

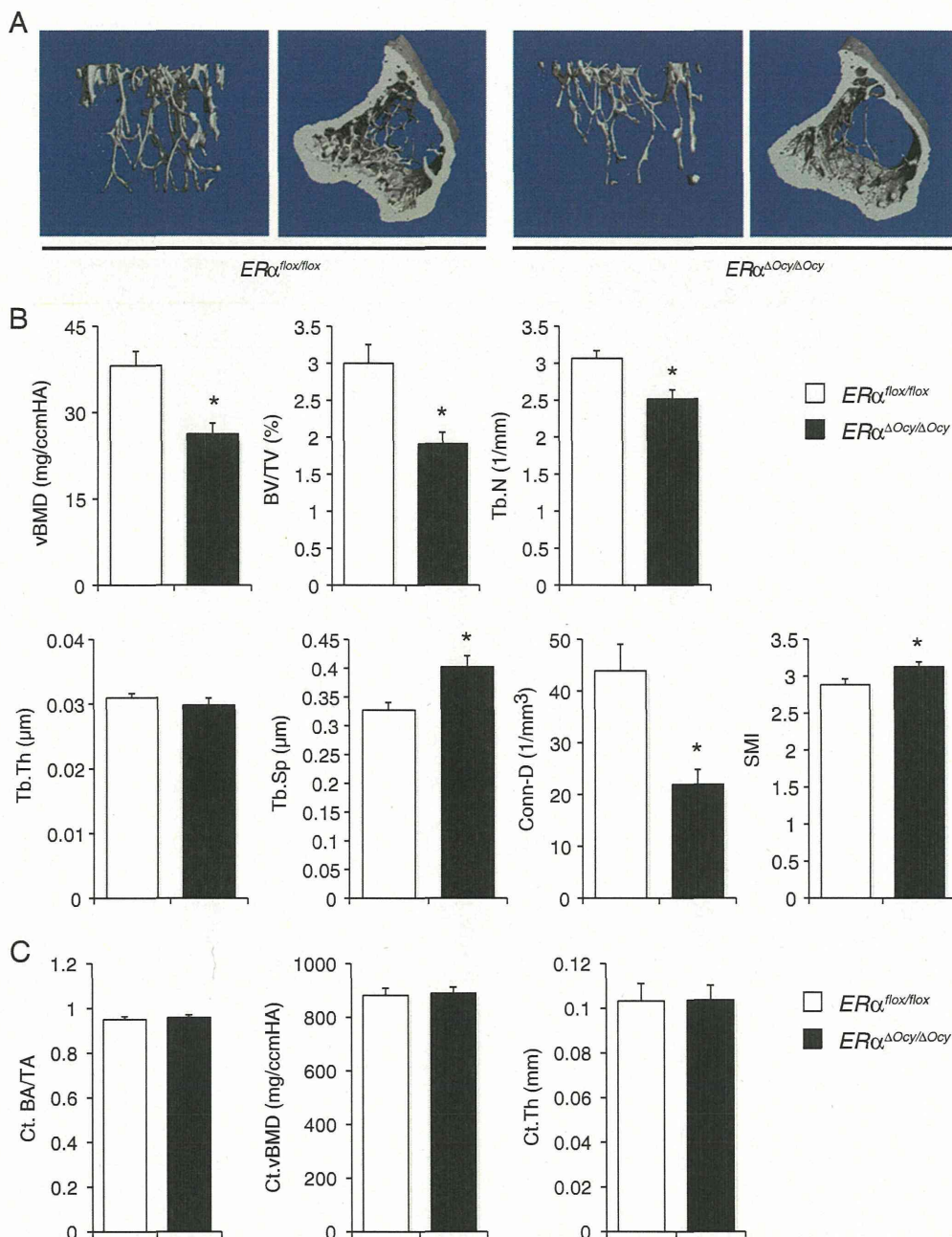


Fig. 2. μ CT analyses of the mice lacking $ER\alpha$ in osteocytes. (A) Representative μ CT views. (B) 3D measurements of proximal tibiae from $ER\alpha^{flx/flx}$ and $ER\alpha^{\Delta Ocy/\Delta Ocy}$ mice. Data are represented as mean $\pm$ SEM ($n = 10$). * indicates $p < 0.05$.

gene) in GFP+ cells was about 25 times higher than in GFP− cells, while the expression of keratocan, Kera, (osteoblast marker gene) in GFP− cells was about 25 times higher than in GFP+ cells (Fig. 4B). Extracted total RNA from $Dmp1$ -GFP+; $ER\alpha^{\Delta Ocy/\Delta Ocy}$ ($n = 3$) and $Dmp1$ -GFP+; $ER\alpha^{flx/flx}$ ($n = 3$) was subjected to a gene expression microarray analysis with GeneChip Mouse Genome 430 2.0 (Affymetrix). There were 276 genes found to be significantly differentially expressed between $ER\alpha^{\Delta Ocy/\Delta Ocy}$ and $ER\alpha^{flx/flx}$ ($p < 0.01$). Among them, 76 genes were significantly down-regulated and 200 genes were up-regulated (Fig. 4C). Gene ontology analyses revealed that 'secreted' was listed top in the Keyword analysis when sorted by p -value (Fig. 4D). Among

these genes, *Mdk* (Midkine) and *Sostdc1* (Sclerostin domain containing 1) were significantly up-regulated in $ER\alpha^{\Delta Ocy/\Delta Ocy}$ although there were no significant differences in *Sost* or β -catenin (*Ctnnb1*) gene expression (Fig. 4E). Up-regulation of mRNA of *Mdk* and *Sostdc1* in $ER\alpha^{\Delta Ocy/\Delta Ocy}$ was also validated when determined by RT-qPCR (Fig. 4F). From the results of functional annotation in differentially expressed genes between $ER\alpha^{\Delta Ocy/\Delta Ocy}$ and $ER\alpha^{flx/flx}$, osteocytic $ER\alpha$ could regulate the expression of secretory protein genes such as *Mdk* and *Sostdc1*, which have been shown to be inhibitors of Wnt signaling-related bone formation [44–46]. However, the expression levels of *Mdk* and *Sostdc1* were not significantly altered when late-stage primary cultured osteoblasts

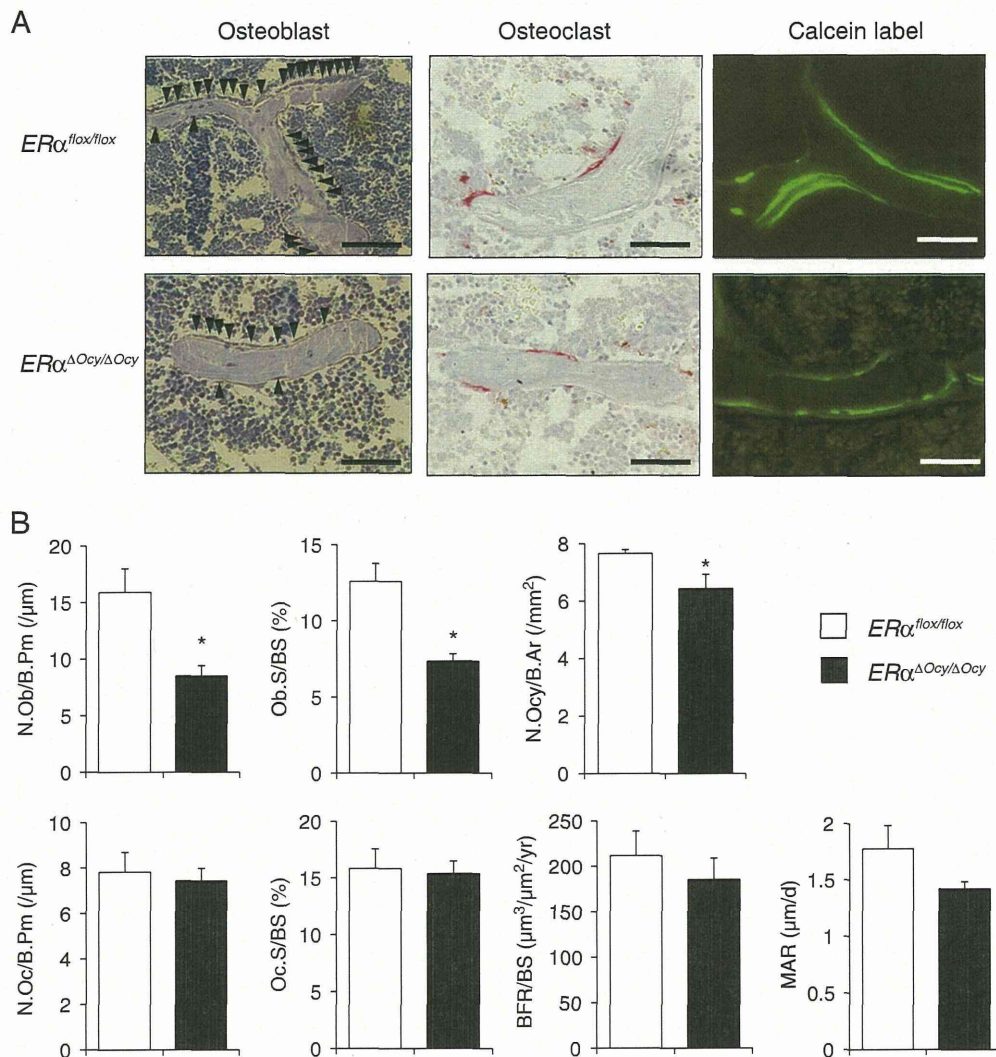


Fig. 3. $ER\alpha^{\Delta Ocy/\Delta Ocy}$ mice exhibit decreased bone formation. (A) Representative views of Toluidine blue staining for mononuclear cuboidal osteoblasts (arrowhead), TRAP staining for multinuclear TRAP-positive osteoclasts and calcein labeling for dynamic parameters are shown. Bars indicate 50 μm . (B) Data are represented as mean $\pm$ SEM (n = 6). * indicates $p < 0.05$.

were treated with 17 β -estradiol for 2 or 6 h (Supplemental Fig. S1), indicating that *Mdk* and *Sostdc1* might not be early responsive genes, but be indirect target genes.

Trabecular bone loss is exacerbated in $ER\alpha^{\Delta Ocy/\Delta Ocy}$ in response to unloading while cortical bone is resistant to unloading-induced bone loss

$ER\alpha$ has been reported to be involved in mechanosensing and increasing cortical bone formation under overloading conditions [11]. The hindlimb tail suspension model is a well-known model for unloading (or immobilization) and it is also reported that tail suspension-induced bone loss is significantly enhanced by ovariectomy [47]. To determine whether osteocytic $ER\alpha$ plays any roles in unloading-induced bone loss, a hindlimb suspension experiment was performed for female $ER\alpha^{\Delta Ocy/\Delta Ocy}$ and $ER\alpha^{flox/flox}$ for 4 weeks starting at 8 weeks of age. Control mice were chained to the cage top during the same period but allowed to load their hindlimbs to control for stress related effects.

During the 4-week experimental period, the average body weight of the experimental group increased 1 g, whereas the control group increased 2 g (Supplemental Fig. S2). Although there was a significant difference in body weight increase over the four weeks between the experimental and control groups, there was no significant difference

in body weight between $ER\alpha^{\Delta Ocy/\Delta Ocy}$ and $ER\alpha^{flox/flox}$ within each group at the end of the experiment (Supplemental Fig. S2). Femoral diaphysis and distal metaphysis of the unloaded and loaded groups of both genotypes ($ER\alpha^{\Delta Ocy/\Delta Ocy}$ and $ER\alpha^{flox/flox}$) were measured using μCT . vBMD in the femoral diaphysis of tail suspended female $ER\alpha^{\Delta Ocy/\Delta Ocy}$ was significantly higher than that of $ER\alpha^{flox/flox}$ (Figs. 5A and B), although there were no significant differences in bone area or cortical thickness between genotypes. Upon further analysis, it was found that the trabecular bone mass was decreased in unloaded mice regardless of genotypes, and tail suspension induced trabecular bone loss in $ER\alpha^{\Delta Ocy/\Delta Ocy}$ was greater than that in $ER\alpha^{flox/flox}$ (Figs. 5C and D). These data indicate that osteocytic $ER\alpha$ is protective against trabecular bone loss due to unloading.

Discussion

Based on reports on the functions of $ER\alpha$ in bone, estrogens are osteoprotective by regulating the life span of osteoclasts through osteoclastic and osteoblastic $ER\alpha$ and also by inhibiting apoptosis of osteoblasts and osteocytes [8,9,48–50]. Recently, it was reported that osteoblastic $ER\alpha$ has an osteoprotective function [12,14,15], however, little is known about the role of osteocytes in the osteoprotective actions

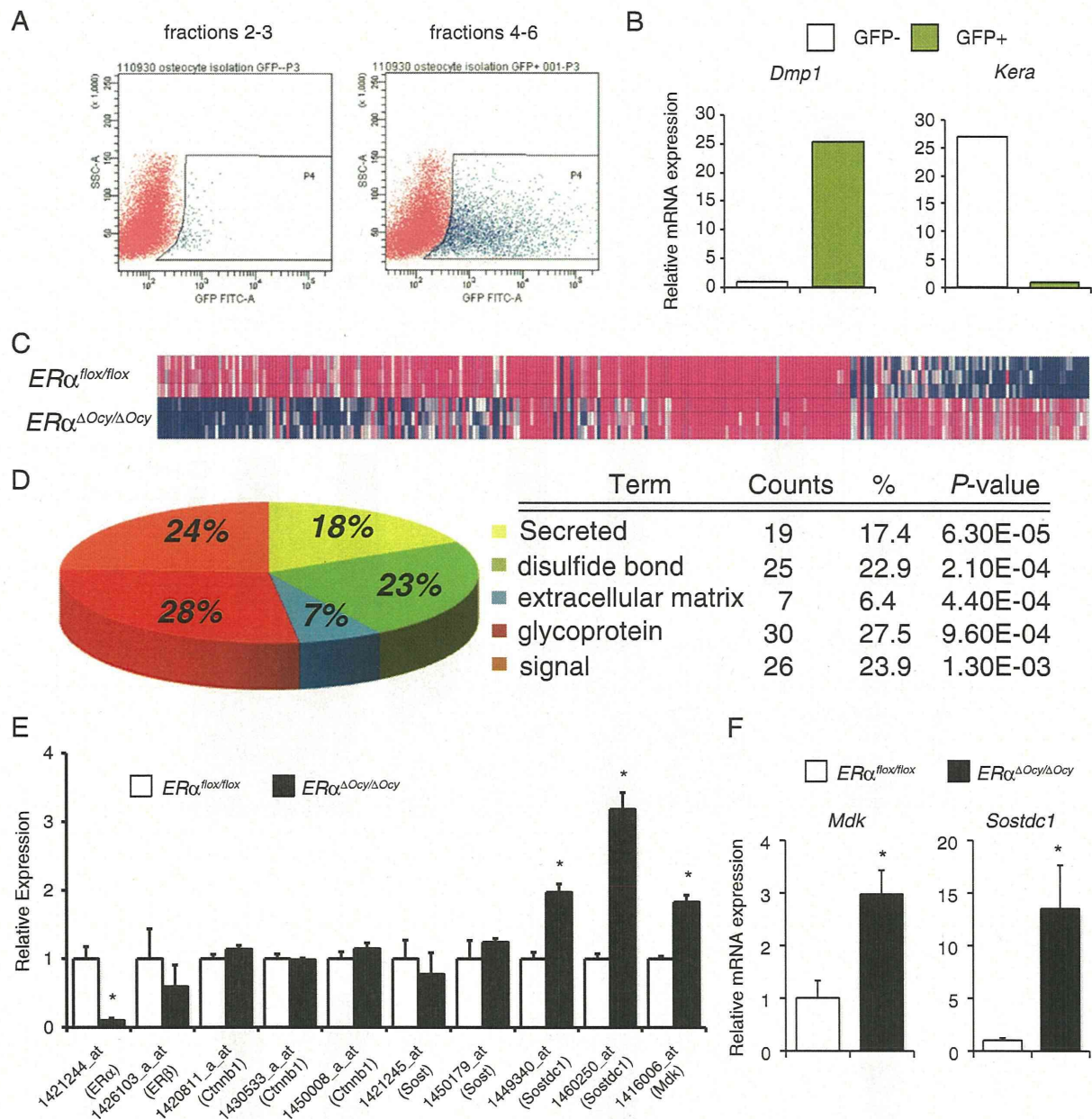


Fig. 4. Osteocytes lacking ERα show increased Mdk and Sostdc1 expression. (A) Two-dimensional dot plot of cells obtained from sequential enzymatic digestion of calvariae of mice expressing Dmp1-GFP. Left: fractions 2–3, right: fractions 4–6. (B) Expression of osteocyte (Dmp1) and osteoblast (Kera) marker genes in the GFP– and GFP+ population of isolated cells. (C) Heat map of significantly regulated genes in the gene expression microarray using total RNA from isolated GFP+ cells of *ERα^{flx/flx}* and *ERα^{ΔOcy/ΔOcy}* mice harboring Dmp1-GFP (*n* = 3). Red: high expression. Blue: low expression. (D) Functional annotation clustering of Keywords by DAVID Bioinformatic Resources. (E) Relative microarray intensity of each probe for ERα, ERβ, Ctnnb1 (β-catenin), Sost, Sostdc1 and Mdk. Data are represented as mean ± SEM (*n* = 3). (F) RT-qPCR for Mdk and Sostdc1 as same as panel E. * indicates *p* < 0.05.

of estrogens in skeletal homeostasis. To decipher the direct functions of ERα in osteocytes, the most abundant bone cell type in the adult skeleton, mice lacking ERα in osteocytes were genetically generated and their bone phenotype were analyzed in this study. ERα in osteocytes was found to play a significant role in maintaining bone mass by regulating osteoblastic bone formation only in females. It was further revealed that ERα in osteocytes is supportive for maintaining trabecular bone mass not only under normal loading conditions but also under tail suspension-induced unloading, which can be considered as experimental recapitulation of immobilization or space flight. However, the absence of this receptor protected against cortical bone loss. These results are consistent with a previous report in which bone mass adaptation induced by

mechanical loading was impaired in ERα null mice [11]. Together, these results indicate that osteocyte mechanosensations at least in part via osteocytic ERα.

Maatta et al. and Melville et al. suggested that ERα in mature osteoblasts plays a role in maintaining trabecular bone mass in females based on analyses of mice lacking ERα in mature osteoblasts using Osteocalcin-Cre mice [12,15]. Almeida et al. suggested that ERα in osteoblast progenitors, but not in mature osteoblasts or osteocytes, is essential for regulation of female cortical bone [14]. As mentioned above, the functions of ERα in osteoblast lineage cells *in vivo* are still controversial and it is important to combine knowledge from various studies. All female mice exhibited an osteopenic phenotype in both the osteoblast-specific ERα

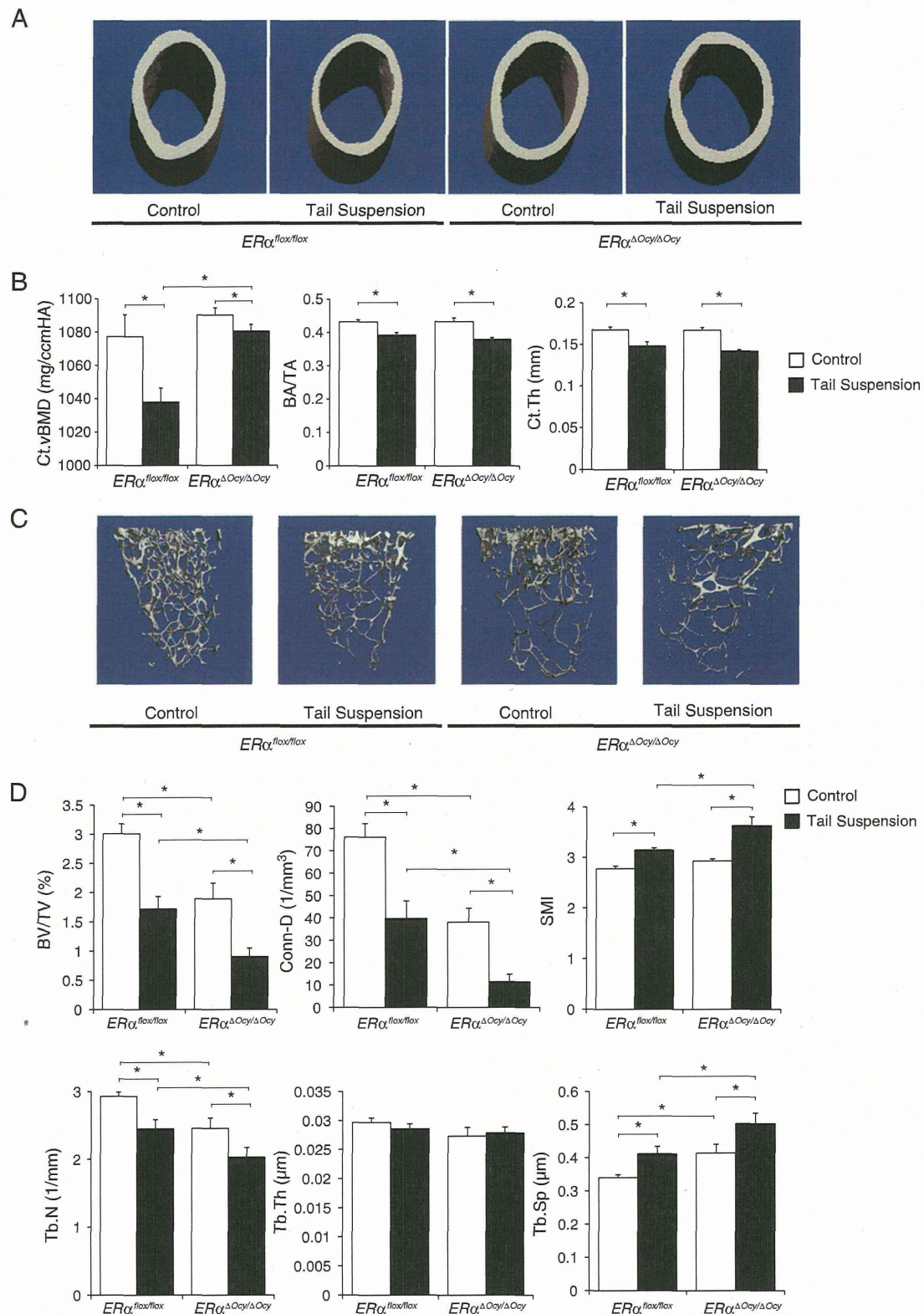


Fig. 5. Effects of unloading on trabecular and cortical bone in mice with targeted deletion of $ER\alpha$ in osteocytes. (A and C) Representative μ CT views. (B) 3D measurements of femoral distal trabecular area and (D) 3D measurements of femoral diaphyses from $ER\alpha^{flx/flx}$ and $ER\alpha^{\Delta Ocy/\Delta Ocy}$ mice subjected or not subjected to tail suspension. Data are represented as mean $\pm$ SEM ($n = 6$). * indicates $p < 0.05$.

knockout mice by Maatta et al. and Almeida et al., and in previous reports regarding osteoclast-specific ER α knockout mice [8,9]. As would be predicted, androgen receptor knockout mice (ARKO), including both systemic ARKO [51] and osteocyte conditional ARKO [52], exhibited bone loss in male mice. These gender-specific phenotypes are probably caused by differences in concentration of circulating sex steroids, estrogens and androgens. In contrast to these studies and our present study, a recent report showed that mice lacking ER α using the same Dmp1-Cre mouse exhibited trabecular bone loss only in male mice, but not in female mice [13]. In this report, Windahl et al. proposed that the physiological trabecular bone-sparing effect of estrogen is mediated via ER α in osteocytes in males, but also via ER α in osteoclasts in females [13]. At present, it is difficult to provide a convincing explanation to describe the discrepancies between our current study and this report [13]. However, one possible reason may be differences in the genetic background of the mouse strain of the ER α -floxed mice since the Dmp1-Cre mice were identical. The ER α -floxed mice used in our study have been registered as *Esr1^{tm1Mma}* and originated from 129S2/SvPas mixed background, and published in 2000 [5], then backcrossed with C57BL6 line for more than 10 times. On the other hand, the ER α -floxed mice used in the study by Windahl et al. have been registered as *Esr1^{tm1Gust}* and originated from 129X1/SvJ mixed background, and published in 2012 [53]. These differences might be responsible for the discrepancies between the two studies. Regardless, the results of these two studies suggest that osteocytic ER α may have a role in maintenance of trabecular bone homeostasis regardless of gender.

To investigate the possible molecular basis underlying ER α function in osteocytes, we performed an osteocyte isolation technique using FACS analysis of Dmp1-GFP positive cells from conditional null mice and their controls. The results obtained from the Functional Annotation Clustering of differentially expressed genes suggested that osteocytic ER α might regulate transcription of the genes related to secretory proteins, which may regulate osteoblastic bone formation and contribute to maintenance of bone homeostasis. In fact, *Sostdc1*, an antagonist of the Wnt signaling [45,54], was elevated as a downstream gene of osteocytic ER α . *Sostdc1* is a gene also called *Wise* or *Ectodin* whose domain is similar to *Sost* (Sclerostin). *Sost* and *Sostdc1* bind to Wnt co-receptors called *Lrps* and regulate the Wnt/ β -catenin pathway negatively [55]. Wnt signal proteins are reported to modulate bone mass *in vivo* by acting directly on mesenchymal stem cells [56–59]. Genes involved in the Wnt signaling are known to regulate the cell proliferation, differentiation, and apoptosis of osteoblasts [60]. Interaction between β -catenin and ER α has been previously reported [61] and the expressions of some Wnt family genes are important for responding to mechanical stress and are reportedly regulated by ER α [32]. Conventional *Sostdc1* KO mice are reported to exhibit abnormal tooth development, which has similar characteristics as bone [45,54]. Also, it has been reported that estradiol regulates mRNA levels of *Sostdc1* in U2OS cells [62]. In addition, a meta-analysis of BMD in a female Chinese population revealed that a mutation in the *Sostdc1* coding region was correlated with BMD, suggesting that *Sostdc1* might play a role in homeostasis of bone metabolism [46].

Also, Midkine, Mdk, was elevated as a downstream molecule of ER α in mice with this targeted deletion. Mdk is a member of a family of heparin-binding growth factors known primarily for their effects on neural cells [63]. Mdk expression is reported to increase during the course of primary osteoblast differentiation. Mdk has been shown to bind to a complex of protein tyrosine phosphatase zeta (Ptp α), low-density lipoprotein receptor-related protein-6 (Lrp6), and exert negative effects on Wnt signaling [64]. Conventional Mdk null mice exhibit increased bone formation, suggesting Mdk is a negative regulator of osteoblastic bone formation. Furthermore, Mdk KO mice are resistant to OVX-induced bone loss and sensitive to mechanical loading induced cortical bone increase [44]. In addition, the expression of ALP and the induction of canonical Wnt signaling in MC3T3E1, an osteoblastic cell line, were inhibited by Mdk treatments [64]. These reports and the results

from our current study suggest that *Sostdc1* and Mdk might be responsible for a component of estrogen's osteoprotective actions.

However, questions remain regarding how ER α negatively regulates the transcription of these genes because there are no reports of a negative transcriptional regulation of the estrogen receptor response element (negative ERE), although details of a negative glucocorticoid receptor response element (nGRE) have been reported [65]. Alternatively, it is possible that the expression of these factors might be regulated by an ER α -dependent miRNA. The precise molecular basis of transcriptional regulation or mRNA stabilization of these genes must be clarified in future studies. Neutralizing or deletion studies of these two proteins in this mouse model could provide possible answers for these questions.

In conclusion, osteocytic ER α might play a role in estrogen's osteoprotective action by controlling the expression of Wnt antagonists, which regulate osteoblastic bone formation in trabecular bone.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bone.2013.12.005>.

Conflict of interest

All authors state that they have no conflicts of interest.

Acknowledgments

The authors thank Dr. S. Kato for his general support and discussion, Ms. Noriko Moriyama for her technical support (microarray). This work was supported by Grant-in-Aids from Japan Society for the Promotion of Science (Research fellowship for young scientist to SK), NIH NIAMS PO1 AR046798 (to LFB) and JSPS KAKENHI (Grant numbers 23689066 and 23659712 to YI).

References

- [1] Nelson HD. Menopause. *Lancet* 2008;371:760–70.
- [2] Imai Y, Kondoh S, Kouzmenko A, Kato S. Minireview: osteoprotective action of estrogens is mediated by osteoclastic estrogen receptor- α . *Mol Endocrinol* 2010;24:877–85.
- [3] Sun L, Peng Y, Sharrow AC, Iqbal J, Zhang Z, Papachristou DJ, et al. FSH directly regulates bone mass. *Cell* 2006;125:247–60.
- [4] Pacifici R. Estrogen deficiency, T cells and bone loss. *Cell Immunol* 2008;252:68–80.
- [5] Dupont S, Krust A, Gansmuller A, Dierich A, Chambon P, Mark M. Effect of single and compound knockouts of estrogen receptors α (ER α) and β (ER β) on mouse reproductive phenotypes. *Development* 2000;127:4277–91.
- [6] Windahl SH, Andersson G, Gustafsson JA. Elucidation of estrogen receptor function in bone with the use of mouse models. *Trends Endocrinol Metab* 2002;13:195–200.
- [7] Sims NA, Dupont S, Krust A, Clement-Lacroix P, Minet D, Resche-Rigon M, et al. Deletion of estrogen receptors reveals a regulatory role for estrogen receptors- β in bone remodeling in females but not in males. *Bone* 2002;30:18–25.
- [8] Nakamura T, Imai Y, Matsumoto T, Sato S, Takeuchi K, Igarashi K, et al. Estrogen prevents bone loss via estrogen receptor α and induction of Fas ligand in osteoclasts. *Cell* 2007;130:811–23.
- [9] Martin-Millan M, Almeida M, Ambrogini E, Han L, Zhao H, Weinstein RS, et al. The estrogen receptor- α in osteoclasts mediates the protective effects of estrogens on cancellous but not cortical bone. *Mol Endocrinol* 2010;24:323–34.
- [10] Tomkinson A, Reeve J, Shaw RW, Noble BS. The death of osteocytes via apoptosis accompanies estrogen withdrawal in human bone. *J Clin Endocrinol Metab* 1997;82:3128–35.
- [11] Lee K, Jessop H, Suswillo R, Zaman G, Lanyon L. Endocrinology: bone adaptation requires oestrogen receptor- α . *Nature* 2003;424:389.
- [12] Maatta JA, Buki KG, Gu G, Alanne MH, Vaaranen J, Liljenback H, et al. Inactivation of estrogen receptor α in bone-forming cells induces bone loss in female mice. *FASEB J* 2012;27:478–88.
- [13] Windahl SH, Borjesson AE, Farman HH, Engdahl C, Moverare-Skrtic S, Sjogren K, et al. Estrogen receptor- α in osteocytes is important for trabecular bone formation in male mice. *Proc Natl Acad Sci U S A* 2013;110:2294–9.
- [14] Almeida M, Iyer S, Martin-Millan M, Bartell SM, Han L, Ambrogini E, et al. Estrogen receptor- α signaling in osteoblast progenitors stimulates cortical bone accrual. *J Clin Invest* 2013;123:394–404.
- [15] Melville KM, Kelly NH, Khan SA, Schimenti JC, Ross FP, Main RP, et al. Female mice lacking estrogen receptor- α in osteoblasts have compromised bone mass and strength. *J Bone Miner Res* 2013 (in press) (Epub ahead of print).
- [16] Burger EH, Klein-Nulend J. Mechanotransduction in bone—role of the lacuno-canalicular network. *FASEB J* 1999(13 Suppl.):S101–12.

- [17] Robling AG, Niziolek PJ, Baldrige LA, Condon KW, Allen MR, Alam I, et al. Mechanical stimulation of bone in vivo reduces osteocyte expression of Sost/sclerostin. *J Biol Chem* 2008;283:5866–75.
- [18] Skerry TM, Bitensky L, Chayen J, Lanyon LE. Early strain-related changes in enzyme activity in osteocytes following bone loading in vivo. *J Bone Miner Res* 1989;4:783–8.
- [19] Gluhak-Heinrich J, Ye L, Bonewald LF, Feng JQ, MacDougall M, Harris SE, et al. Mechanical loading stimulates dentin matrix protein 1 (DMP1) expression in osteocytes in vivo. *J Bone Miner Res* 2003;18:807–17.
- [20] Gluhak-Heinrich J, Pavlin D, Yang W, MacDougall M, Harris SE. MEPE expression in osteocytes during orthodontic tooth movement. *Arch Oral Biol* 2007;52:684–90.
- [21] Thompson DL, Sabbagh Y, Tenenhouse HS, Roche PC, Drezner MK, Salisbury JL, et al. Ontogeny of Phex/PHEX protein expression in mouse embryo and subcellular localization in osteoblasts. *J Bone Miner Res* 2002;17:311–20.
- [22] Nampei A, Hashimoto J, Hayashida K, Tsuboi H, Shi K, Tsuji I, et al. Matrix extracellular phosphoglycoprotein (MEPE) is highly expressed in osteocytes in human bone. *J Bone Miner Res* 2004;22:176–84.
- [23] Liu S, Zhou J, Tang W, Jiang X, Rowe DW, Quarles LD. Pathogenic role of Fgf23 in Hyp mice. *Am J Physiol Endocrinol Metab* 2006;291:E38–49.
- [24] Feng JQ, Ward LM, Liu S, Lu Y, Xie Y, Yuan B, et al. Loss of DMP1 causes rickets and osteomalacia and identifies a role for osteocytes in mineral metabolism. *Nat Genet* 2006;38:1310–5.
- [25] Bonewald LF. Osteocytes as dynamic multifunctional cells. *Ann N Y Acad Sci* 2007;1116:281–90.
- [26] Bergmann P, Body JJ, Boonen S, Boutsen Y, Devogelaer JP, Goemaere S, et al. Loading and skeletal development and maintenance. *J Osteoporos* 2010;2011:786752.
- [27] Lloyd SA, Bandstra ER, Willey JS, Riffle SE, Tirado-Lee L, Nelson GA, et al. Effect of proton irradiation followed by hindlimb unloading on bone in mature mice: a model of long-duration spaceflight. *Bone* 2012;51:756–64.
- [28] Lee KC, Maxwell A, Lanyon LE. Validation of a technique for studying functional adaptation of the mouse ulna in response to mechanical loading. *Bone* 2002;31:407–12.
- [29] Bonewald LF, Johnson ML. Osteocytes, mechanosensing and Wnt signaling. *Bone* 2008;42:606–15.
- [30] Robinson JA, Chatterjee-Kishore M, Yaworsky PJ, Cullen DM, Zhao W, Li C, et al. Wnt/beta-catenin signaling is a normal physiological response to mechanical loading in bone. *J Biol Chem* 2006;281:31720–8.
- [31] Javaheri B, Stern A, Lara N, Dallas M, Zhao H, Liu Y, et al. Deletion of a single beta-catenin allele in osteocytes abolishes the bone anabolic response to loading. *J Bone Miner Res* 2013 (in press) (Epub ahead of print).
- [32] Armstrong VJ, Muzylak M, Sinters A, Zaman G, Saxon LK, Price JS, et al. Wnt/beta-catenin signaling is a component of osteoblastic bone cell early responses to load-bearing and requires estrogen receptor alpha. *J Biol Chem* 2007;282:20715–27.
- [33] Lu Y, Xie Y, Zhang S, Dusevich V, Bonewald LF, Feng JQ. DMP1-targeted Cre expression in odontoblasts and osteocytes. *J Dent Res* 2007;86:320–5.
- [34] Kalajzic I, Braut A, Guo D, Jiang X, Kronenberg MS, Mina M, et al. Dentin matrix protein 1 expression during osteoblastic differentiation, generation of an osteocyte GFP-transgene. *Bone* 2004;35:74–82.
- [35] Bouxsein ML, Boyd SK, Christiansen BA, Guldberg RE, Jepsen KJ, Muller R. Guidelines for assessment of bone microstructure in rodents using micro-computed tomography. *J Bone Miner Res* 2010;25:1468–86.
- [36] Sato S, Hanada R, Kimura A, Abe T, Matsumoto T, Iwasaki M, et al. Central control of bone remodeling by neuromedin U. *Nat Med* 2007;13:1234–40.
- [37] Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, et al. Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. *J Bone Miner Res* 1987;2:595–610.
- [38] Paic F, Igwe JC, Nori R, Kronenberg MS, Franceschetti T, Harrington P, et al. Identification of differentially expressed genes between osteoblasts and osteocytes. *Bone* 2009;45:682–92.
- [39] Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 2009;4:44–57.
- [40] McCarthy JJ, Fox AM, Tsika GL, Gao L, Tsika RW. Beta-MHC transgene expression in suspended and mechanically overloaded/suspended soleus muscle of transgenic mice. *Am J Physiol* 1997;272:R1552–61.
- [41] Tsika G, Ji J, Tsika R. Sp3 proteins negatively regulate beta myosin heavy chain gene expression during skeletal muscle inactivity. *Mol Cell Biol* 2004;24:10777–91.
- [42] Xiong J, Onal M, Jilka RL, Weinstein RS, Manolagas SC, O'Brien CA. Matrix-embedded cells control osteoclast formation. *Nat Med* 2011;17:1235–41.
- [43] Heine PA, Taylor JA, Iwamoto GA, Lubahn DB, Cooke PS. Increased adipose tissue in male and female estrogen receptor-alpha knockout mice. *Proc Natl Acad Sci U S A* 2000;97:12729–34.
- [44] Neunaber C, Catala-Lehnen P, Beil FT, Marshall RP, Kanbach V, Baranowsky A, et al. Increased trabecular bone formation in mice lacking the growth factor midkine. *J Bone Miner Res* 2010;25:1724–35.
- [45] Ahn Y, Sanderson BW, Klein OD, Krumlauf R. Inhibition of Wnt signaling by Wise (Sostdc1) and negative feedback from Shh controls tooth number and patterning. *Development* 2010;137:3221–31.
- [46] He JW, Yue H, Hu WW, Hu YQ, Zhang ZL. Contribution of the sclerostin domain-containing protein 1 (SOSTDC1) gene to normal variation of peak bone mineral density in Chinese women and men. *J Bone Miner Metab* 2011;29:571–81.
- [47] Tou JC, Foley A, Yuan YV, Arnaud S, Wade CE, Brown M. The effect of ovariectomy combined with hindlimb unloading and reloading on the long bones of mature Sprague–Dawley rats. *Menopause* 2008;15:494–502.
- [48] Almeida M, Martin-Millan M, Ambrogini E, Bradsher 3rd R, Han L, Chen XD, et al. Estrogens attenuate oxidative stress and the differentiation and apoptosis of osteoblasts by DNA-binding-independent actions of the ERalpha. *J Bone Miner Res* 2010;25:769–81.
- [49] Tomkinson A, Gevers EF, Wit JM, Reeve J, Noble BS. The role of estrogen in the control of rat osteocyte apoptosis. *J Bone Miner Res* 1998;13:1243–50.
- [50] Krum SA, Miranda-Carboni GA, Hauschka PV, Carroll JS, Lane TF, Freedman LP, et al. Estrogen protects bone by inducing Fas ligand in osteoblasts to regulate osteoclast survival. *EMBO J* 2008;27:535–45.
- [51] Kawano H, Sato T, Yamada T, Matsumoto T, Sekine K, Watanabe T, et al. Suppressive function of androgen receptor in bone resorption. *Proc Natl Acad Sci U S A* 2003;100:9416–21.
- [52] Sinnesael M, Claessens F, Laurent M, Dubois V, Boonen S, Deboel L, et al. Androgen receptor (AR) in osteocytes is important for the maintenance of male skeletal integrity: evidence from targeted AR disruption in mouse osteocytes. *J Bone Miner Res* 2012;27:2535–43.
- [53] Antonson P, Omoto Y, Humire P, Gustafsson JA. Generation of ERalpha-floxed and knockout mice using the Cre/LoxP system. *Biochem Biophys Res Commun* 2012;424:710–6.
- [54] Cho SW, Kwak S, Woolley TE, Lee MJ, Kim EJ, Baker RE, et al. Interactions between Shh, Sostdc1 and Wnt signaling and a new feedback loop for spatial patterning of the teeth. *Development* 2011;138:1807–16.
- [55] Itasaki N, Jones CM, Mercurio S, Rowe A, Domingos PM, Smith JC, et al. Wise, a context-dependent activator and inhibitor of Wnt signalling. *Development* 2003;130:4295–305.
- [56] Liu G, Vijayakumar S, Grumolato L, Arroyave R, Qiao H, Akiri G, et al. Canonical Wnts function as potent regulators of osteogenesis by human mesenchymal stem cells. *J Cell Biol* 2009;185:67–75.
- [57] Takada I, Kouzmenko AP, Kato S. Wnt and PPARgamma signaling in osteoblastogenesis and adipogenesis. *Nat Rev Rheumatol* 2009;5:442–7.
- [58] Augello A, De Bari C. The regulation of differentiation in mesenchymal stem cells. *Hum Gene Ther* 2010;21:1226–38.
- [59] Baron R, Kneissel M. WNT signaling in bone homeostasis and disease: from human mutations to treatments. *Nat Med* 2013;19:179–92.
- [60] Westendorf JJ, Kahler RA, Schroeder TM. Wnt signaling in osteoblasts and bone diseases. *Gene* 2004;341:19–39.
- [61] Kouzmenko AP, Takeyama K, Ito S, Furutani T, Sawatsubashi S, Maki A, et al. Wnt/beta-catenin and estrogen signaling converge in vivo. *J Biol Chem* 2004;279:40255–8.
- [62] Tee MK, Rogatsky I, Tzagarakis-Foster C, Cvorc A, An J, Christy RJ, et al. Estradiol and selective estrogen receptor modulators differentially regulate target genes with estrogen receptors alpha and beta. *Mol Biol Cell* 2004;15:1262–72.
- [63] Nakamura E, Kadomatsu K, Yuasa S, Muramatsu H, Mamiya T, Nabeshima T, et al. Disruption of the midkine gene (Mdk) resulted in altered expression of a calcium binding protein in the hippocampus of infant mice and their abnormal behaviour. *Genes Cells* 1998;3:811–22.
- [64] Liedert A, Mattausch L, Rontgen V, Blakytyn R, Voge D, Pahl M, et al. Midkine-deficiency increases the anabolic response of cortical bone to mechanical loading. *Bone* 2011;48:945–51.
- [65] Surjit M, Ganti KP, Mukherji A, Ye T, Hua G, Metzger D, et al. Widespread negative response elements mediate direct repression by agonist-liganded glucocorticoid receptor. *Cell* 2011;145:224–41.

BRIEF COMMUNICATION

Effect of dietary eugenol on xenobiotic metabolism and mediation of UDP-glucuronosyltransferase and cytochrome P450 1A1 expression in rat liver

Hidetomo Iwano¹, Wakako Ujita¹, Miyu Nishikawa¹, Satomi Ishii², Hiroki Inoue³, and Hiroshi Yokota¹

¹Laboratory of Veterinary Biochemistry, Graduate School of Veterinary Medicine, ²Department of Food Science, Faculty of Dairy Science, and

³Laboratory of Environmental Biochemistry, Faculty of Environmental System, Rakuno Gakuen University, Ebetsu, Hokkaido, Japan

Abstract

Xenobiotic-metabolizing enzymes (XMEs) play an important role in the elimination and detoxification of xenobiotics and drugs. A variety of natural dietary agents are known to protect against cancer by inducing XME. To elucidate the molecular mechanism of XME induction, we examined the effect of dietary eugenol (4-allyl-1-hydroxy-2-methoxybenzene) on xenobiotic metabolism. In this study, rats were administered dietary eugenol for 4 weeks to investigate the various effects of UDP-glucuronosyltransferase (UGT) and cytochrome P450 (CYP) expression. In rats administered dietary eugenol, expression levels of hepatic *CYP1A1* were reduced to 40% than of the controls, while expression of hepatic *UGT1A6*, *UGT1A7* and *UGT2B1* increased to 2–3 times than observed in the controls. Hepatic protein levels of *UGT1A6* and *2B1* were also elevated in the eugenol-treated rats. These results suggest that the natural compound eugenol improves the xenobiotic-metabolizing systems that suppress and induce the expression of *CYP1A1* and *UGT*, respectively.

Keywords

Cytochrome P450, eugenol,
UDP-glucuronosyltransferase,
xenobiotic-metabolizing enzymes

History

Received 8 April 2013
Revised 4 September 2013
Accepted 13 September 2013
Published online 21 October 2013

Introduction

In addition to affecting a wide variety of biological events, environmental contaminants from food and water affect can also cause a variety of diseases. While avoiding the ingestion of these contaminants may be difficult, our bodies are capable of metabolizing most of these substances using a variety of xenobiotic-metabolizing enzymes (XMEs). These XMEs can be classified into three main categories. The first category consists of phase I enzymes, mainly the cytochrome P450 (CYP) enzymes (Nebert & Gonzalez, 1987; Nelson et al., 1993). Most drugs are metabolized by CYP, either as part of detoxification or due to activation of a pathway for an inactive prodrug. The second category is referred to as phase II enzymes (Di Pietro et al., 2010; Mackenzie et al., 1997, 2003). These enzymes usually conjugate phase I products, but can also conjugate other intermediate compounds and intracellular substrates, such as steroids and bilirubin. The last category consists of drug transporters that are membrane-bound proteins involved in drug uptake or excretion (Barry et al., 2003; Hediger et al., 2004). Regulation of these XME is an important area of study because their gene expression is influenced by a wide variety of drugs and chemicals derived from food (Kong et al., 2001; Rushmore & Kong, 2002; Xu et al., 2005). Since disruption of the homeostatic control of XME can cause cancer and a wide variety of diseases, considerable research

has been undertaken in the area of XME metabolism. Of the treatment methods currently available, stimulating the excretion of xenobiotic substances has been shown to restore the health of a subject (Aggarwal, 2008; Aggarwal & Shishodia, 2006). Eugenol (4-allyl-1-hydroxy-2-methoxybenzene) is weakly acidic, slightly soluble in water and soluble in organic solvent (Kamatou et al., 2012). It has been identified in several aromatic plants such as clove oil, cinnamon, basil and nutmeg as a food-flavoring agent; but thus far, the medical application of pure eugenol has been limited to the field of dentistry (Kamatou et al., 2012). In recent years, eugenol has been reported to have antioxidant and anti-inflammatory effects, and recent studies have shown that eugenol is capable of inhibiting melanoma cancer cell proliferation (Ghosh et al., 2005), inducing apoptosis in melanoma (Pisano et al., 2007) and human promyelocytic leukemia cells (HL-60) (Yoo et al., 2005), and is involved in a rat model of gastric carcinogenesis (Manikandan et al., 2010). These authors suggested that eugenol may be a candidate for putative chemopreventive and chemotherapeutic agents. Eugenol is a main component of clove oil and is also present in the essential oils of many other plant species, including cinnamon, basil and nutmeg (Aggarwal, 2008; Aggarwal & Shishodia, 2006). Thus, although eugenol is considered to be well suited for use as an anticancer drug, the mechanisms and molecules involved in the metabolism of this compound are currently unknown (Aggarwal, 2008; Aggarwal & Shishodia, 2006).

This study demonstrates that eugenol influenced the transcription of isoforms of the XMEs CYP (EC 1. 14. 14. 1) and UDP-glucuronosyltransferase (UGT) (EC 2. 4. 1. 17), suggesting that eugenol could potentially be used as an anticancer drug through maintaining the homeostasis of xenobiotic metabolism.

Correspondence: Hiroshi Yokota, Laboratory of Veterinary Biochemistry, Graduate School of Veterinary Medicine, Rakuno Gakuen University, 582 Bunkyo-dai-Midorimachi Ebetsu, Hokkaido 069-8501, Japan. Tel/Fax: +81-11-388-4743. E-mail: h-yokota@rakuno.ac.jp

Table 1. Food composition of basal diet NIH-07PLD in 100 g showed A eugenol diet was produced by adding eugenol (5% w/w) to the basal pelleted diet NIH-07PLD (Oriental Yeast Co., Ltd).

Composition of NIH-07PLD	
Formula	g/100 g
White fish meal	14.00
Corn gluten meal	8.00
Corn	28.50
White flour	40.62
Brewery yeast	2.00
Cane molasses	0.75
Corn oil	2.50
Vitamin and mineral mixture	3.63

Methods

Animal diets

Eugenol was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). A eugenol diet was produced by adding eugenol (5% w/w) to the basal pelleted diet NIH-07PLD (Oriental Yeast Co., Ltd, Tokyo, Japan). NIH-07PLD is an open-formula diet designed to contain as little phytoestrogenic substances as possible. In order to maintain a comparable levels of protein, the levels of gluten meal and white fish meal have been increased (Table 1).

Animals

Male Sprague-Dawley rats (7 weeks old; $n=6$) were housed under standard conditions and provided free access to water and NIH-07PLD pelleted diet (Oriental Yeast Co., Ltd). At 8 weeks of age, the rats were randomly divided into two groups: a control diet group (NIH-07PLD only) and a eugenol diet group (NIH-07PLD containing 5% eugenol: w/w). Body weights and food consumption were recorded every 2 days. After 4 weeks, three of the rats were sacrificed, their livers were excised and frozen immediately in liquid nitrogen before being stored at -80°C until analysis. The section of the intestines corresponding to the jejunum was also removed and stored at -80°C . All of the animals were treated according to the Laboratory Animal Control Guidelines at Rakuno Gakuen University, which is based on the *Guide for the Care and Use of Laboratory Animals of the U.S. National Institutes of Health*.

RNA isolation and semi-quantitative reverse-transcription polymerase chain reaction

Total RNA was isolated from livers and intestines using an RNeasy Mini kit (Qiagen, Hilden, Germany), and DNase digestion was performed using an RNase-Free DNase kit (Qiagen NV, Hilden, Germany). Complementary DNA (cDNA) was synthesized from 1 μg of tRNA with ReverTra Ace reverse transcriptase (TOYOBO, Osaka, Japan) and oligo dT primers (Toyobo) according to the manufacturer's instructions. The coding regions of the respective cDNA species were amplified by polymerase chain reaction (PCR) with oligonucleotide primers (Supplementary Table 1).

Western blot analysis

Liver microsomal protein samples (50 μg) were subjected to gel electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gels. The polypeptide bands were transferred to a nitrocellulose membrane, and immunoreactive bands were detected using polyclonal antibodies against UGT1A6 and UGT2B1 (Ikushiro

et al., 1997; Iwano et al., 1997) and followed by incubation with peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA).

Quantitative RT-PCR analysis

cDNA that had been diluted for the amplification of the selected genes, which were *UGT1A1*, *UGT1A6*, *UGT1A7*, *UGT2B1* and *CYP1A1*, was used for quantitative reverse-transcription polymerase chain reaction (RT-PCR) analysis. A standard curve for each gene was produced using 100-fold serial dilutions of the genes as a template (10^8 – 10^2 copies). The reaction was performed using a QuantiTect SYBR Green PCR kit (Qiagen) and a Light Cycler (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions. We used same oligonucleotide primers in semi-quantitative and quantitative RT-PCR (Supplementary Table 1). The copy number of each gene expressed in the rat liver and intestine was calculated from a standard curve and normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The quantitative values are shown as the mean $\pm$ SE for mice on the control diet ($n=3$) and mice on the eugenol diet ($n=3$).

Statistical analysis

Comparisons were made by the Student's *t* test, and a *p* value of 0.05 was taken to be significant. All values are presented as the mean $\pm$ SE.

Results

To clarify the mechanism by which eugenol influenced these genes, screening was performed by semi-quantitative RT-PCR using hepatic and intestinal total RNA. Body weights and food consumption were recorded every 2 days; these values did not differ between the two diet groups (data not shown). Hepatic and intestinal messenger RNA (mRNA) were analyzed by RT-PCR using specific primers for *CYP1A1*, *1A2*, *2B1*, *2B2*, *2C6*, *2C11*, *2D*, *2E1*, *3A1*, *3A2*, *4A1* and *UGT 1A1*, *1A2*, *1A3*, *1A6*, *1A7*, *1A8*, *1A9*, *2B1*. In the *CYP* family, eugenol treatment caused a slight reduction in the expression of *CYP1A1* in rat livers and a slight increase in the expression of *CYP2B2* in the intestines (Supplementary Figure 1). In the *UGT* family, *UGT1A1*, *UGT1A6*, *UGT1A7* and *UGT2B1* were all strongly expressed in the eugenol-treated rat livers, and these all genes were expressed at approximately the same levels in the intestines (Supplementary Figure 2). The results of the quantitative analysis using real-time PCR are shown in Figure 1. Interestingly, the level of hepatic *CYP1A1* expression in rats fed the eugenol diet decreased to 40% that observed in the rats fed the control diet. Conversely, expression levels of hepatic *UGT1A6*, *UGT1A7* and *UGT2B1* in rats fed the eugenol diet increased by 2–3 times than observed in the control rats. While the levels of intestinal *UGT1A1*, *UGT1A6* and *UGT1A7* expression were slightly increased in both groups of rats, no statistically significant differences were observed between the control and eugenol diet groups (data not shown). These results show that eugenol specifically induces the mRNA expression of the *UGT* gene family, particularly hepatic *UGT1A6*, *UGT1A7* and *UGT2B1*, and also that it reduces the expression of hepatic *CYP1A1*. The protein levels of hepatic UGT 1A6 and UGT2B1 were also elevated in all rats administered the eugenol diet (Figure 2).

Discussion

In this study, we observed down regulation of hepatic *CYP1A1* expression and induction of hepatic *UGT1A6*, *UGT1A7* and *UGT2B1* expression by eugenol administration. Phase I enzymes,

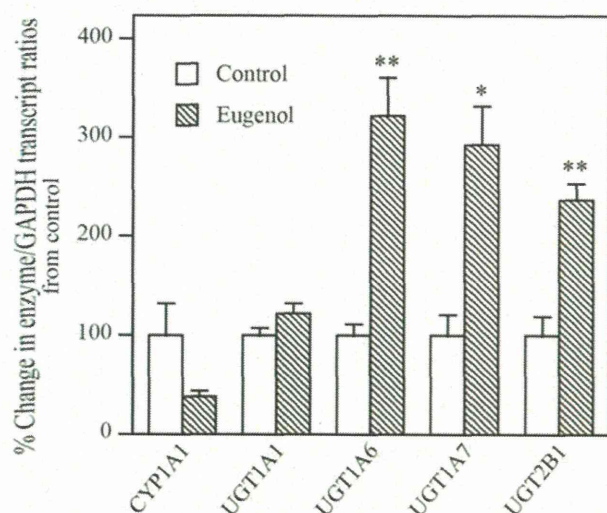


Figure 1. Quantitative analysis of gene expression by real-time RT-PCR. Quantitative analysis of mRNA expression of *Cyp1A1*, *UGT1A1*, *UGT1A6*, *UGT1A7* and *UGT2B1* in the liver of control and eugenol dietary groups were examined by real-time RT-PCR ($n = 3$, respectively). Expression of each gene in control mice was normalized to 100%. Quantitative values are shown as means $\pm$ SE. * $p < 0.05$; ** $p < 0.01$ versus control.

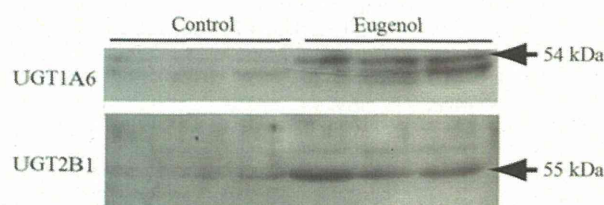


Figure 2. Effect of eugenol on levels of UGT isoforms in rat liver microsomes. Content of UGT isoforms in microsomal fractions of control- and eugenol-treated liver samples were compared. The three lanes on the left contain control liver microsomes and the three lanes on the right contain eugenol-treated liver microsomes. Each lane was loaded with 50 μ g of protein purified from individual animals. Arrows indicate positive bands corresponding to UGT1A6 and UGT2B1.

such as CYP1A1, are generally considered to represent an activating system for many natural and synthetic carcinogens, including polycyclic aromatic hydrocarbons (e.g. benzo[a]pyrene (BaP) and benzene) and polyhalogenated aromatic hydrocarbons (e.g. dioxin-like compounds and polychlorinated biphenyls) (Nebert & Dalton, 2006). Conversely, phase II enzymes, such as UGT (Mackenzie et al., 1997, 2003) and glutathione S-transferase (GST) (Di Pietro et al., 2010), often act to detoxify harmful natural or activated carcinogens. Consequently, inducers such as eugenol, which preferentially elevate the transcription of phase II enzymes, are expected to be beneficial because they can stimulate the detoxification of potentially carcinogenic chemicals (Kong et al., 2001; Rushmore & Kong, 2002; Xu et al., 2005). By catalyzing the transfer of glucuronic acid from UDP-glucuronic acid to predominantly hydrophobic chemicals, UGT facilitates the excretion of endogenous and xenobiotic compounds, such as bilirubin, steroids and environmental pollutants as water-soluble conjugates (Mackenzie et al., 1997, 2003). UGT 1A1 mainly catalyzes the glucuronidation of bilirubin, and absence of UGT1A1 causes Crigler-Najjar syndrome (Iyanagi et al., 1989). UGT1A6 and UGT1A7 catalyze the glucuronidation of similar substrates, such as planar phenols and BaP (Iwano et al., 1997;

Mackenzie et al., 1997, 2003). UGT2B1 catalyzes the glucuronidation of a number of xenobiotics and endobiotics (steroids), such as estrogen and morphine (Mackenzie et al., 1997, 2003). We previously showed that the hepatic enzymatic activities of UGT on a variety of substrates are elevated by eugenol administration (Yokota et al., 1988). We also reported that hepatic microsomal BaP hydroxylase activity was reduced, and that the mutagenicity of BaP in the Ames test was suppressed by eugenol administration (Yokota et al., 1986). These previous observations are consistent with the present results, which showed that eugenol induced *UGT1A1*, *UGT1A6*, *UGT1A7* and *UGT2B1* expression, and reduced *CYP1A1* expression.

The expression of XME is regulated by a variety of nuclear receptors and orphan receptors, including aryl hydrocarbon receptor (AhR), constitutive androstane receptor and pregnane X receptor (Mackenzie et al., 2003; Nebert & Dalton, 2006). It has been well established that activation of AhR by exogenous compounds, such as 3-methylcholanthrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, promotes the nuclear transport and transcriptional activation of *CYP1A1* (Kawajiri & Fujii-Kuriyama, 2007; Pollenz, 2002). It is also been reported that the expression of hepatic CYP is reduced by lipopolysaccharide and cytokines, such as interleukin-1 β and tumor necrosis factor- α , through the nuclear factor kappa B signaling pathway (Ke et al., 2001; Park et al., 2007). Indeed, further investigations need to be conducted to elucidate the detailed mechanisms underlying the suppression of CYP1A1 expression by eugenol. The induction of phase II enzymes by eugenol may also be mediated by XME receptors other than AhR, even though a specific receptor for eugenol has not yet been identified. Recent findings suggest that the *cis*-acting regulatory element and the antioxidant response element (ARE)/ electrophile response element (EpRE), play important roles in the transcriptional regulation of phase II genes, such as *quinone oxidoreductase* (EC 1.6.5.2), *GST* (EC 2.5.1.18) and *UGT*, by phenolic antioxidants (Kong et al., 2001; Rushmore & Kong, 2002; Xu et al., 2005). Several ARE/EpRE-binding proteins have been identified, including the members of basic leucine zipper transcriptional factor family, nuclear factor erythroid 2-related factor 1, 2 (Nrf1, Nrf2) and small Maf proteins (Buetler et al., 1995; Hu et al., 2006). Nrf2 is the most likely transcriptional factor involved in the transcriptional activation of ARE-mediated phase II genes by a phenolic antioxidant in Nrf2-/- mice studies (Maher & Yamamoto, 2010). Indeed, elucidating which XME intermediates are involved in the eugenol signaling pathway should be investigated in the future. Eugenol has been demonstrated to inhibit prostaglandin biosynthesis and to block COX-2 activity (Kim et al., 2003) and has been shown to be a potent inhibitor of melanoma cell proliferation through the suppression of the E2F transcription factor family (Ghosh et al., 2005). These studies suggest that dietary eugenol is beneficial and that it has anti-inflammatory and anti-cancer properties. In long-term carcinogenicity experiments conducted by various groups in CD-1 mice and F344 rats, eugenol was not associated with tumor formation. In addition, the National Toxicology Program concluded that eugenol was not carcinogenic to rats and that there was no evidence that unequivocally proved the carcinogenic nature of eugenol in mice (Report, 1983).

Conclusions

This study demonstrates that the natural compound eugenol improves the xenobiotic-metabolizing systems that suppress and induce the expression of *CYP1A1* and *UGT*, respectively. These suggesting that eugenol could potentially be used as an anticancer drug through maintaining the homeostasis of xenobiotic metabolism.

Declaration of interest

H. Iwano participated in study design and organized all experiments. W. U. and M. N. treated animal and performed quantitative RT-PCR analysis. S. I. helped to draft the manuscript. H. Inoue helped statistical analysis. All authors have read and approved the manuscript.

This work was partially supported by Grants-in-Aid to cooperative Research from Rakuno Gakuen University. Rakuno Gakuen University Dairy Science Institute, 2006 and a Feasibility Study among Fundamental Studies under the framework of EXTEND 2010 (Extended Tasks on Endocrine Disruption 2010) funded by the Ministry of the Environment, Japan.

Reference

- Aggarwal BB, Kunnumakara AB, Harikumar KB, Tharakan ST, Sung B, Anand P. 2008. Potential of spice-derived phytochemicals for cancer prevention. *Planta Medica* 74:1560–1569.
- Aggarwal BB, Shishodia S. 2006. Molecular targets of dietary agents for prevention and therapy of cancer. *Biochem Pharmacol* 71:1397–1421.
- Barry H, Cole SPC, Kuchler K, Higgins CF. 2003. ABC proteins: from bacteria to man. Academic Press.
- Buetler TM, Gallagher EP, Wang C, Stahl DL, Hayes JD, Eaton DL. 1995. Induction of phase I and phase II drug-metabolizing enzyme mRNA, protein, and activity by BHA, ethoxyquin, and oltipraz. *Toxicol Appl Pharmacol* 135:45–57.
- Di Pietro G, Magno LA, Rios-Santos F. 2010. Glutathione S-transferases: an overview in cancer research. *Expert Opin Drug Metab Toxicol* 6: 153–170.
- Ghosh R, Nadiminty N, Fitzpatrick JE, Alworth WL, Slaga TJ, Kumar AP. 2005. Eugenol causes melanoma growth suppression through inhibition of E2F1 transcriptional activity. *J Biol Chem* 280: 5812–5819.
- Hediger MA, Romero MF, Peng JB, Rolfs A, Takanaga H, Bruford EA. 2004. The ABCs of solute carriers: physiological, pathological and therapeutic implications of human membrane transport proteins. *Pflügers Arch* 447:465–468.
- Hu R, Shen G, Yerramilli UR, Lin W, Xu C, Nair S, Kong AN. 2006. In vivo pharmacokinetics, activation of MAPK signaling and induction of phase II/III drug metabolizing enzymes/transporters by cancer chemopreventive compound BHA in the mice. *Arch Pharm Res* 29: 911–920.
- Ikushiro S, Emi Y, Iyanagi T. 1997. Protein–protein interactions between UDP-glucuronosyltransferase isozymes in rat hepatic microsomes. *Biochemistry* 36:7154–7161.
- Iwano H, Yokota H, Ohgiya S, Yotumoto N, Yuasa A. 1997. A critical amino acid residue, asp446, in UDP-glucuronosyltransferase. *Biochem J* 325:587–591.
- Iyanagi T, Watanabe T, Uchiyama Y. 1989. The 3-methylcholanthrene-inducible UDP-glucuronosyltransferase deficiency in the hyperbilirubinemic rat (Gunn rat) is caused by a-1 frameshift mutation. *J Biol Chem* 264:21302–21307.
- Kamatou GP, Vermaak I, Viljoen AM. 2012. Eugenol – from the remote Maluku islands to the international market place: a review of a remarkable and versatile molecule. *Molecules* 17:6953–6981.
- Kawajiri K, Fujii-Kuriyama Y. 2007. Cytochrome P450 gene regulation and physiological functions mediated by the aryl hydrocarbon receptor. *Arch Biochem Biophys* 464:207–212.
- Ke S, Rabson AB, Germino JF, Gallo MA, Tian Y. 2001. Mechanism of suppression of cytochrome P-450 1A1 expression by tumor necrosis factor-alpha and lipopolysaccharide. *J Biol Chem* 276:39638–39644.
- Kim SS, Oh OJ, Min HY, Park EJ, Kim Y, Park HJ, Nam Han Y, Lee SK. 2003. Eugenol suppresses cyclooxygenase-2 expression in lipopolysaccharide-stimulated mouse macrophage RAW264.7 cells. *Life Sci* 73:337–348.
- Kong AN, Owuor E, Yu R, Hebbar V, Chen C, Hu R, Mandlekar S. 2001. Induction of xenobiotic enzymes by the MAP kinase pathway and the antioxidant or electrophile response element (ARE/EpRE). *Drug Metab Rev* 33:255–271.
- Mackenzie PI, Gregory PA, Gardner-Stephen DA, Lewinsky RH, Jorgensen BR, Nishiyama T, Xie W, Radominska-Pandya A. 2003. Regulation of UDP glucuronosyltransferase genes. *Curr Drug Metab* 4: 249–257.
- Mackenzie PI, Owens IS, Burchell B, Bock KW, Bairoch A, Belanger A, Fournel-Gigleux S, et al. 1997. The UDP glycosyltransferase gene superfamily: recommended nomenclature update based on evolutionary divergence. *Pharmacogenetics* 7:255–269.
- Maher J, Yamamoto M. 2010. The rise of antioxidant signaling – the evolution and hormetic actions of Nrf2. *Toxicol Appl Pharmacol* 244: 4–15.
- Manikandan P, Murugan RS, Priyadarsini RV, Vinothini G, Nagini S. 2010. Eugenol induces apoptosis and inhibits invasion and angiogenesis in a rat model of gastric carcinogenesis induced by MNNG. *Life Sci* 86:936–941.
- Nebert DW, Dalton TP. 2006. The role of cytochrome P450 enzymes in endogenous signalling pathways and environmental carcinogenesis. *Nat Rev Cancer* 6:947–960.
- Nebert DW, Gonzalez FJ. 1987. P450 genes: structure, evolution, and regulation. *Ann Rev Biochem* 56:945–993.
- Nelson DR, Kamataki T, Waxman DJ, Guengerich FP, Estabrook RW, Feyereisen R, Gonzalez FJ, et al. 1993. The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA Cell Biol* 12:1–51.
- Park KR, Lee JH, Choi C, Liu KH, Seog DH, Kim YH, Kim DE, et al. 2007. Suppression of interleukin-2 gene expression by isoeugenol is mediated through down-regulation of NF-AT and NF-kappaB. *Int Immunopharmacol* 7:1251–1258.
- Pisano M, Pagnan G, Loi M, Mura ME, Tilocca MG, Palmieri G, Fabbri D, et al. 2007. Antiproliferative and pro-apoptotic activity of eugenol-related biphenyls on malignant melanoma cells. *Mol Cancer* 6:8–19.
- Pollenz RS. 2002. The mechanism of AH receptor protein down-regulation (degradation) and its impact on AH receptor-mediated gene regulation. *Chem Biol Interact* 141:41–61.
- Rushmore TH, Kong AN. 2002. Pharmacogenomics, regulation and signaling pathways of phase I and II drug metabolizing enzymes. *Curr Drug Metab* 3:481–490.
- Xu C, Li CY, Kong AN. 2005. Induction of phase I, II and III drug metabolism/transport by xenobiotics. *Arch Pharm Res* 28:249–268.
- Yokota H, Hashimoto H, Motoya M, Yuasa A. 1988. Enhancement of UDP-glucuronosyltransferase, UDP-glucose dehydrogenase, and glutathione S-transferase activities in rat liver by dietary administration of eugenol. *Biochem Pharmacol* 37:799–802.
- Yokota H, Hoshino J, Yuasa A. 1986. Suppressed mutagenicity of benzo[a]pyrene by the liver S9 fraction and microsomes from eugenol-treated rats. *Mutat Res* 172:231–236.
- Yoo CB, Han KT, Cho KS, Ha J, Park HJ, Nam JH, Kil UH, Lee KT. 2005. Eugenol isolated from the essential oil of *Eugenia caryophyllata* induces a reactive oxygen species-mediated apoptosis in HL-60 human promyelocytic leukemia cells. *Cancer Lett* 225:41–52.
- Report. 1983. Carcinogenesis studies of eugenol in F344/N rats and B6C3F1 mice. National Toxicology Program Technical Report Series, No. 223.

Supplementary material available online

Supplementary Table 1 and Figures 1 and 2.