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PROSTATE GLAND GROWTH DURING DEVELOPMENT IS STIMULATED IN BOTH MALE AND FEMALE RAT FETUSES BY INTRAUTERINE PROXIMITY TO FEMALE FETUSES

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ABSTRACT

In rodents, steroid hormones are transported between adjacent fetuses, and male or female fetuses that develop in utero between female fetuses (2F males or 2F females) have higher serum levels of estradiol and lower serum levels of testosterone relative to siblings of the same sex that develop between two male fetuses (2M males or 2M females). The present study was prompted by the prior unexpected finding that as adults, 2F male mice have an enlarged prostate, and increased numbers of prostatic androgen receptors relative to 2M males. We examined prostate development in both male and female rat fetuses from different intrauterine positions using computer-assisted, 3-dimensional reconstruction of the urogenital complex. In males, this included the prostate, seminal vesicles and utricle (a remnant of the Müllerian ducts), while in females it included development of prostatic glandular buds. The mean cross-sectional area of developing prostatic epithelial buds, utricle and seminal vesicles was significantly increased in 2F male relative to 2M male fetuses. In female fetuses, prostatic bud development was significantly more likely to occur in 2F (67%) than in 2M (29%) animals. These findings suggest that the transport of a small supplement of estrogen from adjacent female fetuses enhances androgen-dependent accessory organ development. We also found that mRNAs encoding receptors for both estrogen and androgen were located in the mesenchyme of the developing male prostate. The localization of estrogen and androgen receptor mRNA in this region further suggests that the mesenchymal induction of prostatic epithelial growth involves both hormones. The cranial dorsolateral prostatic buds exhibited the greatest enlargement in 2F males. This region of the developing prostate in rats is comparable (that is the embryonic homologue) to the region exhibiting benign prostatic hyperplasia (BPH) during aging in men. We propose that the potential for pathological regrowth of the prostate during aging is imprinted by estradiol during fetal development.

KEY WORDS: rat, prostate, fetal development, intrauterine position

Growth and differentiation of the prostate is primarily under the control of androgen. Expression of the androgen metabolizing enzyme, 5α-reductase, within prostatic mesenchyme cells is also necessary for normal development of the prostate.[1] Urogenital sinus mesenchyme, which demonstrates estrogen as well as androgen binding,[2,3] appears to regulate differentiation of the prostatic duct epithelium during fetal and neonatal life in mice and rats.[4,5]

The purpose of this study was to examine the consequence for fetal prostate development of an animal's intrauterine position (IUP), which leads to naturally occurring differences in serum levels of the gonadal steroids estradiol and testosterone. An animal's IUP is defined by the sex of contiguous fetuses in the litter. Gestation between two males (M) is designated as 2M (M*F*M. or M*M*M) and between two females (F) as 2F (F*F*F or F*M*F). The basis for comparing 2F and 2M fetuses (both males and females) is that in both mice and gerbils, 2F male fetuses have higher serum levels of estradiol and lower serum levels of testosterone than 2M males, and 2F female fetuses have higher serum levels of estradiol and lower serum levels of testosterone than 2M females. Also, differences in morphology and functioning of reproductive organs, as well as behavior, related to prior intrauterine position, have been found in rats and gerbils, in addition to mice.[6–8] Because differences in circulating steroid levels due to intrauterine position occur naturally, they fall within a physiological rather than a pharmacological range. This is important, since high and low doses of natural and synthetic estrogens can produce opposite effects on the developing prostate.[9]

The possibility that estrogen might be involved in modulating the effects of androgen on prostatic development during early life has been the subject of speculation for over sixty years.[9–12] In addition, estrogen has been implicated in the etiology of prostatic disease during senescence.[13–15] We undertook the present study due to the prior finding that adult 2F male mice had enlarged prostates, associated with an increased number of prostatic androgen receptors, relative to 2M males,[16] suggesting that the elevated serum estradiol levels in 2F males had permanently increased prostate size. This was subsequently confirmed in a study in which estradiol was experimentally increased in male mouse fetuses and prostate size and numbers of prostatic androgen receptors were permanently increased.[9]

In this study we examined the fetal development of the rat accessory reproductive organs using computer-assisted three-dimensional (3D) reconstruction of serial histological

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sections as described by Timms et al.[17] We compared accessory reproductive organs in both male and female fetuses from different IUPs. Because prostatic bud development occurs in some female rat fetuses,[18] we also sought to determine whether IUP would influence prostatic bud formation in female fetuses. Prostatic buds begin forming from the rat urogenital sinus (UGS) on day 18.5 of gestation (approximately 3 days prior to birth), and give rise to distinct dorsolateral and ventral regions.[19] The three-dimensional pattern of prostatic bud outgrowths in rat and mouse (as well as human) fetuses shows substantial similarities, which have been previously described.[17]

MATERIALS AND METHODS

Animals. Sprague Dawley rats were time-mated as previously described.[20] Rats were killed on both day 20 (n = 5) and 21 (n = 9) of gestation (mating = Day 0). Fetuses were removed, and sex was determined by initially measuring anogenital distance and subsequently verified by examination of internal genitalia. Both male and female fetuses residing in utero between male fetuses (2M) and between female fetuses (2F) were examined. Fetuses developing next to one male and one female fetus are intermediate between 2M and 2F fetuses in all previously examined traits and were not examined in this study.[7]

Morphometric parameters. It is important to note that it was not possible to represent (in the measurements listed below) the complexity of geometric shapes of the different prostatic buds and associated UGS structures. In addition, we could not obtain true volume data by dissection techniques normally used for adult tissue collection. However, the morphometric parameters we report provide an estimate of length, diameter and volume, and these measures appear to be sensitive markers of hormonal effects on prostate budding morphogenesis. The morphometric terms used in this study are based upon the following descriptions:

Mean cross-sectional area (MCSA; in $\mu m.^2$): calculated by first adding together the cross-sectional area data from each section, then dividing by the number of sections per structure to calculate the mean value. These values were calculated for the accessory sex gland primordia, as illustrated in fig. 1.

Total area of budding (TA; in $\mu m.^2$): the software used for this study did not allow surface rendering for true volume analysis, which would be equivalent to a weight measurement. Instead, the total area of buds was calculated as the sum of all of the individual cross-sectional areas in a specific region. This parameter served as an estimate of volume.

Length of budding (LB; in $\mu m.$): the length of the UGS along which prostatic buds and utricle were present. This was calculated from the number of sections (and thickness of sections; 7 μm.) associated with the region of the prostate, since transverse sections through the UGS were examined. For the seminal vesicles, this measure was the length of the developing structure, because the direction of growth was parallel to the plane of section.

Description of urogenital complex structures in male and female fetuses—males. The male urogenital complex, which comprises the bladder, associated urogenital sinus and developing accessory sex structures (fig. 1), was dissected and processed for histology and serial section reconstruction as previously described.[17] Serial sections of the urogenital complex were traced, digitized and reconstructed using computer-assisted 3D morphometric analysis. Epithelial outgrowths of the UGS in 20-day-old male fetuses, which occur as solid cords of cells, and termed prostatic buds, were categorized into anatomical regions: dorsocranial (coagulating

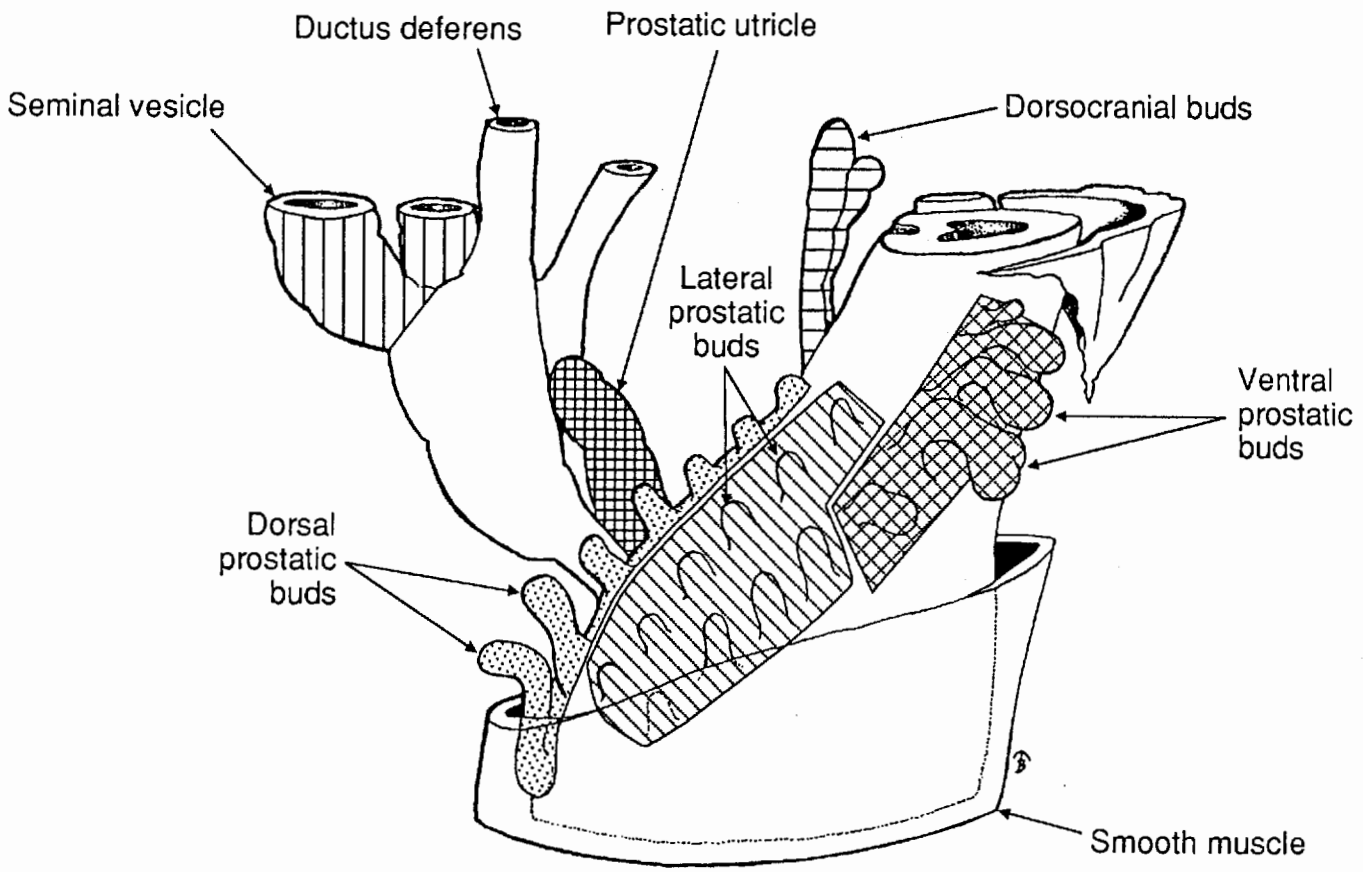

FIG. 1. Schematic illustration of budding pattern in early stages of rat prostate development in males, modified from Timms et al.[17] Hatched and stippled areas represent anatomical regions of urogenital sinus analyzed in this study and used in fig. 2 for data for 2F males.

glands), dorsal (excluding the dorsocranial buds), lateral, and ventral. Seminal vesicle outgrowths, which form from the lower segment of each Wolffian duct, and the prostatic utricle (a remnant of the Müllerian ducts), were also examined.

Females. In female fetuses in which we observed epithelial outgrowths from the UGS, the budding pattern mimicked, on a smaller scale, the budding pattern seen in the craniocaudal, lateral axis in males.[17] There is a different temporal pattern of prostatic outgrowths, as well as a lower incidence and area of outgrowths from the UGS, in females than in males.[18] We thus selected a later stage of fetal development (gestation day 21), and a different classification scheme in females (cranial, middle and caudal prostatic buds) than that used for males. Because the UGS in females is narrower than that of the male, the occurrence of buds on the ventral-dorsal axis is less pronounced. Buds were thus not classified in females as being ventral, lateral, or dorsal, to avoid confusion with the defined ductal tracts present in males.

In situ hybridization. Oligomeric probes were synthesized for a 48-base sequence within the steroid-binding domain of the mRNAs encoding both estrogen[21] and androgen[22] receptors. A 48-base sense probe to the estrogen receptor was used as a control for the in situ procedure. Each 12-μm. cryosection was covered with 25 μl. of hybridization buffer, containing the appropriate ^{35}S-labeled probe, under Parafilm coverslips at 37C in a humidified chamber for approximately 18 hours. Hybridization buffer contained 4XSSC, 50% (vol/vol) formamide, 10% (wt/vol) dextran sulfate, 250 μg./ml. yeast transfer RNA, 500 μg./ml. sheared single-stranded salmon sperm DNA, 1X Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone and 0.02% BSA) and 100 mM dithiothreitol. After the overnight hybridization, sections were washed in four 15-minute changes of 50% (vol/vol) formamide/2XSSC at 40C followed by two changes of 1XSSC for 1 hour each at room temperature. After a brief water rinse and a 5-minute rinse in 70% ethanol, sections were dried. The slides were then dipped in Kodak NTB3 emulsion and exposed at 4C for an appropriate length of time (1 week for the androgen receptor mRNA probe and 2 weeks for the estrogen receptor mRNA probe and control, sense probe). Emulsions were developed and counterstained with toluidine blue, and photographed using darkfield or brightfield illumination.

Data analysis. The data are presented as the mean cross-sectional area and total cross-sectional area of outgrowths (and length along the urethra) in each anatomical region. Variation within groups is estimated by the standard error of the mean (± SEM). Statistical analysis (ANOVA) was performed using the Statistical Analysis System, GLM procedure for comparisons of 2F and 2M males, since males in each group were from separate litters. The null hypothesis was rejected at $p < 0.05$. Statistical analyses were not conducted on the size and length data for females, since too few 2M females showed evidence of prostate development for quantitative comparisons.

RESULTS

Males. Comparisons were made based on examination of histological sections from 5–2M and 5–2F male fetuses produced in 5 litters. To control for variance due to maternal effects, a 2F and 2M male was selected from each litter. There was a significant relationship between intrauterine position and the mean cross-sectional area (MCSA) of epithelial buds that form the accessory reproductive organs in males ($p < 0.001$). Comparisons of MCSA means for corresponding prostatic regions of males shown in fig. 2 reveal that, with the exception of the ventral region of the prostate, MCSA of the regional prostatic buds in 2F males was significantly greater than in 2M males. This relationship between IUP and MCSA was also observed in the seminal vesicles and prostatic utricle. In addition, for the prostatic urethra, the MCSA measure was again significantly greater in 2F males (92,549 ± 9,012 μm. ^{2}) than in 2M males (72,127 ± 3,333 μm. ^{2}; $p < 0.05$).

There was no significant difference in the length of budding (LB) measure for each region, except the dorsocranial prostate (coagulating glands), where this measure was significantly smaller in 2F males (466.2 ± 59.5 μm.) than in 2M males (597.8 ± 16.8 μm.; $p < 0.05$). In contrast, the MCSA measurement for the dorsocranial prostate was significantly greater for 2F than 2M males. These observations suggest that the developing dorsocranial prostate gland in 2F males was shorter, but of larger diameter, than that in 2M males. Thus, while the shape of these buds in 2M and 2F males was different, the TA measure, which incorporates both MCSA and LB, was not significantly different. For buds observed in all other regions examined, there were no significant differences between 2M and 2F fetuses for the TA or the LB measures, although, as above, there were observable shape differences. There were also no significant differences ($p > 0.1$) between 2F and 2M males in any region of the developing prostate in the number of buds. For the dorsal, lateral and ventral prostate, the mean (± SEM) number of buds for 2F males was 12.0 ± 1.4, 29.6 ± 4.4 and 34.0 ± 6.8, and for 2M males was 13.2 ± 0.6, 36.8 ± 2.2 and 29.8 ± 2.6.

Females. The urogenital complex was analyzed in 2F ($n = 14$) and 2M ($n = 12$) females from 9 litters on Day 21 of pregnancy. In females, buds were located on a lateral-dorsal plane with the cranial buds somewhat ventral to the caudal buds (fig. 3, inset). All buds were located along the lateral aspect of the UGS, but there were no buds observed below the point of fusion between the UGS and the lower portion of the Müllerian ducts (the vaginal plate). The latter becomes the upper part of the vagina in females and persists, to varying degrees, as the prostatic utricle in males. We consider these urothelial glandular outgrowths in the female rat to be homologous with the periurethral glands in the human female, based on their proximity to the neck of the bladder and the fact that they are located cranial to the vaginal plate.[23]

Prostatic buds were not observed in all females; 10/14 (67%) 2F females and 4/12 (29%) 2M females had buds in at least one region (cranial, middle or caudal; $p < 0.05$, Chi Square). The mean (± SEM) number of buds for the 10-2F females that showed budding was 3.7 ± 0.6, and for the 4 to 2M females with buds was 2.5 ± 0.8. The results shown in fig. 3 reveal that for the 10–2F and 4–2M females that had buds, 2F females appeared to exhibit a greater MCSA than the 2M females in the cranial and caudal regions. The TA measurements for the middle and caudal regions of 2F females also appeared greater than those of 2M females (table). There appeared to be no difference between 2F and 2M females in the MCSA of the urethra or for any region in the LB measure. Thus, while the length of the line of buds did not differ in 2F and 2M females, the frequency of occurrence and size of the buds appeared to differ.

In situ hybridization. In situ hybridization was performed on parasaggital sections of UGS to examine the localization of mRNAs encoding androgen and estrogen receptors within tissues comprising the urogenital complex (mesenchyme and epithelium). Data were obtained from tissues collected from two male fetuses, each located in utero between a male and female fetus on day 20 of gestation (to control for any IUP differences between 2F and 2M males). The results shown in fig. 4 reveal that for both androgen and estrogen receptor mRNAs, cells in the urogenital mesenchyme were specifically labeled. In contrast there was only background labeling in the urogenital epithelium and prostatic buds for estrogen receptor mRNA, while androgen receptor mRNA was detected at low levels in urogenital epithelium. The sense probe for estrogen receptor mRNA showed only background levels of labeling in both mesenchyme and epithelium. These findings are consistent with prior observations, based on steroid

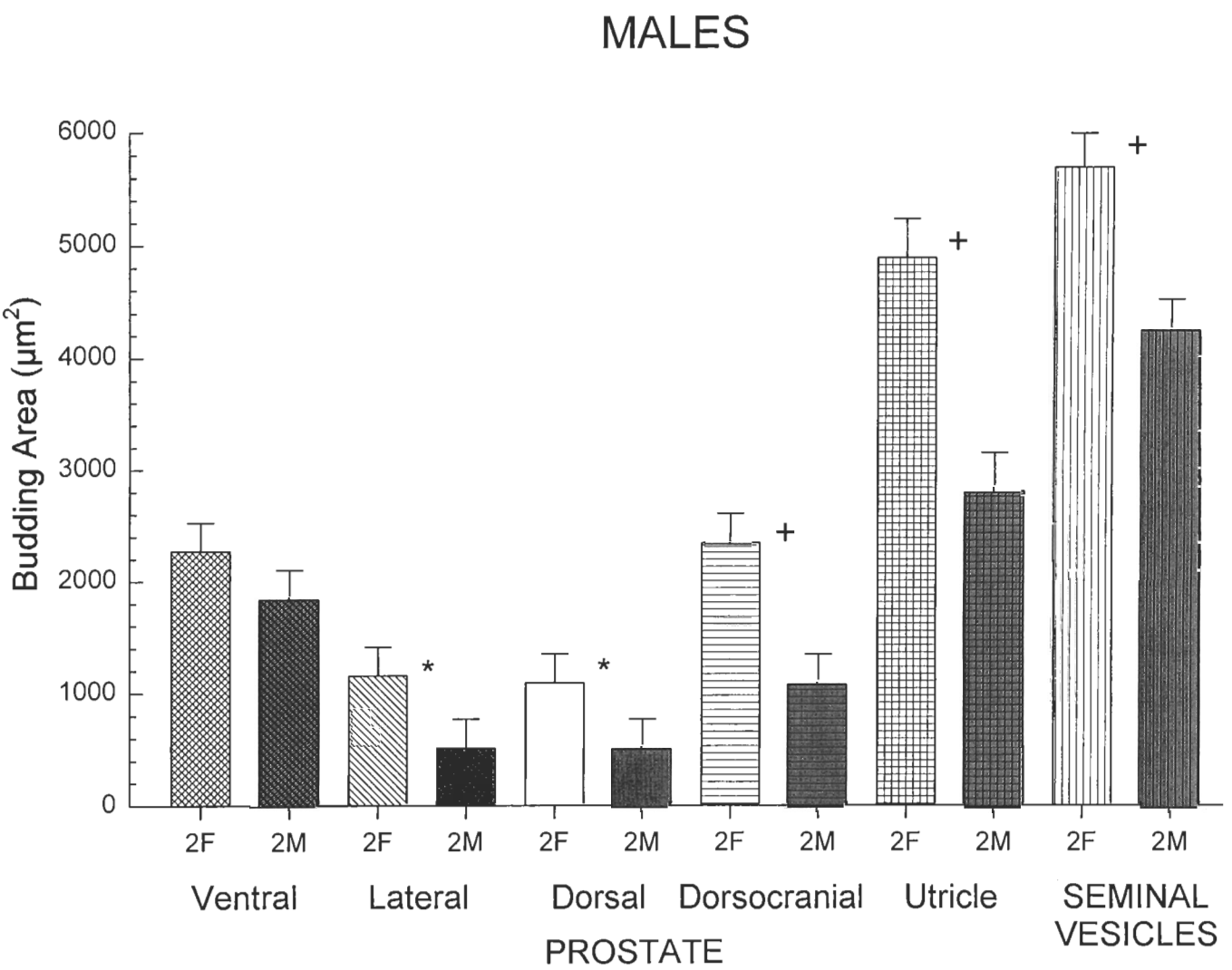

FIG. 2. Mean (±SEM) area (in sq. microns) of budding from urogenital sinus in 20 days-old male fetuses located between two females (2F) or two males (2M). Regions of prostate and associated structures correspond to areas identified in fig. 1. Comparisons of 2M (n = 5) and 2F (n = 5) male fetuses were made by analysis of variance using 95% confidence level for statistical significance. * = $p < 0.05$; + = $p < 0.001$; *t* test.

autoradiography and in situ hybridization analysis, which demonstrated that mesenchyme expresses receptors for both androgen and estrogen in the fetal UGS.[2,3,22]

DISCUSSION

The major finding from this study is that development of the urogenital system in male and female rat fetuses is influenced by their intrauterine proximity to fetuses of the same or opposite sex. Males located between female fetuses (2F males) had enlarged accessory reproductive organs (seminal vesicles as well as dorsocranial, dorsal and lateral prostate) relative to 2M males. 2F females were more likely to show prostatic budding than were 2M females, and the prostatic buds that formed in 2F females appeared to be larger than those that were formed in 2M females. These findings suggest that exposure to supplemental estradiol (due to being positioned between two female fetuses) induces prostatic bud development in females and enhances growth of prostatic buds in both males and females.

Because of the similarities of IUP effects on the regional development of the mouse and rat prostate, and since these effects in mice have been shown to be mediated by estrogen,[9] our hypothesis is that in rats, IUP differences are mediated by differences in estradiol, similar to mice. Future studies are needed to test this hypothesis. Specifically, in a previous study conducted with mice, circulating estradiol levels were increased by 50% in male mouse fetuses by implanting pregnant females with a silastic capsule containing estradiol. We performed 3D reconstructions on gestation day 18, and similar to our findings here, we observed a significant increase in the MCSA for the dorsal but not the ventral region of budding in males exposed to estrogen, relative to controls. Subsequently, in adulthood, males that had been exposed to experimentally elevated levels of estradiol during fetal life showed enlarged (hyperplastic) prostates relative to untreated males.[9]

One explanation for our findings is that estradiol enhances androgen-induced regulation of prostatic gland genesis, as well as glandular growth, during fetal life in rats. During fetal life 2F male mice have 30% higher serum estradiol levels than 2M males, and in adulthood, 2F male mice show an enlarged prostate associated with a 3-fold higher number of prostatic androgen receptors relative to 2M males.[16] When estradiol was increased in male mouse fetuses by 50%, prostate size was permanently increased and a 6-fold increase in androgen receptors was observed.[9] Elevated blood levels of estradiol experienced by 2F male fetuses may permanently imprint the growth characteristics of the prostate by increas-

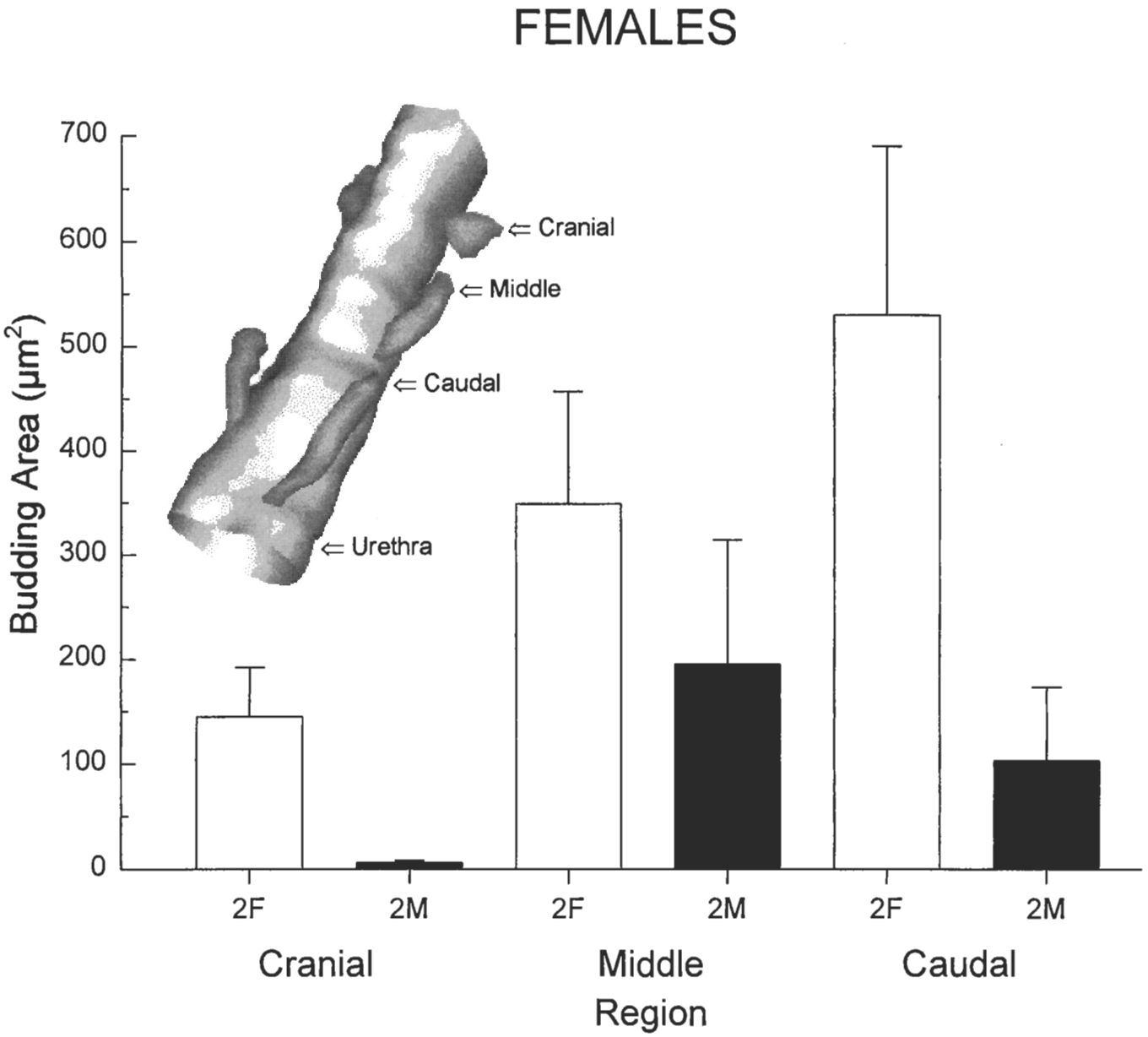

FIG. 3. Mean (±SEM) area (in sq. microns) of budding from urogenital sinus in 2F and 2M female fetuses on day 21 of fetal life. Inset: representative serial section reconstruction of budding pattern in 2F female (SURFdriver ® reconstruction software, University of Alberta, Edmonton, Alberta, Canada).

Total area of budding for 4-2M and 10-2F female fetuses in which prostatic buds were observed in cranial, middle and caudal segments of prostatic region of urogenital sinus, beginning caudal to bladder. No buds were observed below point of fusion between urogenital sinus and lower portion of Müllerian ducts (the vaginal plate)

	Cranial		Middle		Caudal	
	# Buds	TA	# Buds	TA	# Buds	TA
2M	6	1037 ± 357	4	641 ± 243	5	2202 ± 956
2F	11	3197 ± 817	12	5487 ± 1286	14	6245 ± 1278

ing prostatic androgen receptors and thus sensitivity to androgen. Estradiol increases the sensitivity of tissues to other hormones via increasing receptor numbers; this effect is well characterized with regard to uterine oxytocin receptors and both uterine and brain progesterone receptors.[30,31] Estradiol has also been shown to influence hypothalamic androgen receptors in adult male rats.[32]

In the present study, mRNA for androgen receptors was primarily localized in mesenchyme, and estrogen receptor mRNA was only located in mesenchyme, which is consistent with findings for androgen and estrogen receptor protein.[2,3] These findings suggest that the modulating effect of estradiol on epithelial glandular budding and growth is mediated by factors produced by mesenchymal cells.[24]

The finding of enhanced development of prostatic buds in the dorsolateral but not ventral region of the urogenital sinus in 2F male fetuses, relative to 2M males, supports the concept of differential inductive mechanisms of positional mes-

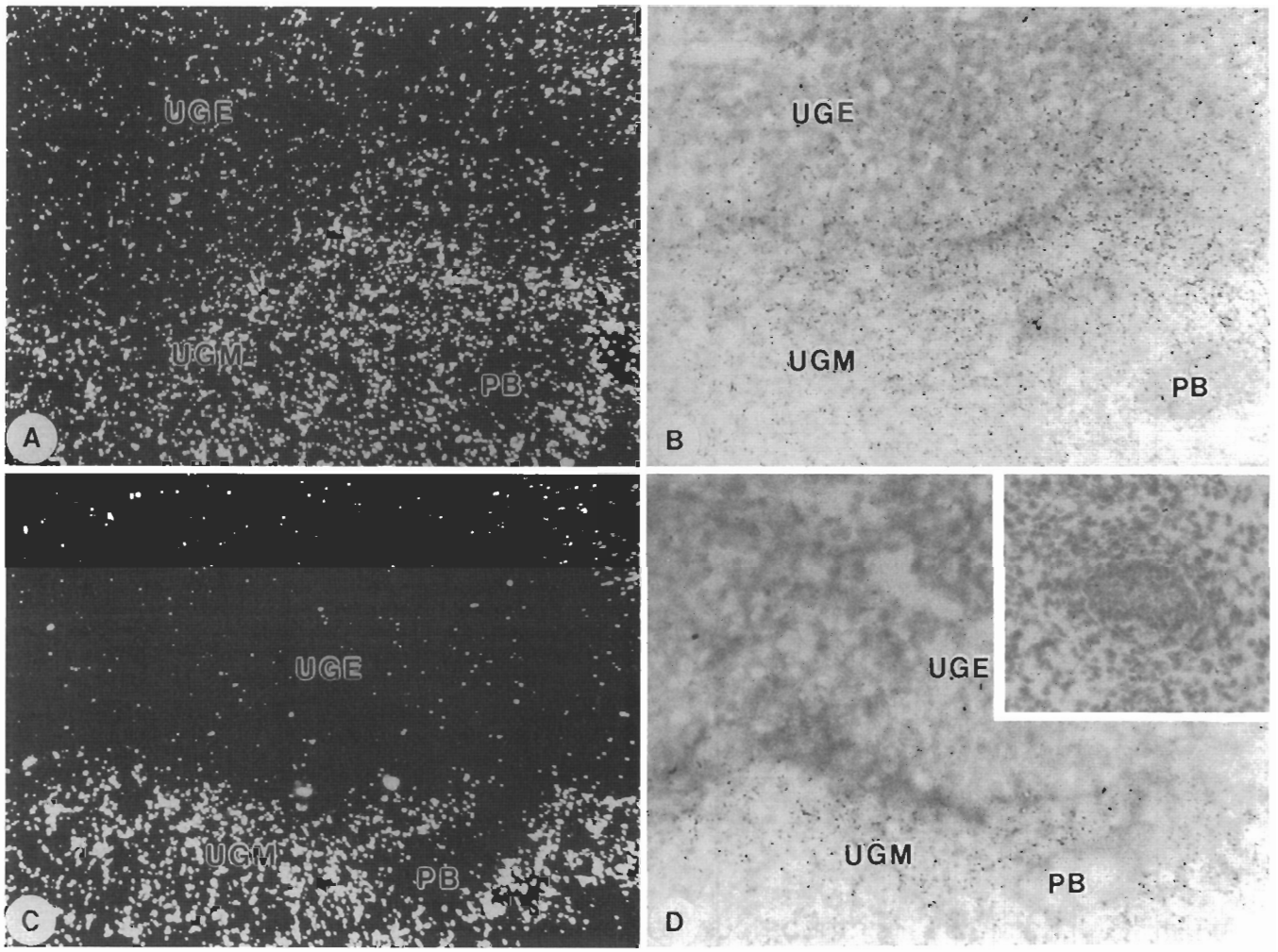

FIG. 4. Localization of androgen (*A, B*) and estrogen (*C, D*) receptor protein mRNAs in serial cryosections of male urogenital sinus from 20 days fetal rats. Silver grains are predominantly associated with urogenital mesenchyme (UGM) for both probes. Relative to mesenchyme, lower level of labeling was observed for androgen receptor mRNA in epithelium, while no specific labeling above background levels was observed for estrogen receptor mRNA. UGE: Urogenital epithelium; PB: prostatic bud. Dark-field (*A, C*) and brightfield illumination (*B, D*) photomicrographs are shown. Control section labeled with sense probe for estrogen receptor is shown in inset (*D*). Original magnification x 245.

enchyme.[4,5] This finding is of particular interest, because the ventral region of buds regresses and does not persist into adulthood in humans. The human prostate thus consists only of glands that originate in the dorsal and lateral regions of the urogenital sinus. Previous observations of comparative fetal ductal budding patterns in a variety of species have suggested that the prostatic buds on the cranial-caudal axis in the dorsal area of the urogenital sinus are homologous with the regions in the human male which develop benign prostatic hyperplasia (cranial origin) and cancer (caudal origin).[17,25]

Estradiol has been implicated in the etiology of prostate hyperplasia during senescence through a mechanism of stromal-induced proliferation of prostatic epithelium.[14] More than seventy years ago, Reischauer[26] proposed that stroma was associated with the formation of new ducts in benign prostatic hyperplasia (BPH). The hypothesis of a reawakening of inductive interactions between stroma and epithelium associated with BPH has also been proposed by McNeal.[27] Between young adulthood (4 months old) and middle age (18 months old) the prostate of CF-1 mice doubles in size.[14] It is possible that exposure to a small increase in estradiol during early development may predispose the prostate of 2F rodent males for altered growth parameters during senescence. This also leads to the hypothesis that an increase in estrogenic activity in the blood of male fetuses in humans, due to either endogenous estradiol or environmental estrogens,[28] might also be a factor in predisposing men to an early onset of BPH. The results of our studies suggest a high degree of sensitivity of the developing urogenital sinus to very small changes in estradiol. Low concentrations of estradiol have been reported to stimulate cell proliferation of human tissue in vitro.[29]

Recent interest in environmental chemicals with estrogenic activity has prompted speculation about the possible effects such compounds may have on the etiology of a variety of diseases, particularly when exposure occurs during fetal development.[28,33] For example, Nagel et al[34] reported that ingestion by pregnant mice of a low dose of the estrogenic chemical bisphenol A (used to make polycarbonate plastic) led to a permanent enlargement of the prostate in male offspring, similar to effects of low doses of estradiol and diethylstilbestrol (DES).[9] These findings add further credence for our hypothesis that exposure to a very small increase in estrogen during fetal life predicts prostatic size in adulthood.

The present experiment offers a significant contrast to most studies of the effects of estrogen on prostate development that have involved the administration of very high, pharmacological doses of estrogen. A consistent observation in this regard has been that exposure to estrogen inhibits normal development of the prostate.[35–37] An opposite effect of low and high doses of estrogen on prostate development

was recently demonstrated in a study in which male mouse fetuses were exposed (via maternal ingestion) to very low (parts per trillion) doses of DES during fetal development, and a permanent increase in prostate size was observed. This outcome was opposite to effects of much higher doses of DES, which permanently inhibited normal prostate development. Specifically, across a 5-log range of doses of DES, an inverted-U dose-response relationship between fetal exposure to DES and adult prostate weight was observed.[9]

We found that 2F females were more likely to develop prostate glandular buds, and the buds that formed were larger, in comparison to 2M females. A prostate gland is found in the female of several species, including rodents and humans.[38–40] Based on studies showing that the female UGS could be induced by exogenous testosterone to form prostatic buds,[41] it had been anticipated that 2M females would be more likely than 2F females to exhibit glandular morphogenesis. Interestingly, through selective breeding protocols, a strain of Wistar rats was found to have prostate development in female fetuses.[18] It is possible that prostate development in these female rats was due to selective breeding for females with elevated estradiol levels during fetal life. In women, periurethral glands grow in a lower androgen environment relative to that in male fetuses, which also suggests that factors other than androgen play a role in the development and maintenance of glands that originate in the cranial region of the developing dorsolateral UGS.[23]

In summary, results from this study add credence to the concept that development of the prostate and other accessory reproductive organs in males is regulated by a finely tuned interaction between estrogen-mediated and androgen-mediated mechanisms within the mesenchyme of the urogenital complex. Physiological levels of estradiol may stimulate regional epithelial glandular growth via factors secreted by mesenchyme. The stimulating effect of an increase in estradiol within a physiological range was not predicted by prior studies involving administration of supraphysiological doses of estrogenic compounds. The development of glandular buds that form the ventral lobe of the prostate in male rats was unaffected by fetal intrauterine position, while the dorsocranial, dorsal and lateral region of buds were all affected. These findings provide further evidence for regional differences in response of the urogenital complex to the hormonal control of differentiation and growth.

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