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Sexual Differentiation in the Male and Female Mouse from Days 0 to 21: A Detailed and Novel Morphometric Description

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Abstract

Purpose—We hypothesized that a significant portion of sexual differentiation of mouse external genitalia occurs postnatally. To establish a baseline of normal development to which genital abnormalities could be compared, we determined morphometric measurements and morphological descriptions of the postnatal period of sexual differentiation in mice from birth to 21 days postnatally.

Materials and Methods—The external genitalia of male and female CD-1® mice were serially sectioned for histological staining. Mice were studied by age group, including ages 0 to 1, 2 to 3, 4 to 5, 10 and 21 days. Three-dimensional reconstructions were created to visually compare differences. Morphometric measurements were made of the distal mouse external genitalia and compared across age groups, and between males and females.

Results—The morphology of male and female mouse external genitalia is similar at 0 to 1 days but undergoes dramatic changes during 21 days. The changes include the development of mesenchymal extensions, which in males form the male urogenital mating protuberance and erectile bodies, as well as comparable structures in females. Differences in morphometric measurements in homologous males and females became pronounced during 0 to 21 days, serving as a baseline for recognizing the genesis of malformations of the mouse external genitalia.

Conclusions—Male and female mouse external genitalia are similar morphologically and morphometrically at birth but achieve dramatic differences by age 21 days, suggesting that a significant portion of sexual differentiation in the mouse occurs after birth. We described these changes using novel, accurate morphometric measurements and 3-dimensional reconstruction. These results will be useful for studying abnormal sexual development of the mouse external genitalia.

Keywords

genitalia, female; genitalia, male; sex differentiation; anatomy and histology; growth and development

Sexual differentiation of the ExG is a complex process. In humans the process of sexual differentiation of the ExG starts around week 8 of gestation and is complete by the early second trimester.^{1–3} Defects in this process in males can lead to penile abnormalities such as hypospadias.^{4,5}

In mice ExG development begins with assembly of the ambisexual GT from embryonic days 12 to 16. Sexual differentiation into male and female phenotypes begins at 16 days of gestation. While male and female mice can be sexed visually at birth based on the longer anogenital distance and larger size of the GT, by histological criteria the male and female ExG appear undifferentiated at birth.

We previously described the adult mouse penis, which is the end point of sexual differentiation, using morphometric measurements, histology, 3DR and scanning electron microscopy.^{6–8} We reported the MUMP, MUMP ridge, urethral flaps and MUMP corpus cavernosum, and confirmed previously described structures such as the CCG, corpus cavernosum urethrae and os penis. , Our initial histological analysis of serially sectioned male and female ExG at day 21 revealed that all elements of adult structures were present. In contrast, initial viewing of serial sections of the GT at day 0 revealed strikingly similar male and female morphology with several structures completely absent in each gender compared to day 21 of life or adulthood. This suggests that most sexual differentiation develops after birth.

We hypothesized that a significant portion of sexual differentiation of the mouse ExG occurs postnatally. We describe the normal anatomy and morphological changes of the distal ExG in male and female mice from day 0 to 21 of life using serial histological sections, morphometric measurements, statistical analysis and 3DRs. We present our system of morphometric measurements and analysis, which can be used in future studies to measure and report morphometric changes related to abnormalities in mouse sexual differentiation, such as hypospadias.

MATERIALS AND METHODS

Animal Use and Specimen Preparation

The University of California-San Francisco animal care committee approved the protocol. Pregnant CD-1 mice were housed in polycarbonate cages with free access to Purina LabDiet® and tap water. CD-1 mice were chosen because they are outbred and a commonly used strain. Day of birth was designated as day 0. Pups were sacrificed at days 0 to 1, 2 to 3, 4 to 5, 10 and 21 of life with at least 2 litters sacrificed per age range. The ExG were dissected, fixed in formalin, embedded in paraffin, serially sectioned transversely at 7 µm and stained with hematoxylin and eosin.

Morphometric Measurements

We measured structures of the distal ExG by counting the number of consecutive serial transverse sections that contained the structure of interest. Relational measures were obtained by counting the number of serial transverse sections from 1 point of interest to

another. The length of measurements in μm was determined by multiplying the number of sections by 7 μm .

Only well aligned specimens were included. We defined a well aligned specimen as one in which the penile or clitoral body was a circle or ellipse in the horizontal plane, paired right and left structures appeared within 5 serial sections and all structures could be measured. A specimen in which the penile or clitoral body was an ellipse in the vertical plane suggested that the specimen was tilted significantly in the vertical plane, which affects the results of relational measurements. A specimen in which paired right and left structures did not appear around the same serial section suggests that the specimen was tilted significantly in the horizontal plane. A total of 88 male specimens were sectioned with 47 (53%) included in study and 92 female specimens were sectioned with 47 (51%) included, representing 10, 13, 9, 8 and 7 males, and 11, 14, 10, 8 and 4 females at ages 0 to 1, 2 to 3, 4 to 5, 10 and 21 days, respectively.

Three-Dimensional Reconstructions

Computer generated 3DRs were generated from sets of serial sections using BioVis3D reconstruction software (BioVis3D, Montevideo, Uruguay). A digital image was obtained of each section and aligned with the previous section using the software. Structures of interest in the distal ExG were outlined in aligned serial sections using each section and 3DRs were generated.

Statistical Analysis

Morphometric measurements were compared across age groups using 1-way ANOVA with the Bonferroni correction for multiple comparisons across groups. Select morphometric measurements were compared between males and females using the 2-tailed t test for different age groups.

RESULTS

CD-1 Mice

Males—Figure 1 shows a 3DR and transverse sections of the ExG of a 4 to 5-day-old male mouse to illustrate the relational measurements that were made. A critical point was to define the start of the penile urethra. This is important because there are 2 epithelial lined tubes in developing and adult male ExG, namely the preputial space and urethra, which are continuous with each other at the urethral meatus. The prepuce detaches from the penis distal by delamination of the solid preputial lamina, which has attachments to epithelia circumscribing other structures (fig. 1, A to D). The start of the penile urethra was defined as the point where an epithelium lined tube was completely surrounded by penile stroma in transverse section (fig. 1, E). The overall length of the distal penis was measured from the tip of the bifid extension of the MUMP to the point where the only epithelium remaining in the penis was urethral epithelium on transverse section (fig. 1, F). Ventral confluence of the prepuce was defined as the point in serial sections where preputial stroma became confluent across the ventral midline (fig. 1, A).

Figure 2 shows a 10-day-old CD-1 mouse penis with an intact prepuce. The overlying 3DRs are approximately to scale and show the relationship between the prepuce and penis.

The table lists measurements of the developing mouse penis by age category. Penile length increased significantly from days 0 to 21 (fig. 1). Morphogenesis of the distal male ExG was characterized in part by the development of 3 paired mesenchymal extensions of the central penile mesenchyma, including paired distal mesenchymal extensions from the bifid MUMP tip, paired lateral mesenchymal extensions from the MUMP corpus cavernosum and paired ventral mesenchymal extensions that formed the corpus cavernosum urethrae. The length of these 3 paired extensions increased significantly from day 0 to 21 (seeTable). Relational measurements of interest included the distance from the MUMP tip to the ventral mesenchymal extension tip, to the lateral mesenchymal extension tip, to the beginning of the urethra and to the ventral preputial confluence (seetable). The relational measurement that changed the most from day 0 to 21 was the point of ventral preputial confluence in relation to the MUMP tip. Ventral preputial fusion was proximal to the MUMP tip at day 0 to 1 and quite distal to the MUMP tip by day 21, as denoted by negative values (seetable). An additional measurement made at day 21 but not at earlier time points was the length of the ventral cleft in the MUMP ridge. At 21 days the mean $\pm$ SD length of the ventral cleft of the MUMP ridge was $283 \pm 99 \mu\text{m}$.

Figure 3 shows the development of the ExG of male CD-1 mice from birth to 10 days, as represented by 3DRs with the prepuce removed. In contrast, figure 2 shows the prepuce. Morphological changes in the distal male mouse ExG were characterized by an overall increase in the length of all parameters and the development of the 3 paired mesenchymal extensions (fig. 3). The morphology of a male CD-1 mouse at age 21 days included the MUMP ridge ventral cleft (fig. 4). The MUMP ridge ventral cleft was not discernible at earlier ages.

Females—Figure 5 shows a 3DR and transverse sections of ExG structures measured in 10-day old female CD-1 mice. The fully formed clitoral body was defined as the point where a U-shaped clitoral epithelial lamina was fully formed and, therefore, not connected to any other epithelia (fig. 5, F). The FUMP was defined as a midline distal mesenchymal structure with a bifid tip extending distal to the fully formed clitoral body, appearing homologous to the MUMP (fig. 5, B and C). The overall length of the distal ExG in females was measured from the FUMP tip to the fully formed clitoral body. The start of the female urethra was defined as the point where the urethral epithelial tube was completely surrounded by stroma and not attached to other epithelial structures (fig. 5,E). The ventral confluence of the female prepuce was defined as the point in transverse section where stroma were confluent across the ventral preputial midline.

The table lists ExG measurements of female CD-1 mice by age category. The distal ExG in female CD-1 mice did not change as dramatically as in males in overall length from day 0 to 21. As in males, the development of the distal ExG in females was characterized by 3 paired mesenchymal extensions of the central clitoral mesenchyma, including paired distal extensions of the FUMP, paired lateral mesenchymal extensions and paired ventral mesenchymal extensions. The length of these 3 paired mesenchymal extensions increased

significantly from day 0 to 21 (seetable). The relational measurement with the most dramatic change from day 0 to 21 was the point of ventral preputial confluence across in relation to the FUMP tip, while the ventral preputial confluence was proximal to the FUMP tip at day 0 to 1 and then distal to the FUMP tip by day 21 (seetable). Figure 3 shows the development of the distal ExG in female CD-1 mice from age 0 to 10 days as 3DRs with the preputial skin and stroma removed.

Male and Female CD-1 Mouse ExG Comparison

The male and female murine ExG were most similar in appearance at day 0 to 1, as shown by 3DRs (fig. 3). Statistical comparisons were made between the ExG of male and female mice in overall length, the length from the MUMP or FUMP tip to the urethra, the ventral preputial stromal confluence in relation to the MUMP or FUMP tip and the length of the 3 paired mesenchymal extensions. There was no significant difference in overall distal ExG length between males and females at day 0 to 1 ($p = 0.12$). However, the male ExG were significantly longer in all other age groups ($p < 0.001$). The bifid mesenchymal extensions of the MUMP were longer than the homologous bifid FUMP mesenchymal extensions in all age groups, attaining statistical significance on days 2 to 3, 4 to 5 and 21 ($p < 0.01$). Lateral and ventral mesenchymal extensions were significantly longer in males than in females at all ages ($p < 0.01$). The point of ventral preputial stromal confluence in relation to the distal tip of the FUMP or MUMP was significantly more distal in males. The smallest difference between males and females was seen at day 0 to 1. Figure 6 shows how morphometric measurements evolved with time in male and female mice. In general, male and female ExG measurements and 3DR appearance were most similar in the youngest age group at day 0 to 1.

DISCUSSION

We describe normal morphogenesis of the distal mouse ExG from birth to day 21. The slight morphological differences between males and females at birth, and the dramatic differences at day 21 support our hypothesis that a significant portion of ExG sexual differentiation in mice develops after birth, which we suggested previously. Using simple morphometric methods requiring well oriented serial sections and the ability to recognize and count structures of interest, we prepared a detailed morphometric baseline of the process by which the undifferentiated GT of newborn mice gradually develops into mature male and female ExG by day 21. Our morphometric measurements and morphological descriptions of normal development will be helpful in future studies of abnormal ExG development in mice, including hypospadias.

Hypospadias in humans is defined by an abnormal proximal location of the urethral meatus, deficient ventral foreskin and ventral penile curvature. A difficulty in using a mouse model of hypospadias is that the mouse penis is anatomically different from the human penis. Accordingly, the term mouse hypospadias was uncritically applied to a range of ventral defects of the embryonic GT in numerous studies.^{8,10–12} Many of these mouse GT malformations reported previously as hypospadias were surely defects in the prepuce but whether they progress to adult penile malformations has been rarely reported in mice.

Clearly, diagnosing mouse hypospadias based only on analysis of gross morphology and transverse sections of embryonic GTs^{10–12} is not an adequate measure of mouse hypospadias since we noted that significant changes develop after birth in normal mouse sexual development. We suggest that a diagnosis of mouse hypospadias or other abnormalities after exposure or genetic manipulation should be confirmed by morphometric measurements of relevant anatomical structures in control and exposure groups at age 21 days or older. Since changes in morphology may be subtle after experimental exposure in mice, it is important to have an accurate, reliable method to measure and quantify morphology.

In humans a proximal urethral meatus is a feature of hypospadias. The urethral meatus of the mouse is defined by the MUMP ridge, which has an open ventral cleft. Accordingly, mouse hypospadias would likely include an increased proximal-distal length of the ventral cleft in the MUMP ridge or distance from MUMP tip to penile urethra. A deficient ventral foreskin is a feature of hypospadias in humans. Thus, the distance from the MUMP tip or from the penile urethra to the ventral preputial stromal confluence may be a feature of mouse hypospadias.

Limitations

An aspect to consider that can be a limitation when using transverse sections to make morphometric measurements is that specimen misalignment adds to measurement error. Therefore, only well aligned specimens should be used for morphometric measurements and an adequate number of samples should be measured. We can achieve adequate alignment about 50% of the time when embedding is performed with a dissecting microscope.

CONCLUSIONS

Male and female mouse ExG morphology is morphologically and morphometrically similar at birth but becomes dramatically different in the next 21 days, suggesting that a significant portion of sexual differentiation in the mouse develops after birth. We describe these changes using novel, simple morphometric measurements and 3DRs. Understanding this period of sexual differentiation and the use of morphometric measurements will be useful for studying abnormal sexual development of the mouse ExG.

Abbreviations and Acronyms

3DR	3-dimensional reconstruction
CCG	corpus cavernosum glandis
ExG	external genitalia
FUMP	female urogenital mating protuberance
GT	genital tubercle
MUMP	male urogenital mating protuberance.

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Figure 1.

Transverse sections (A to F) of penis of 5-day-old CD-1 mouse in dorsal (1) and lateral (2) views, and 3DRs without prepuce elongated by factor of 3. Red lines indicate where transverse sections correspond to 3DRs. *a*, bifid distal extension of MUMP (A, yellow outlines). *b*, lateral mesenchymal extension (C, purple outlines). *c*, ventral mesenchymal extension (D, red outlines). *d*, MUMP tip to ventral extension. *e*, MUMP tip to lateral extension. *f*, MUMP tip to penile urethra. *g*, MUMP tip to CCG (E, green outlines). *h*, CCG length. *i*, MUMP tip to penile body. Red arrows (A to D) indicate preputial space not shown on 3DRs. Yellow double arrows (A to F) indicate prepuce stroma not shown on 3DRs. Orange arrows and outlines indicate penile urethra (E and F). Black arrows indicate where penile stroma completely surround epithelial tube, indicating penile urethra (E). Blue outlines indicate central penile body (B to F). Scale bar indicates 500 μm (A to F).

Figure 2.

Lateral (A) and dorsal (B) 3DRs of 10-day-old male CD-1 mouse superimposed on penile dissections with intact prepuce. Note how prepuce extends distal to MUMP tip so that most elevation on perineum is prepuce. At age 10 days ventral confluence of preputial is distal to MUMP tip. Composite provides reasonably accurate representation of relative position of developing penis to hair bearing elevation in perineum, which is prepuce. Scale bar indicates 500 μm.

Figure 3. Dorsal and lateral 3DRs of male and female CD-1 mice ExG without prepuce by age group with 3DRs elongated by factor of 3. Scale bar indicates 500 μ m.

Figure 4.

Transverse sections (A to D) and 3DRs (1 to 3) elongated by factor of 3 show MUMP ridge ventral cleft in 21-day-old CD-1 male mouse. Red lines indicate where 3DRs correspond to transverse sections. Note lateral (1) and ventral (2) 3DR views. *a*, length of MUMP ridge ventral cleft (2, yellow arrows). Semitransparent penile body and CCG demonstrate ventral and lateral mesenchymal structures and extensions, and distal tips of ventral mesenchymal extensions proximal to ventral stromal confluence of MUMP ridge (3). Black arrow (D) indicates point of ventral stromal confluence of MUMP ridge, which is end of MUMP ridge ventral cleft. Yellow outlines indicate bifid MUMP tip (A). Red arrow indicates preputial space (A and B). Double yellow arrows denote preputial stroma (A to D). Blue outlines indicate central penile mesenchyma (B to D). Green outlines indicate CCG (C and D). Orange outlines and arrow indicate penile urethra (C and D). Scale bar indicates 500 μ m (A to D).

Figure 5.

Dorsal (1) and lateral (2) 3DRs elongated by factor of 3 without prepuce, and transverse sections (A to F) demonstrate morphometric measurements in 10-day-old CD-1 female mouse. Red lines indicate where transverse sections correspond to 3DRs. a, bifid distal extension of FUMP (A, yellow outlines). b, lateral mesenchymal extension (D, purple outlines). c, ventral mesenchymal extension (B to D, red outlines). d, FUMP tip to ventral extension. e, FUMP tip to lateral extension. f, MUMP tip to CCG. g, CCG length. h, total length. i, FUMP tip to urethra. Red arrows indicate preputial space (A to D). Yellow double arrows indicate preputial stroma (A to F) shown in 3DRs. Orange outlines and arrows indicate urethral stroma and urethra (D to F). Black arrows indicate point where urethral epithelium is not in contact with other epithelium, indicating start of female urethra (E). Scale bar indicates 500 μm (A to F).

Figure 6.

Measurement changes with time in 10, 13, 9, 8 and 7 males, and 11, 14, 10, 8 and 4 females on days 0–1, 2–3, 4–5, 10 and 21, respectively. Relational measures in male and female CD-1 mice (A). Negative measurements indicate ventral stromal confluence distal to MUMP or FUMP tip. *Length*, overall length. *Tip to urethra*, MUMP or FUMP tip to start of urethra. *Tip to prepuce confluence*, MUMP or FUMP tip to ventral confluence of preputial stroma. Length of paired mesenchymal extensions (B). *Distal bifid*, distal bifid extension of MUMP

or FUMP. *Lateral*, lateral mesenchymal extension. *Ventral*, ventral mesenchymal extension. Error bars indicate $2 \times \text{SE}$.

Male and female morphometric measurements by age category

	Day 0-1*	Day 2-3	Day 4-5	Day 10	Day 21
No. males	10	13	9	8	7
Mean $\pm$ SD morphometric measurement (μm):					
MUMP bifid distal extension [†]	3.5 $\pm$ 6.2	26.4 $\pm$ 13.2 [‡]	29.6 $\pm$ 13.1 [‡]	72.6 $\pm$ 35.4 [§]	146.5 $\pm$ 39.9
Lateral mesenchymal extension [†]	2.8 $\pm$ 4.2	31.8 $\pm$ 24.0 ^{*‡}	52.1 $\pm$ 20.0 [‡]	169.8 $\pm$ 55.8 [§]	278.5 $\pm$ 62.2
Ventral mesenchymal extension [†]	17.2 $\pm$ 8.6	52.5 $\pm$ 33.0 [‡]	103.4 $\pm$ 18.2 [§]	172.4 $\pm$ 22.0	254.0 $\pm$ 45.3
MUMP tip-ventral extension [†]	207.2 $\pm$ 34.0	237.7 $\pm$ 50.8 [*]	252.0 $\pm$ 23.2 [*]	378.9 $\pm$ 113.8 [‡]	826.5 $\pm$ 218.1 [§]
MUMP tip-lateral extension [†]	126.4 $\pm$ 23.0	168.8 $\pm$ 31.2 ^{*‡}	194.4 $\pm$ 30.9 ^{‡§}	250.7 $\pm$ 53.2 [§]	492.0 $\pm$ 128.7
MUMP tip-penile urethra	256.2 $\pm$ 47.8	303.7 $\pm$ 49.4 ^{*‡}	370.2 $\pm$ 24.6 [‡]	576.6 $\pm$ 123.9 [§]	1111.0 $\pm$ 251.7
MUMP tip-CCG	180.6 $\pm$ 31.3	234.8 $\pm$ 49.3 ^{*‡}	311.9 $\pm$ 33.6 [‡]	454.1 $\pm$ 58.3 [§]	706.0 $\pm$ 206.2
CCG length	180.6 $\pm$ 55.1	209.5 $\pm$ 85.5 ^{*‡}	172.7 $\pm$ 45.0 [*]	308.9 $\pm$ 151.3 [‡]	307.0 $\pm$ 76.9 [‡]
MUMP tip-penile body	361.9 $\pm$ 48.7	459.3 $\pm$ 73.8 [*]	512.6 $\pm$ 25.4 [*]	791.0 $\pm$ 144.9 [‡]	1184.0 $\pm$ 244.3 [§]
MUMP tip-ventral preputial confluence ^{**}	119.7 $\pm$ 60.9	-6.4 $\pm$ 71.4 [*]	-92.6 $\pm$ 68.5 [‡]	-381.5 $\pm$ 66.5 [§]	-949.0 $\pm$ 276.7
No. females	11	14	10	8	4
Mean $\pm$ SD morphometric measurement (μm):					
FUMP bifid distal extension [†]	1.0 $\pm$ 2.5	2.0 $\pm$ 3.7 [*]	10.5 $\pm$ 8.6 [*]	64.8 $\pm$ 19.5 [‡]	97.1 $\pm$ 22.9 [§]
Lateral mesenchymal extension [†]	0 $\pm$ 0	1.0 $\pm$ 2.5 [*]	14.4 $\pm$ 8.6 [‡]	32.4 $\pm$ 17.5 [§]	26.8 $\pm$ 21.4 [§]
Ventral mesenchymal extension [†]	0 $\pm$ 0	2.3 $\pm$ 3.3 [*]	14.4 $\pm$ 9.2 [*]	131.7 $\pm$ 29.1 [‡]	210.0 $\pm$ 84.8 [§]
FUMP tip-ventral extension ^{†***}	204.3 $\pm$ 29.0	200.8 $\pm$ 24.3 [*]	207.2 $\pm$ 37.8 [*]	68.3 $\pm$ 23.2 [‡]	-0.9 $\pm$ 107.0 [§]
FUMP tip-lateral extension [*]	86.9 $\pm$ 15.7	91.5 $\pm$ 16.6 [*]	112.7 $\pm$ 44.7 [*]	150.9 $\pm$ 37.2 [‡]	166.3 $\pm$ 41.3 [‡]
FUMP tip-CCG	94.8 $\pm$ 18.4	92.5 $\pm$ 39.8 [*]	112.0 $\pm$ 51.0 [*]	250.3 $\pm$ 60.9 [‡]	266.0 $\pm$ 80.2 [‡]
CCG tipeU-shaped clitoris	230.4 $\pm$ 62.9	206.0 $\pm$ 46.7 [*]	193.9 $\pm$ 72.7 ^{*‡}	133.0 $\pm$ 21.2 [‡]	175.0 $\pm$ 12.8 ^{*‡}
FUMP tipeU-shaped clitoris	325.2 $\pm$ 58.9 ^{‡§}	298.5 $\pm$ 30.6 [‡]	305.9 $\pm$ 51.2 ^{*‡}	380.6 $\pm$ 52.2 [§]	441.0 $\pm$ 72.4
FUMP tip-urethra	325.2 $\pm$ 53.9 [§]	298.5 $\pm$ 30.6 [*]	305.9 $\pm$ 51.2 [*]	225.8 $\pm$ 19.0 ^{‡§}	246.8 $\pm$ 31.5 [§]
FUMP tip-ventral prepuce fusion [†]	337.9 $\pm$ 82.1	333.0 $\pm$ 79.5 [*]	288.4 $\pm$ 90.2 [*]	17.5 $\pm$ 64.4 [‡]	-136.5 $\pm$ 165.0 [‡]

* Measurement significantly differed among age groups (ANOVA with Bonferroni correction $p < 0.05$).

[†] Two measurements per mouse for paired structures.

[‡] Measurement significantly differed among age groups (ANOVA with Bonferroni correction $p < 0.05$).

[§] Measurement significantly differed among age groups (ANOVA with Bonferroni correction $p < 0.05$).

^{||} Measurement significantly differed among age groups (ANOVA with Bonferroni correction $p < 0.05$).

[¶] Measurement significantly differed among age groups (ANOVA with Bonferroni correction $p < 0.05$).

^{**} Negative value indicates ventral preputial confluence distal to MUMP tip in males and ventral preputial confluence or ventral extension tip distal to FUMP tip in females.