0013-7227/96/$03.00/0
Endocrinology

Vol. 137, No. 5
Printed in U.S.A.

Alterations of Maternal Estrogen Levels During Gestation Affect the Skeleton of Female Offspring*

SILVIA MIGLIACCIO, RETHA R. NEWBOLD, BILL C. BULLOCK, WENDY J. JEFFERSON, FRANK G. SUTTON JR., JOHN A. MCLACHLAN, AND KENNETH S. KORACH

Receptor Biology Section (S.M., F.G.S., K.S.K.), Laboratory of Reproductive and Developmental Toxicology, and Developmental Endocrinology Section (R.R.N., W.J.J.), Reproductive Toxicology Group, Toxicology Branch, Environmental Toxicology Program, National Institute of Environmental Health Science, Research Triangle Park, North Carolina 27709; II Division of Endocrinology (S.M.), Department of Experimental Medicine, University of Rome "La Sapienza," 00191 Rome, Italy; the Department of Comparative Medicine (B.C.B.), Bowman-Gray School of Medicine, Wake Forest University, Winston-Salem, North Carolina 27109; and the Center for Bioenvironmental Research (J.A.M.), Department of Pharmacology, Tulane University, New Orleans, Louisiana 70112-2699

ABSTRACT

Estrogens have important effects on bone turnover in both humans and experimental animal models. Moreover, the decreased level of estrogens after menopause appears to be one of the key factors in determining postmenopausal osteoporosis. The presence of estrogen receptor in both osteoblasts and osteoclasts has suggested a direct role of these steroid hormones on bone tissue. Thus, this tissue is now regarded as a specific estrogen target tissue. Exposure to estrogens during various stages of development has been shown to irreversibly influence responsive target organs. We have recently shown that transient developmental neonatal exposure (days 1–5 of life) of female mice to estrogen resulted in an augmented bone density in the adult animals. The aim of the present study was to evaluate whether short-term modification of maternal estrogen levels during pregnancy would induce changes in the skeleton of the developing fetuses and to identify any long-term alterations that may occur. Pregnant mice were injected with varying doses (0.1–100 μg/kg maternal BW) of the synthetic estrogen diethylstilbestrol (DES) from day 9–16 of pregnancy. Offspring were weaned at 21 days of age, and effects on bone tissue of the female mice were evaluated in adulthood (6–9 months of age). Prenatal DES treatment(s) did not significantly affect BW. However, a dose-dependent increase in bone mass, both in the trabecular and cortical compartments, was observed in the prenatal DES-exposed female offspring. Furthermore, long bones of DES-exposed females were shorter than controls. Normal skeletal mineralization accompanied these changes in the bone tissue, as shown by a parallel increase in skeletal calcium content. Double tetracycline labeling performed in 6-month-old DES-exposed animals showed an increase in mineral apposition rate in adult DES-exposed mice as compared with untreated control animals, although no significant difference in the circulating estrogen levels was found in animals of this age. Experiments were then performed to evaluate whether perturbation of the estrogen surge at puberty in these diethylstilbestrol (DES)-exposed mice could reverse the observed changes. Femur length was chosen as a marker of potential estrogenic effect. Prepubertal ovariectomy of the prenatally DES-treated animals could only partially reverse the effects observed in the skeleton of the DES-treated animals. Further experiments were performed to evaluate whether these changes could have occurred *in utero*. CD-1 pregnant female mice were injected with DES (100 μg/kg maternal BW) from days 9–15 of gestation. On day 16 of gestation, fetuses were examined and stained by a standard Alizarin Red S and Alcian Blue procedure to visualize calcified and uncalcified skeletal tissue. Estrogen treatment induced an increase in the amount of calcified skeleton as compared with untreated controls and also a decrease in the length of long bones, strongly suggesting a change in both endochondral ossification and endosteal and periosteal bone formation. In summary, these data show, for the first time, that alterations in the maternal estrogenic levels during pregnancy can influence early phases of fetal bone tissue development and subsequently result in permanent changes in the skeleton. Finally, the effect of this short-term estrogen treatment can be seen in the fetal skeleton, suggesting an estrogen-imprinting effect on bone cell-programming in fetal life because treatment effects on bone cell turnover can be observed later in adult life. (*Endocrinology* **137:** 2118–2125, 1996)

ESTROGENS exert their biological actions on target organs through binding by a specific estrogen receptor (ER), a member of the steroid receptor superfamily (1–3). The equilibrium of the circulating levels of these steroid hormones and a normal expression of ER in target organs is of primary importance for the normal homeostasis of target tissues, *i.e.* uterus (4–6). Furthermore, an imbalance and/or an increase of the circulating levels of these steroids is also believed to be crucial for the development of hyperplasia/hypertrophy of the target organs such as the uterus (7). Specifically, it has been postulated that estrogens may have different effects on estrogen target tissues depending on the age and the length of exposure (8–10). Several experimental animal models have been developed (8, 9, 11, 12) on the basis of potential human exposure to estrogenic compound(s) (7, 10, 13) at different periods of organogenesis to further evaluate the mechanism of action of estrogens in target organs during critical periods of body maturation.

Interestingly, estradiol (E_2) has been described to modulate bone cell activity *in vitro* (14). Beside these *in vitro* effects of E_2, it is well known that changes in estrogen levels can dramatically modify bone tissue turnover in both postmeno-

Received October 6, 1995.
Address all correspondence and requests for reprints to: Dr. Silvia Migliaccio, Histology and General Embryology, Via A. Scarpa 14, University of Rome "La Sapienza," 00161 Rome, Italy.

* Part of these studies was presented at the XI International Conference of Calcium Regulating Hormones, Florence, Italy, 1992.

pausal women (15, 16) and adult experimentally induced postmenopausal animals (17, 18). In addition, long-term exposure of adult animals to E_2 or DES induces hyperplasia of bone tissue (18–19). There is little doubt that estrogens are considered the most effective treatment for postmenopausal osteoporosis (15, 16, 21). Lately, biologically active ER has been recently described in bone cells (22, 23), leading to the hypothesis that estrogens modulate bone cell homeostasis through specific receptor-mediated mechanism(s) (22–25). Thus, these data strongly suggest that bone tissue directly responds to estrogens as other estrogen target tissues do, and, therefore, bone tissue is now regarded as an estrogen target organ. However, there are no definitive data on the biological role that estrogens play in bone tissue physiological regulation during the period preceding maturity. In fact, few studies have investigated how disruption of estrogen levels before puberty can influence bone turnover and bone mass in girls (26, 27). Other studies have dealt with estrogen effects on growing postpubertal rats (28).

In previous studies, we have developed an animal model to elucidate the role that estrogens play in early phases of life on the skeletal formation and on the final adult peak bone density (29). The results of these earlier studies have shown that transient neonatal exposure of female mice to estrogens increased bone mass in the animals during adulthood (29). Furthermore, a similar animal model has been reported by Iguchi and colleagues (30), who showed that sex steroids can influence the development of the dimorphism in the mouse innominate bone. Thus, these data strongly suggest that alterations of estrogen levels before puberty, in early phases of development, can dramatically influence skeletal maturation, including final peak bone density (29).

The aim of the present study was to determine whether perturbations in the maternal estrogen levels during pregnancy can induce modifications to the developing fetal skeleton in a permanent manner and to evaluate if these effects could also be observed in both prenatal and postnatal periods.

Materials and Methods

Animals

Outbred CD-1 mice were purchased from Charles River Breeding Laboratories (Wilmington, MA), or bred to male mice of the same strain at our animals quarters at the NIEHS breeding colony (Research Triangle Park, NC). Detection of vaginal plug was considered day 0 of pregnancy. Animals were housed in groups of four (pregnant mice were housed individually) in a temperature-controlled room (21–22 C) with 14-h light and 10-h dark periods and were provided with synthetic bedding, fresh water, and NIH 31 laboratory mouse chow *ad libitum*.

Chemicals and materials

DES, demeclocycline, and oxytetracycline were purchased from Sigma Chemical Co. (St. Louis, MO). Purity of DES was checked by HPLC chromatography and mass spectrometry. Decalcifying solution was purchased from Baxter Scientific (Charlotte, NC).

Animal experiments

Pregnant mice were treated from days 9–16 of gestation in accordance with ethical guidelines and an approved NIEHS animal protocol. The daily doses of DES used were 0.1, 2.5, 5, 10, 50, and 100 μg/kg maternal BW. DES was dissolved in corn oil (DES groups) or corn oil alone as control (CTL groups) as previously described (8). Treatments were administered sc in the pregnant female. At birth, litter sizes were standardized to eight offspring. At 25 days of age, mice were weaned and housed four per cage for 7–9 months. On the day of sacrifice, animals were weighed and killed by decapitation. Blood was collected to evaluate steroid hormones levels. Blood samples were centrifuged for serum isolation (800 $\times$ *g*, 4 C, 30 min) and serum samples were kept at -20 C until the RIA was performed. After gross anatomical observation, samples from different skeletal sites were collected and stored in 10% formalin or saline for later evaluation. Additionally, experiments were performed to evaluate whether this effect was solely related to this prenatal short-term exposure with DES or whether was related to the endogenous onset of estrogen at puberty. To address this point, a group of prenatally DES-treated mice and vehicle-treated mice were ovariectomized at 17 days of age to prevent the physiological onset of estrogens at puberty, which, in thse animals, occurs approximaly at 21 days of age. The total number of female mice used in each experimental group in the different experiments is reported in the tables or figure legends.

Physical and biochemical measurements

After removal of soft tissue, left femurs were collected by dissection, defatted, and processed (28). Bones were extracted for 8–12 h with absolute ethanol and then with ether. At the end of this procedure, femurs were weighed and their length measured from the great trochanter to the external condyle. Dried femurs were then ashed at 600 C for 24 h and weighed. The ashes were dissolved in 5 M HCl, then diluted in distilled water, to perform total calcium (Ca) measurements (28). Total Ca was measured by using a Calcette analyzer system.

Histological evaluations

Right femurs and lumbar vertebrae were fixed in 10% neutral buffered formalin, decalcified, embedded in paraffin, and sectioned at 6 μm. Tissue sections were stained with hematoxylin and eosin (H&E) and evaluated using a light microscope.

Computer-assisted analysis and histomorphometric measurements

The same tissue sections prepared for histological analysis were evaluated for histomorphometric measurements using a SMI-Microcomp image-analysis system (Southern Micro Instruments, Inc., Atlanta, GA) interfaced with an IBM computer and an Olympus BH-2 microscope. Areas of interest were selectively traced; areas bounded by tracing were automatically calculated by the computer as previously described (28). Corresponding lumbar vertebrae sections were chosen from control and DES-treated animals. Triplicate sections were measured and the mean determined for each mouse analyzed in the present study.

Double tetracycline labeling

Six month old female CD-1 mice, who were exposed to DES 100 μg/kg maternal BW during the prenatal period, and unexposed control mice were injected intraperitoneally with demeclocycline and oxytetracycline (20 mg/kg BW) 14 and 7 days before sacrifice. Twenty four h after the last administration, animals were killed. On day of sacrifice, femurs from control and prenatally DES-treated animals were fixed in 70% ethanol. Samples were then embedded in hard plastic and sections obtained using a heavy-duty microtome. Double tetracycline staining was then evaluated using a SMI Microcomp image system interfaced with an IBM computer connected with an Olympus BH-2 microscope under epifluorescent light. Measurements were performed at the level of distal epiphysis of the femurs, at least 2-mm distal from the epiphyseal plate as previously described (18). Mineral apposition rate was obtained by dividing the distance between the two labeled surfaces by the days of the labeling period.

Alizarin red S and alcian blue staining for fetal skeletal alterations

Fetuses were collected on the morning of the 16th day of pregnancy by Caesarian section. They were examined to determine their gender and then were placed in 95% ethanol (EtOH) for 24 h. EtOH was then discarded and fetuses were placed in Alcian Blue stain overnight. The following morning Alcian blue stain was discarded and replaced by 95% EtOH for the after 24 h. At the end of this period, fetuses were placed in Alizarin Red S for an additional 8 h, after which they were transferred to 0.5% potassium hydroxide (KOH) overnight. Fetuses were then analyzed by an using an Olympus Microscope interfaced with an Image Analysis system software.

Statistical analysis

Means of each independent exposed group were statistically evaluated by Student's t test or by one-way ANOVA followed by nonparametric Dunnett's test (26). Significant differences were considered when a $P < 0.05$ was found.

Results

Because our previous studies showed that developmental exposure to estrogens could significantly augment bone mass at the level of both trabecular and cortical compartments in adult mice, similar analysis was performed to examine bone features of these prenatally DES-exposed animals at 6–9 months of age. Cross-sections of lumbar vertebrae of control and prenatally DES-exposed animals were evaluated by histological analysis. Figure 1 shows histological samples from control mice and mice prenatally exposed to different doses of DES. These data show that maternal modifications of estrogens during pregnancy, a period in which major fetal organogenesis is occurring in mice, resulted in an increase in the bone mass of these adult female mice. Nevertheless, the body weight of DES-exposed mice was not significantly different from unexposed mice (Table 1). Additionally, no major macroscopic modifications of the reproductive tract, except at the high DES dose, were observed at the age at which the animals were analyzed.

Vertebral bone samples were also evaluated by a computerized image-analysis system to accurately quantify the bone mass. Figure 2 depicts the dose-dependent increase in the bone mass of DES-exposed mice as compared with control animals. Maximal effects were observed at doses of 5 and 10 μg/kg/maternal BW of DES. Higher doses did not induce further increase in bone mass.

In addition, femurs were evaluated for other physical skeletal characteristics of these mice and for skeletal mineralization. Prenatally DES exposed mice had shorter femurs than age-matched control animals. As described for the increase in bone mass, this event occurs in a dose-dependent manner (Table 1). Evaluation of the dry weight and the ash content of the femurs of control and DES-exposed animals was also performed as shown in Table 1. A minimal dose-dependent increase of these parameters can be observed with maximal effect reached at doses between 2.5 and 10 μg/kg maternal BW, in accordance

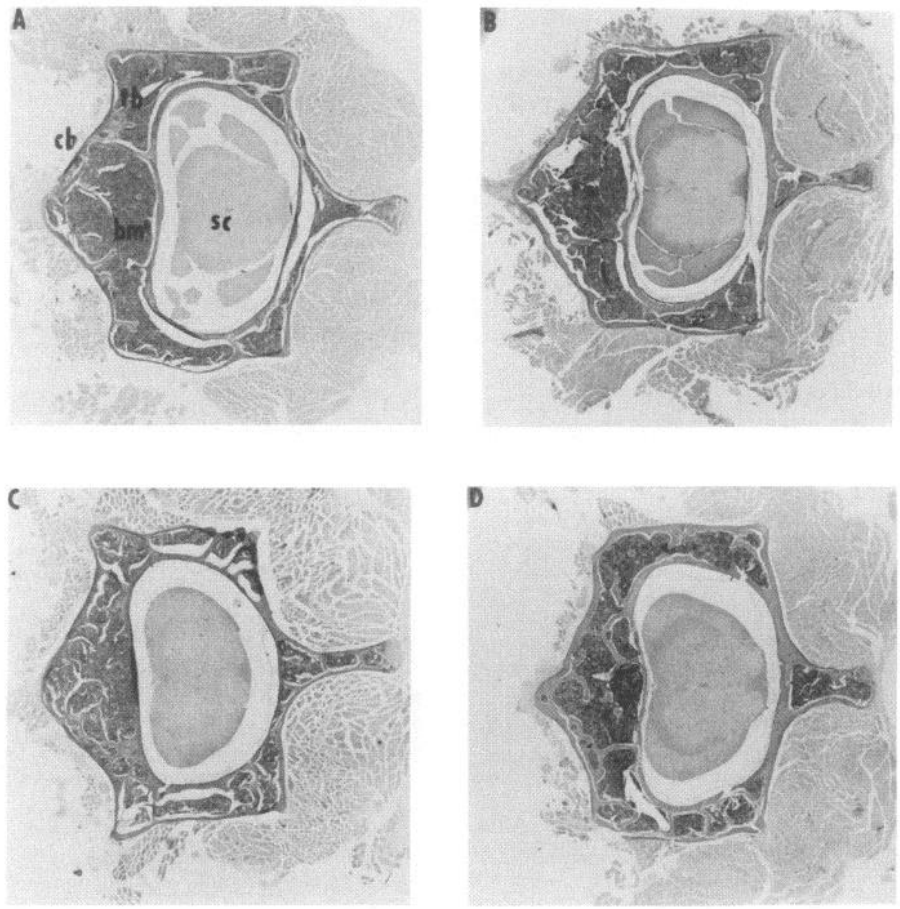

FIG. 1. Histological bone sections of DES-exposed and control mice. Cross-sections of lumbar vertebrae from (A) control and animals exposed to (B) DES 2.5 μg/kg/maternal BW, (C) 10 DES μg/kg/maternal BW and (D) DES 50 μg/kg/maternal BW from days 9–16 of fetal life. H&E ×40. Cb, compact bone; tb, trabecular bone; sc: spinal cord.

TABLE 1. Physical characteristics and skeletal biochemical parameters of 7- to 9-month-old female mice exposed to different doses of DES during gestation

Group	Body weight (g)	Femur length (mm)	Dry weight (mg/mm)	Ash (mg/mm)
Control (15)	49.13 ± 1.07	15.64 ± 0.15	3.77 ± 0.07	2.27 ± 0.04
0.1 (5)	55.94 ± 4.11	15.79 ± 0.27	4.04 ± 0.11	2.48 ± 0.01
2.5 (5)	49.54 ± 1.87	15.77 ± 0.42	3.67 ± 0.14	2.09 ± 0.13
5 (9)	55.91 ± 3.29	15.72 ± 0.23	4.25 ± 0.15	2.59 ± 0.10
10 (12)	48.42 ± 1.71	15.39 ± 0.12	4.00 ± 0.10	2.34 ± 0.06
50 (5)	54.59 ± 2.52	15.08 ± 0.30	4.16 ± 0.13	2.50 ± 0.09
100 (15)	49.27 ± 1.68	14.93 ± 0.22[a]	3.86 ± 0.12	2.33 ± 0.07

Doses of DES shown are considered as μg/kg maternal BW (days 9–16 of gestation). Length was measured from the great trochanter to the external condile of right femurs. Dry weight of same femurs were assessed after 9–12 h EtOH and 9–12 h of ether soxlet extractions, as described in *Materials and Methods*. Ashes were estimated after femurs were placed for 24 h at 600 C. Both weight and ash values were normalized per femur length. Results are expressed as mean ± SE. Numbers in parentheses represent animals evaluated in each group.

[a] $P < 0.05$ *vs.* control group by Kruskal-Wallis followed by nonparametric Dunnett's test.

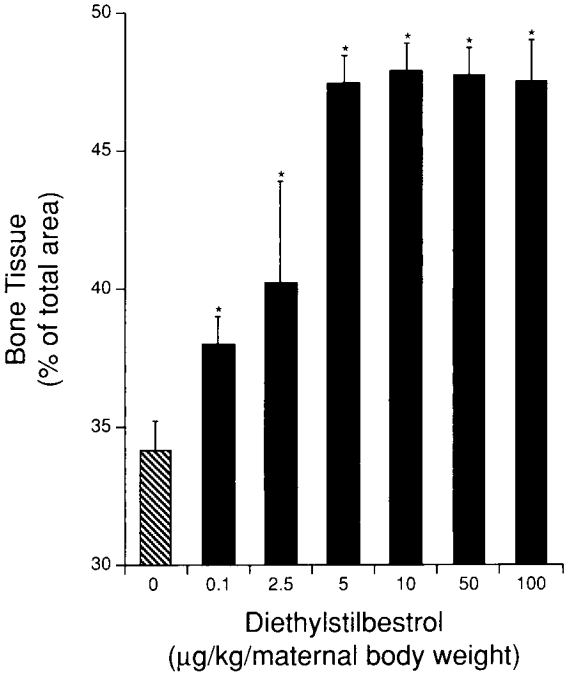

FIG. 2. Dose-dependent increase of trabecular bone mass. Value obtained from the computer image analysis of vertebral sections were transformed in percentage of bone tissue in the total area of bone considered. Data are represented as mean ± SE. *Open bar*, Control, *crosshatch bars*, prenatally DES-treated groups. *, $P < 0.05$ *vs.* control. Doses of prenatally DES-exposed mice are expressed as μg of DES/kg maternal BW (days 9–16 of uterine life).

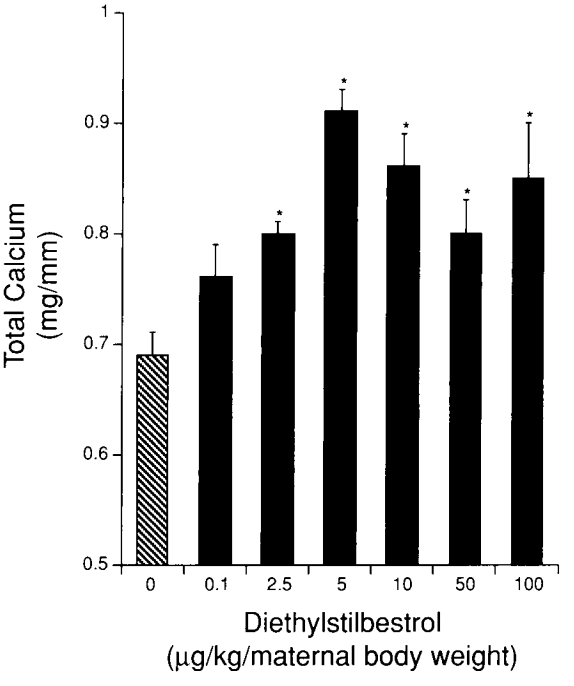

FIG. 3. Calcium content in femurs of prenatal DES-exposed animals. Values are reported after normalization to length of bone. *Open bar*, control; *crosshatch bar*, prenatally exposed group. Data are expressed as mean ± SE. Number of animals considered in each group is reported in Table 1. *, $P < 0.05$ *vs.* control group.

with femur length and bone tissue mass. However, these differences do not appear statistical different.

Calcium content in the femurs was also evaluated as an useful and valuable marker of total skeletal mineralization (32). Calcium content of the femur showed a dose-dependent increase in the femurs of the DES-exposed animals (Fig. 3) with a maximal effect as already shown for bone mass. The increase in the calcium content shows a similar profile to the increase in bone mass (Figs. 2 and 3), strongly suggesting a normal mineralization in the skeleton of the DES-exposed animals. Double-tetracycline labeling was performed in adult prenatally DES-exposed and control mice to evaluate potential alteration in the rate of mineral apposition rate (MAR), which could explain the increase in the bone mass observed. This analysis showed a significant difference in the

TABLE 2. Histomorphometric measurements in 6-month-old control and prenatally DES-exposed female mice

Group	Double-labeling width (μm)	Mineral Apposition Rate (μm/day)
Control (3)	164.72 ± 10.70	23.53 ± 1.52
DES (3)	324.89 ± 20.50[a]	46.41 ± 2.92[a]

Measurements were performed on the endosteal surface of a standardized area at least 2 mm from the metaphyseal cancellous bone of the distal femur. Data are considered mean ± SE.

[a] $P < 0.05$ versus control by Student's *t* test. Data refer to 6-month-old control (CTL) and prenatally DES-exposed mice (100 μg/maternal BW). Treatment and double-tetracycline labeling was performed as described in *Materials and Methods* section.

double labeled width (μm) and the MAR (μm/day) between control and prenatally DES-treated female mice (Table 2). Because higher levels of circulating estrogens have been linked to change in bone turnover and increased bone for-

mation, estrogen circulating levels were evaluated in the adult animals. Levels of estrogens in 6- to 9-month-old mice, prenatally DES-exposed, did not show any modifications in comparison with age-matched control mice (CTL, 26.65 ± 3.85 pg/ml; P-DES: 19.85 ± 1.71 pg/ml). Because estrogen levels in the adult animals appeared unmodified, our interest was to evaluate whether these bone cell effects developed earlier in life and whether the estrogen surge at puberty played a role in such changes.

To address this issue, mice exposed to DES before birth and corresponding control animals were ovariectomized on day 17 of age, to prevent the onset of puberty. Animals were then evaluated at 6–9 months of age. Differences in femur length were used as a marker of the estrogenic effect. As expected, length of the femurs of control ovariectomized mice were longer than control intact animals. A similar difference was detected in ovariectomized prenatally DES-treated mice *vs.* intact prenatally DES-treated mice (Fig. 4), although the difference was much smaller. Thus, ovariectomy of prenatally DES-treated animals partially reversed the shorter femur length compared with intact control animals. A significant difference in femur length, however, persisted between ovariectomized control animals and prenatally DES-treated animals (Fig. 4). Because these results suggested that the absence of the pubertial estrogen surge did not abrogate the effect induced by prenatal DES-treatment, a set of experiments were performed to evaluate whether some of the changes observed in adulthood could already appear during fetal life.

Pregnant CD-1 mice were treated with DES (100 μg/kg maternal BW) from days 9–15 of gestation. Sixteen-day-old fetuses were obtained by Caesarian section and were stained with Alizarin Red S and Alcian Blue. The differential staining allowed distinction between mineralized tissue and unmineralized cartilage in the skeleton of the embryos. Red staining delineates calcified cartilage as compared with the blue stained cartilage. The staining of these 16-day-old DES-exposed fetuses suggested a qualitative increase in the amount of mineralized skeletal tissue, as compared with unexposed fetuses (Fig. 5). Flat bones of the skull showed an increased calcification pattern as compared with control unexposed mice. Control fetuses showed also an abundance of blue staining cartilagineous skeletal tissue (Fig. 5).

Moreover, femurs and tibiae of these embryos were measured using the calcified part of the diaphysis as reference points (Fig. 5). The measurements showed a shorter length of both tibiae and femurs in DES-treated fetuses as compared with the control fetuses (Table 3). It is also interesting to note that this early alteration in the maternal hormonal levels did not induce any major abnormal development of bone tissue or any teratogenic effect on the skeleton of the offsprings (Fig. 5).

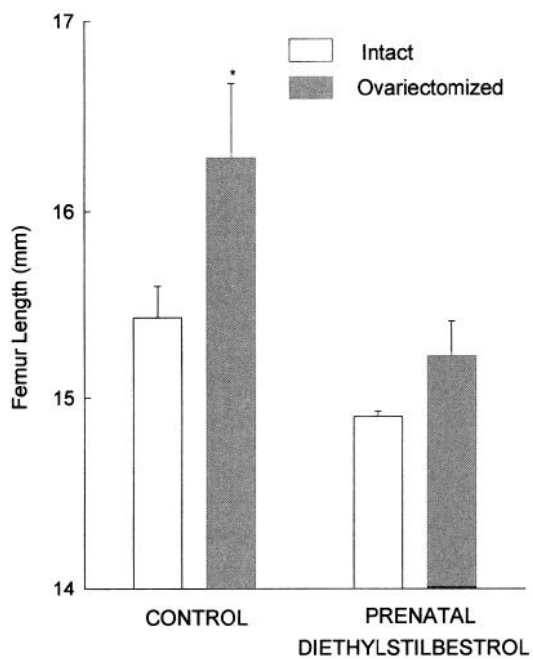

FIG. 4. Femur length in 6-month-old control and prenatally DES-exposed female mice ovariectomized or sham-operated on day 17 to prevent pubertial estrogen surge. *Open bar*, intact female; *crosshatch bar*, ovariectomy. Length was measured from the great trochanter to the external condile of right femurs. Analysis was performed as described in *Materials and Methods* section. Data are expressed as mean ± SE.

Discussion

The present study has been performed to further extend our previous investigations on the effects of changes of estrogen levels during early developmental stages on the skeleton of mice. The present results demonstrate, for the first time, that, indeed, maternal alterations of estrogen levels during gestation can dramatically influence the skeletal tissue of developing female fetuses. In fact, a significant increase in the bone mass of these animals is observed in adulthood. The increase in bone mass after this transient prenatal exposure to estrogens is analogous to the increase of bone mass previously observed in female mice postnatally exposed to DES from days 1–5 after birth (29). Shorter femur length was also noted. Both types of changes occur in a dose-dependent manner, suggesting the possibility of a specific direct effect of these sex steroids by a direct ER-mediated mechanism. The presence of ER in osteoblast-like cells (22–25) further supports the hypotheses that the observed events may occur by a direct ER-mediated mechanism. It is well known that changes in estrogen levels during adulthood can influence bone mass and the decrease in estrogen levels in postmenopausal women and ovariectomized animals result in severe osteopenia (15–17). Estrogen replacement therapy (ERT) prevents such a condition (15–17). The mechanism by which estrogen exerts these effects is not fully understood. In fact, estrogen has been shown to increase bone formation, to reduce bone resorption, to affect both, or to actually reduce and down-regulate all markers of bone turnover in postpubertal animals (16–18, 33–34). Although all these data may appear contradictory, they apparently all occur. Different doses of the hormone, route of administration, age of subjects, periods after either menopause or ovariectomy probably influence the different outcomes reported. In the model described in this paper, MAR is increased in the adult animals after the short-term prenatal exposure to estrogen as shown by the results presented by other authors (18, 33–34).

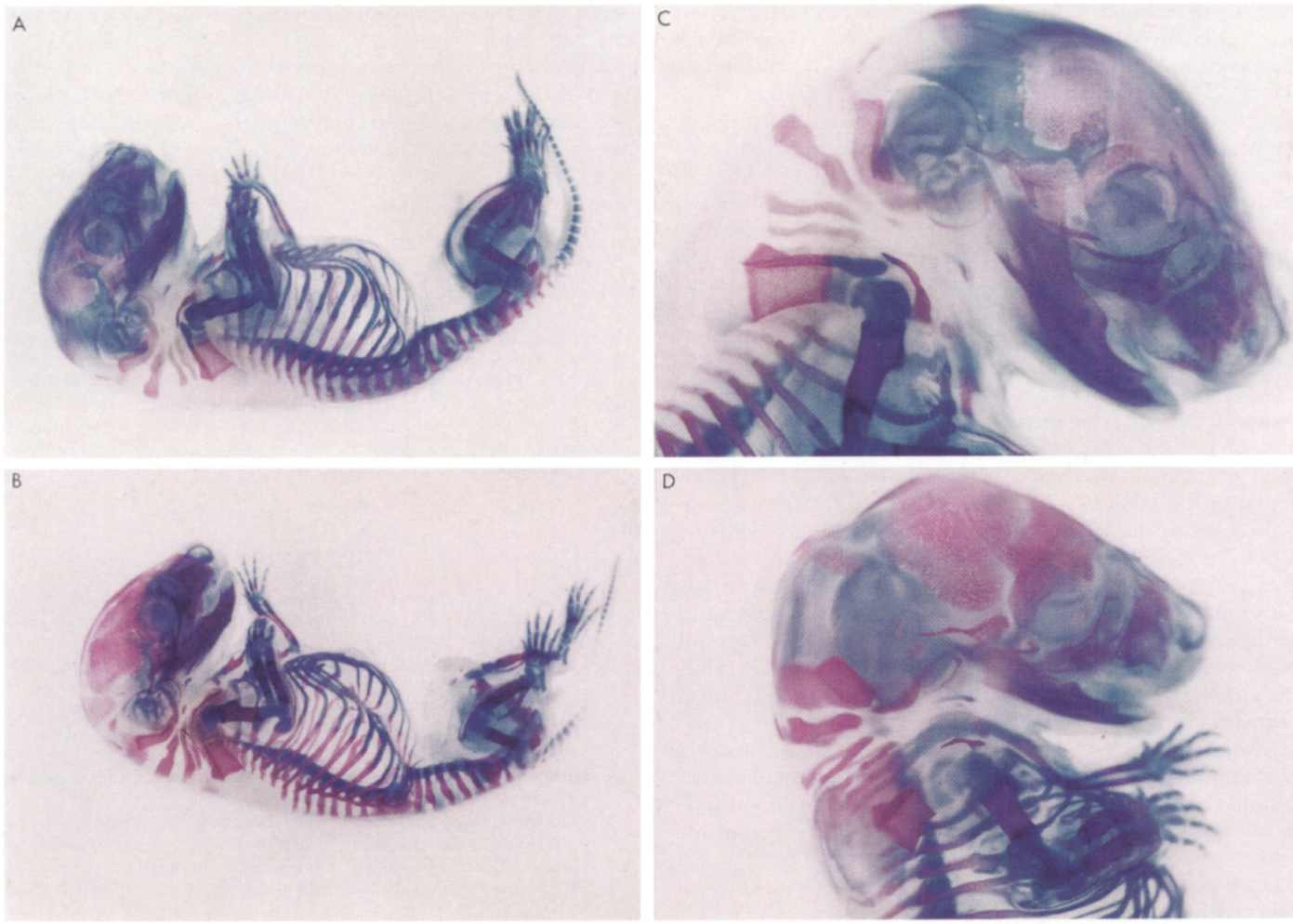

FIG. 5. Control and prenatally DES-treated mice were examined on day 16 of gestation. A and C, Control (CTL); B and D, diethylstilbestrol (DES) exposed mice. CTL and DES mice shown in the pictures are one representative of three different litters for each treatment. Alcian Blue stains for cartilagineous unmineralized skeleton; Alizarin Red stains for calcified skeleton. Higher magnification of the skull of 16-old-day CTL (C) and DES-exposed (D) mice. The picture shows the different calcification pattern of the flat bone of the skull in CTL (C) and DES (D) fetuses. Mice shown in the pictures are representative of three different litters analyzed for each treatment. Staining was obtained as previously described in *Materials and Methods*.

TABLE 3. Femurs and tibiae length of 16 day old embryos mice from control and DES-treated pregnant female

Group	Femur length (mm)	Tibia Length (mm)
Control (4)	1.46 ± 0.03	2.21 ± 0.08
DES (4)	1.26 ± 0.07[a]	1.85 ± 0.1[a]

Pregnant CD-1 female mice have been treated with 100 μg/kg maternal BW of DES from days 9–15 of pregnancy. Embryos were collected on day 16. Bone length has been measured considering the mineralized part of the diaphysis of long bones of the animals (see *red staining* in Fig. 5). Fetuses have been taken from different litters to reflect litter variation in size and number. Data are considered as mean ± SE.

[a] $P < 0.05$ *vs.* control by Student's *t* test.

It is also interesting to note that in our neonatal model, postpubertal female mice show both an increase in MAR and a decrease in osteoclast number and in the tartrate resistant acid phosphatase activity, which is strongly suggestive of a role of estrogen in both increasing bone formation but also decreasing bone resorption (Migliaccio, S., and K. S. Korach, manuscript in preparation). Experiments are ongoing to evaluate a similar effect on the osteoclastic cells in the prenatal estrogen model.

Changes in bone turnover induced by estrogens reported by other authors were obtained by modulating the levels of estrogens, either by ovariectomizing the animals or treating them with estrogens during adulthood (17, 18). The unchanged levels of estrogens in the adult prenatally DES-exposed mice and the partial failure by prepubertal ovariectomy in blunting the described changes in the skeleton, suggest that the skeletal alterations in adults may be related to an estrogen programming of bone cells as a consequence of the altered estrogen levels during the period in which major organogenesis of estrogen target organs occur. The effect on the decreased length of long bones and the acceleration in the calcification process observed in the DES-exposed fetuses support this hypothesis. These events are probably due to a differential action of estrogens on both endochondral and endosteal/periosteal bone formation. These data also suggest an effect of estrogen on both cartilage and bone cells, supported by the differential effect on bone

length and on bone mass. The specific direct biological effect of estrogen on both bone and cartilage cells has been already described in studies both *in vitro* (14, 22–25, 33) and *in vivo* (17, 18, 29, 34–41). Our data are in accordance with previous reports that described an enhancement of bone formation and calcification in long bone of fetal mice exposed to estrogens *in vitro* (33). However, our report is the first to show this specific effect of estrogen on the skeleton of mice *in utero*. Likewise, our data and the previous reports suggest that alterations of estrogenic levels during these developmental periods may be of dramatic importance for the skeleton homeostasis. Our data also suggest that this early estrogen exposure could be one of the key factors for the final development of peak bone mass.

Although accumulating data show an action of estrogen on bone cells and bone turnover, the biological mechanism(s) of action of these hormones in bone cell homeostasis is not fully elucidated. The lack of a pure antagonistic compound active in the bone (42) has made it difficult to prove a definitive direct involvement of ER in the experiments *in vivo*. Moreover, the use of high concentrations of an estrogen antagonist during pregnancy would increase the rate of miscarriages in the animals (7–10). However, recent clinical studies have indicated that the lack of functional ER has a dramatic effect on bone density in an adult patient (43). Thus, these data strongly suggest a direct role of the ER in bone cell homeostasis.

Thus, it is not clear as yet whether estrogens are the actual modulator or whether they trigger the production of growth factors (GF) that may induce the observed effects on bone tissue. In fact, whereas GFs and cytokines are present in bone and modulate this tissue homeostasis (44–47), new evidences have also shown an interaction of multiple intracellular pathways in estrogen target cells including bone cells (48, 49). Growth factors can mimic biological estrogenic responses by coupling the ER (50), so intracellular kinases can also modulate the responsiveness of target cells. These interactions appear important, but further studies are needed to elucidate their role in the biological regulation of specific estrogen target tissues.

Regardless of the intracellular mechanism(s) of action involved in these events, it is important to point out that this early modification in estrogen levels leads to a gain in the final peak bone mass of the DES-exposed mice as compared with controls. Thus, early modifications of estrogens levels may be one of the key factors in determining the final bone mass in adulthood. It is believed that humans usually accumulate bone mass during the early years of life through the second decade of life (51–53). Recent studies have demonstrated, for example, how differential concentrations of calcium intake during early development can lead to a difference in the bone mass later in life (54). Additionally, it is well known that several maternal factors occurring during pregnancy influence the offspring's development (55, 56). Nutrition, smoking, drinking have all been demonstrated to influence fetal development (55, 56).

Our data add some useful information describing estrogens as a factor that plays a role in the early stages of skeleton development. Furthermore, our present results show that maternal hormone levels during pregnancy influence, and even determine, the peak bone mass in the offspring later after maturity.

In conclusion, our data support the hypothesis that estrogen during early phases of life, among other factors, plays a leading role in the determination of the final peak bone mass reached in adult life. Our results suggest that these events may be related to an estrogen programming of bone cell activity in female mice.

Acknowledgments

Authors are grateful to Rita Hanson (EHP, NIEHS) for skillfull technical assistance and to the Histology Laboratory at NIEHS for technical assistance in tissue section preparation. We thank Dr. Philip Hirsch (Dental School, University of North Carolina at Chapel Hill, NC) for the use of the calcium analyzer in his laboratory and Dr. Charles Eldridge (Bowman Gray School of Medicine, Winston-Salem, NC) for hormone assays.

References

1. **Beato M** 1989 Gene regulation by steroid hormones. Cell 56:335–344
2. **Evans RM** 1988 The steroid and thyroid hormone receptor superfamily. Science 240:889–895
3. **Carson-Jurica MA, Schrader WT, O'Malley BW** 1990 Steroid receptor family: structure and functions. Endocr Rev 11:201–220
4. **Korach KS** 1979 Estrogen action in the mouse uterus: characterization of the cytosol and nuclear receptor system. Endocrinology 104: 1324–1332
5. **Gorski J, Toft D, Shymala G, Smith D, Notides A** 1968 Recent Prog Horm Res 24:45–50
6. **Quarmby V, Korach KS** 1984 The influence of 17 beta estradiol on patterns of cell division in the uterus. Endocrinology 114:694–702
7. **Herbst AL** 1981 Developmental effects of diethylstilbestrol (DES) in pregnancy Herbst AL, Bern HA (eds) Thieme-Stratton Inc., New York, 318–320
8. **McLachlan JA, Newbold BC, Bullock BC** 1980 Long-term effects on the female mouse genital tract associated with the prenatal exposure to diethylstilbestrol. Cancer Res 40:3988–3999
9. **Newbold RR, Bullock BC, McLachlan JA** 1990 Uterine adenocarcinoma in mice following developmental treatment with estrogens: a model for hormonal carcinogenesis. Cancer Res 50:7677–7681
10. **Herbst AL, Poskanzer DC, Robboy SJ, Friedlander L, Scully RE** 1975 Prenatal exposure to stilbestrol. A prospective comparison of exposed female offspring with unexposed controls. N Engl J Med 292:334–339
11. **Iguchi T, Ostrander PL, Mills KT, Bern HA** 1985 Induction of abnormal epithelium changes by estrogen in neonatal mouse vaginal transplants. Cancer Res 45:5688–5693
12. **Takasugi N, Bern HA** 1964 Tissue changes in mice with persistent vaginal cornification induced by early postnatal treatment with estrogn. J Natl Cancer Inst 33:855–865
13. **Herbst AL, Ulfeder H, Poskanzer DC** 1971 Adenocarcinoma of the vagina. Association of maternal stilbestrol therapy with tumor appearance in young women. N Engl J Med 284:878–881
14. **Gray TK, Flynn TC, Gray KM, Nabell LM** 1987 17-β-Estradiol acts directly on the clonal osteoblastic cell line UMR-106. Proc Natl Acad Sci USA 84:6267–6271
15. **Albright F, Bloomberg F, Smith PH** 1940 Postmenopausal osteoporosis. Trans Assoc Am Physicians 55:298–305
16. **Lindsay R, Hart DM, Aikken JM, McDonald EB, Anderson JB, Clark AC** 1976 Long-term prevention of post-menopausal osteoporosis by estrogen: evidence of an increased bone mass after delayed onset of estrogen treatment. Lancet 1:1038–1040
17. **Wronsky TJ, Cintron M, Doherty Al Dann LM** 1988 Estrogen treatment prevents osteopenia and depresses bone turnover in ovariectomized rats. Endocrinology 123:681–686
18. **Yamamoto TT, Rodan GA** 1991 Direct effects of 17 beta estradiol on

trabecular bone in ovariectomized rats. Proc Natl Acad Sci USA 87:2172–2176
19. **Greenman Dl Delongchamp RR** 1986 Interactive responses to DES in C113 mice. Food Chem Toxicol 24:931–934
20. **Highman B, Roth SI, Greenman DL** 1981 Osseus changes and osteosarcomas in mice continuously fed diets containing DES or 17-β-estradiol. J Natl Cancer Inst 67:653–662
21. **Balfour JA, McTavish** 1992 Transdermal estradiol. A review of its pharmacological profile, and therapeutic potential in the prevention of postmenopausal osteoporosis. Drugs Aging 2:487–507
22. **Komm BS, Terpening CM, Benz DJ, Graeme KA, Gallegos A, Kore M, Greene GL, O'Malley BW, Haussler MR** 1988 Estrogen binding, receptor mRNA, and biologic response in osteoblast-like osteosarcoma cells. Science 241:81–84
23. **Eriksen EF, Colvard DS, Berg NJ, Graham ML, Mann KG, Spelsberg TC, Riggs BL** 1988 Evidence of estrogen receptors in normal human osteoblast-like cells. Science 241:84–86
24. **Ernst M, Parker MG, Rodan GA** 1991 Functional estrogen receptor in osteoblastic cells demonstrated by transfection with a reporter gene containing an estrogen response element. Mol Endocrinol 5:1597–1606
25. **Migliaccio S, Davis VL, Gibson MK, Gray KT, Korach KS** 1992 Estrogens modulate the responsiveness of osteoblast-like cells (ROS 17/2.8) stably transfected with estrogen receptor. Endocrinology 130:2617–2624
26. **Rosenfield RL** 1991 Puberty and its disorders in girls. Endocrinol Metab Clin North Am 20:15–42
27. **Ross JL, Long LM, Skerda M, Cassorla F, Kurtz D, Loriaux DL, Cutler GB Jr** 1986 Effects of low doses of estradiol on 6-months growth rate and predicted height in patients with Turner syndrome. J Pediatr 109:950–953
28. **Turner RT, Vandersteenhoven JJ, Bell NH** 1987 The effects of ovariectomy and 17-β Estradiol on cortical bone hystomorphometry in growing rats. J Bone Miner Res 2:115–122
29. **Migliaccio S, Newbold RR, Bullock BC, McLachlan JA, Korach KS** 1992 Developmental exposure to estrogens induces persistent changes in skeletal tissue. Endocrinology 130:1756–1758
30. **Yasuo U, Taguchi O, Noumura T, Iguchi T** 1992 Effects of sex steroids on the development of sexual dimorphism in mouse innominte bone. Anat Rec 234:541–548
31. **Peng TC, Garner SC, Hirsch PF, Posillico JT** 1986 Cytochemical bioassay of circulating concentration of rat parathyroid hormone: application to a study of age and sex. J Bone Miner Res 1:351–357
32. **Cox AC, Balloun SL** 1971 Prediction of total skeleton mineralization from individual bone data. Poultry Sci 50:186–194
33. **Schwartz Z, Soskkolne WA, Neubauer T, Goldstein M, Adi S, Ornoy A** 1991 Direct and sex specific enhancement of bone formation and calcification by sex steroids in fetal mice long bone *in vitro* (biochemical and morphometric study). Endocrinology 129:1167–1174
34. **Edwards MW, Bain SD, Bailey MC, Lantry MM, Howard GA** 1992 17-β- estradiol stimulation of endosteal bone formation in the ovariectomized mouse: an animal model for the evaluation of bone targeted estrogens. Bone 13:29–34
35. **Simmons DJ** 1966 Collagen formation and endochondral ossification in estrogen treated mice. PSEBM 121:1165–1168
35. **Ostadalova I** 1976 The effect of single dose of oestrogens administered during the early postnatal period on the DNA content of rat bone epiphyses. Physiol Bohemosl 25:97–102
37. **Corvol MT, Carrascosa A, Tsagris L, Blanchard D, Rappaport R** 1987 Evidence for a direct *in vitro* action of sex steroids on rabbit cartilage cells during skeletal growth: influence of age and sex. Endocrinology 120:1422–1429
38. **Dayani N, Paul MI, Robel P, Eychenne B, Moncharmont B, Tasgris L, Rappaport R** 1988 Estrogen receptor in cultured rabbit articular chondrocytes. Influence of age. J Steroid Biochem 31:351–356
39. **Wronsky TJ, Cintron M, Doherty Al Dann LM** 1988 Estrogen treatment prevents osteopenia and depresses bone turnover in ovariectomized rats. Endocrinology 123:681–686
40. **Turner RT, Riggs LB, Spelsberg TC** 1994 Skeletal effects of estrogen. Endocr Rev 15:275–300
41. **Chow J, Tobias JH, Colston KW, Chambers TJ** 1992 Estrogen maintains trabecular bone volume in rats not only by suppression of bone resoption but also by stimulation of bone formation. J Clin Invest 89:74–78
42. **Takeuchi M, Tokin M, Nagata K** 1995 Tamoxifen directly stimulates the mineralization of human osteoblast-like osteosarcoma cells through a pathway independent of estrogen response element. Biochem Biophys Res Commun 210:295- 301
43. **Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB, Korach KS** 1994 Estrogen resistance caused by a mutation in the estrogen receptor gene in a man. N Engl J Med 331:1056–1061
44. **Raisz LG** 1993 Local and systemic factors in the pathogenesis of osteoporosis. World Rev Nutr Diet 72:92–101
45. **Raisz LG** 1990 The role of prostaglandins in the local regulation of bone metabolism. Prog Clin Biol Res 332:195–203
46. **Canalis E** 1993 Insulin like growth factors and the local regulation of bone formation. Bone 14:273–276
47. **Finkelman RD** 1992 Growth factors in bones and teeth. J Calif Dent Assoc 20:23–29
48. **Chalbos D, Philips A, Rochefort H** 1994 Genomic cross-talk between the estrogen receptor and growth factor regulatory pathways in estrogen target tissues. Semin Cancer Biol 5:361–368
49. **Migliaccio S, Wetsel WC, Fox WM, Washburn TF, Korach KS** 1993 Endogenous protein kinase C activation in osteoblast-like cells modulates responsiveness to estrogen and estrogen receptor levels. Mol Endocrinol 7:1133–1143
50. **Ignar-Trowbridge DM, Nelson KG, Bidwell MC, Curtis SW, Washburn TF, McLachlan JA, Korach KS** 1992 Coupling of dual signal signaling pathways: Epidermal growth factor action involves the estrogen receptor. Proc Natl Acad Sci USA 89:4658–4662
51. **Raisz LG** 1994 Assessment of the risk of osteoporosis at the menopause: therapeutic consequences. Osteoporosis Int. 4:53–57
52. **Kelly PJ, Twomey L, Sambrook PN, Eisman JA** 1990 Sex differences in peak adult bone mineral density. J Bone Miner Res 5:1169–1175
53. **Johnston Jr CC, Miller JZ, Slemenda CW, Reister TK, Hui S, Christian JC, Peacock M** 1992 Calcium supplementation and increases in bone density in children. New Engl J Med 327:82–87
54. **Weinberg J, D'Alquen G, Bezio S** 1990 Interactive effects of ethanol intake and maternal nutritional status on skeletal development of fetal rats. Alcohol 7:383–388
55. **Lee M, Leichter J** 1983 Skeletal development in fetuses of rats consuming alcohol during gestation. Growth 47:254–262
56. **Balansky RM, Blagoeva PM** 1989 Tobacco smoke-induced clastogenicity in mouse fetuses and in newborn mice. Mutat Res 223:1–6