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Developmental Biology / Research Article

Cells Tissues Organs
DOI: 10.1159/000541805

Received: May 13, 2024
Accepted: October 1, 2024
Published online: October 4, 2024

Comparative Analyses Reveal Conserved and Modified Steps in the Testis Descent and Scrotum Development in Mouse and Opossum

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Keywords
Scrotum · Testis descent · Marsupial · Cryptorchidism · *Gsc1*

Abstract
Introduction: In many mammals, the testes descend from its abdominal position on the mesonephric kidney and are housed in the scrotum. It has been speculated that metatherians and eutherians might have acquired the scrotal testis independently because metatherians have the scrotum cranially to the phallus, while eutherians, such as humans and mice, possess it caudally. Rather, recent studies based on sequence comparisons of testis-descent-related genes indicate that the metatherian-eutherian common ancestor might already possess the descent mechanisms. To further elucidate the path of scrotal testis evolution, it is informative to compare the processes of the descent and scrotum development between metatherian and eutherian model animals. ***Methods:*** In this study, we histologically and molecularly compare these processes in gray short-tailed opossum (*Monodelphis domestica*), the most commonly used metatherian experimental model, and compare them with those in mouse. ***Results:*** Our observations indicate that, while transabdominal phase of the descent appears to be largely similar, scrotal phase differs due to their distinct scrotum positions. Our cell-labeling analyses and dynamic expression of *Gsc1* reveal extensive cell/tissue rearrangements in murine scrotal development. In contrast, *Gsc1* is not expressed in the developing genitalia and scrotal primordium of the opossum. ***Conclusion:*** Our results suggest recruitment of new regulatory pathways for the scrotum development and the scrotal phase of the testis descent during the evolution of eutherian mammals.

Plain Language Summary
In many mammals, the testes are housed into the scrotum. While marsupials, such as kangaroo and opossum, have the scrotum cranially to the phallus, placentals, such as human and mouse, have it caudally. In this study, we compare the process of testis descent and scrotum development in opossum and mouse. We show extensive cell/tissue rearrangements in the mouse scrotal development. Our results

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suggest recruitment of new regulatory pathways for the scrotum development and the testis descent during the evolution of placental mammals.

Introduction

In many mammals, the testes descend from their abdominal position on the mesonephric kidneys and are housed in the scrotum [1–3]. While evolutionary and physiological roles of scrotal testis have been under debate for a long time [3, 4], failure of the testes to descend often leads to male infertility. In humans, for instance, cryptorchidism is a major congenital anomaly, occurring in 2–4% of infants [1, 2]. Livestock mammals, such as horse, pig, and sheep, also show similar prevalence of the cryptorchidism [2]. Thus, understanding mechanisms of the testis descent has significant implications for human health and agriculture.

Among mammals, metatherians (marsupials) and many eutherians (placentals), but not prototherians (monotremes), possess the scrotal testes. Degree of the testis descent in eutherians varies from no descent (testicondy) to full descent into the scrotum [3]. While most Euarchontoglires species (including rodents and primates) possess the scrotal testis, many species in Laurasiatheria, including entire Eulipotyphila, as well as some aquatic mammals, such as hippopotamus, whale, and seal, have partially descended ascrotal testis. Because Afrotherian mammals – a stem group of eutherians including elephant, hyrax, and tenrec – do not possess the scrotal testis, the descent mechanism might have been acquired in a non-Afrotherian eutherian ancestor after segregation from Afrotherians [3, 4]. A recent study, however, revealed that *insulin-like 3* (*Insl3*) and *relaxin family peptide receptor 2* (*Rxfp2*, encoding a receptor for Insl3) genes, which are required for the testis descent (see also below), have been independently inactivated by loss-of-function mutations in Afrotherian species, suggesting that the ancestor of Afrotherians might also possess a mechanism of the descent [5]. A recent computational analysis of genome sequences relative to states of the testis descent and scrotal/ascrotal testis in mammalian species suggested that the scrotal testis might be the ancestral state for mammals [6]. Some researchers have speculated that metatherians might have acquired the testis descent independently because their scrotums are located cranially to the phallus unlike eutherian counterparts [4] (see also online suppl. Fig. 1; for all online suppl. material, see https://doi.org/10.1159/000541805). To further elucidate the path of scrotal testis evolution, it is informative to compare the processes of the descent and scrotum development between metatherian and eutherian model animals.

In eutherian mammals, gonads initially develop on urogenital ridge of the mesonephros. Mesonephric peritoneums cranial and caudal to the gonad give rise to supporting cords (cranial suspensory ligament, CSL, and gubernaculum, gub, respectively) [1, 2]. In females, to tether the ovary to the abdominal wall, the CSL gives rise to a suspensory ligament (ligamentum suspensorium ovarii), and the gub develops into an ovarian ligament (ligamentum ovarii proprium) and a round ligament (ligamentum teres uteri). In males, the CSL degenerates under the influence of Insl3 signaling, while the gub tethers descending testis to the body wall at the position of future internal inguinal ring [1–3, 7]. As the testes detach from the degenerating mesonephros and the body wall grows, a distal part of the gub becomes prominent as the gubernacular bulb by extensive cell proliferation. Although detailed mechanisms are unknown, the body wall invaginates at the gub attaching site as a vaginal process (processus vaginalis), and the gubernacular bulb penetrates the body wall, leading to testicular and epididymal migration through the inguinal canal. After such externalization from the body cavity, the testis is further transported into the scrotum. In previous descriptions, this process was mechanistically divided into an *Insl3*-dependent transabdominal phase and an androgen-dependent inguino-scrotal phase [1, 3, 8]. Some eutherian species such as hedgehog possess inguinal testis [4, 9]. As is the case for horses with the cryptorchidism, the testis descent can be perturbed within the inguinal canal [2, 10]. In the majority of cryptorchidism human patients, the descent is often perturbed outside of the body wall during testis translocation from external inguinal ring to scrotum neck [2, 11]. Therefore, the inguino-scrotal phase has been suggested to be further subdivided into trans-inguinal phase, in which the descending testis is passing the body wall through the inguinal canal, and subsequent scrotal phase, in which the testis is translocating into the scrotum [2, 12].

Compared to the testis descent, little is known about the scrotum development. In eutherian mammals, including mouse and human, signaling molecules, such as fibroblast growth factor 8 (Fgf8), bone morphogenetic protein 4 (Bmp4), and sonic hedgehog (Shh), are emitted mostly from the midline to pattern the genital tubercle (GT) [4, 13]. In males, the central part of the GT develops into the phallus, while bilateral parts of the genitalia become labioscrotal folds. As the GT grows cranially, the

relative position of the scrotal folds shifts caudally, and these folds fuse at the midline (raphe) to form the scrotum.

In metatherians, males possess their scrotum cranially to the phallus, at the position of caudal mammary primordia in females (online suppl. Fig. 1). To house the testis in the scrotum, therefore, both the genital patterning and descent process are expected to differ from those in eutherians. While development of genital tissues/organs have been reported in a few metatherian species [14–20], details of the testis descent and scrotum development of gray short-tailed opossum (*Monodelphis domestica*), the most commonly used metatherian experimental model, remain unclear. In this study, we describe these processes in this opossum species and compare them with those in the mouse. Our observations indicate that the transabdominal phase seems to be largely similar in opossum and mouse. We also show dynamic expression of *Goosecoid1* (*Gsc1*) in murine scrotal development. Along with our cell-labeling experiments, a group of *Gsc1*-expressing somatopleure mesenchyme cells located proximal caudal to the hindlimb bud contribute to the labioscrotal folds. In contrast, *Gsc1* is not expressed in the scrotum primordium of the opossum, suggesting that the underlying regulatory mechanisms of the scrotum development may differ between metatherian and eutherian mammals. We suggest, therefore, that the descent itself might have already been acquired in the common ancestor of metatherian and eutherian mammals but that mechanisms for scrotum positioning and testis transportation to the scrotum might have been modified after the segregation of metatherian and eutherian lineages.

Materials and Methods

Experimental Animal

Pregnant C57BL/6 J mice were purchased from CLEA Japan, and midday of vaginal plug was designated as embryonic day 0.5 (E0.5). Breeding colonies of gray short-tailed opossum were maintained at Nihon University School of Dentistry at Matsudo and at RIKEN Center for Biosystems Dynamics Research. Opossum embryos were obtained from pregnant females, with matings video recorded and visually confirmed [21].

In situ Hybridization

Whole-mount and section in situ hybridizations were performed as described previously [22–24]. DNA fragments of opossum *Gsc1* (forward primer 1: CTGGCA TGTTCAGCATCGAC, reverse primer 1: ACCTGAGGA TGGAACGCAGG, forward primer 2: CCCACAGAA GGATATGGCAG, reverse primer 2: TCCCTTCTGATC CCATCACC) and *Isl1* (forward primer: CTCCAACCT CCTCTCTGGTC, reverse primer: TAGGACTGGCTA CCATGCTG), corresponding to their exons, were PCR amplified from genomic DNA of opossum adult liver [23] and subcloned into *pBluescriptII* (Stratagene). Probes of opossum *Sox9* [23], mouse *Gsc1* [25], and mouse *Alx4* [26] have been described previously. A cDNA clone of mouse *Isl1* [27] was a kind gift from Dr. Sandra Hoffman.

Histological Analysis

Postnatal opossums and mouse embryos were fixed in 4% PFA/PBS. The lower half of the body was embedded in paraffin. Serial sections (10 μm thickness) were stained with hematoxylin and eosin with a standard protocol. Bright field images were captured by AxioCam CCD camera on Axioplan2 microscope (Zeiss).

DiI-Labeling and Explant Culture

DiI solution (0.01% in dimethyl sulfoxide) was injected by microcapillary to the somatopleural mesenchyme proximal caudal to the hindlimb bud of E10.5 and E11.5 mouse embryos. The torso was dissected into halves, and the caudal half was cultured in 10% rat serum/DMEM with 95% O_2 + 5% CO_2 for 24 h with gentle rotation. Cultured tissue was fixed in PFA/PBS, and whole-mount images were captured by CCD camera (LEICA DFC7000T) on a fluorescence dissection microscope (Leica M165 FC). Cryo-sections of PFA-fixed explants were counterstained with DAPI (Sigma) to visualize nuclei and mounted with VectaShield mounting medium (Vector Laboratories). Fluorescent images were captured by AxioCam CCD camera on Axioplan 2 microscope (Zeiss).

Amino Acid Sequence Comparisons

Amino acid sequences of Insl3 and Rxfp2 proteins were aligned using the CLUSTALW platform (https://www.genome.jp/tools-bin/clustalw). NCBI reference numbers of the sequences for alignments are as follows. Platypus (*Ornithorhynchus anatinus*) Insl3: NM_001122688.1, Tasmanian devil (*Sarcophilus harrisii*) Insl3: XM_003762760.4, gray short-tailed opossum (*Monodelphis domestica*) Insl3: XM_007489659.3, human (*Homo sapiens*) Insl3: NM_005543.4, mouse (*Mus musculus*) Insl3: BC049540.1, horse (*Equus caballus*) Insl3: XM_023625458.1, hedgehog (*Erinaceus europaeus*) Insl3: XM_007533220.1, Platypus Rxfp2: XM_039915010.1, Tasmanian devil Rxfp2: XM_003764504.3,

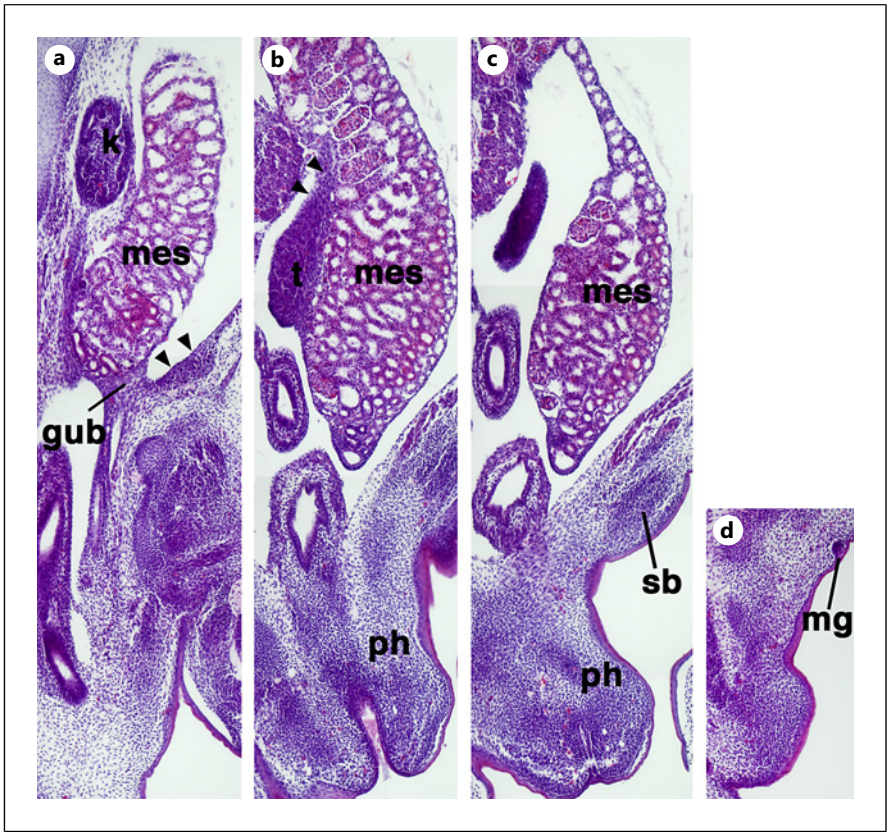

Fig. 1. Gubernaculum and scrotum development in E14.5 opossum embryos. HE-stained para-sagittal sections of E14.5 opossum male (**a–c**) and female (**d**) embryos are shown. Sections shown in (**a–c**) are prepared from the same sample. **a** Future gubernaculum (gub) is connecting mesonephros (mes) to the body wall. Condensed mesenchyme cells are observed cranially to the gub (arrowheads). **b** Testis (t) tightly adheres to mesonephros. Prospective CSL cells are indicated by arrowheads. **c** Scrotal bulge (sb) is observed cranially to the phallus. **d** Developing mammary gland (mg) is observed at a similar position to the scrotal bulge in (**c**). k, metanephric kidney; ph, phallus.

gray short-tailed opossum Rxfp2: XM_007495281.3, human Rxfp2: NM_130806.5, mouse Rxfp2: NM_080468.2, horse Rxfp2: XM_046663756.1, hedgehog Rxfp2: XM_007520292.2.

Results

Scrotum Development and Testis Descent in Opossum

Previous studies described aspects of testis descent and genital development in metatherian species [16, 18]. In this study, to compare the process of the descent and scrotum development between opossum and mouse, we first histologically examined embryonic and postnatal stages of opossum. At embryonic day 12.5 (E12.5) and E13.5, in embryos with *SRY-box transcription factor 9* (*Sox9*)-positive male gonad, there is no sign of scrotal protrusion cranially to developing GT (not shown). At E14.5, just prior to birth, small swellings of the scrotal bulge with mesenchymal condensation are observed cranially to the phallus of male samples (Fig. 1), as previously described in P0 opossum [17], or P2 tammar wallaby [20]. At these stages, the developing gonads are adhered to the mesonephros, and mesenchymal cells are observed cranially and caudally to the gonad along the mesonephros ridges (Fig. 1a, b). Those cells might eventually form the CSL and the gub, respectively. At E14.5, the gub is observed as mesenchymal cells connecting the mesonephros and testis to the body wall (Fig. 1a). Mesenchymal condensation is observed cranially to the gub attachment site, which may give rise to the inguinal canal (Fig. 1a). At this stage, the scrotal bulge is also observed cranially to the phallus (Fig. 1c). In females at this stage, mammary gland primordia are observed at a similar position to the male scrotum (Fig. 1d; see also [14]).

At postnatal day 8 (P8), the testes remain associated with the degenerating mesonephros within the abdomen (Fig. 2a, b). The gub has passed through the body wall via the inguinal canal, forming the processus vaginalis caudal to epipubis (Fig. 2b). At this stage, specification of the gub and surrounding tissues are observed at/around the distal end of the gub (Fig. 2c), as previously described in wallaby [16, 17]. In the scrotal bulge, mesenchyme cells appear to be aligned from the neck to the surface of the scrotum (Fig. 2d, d'). These cells may correspond to the "tough fibrous tissue" described in postnatal tammar wallaby [17]. At P10, the distal tip of the gub has reached the lateral part of the scrotal wall, leading the testis into the scrotum (Fig. 2e). At P15, both testis and epididymis have already passed through the inguinal canal at the position

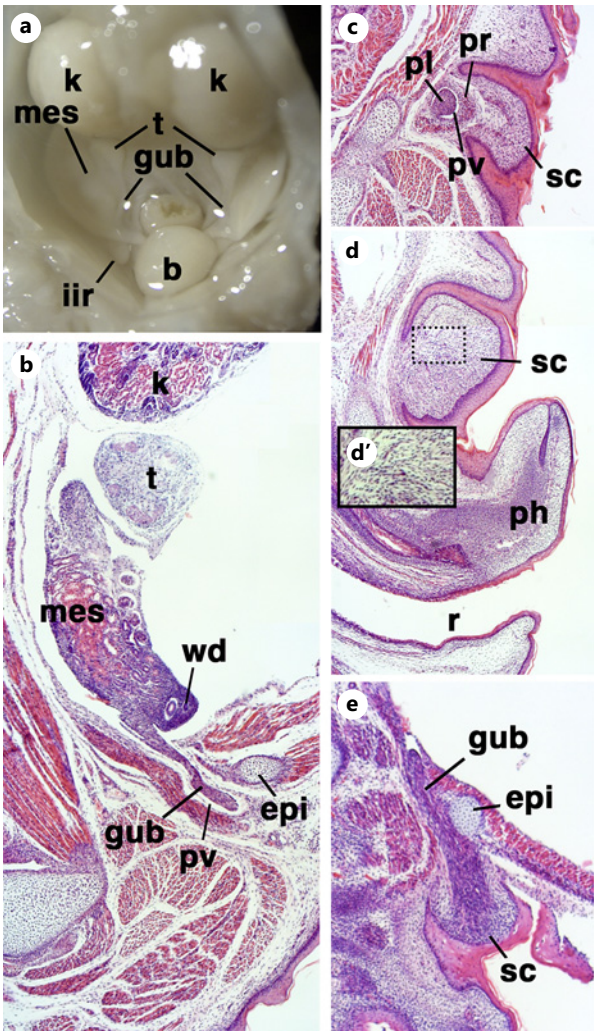

Fig. 2. Gubernaculum (gub) and scrotum (sc) development in P8 and P10 male opossum. **a** Dissected abdominal region of P8 male opossum showing the urogenital organs. The gub remains associated with mesonephros. **b–d** HE-stained para-sagittal sections of P8, prepared from the same sample. **b** The gub penetrates through the body wall at a position caudal to developing epipubis (epi). **c** Caudal tip of the gub enters the sc. **d** Scrotal mesenchyme cells appear to be oriented from the neck to the surface of the sc. A higher magnification view of the scrotal mesenchyme boxed with dotted line is indicated in (**d'**) as an inset. **e** Para-sagittal section at P10, showing that the distal tip of the gub reaches scrotal periphery. b, bladder; iir, inner inguinal ring; k, kidney; mes, mesonephros; t, testis; pv, processus vaginalis; wd, Wolffian duct; pl, plica gubernaculi; pr, pars vaginalis gubernaculi; ph, phallus; r, rectum.

caudal to epipubis (Fig. 3a), without entering into the scrotum (Fig. 3b). Similar to the P10 samples (Fig. 2e), aligned mesenchymal cells are observed in the scrotum (Fig. 3c, c'). At P20, the testis is entering the scrotum, and many eosin-stained cremaster muscle cells are observed along the gub (Fig. 3d).

Gsc1 *Expression in the Mouse Scrotal Development*

In eutherian mammals, such as mouse, the scrotum is derived from the bilateral swellings adjacent to the GT (labioscrotal folds), which are absent in metatherians. In males, these scrotal folds caudally translocate and centrally fuse to form scrotal raphe caudally to the phallus and cranially to the anus. At E15.5, while the testis remains internally within the body cavity at the transabdominal phase (Fig. 4a), the developing scrotum has already located caudal to the phallus (Fig. 4b). The distal portion of the gub adheres to the body wall as the future internal inguinal ring becomes prominent (Fig. 4c, asterisk). While the internal and superficial cells of the distal gub are morphologically distinguishable, the boundary of the gub mesenchyme and body wall mesenchyme appears unclear (Fig. 4c, asterisk). At this stage, there is a significant distance between the exit point of the testis (asterisk in Fig. 4c) and the scrotum (sc in Fig. 4c).

While molecular/genetic aspects of the scrotum development have not been well documented, previous studies reported *Goosecoid1* (*Gsc1*) expression in the developing mouse scrotum [25, 28]. Accordingly, we confirmed *Gsc1* expression in the scrotal mesenchyme of E15.5 male mouse embryos (Fig. 4d). In addition, we observed the outer layer of gub expressing *collagen type XIII alpha 1* (*Col13a1*) at this stage (Fig. 4e) in embryos with *Sox9*-expressing testis (Fig. 4f), suggesting specification of the distal gub cells into at least 2 lineages.

Next, we examined the *Gsc1* expression at earlier developmental stages. As reported [29], the *Gsc1* expression is detected in the proximal part of hindlimb bud mesenchyme of E10.5 and E11.5 embryos on whole-mount preparations (Fig. 5a, b). Interestingly, at E11.5 a stream of *Gsc1*-positive cells is observed extending from the posterior margin of the proximal hindlimb bud along the lateral side of the GT (Fig. 5b). There was small but distinct overlap of *Gsc1*-expressing domain with the lateral part of genitalia expressing *Islet1* (*Isl1*), encoding a LIM homeodomain transcription factor (Fig. 5b', c, d; see also [30–32]). At E12.5, the *Gsc1* expression is observed in the mesenchyme cranial and lateral to the GT (online suppl. Fig. 2a–c). At E13.5 and E14.5, as seen at E11.5, there was a distinct overlap of *Gsc1* and the *Isl1* expressions at the lateral region of the GT (Fig. 5g, h; see also Fig. 5f for the external view of the genital region) but not with the medial GT expressing *homeobox protein aristaless-like* 4 (*Alx4*, Fig. 5i; see also [26]), suggesting that this *Gsc1* expression lateral to the GT may

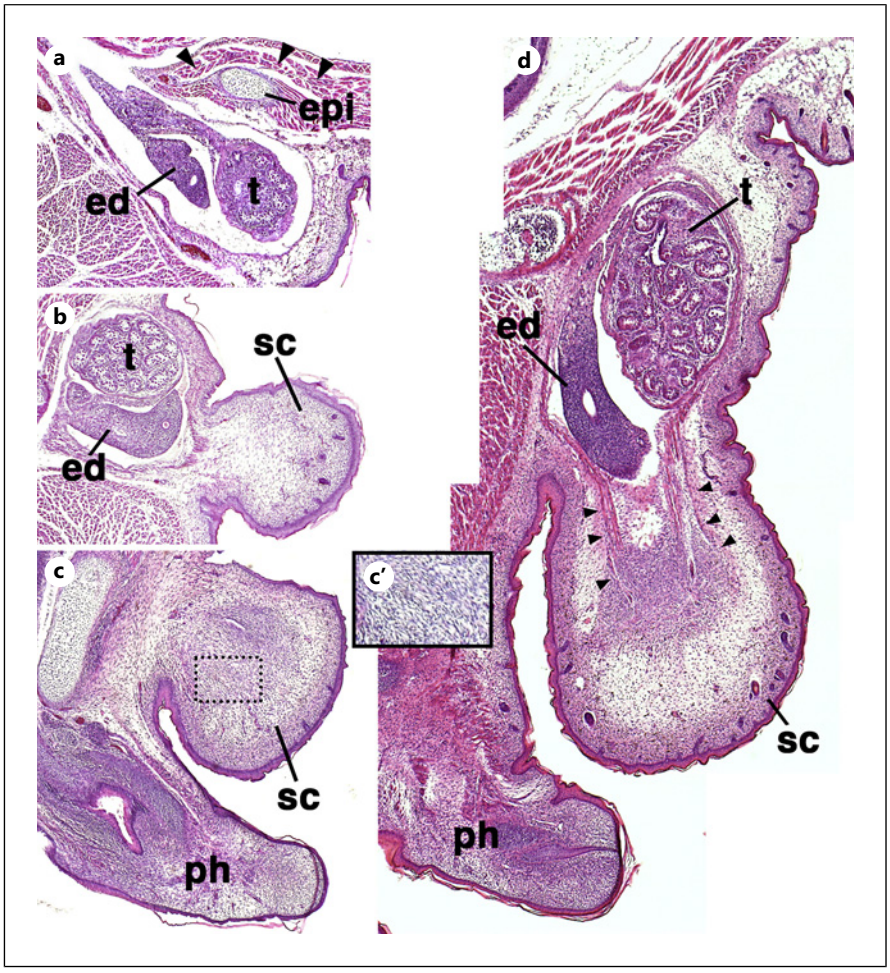

Fig. 3. Urogenital organ development of P15 and P20 opossum shows scrotal phase of the testis descent. **a–c** HE-stained para-sagittal sections of P15, prepared from the same sample. **a**, **b** Both testis (t) and epididymis (ed) are found outside of the abdominal wall. Body wall muscles are indicated by arrowheads in (**a**). **c** Scrotum (sc) is filled with mesenchyme cells oriented from the neck to the surface of the sc. A higher magnification view of the scrotal mesenchyme boxed with dotted line is indicated in (**c**'). **d** HE-stained para-sagittal section of P20. Both testis (t) and epididymis (ed) are entering the sc, led by cremaster muscles (arrowheads). epi, epipubis; ph, phallus.

correspond to the labioscrotal folds. At E14.5, the *Gsc1* expression is also observed in the mesenchyme cranial to the GT including the developing pubis (online suppl. Fig. 2d) and in scrotal swellings caudal to the GT (online suppl. Fig. 2d). At E15.5, *Gsc1* expression is highly restricted to the developing scrotum caudal to the growing phallus, as described above (Fig. 4d; see also [25]). Based on these observations, we hypothesized that the *Gsc1* expression domain posterior to the hindlimb bud and lateral to the GT might indicate caudal translocation of scrotal mesenchyme cells.

Cell Tracing Analysis of the Scrotal Fold Mesenchyme in Mouse Embryos

As mentioned above, we speculated that somatopleure cells of the body wall posterior to the hindlimb bud might give rise to the scrotal fold mesenchyme and eventually to the scrotal mesenchyme in mouse. To test this hypothesis, a group of somatopleure cells posterior proximal to hindlimb bud of mouse embryos were labeled with the fluorescent dye DiI, and the caudal halves of the trunk were subsequently cultured for 24 h (Fig. 6a; see also Materials and Methods). After labeling at E10.5 (Fig. 6b-b″), labeled cells spread caudally, lateral to the GT (n = 5/18, Fig. 6c-c″; see also online suppl. Fig. 3), or both lateral to the GT and medially in front of the GT (n = 10/18, Fig. 6d-d″). In contrast, when labeled at E11.5 (Fig. 6e-e″), labeled cells largely remain at the initial position (n = 6/6, Fig. 6f-f″). These results indicate that there are morphogenetic cell movements/positional rearrangements that form the scrotal folds lateral to the GT between E10.5 and 11.5 (see also online suppl. Fig. 4).

Gsc1 *Expression in the Opossum Scrotum Development*

As shown above, the somatopleure *Gsc1* expression might reflect spatiotemporal changes in the scrotal mesenchyme during mouse genital development. Next, we examined the *Gsc1* expression in opossum embryos. At E12.5 (Fig. 7a), *Isl1* expression is detected both in the proximal hindlimb buds and in the GT (Fig. 7b, d). At this stage, the *Gsc1* expression is highly restricted to the proximal part of the hindlimb bud

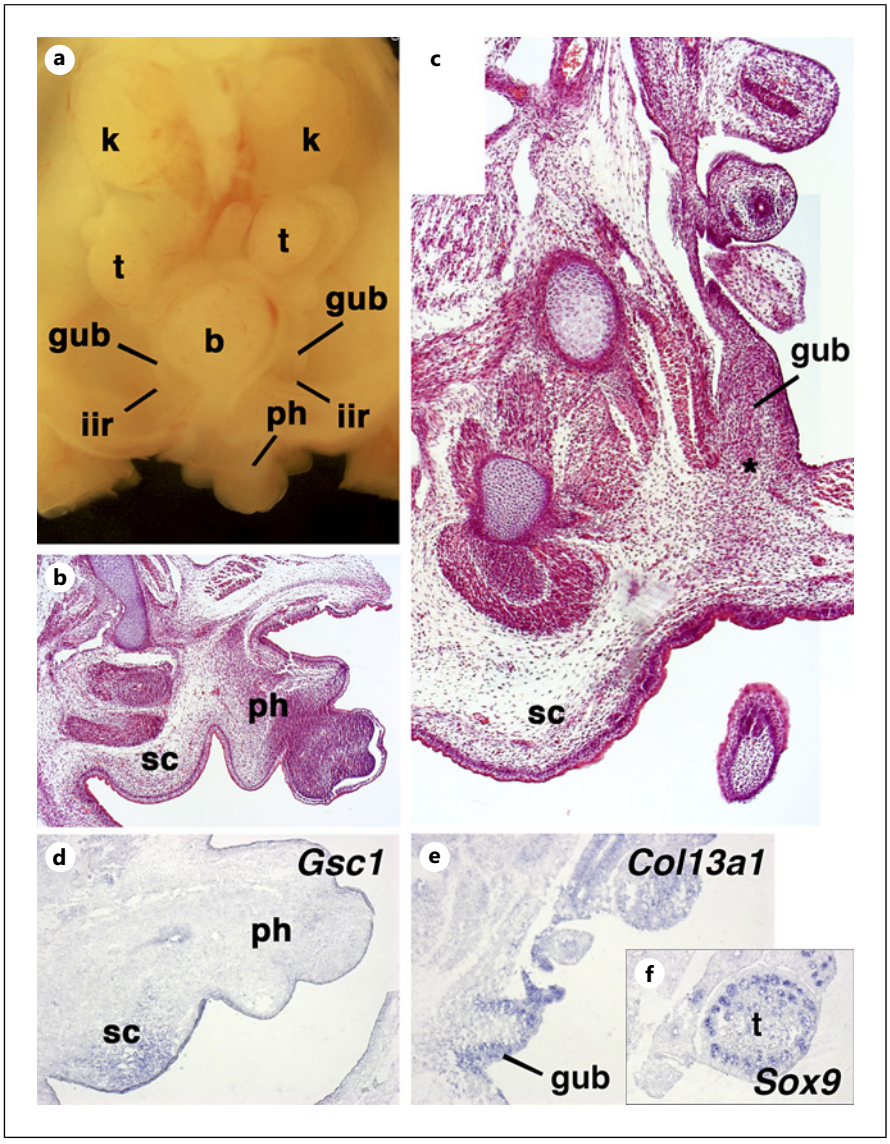

Fig. 4. Urogenital organ development of E15.5 male mice, showing differentiation of scrotal and gubernacular mesenchyme cells. **a** Dissected abdominal region of E15.5 male mouse showing the urogenital organs. **b** HE-stained sagittal section of P15.5, showing scrotum (sc) caudal to the phallus (ph). **c** HE-stained parasagittal section, showing gubernaculum (gub). Note that the internal mesenchyme of the gub is continuous with the scrotal mesenchyme. An asterisk indicates future exit point of the testis. **d** *Gsc1* mRNA expression in the sc mesenchyme. **e** *Col13a1* mRNA expression indicates differentiation of the outer and inner layers of gub. **f** *Sox9* mRNA expression in the gonad of a nearby section of (**d**) and (**e**), indicating that this embryo is male. b, bladder; gub, gubernaculum; iir, inner inguinal ring; k, kidney; t, testis.

mesenchyme with a small overlap with the *Isl1*-positive domain in the genital area (Fig. 7c, e), similar to observations in E10.5 mouse embryos (Fig. 5c, d). The *Isl1*-positive mesenchyme cells between the GT and umbilical cord (asterisk in Fig. 7b) do not express *Gsc1* (asterisk in Fig. 7c). At E13.5 (Fig. 7f), the *Gsc1*-positive region only overlaps with the *Isl1*-positive region at the proximal part of the hindlimb bud mesenchyme cranial to the GT (Fig. 7g–j), and the *Isl1*-positive mesenchyme cells between the GT and the umbilical cord (asterisk in Fig. 7g) do not express *Gsc1* (asterisk in Fig. 7h). At E14.5 (Fig. 7k), scrotal bulges with mesenchyme condensations (see also Fig. 1c) are observed cranially to the *Isl1*-positive GT (Fig 7l, n). Unlike the developing scrotal fold/scrotum in mice (Fig. 4d, 5h; online suppl. Fig. 2d), the *Gsc1* expression is not detected in the scrotal bulge (Fig. 7m, o). Taken together, no *Gsc1* expression was detected in the mesenchyme between the GT and the umbilical cord at any stages examined (Fig. 7c, e, m, o).

Discussion

Acquiring Scrotal Testis in Mammalian Evolution

It has been debated at which stage of mammalian evolution the descent mechanisms were acquired to house the testes inside the scrotum [4–6]. In this study, we have intensively observed the descent process in opossum development. As previously described for

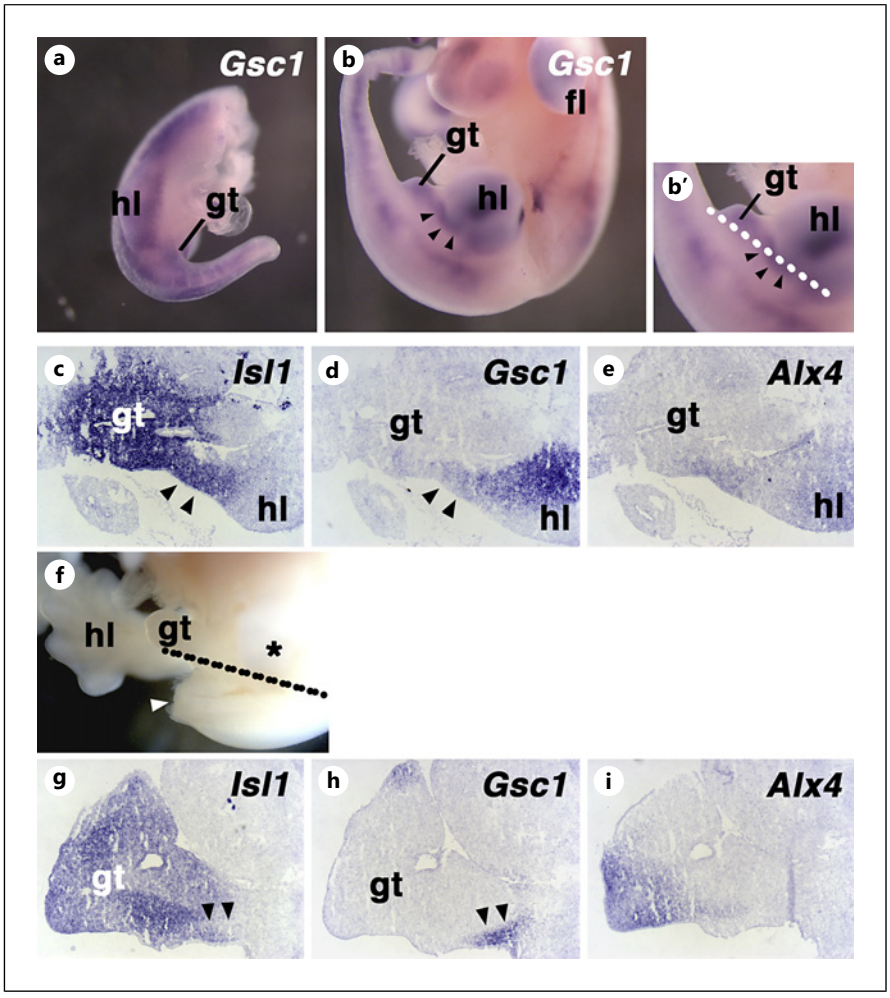

Fig. 5. *Gsc1* expression in developing labioscrotal folds of mouse embryos. **a**, **b** Whole-mount detection of *Gsc1* mRNA expression in E10.5 and E11.5 mouse embryos. **a** At E10.5, the *Gsc1* expression is detected in the proximal region of the hindlimb bud (hl) and genital tubercle (GT). **b** At E11.5, *Gsc1* is expressed in the area connecting the proximal-caudal part of hindlimb bud and tissues lateral to the GT (arrowheads). B' Higher magnification view of (**b**), showing the direction of sections indicated in (**c–e**). **c–e** Neighboring transverse sections of an E11.5 mouse embryo, showing *Isl1* expression as a genital marker (**c**), *Gsc1* (**d**), and *Alx4* (**e**). The *Gsc1*-positive somatopleure cells residing posterior to hindlimb bud coexpress *Isl1* (arrowheads). **f** External view around the genital region of a mouse embryo, indicating the direction of sections in (**g–i**) (dotted line). Distal part of the left hind limb (*) and tip of the tail (arrowhead) are removed to show around the genitalia. **g–i** Neighboring transverse sections of an E14.5 mouse embryo, showing overlapping *Isl1* (**g**) and *Gsc1* (**h**) expression in bilateral parts of the GT (arrowheads), which lacks *Alx4* expression marking the medial part of the GT (**i**). fl, forelimb bud.

wallaby [17, 20], the transabdominal phase of the descent in opossum seems to be quite similar to that of eutherians, suggesting that the common ancestor of these groups (therian mammal) already had the descent mechanisms. Consistently, metatherian species possess both *Insl3* and *Rxfp2* genes (online suppl. Fig. 5; see also [33]). If the descent mechanisms had already been acquired in the ancestral therians, where did they possess their scrotum?

In mouse embryos, we showed that a group of somatopleure cells proximal caudal to the hindlimb bud contributes to the scrotal fold mesenchyme (Fig. 6; online suppl. Fig. 3, 4). A previous cell-labeling study showed that mesenchymal cells cranial to the GT are integrated into the growing phallus [25]. Thus, while the medial mesenchymal cells are integrated into the GT, lateral cells may maintain their relative position, such that these cells will localize laterally to the GT. Alternatively, if not exclusively, these cells may actively migrate to form the labioscrotal folds and eventually the scrotum in male. Compared to these drastic morphogenetic rearrangements to position the scrotum caudally to the phallus, relatively simple scrotal development and short distance of testis translocation in the scrotal phase, the developmental process of the metatherian scrotum may be more ancestral. In eutherian evolution, nonetheless, the integration of mesenchyme cells has reduced the space and components between the umbilical cord and the phallus, and the caudal translocation of the scrotal fold mesenchymal cells has increased the cell number and space between the phallus and anus, as perineum.

Gsc1 *in Scrotum Development*

In previous studies, the *Gsc1* expression has been described in the developing scrotum of mouse embryos [25, 28]. In this study, we examined the *Gsc1* expression in earlier developmental stages and found that the *Gsc1* expression could be correlated to the abovementioned cell movement between E10.5 and E11.5 of the mouse embryos (Fig. 5; online suppl. Fig. 4). At these stages of

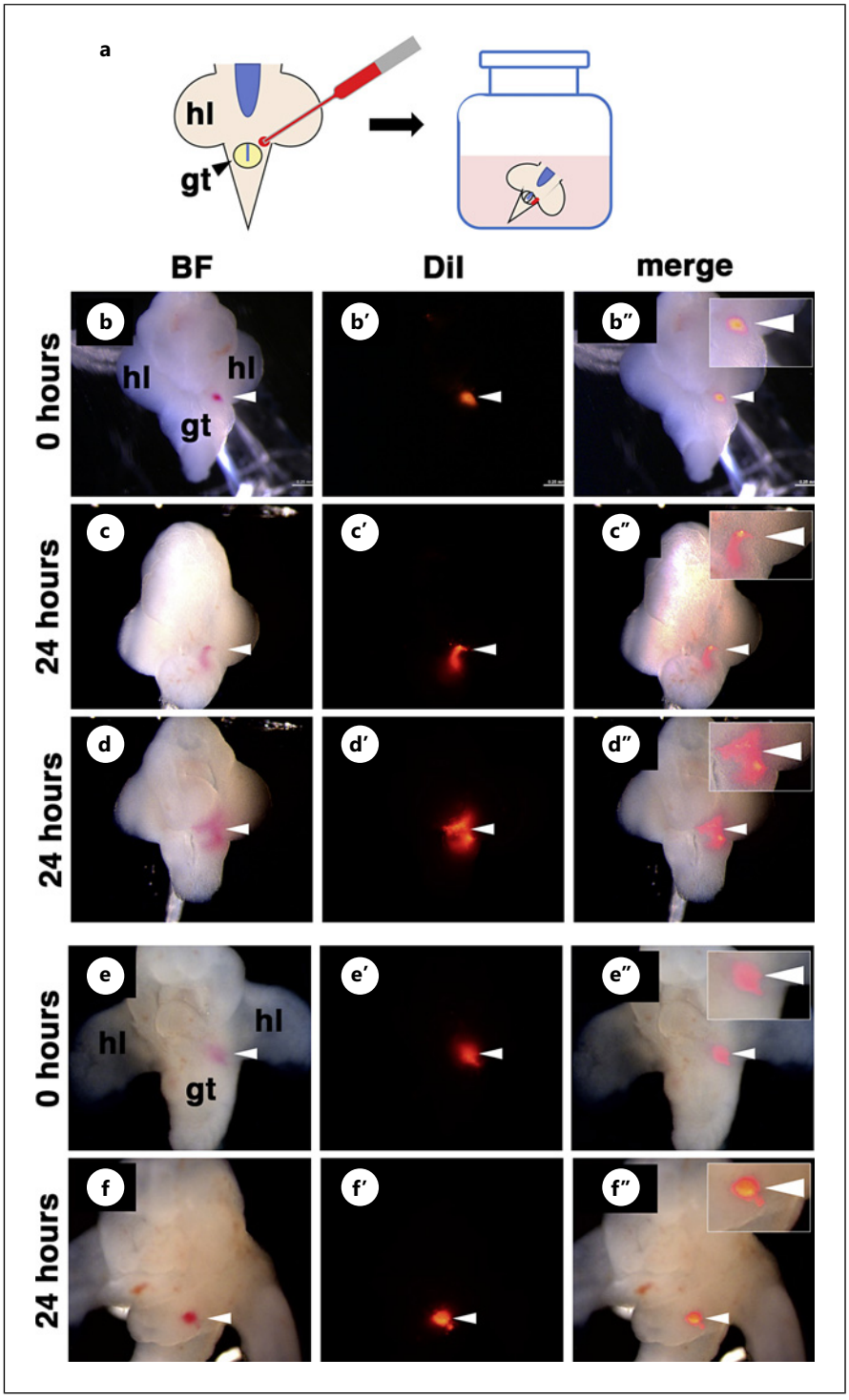

Fig. 6. DiI-labeling reveals posterior migration/translocation of mesenchymal cells around inguinal region. **a** Schematic drawing of cell labeling and organ culture. A group of somatopleure cells located cranially to genital tubercle (GT) and caudally to hindlimb bud (hl) were DiI-labeled. The caudal half of labeled embryos were cultured for 24 h in a bottle (see also Materials and Methods). **b-b″** Ventral view of the lower half of an E10.5 mouse embryo, showing the position of labeling. **c-c″**, **d-d″** Representative examples labeled at E10.5 and subsequently cultured for 24 h. In (**c-c″**), the labeled cells spread caudally. In (**d-d″**), the labeled cells spread both caudally and medially. **e-e″** Lower half of E11.5 mouse embryo, showing the position of DiI-labeling. A group of cells located cranially to GT and caudally to hindlimb bud were labeled. **f-f″** Representative example labeled at E11.5 and cultured for 24 h. The labeled cells do not show prominent migration/translocation toward any direction. Arrowheads indicate position of DiI injection. Insets in (**b″**, **c″**, **d″**, **e″**, **f″**) indicate higher magnification views of the labeled areas.

genital development, several signaling events and tissue interactions actively pattern the developing external genitalia [1, 13, 34]. Thus, the labioscrotal fold-restricted *Gsc1* expression may also be under the influence of such signaling.

We showed that a significant fraction of the DiI-labeled somatopleure cells also spreads medially (Fig. 6; online suppl. Fig. 4). In birds, somatopleure cells proximal to the hindlimb bud give rise to the pelvic girdle including the pubis [35, 36], and a misexpression of *Gsc1*

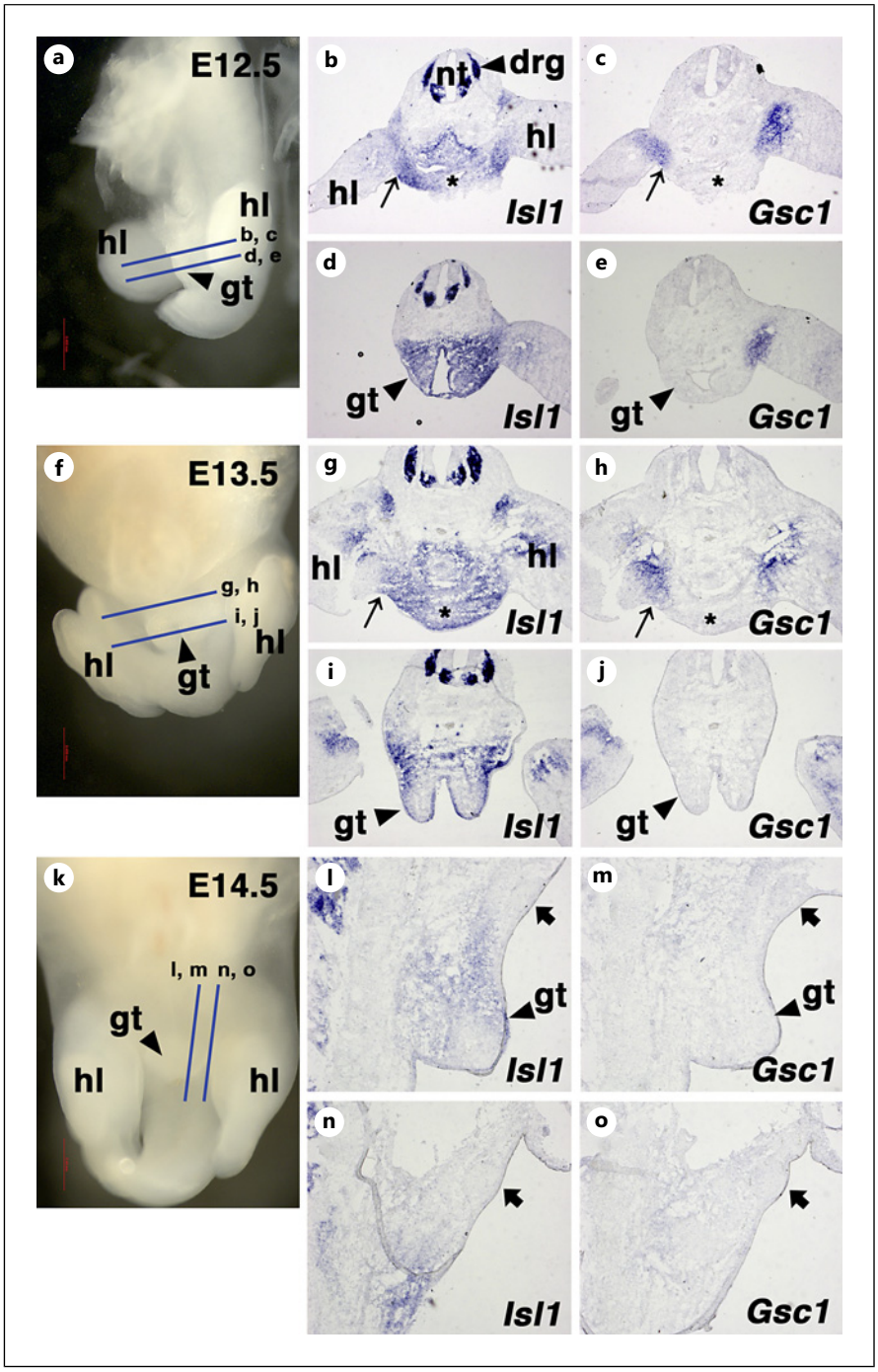

Fig. 7. Lack of *Gsc1* expression in developing scrotal bulge of opossum embryos. **a** External view of the lower half of an E12.5 opossum embryo. Planes of sectioning for (**b–e**), are indicated. **b–e** Transverse sections cranial to (**b**, **c**) and at the middle of the genital tubercle (gt) (**d**, **e**), showing expression of *Isl1* (**b**, **d**) and *Gsc1* (**c**, **e**). Both *Isl1* and *Gsc1* are expressed in the proximal part of hindlimb buds, with limited overlap at the lateral part of the gt (arrows). Asterisks indicate *Isl1*-positive, *Gsc1*-negative mesenchyme between umbilical cord and the gt. **f** External view of the lower half of E13.5 opossum embryo. Planes of sectioning for (**g–j**) are indicated. **g–j** Limited overlap of *Isl1* and *Gsc1* expression is observed at the proximal mesenchyme of the hindlimb buds (arrows) at the level cranial to the gt (**g**, **h**) but not at the level of the gt (**i**, **j**). Asterisks indicate *Isl1*-positive, *Gsc1*-negative mesenchyme between the umbilical cord and the gt. **k** External view of the lower half of E14.5 opossum embryo. Planes of sectioning for (**l–o**) are indicated. While the gt (arrowheads) expresses *Isl1* (**l**, **n**), the scrotal bulge (arrows) is negative for *Isl1* or *Gsc1* expression (**m**, **o**). hl, hindlimb bud; nt, neural tube; drg, dorsal root ganglia.

in chicken embryos affected the pelvis morphogenesis [37]. Since *Gsc1* is expressed in the mesenchymal cells of the proximal hindlimb bud, developing pubis, and labioscrotal fold, the *Gsc1* expression might indicate the shared origin of these structures. Consistently, *Gsc1*-knockout mice showed morphological abnormalities in the pelvis and hip joints [25]. Interestingly, muscle precursor cells in the hindlimb bud have been shown to migrate medially and caudally to form cloacal/perineal musculature at slightly later stages of mouse development [38] possibly following the path of the somatopleure cells around the genital area. To fully understand the behavior of *Gsc1*-expressing cells, nevertheless, extensive cell lineage analyses will be required.

Consistent with the *Gsc1* expression in mouse embryos, patients with mutations in *GSC1* develop cryptorchidism without scrotal sac [28]. However, defects in testis descent and scrotum development have not been observed in the *Gsc1*-knockout mice [25]. In these mutant mice, the loss of *Gsc1* function may be compensated by orthologous *Gsc2* [39]. In opossum, although we found a small overlap of *Gsc1* and *Isl1* expression in the somatopleure cells proximal caudal to the hindlimb bud, no *Gsc1* expression was observed in the mesenchyme between the umbilical cord and the phallus at the stage before the scrotal primordium emerged (Fig. 7b, c, g, h). The *Gsc1* expression was also absent in the scrotal mesenchyme afterward (Fig. 7m, o), suggesting that *Gsc1* may not be involved in the scrotum development of metatherian mammals. The scrotal *Gsc1* expression in mouse may indicate recruitment of new regulatory pathways for the scrotum development and the scrotal phase of the testis descent in the eutherian mammals. Future studies on regulatory mechanisms of the *Gsc1* expression in the scrotum development will provide a clue to uncover genetic alterations for the scrotum development after metatherian-eutherian segregation.

Acknowledgments

We thank Dr. Thomas Maynard for proofreading and editing the manuscript. We also thank Dr. Sandra Hoffman, Riko Yoshimi, and Haruka Endo for the mouse *Isl1* plasmid, opossum sample preparations, and E14.5 mouse embryo, respectively.

Statement of Ethics

Animal experiments were conducted in accordance with guidelines of Tohoku University (Regulations for Animal Experiments and Related Activities at Tohoku University) and with approval of the Tohoku University Medical School Animal Experiment Committee (2015MdA-129-1, 2017MdA-209, 2018MdA-063). Experiments with gray short-tailed opossum at Nihon University School of Dentistry at Matsudo and at RIKEN Center for Biosystems Dynamics Research were approved by Nihon University Animal Care and Use Committee (No. AP12MD015, AP14MD014), and Institutional Animal Care and Use Committee of RIKEN Kobe Branch (Approval No. A2001-03-83) according to RIKEN Regulations for Animal Experiments, respectively.

Conflict of Interest Statement

The authors have no conflict of interest, which might affect this work.

Funding Sources

This work was supported in part by a grant to Y.W. from JSPS (KAKENHI Grant No. 20K06651, 15K07070) and to K.S. (KAKENHI Grant No. 20592154).

Author Contributions

Y.W. designed and performed experiments and wrote the manuscript. Y.T. assisted paraffin sectioning. K.S. and H.K. provided opossum samples. K.T. and G.Y. edited the manuscript. Y.W., K.T., and G.Y. contributed to the conceptualization of this study.

Data Availability Statement

All data obtained in this study are included in this article and its online supplementary material files. Further inquiries can be directed to the corresponding author.

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