Raptor<sup>fl/fl</sup> pups, washed with PBS, and then digested with 1.2 mg/ml protease (Sigma) dissolved in PBS at 37°C for 30 min. This was followed by incubation with 3 mg/ml collagenase (Sigma) in DMEM for 60 min at 37°C. The soft tissues were then carefully removed.

For P-S6 immunostaining, paraffin sections of embryonic limbs were deparaffinized, immersed in boiling 10 mM sodium citrate buffer (pH 6.0) for 20 min, and blocked with 5% goat serum before incubation with primary antibody overnight. The sections were then washed three times in PBS, and incubated for 1 h at room temperature with Alexa Fluor 594-conjugated goat anti-rabbit IgG secondary antibody (diluted 1:250 in PBS; Life Technologies, catalog code A-11012). Stained sections were mounted with VECTASHIELD mounting medium containing DAPI (Vector Laboratories).

Statistics

All quantitative data are presented as mean±s.d. from a minimum of three independent samples. *P<0.05 (two-tailed Student’s t-test) is considered statistically significant.

Competing interests

The authors declare no competing financial interests.

Author contributions

J.C. and F.L. conceived the project; J.C. conducted experiments; J.C. and F.L. analyzed data and wrote the paper.

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Supplementary material

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.108811/-/DC1

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**Figure S1. mTOR is critical for embryonic skeletal development.** (A-B) Gross appearance of E18.5 wild-type (A) versus mTORCKO (B) littermates. (C-D) Whole-mount skeletal staining of E18.5 WT (C) and mTORCKO (D) littermates. (E-L) Higher magnification of calvaria (E,F), and sterna (G,H) from E18.5 WT (E, G) and mTORCKO (F, H) littermates. (I-J) H&E staining of longitudinal sections through the ulna from E18.5 WT (I) and mTORCKO (J) littermates. Green arrows: parietal bone; red arrows: sternum. BM: bone marrow; *: hypertrophic cartilage.

**Figure S2. Raptor critically regulates embryonic skeletal development.** (A) Western blot analyses with forelimb buds from E12.5 WT versus RapCKO littermates. (B) P-S6 immunofluorescence staining on longitudinal humeral sections from E16.5 WT versus RapCKO embryos. P-S6: red; DAPI: blue. (C-D) Gross appearance of E18.5 wild-type (C) versus RapCKO (D) littermates. (E-F) Whole-mount skeletal staining of E18.5 WT (E) and RapCKO (F) littermates. (G-J) Higher magnification of calvaria (G, H), and sterna (I, J) from E18.5 WT (G, I) and RaptorCKO littermates (H, J). Green arrows: parietal bone; red arrows: sternum.
Figure S3. mTORC1 regulates chondrocyte hypertrophy. (A, B) Alcian Blue/Picrosirius Red staining of longitudinal humeral sections from E14.5 WT versus RapCKO littermates. Red lines indicate the length of hypertrophic zone. (C, D) In situ hybridization of Col10a1 on longitudinal humeral sections from E13.5 littermates. Hybridization signal in red. Asterisk denotes Col10a1-expressing domain in WT sample.

Figure S4. mTORC1 regulates chondrocyte size and amount of extracellular matrix. (A-B) H&E staining of longitudinal humeral sections from E13.5 WT (A) versus RapCKO (B) littermates. Areas in colored boxes shown below at higher magnification. (C) Quantification of chondrocyte density in round and columnar regions. *: p<0.05, n=3.