J. Biochem. 2010;148(1):29–34 doi:10.1093/jb/mvq030

JB THE JOURNAL OF BIOCHEMISTRY

# Development of evaluation system for bioactive substances using human artificial chromosome-mediated osteocalcin gene expression

Received February 22, 2010; accepted March 8, 2010; published online March 17, 2010

**Yusuke Takahashi[1,2], Saori Tsuji[3], Yasuhiro Kazuki[3,4], Makoto Noguchi[2], Ichiro Arifuku[2], Yukihiro Umebayashi[2], Tomoko Nakanishi[1], Mitsuo Oshimura[3,4] and Kenzo Sato[1,4,*]**

[1]Department of Molecular Biology, School of Life Sciences, Faculty of Medicine, Tottori University, Nishi-cho, Yonago, 683-8503; [2]Department of Applied Biotechnology, Food Developing Laboratory, Tottori Institute of Industrial Technology, 2023-3 Nakano-cho, Sakaiminato, 684-0041; [3]Department of Molecular Cell Genetics, Human Genome Sciences, and Biomedical Science, Institute of Regenerative Medicine and Biofunction, Graduate School of Medicine, Tottori University, Nishi-cho, Yonago, 683-8503; and [4]Tottori University Chromosome Engineering Research Center, Nishi-cho, Yonago, 683-8503, Japan

*Kenzo Sato, Department of Molecular Biology, School of Life Sciences, Faculty of Medicine, Tottori University, Yonago, 683-8503, Japan, Tel: +81 859 38 6201, Fax: +81 859 38 6200, email: kensato@med.tottori-u.ac.jp

**Bioactive substances in daily food and supplements are expected to prevent various lifestyle-related diseases. Recently, many evaluation systems for bioactive substances were developed with cell lines integrated with green fluorescence protein (GFP) reporter gene. To evaluate osteogensis activity in functional food, we developed a novel cell line that reports *osteocalcin* gene expression using the human artificial chromosome (HAC) vector. HAC vectors are able to avoid various problems in usual plasmid vector such as difficulty in control of transgene copy number. HAC is transmitted to cells as an independent chromosome from host chromosomes, and expresses transgenes depending on host cell circumstances. We established Chinese hamster ovary cell lines that carried GFP gene regulated by *osteocalcin* gene promoter on the HAC. Expression of GFP was responded to vitamin $D_3$ [1α,25(OH)$_2$D$_3$]. Furthermore, we constructed HAC vector bearing tandem repeats of reporter gene unit, to enhance intensity of gene expression. GFP expression in these reporter cells is related to the copy number of reporter gene units. Using the evaluation system for bioactive substances, we could show osteogenic activity in some fish oils.**

*Keywords*: Bioactive substance/GFP gene/human artificial chromosome vector/osteocalcin gene/vitamin D3.

*Abbreviations*: CHO, chinese hamster ovary; CMV, cytomegaro virus; 1α, 25(OH)$_2$D$_3$, 1α,25-dihydroxy vitamin $D_3$; GFP, green fluorescent protein; HAC, human artificial chromosome; OC, osteocalcin; OSE, osteacalcin specific element; VDRE, vitamin D responsive element.

Osteoporosis is now a major threat against public health in countries with population ageing. In fact, the number of osteoporosis and latent patients are estimated dramatically increase in the coming decades. Various functional foods, nutrition or supplements were developed to prevent us from this disease, as well as from lifestyle-related disease. For instance, plant derived phytoestrogens can stimulate osteoblast differentiation (*1*), and positive effect of phytoestrogens on bone were reviewed (*2*). In a field of nutrition study, more bioactive substances should be confirmed physiological effects on human health and non-communicable diseases by various evaluation systems.

We previously reported bioactive compounds from fish can reduce cell proliferation, and induce growth factor production (*3*, *4*). Expression of the reporter gene, such as Green fluorescent protein (GFP) genes is controlled by transcriptional regulatory elements (enhancer and promoter) of the interest gene or tissue and cell specific genes. However, transduction of the reporter genes into host cells is accompanied with various uncertainties. In this study, we established a reporter cell line carried human artificial chromosome (HAC) vector to develop a high throughput evaluating and screening system of food derived natural compounds that can reduce risk of osteoporosis and lifestyle-related diseases.

Artificial chromosome vectors, which were introduced into cells by microcell mediated chromosome transfer (*5*), rely on the mechanisms of governing replication (*6*) and accurate segregation of natural chromosomes, and offers new approach for gene delivery vectors. Since, HAC is maintained as independent chromosomes in host cells, HAC mediated gene delivery has several advantages: (i) Well defined transgene copy number, (ii) Few risk of transgene integration into host genome, (iii) No interference by host genome, (iv) Huge capacity of transgene insertion. Thus, HAC vector is very reliable and useful tool for gene delivery (*7*). In a previous study, an EGFP gene driven by osteoblast specific osteopontin gene promoter was expressed in mesenchymal stem cell that differentiated into osteocytes (*8*).

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 27, 2012

© The Authors 2010. Published by Oxford University Press on behalf of the Japanese Biochemical Society. All rights reserved

Osteocalcin (OC), which is a non-collagenous protein found in bone matrix involving in mineralization and calcium ion homeostasis, is one of the major bone morphogenetic marker and used in evaluation of osteoblast maturation and osteogenic activation (*9–11*). *OC* gene is regulated by various growth factors, hormones, cytokines and vitamins. Basic fibroblast growth factor (bFGF) (*12*), bone morphogenetic proteins 2 and 4 (BMP2/4) (*13*, *14*) and parathyroid hormone (PTH) (*15*) are major positive regulatory factors of *OC* gene expression, as well as vitamin D. Therefore, we developed evaluation system for osteogenetic activity of bioactive substances by monitoring GFP fluorescence regulated by OC gene enhancer/promoter in HAC vector.

## Materials and Methods

### Cell culture

21ΔpqHAC vector was constructed by telomere-directed chromosome breakage, and contains single loxP sequence for site specific insertion of circular DNA by Cre/loxP system (*6*). CHO-21Δpq HAC cell, which is CHO cell line bearing a single copy of 21ΔpqHAC (*6*), was maintained Ham's F12 medium (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum and 10 mg/ml of blasticidin S hydrochloride. Recombinant CHO-HACxOG cell lines that contain *OC* promoter-driven GFP expression unit(s) were maintained with Ham's F12 medium containing 750 μg/ml G418 (Invitrogen, Carlsbad, CA, USA).

### Plasmid

OC gene expression reporter unit (OG unit) was constructed by insertion of the human *OC* gene promoter region (−550 bp/+10 bp from transcription initiation site) into a promoterless GFP reporter plasmid (Fig. 1A) (Clontech Laboratories, Inc. Mountain View, CA, USA). The human *OC* promoter region was amplified from human hepatoma cell line HepG2 by PCR using specific primers (5′-atgctgcagggcagggctgaccgt-3′, 5′-agcctgcagactgtttataccct-3′). HAC recombination plasmids, pHAC-OGs, which were intermediate plasmids for transferring OG units to HAC vector (21ΔpqHAC) (Fig. 1B), was constructed with tandem repeated OG units (one copy of OG, ×1OG; two copies; ×2OG, three copies, ×3OG) and three-repeat of chicken β-globin insulator sequence (*6*) in both end of OG units (Fig. 1B). pHAC-OGs also contains loxP sequence for recombination to 21ΔpqHAC loxP site, and human cytomegalovirus (CMV) promoter-driving 5′ fragment of neomycine resistance gene (5′ Neo) for recombinants screening.

### Transfection and isolation of recombinant HAC cells

CHO–HAC cells, which retain 21ΔpqHAC empty vector, were cultured at $5 \times 10^4$ in 60 mm dish, and were co-transfected with 5 μg of pHAC-OG plasmids and 1 μg of Cre recombinase expressing plasmid, using 12 μl of FuGene6 transfection reagent (Roche Diagnosyics GmbH, Mannheim, Germany). After 18 h of culture in Ham's F12 medium, cells were trypsinized and cultured in 100-mm culture dish in the presence of G418 (750 μg/ml). G418 resistant colonies, which were proceeded Cre recombination at loxP sites of 21ΔpqHAC and pHAC-OGs, were isolated and expanded for further analysis (Fig. 1B).

### Genomic DNA preparation and PCR analysis

Genomic DNA was prepared from G418 resistant CHO HAC OG colonies using QIAmp DNA Mini Kit (QIAGEN Inc., Valencia, CA, USA). Cre recombination in CHO HAC clones were determined by genomic PCR generated 300-bp fragment from 3′-Neo to CMV promoter (primers: 5′-cgtaacaactccgccccatt-3′ and 5′-gcagccgattgtctgttgtg-3′), and 650-bp fragment from OC gene to GFP (primers: 5′-atgctgcagggcagggctgaccgt-3′ 5′-gtccagctcgaccaggat-3′) (schemed in Fig.1B). PCR products were separated by polyacrylamide gel electrophoresis.

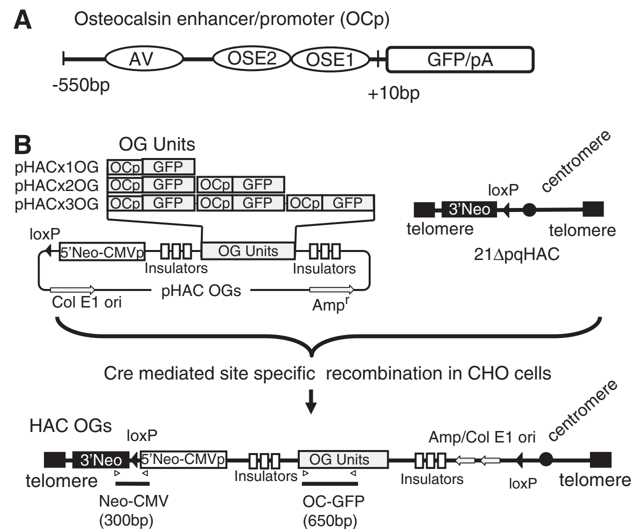


**Fig. 1 Construction of HAC vectors.** (A) Structure of osteocalsin promoter (OCp)-driven GFP expression unit (OG unit). −550 to +10-bp region of *OC* promoter (OCp) amplified by PCR contains two osteoblast specific element (OSE1, 2) and AP-1/VDRE element (AV). GFP gene with polyA signal (pA) was flanked on downstream of *OC* promoter fragment. (B) Construction of pHAC recombination plasmids and predicted structure of HAC OGs. OCp-GFP reporter unit (OG unit) tandem repeated was flanked on both sides with three copies of insulators, CMV promoter-linked 5′ fragment of Neo gene and loxP sequence. 21ΔpqHAC (empty HAC vector) contains centromere and both end telomeres from human chromosome 21 and 3′ fragment of Neo gene interrupted by loxP sequence. Co-transfection of CHO hybrids harboring 21ΔpqHAC empty vector with the pHAC OGs plasmid and Cre recombinase expression vector yielded predicted recombinant HAC OGs shown in the bottom of figure. Arrows indicate specific PCR primers for detecting Neo-CMV (300 bp) junction and OG unit (650 bp).

### Determination of intensity of fluorescence from CHO HAC OGs cells

CHO HAC OGs cells were cultured in Ham's F12 medium with 10% fetal calf serum, and treated with active form of vitamin D {1α,25-Dihydroxyvitamin $D_3$ [1α,25$(OH)_2D_3$]}(Merck KGaA, Darmstadt, Germany), or bioactive substances in serum-free medium. Intensities of fluorescence from the treated cells were determined by three types of analysis as follows:

(i) Flow cytometric analysis: CHO HAC OGs cells were harvested by trypsin-treatment and fixed by 4% paraformaldehyde. Intensities of fluorescence were determined by flow cytometer.
(ii) Plate reader analysis: CHO HAC OGs cells cultured in 96-well black plate and treated with bioactive substances were directly measured by Infinite F200 multiplate reader (TECAN, Männedorf, Switzerland).
(iii) Imaging analysis: CHO HAC OGs cells cultured in six-well plate and treated with bioactive substances were tripsinized and fixed with 4% paraformaldehyde. Fluorescence photograph image was obtained using microscopic imaging system, and the intensities of fluorescence were determined by Image pro analyzer (Media Cybernetics Inc. Bethesda, MD, USA).

### RNA extraction and real-time reverse-transcription–PCR

Total RNA was extracted from CHO cells using the Total RNA Mini kit (Viogene, Sunnyval, CA, USA). Following reverse transcription (RT), aliquots of cDNA were subjected to quantitative real-time PCR using a 7500 real-time PCR system (Applied Biosystems, Foster City, Ca, USA). The primer sequences used are TGAGAGCCCTCACACTCCTC and ACCTTTGCTGGACTCT GCAC designed by human OC mRNA. The mRNA levels were

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 27, 2012

normalized relative to the mRNA level of the β-actin gene (GGCCA GGTCATCACCATT and GGATGCCACAGGACTCCAT).

### *Extraction of fish oil and determination of vitamin D3*

Fish oils were extracted from chopped fishes (Spanish mackerel, Horse mackerel, Sandfish and mild-dried young sardines) by 10 vol. of chloroform:methanol (2 : 1), and the chloroform:methanol was evaporated from the extract. Fish oils dissolved in 100% ethanol were applied to CHO HAC×3OG cells culture. Determination of vitamin D3 was performed by 25-OH Vitamin D EIA Kit (Immundiagnostic AG, Bensheim) as described in the manufacturer's manual.

## Results and Discussion

### *Construction of HAC vector carrying OC promoter GFP expression unit*

OC is one of the major bone marker used in evaluation of osteoblast maturation and osteogenic activation *in vitro* (*9*, *10*). To establish a novel system based on HAC mediated *OC* gene expression for evaluation of bioactive substances, at first, we constructed GFP reporter gene driven by *OC* promoter −550 to +10 bp (OG unit) (Fig. 1A). This promoter region contain some transcriptional regulatory element, such as AP-1/VDRE (AV) element that is composed of vitamin D responsive element (VDRE), retinoic acid responsive element (RE) and Jun-Fos responsive AP-1 (*16*), and osteoblast specific factor binding elements (OSE1 and OSE2) (*17*). Especially, −513 to −493-bp region of OC promoter is essential for positive regulation of transcription by vitamin D (*18*, *19*).

Furthermore, to enhance the responsiveness to bioactive substances, we also constructed expression vector (HAC recombination plasmid) carrying two or three times tandem repeat of the OG unit (pHAC × 1OG, pHAC × 2OG, pHAC × 3OG) (Fig. 1B). To make these expression units independent of surrounding transcriptional regulatory elements, we put three times tandem repeats of insulator sequences in the both end of expression units (OG units) in these HAC recombination plasmids. HAC recombination plasmids are,

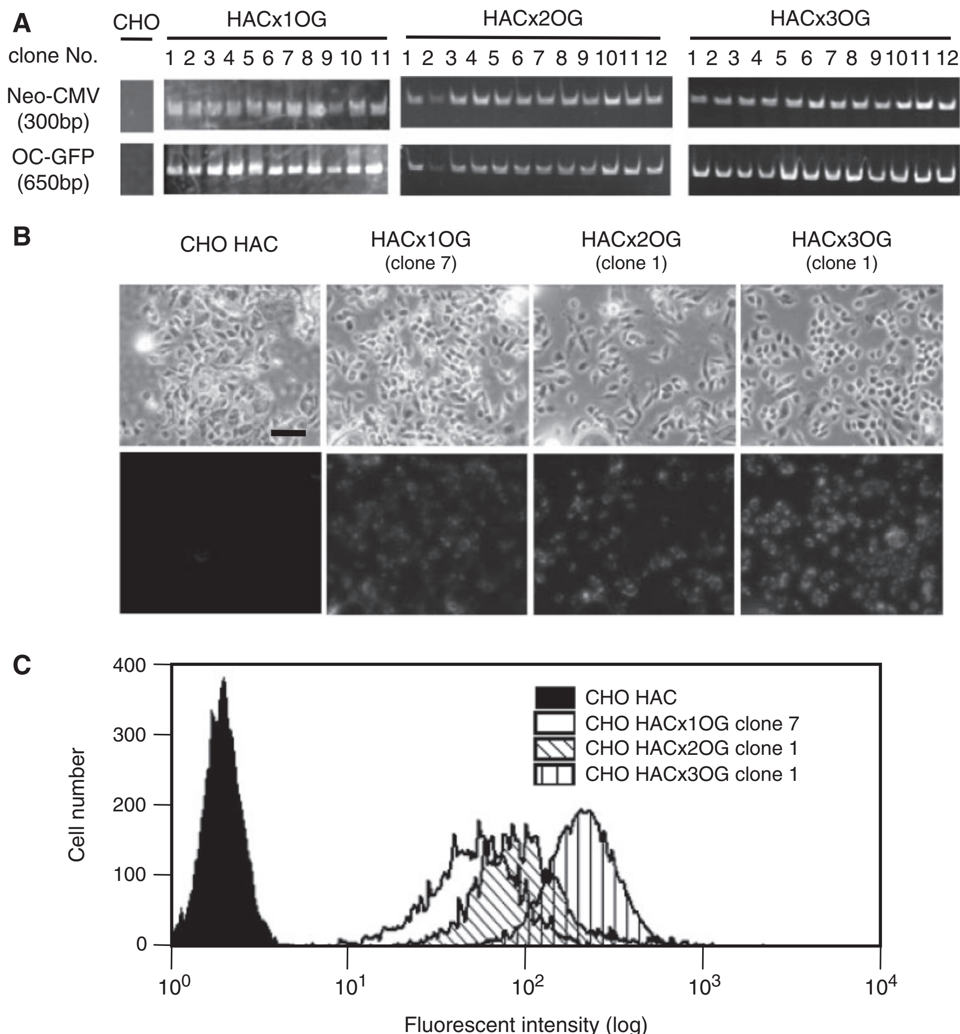


**Fig. 2 Isolation of CHO HAC OGs clones.** (A) Co-transfection with pHAC OGs and Cre-expression plasmid into CHO HAC empty vector cells generated Neo (G418)-resistant clone. A total of 11–12 clones were isolated from each transfection (CHO HAC×1OG, HAC×2OG and HAC×3OG) and determined the correct recombination by genomic PCR (Neo-CMV, 300 bp and OC-GFP, 650 bp) with probes shown in Fig 1B. DNA from CHO cells were used as a negative control. (B) GFP expression of representative clones and CHO–HAC empty vector cell. Upper panels show phase contrast microscopic view. Lower panels show fluorescence images of identical field as depicted upper panel. Scale bar shows 0.1 mm (C) Flowcytometric analysis of representative clones.

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 27, 2012

furthermore, carrying loxP sequence for Cre recombination with loxP site of 21ΔpqHAC and CMV-promoter driven 5′ fragment of Neo gene for screening correct recombinants that were completed connecting with 3′ fragment of Neo gene in 21ΔpqHAC in CHO HAC cell by Cre recombination.

### *Isolation of CHO HAC-OG clones by Cre recombination*

By co-transfection of HAC recombination plasmid (pHAC-OGs) and Cre expression plasmid in CHO HAC cells that maintain a single copy of 21ΔpqHAC empty vector (*6*), we generated recombinant CHO cell lines bearing HAC OGs expression units, and we obtained each 12 clones from the co-transfection (CHO HAC×1OG clones 1–11, CHO HAC×2OG clones 1–12, CHO HAC×3OG clone 1–12), that were resistant to G418 (neomycin) mediated by Cre recombination (Fig. 2A).

In these clones, integration of transgene was confirmed by genomic PCR with primers sets (Noe-CMV, 300 bp and OC-GFP, 650 bp) shown in Fig. 1B. Cre mediated site specific gene recombination produces connection of 5′Neo and 3′Neo, resulting in amplification of Neo-CMV 300 bp fragment across loxP sequence. As shown in Fig. 2A, Neo-CMV PCR product was amplified in all G418 resistant clones. Presence of expression unit (OG unit) in the HAC vector was determined by amplification of OC-GFP 650 bp, suggesting that all G418-resistant clones carried OG units resulted from successful integration into 21ΔpqHAC by Cre recombination. Moreover, microscopic observation showed GFP reporter gene expression in representative clones (HAC×1OG clone 7, HAC×2OG clone 1, and HAC×3OG clone 1) (Fig. 2B). Fluorescence intensities from these clones were shown to depend on the copy number of tandem repeat of OG units by flow cytometry analysis (Fig. 2C), suggesting that each OG unit repeated in HAC vector were independently regulated gene expression.

### *Enhancement of GFP expression in OG unit by vitamin $D_3$ [1α,25(OH)$_2$D$_3$]*

To assess GFP reporter gene expression in these CHO HAC OGs cell lines, these cells were treated with vitamin $D_3$ [1α,25(OH)$_2$D$_3$]. CHO cell was reported to express vitamin D receptor and respond to vitamin D (Fig. 3A) (*20*). Because the *OC* promoter contains VDRE (*16–19*), these cells should be enhanced expression of GFP depending on vitamin $D_3$ treatment. As shown in Fig. 3B, vitamin $D_3$-treated cells, especially CHO HAC×3OG clone 1, significantly showed GFP fluorescence expression in dose dependent manner by microplate reader analysis. However, CHO HAC×2OG clones slightly showed enhancement of GFP expression by vitamin D, and CHO HAC×1OG clones exhibited very little effect by vitamin $D_3$ in high dose. Furthermore, these GFP expressions in CHO HAC OGs every clone were continuously enhanced by vitamin $D_3$ up to 48 h (Fig. 3C).

Using microplate fluorescence reader, we achieved high throughput measurement of fluorescent intensity from these CHO HAC OGs cells, which maintained precise copy number of expression units (OG unit), such as single copy, two copies and three copies of OG unit. These observations showed these cell lines are a reliable evaluation system for bioactive substances.

### *Evaluation of bioactivity of fish oil*

In Japan, boiled and dried baby sardine is generally eaten and believed to be valid to bone

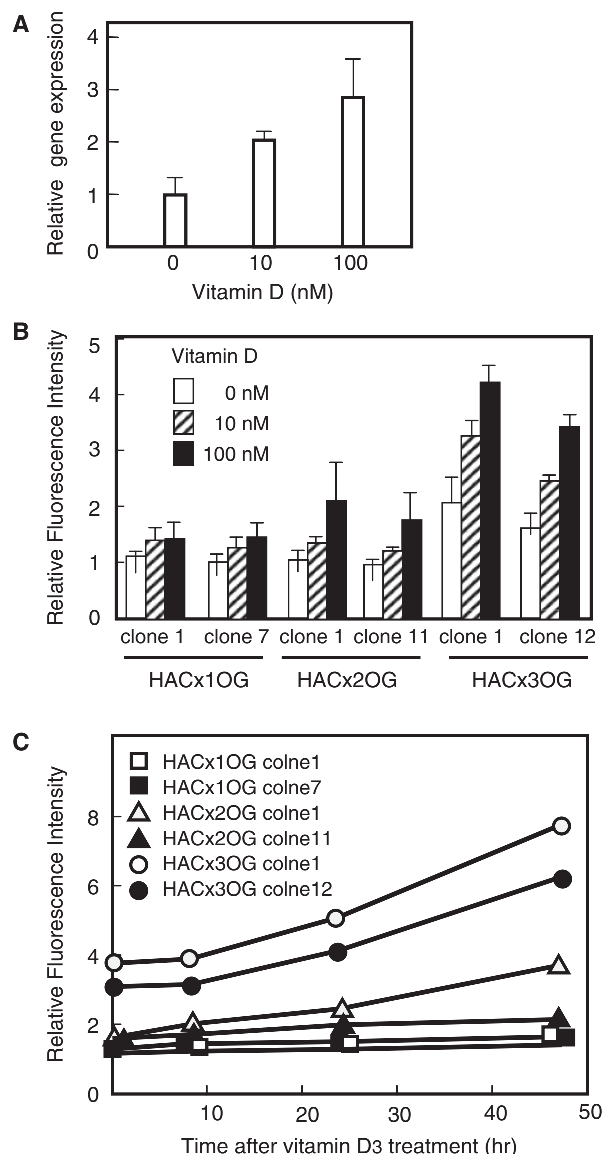


**Fig. 3 Enhancement of GFP expression of CHO HAC OGs clones by active form of vitamin D [1α,25(OH)$_2$D$_3$].** (A) Endogenous osteocalcin gene expression were determined by real-time RT–PCR. (B) Dose dependent induction of fluorescent intensity of representative HAC OGs clones. Cells in 96 well plate were treated 0, 10, 100 nM vitamin $D_3$ for 24 h, and was measured fluorescent intensity by microplate reader. Fluorescent intensity was shown by relative fluorescent intensity as against fluorescence from the non-treated HAC×1OG clone 1. Bars indicating mean ± SD. ($n = 4$) (C) Time course of GFP expression in the representative HAC OG clones by vitamin $D_3$-treatment (100 nM). Fluorescent intensity was measured by microplate reader.

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 27, 2012

development, because of its large content of vitamin $D_3$ (Noguchi, M., Watanabe, S., Japan patent P2005-253323A). Hence, evaluation of bioactivity in oil extracts from some fishes (Spanish mackerel, horse mackerel, sandfish and mild-dried young sardine) was performed with CHO HAC×3OG clone 1. As shown in Fig. 4A, the reporter cells treated with fish oil from Spanish mackerel, sandfish and young sardine were enhanced GFP expression, particularly the oil extracted from young sardine showed strong increasing effect on GFP expression. While oil from horse mackerel hardly increased in GFP fluorescence in the reporter cells. Interestingly, contents of active form of vitamin D3 in these oils were not consistent with the intensities of GFP fluorescence from the cells treated with these oils (Fig. 4B). Oil from horse mackerel contains low level of vitamin D3 and fluorescence, while, oil from Spanish mackerel shows high level of vitamin D3 and increased fluorescence. However, although oils from sandfish and Mild-dried young sardine contained low level of vitamin D3, these oils showed high activities in induction of GFP fluorescence in the treated cells, suggesting the presence of unknown bioactive factor(s) in these oils. These results suggested that evaluation system using CHO HAC OGs cell is available for screening of bioactive substances in functional foods as well as active form of vitamin D3.

## Conclusion

Human bone density is increased until around 20-years old, thereafter gradually decreased and rapidly declined after climacteric stage in females. Decrease in bone density is closely related to osteoporosis. Therefore, fracture-healing slows with age; osteoporosis is now a major threat against public health in countries with population ageing. In this study, we established an evaluation system for osteogenesis by monitoring OC gene expression mediated HAC vector that is maintained as single copy independent of host chromosomes. We have achieved enhancement of sensitivity to bioactive substances by tandem repeat

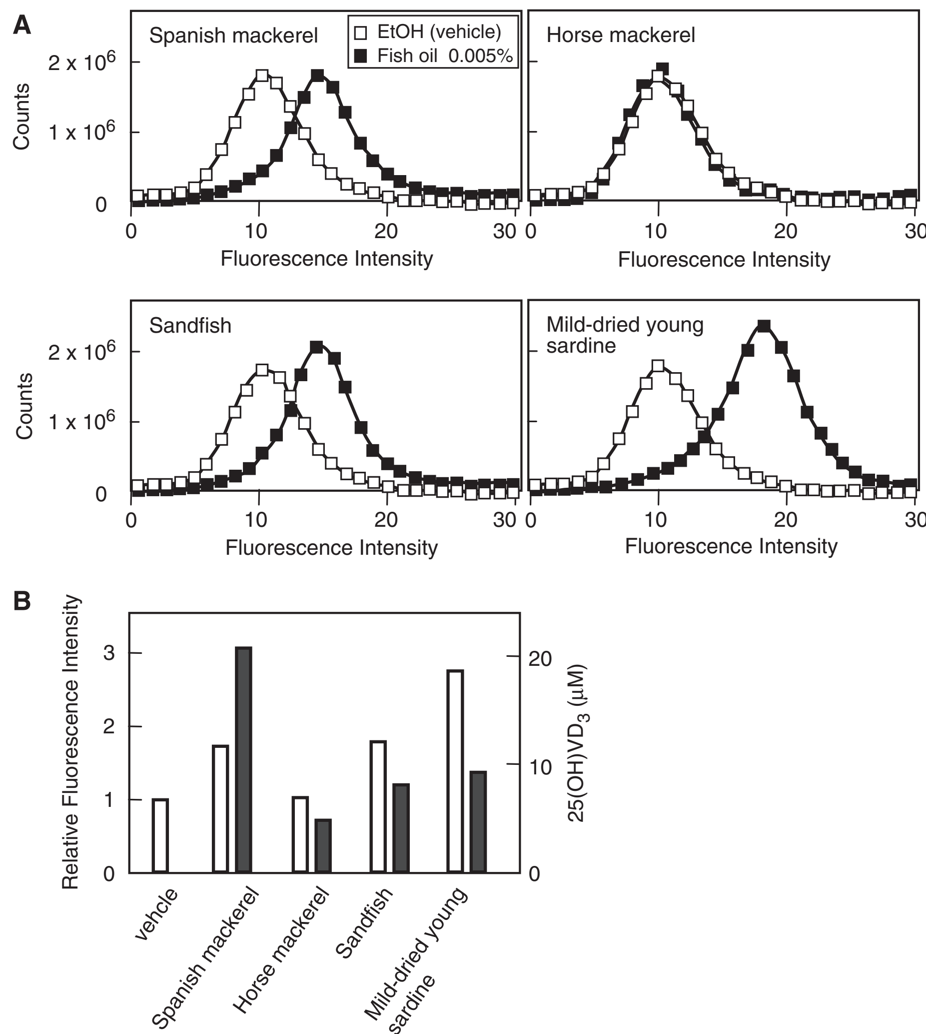


**Fig. 4 Evaluation of bioactivities of fish oil extracts.** (A) CHO HACx3OG clone 1 cells in 24-well plate were treated with 0.005% fish oils (closed box) or vehicle ethanol (open box) suspended in F12 medium for 24 h. The fish oil-treated cells were fixed by 4% paraformaldehyde, and intensities of fluorescence in photographs were analysed by imaging software. (B) Comparison of fluorescence intensity and content of vitamin D3 in various oils. GFP fluorescence by CHO HAC×3OG cells treated with fish oils were quantified from (A) and show by open columns (by left axis). Concentrations of vitamin D3 contained in the oils were showed by gray columns (by right axis).



Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 27, 2012

of reporter gene units (OG units). This evaluation system is feasible to detect osteogenic activity in development of functional foods and medicines.

## Acknowledgements

We thank Dr Naohiro Hori for their helpful discussion and Mr Koichi Adachi for his excellent technical assistance.

### Funding
This research was supported by a City Area Program (Basic Stage) from Ministry of Education, Culture, Sports, Science and Technology of Japan.

### Conflict of interest
None declared.

## References

1. Morris, C., Thorpe, J., Ambrosio, L., and Santin, M. (2006) The Soybean Isoflabone and Genistein induces differentiation of MG63 human osteosarcoma osteoblasts. *J. Nutr*. **136**, 1166–1170
2. Setchell, K.DR. and Lydeking-Orsen, E. (2003) Dietary phytoestrogens and their effect on bone: evidence from in vitro and in vivo, human observational and dietary intervention studies. *Am. J. Clin. Nutr*. **78**, 593S–609S
3. Noguchi, M., Arifuku, I., Yamashita, A., and Sato, K. (2003) Induction of hepatocyte growth factor and inhibition of proliferation by extracellular matrix component derived from fish skin of *Rreinharardtius hippoglossoides*. *Fish. Sci*. **69**, 401–407
4. Noguchi, M., Takahashi, Y., Taniguchi, M., Isa, S., Arifuku, I., Yamashita, A., and Sato, K. (2003) Induction of hepatocyte nuclear factor 3$\gamma$ gene expression by extracellular matrix component from fish skin. *Yonago Acta medica*. **46**, 103–108
5. Kugoh, H., Mitsuya, K., Meguro, M., Shigenami, K., Schulz, T.C., and Oshimura, M. (1999) Mouse A9 cells containing single human chromosomes for analysis of genomic imprinting. *DNA Res*. **6**, 165–172
6. Katoh, M., Ayabe, F., Norikane, S., Okada, T., Masumoto, H., Horike, S., Shirayoshi, Y., and Oshimura, M. (2004) Construction of a novel human artificial chromosome vector for gene delivery. *Biochem. Biophys. Res. Commun*. **321**, 280–290
7. Kakeda1, M., Hiratsuka, M., Nagata1, K., Kuroiwa1, Y., Kakitani1, M., Katoh, M., Oshimura, M., and Tomizuka, K. (2005) Human artificial chromosome (HAC) vector provides long-term transgene expression in normal human primary fibroblasts. *Gene Ther*. **12**, 852–856
8. Ren, X.R., Katoh, M., Hoshiya, H., Kurimasa, A., Inoue, T., Ayabe, F., Shibata, K., Toguchida, J., and Oshimura, M. (2005) A novel human artificial chromosome vector provides effective cell lineage-specific transgene expression in human mesenchymal stem cells. *Stem Cells* **23**, 1608–1616
9. Locklin, R.M., Oreffo, R.C.O., and Triffitt, J.T. (1998) Modulation of osteogenic differentiation in human skeletal cells *in vitro* by 5-azacytidine. *Cell Biol. Int*. **22**, 207–215
10. Lemonnier, J., Ghayor, C., Guicheux, J., and Caverzasio, J. (2004) Protein kinase C-independent activation of protein kinase D is involved in BMP-2-induced activation of stress mitogen-activated protein kinases JNK and p38 and osteoblastic cell differentiation. *J. Biol. Chem*. **279**, 259–264
11. Ferrari, S.L., Ahn-Luong, L., Garnero, P., Humphries, S.E., and Greenspan, S.L. (2003) Two promoter polymorphisms regulating interleukin-6 gene expression are associated with circulating levels of C-reactive protein and markers of bone resorption in postmenopausal women. *J. Clin. Endocrinol. Metab*. **88**, 255–269
12. Bodo, M., Lilli, C., Bellucci, C., Carinci, P., Calvitti, M., Pezzetti, F., Stabellini, G., Bellocchio, S., Balducci, C., Carinci, F., and Baroni, T. (2002) Basic fibroblast growth factor autocrine loop controls human osteosarcoma phenotyping and differentiation. *Mol. Med*. **8**, 393–404
13. Yamaguchi, A., Ishizuya, T., Kintou, N., Wada, Y., Katagiri, T., Wozney, J.M., Rosen, V., and Yoshiki, S. (1996) Effects of BMP-2, BMP-4, and BMP-6 on osteoblastic differentiation of bone marrow-derived stromal cell lines. *Biochem. Biophys. Res. Commun*. **220**, 366–371
14. Yamaguchi, A., Komori, T., and Suda, T. (2000) Regulation of osteoblast differentiation mediated by bone morphogenetic proteins, hedgehogs, and Cbfa1. *Endcr. Rev*. **21**, 393–411
15. Yu, X.P. and Chandrasekhar, S. (1997) Parathyroid hormone (PTH 1-34) regulation of rat osteocalcin gene transcription. *Endocrinology* **138**, 3085–3092
16. Schüle, R., Umesono, K., Mangelsdorf, D.J., Bolado, J., Wesley Pike, J., and Evans, R.M. (1990) Jun-Fos and receptors for vitamins A and D recognize a common response element in the human osteocalcin gene. *Cell* **61**, 497–504
17. Yeung, F., Law, W.K., Yeh, C-H., Westendorf, J.J., Zhang, Y., Wang, R., Kao, C., and Chung, L.W.K. (2002) Regulation of human osteocalcin promoter in hormone-independent human prostate cancer cells. *J. Biol. Chem*. **277**, 2468–2476
18. Kerner, S.A., Scott, R.A., and Wesley Pike, J. (1989) Sequence elements in the human osteocalcin gene confer basal activation and inducible response to hormonal vitamin$D_3$. *Proc. Natl Acad. Sci. USA* **86**, 4455–4459
19. Morisson, N.A., Shine, J., Fragonas, J-C., Verkest, V., Mcmenemy, M.L., and Eisman, J.A. (1989) 1,25--dihydroxyvitamin D-responsive element and glucocorticoid repression in the osteocalcin gene. *Science* **246**, 1158–1161
20. Dokoh, S., Donaldson, C.A., Marion, A.L., Pike, J.W., and Haussler, M.R. (1983) The ovary: a target organ for 1,25-dihydroxyvitamin $D_3$. *Endocrinology* **112**, 200–206

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on September 27, 2012