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Original Article

Prenatal modulation of breast density and breast stem cells by insulin-like growth factor-1

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Abstract: Biological determinants of breast density, a strong predictor of human breast cancer risk, are postulated to be influenced by prenatal exposures to mitogens. We investigated the extent to which prenatal exposures to insulin-like growth factor-1 (IGF-1) would affect body weight, breast density, and levels of breast stem/progenitor cells in the prepubescent offspring of wild type C57BL/6J and IGF-1 deficient mice. We found that administration of IGF-1 to pregnant mice resulted in significantly heavier birth and postnatal body weights of the offspring when compared to PBS controls. Morphometric analysis of whole mount carmine alum staining of the left fourth inguinal mammary gland revealed that a prenatal dose of 5 μg IGF-1 resulted in significantly longer ductal elongation in wild type mice and significantly higher breast density in both mouse strains. Furthermore, 5 μg IGF-1 also resulted in the highest number of putative $CD49f^+CD24^+$ and $CD49f^+CD24^+CD29^+$ breast stem/progenitor cells in the wild type offspring when compared to PBS controls, as assessed by flow cytometric analysis of dissociated cells from the right fourth inguinal mammary gland, while significantly higher numbers of these cell populations as well as $CD24^+CD29^+$ and $CD49f^+EpCAM^+$ cells were observed in IGF-1 deficient mice. These findings provide direct evidence for a prenatal modulation of breast density in the offspring by IGF-1, possibly involving populations of breast stem/progenitor cells.

Keywords: Alveolar bud, birth weight, breast cancer risk, epithelial ducts, fetal origin of disease, flow cytometry, *in utero* environment, mammary gland, mouse model, terminal end bud

Introduction

There is an emerging consensus that breast cancer begins in the womb [1-7]. In an attempt to dissect the perinatal influences pertinent to this etiology, population-based studies have shown that the positive association between *in utero* levels of insulin-like growth factor-1 (IGF-1) and birth weight [8-11], a known significant predictor of adult breast cancer risk [12, 13], is mediated by intrauterine stem cell pools, including that of hematopoietic [14-16] and possibly breast stem cells [17]. In adult life, birth weight is in turn positively associated with breast density [18, 19], one of the strongest risk factors for breast cancer [20-24].

Since *in utero* levels of IGF-1 affect intrauterine stem cell pools [14, 15], and maternal levels of IGF-1 have been reported to be positively associated with breast density [25-27] and breast cancer risk [28], we hypothesize that *in utero* exposure to IGF-1 may also be a major determinant for the number of mammary tissue-specific stem cells of the adult breast – the higher the levels of IGF-1, the greater the number of breast stem cells which in turn contribute to a denser breast. There has been no report demonstrating the effects of *in utero* levels of IGF-1 on breast stem cells and breast density in adults, primarily due to the limitation of human studies.

However, prenatal modulation studies have been performed using rodents. Pregnant mice treated with subcutaneous or intraperitoneal (i.p.) injections of diethylstilbestrol produced reproductive anomalies including tumors in the offspring [29-31]. Daily i.p. injections of the endocrine disruptors methoxychlor, a pesticide

with estrogenic properties, or vinclozolin, a fungicide with antiandrogenic properties, to pregnant rats decreased spermatogenic capacity leading to increased infertility in the male offspring [32-34]. Of note, prenatal exposures to the xenoestrogen bisphenol A in rodents have been found to alter the development and tissue organization of the mammary gland of the offspring, including an increase in the density of mammary structures [35-38], which led to an increased susceptibility to breast cancer [37]. Similarly in models of nutrition, a maternal diet that is high in fats [39, 40] or containing alcohol [41] during pregnancy can increase estrogen levels leading to increased mammary tumorigenesis in the female offspring of rats. To our knowledge, prenatal modulation studies of IGF-1 in rodents have so far only examined the effects on fetal growth [42].

In the present study, we established animal models by the i.p. administration of different doses of IGF-1 into pregnant wild type and IGF-1 deficient mice, followed by body weight measurements, morphometric analyses of histologically-stained mammary glands, and flow cytometric quantification of breast stem cell populations of the prepubescent offspring. We report here a prenatal modulation of body weight, breast density, and breast stem cell numbers by IGF-1.

Materials and methods

Mice

Pregnant wild type C57BL/6J at 8 weeks of age and non-pregnant IGF-1-deficient (known as IGF-1$^{m/m}$, or IGF-1 Midi, or *Igf1tm2Ts/*ImJ) mice were purchased from Jackson Laboratory (Bar Harbor, ME). Animals were housed in clear plastic cages in a temperature- and light-controlled (12 hour:12 hour light dark cycle) room with food and water *ad libitum*. Timed pregnancies of IGF-1$^{m/m}$ were performed by mating one male with one female, with gestational day 0 (GD0) denoted as the day that a vaginal plug was observed in a female after overnight mating. Offspring were delivered naturally but to ensure consistency in the duration of gestation, offspring that delivered before GD19 were excluded from the study. Offspring were weaned from their mothers at postnatal day 21 (P21). For harvest of mammary tissues, prepubescent female offspring were sacrificed between P27 to P31 with an overdose of sodium pentobarbital and at the same time of day to account for circadian effects. All procedures on live animals were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School (Permit Number: A-3306-01) and the Animal Care and Use Review Office of the US Army Medical Research and Materiel Command.

IGF-1 injections

IGF-1 (R&D Systems, Minneapolis, MN) dissolved in 1X phosphate-buffered saline (PBS) (Life Technologies/GIBCO, Grand Island, NY) was administered by daily i.p. injections to pregnant dams from GD10 to GD18 (C57BL/6J mice) or from GD9 to GD17 (IGF-1$^{m/m}$ mice). We investigated 5 doses (2.5 μg, 5 μg, 10 μg, 20 μg, and 50 μg) and 2 doses (5 μg and 20 μg) of total IGF-1 in C57BL/6J and IGF-1$^{m/m}$ mice, respectively. All test and control animals were injected with equal volumes (200 μl) of IGF-1 and 1X PBS, respectively. The experiments were repeated several times to procure a sufficient number of offspring (9 to 15 per group) to allow detection of meaningful differences in the study outcomes. For the treatment of wild type C57BL/6J mice with IGF-1, we report the results from a total of 4 experiments that gave rise to 40 offspring (18 females, 20 males, and 2 died by P3) from 6 dams injected with PBS, 27 offspring (13 females and 14 males) from 5 dams injected with a total of 2.5 μg IGF-1, 21 offspring (12 females and 9 males) from 4 dams injected with a total of 5 μg IGF-1, 28 offspring (13 females, 14 males, and 1 died by P7) from 4 dams injected with a total of 10 μg IGF-1, 20 offspring (10 females, 8 males, and 2 died by P21) from 3 dams injected with a total of 20 μg IGF-1, and 20 offspring (11 females, 8 males, and 1 died after time of wean) from 4 dams injected with a total of 50 μg IGF-1. A small subset (3, 4, and 3 animals in the PBS, 2.5 μg IGF-1, and 10 μg IGF-1 group, respectively) was excluded from the stem cell analysis due to an instrumentation error. For the treatment of IGF-1$^{m/m}$ mice with IGF-1, we report the results from a total of 3 experiments that gave rise to 27 offspring (11 females and 16 males) from 6 dams injected with PBS, 28 offspring (15 females and 13 males) from 6 dams injected with a total of 5 μg IGF-1, and 18 offspring (7 females and 11 males) from 3 dams injected with a total of 20 μg IGF-1.

Body weight measurements

Offspring born from wild type pregnant dams were weighed at birth (P1), P3, P7, P14, and P21 (the day of wean) using a Scout Pro electronic balance (Ohaus, Pine Brook, NJ), while that of IGF-$1^{m/m}$ mice were weighed at P7, P14, and P21 to minimize loss due to handling. Body weight analyses included both female and male offspring and gender-specific analyses were performed at P21.

Mammary gland whole mount staining and morphometric analysis

For each female offspring, the left fourth inguinal mammary gland was dissected out as described by DeOme et al. [43], spread onto a Tissue Path Superfrost Plus Gold slide (Fisher Scientific, Pittsburgh, PA), and allowed to adhere for 30 minutes to an hour. The gland was then subjected to whole mount carmine alum staining as described by Brisken et al. [44]. Briefly, the gland was fixed in a 1:3 mixture of glacial acetic acid/100% ethanol overnight followed by hydration in graded solutions of ethanol. The gland was then stained in a mixture of 0.2% carmine (Sigma-Aldrich, St. Louis, MO) and 0.5% aluminum potassium sulfate dodecahydrate ($AlK(SO_4)_2$) (Sigma-Aldrich) overnight followed by dehydration in graded solutions of ethanol. The gland was then cleared in toluene (Sigma-Aldrich) and cover-slipped in Permount (Fisher Scientific). Mammary structures as defined by Russo et al. [45], including terminal end buds (TEB), lateral buds (LB), alveolar buds (AB), terminal ducts (TD), and the sum of primary, secondary and tertiary ducts (D), were enumerated blind with the help of a SZ61 stereo microscope (Olympus, Nashua, NH). The number of epithelial duct criss-crosses (CC), as a measure of mammary gland branching pattern [46], was also counted. Breast density was determined as a measure of the total number of TEB, LB, AB, TD, D, and CC per unit area (cm^2) of each mammary gland. Additionally, the extent of ductal elongation was measured between the nipple or the edge of the lymph node closest to the nipple, and the furthest extension of the mammary tree.

Mammary gland dissociation and breast stem cell analysis

The right fourth inguinal mammary gland was similarly dissected out and incubated in 3 ml of dissociation solution, consisting of EpiCult-B Complete medium (with Proliferation Supplements added only) + 1X Collagenase/Hyalurondiase mixture + 5% fetal bovine serum (FBS), for 6 hours in a 37°C/5% carbon dioxide incubator. After dissociation, the cells were centrifuged down at 450 *g* and washed with a 1:4 mixture of Hank's Balanced Salt Solution Modified supplemented with 2% FBS and ammonium chloride. The cells were centrifuged down and treated with 2 ml pre-warmed Trypsin-EDTA by gentle pipetting for 1 minute. The cells were then washed with Hank's Balanced Salt Solution Modified supplemented with 2% FBS (HF) and subjected to further dissociation by treatment with 2 ml of 5 mg/ml Dispase and 200 µl of 1 mg/ml DNase for 1 minute. The cell suspension was diluted with 10 ml of HF and filtered through a 40-µm cell strainer. After a final centrifugation, the dissociated mammary cells were resuspended in 350 µl of HF. All dissociation reagents were purchased from StemCell Technologies (Vancouver, Canada). Breast stem cell measurements were determined by flow cytometric analyses as described previously [17]. Briefly, 50 µl of dissociated mammary cells were incubated for 30 minutes on ice in the dark with the following fluorochrome-conjugated antibodies: anti-CD49f-fluorescein isothiocyanate (FITC) (Clone GoH3, StemCell Technologies), anti-CD24-phycoerythrin (PE) (Clone M1/69, BD BioSciences Pharmingen, San Diego, CA), anti-CD29-allophycocyanin (APC) (Clone 265917, R&D Systems), anti-EpCAM-APC (Clone G8.8, BioLegend, San Diego, CA), or the combination of anti-CD49f-FITC, anti-CD24-PE, and anti-CD29-APC, or the combination of anti-CD49f-FITC and anti-EpCAM-APC. Samples treated with no antibody served as negative controls. Cells were washed, fixed with 4% paraformaldehyde and analyzed using a FACSCalibur flow cytometer (BD Biosciences Immunocytometry Systems, San Jose, CA). Putative breast stem/progenitor cell subpopulations ($CD49f^+CD24^+$, $CD49f^+CD24^+CD29^+$, $CD24^+CD29^+$, and $CD49f^+EpCAM^+$) were quantified using the FlowJo software program (Tree Star, Ashland, OR).

Statistical analysis

Means, standard deviations, medians, and ranges of each quantitative outcome measurement were summarized for each treatment

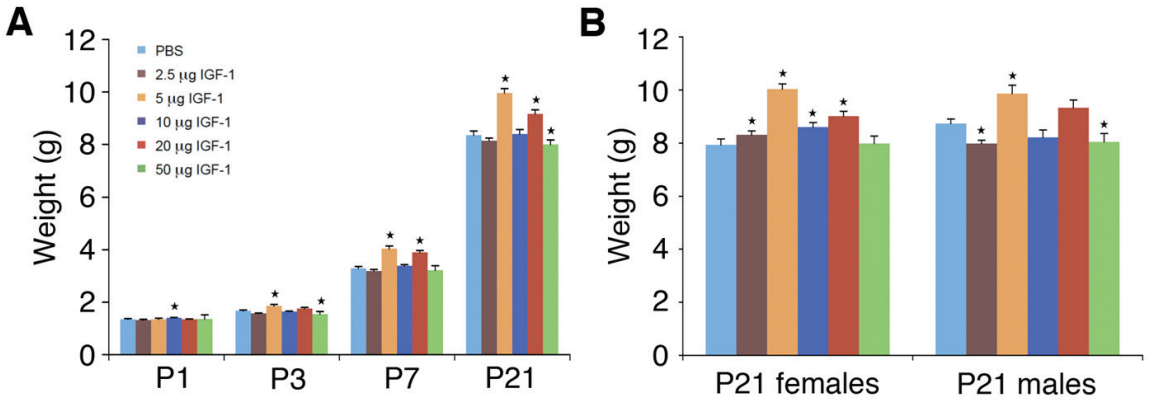

Figure 1. Prenatal modulation of body weight by IGF-1. Histograms showing the mean body weights of (A) female and male C57BL/6J offspring at postnatal day 1 (P1), P3, P7, and P21, and (B) female or male offspring at P21 born to mothers that were injected with PBS (blue, *n* = 38 to 40) or IGF-1 consisting of 2.5 µg (brown, *n* = 27), 5 µg (orange, *n* = 21), 10 µg (dark blue, *n* = 27 to 28), 20 µg (red, *n* = 18 to 20), or 50 µg (green, *n* = 20). Error bars represent standard error of the mean (SEM). ★denotes significance at $P < 0.05$ by Kruskal-Wallis rank test when compared to PBS controls.

group. Groups receiving different doses of IGF-1 were compared with the PBS control group on body weight, mammary gland structures, and stem cell measurements. Differences between groups were analyzed by the Kruskal-Wallis non-parametric rank test. All the *P*-values presented were two-sided.

Results

Establishment of an animal model for the prenatal effects of IGF-1

To study the *in utero* effects of IGF-1, we set out to develop an animal model by giving i.p. injections of IGF-1 to pregnant wild type C57BL/6J. First, we found that daily i.p. injections of IGF-1 were more effective when administered from GD10 to GD18 than from GD10 to GD16. Second, the administration of IGF-1 based on body weight of the mice, i.e., µg of IGF-1 per kg weight of the animal, resulted in the injection of different volumes of IGF-1, which was difficult to control. In experiments reported here, we administered total amounts of IGF-1, i.e., 2.5, 5, 10, 20, or 50 µg of IGF-1 injected per animal over the course of 9 injections, such that for a particular dose, equal volumes of IGF-1 or PBS were used for both test and control animals, respectively. Third, for consistency in the duration of gestational period (as we could never be absolutely sure of a successful copulation), mice that delivered before GD19 were excluded from the study.

Prenatal modulation of body weight by IGF-1 in wild type mice

Only the prenatal i.p. administration of a total amount of 10 µg IGF-1 in pregnant C57BL/6J mice resulted in offspring with significantly heavier birth weights, i.e., body weight at P1, when compared to PBS controls ($P = 0.007$; unless otherwise stated, all *P* values reported were obtained by Kruskal-Wallis rank test). While the administration of 20 µg IGF-1 resulted in significantly heavier body weights at P7 and P21 ($P < 0.0001$ and $P = 0.002$, respectively) when compared to PBS controls, the administration of 5 µg IGF-1 resulted in significantly heavier body weights of the offspring at P3 ($P = 0.001$), P7 ($P < 0.0001$), and P21 ($P < 0.0001$) (**Figure 1A**). The significantly heavier body weight at P21 by the treatment of 5 µg IGF-1 was observed in, and hence contributed by, both female and male offspring (females: $P = 0.0001$; males: $P = 0.008$) (**Figure 1B**). Interestingly, the administration of 50 µg IGF-1 resulted in significantly lowered body weights at P3 and P21 ($P = 0.02$ and 0.05, respectively) when compared to PBS controls.

Prenatal modulation of mammary gland structures and breast density by IGF-1 in wild type mice

Female offspring were sacrificed at approximately 4 weeks of age and their left fourth inguinal mammary glands were subjected to

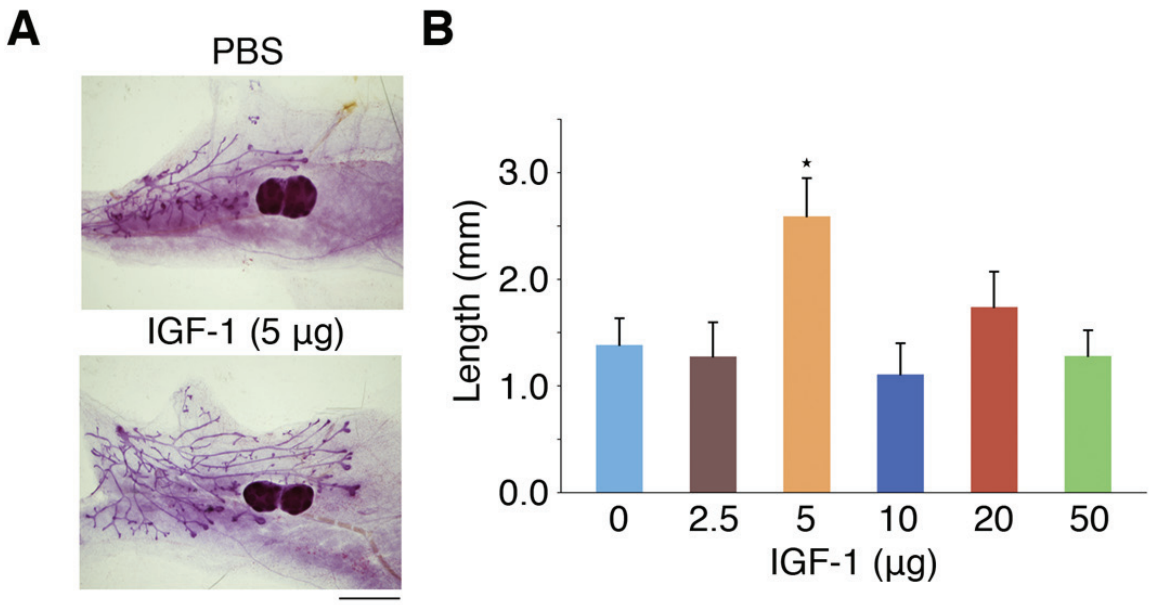

Figure 2. Prenatal modulation of mammary duct elongation by IGF-1. A: Whole mount carmine alum staining showing the extent of ductal elongation from the mammary gland of a C57BL/6J female offspring born to a mother that were injected with PBS (top) or 5 µg of IGF-1 (bottom). Scale bar represents 2 mm. B: Histograms showing the extent of ductal elongation in mammary glands of C57BL/6J female offspring born to mothers that were injected with PBS (blue, n = 18) or IGF-1 consisting of 2.5 µg (brown, n = 13), 5 µg (orange, n = 12), 10 µg (dark blue, n = 13), 20 µg (red, n = 10), or 50 µg (green, n = 11). Error bars represent standard error of the mean (SEM). ⋆denotes significance at $P < 0.05$ by Kruskal-Wallis rank test when compared to PBS controls.

whole mount staining with carmine alum solution. We performed morphometric analyses on the extent of ductal migration, the number of different mammary structures as characterized by Russo et al. [45], and the number of crossing epithelial ducts as a measure of mammary gland branching pattern [46]. First, only mammary glands of the offspring prenatally treated with 5 µg IGF-1 displayed a faster rate of IGF-1-induced growth (P = 0.007), as evidenced by a significantly longer ductal elongation of the mammary tree when compared to controls (**Figure 2A** and **2B**). Among the different mammary gland structures enumerated (**Figure 3A**), prenatal treatment with 5 µg IGF-1 also resulted in significantly higher numbers of lateral buds (LB), ducts (D), and criss-crosses (CC) when compared to controls (P = 0.007, 0.0006, and 0.03, respectively) (**Figure 3B**). By rationalizing that breast density is a measure of the total number of mammary structures, including number of ductal crossings, per unit area per gland, we found that prenatal treatment with 5 µg IGF-1 resulted in significantly denser breasts when compared to PBS controls (P = 0.001) (**Figure 3C**).

Prenatal modulation of breast stem cells by IGF-1 in wild type mice

To examine the extent to which a denser breast could be attributed to the number of breast stem cells, contra-lateral fourth inguinal mammary glands of the same pups that were used for morphometric analyses were enzymatically dissociated into single cells for the detection of putative breast stem/progenitor cell markers by flow cytometry. While we were able to detect the putative markers of breast stem/progenitor cells proposed for analysis, including the $CD49f^+CD24^+$ [47], $CD49f^+CD24^+CD29^+$ [48], $CD24^+CD29^+$ [49], and $CD49f^+EpCAM^+$ [50] subpopulations (**Figure 4A**), only the prenatal treatment of 5 µg IGF-1 resulted in a significant increase in the $CD49f^+CD24^+$ and $CD49f^+CD24^+CD29^+$ subpopulations when compared to PBS controls (P = 0.01 and 0.03, respectively) (**Figure 4B**).

Prenatal IGF-1 modulation in IGF-1 deficient mice

To address the concern that the effects from exogenously administered IGF-1 in wild type

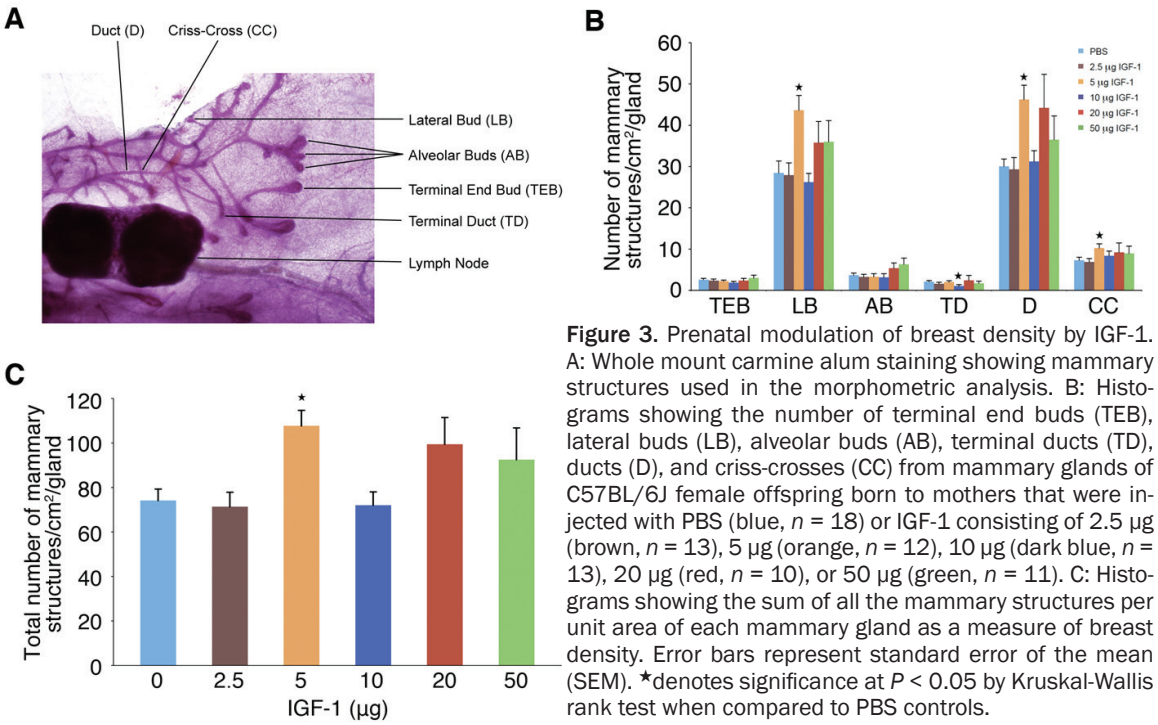

Figure 3. Prenatal modulation of breast density by IGF-1. A: Whole mount carmine alum staining showing mammary structures used in the morphometric analysis. B: Histograms showing the number of terminal end buds (TEB), lateral buds (LB), alveolar buds (AB), terminal ducts (TD), ducts (D), and criss-crosses (CC) from mammary glands of C57BL/6J female offspring born to mothers that were injected with PBS (blue, n = 18) or IGF-1 consisting of 2.5 µg (brown, n = 13), 5 µg (orange, n = 12), 10 µg (dark blue, n = 13), 20 µg (red, n = 10), or 50 µg (green, n = 11). C: Histograms showing the sum of all the mammary structures per unit area of each mammary gland as a measure of breast density. Error bars represent standard error of the mean (SEM). ★denotes significance at $P < 0.05$ by Kruskal-Wallis rank test when compared to PBS controls.

mice might be masked or dampened by the endogenous levels of IGF-1, we repeated our experiments in a strain of mice homozygous for the Igf1^{tm2Ts} mutation in which a targeting construct consisting of the neomycin-resistance thymidine kinase gene and vector sequences was inserted just upstream of the 5' end of exon 3 of the *Igf1* gene. These IGF-1$^{m/m}$ mice are fertile and viable but with body weights 60 to 65%, serum IGF-1 levels 30%, and mammary ductal branching 50% that of wild type mice [51, 52]. Using these IGF-1 deficient mice, we continued to observe similar effects where prenatal treatments with 5 µg IGF-1 induced significantly higher body weights (P7 and P14: P = 0.002, P21: P = 0.0001) (**Figure 5A**) that were contributed by both female and male offspring at P21 (females: P = 0.0008, males: P = 0.004) (**Figure 5B**). This dose of IGF-1 also resulted in significantly denser breasts in the female offspring (P = 0.03) when compared to PBS controls (**Figure 5D**), with significantly more terminal end buds (TEB) (P = 0.008), not previously observed in the wild type mice, and lateral buds (LB) (P = 0.03) (**Figure 5C**). However, there was no significant difference in ductal elongation. More significantly, 5 µg IGF-1 induced significantly higher number of cells with putative breast stem/progenitor cell markers in all the populations examined (P = 0.008, 0.006, 0.001, and 0.003, for CD49f$^+$CD24$^+$, CD49f$^+$CD24$^+$CD29$^+$, CD24$^+$CD29$^+$, and CD49f$^+$EpCAM$^+$ subpopulations, respectively) when compared to PBS controls (**Figure 5E** and **5F**).

Discussion

The role of *in utero* levels of IGF-1 in increased breast cancer risk has been implicated in epidemiological studies. Our animal study data ostensibly demonstrate the effects of prenatal exposures to IGF-1 on increased body weight and breast density, that may be due to an increased in the number of mammary gland-specific stem cells. The findings support a paradigm for a prenatal mechanism affecting adult breast cancer risk due to the influence of fetal mitogens on stem cell load.

Although successful prenatal influences via different routes of administration have been reported, we decided to model our experiments after Block et al. [31] with the rationale that i.p. administered IGF-1 will be readily accessible to the developing embryos since the uterine horns are located in the peritoneal cavity. Because mammary buds of rodents are first evident in embryos at GD10 to GD11 and rapid proliferation of the epithelial components occurs from GD16 to birth [53], we maximized the availabil-

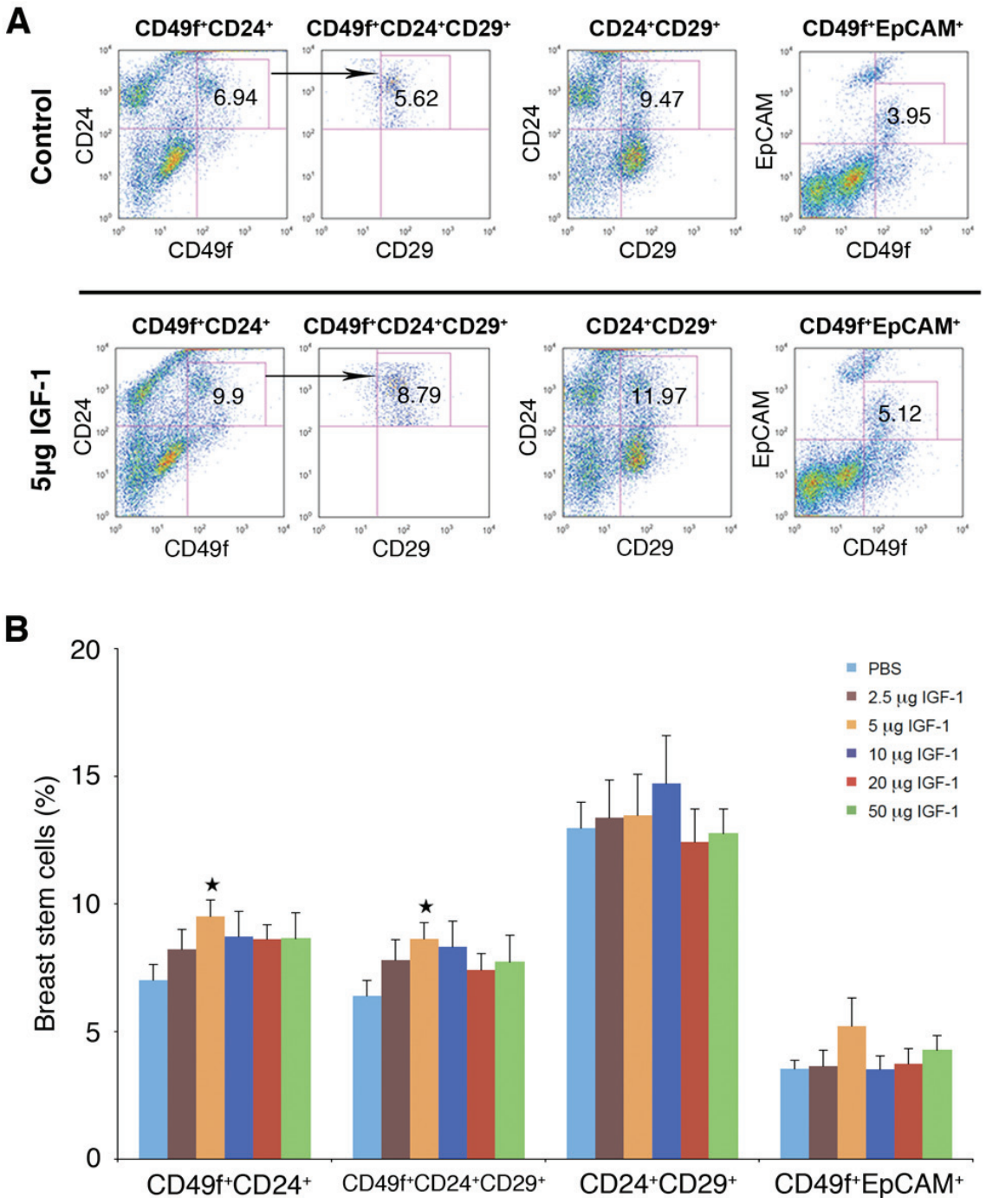

Figure 4. Prenatal modulation of breast stem cells by IGF-1. A: Representative flow cytometric pseudocolor plots showing the detection of $CD49f^+CD24^+$, $CD49f^+CD24^+CD29^+$, $CD24^+CD29^+$, and $CD49f^+EpCAM^+$ subpopulations of putative breast stem/progenitor cells (boxed, with percentage of cells indicated) from a mammary gland of C57BL/6J female offspring born to a mother that were injected with PBS (top) or 5 μg IGF-1 (bottom). The arrows indicate that the triple positive population was derived from the double positive population as shown. B: Histograms showing the percentages of putative breast stem/progenitor cell populations, as quantified by flow cytometry, from mammary glands of C57BL/6J female offspring born to mothers that were injected with PBS (blue, $n = 15$) or IGF-1 consisting of 2.5 μg (brown, $n = 9$), 5 μg (orange, $n = 11$), 10 μg (dark blue, $n = 10$), 20 μg (red, $n = 10$), or 50 μg (green, $n = 11$). Error bars represent standard error of the mean (SEM). ★denotes significance at $P < 0.05$ by Kruskal-Wallis rank test when compared to PBS controls.

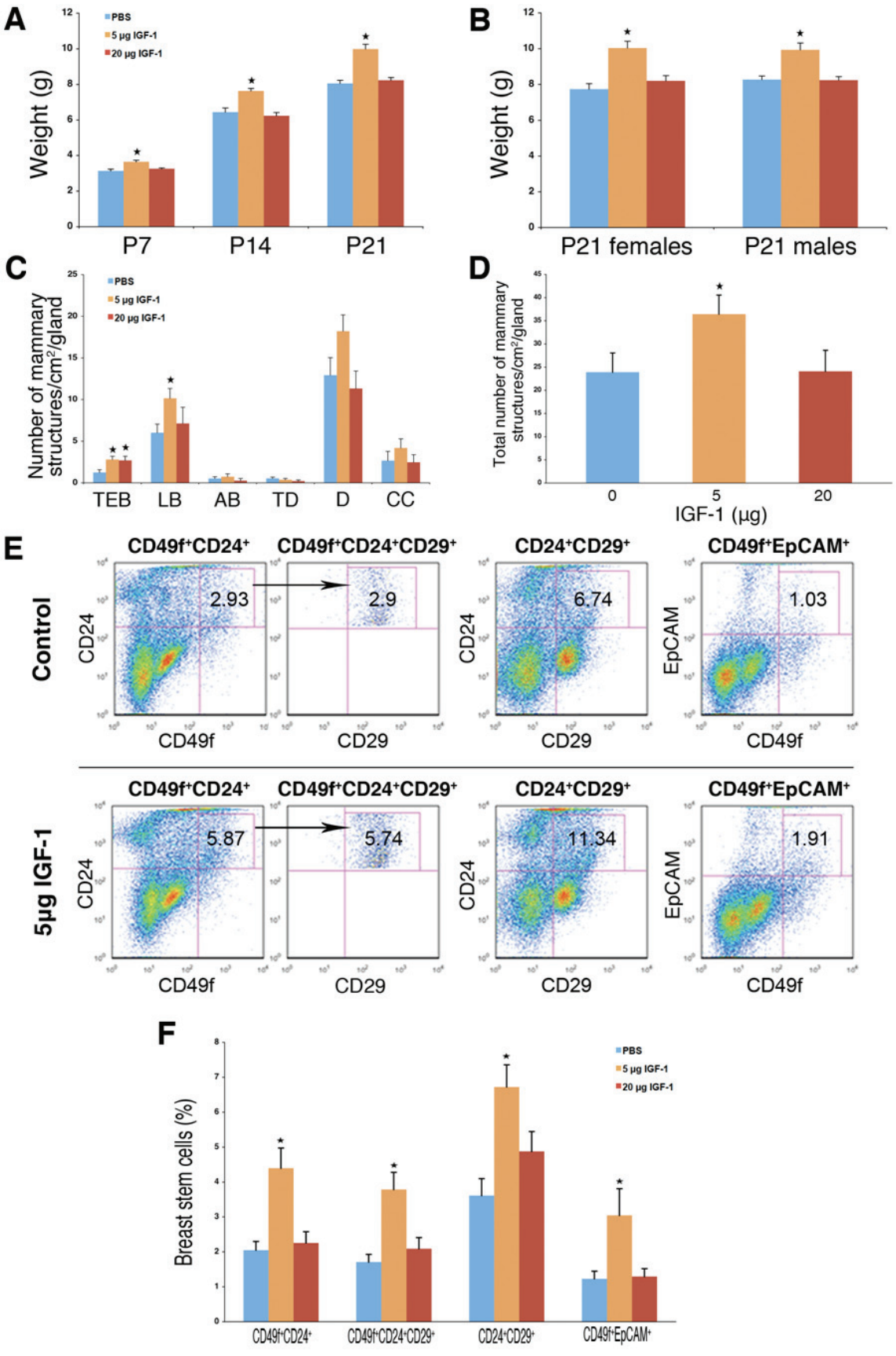
A
PBS
5 μg IGF-1
20 μg IGF-1
Weight (g)
P7
P14
P21
B
Weight (g)
P21 females
P21 males
C
PBS
5 μg IGF-1
20 μg IGF-1
Number of mammary structures/cm²/gland
TEB
LB
AB
TD
D
CC
D
Total number of mammary structures/cm²/gland
IGF-1 (μg)
E
Control
5μg IGF-1
CD49f⁺CD24⁺
CD49f⁺CD24⁺CD29⁺
CD24⁺CD29⁺
CD49f⁺EpCAM⁺
2.93
2.9
6.74
1.03
5.87
5.74
11.34
1.91
CD24
CD49f
CD29
EpCAM
F
Breast stem cells (%)
PBS
5 μg IGF-1
20 μg IGF-1
CD49f⁺CD24⁺
CD49f⁺CD24⁺CD29⁺
CD24⁺CD29⁺
CD49f⁺EpCAM⁺

Figure 5. Prenatal modulation by IGF-1 in IGF-1$^{m/m}$ mice. Histograms showing the mean body weights of (A) female and male IGF-1$^{m/m}$ offspring at postnatal day 7 (P7), P14, and P21, and (B) female or male offspring at P21, born to mothers that were injected with PBS (blue, *n* = 27) or IGF-1 consisting of 5 μg (orange, *n* = 28), or 20 μg (red, *n* = 18). C: Histograms showing the number of terminal end buds (TEB), lateral buds (LB), alveolar buds (AB), terminal ducts (TD), ducts (D), and criss-crosses (CC) from mammary glands of IGF-1$^{m/m}$ offspring born to mothers that were injected with PBS (blue, *n* = 11) or IGF-1 consisting of 5 μg (orange, *n* = 15), or 20 μg (red, *n* = 7). D: Histograms showing the sum of all the mammary structures per unit area of each mammary gland as a measure of breast density. E: Representative flow cytometric pseudocolor plots showing the detection of CD49f$^+$CD24$^+$, CD49f$^+$CD24$^+$CD29$^+$, CD24$^+$CD29$^+$, and CD49f$^+$EpCAM$^+$ subpopulations of putative breast stem/progenitor cells (boxed, with percentage of cells indicated) from a mammary gland of IGF-1$^{m/m}$ female offspring born to a mother that were injected with PBS (top) or 5 μg IGF-1 (bottom). The arrows indicate that the triple positive population was derived from the double positive population as shown. F: Histograms showing the percentages of putative breast stem/progenitor cell populations, as quantified by flow cytometry, from the mammary glands of IGF-1$^{m/m}$ female offspring born to mothers that were injected with PBS (blue, *n* = 11) or IGF-1 consisting of 5 μg (orange, *n* = 15), or 20 μg (red, *n* = 7). Error bars represent standard error of the mean (SEM). ★denotes significance at $P < 0.05$ by Kruskal-Wallis rank test when compared to PBS controls.

ity of IGF-1 by giving daily injections to pregnant dams from GD10 to GD18. In our animal model, virgin mice were used to avoid the confounding effects of pseudopregnancy and, to simulate the human condition, we did not control for the potential different concentrations of IGF-1 in the milk by cross-fostering.

Epidemiological studies exploring the 'fetal origin of disease' often use birth weight as a variable for predicting adult diseases. As we and others have shown a positive correlation between levels of umbilical cord blood IGF-1 and birth weight [8-11, 16], we expected that a prenatal influence of IGF-1 would result in higher birth weights in our animal model. While only the prenatal treatment of 10 μg IGF-1 in wild type mice resulted in significantly heavier birth weight, there was a constant significant postnatal increase in body weight of the pups born to mothers treated with 5 μg IGF-1 (**Figure 1** and **5**), suggesting an enhanced potential for postnatal growth and consistent with reports exploring the effects of endogenous or exogenous elevation of maternal IGF-1 in mice [42, 54], even though we did not control for potential litter effects by random distribution of pups to different mothers within treatment groups. Of note, a greater body mass index or body size, as indicated by significantly higher IGF-1-induced body weights in our animal model, is also used as a predictor for increased cancer risk [55, 56].

Growth-promoting effects on mammary gland development have been reported from early life exposures to estrogen and the xenoestrogen bisphenol A [7, 35, 38, 46], and a high-fat diet [39, 57]. Since prenatal exposure to IGF-1 had promoted mammary growth resulting in denser breasts, we asked the question whether denser breasts observed could be associated with the presence of a greater number of mammary gland-specific stem cells. As label-retaining putative breast stem cells have been reported to be present in the terminal end buds and ducts of mammary glands [58] and pluripotent stem cells of the developing mammary gland give rise to the luminal and myoepithelial cells of the advancing ducts [59, 60], it follows that the availability of more stem cells would potentially favor formation of a more extensive mammary tree. Indeed, 5 μg IGF-1-treated mammary glands were morphometrically densest with significantly more breast stem cells, which was more evident in an IGF-1 deficient background (**Figure 4** and **5**). Intriguingly, we could detect putative breast stem cells that are positive for CD49f and EpCAM in our mouse model since these populations have previously only been reported in humans [50, 61-63].

In the detection of putative breast stem cells, we have opted for not doing the depletion of non-epithelial cells to enhance the yield and viability of cells that have already gone through an enzymatic dissociation treatment. However, since some stromal cells in young C57BL/6J mice express CD24 [64], the purity of the stem cell fraction in our study is decreased and the different sub-populations of cells as quantified by flow cytometry do contain a small percentage of non-epithelial cells. Since the goal of the present study is to compare the number of mammary stem cells from mice treated with IGF-1 with that of PBS controls, we make the assumption that IGF-1 or PBS treated samples have similar number of contaminating non-epi-

thelial cells or, more likely, that IGF-1 treatments enhanced homogenously the proliferation of cells with the markers under study.

It is interesting that administration of 5 μg IGF-1 to pregnant dams consistently resulted in significantly increased body weight, breast density, and number of cells with breast stem/progenitor cell markers in the offspring when compared to vehicle controls, but not administration of lower or higher IGF-1 doses. It is plausible that a narrower range of IGF-1 dose, e.g., 3.75 to 7.5 μg IGF-1, could show similar prenatal modulated effects and this needs to be verified in future studies. We speculate that this optimum dose of IGF-1 is above the threshold concentration required to elicit signaling pathways pertaining to cell proliferation. Higher concentrations of IGF-1 did not result in a corresponding increase in measurable outcomes and in the case of body weight analysis, gave an inverse association. This could be due to saturation of receptors, including IGF-1 receptors (IGF-1R), sequestration by IGF-binding proteins, or feedback loops [65]. Of note, in an animal model examining the effects of the xenoestrogen bisphenol-A, perinatal exposures to low doses, but not higher doses, affected body weight [66] and development of mammary gland structures [35].

While IGF-1 is recognized to play an important role in mediating fetal and postnatal growth [67, 68], the precise mode of IGF-1 action in our prenatal animal model is unclear. In humans, both maternal and fetal serum IGF-1 increased significantly from 18 to 40 weeks of gestation, although fetal IGF-1 levels are four to five times lower than those in the maternal circulation [69]. In spite of the lower fetal IGF-1 levels, IGF-1R are expressed five-fold more on the maternal-facing microvillous membrane than the fetal-facing basal membrane of the syncytiotrophoblast [70], suggesting that maternal IGF-1 plays a role in placenta function, perhaps by enhancing transplacental transport of nutrients to the fetus and thereby controlling fetal growth [54, 71], even though IGF-1 is not known to cross the placenta [72]. In our mouse model, we surmise that IGF-1 administered into the peritoneal cavity might have enhanced transplacental transport of nutrients and/or crossed the wall of the uterus by an unknown mechanism to bathe the embryos directly and gain access into the bloodstream of the embryos. Ultimately, exogenously administered IGF-1 would act in concert with endogenous fetal IGF-1 and upon binding to IGF-1R, will trigger IGF-1-induced signal transductions that include mitogenic and anti-apoptotic effects and, in some tissues, differentiation [68].

Taken together, a prenatal treatment of IGF-1 resulted in significantly denser breasts in the prepubescent offspring, an outcome that is indicative of enhanced morphogenesis due to a higher proliferative response of subpopulations of breast stem cells. Stated simply, this is the first demonstration of a prenatal effect on stem cell load of the offspring due to an aberrant *in utero* environment. We hypothesize further that increased cellular proliferation, especially that of stem cells including breast stem cells, induces genetic and/or epigenetic changes (such as DNA methylation and/or histone modification events) at gene promoter regions that affect gene transcription leading to heavier body weights and denser breasts. If breast stem cells and their progenies are indeed the targets of genetic and/or epigenetic changes during cycles of proliferation, then by inference breast stem cells will participate in mammary gland carcinogenesis. Consistent with a fetal programming hypothesis [5], cells that have acquired IGF-1-induced genetic and/or epigenetic changes in early life will potentially play a role in host susceptibility by persisting in the mammary genome to increase the risk of breast cancer in adulthood, perhaps after multiple hits over the course of life. This risk may also be exacerbated by the postnatal continuation of morphological development of the mammary gland under the influence of the growth hormone-IGF-1 axis and estrogens and by the cycles of proliferation, differentiation, and tissue remodeling unique to the stages of puberty and pregnancy [73, 74]. Additionally, the prenatal actions of IGF-1 may be mediated by changes in other growth regulatory fetal hormones [75] that can further contribute to carcinogenesis. In future studies, the prenatal effects by IGF-1 will be examined in adult mice 12 weeks of age or older to better represent the human condition.

In summary, we have established a model to study the prenatal modulation effects of IGF-1 and provided supporting evidence for mammary stem cell number as a determinant of breast cancer risk. The prenatal-induced developmen-

tal effects of the mammary gland could potentially be shown in other organs such as the brain and gut, where tissue-specific stem cells have been reported. More broadly, this study sets the stage for the investigation of prenatal influences on other health outcomes (obesity, diabetes) that might potentially involve the role of tissue-specific stem cells.

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