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Activation of Nrf2 by sulfuretin stimulates chondrocyte differentiation and increases bone lengths in zebrafish

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Elongation of most bones occur at the growth plate through endochondral ossification in postnatal mammals. The maturation of chondrocyte is a crucial factor in longitudinal bone growth, which is regulated by a complex network of paracrine and endocrine signaling pathways. Here, we show that a phytochemical sulfuretin can stimulate hypertrophic chondrocyte differentiation *in vitro* and *in vivo*. We found that sulfuretin stabilized nuclear factor (erythroid-derived 2)-like 2 (Nrf2), stimulated its transcriptional activity, and induced expression of its target genes. Sulfuretin treatment resulted in an increase in body length of zebrafish larvae and induced the expression of chondrocyte markers. Consistently, a clinically available Nrf2 activator, dimethyl fumarate (DMF), induced the expression of hypertrophic chondrocyte markers and increased the body length of zebrafish. Importantly, we found that chondrocyte gene expression in cell culture and skeletal growth in zebrafish stimulated by sulfuretin were significantly abrogated by *Nrf2* depletion, suggesting that such stimulatory effects of sulfuretin were dependent on Nrf2, at least in part. Taken together, these data show that sulfuretin has a potential use as supporting ingredients for enhancing bone growth. [BMB Reports 2023; 56(9): 496-501]

INTRODUCTION

The precise regulation of chondrocyte differentiation and function can be achieved by multiple secreted factors, including insulin-like growth factor (IGF), thyroid hormone, parathyroid hormone-related peptide (PTHrP), Indian hedgehog (IHH), fibroblast growth factor (FGF), wingless-related integration site (Wnt), transforming growth factor-β (TGF-β), and C-type natriuretic peptide (1-3). Chondrocyte differentiation is also regulated by intracellular signaling mediators such as MAP kinase, PI3K/Akt, and GTPase and various transcriptional regulators including Sox9, β-catenin, Smad, Runx, Mef2, and histone deacetylase HDAC4 (1, 4).

Sulfuretin, a major flavonoid isolated from *Rhus verniciflua* Stokes (RVS), has been shown to inhibit oxidative stress, inflammation, and obesity related metabolic defects (5-7). Sulfuretin can promote osteoblast differentiation *in vitro* and bone regeneration *in vivo* through activation of BMP, mTOR, Wnt/β-catenin, and Runx2 signaling (8). Sulfuretin can also inhibit osteoclast differentiation induced by macrophage colony stimulating factor and receptor activator of NF-κB ligand released from bone marrow macrophages by inhibiting NF-kB signaling (9, 10). These findings suggest that sulfuretin can facilitate bone formation by stimulating osteoblastogenesis and concurrently inhibiting osteoclastogenesis. However, whether sulfuretin plays a role in chondrocyte differentiation remains to be elucidated.

Transcription factor nuclear factor erythroid 2 (NF-E2) p45-related factor 2 (Nrf2) is a player that can sense oxidative stress and provide antioxidant responses (11). A ubiquitously detected Nrf2 can recognize the antioxidant response element (ARE), a *cis*-acting sequence found in the 5′-flanking region of target genes (12). Nrf2 has been implicated in bone remodeling (6). In particular, Nrf2 is detected in chondrocyte layers of tibia from postnatal mouse (13). Nrf2 overexpression in ATDC5 cells can inhibit chondrocyte differentiation (13). Nrf2 also inhibits apoptosis in human T/C28a2 chondrocytes induced by a high shear flow (14). Plant-derived natural antioxidants andrographolide and astaxanthin can protect chondrocytes against oxidative stress through Nrf2 activation (15, 16). These data suggest a critical role of Nrf2 in chondrocytes.

Signaling cascades of bone formation in higher vertebrates are well conserved in zebrafish, rendering zebrafish a useful model to dissect underlying molecular mechanisms and to

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model human skeletal diseases. Zebrafish model can take advantages of tractable genetic manipulations and versatile *in vivo* imaging (17). It can be applied in compound screening for bone homeostasis (18).

In this study, we investigated effects of sulfuretin on bone growth using cell lines and zebrafish models. We showed that sulfuretin stimulated chondrocyte differentiation and increased body length in zebrafish in an Nrf2-dependent manner. These data suggest that sulfuretin-mediated Nrf2 activation has potential applications in bone growth and its related disorders.

RESULTS

Sulfuretin promotes chondrocyte differentiation

Previous findings have suggested that sulfuretin exhibits biological effects in bone cells including osteoblasts and osteoclasts (8). However, its roles in chondrocyte differentiation have not been addressed yet. To investigate effects of sulfuretin on chondrocyte differentiation, we induced a mass culture of C3H10T1/2 cells into chondrocytes for 12 days in the presence of sulfuretin. Matrix proteoglycan production evaluated by Alcian blue staining exhibited stimulatory effects of sulfuretin on chondrocyte differentiation (Fig. 1A). Sulfuretin at tolerated doses (Supplementary Fig. 1) induced the expression of late hypertrophic chondrocyte markers such as *Col10a1*, *Mmp13*, and *Mmp3* without significantly changing the expression of early chondrogenic markers such as *Col2a1*, *Aggrecan*, or *Sox9* (Fig. 1B). Sulfuretin suppressed the expression of adipogenic markers including *Pparg*, *Fabp4*, and *Cd36* (Fig. 1B), consistent with a previous report (7). Sulfuretin also increased expression of *Col10a1* and *Mmp13*, but not early chondrogenic markers (*Col2a1* or *Aggrecan*) in primary chondrocytes isolated from mouse at P7 (Fig. 1C). These data show that sulfuretin can stimulate chondrocyte differentiation.

Sulfuretin directly activates Nrf2 mediated transcription

Various antioxidant phytochemicals isolated from edible or medicinal plants have been shown to increase Nrf2 activities (19). Accordingly, sulfuretin has been shown to affect Nrf2 singling pathway in human SH-SY5Y cells (20) and HepG2 cells (21). Based on this, we investigated effects of sulfuretin on the expression levels of verified Nrf2 dependent target genes, Heme oxygenase 1 (*Hmox1*) and NAD(P)H:quinone oxidoreductase 1 (*Nqo1*) in C3H10T1/2 cells (Fig. 2A). We found that sulfuretin increased the mRNA levels of *Hmox1* and *Nqo1* upto 9.3 and 4.6 folds respectively.

Since Nrf2 stability is increased upon its activation, we measured protein levels of Nrf2. Comparable to the effects of tert-butylhydroquinone (tBHQ), a known Nrf2 activator, sulfuretin dose dependently increased Nrf2 protein levels in C3H10T1/2 cells (Fig. 2B). We transiently co-transfected HEK293T cells with Nrf2 dependent-antioxidant response element (ARE) luciferase reporter constructs along with an expression vector encoding *Nrf2* and treated with DMSO or sulfuretin. Sulfuretin significantly stimulated the luciferase activity in the presence of Nrf2 (Fig. 2C).

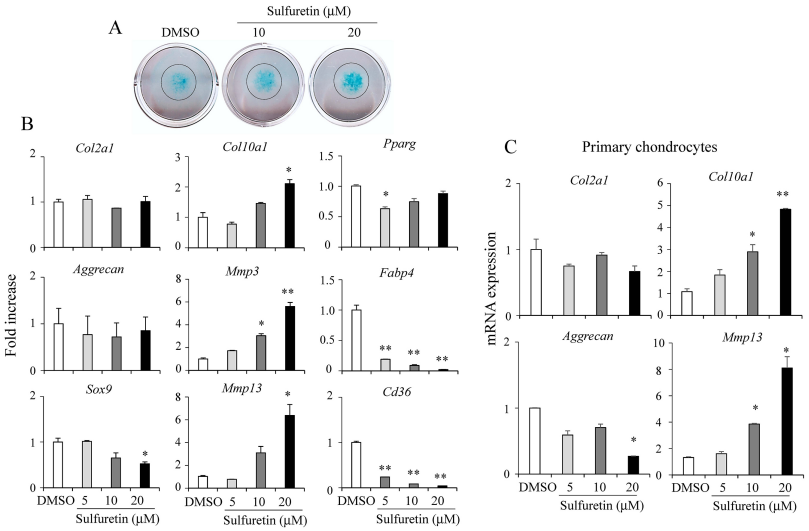

Fig. 1. Sulfuretin stimulates chondrocyte differentiation in cell culture. (A) C3H10T1/2 cells were differentiated into chondrocytes in the presence of sulfuretin (10 or 20 μM) for 12 days followed by Alcian Blue staining. (B) Relative mRNA expression levels of early chondrocyte genes (*Col2a1*, *Aggrecan*, and *Sox9*), late chondrocyte genes (*Col10a1*, *Mmp3*, and *Mmp13*), and adipocyte genes (*Pparg*, *Fabp4*, and *Cd36*) after sulfuretin treatment were determined by real-time PCR. (C) Primary chondrocytes were isolated from mice at postnatal day 7 and treated with sulfuretin for 12 days. Relative mRNA expression was quantified by real-time PCR. Data shown represent mean ± SEM. Statistical significance was determined relative to a control by Student's t-test (*$P < 0.05$; **$P < 0.01$).

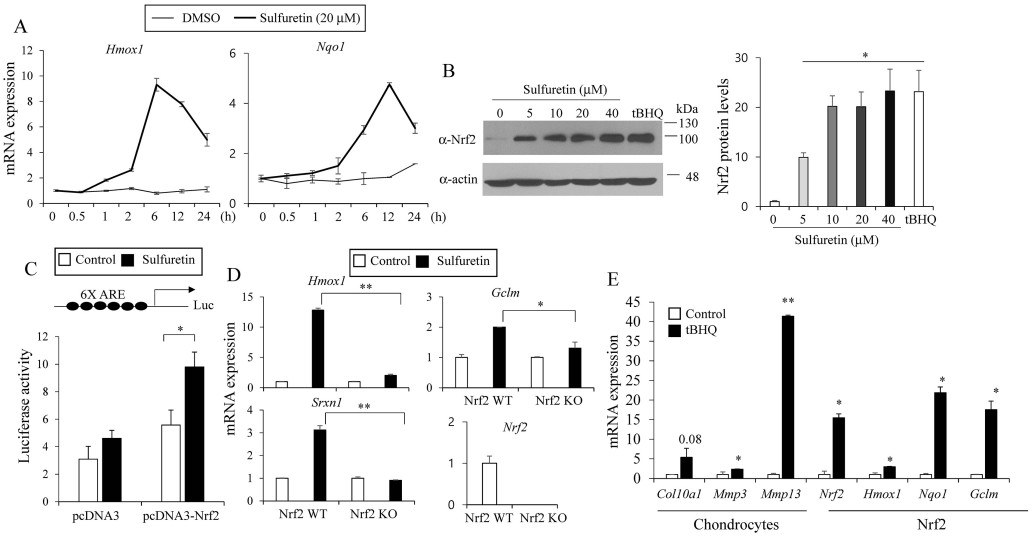

Fig. 2. Sulfuretin stimulates Nrf2 activity. (A) C3H10T1/2 cells were treated with sulfuretin (20 μM) for 0.5, 1, 2, 6, 12, and 24 hours and relative temporal expression levels of *Hmox1* and *Nqo1* were measured by real-time PCR. (B) Sulfuretin treatment at 5-40 μM for 6 hours in C3H10T1/2 cells increased Nrf2 protein levels as determined by western blotting. Actin was used for loading control and tBHQ (50 μM) was used as a control. Representative blot images and relative densitometry bar graph were shown (n = 3). (C) HEK293T cells were transfected with the Nrf2 dependent-antioxidant response element (ARE) luciferase reporter construct (6XARE) and an expression vector coding for Nrf2 (pcDNA3-Nrf2). After 24 hours, cells were treated with DMSO (control) or sulfuretin (20 μM) for 24 hours and luciferase activity was measured. Luciferase signals were normalized with Renilla luciferase activity. (D) Nrf2 wild type (WT) MEF or Nrf2 knockout (KO) MEF were treated with sulfuretin (20 μM) for 12 hours and expression levels of Nrf2 target genes were measured by real-time PCR. (E) C3H10T1/2 cells were differentiated into chondrocytes in the presence of tBHQ (100 μM) and relative levels of hypertrophic genes and Nrf2 target genes were determined by real-time PCR. Data are presented as mean ± SEM (n = 3). Statistical significance was determined by comparison to the control using Student's t-test (*$P < 0.05$; **$P < 0.01$).

To examine the effects of Nrf2 dependency of sulfuretin in inducing Nrf2 target gene expression, we treated sulfuretin into wild type (Nrf2 WT) and *Nrf2* knockout (Nrf2 KO) mouse embryonic fibroblast (MEF) and measured Nrf2-induced gene expression. Sulfuretin induced expression levels of *Nrf2* target genes (*Hmox1*, *Gclm*, and *Srxn1*) in WT MEF. However, these effects were significantly impaired in *Nrf2* KO cells, showing that sulfuretin's effects were Nrf2 dependent (Fig. 2D).

Having observed the effects of sulfuretin in stimulating Nrf2 activity, we tested alternative pharmacological Nrf2 activators to examine their effects on chondrocyte gene expression and bone growth. Similar to effects of sulfuretin, treatments with Nrf2 activator tBHQ in C3H10T1/2 cells for 12 days during chondrocyte differentiation induced *Nrf2*, *Hmox1*, *Nqo1*, and hypertrophic chondrocyte genes *Col10a1*, *Mmp3*, and *Mmp13* (Fig. 2E). Taken together, these data indicate that sulfuretin can stimulate Nrf2 activity.

Sulfuretin increases bone formation in zebrafish

Zebrafish and mammals share conserved mechanisms of bone formation and remodeling (22). Thus, we tested the effects of sulfuretin in zebrafish. Incubating zebrafish embryos starting at 1 day postfertilization (dpf) for 4 or 6 days with sulfuretin (100 μM) significantly ($P < 0.05$) increased body lengths by approximately 5.6% and 3.9%, respectively, compared to control treatment (Fig. 3A, B). In addition, bone formation in the developing spine shown by alizarin red-positive numbers of small bones in the vertebral column was accelerated in sulfuretin-treated larvae than in DMSO-treated larvae (Supplementary Fig. 2). Similar to the effects in cell culture, sulfuretin treatment induced expression of hypertrophic chondrocyte genes including *mmp13*, *col10a1a*, *col10a1b*, *ctgfa*, and *ctgfb* in zebrafish larvae (Fig. 3C). Since ceratohyal and parasphenoid are two hallmarks of bone elements that form through endochondral ossification and intramembranous ossification, respectively, in zebrafish (23), we performed whole mount *in situ* hybridization (WISH) for visualizing *col10a1* and *ctgf* as selective markers of hypertrophied chondrocyte formation (24). Compared to DMSO treatment, 4 days of treatment with sulfuretin (100 μM) increased the length of the center of ceratohyal by approximately 40% (*col10a1* staining) and the total length of ceratohyal (*ctgf* staining) by 20%. However, no difference was observed in the parasphenoid area shown by WISH staining of *col10a1* and *ctgf* (Fig. 3D, E). These data strongly suggest that sulfuretin can specifically stimulate endochondral ossification.

To investigate whether sulfuretin also act on Nrf2 in zebrafish, we measured the Nrf2 target gene expression in zebrafish treated with sulfuretin. In line with the effects of sulfuretin as

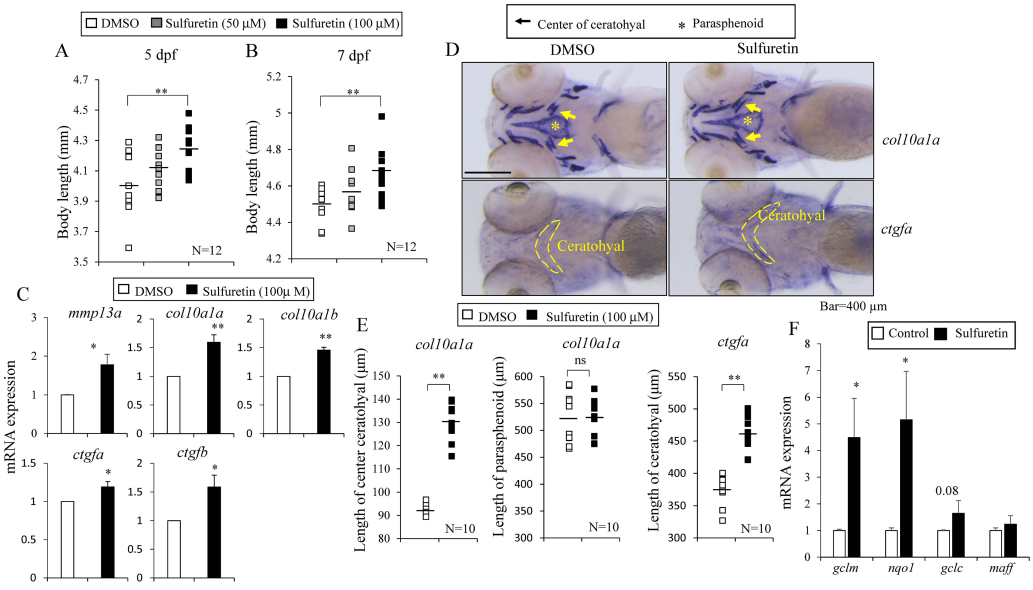

Fig. 3. Sulfuretin treatment increases body length and Nrf2 target gene expression in zebrafish. (A) Zebrafish at 1 dpf were treated with sulfuretin (50 or 100 μM) for 4 days. Body lengths were then measured at 5 dpf. (B) Zebrafish at 1 dpf were treated with sulfuretin for 6 days and body lengths were measured at 7 dpf. (C) Total RNA was isolated from zebrafish after treatment with sulfuretin (100 μM) for 4 days and relative expression levels of *mmp13a*, *col10a1a*, *col10a1b*, *ctgfa*, and *ctgfb* were determined by real-time PCR. (D, E) Sulfuretin (100 μM) treatments increases the length of the ceratohyal (arrow) but not the parasphenoid (*) in zebrafish. (D) Whole mount *in situ* hybridization of *col10a1* and *ctgf*. (E) Quantification of WISH staining in center of ceratohyal (*col10a1*), parasphenoid area (*col10a1*), and total length of ceratohyal (*ctgf*). (F) Zebrafish at 1 dpf were treated with sulfuretin for 4 days and expression levels of *gclm*, *nqo1*, *gclc*, and *maff* were measured by real-time PCR. Data are presented as mean ± SEM (n = 10 per group). Statistical significance was determined by Student's t-test (ns, not significant; *P < 0.05; **P < 0.01).

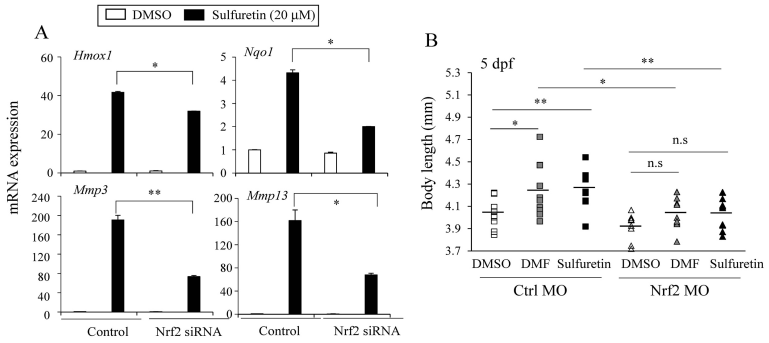

Fig. 4. Nrf2 is necessary for effects of sulfuretin on chondrocyte and bone growth in zebrafish. (A) Induction of chondrocyte genes by sulfuretin is blunted in Nrf2 knockdown cells. C3H10T1/2 cells were transfected with siRNA targeting Nrf2 (Nrf2 siRNA) and differentiated into chondrocytes for 12 days. Expression levels of Nrf2 targets (*Hmox1* and *Nqo1*) and chondrocyte genes (*Mmp3* and *Mmp13*) were measured by real-time PCR. Data are presented as mean ± SEM (n = 3). (B) Effects of sulfuretin and DMF on body lengths were compromised in *nrf2* morpholino injected zebrafish. Sulfuretin (100 μM) or DMF (4 μM) was used to treat control morpholino or *nrf2* morpholino injected zebrafish at 1 dpf for 4 days and their lengths were determined. Data are presented as mean ± SEM (n = 10). Statistical significance was determined by comparison with the control using Student's t-test (ns, not significant; *P < 0.05; **P < 0.01).

an Nrf2 activator, sulfuretin treatment in zebrafish consistently increased expression levels of *nqo1*, *gclc*, and *gclm* (Fig. 3F).

To further verify the effects of Nrf2 in body length, we treated 1 dpf zebrafish embryos with an FDA-approved cysteine-modifying drug dimethyl fumarate (DMF) (25) for 6 days and measured body lengths. Treatment with 1 or 4 μM DMF significantly increased the body length (Supplementary Fig. 3) and alizarin red-positive numbers of small bones in the vertebral

column (Supplementary Fig. 4). DMF also induced the expression of hypertrophic chondrocyte markers similar to effects of sulfuretin in zebrafish (Supplementary Fig. 3). Together, these data show that sulfuretin can stimulate Nrf2 activity and increase body length in zebrafish.

Nrf2 is required for sulfuretin-induced chondrocyte gene expression *in vitro* and increased body length in zebrafish larvae

To assess Nrf2 dependent effects of sulfuretin on chondrocyte gene induction, we transfected C3H10T1/2 cells with *Nrf2* small interfering RNAs (siRNA) or scramble siRNA and differentiated them into chondrocytes in the presence of sulfuretin. While the expression levels of Nrf2 target genes such as *Hmox1* and *Nqo1* upon sulfuretin treatment were dramatically increased in the group treated with scramble siRNA controls, such effects of sulfuretin were significantly compromised in *Nrf2* siRNA transfected cells (Fig. 4A). In addition, induction of hypertrophic chondrocyte genes *Mmp3* and *Mmp13* by sulfuretin was also significantly impaired in *Nrf2* siRNA transfected cells (Fig. 4A).

To further determine the Nrf2-dependent effects of sulfuretin and DMF *in vivo*, we treated *nrf2* morpholino (MO)-injected zebrafish embryos with sulfuretin for 4 days and assessed its effects. Sulfuretin or DMF treatment significantly increased body length of zebrafish by 5.22% or 4.77%, respectively, compared to control treatments. However, such effects were significantly abrogated in *nrf2* MO injected larvae by 3.0% and 3.16% (42% and 33.8% reduction), respectively, compared to effects in control MO injected larvae, corroborating the role of *nrf2* activation in increased body length (Fig. 4B). Together, these data indicate that the ability of sulfuretin to increase chondrocyte differentiation and body length is dependent on Nrf2, at least in part.

DISCUSSION

Sulfuretin, a major flavonoid of *Rhus verniciflua* Stokes, exhibits anti-inflammatory, anti-obese, anti-oxidative, pro-osteogenic, and neuroprotective activities (5, 7, 26). It has been also shown that sulfuretin can induce bone regeneration and inhibit bone resorption (8). In this study, we provided evidence that sulfuretin could increase expression of late chondrocyte genes in C3H10T1/2 cells and primary chondrocytes. We also showed that treatment with sulfuretin increased body lengths of zebrafish larvae by 5.6% likely through activation of Nrf2 signaling. Therefore our data strongly suggest that sulfuretin stimulates chondrocyte differentiation *in vitro* and increases body length in zebrafish.

We presented evidence that sulfuretin could increase chondrocyte gene expression by acting on Nrf2 signaling pathway. In line with this, a previous study has shown that sulfuretin can protect Aβ25-35-induced neurotoxicity through activation of Nrf2 and PI3K/Akt signaling pathways (20). Although the detailed molecular pathways linking Nrf2 to chondrocyte differentiation remain to be determined, it is possible that Nrf2 exhibits functional connections with numerous signaling pathways mediated by nuclear factor-κB (NF-κB), p53, mTOR, adrenergic receptor, and Notch1 (27-29). Growth factors such as IGF, PTHrp, IHH, FGF, Wnt, TGF-β, and BMP signaling are also candidates for Nrf2 targeting as they can affect chondrocyte differentiation (1-3). Indeed, we found that sulfuretin treatment promoted expression of Wnt, Tgf-β, and BMP signaling target genes (Supplementary Fig. 5), suggesting possible links between sulfuretin and these growth factor signaling pathways.

In the current study, sulfuretin increased bone length possibly by acting on chondrocytes. However, we cannot exclude the possibility that sulfuretin can affect multiple cell types including osteoblasts and osteoclasts as they have known to regulate bone lengths in certain animal models (5). In addition, it has been shown that enhancing or decreasing activities of osteoblasts or osteoclasts, respectively, can increase bone length. FGFr2 inactivation in osteoblast can decrease bone growth while osteoblast expression of growth hormone can increase tibial and femoral lengths (30). Therefore, although sulfuretin affects chondrocyte differentiation in a cell-autonomous manner, a limitation of the current study lies in the lack of direct evidence linking the contribution of sulfuretin mediated osteoblast and osteoclast activities to effect on longitudinal bone length *in vivo*. As such, the effects of sulfuretin in bone tissues followed by histological examination on hypertrophic chondrocytes in animal models would be interesting topics for future investigation.

In conclusion, we showed that sulfuretin stimulated chondrocyte differentiation *in vitro* and longitudinal bone growth in zebrafish. We also show that sulfuretin-mediated Nrf2 signaling plays a role in controlling chondrocyte differentiation. Given these *in vitro* and *in vivo* effects, sulfuretin and Nrf2 activators might provide a new approach to treat bone growth problem and bone-related diseases.

MATERIALS AND METHODS

Materials and methods are available in the supplemental material.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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