Activation of mTORC1 in chondrocytes does not affect proliferation or differentiation, but causes the resting zone of the growth plate to become disordered

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1. Introduction

mTOR is a serine/threonine kinase activated by complex formation and this complex (mTORC) 1 coordinates anabolic (protein and lipid synthesis) and catabolic activities (autophagy) (Laplante and Sabatini, 2012). Both the function and sequence of mTOR have been highly conserved during evolution. For example, the yeast homolog of mTOR, TOR, regulates cell growth in response to nutrient supply (Laplante and Sabatini, 2012). The drosophila homolog, dTOR, also controls cell growth, as well as regulating body size (Oldham, 2011). With respect to mammals, mTORC1 signaling controls cell size in isolated systems, including liver, and in both skeletal and cardiac muscle (Lee et al., 2007). Genetic ablation of \textit{MTOR} or RPTOR in the whole limb results in these structures being extremely small (Chen and Long, 2014).

We previously reported that activation of mTORC1 signaling stimulates bone growth in vitro (Newton et al., 2015) and aimed to explore its role in vivo by using Col2-Cre mice to ablate the gene encoding a key inhibitor of mTORC1, tuberous sclerosis 1 (TSC1). Although these mice displayed severe growth retardation starting after 2 weeks of age, we were dismayed that they also developed chronic wasting and seizures associated with premature death, which might be attributed to leakage of Cre in non-cartilaginous tissues. At the same time, a recent study based on this same model came to the conclusion that mTORC1 coordinately chondrocyte growth, proliferation and differentiation, in part via regulation of PTHrP (Yan et al., 2016).

Here, we suggest an alternative explanation to the phenotype described by Yan et al. (2016) based on actions of Tsc1 in cells other than chondrocytes. Furthermore, our present considerations are of key relevance to all experimental approaches involving tissue-specific gene ablation.

2. Results

1. Ablation of Tsc1 using Col2-Cre causes severe developmental abnormalities
We start by making direct comparisons below between our work and the similar experiments performed by Dr. Yan et al. (2016), we refer to our figures in bold letters and those of Yan and colleagues in italics.

Like Yan et al. (2016), we ablated TSC1 in murine chondrocytes utilizing non-inducible Collagen2-driven Cre (Kobayashi et al., 2002) and observed similar growth retardation in the resulting mice (Fig. 1A and B versus Fig. 3c and 6b (Yan et al., 2016)). Our ablation was
A Growth of Col2-Cre Tsc1 floxed metatarsals in culture

B Analysis of Col2-Cre Tsc1 floxed metatarsals

|                         | Before culture | Cultured for 24 hours |
|-------------------------|----------------|-----------------------|
|                         | Control        | Het                   | cKO         | Control        | Het      | cKO      |
| pS6                     |                |                       |             |                |          |          |
| Ki67                    |                |                       |             |                |          |          |
| Col X                   |                |                       |             |                |          |          |
confirmed by PCR (not shown), as well as in rosa26-LacZ reporter mice (Fig. 1C), which demonstrated 99.91% recombination efficiency in the growth plate (2362 cells from two mice). S6 activity in the growth plate of Tsc1cKO mice was elevated (Fig. 1D), similar to Fig. 3b (Yan et al., 2016)). Clearly, our TSC1 ablation was successful. Col2-Cre:Tsc1 fl/+ mice were used as a control for the effect of Col2-Cre recombinase itself (Fig. 1B and F). As it showed no phenotype (Fig. 1B and F), which is in line with previous observations by us and others (Kobayashi et al., 2002; Bouderlique et al., 2015; Vuppalapati et al., 2015), we did not perform further analysis of this specific genotype.

In contrast to Yan et al. (2016), we found no significant changes in the height of the growth plate or of the hypertrophic zone (Figs. 1E, F and 2B versus Fig. 3e (Yan et al., 2016)), the level of BrdU incorporation (Fig. 1G versus Fig. 4b (Yan et al., 2016)) or the expansion of collagen X-producing hypertrophic chondrocytes (Figs. 1H, 2B versus Fig. 5a (Yan et al., 2016)). Yan and co-workers reported increased hypertrophy of chondrocytes in the rib upon Tsc1 ablation (Fig. 4a (Yan et al., 2016)). We did not analyze the ribs, but the size of terminal hypertrophic chondrocytes in the proximal tibia was not changed upon Tsc1 ablation (Fig. 1F). However, we noticed some irregularity in the resting zone of the adult Tsc1cKO mice (Fig. 1E), similar to that shown by Yan and co-workers (Fig. 3d (Yan et al., 2016)).

Our Tsc1cKO mice began dying at approximately 4 weeks of age (not shown), which was also the case in the Yan study (Fig. 6a (Yan et al., 2016)). For ethical reasons, we had to sacrifice these mice at this point, since they began to suffer severely, developing seizures and chronic wastage.

In general, the growth retardation we observed was in contrast to our recent report that activation of mTORC1 signaling stimulated bone growth in vitro (Newton et al., 2015). However, we found that when cultured ex vivo metatarsal bones from Tsc1cKO mice grew longer than control bones (Fig. 2A), which could be detected after the first 24 h in culture (52% increase in growth: control bones grew 150 ± 13 μm; cKO bones grew 229 ± 19 μm, p = 0.0016, n = 18 and this growth increment was sustained over the culture period) albeit no changes in the dose of tamoxifen used throughout. Activation of mTORC1 signaling was confirmed by elevated phosphorylation of S6 by 4.1 folds during embryonic period. The Col2-Cre:Rosa26Tr:Col2-CreERT2 mice obtained were injected with tamoxifen on postnatal day 3 in accordance with protocols established previously (Chagin et al., 2014). As we have extensively characterized this Col2-CreERT system before and never observed the effect of the CreERT transgene per se (Chagin et al., 2014; Hirai et al., 2011; Kaucka et al., 2016; Kaucka et al., 2017; Li et al., 2017), we presented analysis of CreERT positive controls in Fig. 3C and D only. Recombination of TSC1 DNA was confirmed by PCR (Fig. 3A) and sequencing (not shown) and CreERT activity was confirmed in two reporter strains: Rosa26-LacZ (Fig. 3B) and R26-Confetti (Fig. 3C), the latter with growth plate chondrocytes exhibiting a recombination efficiency of 66.36% (3921 cells analyzed from 4 mice) at the dose of tamoxifen used throughout. Activation of mTORC1 signaling was confirmed by elevated phosphorylation of S6 by 4.1 folds (fluorescent intensity 0.003 ± 0.0005 in heterozygotes versus 0.0135 ± 0.0033 arbitrary units, p < 0.05, n = 3; see also Fig. 3D) as well as phosphorylation of 4EBP1 (Fig. 3E), two well-known readouts of mTORC1 activity. No recombination occurred without tamoxifen injection as analyzed in Col2-CreERT:R26-Confetti mice (data not shown).

These mice developed normally (not shown), with normal bones (Fig. 3F) and no alterations in chondrocyte hypertrophy (Fig. 3G and H), the expression of collagen type X (Fig. 2I), Ihh (Fig. 2J) or cell

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**Table 1**

|                      | Cre negative Tsc1 fl/+ | Col2-Cre positive Tsc1 fl/+ | p-Value |
|----------------------|------------------------|-----------------------------|---------|
| Body mass (g)        | 6.00 ± 0.14 (5)        | 6.03 ± 0.07 (3)             | 0.867   |
| Tibial length (mm)   | 11.88 ± 0.11 (5)       | 11.52 ± 0.08 (3)            | 0.070   |
| Femoral length (mm)  | 8.45 ± 0.13 (5)        | 8.58 ± 0.02 (2)             | 0.616   |
| Growth plate height (μm) | 305.2 ± 17.9 (3)      | 324.5 ± 30.8 (3)            |         |
| Hypertrophic zone height (μm) | 133.0 ± 7.0 (3)      | 142.2 ± 10.8 (3)            | 0.511   |
| Terminal hypertrophic cell size (μm) | 24.0 ± 0.9 (3)        | 24.8 ± 0.6 (3)              | 0.532   |

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osteo blast and osteocytes (Vuppalapati et al., 2015), mesenchymal cells and the central nervous system (Long et al., 2001). Our observation of β-galactosidase reporter activity in the brain and kidney of Col2-Cre:Rosa26Tr:Col2-CreERT2 mice was also the case in the Yan study (Yan et al., 2016)), the expression of collagen type X (Nakamura et al., 2006) for postnatal ablation of Tsc1 in the growth plate, which avoids any activity of Cre in non-cartilaginous tissues during embryonic period. The Col2-CreERT:Col2-CreERT2 mice obtained were injected with tamoxifen on postnatal day 3 in accordance with protocols established previously (Chagin et al., 2014). As we have extensively characterized this Col2-CreERT system before and never observed the effect of the CreERT transgene per se (Chagin et al., 2014; Hirai et al., 2011; Kaucka et al., 2016; Kaucka et al., 2017; Li et al., 2017), we presented analysis of CreERT positive controls in Fig. 3C and D only. Recombination of TSC1 DNA was confirmed by PCR (Fig. 3A) and sequencing (not shown) and CreERT activity was confirmed in two reporter strains: Rosa26-LacZ (Fig. 3B) and R26-Confetti (Fig. 3C), the latter with growth plate chondrocytes exhibiting a recombination efficiency of 66.36% (3921 cells analyzed from 4 mice) at the dose of tamoxifen used throughout. Activation of mTORC1 signaling was confirmed by elevated phosphorylation of S6 by 4.1 folds (fluorescent intensity 0.003 ± 0.0005 in heterozygotes versus 0.0135 ± 0.0033 arbitrary units, p < 0.05, n = 3; see also Fig. 3D) as well as phosphorylation of 4EBP1 (Fig. 3E), two well-known readouts of mTORC1 activity. No recombination occurred without tamoxifen injection as analyzed in Col2-CreERT:R26-Confetti mice (data not shown).

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Fig. 2. Col2-Cre:Tsc1 fl/+ metatarsals have a growth advantage ex vivo. (A) Growth curves of metatarsals collected from three-day old mice and cultured for six days ex vivo. (B) Metatarsals were collected immediately before culture or after one day in culture (controlateral controls), then stained for pS6, Ki67 (immunohistochemistry) or COL10A1 (in situ hybridization). For Ki67 staining, the region directly above the hypertrophic zone is expanded (pink box). For COL10A1 staining, the yellow dashed line denotes the border between the cartilage and the primary spongiosa. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Validation of recombination within Col2-CreERT:Tsc1 (tamoxifen administrated at P3)

| DNA recombination | Enzymatic activity of Col2-Cre | Hydrolyzed Xgal | Crossed with R26-Confetti |
|-------------------|-------------------------------|----------------|--------------------------|
| kO (ind)          | Control                       | Cre-positive   | Heterozygous             |
| Control           |                               |                |                          |
| cKO (ind)         |                               |                |                          |
| Control           |                               |                |                          |
| cKO (ind)         |                               |                |                          |
| Control           |                               |                |                          |

Col2-CreERT:Tsc1 (tamoxifen administrated at P3)

D mTORC1 activity was assessed by pS6

E mTORC1 activity was assessed by p4EBP1

F Bone lengths

G Growth plate histology

H Growth plate histomorphometry

I In situ hybridisation of ColX at one month

J In situ hybridisation of Ihh at one month

K BrdU immunohistochemistry with alcian blue counterstaining at one month of age

Fig. 3. Postnatal ablation of Tsc1 in mice does not affect cell proliferation or hypertrophy in the growth plate, but leads to disorganization of the resting zone. Unless otherwise stated, in all these images, data on control (Col2-CreERT-negative) mice are shown to the left and on Tsc1cKO (Col2-CreERT:Tsc1 fl/fl) mice to the right. To achieve recombination, all animals were injected with tamoxifen on P3 and sacrificed at 8 days (A, B, E) or 1 month of age (C, D, F–K). (A) Cartilage was isolated from the elbows, DNA extracted, and PCR performed to identify the product of Tsc1 recombination. The bands are seen exclusively in the Cre-positive Tsc1 fl/fl animals. (B) The enzymatic activity of Cre in sections of growth plate from Col2-CreERT:Rosa26 reporter mice was assessed by staining with X-gal. (C) The R26-Confetti reporter strain (in which Cre recombination leads to the expression of different fluorescent proteins, in a stochastic manner) was crossed with Col2-CreERT and Tsc1-foxed mice and the progeny injected with tamoxifen on P3 and analyzed at 1 month of age. Col2-CreERT:Tsc1 fl/+; R26-Confetti on the left and Col2-Cre:Tsc1 fl/fl; R26-Confetti on the right panel. (D) The level of phosphorylated S6 in cKOind mice was higher than in the control mice (1 month of age). (E) The level of phosphorylated 4EBP1 in cKOind mice was higher than in the control mice (1 month of age). (F) Lengths of the tibia (t) and femur (f) are shown at one month of age. (G, H) Sections of the growth plate were stained with H&E staining and morphometric analysis was conducted. (I) Transcription of COL10A1 and (J) Ihh expression in the growth plate, as detected by in situ hybridization. (K) Immunohistochemical quantification of BrdU (injected 2 h prior to sacrifice). All values presented are means ± SEM (n = 3–8). No significant differences were detected between control and cKO mice utilizing an unpaired t-test (F, H, I and K).
proliferation (Fig. 3K). However, the height of the growth plate was significantly increased (p < 0.05, Fig. 3F, G), as the result of an expansion of the resting zone (34 ± 3 vs 58 ± 8 μm, p = 0.108; n = 4 (heterozygous) or 8 (homozygous mutant)). Indeed, irregularities in the resting zone chondrocytes could be seen in both non-inducible (Fig. 1E) and inducible (Fig. 3G) Tsc1cKO strains, particularly striking when Tsc1 ablation was performed in the clonal R26-Confetti reporter strain (Fig. 3C).

On the basis of our experiments with two different Cre strains, we conclude that ablation of TSCI does not affect chondrocyte proliferation or differentiation, in contrast to the conclusion by Yan et al. (2016). We believe that side-effects associated with Col2-Cre leakage in combination with the general suffering/dying of Tsc1cKO mice after 4 weeks of age (Fig. 6a), the time-point at which Yan and colleagues performed most of their phenotypic analyses (Figs. 3, 4, 5, 6, 53, 54, 56) (Yan et al., 2016), may explain their findings.

3. Discussion

Based on our previous observations (Newton et al., 2015), we had hypothesized that elevation of mTORC1 activity in the growth plate would stimulate chondrocyte hypertrophy and thereby increase longitudinal growth. However, when we ablated Tsc1 in chondrocytes using Col2-Cre and carefully assessed the growth plates of these mice, we found no changes in chondrocyte differentiation or proliferation, in contrast to the report by Yan and colleagues (Yan et al., 2016).

One potential explanation for these discrepancies is that the mice employed in both these studies were of mixed genetic backgrounds (personal correspondence with Dr. Bai). Additionally, both studies used different Cre strains: we used one produced by Dr. Johnson (Kobayashi et al., 2002) whereas Dr. Bai’s laboratory used the strain obtained as a gift from Dr. Yang (presumably described here (Yan et al., 2016; Hao et al., 2002)). Thus, the phenotype of the two strains (Col2-CreJohnson: Tsc1cKO fl/fl and Col2-CreYang:Tsc1 fl/fl) can conceivably be different. However, since the gross phenotypes of the Col2-CreJohnson: Tsc1cKO fl/fl and Col2-CreYang:Tsc1 fl/fl mice are the same it seems likely that the mechanisms by which Tsc1 causes growth retardation and death are also similar. Interestingly, ablation of Tsc1 in neurons (Tsc1cKO-neuro) pheno-coated Col2-Cre:Tsc1 fl/fl mice (Tsc1cKO-cartilage), with similar growth retardation, thoracic lordosis and increased mortality from 3 weeks of age (Meikle et al., 2008; Carson et al., 2012). Tsc1cKO-neuro has reported seizures, which are also observed in our Tsc1cKO-cartilage mice (not reported by Yan et al.). This observation, combined with the absence of such features as growth retardation, seizures and death upon postnatal Tsc1 ablation (Tsc1cKOind) suggest that the growth plate phenotype can be secondary to non-specific activity of Col2-Cre line in the brain during development. Activity in other non-cartilaginous tissues can also cause the described phenotype, since the expression of alpha 1(II) collagen during embryonic development was demonstrated in the heart, epidermis, inner ear, notochord, sclera of developing eye, neural retina, the cornea and conjunctival epithelia, proliferative ventricular cells of the forebrain and midbrain and the cervical spinal cord (Cheeh et al., 1991). The activity of Col2-Cre in non-cartilaginous tissue has also been well reported (Vuppalapati et al., 2015; Ovchinnikov et al., 2000; Kolpakova-Hart et al., 2008). Accordingly, the data should always be interpreted carefully and although the Cre-lox approach is one of the most powerful tools for examining the functions of gene products in target tissues, expression of Cre in non-target cells is a serious drawback and must always be taken into account.

It is clear that mTORC1 is important for limb development (Chen and Long, 2014; Wu et al., 2017; Phornphutkul et al., 2009). However, our present findings with Tsc1cKOind mice indicate that elevated mTORC1 activity in chondrocytes is not in itself sufficient to disrupt their functionality or proliferative activity. We did, however, observe that the resting zone in Tsc1cKOind mice was disorganized, which is also apparent in the non-inducible model. Accordingly, this disorganization, confirmed in experiments with both non- and inducible Col2-Cre strains, is probably due to direct activation of mTORC1 in chondrocytes. Altogether, these observations suggest that mTOR-signaling pathway is not essential, but rather modulates the growth plate chondrocytes. In this regard it is interesting to point out that ablation of mTOR in the adult cartilage poses no histological phenotype in the growth plate (Zhang et al., 2015).

In conclusion, and in contrast to a recent report, elevated mTORC1 signaling in chondrocytes does not alter the proliferation or differentiation of growth plate chondrocytes. The described phenotype is likely caused by aberrant Cre-activity in other non-cartilaginous tissues early in development.

4. Materials and methods

4.1. Mouse strains

All animal work was approved by the Ethical Committee on Animal Experiments (Stockholm North Committee/Norra Djurförsökssetiska Nämnd) and conducted in accordance with The Swedish Animal Agency’s Provisions and Guidelines for Animal Experimentation.

The Col2-Cre strain (Kobayashi et al., 2002) expresses Cre recombinase under the transcriptional control of the Collagen type 2a1 promoter (Col2a1) (Schipani et al., 2001). The Col2a1 gene is active in chondrocytes, which facilitates expression of high levels of Col2-Cre in chondrocytes (Schipani et al., 2001).

In the Col2-CreERT2 strain, developed by Susan Mackem (NIH), expression of CreERT2 protein is controlled by Col2a1 (Nakamura et al., 2006); Cre-mediated DNA recombination is dependent on tamoxifen in both of these strains.

Rosa26-lacZ is a reporter mouse strain in which upon Cre-mediated recombination, beta galactosidase is expressed, allowing visualization of cells in which Cre has acted (Soriano, 1999) (JAX#003309).

In the Tsc1-flxed mice (JAX#005680) were developed by Kwiatkowski and colleagues, Cre-mediated recombination excises the essential exons 17 and 18 (Kwiatkowski et al., 2002).

Rosa26-Confetti (referred to as Confetti, and obtained from the laboratory of Hans Clevers (Hubrecht Institute) is a reporter mouse strain that contains the brainbow 2.1 construct (Livet et al., 2007). Upon Cre-mediated DNA recombination, one of four different fluorescent proteins (nuclear green, cytoplasmic red, cytoplasmic yellow and membrane-bound cyan) is expressed in a stochastic manner from each allele, allowing clonal identification (Snippert et al., 2010). Importantly, in our system we almost never (< 1%) observed the appearance of green fluorescent of confetti colors.

The Col2-CreERT2 strain was crossed with Tsc1-flxed and Confetti mice to obtain inducible, chondrocyte-specific loss of Tsc1 in the developing epiphysial cartilage (Tsc1cKOind). Gene ablation/tracing was induced on P3 by IP injection of 500 μg tamoxifen into each mouse. All control mice were littermates of the cKO mice.

4.2. Confocal microscopy

Confocal imaging was performed with a Zeiss LSM710 fluorescence microscope. The images displayed are of maximal projection. Contrast and brightness have been altered to improve visualization; where test and control are included, adjustments were made in an identical manner.

4.3. Immunofluorescence

Immunofluorescence was conducted to detect pS6 using tissues fixed for 6 h in 4% PFA/PBS, without antigen retrieval. Briefly, after blocking with 3% horse serum in PBS-Triton (PBS + 0.1% Triton), slides were incubated with primary antibody (Cell Signaling...
Technology, 4858S) overnight at 4 °C, washed with PBST (PBS + 0.1% Tween 20) and subsequently incubated with secondary antibody (Alexa fluor 647-conjugated Donkey anti-Rabbit IgG; Jackson laboratories, 711 605 152) for 1 h at room temperature and thereafter protected from light. In this particular experiment, mice were injected with a dose of 0.4 mg of tamoxifen on P3 and tissues collected on P40.

4.4. Quantification of fluorescent signal

Z-stack images were collected by confocal microscopy using the same settings for each slide, and maximum projection images obtained. ImageJ software (NIH, Bethesda, U.S.A.) was used for the digital analysis.

Firstly, to outline the growth plate precisely Freehand Selection tool and the “Edit > Clear Outside” function was used. The area of the growth plate was determined using the “Analyze > Measure” function (“Area” must be selected in the “Analyze > Set Measurements...” function). All spaces surrounding the growth plate were completely filled in black color using the “Edit > Fill” tool. All images were processed in this way.

Secondly, the images were stitched together in a single file (for direct comparison of the signal between the samples). To do this a new ImageJ file was created (File + N) and all images to-be-analyzed were copied and pasted into this new single image from left to right (and the order noted) leaving a gap between each image (Fig. S1A).

Thirdly, every pixel was selected (Control + A) and the signal was converted to a graph using the plot profile tool (Control + K), which generates a profile image (Fig S1B). To quantify the area under the curve for each plot, the Wand tool was used to select a plot and its area measured using the “Analyze > Measure” function. Notice, for Wand tool to select individual plots everything under the curve needs to be manually colored black using the Wand + the Wand tool (Control + K), which

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