



# The bone anabolic carotenoid $\beta$ -cryptoxanthin enhances transforming growth factor- $\beta$ 1-induced SMAD activation in MC3T3 preosteoblasts

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**Abstract.** The xanthophyll  $\beta$ -cryptoxanthin is a member of the carotenoid family of plant-derived pigments endowed with anti-osteoporotic properties *in vivo*.  $\beta$ -cryptoxanthin was demonstrated to stimulate osteoblastic bone formation and simultaneously repress osteoclastic bone resorption *in vitro*. However, the mechanisms of action remain to be elucidated. The SMAD signal transduction pathway is established to play a critical role in osteoblast lineage commitment and differentiation. In this study we used transient transfection assays of a SMAD luciferase reporter to investigate whether  $\beta$ -cryptoxanthin regulates SMAD activation in MC3T3 pre-osteoblastic cells.  $\beta$ -cryptoxanthin did not stimulate basal SMAD activity but amplified transforming growth factor (TGF)- $\beta$ 1-induced SMAD activation. Interestingly,  $\beta$ -cryptoxanthin did not affect bone morphogenetic protein-2 (BMP-2)-induced SMAD activation in osteoblastic cells, suggesting specificity of action on the TGF- $\beta$ 1 pathway. This study suggests that the carotenoid  $\beta$ -cryptoxanthin may promote osteoblast differentiation and activity by amplifying TGF- $\beta$ 1-induced lineage commitment of osteoblast precursors.

## Introduction

Bone homeostasis is maintained by a delicate balance between osteoblastic bone formation and osteoclastic bone resorption. Numerous pathological processes have the capacity to disrupt this equilibrium leading to conditions where the rate of bone resorption outpaces the rate of bone formation leading to osteoporosis, a devastating bone disease that is widely recognized as a major public health threat. Postmenopausal osteoporosis, a consequence of ovarian hormone deficiency, is

the archetypal osteoporotic condition in women after menopause and leads to bone destruction though complex and diverse metabolic and biochemical changes (1).

Pharmacologic and nutritional factors have long been associated with reduced postmenopausal bone loss as chemical compounds present in certain food and plants appear to modulate bone turnover. Food and plant chemical factors can help prevent bone loss but the molecular actions of these factors are poorly understood (2,3).

Found predominantly in fruit and vegetables, carotenoids are a group of plant pigments whose members include  $\beta$ -cryptoxanthin, lutein, lycopene, astaxanthin, and  $\beta$ -carotene. Interestingly,  $\beta$ -cryptoxanthin, which is abundant in Satsuma mandarin orange (*Citrus unshiu* MARC.), has anabolic effects on osteoblastic bone formation (4-7) and suppressive effects on osteoclastic bone resorption *in vitro* (5,8,9), leading to an increase in bone mass. Furthermore, oral administration of  $\beta$ -cryptoxanthin was shown to have anabolic effects on bone turnover in young and aged rats (10), and prevents bone loss in streptozotocin-diabetic rats (11), and ovariectomized rats (12) *in vivo*.

Moreover, the intake of  $\beta$ -cryptoxanthin-reinforced juice for long periods of time was shown to have both stimulatory effects on osteoblastic bone formation and inhibitory effects on osteoclastic bone resorption in healthy humans and in postmenopausal women, as assessed by serum biochemical markers of bone metabolism *in vivo* (13). Epidemiological studies have further suggested that  $\beta$ -cryptoxanthin constitutes a sustainable nutritional approach to improving the bone health of human subjects (14-16).

Although  $\beta$ -cryptoxanthin is widely considered to be an effective nutritional factor for maintaining healthy bone, and for prevention of osteoporosis (17), the molecular mechanisms by which  $\beta$ -cryptoxanthin modulates bone cells are poorly understood.

$\beta$ -cryptoxanthin has been shown to have stimulatory effects on osteoblastic bone formation and mineralization in organ culture *ex vivo* (4,5) and to increase cell number, DNA content, protein content, and alkaline phosphatase activity in osteoblastic cells (6,7), suggesting that  $\beta$ -cryptoxanthin stimulates both preosteoblast proliferation and differentiation.  $\beta$ -cryptoxanthin has a stimulatory effect on the gene expression of various osteoblastic proteins (including Runx2,  $\alpha$ 1 (I) collagen and

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alkaline phosphatase), factors essential for osteoblastic bone formation and mineralization (6,7).

How  $\beta$ -cryptoxanthin promotes osteoblastic differentiation is unclear, however, this factor was shown to stimulate the expression of insulin-like growth factor-I (IGF-I) and TGF- $\beta$ 1 (6), factors implicated in bone growth, and in osteoblast differentiation (18,19).

TGF- $\beta$ 1 has potent activity on osteoblast-lineage commitment, an event that is partly mediated through SMAD transcription factors (23). We reported that NF- $\kappa$ B signaling represses basal osteoblast differentiation and mineralization in MC3T3 cells and antagonizes TGF $\beta$  and BMP-2 mediated MC3T3 mineralization by downregulating SMAD activation (20). Other studies found that NF- $\kappa$ B signaling antagonizes SMAD activation in Saos2 osteosarcoma cells by a mechanism involving induction of the inhibitory SMAD7 (21). Interestingly, we recently reported that  $\beta$ -cryptoxanthin has the capacity to suppress NF- $\kappa$ B activity in MC3T3 preosteoblastic cells (22) suggesting that  $\beta$ -cryptoxanthin leads to enhanced SMAD signaling.

In this study we examined whether  $\beta$ -cryptoxanthin directly stimulates SMAD activation or regulates TGF- $\beta$ 1-induced SMAD activation in osteoblastic cells *in vitro*. Our data show that  $\beta$ -cryptoxanthin potentiates TGF $\beta$ -induced, but not BMP-2-induced, SMAD activation in MC3T3 preosteoblastic cells, suggesting that one mechanism by which  $\beta$ -cryptoxanthin stimulates bone formation is by potentiating the TGF- $\beta$ 1-mediated commitment of preosteoblasts to differentiate along the osteoblastic pathway.

## Materials and methods

**Materials.**  $\alpha$ -Minimal essential medium ( $\alpha$ -MEM) and antibiotics (penicillin and streptomycin) were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was from Hyclone (Logan, UT).  $\beta$ -cryptoxanthin was obtained from Extrasynthase (Lyon-Nord, France). Recombinant TGF- $\beta$ 1 or BMP-2 was purchased from R&D Systems, Inc. (Minneapolis, MN). PD 98059 was obtained from EMD Biosciences, Inc. (La Jolla, CA). Alizarin Red S, and all other chemicals were obtained from the Sigma Chemical Corporation (St. Louis, MO) unless otherwise stated.

**Cell culture.** The preosteoblastic cell line MC3T3-E1, clone 14 (MC3T3) was purchased from the American Type Culture Collection (Manassas, VA) and has been previously described in detail (24). Cells were cultured at 37°C in a humidified 5% CO<sub>2</sub> incubator in plastic dishes containing  $\alpha$ -MEM supplemented with 10% FBS, with 2 mM glutamine, 100 IU/ml penicillin, and 100  $\mu$ g/ml streptomycin. Cells were passaged every 3 days using 0.2% trypsin plus 0.02% EDTA in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free phosphate-buffered saline (PBS).

**Osteoblast differentiation assays and Alizarin Red S staining.** MC3T3 cells (1.0x10<sup>5</sup> cells per well) were cultured for 72 h in  $\alpha$ -MEM (1.0 ml/well) containing 10% FBS in 12-well dishes. Medium was aspirated and changed to mineralization medium [ $\alpha$ -MEM supplemented with 10% FBS, L-ascorbic acid (100  $\mu$ g/ml) and 4 mM  $\beta$ -glycerophosphate] as previously described (7,20). Cells were treated with either vehicle (PBS)

or  $\beta$ -cryptoxanthin (10<sup>-8</sup> or 10<sup>-5</sup> M). Fresh medium was added every 3 days. At 21 days of culture, cells were rinsed with PBS and calcium deposition was visualized by fixing the cells in 75% ethanol for 30 min at 4°C followed by staining with Alizarin Red S (40 mM, pH 6.2) for 30 min at room temperature. Excess stain was removed by copious washing with distilled water.

Mineralization was quantitated following capture on a flatbed scanner (Epson Perfection 1660 Photo) by densitometry using Image J (Rasband, W.S., ImageJ, US National Institutes of Health, Bethesda, MD, USA, <http://rsb.info.nih.gov/ij/>, 1997-2009). Data are expressed in arbitrary units.

**SMAD reporter constructs and luciferase assays.** A SMAD reporter was generated by synthesizing a double-strand oligonucleotide bearing three tandem copies each of the consensus (5'-GTCT) SBE interspersed with its palindrome 5'-AGAC (5'-GTCTAGACGTCTAGACGTCTAGAC) (25) and cloning the oligonucleotide upstream of the luciferase gene in pGL3-BASIC (Promega, Madison, WI) to create pGL3-SMAD.

Reporter plasmids were transfected into MC3T3 preosteoblastic cells using Lipofectamine 2000 reagent (Invitrogen) as previously described (20). Briefly, MC3T3 cells (2x10<sup>4</sup> cells/well) were cultured in 96-well white opaque luminometer plates (Corning Costar, Lowell, MA) for 24 h in  $\alpha$ -MEM, and then changed to  $\alpha$ -MEM without FBS and antibiotics and transfected with pGL3-SMAD reporter plasmid. Medium was changed after 5 h to  $\alpha$ -MEM containing antibiotics and with or without 10% FBS. Wells were treated with vehicle (PBS) or  $\beta$ -cryptoxanthin (10<sup>-8</sup>-10<sup>-5</sup> M), and/or TGF- $\beta$ 1 (1 or 10 ng/ml) for 24 h. In some experiments BMP-2 (0.5  $\mu$ g/ml) was added 16 h after addition of  $\beta$ -cryptoxanthin (10<sup>-7</sup>-10<sup>-5</sup> M), and the cells were cultured for 8 h. Luciferase activity was measured on a microplate luminometer (Turner Designs, Sunnyvale, CA, USA) following cell lysis in Passive Lysis Buffer (Promega).

**Statistical analysis.** Statistical significance was determined using GraphPad InStat version 3 for Windows XP (GraphPad Software). Multiple comparisons were performed by one-way ANOVA with Tukey-Kramer post test. P<0.05 was considered statistically significant. Data are presented as mean  $\pm$ SD of 5 replicate wells and data are representative of two or more independent experiments.

## Results

**$\beta$ -cryptoxanthin stimulates osteoblastic mineralization.** As the effect of  $\beta$ -cryptoxanthin on MC3T3 mineralization has not been previously reported we cultured MC3T3 cells with  $\beta$ -cryptoxanthin (10<sup>-6</sup> or 10<sup>-5</sup> M) for 21 days and stained for calcium deposition with Alizarin Red S. The data show that  $\beta$ -cryptoxanthin had a stimulatory effect on MC3T3 mineralization *in vitro* which was saturated at 10<sup>-6</sup> M (Fig. 1).

**$\beta$ -cryptoxanthin amplifies TGF $\beta$ -induced SMAD activity in MC3T3 cells.**  $\beta$ -cryptoxanthin has been shown to stimulate osteoblastic bone formation and mineralization *ex vivo* (6,7). However, the mechanism by which this carotenoid achieves this action remains unclear. We recently reported that  $\beta$ -cryptoxanthin antagonizes NF- $\kappa$ B signal transduction (22), a potent

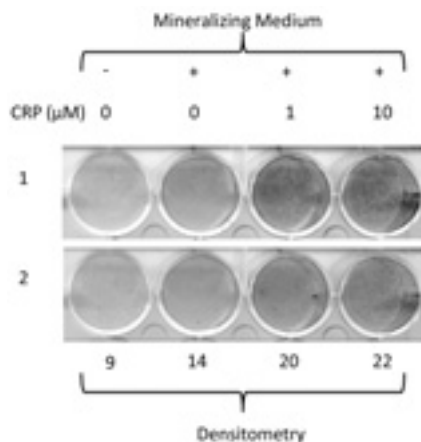


Figure 1.  $\beta$ -cryptoxanthin promotes osteoblastic differentiation and mineralization in preosteoblastic MC3T3 cells. MC3T3 cells were differentiated into mineralizing osteoblasts by culture in  $\alpha$ -MEM supplemented with 10% FBS, ascorbic acid (100 ng/ml) and 4 mM  $\beta$ -glycerophosphate (mineralizing medium) in the presence or absence of  $\beta$ -cryptoxanthin (CRP) at  $10^{-6}$  or  $10^{-5}$  M. After 28 days cells were washed with PBS and stained with Alizarin Red S to visualize calcium deposition. The outcome of 2 independent experiments (labeled 1 and 2) is shown. Average densitometric quantitation is presented below each well in arbitrary units.

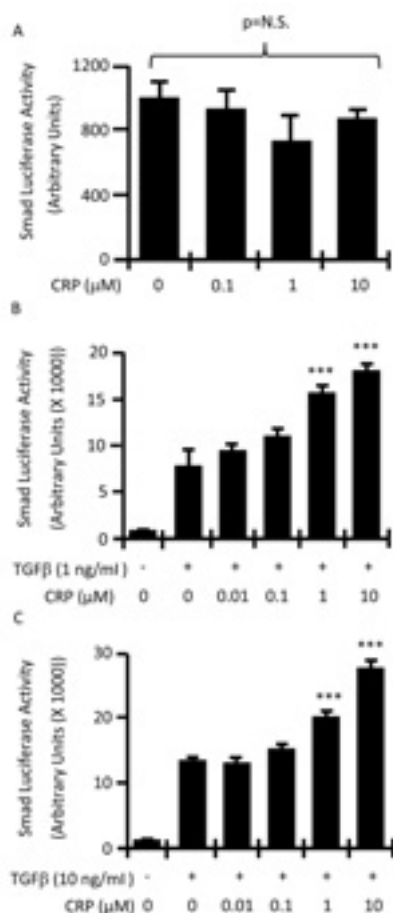


Figure 2.  $\beta$ -cryptoxanthin enhances TGF- $\beta$ 1-induced SMAD activation in preosteoblastic MC3T3 cells. MC3T3 cells were transfected with pGL3-SMAD, a SMAD activity reporter and cells cultured in the presence or absence of (A)  $\beta$ -cryptoxanthin ( $10^{-7}$ - $10^{-5}$  M), (B) TGF- $\beta$ 1 (1 ng/ml) with  $\beta$ -cryptoxanthin ( $10^{-8}$ - $10^{-5}$  M), or (C) TGF- $\beta$ 1 (10 ng/ml) with  $\beta$ -cryptoxanthin ( $10^{-8}$ - $10^{-5}$  M). Luciferase activity was determined after 24 h. Data represent mean  $\pm$ SD of five replicate assays. \*\*\* $P$ <0.001 relative to pGL3-SMAD + TGF $\beta$  only (one-way ANOVA). N.S., no significant difference between data within brackets.

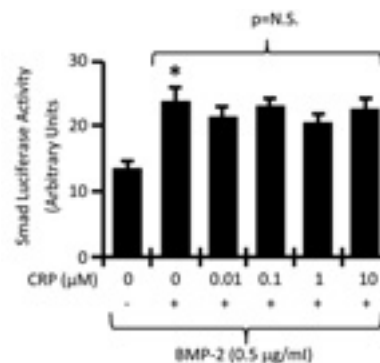


Figure 3.  $\beta$ -cryptoxanthin fails to enhance the BMP-2-induced SMAD activation in preosteoblastic MC3T3 cells. MC3T3 cells were transfected with pGL3-SMAD and cells cultured in the presence or absence of BMP (0.5  $\mu$ g/ml) with or without  $\beta$ -cryptoxanthin ( $10^{-8}$ - $10^{-5}$  M). Luciferase activity was determined after 24 h of BMP-2 addition. Data represent mean  $\pm$ SD of five assays. \*\*\* $P$ <0.001 relative to pGL3-SMAD only (one-way ANOVA). N.S., no significant difference between data within brackets.

inhibitor of osteoblast differentiation, and a suppressor of SMAD activation. We thus investigated whether  $\beta$ -cryptoxanthin is capable of promoting basal or TGF $\beta$  stimulated SMAD activation, using a SMAD responsive reporter (pGL3-SMAD). While  $\beta$ -cryptoxanthin ( $10^{-7}$ - $10^{-5}$  M) was not seen to significantly impact basal MC3T3 SMAD activity (Fig. 2A), it significantly and dose-dependently ( $10^{-8}$ - $10^{-5}$  M) enhanced TGF- $\beta$ 1 (1 and 10 ng/ml)-induced SMAD activation (Fig. 2B and C, respectively).

As BMP-2, another potent pro-osteoblastic factor, also signals via the SMAD pathway (25), we examined whether  $\beta$ -cryptoxanthin could also amplify BMP-2-induced SMAD activation. Interestingly, in contrast to TGF- $\beta$ 1,  $\beta$ -cryptoxanthin ( $10^{-8}$ - $10^{-5}$  M) had no potentiating effect on BMP-2-induced (0.5  $\mu$ g/ml) SMAD activation in MC3T3 cells (Fig. 3).

As  $\beta$ -cryptoxanthin has been reported to promote MC3T3 proliferation and differentiation by means of a MAP kinase kinase (MEK) dependent pathway sensitive to the MEK inhibitor PD98059 (6), we examined whether PD98059 (26,27) ablates the potentiating action of  $\beta$ -cryptoxanthin on TGF $\beta$ -induced SMAD activity. PD98059 ( $10^{-7}$ - $10^{-5}$  M) had no significant effect on basal SMAD activity (Fig. 4A). Furthermore, PD98059 ( $10^{-7}$  and  $10^{-6}$  M) had no significant effect on the capacity of  $\beta$ -cryptoxanthin ( $10^{-5}$  M) to enhance TGF- $\beta$ 1 (10 ng/ml)-induced SMAD activity (Fig. 4B).

## Discussion

$\beta$ -cryptoxanthin has been shown to have a stimulatory effect on osteoblastic bone formation *in vitro* (6,7) and *in vivo* (10-12) by stimulating osteoblastic cell proliferation and differentiation and by regulating osteoblastic gene expression, including Runx2 a master regulator of osteoblast differentiation (26,28), the matrix protein of  $\alpha$ 1 (I) collagen (6,29), and alkaline phosphatase which participates in the mineralization process (6,30). The molecular mechanism by which  $\beta$ -cryptoxanthin promotes osteoblast differentiation is, however, poorly defined. We have previously reported that TGF $\beta$  and/or BMP-2 signaling through SMADS is impeded by basal and stimulated (TNF $\alpha$  or RANKL) induced NF- $\kappa$ B



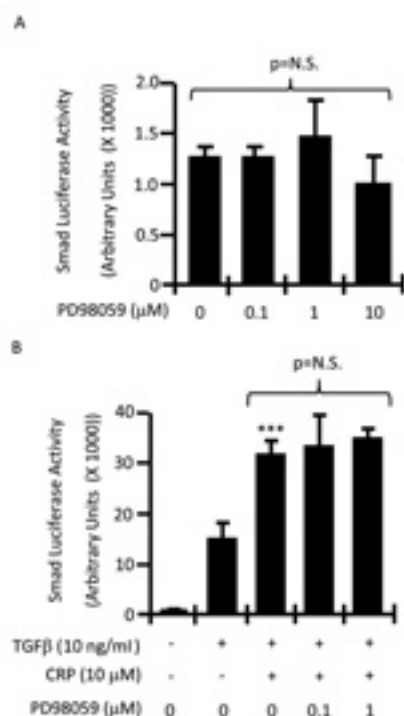


Figure 4. Inhibition of MEK fails to block  $\beta$ -cryptoxanthin augmented TGF- $\beta$ 1-induced SMAD activation in MC3T3 preosteoblastic cells. A) MC3T3 cells were transfected with pGL3-SMAD and treated with PD98059 ( $10^{-7}$ - $10^{-5}$  M) or B) with PD98059 ( $10^{-7}$ - $10^{-6}$  M) with TGF- $\beta$ 1 (10 ng/ml) in the presence or absence of  $\beta$ -cryptoxanthin ( $10^{-5}$  M). Luciferase activity was determined after 24 h. Data represent mean  $\pm$ SD of five assays. \*\*\* $P$ <0.001 relative to pGL3-SMAD only (one-way ANOVA). N.S., no significant difference between data within brackets.

signaling. Consequently, NF- $\kappa$ B antagonists stimulate osteoblast differentiation and mineralization (20). We further demonstrated that  $\beta$ -cryptoxanthin antagonizes NF- $\kappa$ B activity in MC3T3 preosteoblastic cells (22). These observations suggested that one mechanism by which  $\beta$ -cryptoxanthin may stimulate osteoblast differentiation is by promoting SMAD activation. The data presented herein are consistent with this notion. While,  $\beta$ -cryptoxanthin failed to directly stimulate SMAD activity, it did, however, amplify TGF- $\beta$ 1-induced SMAD signaling. Interestingly, BMP-2 mediated SMAD signal transduction was not augmented by  $\beta$ -cryptoxanthin. The reason for this difference in response is presently unclear, however while TGF- $\beta$ 1 specifically activates R-SMADS 2 and 3, BMP-2 utilizes R-SMADS 1, 5 and 8. Upon R-SMAD phosphorylation and activation these SMADS associate with a Co-SMAD (SMAD4) and the complexes translocate to the nucleus where they bind to SMAD binding elements in DNA (23). One possibility is thus that  $\beta$ -cryptoxanthin specifically facilitates SMAD2 or 3 expression or activation. Alternatively,  $\beta$ -cryptoxanthin may specifically interact with, or promote the expression of TGF $\beta$  receptor components. Interestingly,  $\beta$ -cryptoxanthin was previously reported to stimulate the expression of TGF- $\beta$ 1 mRNA in MC3T3 cells (6). Consequently, it is also possible that induction of TGF- $\beta$ 1 expression by  $\beta$ -cryptoxanthin contributes to the amplification of SMAD signaling in our experiments. This is unlikely however, as we show that  $\beta$ -cryptoxanthin enhanced TGF- $\beta$ 1-induced SMAD activity by

about the same amount ( $\sim$ 2-fold) with a low dose of TGF- $\beta$ 1 (1 ng/ml) and with a high dose of TGF- $\beta$ 1 (10 ng/ml).

Previous studies also reported that  $\beta$ -cryptoxanthin has the capacity to promote osteoblast differentiation through MAP kinase pathways, specifically through MEK (6). In the present study the ability of  $\beta$ -cryptoxanthin to enhance TGF- $\beta$ 1-induced SMAD activation was not suppressed by PD98059, a specific MEK inhibitor, suggesting that the amplificatory action of  $\beta$ -cryptoxanthin on TGF- $\beta$ 1-induced SMAD activation is mediated independently of MEK.

Taken together, our data suggest that  $\beta$ -cryptoxanthin stimulates osteoblast differentiation through multiple overlapping pathways, at least two of which converge on the TGF $\beta$  pathway, leading to enhanced TGF- $\beta$ 1 production, and increased sensitivity of the TGF $\beta$ -induced SMAD signal transduction pathway.

$\beta$ -cryptoxanthin is a provitamin A carotenoid that can be converted to vitamin A (retinol), a factor that was reported to independently inhibit the proliferation of osteoblastic MC3T3-E1 cells (31) by binding to RXR nuclear receptors. While it is possible that some of the observed effects of  $\beta$ -cryptoxanthin in our studies are mediated through its conversion to retinol, this is unlikely as previous studies have demonstrated that the stimulation of Runx2 and  $\alpha$ 1(I) collagen mRNA expression in MC3T3 cells by  $\beta$ -cryptoxanthin is not mimicked, or impeded, by retinol (7).

In conclusion, this study demonstrates that the bone anabolic carotenoid  $\beta$ -cryptoxanthin enhances TGF- $\beta$ 1-induced SMAD activation in MC3T3 preosteoblastic cells.

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