

ARTICLE

Increased Bone Mass in Male and Female Mice Following Tamoxifen Administration

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Summary: Tamoxifen is capable of preserving bone mass in gonadectomized rodents as well as intact female mice; however, a detailed 3D quantitative analysis of the structural changes produced in the growing skeleton of intact mice of both genders by this agent is lacking. Employing quantitative microcomputed tomography (μ CT), we assessed the effects of 4-hydroxytamoxifen (OHT) on the femora of C57BL/6J mice administered this agent either for 12 (males and females) or 2 (females) weeks. In mice of either gender, but especially in females, 12 weeks of OHT exposure led to dramatic increases in both cortical and trabecular bone. Females exposed to OHT for either 2 or 12 weeks demonstrated significantly increased cortical wall thickness, trabecular bone volume, connectivity, and number, as well as decreased trabecular separation. Significant increases in several of these parameters were also evident in males after 12 weeks of OHT administration. In view of the expanding use of OHT to induce Cre-mediated recombination events, our findings suggest that care should be exercised when interpreting the skeletal phenotypes of mice exposed to this agent, particularly in situations where the effects of OHT might synergize with the phenotypic outcome of a specific genetic alteration. *genesis* 45:229–235, 2007. © 2007 Wiley-Liss, Inc.

Key words: tamoxifen; microcomputed tomography (μ CT); trabecular bone; cortical bone; bone mass; Cre recombinase; estrogen receptor; Cre-ER; conditional mutagenesis

INTRODUCTION

Upon estrogenic steroid binding, the two known estrogen receptors (ER), ER α and ER β , either translocate to the nucleus to interact with corepressors and coactivators to modulate gene expression patterns, or they activate “non-genomic” cytosolic signal transduction pathways (McDonnell and Norris, 2002). Binding of selective ER modulators (SERMs) to ERs, on the other hand, can have either antagonistic or partial agonistic effects,

depending on tissue type and the ER isoform(s) expressed therein. For example, tamoxifen, a first generation SERM exhibits potent antiestrogenic effects on the mammary epithelium, but partial agonistic effects on bone and uterus. In vivo, tamoxifen is hydroxylated to the primary active metabolites 3,4-dihydroxy tamoxifen and 4-hydroxy tamoxifen (OHT), the latter being ~100-fold more potent as an antiestrogen than tamoxifen (Borgna and Rochefort, 1981).

The effects of tamoxifen in humans depend not only on the cell type, the spectrum of gene promoter estrogen response elements available for receptor interaction, and the ER subtype(s) expressed, but also the status of ovarian function (Watanabe *et al.*, 1997). Thus, although in postmenopausal women tamoxifen exhibits a mild agonistic activity, preserving and even enhancing bone mineral density of the lumbar spine (Kristensen *et al.*, 1994; Love *et al.*, 1992), in premenopausal women tamoxifen interferes with the positive skeletal effects of endogenous estrogens, thus promoting the development of osteoporosis (Powles *et al.*, 1996). How does tamoxifen bring about changes in bone? There is evidence from in vitro experiments that tamoxifen stimulates osteoblast proliferation and differentiation (Qu *et al.*, 1999); however, as ER α , ER β , and androgen receptors are also present in chondrocytes, bone marrow stromal cells, as well as osteoclasts and their progenitors (Bellido *et al.*, 1995; Benz *et al.*, 1991; Couse and Korach, 1999; Eriksen *et al.*, 1999; Komm *et al.*, 1988), tamoxifen-mediated effects on these cell populations may have an in vivo role.

The effects of tamoxifen on bone have also been investigated in animal models. Studies employing conven-

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tional histological and histomorphometry techniques on both ovariectomized (Turner *et al.*, 1988) and orchidectomized (Broulik, 2000; Fitts *et al.*, 2004) rats have shown that tamoxifen exhibits an antiresorptive effect on cancellous bone. A study of intact (nonovariectomized) young female mice demonstrated increased trabeculae formation in long bones upon tamoxifen administration (Perry *et al.*, 2005), a finding that is opposite to that observed in premenopausal women treated with this agent. The animal models reported thus far to study tamoxifen effects on bone have been based primarily on treatment of ovariectomized mice or rats. There is scanty evidence regarding the effects of tamoxifen in intact male mice (Wakley *et al.*, 1988), and to our knowledge there is only one study in which the effects of tamoxifen were evaluated using conventional methods in intact female mice (Perry *et al.*, 2005).

It is of importance to quantify the effects of tamoxifen in skeletal tissue of intact mice, as tamoxifen is increasingly being used to induce Cre recombinase-mediated genetic alterations in specific cell types. In the case of osteoclast or osteoblast specific alterations (cells with a high turnover rate particularly during early skeletogenesis and remodeling), tamoxifen might need to be administered for an extended period of time in order for sufficient "genetically-altered" cells to accumulate and produce a bone phenotype. Furthermore, since a quantitative 3D analysis of the skeletal changes in response to this agent had not been reported, we employed microcomputed tomography (μ CT) to investigate the effects of tamoxifen administration on the microstructure of growing skeletons of intact C57BL/6J mice of both genders.

MATERIALS AND METHODS

Animals and Treatment Protocol

Twenty four-week-old C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, MA). Ten control mice consisting of five mice of each gender were injected intraperitoneally two times per week with 100 μ l of corn oil vehicle (Acros Organics, Geel, Belgium). Ten experimental mice (five of each gender) received bi-weekly intraperitoneal injections of 0.1 mg OHT (Sigma Aldrich, Oakville, ON) dissolved in corn oil. Injections started at 5 weeks of age and continued for 12 weeks. This dosage was based on the publication by Indra *et al.* (1999), where the OHT was shown to cause complete nuclear translocation of the CreER^{T2} recombinase protein. The period of administration was chosen, as this might be required when studying tamoxifen-inducible gene alterations in relatively short-lived skeletal cells, such as osteoclasts. To study the effects of a shorter period of OHT exposure, female mice (6 weeks old) were injected with OHT (12 mice), or vehicle (12 mice), three times per week for 2 consecutive weeks; then, after a 2-week recovery period, the mice were analyzed. All mice were maintained in a barrier facility in

accord with University of Calgary Animal Care Committee and Canadian Council on Animal Care guidelines. At the end of the treatment periods mice were euthanized by CO₂ inhalation. The right lower limb was removed at the level of the hip in preparation for μ CT scanning.

Microcomputed Tomography

μ CT scanning was performed (*vivaCT 40*, Scanco Medical, Bassersdorf, Switzerland) on each femur at a nominal isotropic resolution of 10 μ m at two locations on the femur (55 kVp, 109 μ A, 400 ms integration time, 2,000 projections on 360°, 2048 CCD detector array, and cone-beam reconstruction). The scan for trabecular analysis was located in the metaphyseal region of the distal femur and included a 1 mm region (100 slices) located 0.1 mm below the growth plate-metaphyseal junction. For the cortical analysis, a 1.5 mm region of the midshaft of the femur was scanned.

Morphological Analysis

Trabecular and cortical regions were separated by semi-automatically drawn contours (Laib *et al.*, 2000, 2001), and the complete secondary spongiosa of the distal femur as well as the cortical bone in the midshaft were evaluated. The resulting gray-scale images were Gaussian filtered ($\sigma = 1.2$, support = 1) and globally thresholded (15% of maximum gray value) to form binarized images on which morphological analyses were performed. Three-dimensional analysis techniques were utilized to assess bone volume ratio (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular number (Tb.N) (Hildebrand and Rüeggsegger, 1997), and connectivity density (Conn.D) (Odgaard and Gundersen, 1993).

RESULTS

Qualitative and Quantitative μ CT Analysis of OHT-Treated Mice

Using μ CT, morphological analyses of trabecular and cortical bone structure were performed on (i) female and male mice after 12 weeks of OHT treatment, as well as on (ii) female mice injected with OHT for 2 weeks followed by a 2-week recovery period. For the latter study, females were selected as they showed the most dramatic responses to OHT in the 12-week study described immediately below. Increases in bone mass were observed by μ CT after 12 weeks of treatment with OHT in both female and male mice (Fig. 1). Trabeculae at the distal metaphysis of the femurs were increased in volume, number, and thickness, and cortical bone of the midshaft region was obviously thickened, especially in female mice (Fig. 1a,b). Trabecular bone in the distal femoral metaphysis was increased in both the male and female mice after 12 weeks of OHT treatment, as well as in female mice treated with OHT for only 2 weeks (Fig. 2). Similarly, cortical wall thickness was increased in all OHT-treated groups as compared with the corn oil

(vehicle)-only injected groups (Fig. 2f). A quantitative morphological assessment of the trabecular bone revealed significantly increased bone volume ratio in the

group treated with OHT for 2 weeks ($P < 0.05$), and this difference was even more pronounced after the 12-week OHT exposure period ($P < 0.002$) (Fig. 2a). Other significant changes to the trabecular bone included elevated trabecular number (Tb.N) and reduced trabecular separation for all groups ($P < 0.05$) (Fig. 2c,e). Although an increase in connectivity density (Conn.D) was observed in the female mice after both the 2 and 12 weeks of OHT treatment ($P < 0.05$), this was not evident in the male mice ($P = 0.063$) (Fig. 2b). The increase in the trabecular thickness of the OHT-treated mice was significant for the male and female cohorts treated with OHT for 12 weeks ($P < 0.05$), but was not for the female mice exposed to OHT for 2 weeks ($P = 0.350$) (Fig. 2d). The lack of increased trabecular thickness after the 2-week OHT exposure may indicate that elevated trabecular bone volume ratio was because of new trabeculae being formed, rather than to thickening of existing trabeculae. This notion was supported by the observed increased connectivity density and trabecular number in the 2-week OHT cohort (Fig. 2b,c). The trend toward increased trabecular bone in the OHT group was paralleled at the level of the midshaft cortex, where there was a trend toward cortical thickening (Ct.Th; $P < 0.05$) in all the OHT exposed groups. Although this reached significance in both female cohorts ($P < 0.05$), it was not significant in the male cohort ($P = 0.057$), likely a reflection of the relatively small sample size.

DISCUSSION

This μ CT study demonstrates that OHT administration to C57BL/6J mice over a 12-week period, at a dose typical of those used to activate Cre-ER fusion protein activity in transgenic lines (Indra *et al.*, 1999), leads to striking microarchitectural changes in femoral bones of intact male and female mice (Fig. 1). In female mice, the trabecular and cortical parameters were all significantly increased by OHT. Although OHT produced similar incremental effects on the trabecular bone parameters of males, a significant difference in cortical wall thickness was not observed, and only a positive trend was evident. Furthermore, quantitative analysis of the μ CT data demonstrated significant changes in the femoral bone structure of female mice stemming from only a 2-week OHT treatment schedule. This latter result is particularly important given that OHT dosing regimens for most applications involving activation of Cre recombinase-ER fusion proteins in mice with “floxed” alleles

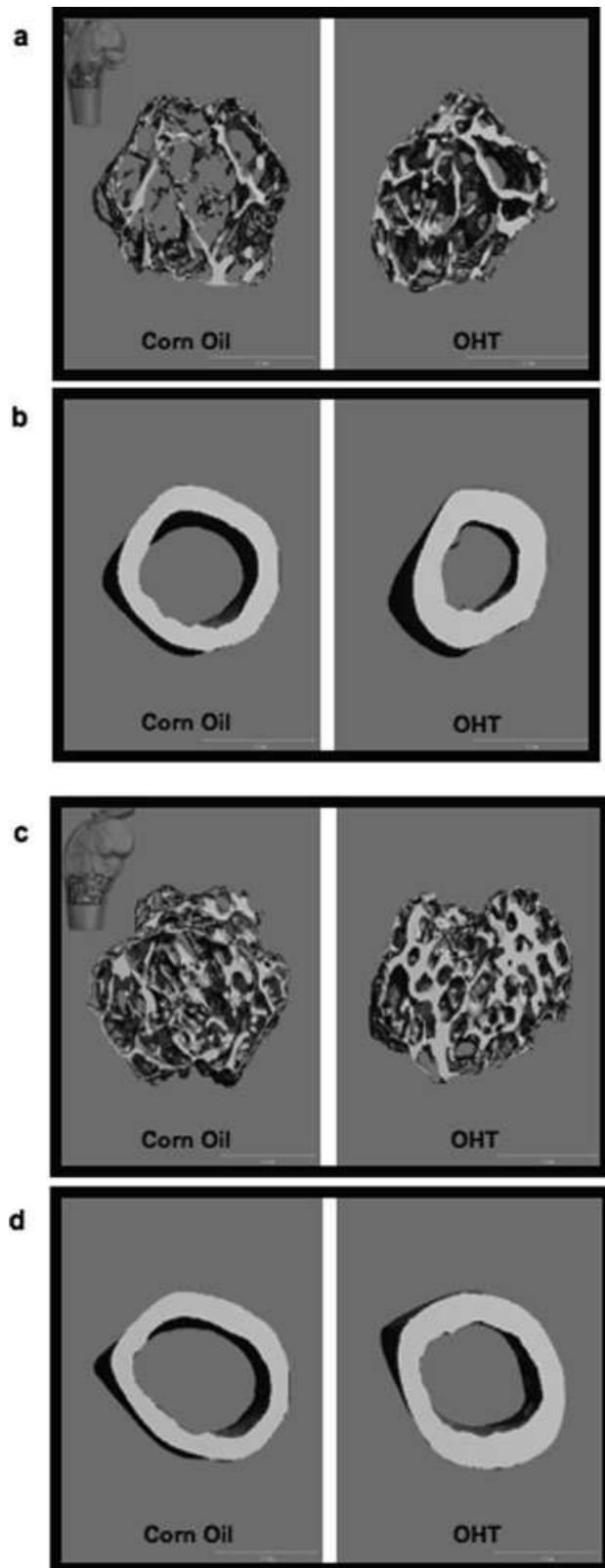


FIG. 1. Representative μ CT images of female (a, b) and male (c, d) C57BL/6J mouse trabeculae of the distal metaphysis of the femur and femoral midshaft cortical region from each of the 12-week treatment groups (males versus females, OHT in corn oil versus corn oil). (a, c): region employed in the trabecular analysis is shown in upper left corner. Note the increased trabecular bone volume, number, and thickness subsequent to OHT administration in both sexes. (b, d): cortical bone of femoral midshaft thickness with and without OHT administration. In both sexes, and especially in the females, cortical thickening is observed following OHT treatment.

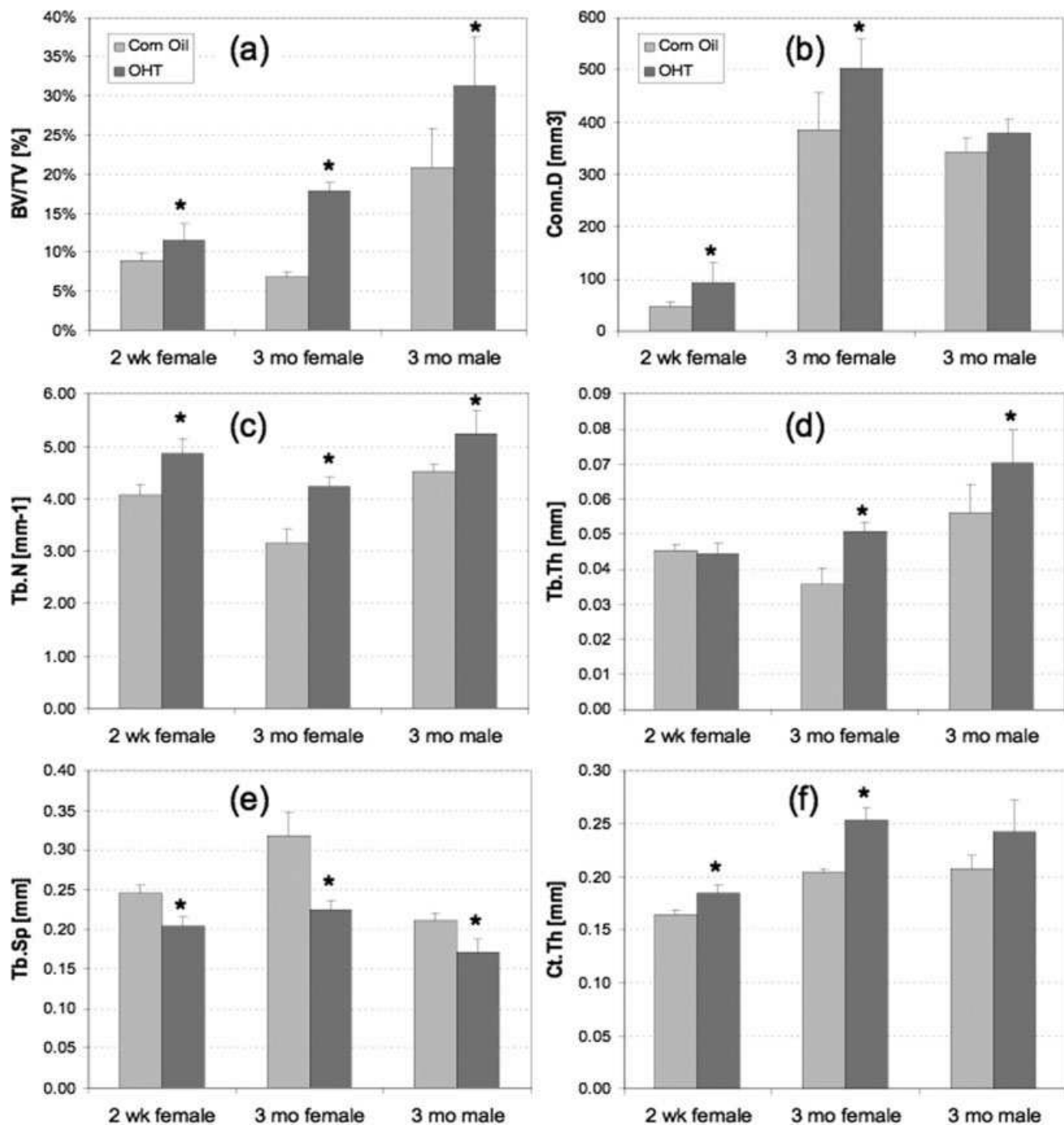


FIG. 2. Morphological analysis by quantitative μ CT of the metaphyseal (a–e) and midshaft cortical (f) regions of C57BL/6J femora. Results are shown for male and female mice injected with either OHT in corn oil versus corn oil (vehicle controls) for 3 months (12 weeks), or for females treated for 2 weeks followed by a 2-week recovery period. Differences between groups (corn oil versus OHT in corn oil) were analyzed using an unpaired *t*-test. Significant differences are noted by “*” ($P < 0.05$). Compared with the corn oil control groups, the OHT treated animals exhibited increased bone volume ratio (BV/TV), connectivity density (Conn.D), trabecular number (Tb.N), trabecular thickness (Tb.Th) and midshaft cortical wall thickness (Ct.Th), and decreased trabecular separation (Tb.Sp).

would generally be administered over a 1–3 week period. Given that males showed less pronounced increases in bone after the 12 weeks of OHT, it is unlikely that male mice exposed to OHT over a 2-week period would show significant changes in bone structure.

The mechanism(s) through which tamoxifen brings about its skeletal effects remains somewhat nebulous. There is *in vitro* evidence that osteoclast functions are inhibited by tamoxifen in an ER-independent manner; this is thought to occur by inhibition of H^+ -ATPase activity

as well as disruption of calmodulin dependent signaling processes (Williams *et al.*, 1996; Zhang *et al.*, 2003). Tamoxifen has also been found to induce apoptosis in osteoclasts by interfering with calmodulin binding to the Fas death receptor (Wu *et al.*, 2005). In addition, tamoxifen may also act to increase skeletal growth through ER-mediated pathways. Thus, in osteoblasts, tamoxifen may act via stimulation of ER α , since estrogen-induced bone formation in intact mice requires activation function-1 domain of ER α (McDougall *et al.*, 2003). Furthermore, tamoxifen appears to inhibit the actions of bone resorption-promoting cytokines such as IL-1, IL-6, and TNF α on osteoblasts, likely via a trans-repression of NF- κ B as a result of ER α activation (Quaedackers *et al.*, 2001).

To try to define the *in vivo* roles of the individual ER receptors (α and β) on skeletal remodeling, mice having either targeted disruptions of each receptor, or both receptors, have been generated. It was demonstrated that ER α was responsible for regulating bone remodeling in male mice, whereas both ER α and ER β were involved in bone remodeling in females and were able to compensate for each other (Sims *et al.*, 2002). Although the increased bone mass of males is primarily attributed to the effects of androgens, it is also evident that estrogenic steroids play a key role in the maintenance of bone mass. Hence, osteoporosis was observed in human and rodent males deficient in aromatase (Bilezikian *et al.*, 1998; Carani *et al.*, 1997; Oz *et al.*, 2000), as well as in a man expressing a truncated, nonfunctional ER α (Smith *et al.*, 1994). When both estrogens and androgens were depleted in male mice by the combination of orchidectomy (ORX) and genetic aromatase deficiency (ArKO), the resulting defect in femoral trabecular bone of the ORX/ArKO mice was more severe than that of ORX-alone. The latter suggested a role for estrones derived from extragonadal sources in the maintenance of cancellous bone in males (Matsumoto *et al.*, 2006).

Our study shows that OHT administered to nongonadectomized mice during puberty and early adulthood for a period of 3 months increases femoral cortical bone thickness, as well as trabecular bone volume, number, and thickness in both males and females. Previous studies have shown that tamoxifen was able to prevent bone loss in immobilized (Wakley *et al.*, 1988), as well as in gonadectomized male rats (Broulik, 2000; Fitts *et al.*, 2004). To our knowledge, however, this is the only study demonstrating the effects of OHT on the cortical and trabecular bone of the femurs of intact growing male mice, and the first application of quantitative 3D μ CT to the study effects of the responses of mouse femora to this agent. It is important to note that our study has been confined to analysis of a long bone, and as such may not necessarily reflect changes that might be occurring in response to OHT at other sites, such as vertebrae, membranous bones, or bones of neural crest origin.

The results of this study have important implications regarding the use of transgenic animals to study the musculoskeletal system. Specifically, our findings will be relevant to the use of tamoxifen-inducible Cre-*loxP* technol-

ogy to carry out conditional gene alterations in skeletal cells. Such approaches make use of either tissue-specific or ubiquitously-expressed transgenes encoding a protein that consists of the bacteriophage-derived Cre recombinase fused to the ER ligand-binding domain (Cre-ER) (Danielian *et al.*, 1998; Feil *et al.*, 1996; Metzger and Chambon, 2001; Schwenk *et al.*, 1998). Similar to the ER, the Cre-ER fusion protein is predominantly localized to the cytosol, until ligand (OHT) binding triggers its translocation into the nucleus where *loxP*-containing gene recombinations can be triggered.

If gene alterations are to be induced in long-lived cells such as those lining the kidney collecting ducts (Yang *et al.*, 2006), neurons, cardiomyocytes (Li *et al.*, 2005), or skeletal muscle cells (Schuler *et al.*, 2005), the transient (1–3 weeks) administration of tamoxifen to achieve permanent gene alterations in the target population minimizes the possibility of unwanted effects being introduced by the inducer itself. On the other hand, when studying cell populations that are rapidly “turning-over”, such as epidermal cells (Stratis *et al.*, 2006), enterocytes, neutrophils, and importantly, osteoblasts and osteoclasts (particularly during rapid phases of skeletal growth), tamoxifen may need to be administered for protracted periods of time, in the order of many weeks, depending on the expected phenotype. The downside of long term OHT administration is obviously the potential for disturbances in the wide range of physiological parameters that are normally regulated by endogenous estrogenic steroids. While the necessity for long-term OHT administration could theoretically be obviated by the use of transgene promoters that result in Cre-ER expression that is restricted to stem cell populations, such cells can give rise to multiple cell types (for example, mesenchymal stem cells that generate various cell types, or the multiple lineages generated by hematopoietic stem cells) making this strategy unworkable for the analysis of gene alterations in specific subsets of proliferating or terminally-differentiated cells.

In the case of Cre-ER fusion proteins expressed in developing osteoblasts and odontoblasts (Kim *et al.*, 2004) in particular, it will be important to determine whether a given phenotype is due either to the specific *floxed* gene alteration being studied, to the tamoxifen used to induce the alteration (done via the use of tamoxifen-treated controls), or, importantly, to a combination of the two. The latter could include, for example, a unique skeletal phenotype stemming from the combination of tamoxifen's ability to inhibit osteoclasts and an osteoblast-specific gene deletion. Such a scenario might require the use of an alternative method for inducing an osteoblast-specific gene deletion, enabling the phenotypic effects of the specific gene alteration to be evaluated in isolation from the contribution of tamoxifen.

Lastly, the duration (12 weeks) of tamoxifen administration in our comparative study of male and female mice was relatively long, and the increased bone we observed on the murine femurs was correspondingly dramatic. The latter result raised the possibility that

shorter, more typical, exposures of OHT might also lead to quantifiable changes in long bone structure. Consistent with this, we found that even a 2-week course of OHT administration to young female mice was capable of significantly affecting femoral skeletal morphology. As for how long it would take for the skeletal changes to return to baseline after cessation of OHT administration, this would depend on the rate of bone remodeling in the region being studied, which, in turn would depend in part on the mechanical stresses in that region. Taken together, our findings show that caution should be used when interpreting bone phenotypes in situations where OHT is used as the inducer of a genetic alteration relevant to the skeleton, particularly in situations where appropriate controls are difficult to achieve, such as when a synergy exists between OHT and the phenotype stemming from the loss of specific “*floxed*” gene. It would be prudent to use male mice for skeletal studies, as we found that the effects of OHT on femoral bone in this gender were less striking. Furthermore, prior to using OHT to trigger genetic alterations, investigators should determine the minimal duration of OHT exposure required to achieve the desired gene modification in the target cell population.

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