Expression patterns of *Fgf8* and *Shh* in the developing external genitalia of *Suncus murinus*

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Abstract

Reciprocal epithelial–mesenchymal interactions and several signalling pathways regulate the development of the genital tubercle (GT), an embryonic primordium of external genitalia. The morphology of the adult male external genitalia of the Asian house musk shrew *Suncus murinus* (hereafter, laboratory name: suncus) belonging to the order Eulipotyphla (the former order Insectivora or Soricomorpha) differs from those of mice and humans. However, the developmental process of the suncus GT and its regulatory genes are unknown. In the present study, we explored the morphological changes and gene expression patterns during the development of the suncus GT. Morphological observations suggested the presence of common (during the initial outgrowth) and species-specific (during the sexual differentiation of GT) developmental processes of the suncus GT. In gene expression analysis, fibroblast growth factor 8 (*Fgf8*) and sonic hedgehog (*Shh*), an indicator and regulator of GT development in mice respectively, were found to be expressed in the cloacal epithelium and the developing urethral epithelium of the suncus GT. This pattern of expression specifically in GT epithelium is similar to that observed in the developing mouse GT. Our results indicate that the mechanism of GT formation regulated by the FGF and SHH signalling pathways is widely conserved in mammals.

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Introduction

Mammalian external genitalia, which show morphological differences among species, are reproductive organs facilitating efficient copulation and internal fertilisation (Yamada *et al.* 2003, Murashima *et al.* 2015). Furthermore, external genital malformations, known as human birth defects (i.e. hypospadias and ambiguous external genitalia), are caused by a combination of genetic and environmental factors (Yamada *et al.* 2006, van der Zanden *et al.* 2012, Sinclair *et al.* 2016a). The genital tubercle (GT) is an embryonic primordium of the external genitalia; it differentiates into the penis in males and the clitoris in females (Yamada *et al.* 2003, 2006, Cohn 2011). GT morphogenesis is divided into two processes: (a) initial outgrowth and (b) sexual dimorphism characterised by the differentiation of mesenchyme and urethra (Suzuki *et al.* 2002, Yamada *et al.* 2003). Male GT is masculinised by androgens secreted from the testes (Miyagawa *et al.* 2009b, Murashima *et al.* 2015). GT consists of endodermal and ectodermal epithelia and

mesenchyme, and epithelial–mesenchymal interactions play an essential role in its development (Haraguchi *et al.* 2000, Yamada *et al.* 2003, Iipulan *et al.* 2016). The entire urethra, without ectoderm contribution, originates from the endoderm (Seifert *et al.* 2008, Iipulan *et al.* 2016). In mice, the tube formation of the most urethra occurs through the canalisation process of the urethral plate from the proximal to distal direction, whereas the distal aspect of the urethra and especially the urethral meatus form through fusion of the preputial–urethral folds (Seifert *et al.* 2008, Mahawong *et al.* 2014, Georgas *et al.* 2015, Sinclair *et al.* 2016a,b). Several signalling pathways, including the fibroblast growth factor (FGF) (Haraguchi *et al.* 2000, Miyagawa *et al.* 2009a, Lin *et al.* 2013, Gredler *et al.* 2015, Harada *et al.* 2015), sonic hedgehog (SHH) (Haraguchi *et al.* 2001, Perriton *et al.* 2002, Lin *et al.* 2009, Miyagawa *et al.* 2009a, Seifert *et al.* 2009a) and bone morphogenetic protein (BMP) (Suzuki *et al.* 2003, 2008, Wu *et al.* 2009) pathways, control mouse GT formation. Phenotypic analyses of genetically modified or endocrine-modulated mice *in vivo* and their GTs

cultured *in vitro* partially uncovered the molecular mechanism involved in the embryonic development of external genitalia (Ipulan *et al.* 2016). However, comparative analyses between mammalian species using various non-rodent models are required to fully understand the mechanisms underlying the morphological diversity observed in the mammalian external genitalia.

The Asian house musk shrew *Suncus murinus* (hereafter, suncus) belongs to the order Eulipotyphla (the former order Insectivora or Soricomorpha), which is classified into the superorder Laurasiatheria. In contrast, the orders Primates and Rodentia are classified into the superorder Euarchontoglires (Bininda-Emonds *et al.* 2007). Thus, suncus is a phylogenetically distinct species from mice and humans. Previous studies have reported morphological analysis of the external genitalia of the adult male suncus (Bedford *et al.* 1997, Kamikawa-Miyado *et al.* 2005), and Kitoh and coworkers (1985) have reported that the urethral and rectal orifices of the suncus are encompassed by a cavity called the ostium urogenitoanal. We have elucidated one of the primary characteristic features of the adult suncus external genitalia: the presence of musculus ischiocavernosus dorsalis (Kamikawa-Miyado *et al.* 2005). Notably, several morphological features of the external genitalia of the adult male suncus are distinct from those of mouse and human (Table 1) (Glucksmann *et al.* 1976, Murakami 1987, Yamada *et al.* 2003, Kamikawa-Miyado *et al.* 2005, Rodriguez *et al.* 2012, Weiss *et al.* 2012, Sinclair *et al.* 2016c). In both humans and the adult male suncus, the mid-shaft of male external genitalia possesses a penile urethra, a corpus spongiosum urethrae (also known as a corpus cavernosum urethrae) and a corpus cavernosum penis covered with an extremely thick tunica albuginea (Yamada *et al.* 2003, Kamikawa-Miyado *et al.* 2005). The middle part of the adult male mouse external genitalia possesses a penile urethra,

a corpus spongiosum urethrae, a circumferential corpus cavernosum glandis and a penile bone (Table 1) (Glucksmann *et al.* 1976, Murakami 1987, Yamada *et al.* 2003, Rodriguez *et al.* 2012, Weiss *et al.* 2012, Sinclair *et al.* 2016c). However, the processes involved in the development of the embryonic GT in the suncus remain unclear.

In the present study, we investigated the changes in morphological features and gene expression patterns during the development of the suncus GT.

Materials and methods

Animals

All animal experiments were approved by the Animal Care Committee of the National Research Institute for Child Health and Development (project number: A2007-001) and Kumamoto University. All experiments were performed in accordance with the institutional guidelines of the care and use of laboratory animals. Adult female and male suncus of an outbred KAT strain were maintained at 25°C under a 12-h light and 12-h darkness cycle, with food (CIEA-305; Clea, Japan) and water *ad libitum*.

Embryo preparation

For mating, the female was housed with the male for 2 h. The day after mating was denoted as embryonic day 0 (E0). The gestation period was approximately 30 days, and pregnant females were killed to collect embryos staged at E14–E26. The embryonic stage was estimated according to the morphological features of embryos, as described previously (Inouye *et al.* 1985). After E20, the sex of the embryo was determined based on the gonadal shape and size. Tissue samples were fixed with 4% paraformaldehyde (PFA), dehydrated with methanol and stored at –20°C until used in further experiments. Before being frozen in OCT, the tissues were fixed with 4% PFA and cryoprotected in 20% sucrose. The main three body axes and the corresponding GT axes are illustrated in Fig. 1.

Table 1 Structural comparison of adult male external genitalia and testis.

	Suncus	Mouse	Human
Orifices of urethra and rectum	Share of a large common cavity (ostium urogenitoanal)	Separation	Separation
Urethra completely within penis	Present	Present	Present
Defined erectile bodies	Present	Present	Present
Distal-dorsal loose connective tissue	Present	Absent	Absent
Distal fibrocartilage	Absent	Present	Absent
Proximal hyaline cartilage	Absent	Present	Absent
Penile bone	Absent	Present	Absent
Skeletal muscle within the penile shaft	Present	Absent	Absent
Pair of smooth muscles within the penile shaft	Present	Absent	Absent
Extremely thick tunica albuginea	Present	Absent	Present
Circumferential penile epithelium	Present	Present	Present
Keratinised epithelial spines	Present	Present	Absent
No tethering, freely mobile	Present	Present	Present
Prepuce	Present	Present	Present
Preputial gland	Verification required	Present	Absent
Testes position	Abdominal cavity	Scrotum	Scrotum

Figure 1 Development of the suncus genital tubercle (GT). (A) The cloacal region before appearance of the GT. (B) Ventral view of the developing GT. Dashed lines show the abdominal median line and the midline of the GT respectively. The orange-coloured region shows the cloacal region. hl, hind limb; t, tail; uc, umbilical cord. Scale bars, 200 µm.

Scanning electron microscope (SEM) analysis

Embryos stored at -20°C were critical-point dried after tails were removed, and methanol in the samples was replaced with isoamyl acetate. The treated samples were mounted on an aluminium stub using carbon conductive tape and a silver paste and were coated with gold particles. The sample surface was observed using an SEM (S-800; Hitachi) and photographed using a film camera. At least two embryos were examined for each stage and sex.

Analysis of gene expression patterns

Whole-mount *in situ* hybridisation was performed as described previously (Ogi *et al.* 2002, Miyado *et al.* 2007). Stained samples were embedded in albumin and sectioned into 30-µm-thick slices using a vibratome. At least three embryos were examined for each gene, stage and sex. *In situ* hybridisation was performed using sections (14-µm) of frozen tissue from two male GTs staged at E22, according to standard procedures.

Histological analysis

Embryos stored at -20°C were immersed in ethanol and xylene, embedded in paraffin and sectioned. Serial 6-µm-thick sections were mounted on slides and stained with hematoxylin–eosin. At least three embryos were examined for each stage and sex.

Results

Features for initial outgrowth of the suncus external genitalia

To examine the morphological features of the suncus external genitalia, we performed SEM analysis of the external genitalia from E14 (before the appearance of the GT) to E18 (before sexual differences appeared morphologically; Fig. 2). In E14 embryos, outgrowth of the GT was not detected in the abdominal median line of the embryo (white arrowheads in Fig. 2A). At

Figure 2 Electron microscopic observation of the genital tubercle (GT) during initial outgrowth. The cloacal region at E14 (A) and outgrowing GT at E15 (B), E16 (C) and E18 (D). Photographs in A, C and D were arranged with the tail bud oriented toward the bottom left of the photo. Photographs in B were arranged with the tail bud oriented toward the bottom of the photo. In A and B, white arrowheads indicate the cloacal region and genital swellings respectively. In B, C and D, white arrows indicate a small distal protrusion at the tip of the GT. The white-dashed line shows the abdominal median line. abd, abdomen; hl, hind limb; t, tail. Scale bars, 100 µm.

E15, slight GT outgrowth was observed between the abdomen and tail (white arrowheads in Fig. 2B), and the cloaca was divided into a urogenital sinus and rectum. At E16 and E18, the GT was prominently elongated, and a small distal protrusion was observed at its tip (white arrows in Fig. 2C and D). No sex-specific morphological differences were observed in any of the stages examined here.

Emergence of sexual dimorphism of the suncus external genitalia

We also performed SEM analysis of the GT at E20 and later stages (Fig. 3). At E20, no sex-specific differences were detected at the distal region of the GT, whereas the signs of sexual dimorphism were visualised at the proximal region (white arrows in Fig. 3A1 and A2). The anogenital distance, which is one of the signs of sexual dimorphism, was longer in males than in females. At E22, the proximal region of the GT showed greater sex-specific differences than at E20, and the anogenital distance was more longer in males than that in females (white arrows in Fig. 3A3 and A4). At E24, the glans of the GT was round in males and egg-shaped in females (blue-coloured regions in Fig. 3A5 and A6), and the GT was beginning to be engulfed in the preputial skin in both sexes (Fig. 3A5 and A6). At E26, the GT was covered with the skin in both sexes (Fig. 3B1 and B2). The urethral and rectal orifices in adult males and the urethral, rectal and vaginal orifices in adult females share a cavity termed the ostium urogenitoanal

(Kitoh *et al.* 1985, Kamikawa-Miyado *et al.* 2005). At E26, the ostium urogenitoanal was incompletely formed (Fig. 3B1 and B2).

Gene expression during the suncus GT development

During the developmental stages of mouse external genitalia, *Fgf8* is expressed in the cloacal epithelium and the distal urethral epithelium of the GT and serves as an indicator of budding and outgrowth of the GT (Haraguchi *et al.* 2000, Ipulan *et al.* 2014). Therefore, we first examined the expression of *Fgf8* in the suncus GT (Fig. 4A). In the absence of outgrowth of the GT, *Fgf8* was expressed in the abdominal median line,

particularly in the cloacal epithelium at E14 (white arrows in Fig. 4A1, A2 and A3 and Supplementary Fig. 1B and C, see section on supplementary data given at the end of this article). Furthermore, during GT outgrowth at E15 and E18, *Fgf8* was expressed in the distal GT region (white arrows in Fig. 4A4, A5, A6 and A7). At E20, *Fgf8* was expressed at the GT tip, but its signal was weak (white arrows in Fig. 4A8 and A9). These expression patterns are similar to those observed during GT outgrowth in mice. Previous studies have shown that *Shh* is expressed in the endodermal cloacal epithelium and the urethral epithelium and is involved in the dual processes of GT outgrowth and urethral tube formation in mice (Haraguchi *et al.* 2001,

Figure 5 Histological analysis of the developing genital tubercle (GT). (A) Transverse (A1 and A2) and sagittal sections of the cloacal region at E14. (B) Transverse sections of the GT at E18. The left and right panels correspond to the regions of the distal and proximal GT respectively. (C) Transverse sections of the GT at E22. Black-dashed lines indicate the epithelial–mesenchymal boundary. In B, black arrows indicate the urethral plate epithelium. In B and C, the regions enclosed by boxes show the enlarged views of the lower panels. cc, anlage of corpus cavernosum penis or clitoris; cl, cloaca; cm, cloaca membrane; cp, anlage of corpus spongiosum urethrae; hl, hind limb; re, prospective rectum; t, tail; u, urethra. Scale bars, 100 μ m.

Miyagawa *et al.* 2009a). Therefore, we next examined the region expressing *Shh* in the suncus GT (Fig. 4B). *Shh* was expressed in the abdominal median line, exclusively in the cloacal epithelium, before GT outgrowth at E14 (white arrows in Fig. 4B1, B2 and B3 and Supplementary Fig. 1B' and C'). At E18 and E20, *Shh* was widely expressed in the ventral midline of the GT from the proximal to distal regions (white arrows in Fig. 4B4, B5, B6 and B7). At E22, *Shh* was also expressed in the ventral midline of the GT in both sexes (white arrows in Fig. 4B8 and B9), although sex-specific expression pattern was observed in the proximal region. *Shh* expression was not visible in the proximal side of the male GT (white-dashed line in Fig. 4B8). In contrast, *Shh* was expressed in the proximal aspect of the female GT (black arrow in Fig. 4B9). Expression of *Shh* was detected in the urethral tube epithelium on the proximal side of the male GT staged at E22 by detailed additional *in situ* hybridisation analyses using frozen tissue sections (inset in Fig. 4B8). These expression patterns observed are quite similar to those reported during the outgrowth of the mouse GT and in urethral tube formation in the mouse GT.

Histological features of the suncus GT

We also conducted histological examination of the developing suncus GT (Fig. 5). At E14, the cloacal membrane was formed by the contact region between ectodermal and endodermal epithelia (Fig. 5A). The ventral cloacal epithelium was thicker than the dorsal cloacal epithelium on the dorsal aspect of the GT. At the distal and proximal regions of the GT, the mesenchymal

layers were undifferentiated and primordial, even at E18 (Fig. 5B). The distal urethral plate appeared as more condensed epithelium than the urethral plate at the proximal region of the GT (arrows in Fig. 5B). At E22, the urethra was already formed as a ductal structure at the proximal region of the male GT ('u' in Fig. 5C), whereas no urethral structure was observed at the proximal region of the female GT (arrowhead in Fig. 5C). At E22, mesenchymal condensations were observed as the prospective corpus cavernosum of the penis and the clitoris ('cc' in Fig. 5C), and a mesenchymal condensation surrounding the forming penile urethra was also noted ('cp' in Fig. 5C).

Discussion

In this study, we demonstrated the expression of the essential growth factors *Fgf8* and *Shh* and characterised the morphological features of the embryonic GT, a common anlage of the external genitalia, of the suncus. We determined the time course of GT development and the emergence of sexual dimorphism, including urethral development. As reported previously in mouse, the GT develops from the cloacal region starting at E10.5 (Haraguchi *et al.* 2000, Yamada *et al.* 2006, Georgas *et al.* 2015), indicating that GT outgrowth is a consequence of mesenchymal swelling around the cloaca. During mouse GT development, *Fgf8* is expressed from E10.5, shortly before GT outgrowth, and up to E14.0, when GT outgrowth is prominent; this *Fgf8*-expressing region is termed the distal urethral epithelium (Haraguchi *et al.* 2000, Perriton *et al.* 2002). Removal of this region reduces the expression of mesenchymal genes such as *Fgf10*, suppressing GT

outgrowth (Haraguchi *et al.* 2000). Therefore, interaction between the distal urethral epithelium and its peripheral mesenchymal region is thought to play a major role in both swelling and outgrowth (Haraguchi *et al.* 2000). As shown in Fig. 2A, the formation of the cloacal membrane was unclear in the abdominal median line in the suncus, whereas the cloacal membrane is formed clearly as a groove in mice (Suzuki *et al.* 2002). The difference in cloaca morphology emerged before the GT outgrowth, possibly due to the morphological diversity of the external genitalia. Despite the morphological differences between the mouse and suncus, we assumed that the cloacal epithelium may control initial GT outgrowth in the suncus because *Fgf8* was expressed in this region. We also observed the small distal protrusion at the tip of the suncus GT, which may correspond to the distal urethral epithelium reported in mouse GT (Haraguchi *et al.* 2000). The region emerged at E15 and was clearly distinguishable prior to at E20, when *Fgf8* was expressed marginally. *Fgf8* conditional knockout and *Fgf4/8* compound-mutant mice have been shown to exhibit normal GT outgrowth, although FGF8 induces extracellular signal-regulated kinase 1/2 phosphorylation and cell proliferation in the mouse GT *in vitro* (Miyagawa *et al.* 2009a, Seifert *et al.* 2009b). In mouse GT development, the presence of regulatory mechanisms overlapped among multiple FGFs, which are functionally redundant from phenotypic analyses of their knockout mice, has been discussed (Miyagawa *et al.* 2009a). These findings indicate that FGF signalling serves as an important regulator of GT outgrowth not only in the mouse but also in the suncus. Because redundant and outgrowth-regulating functions of FGFs have been well studied in the mouse GT (Miyagawa *et al.* 2009a), the current finding indicates a potentially intriguing possibility of similar/divergent functions of FGF signals in the suncus, which should necessarily be examined by further studies.

As shown in Fig. 4B, *Shh* was expressed in the cloacal epithelium and the developing urethral epithelium of the suncus GT, and its expression pattern is quite similar to that in the mouse GT. In the ventral midline of the mouse GT, endodermal and ectodermal epithelia and the mesenchyme contact each other, and the urethral tube except for the distal aspect of the urethra is formed by canalisation of the urethral plate (Seifert *et al.* 2008, Miyagawa *et al.* 2009b, Herrera & Cohn 2014, Mahawong *et al.* 2014, Sinclair *et al.* 2016a,b). In fact, a tubular urethra was recognised within the proximal urethral plate epithelium of the male suncus GT staged at E22. Although the tube formation of the distal aspect of the urethra needs to be validated by further studies, it is likely that the most urethra of the suncus may be formed in a direction from the proximal urethral plate to the distal plate, similar to that in the mouse (Seifert *et al.* 2008, Mahawong *et al.* 2014, Georgas *et al.* 2015, Sinclair *et al.* 2016a,b).

In mice, several growth factors have been reported to control GT outgrowth and urethral tube formation (Yamada *et al.* 2006, Ipulan *et al.* 2016). For example, *Shh* is expressed in the cloacal and urethral epithelium of the GT and plays critical roles in both GT outgrowth initiation and mesenchymal differentiation of the GT (Haraguchi *et al.* 2001, 2007, Perriton *et al.* 2002, Miyagawa *et al.* 2009a). SHH signalling is also involved in the patterning and development of the male urethra in humans (Shehata *et al.* 2011). Our results indicate that SHH plays similar roles in the mouse and the suncus during GT development.

The suncus has been increasingly used as a model species to analyse organogenesis including genitalia, craniofacial and tooth development during the prenatal period (Niida *et al.* 1994, Ogi *et al.* 2002, Miyado *et al.* 2007, Yamanaka *et al.* 2007). Epithelial–mesenchymal interactions widely promote embryogenesis and also GT and tooth development in mice. Owing to the morphological diversity, different molecular mechanisms exist in the GT and tooth germ (Thesleff 2003). However, SHH signalling is required for the early development and patterning of both GT and tooth germ, and the expression patterns of many genes in both organs are quite similar. As described previously (Miyado *et al.* 2007, Yamanaka *et al.* 2007), the suncus tooth germ expresses *Shh*, indicating that SHH function in tooth development is widely conserved among mammals. As reported previously for *Shh*-expressing organs in mice, SHH signalling presumably plays a critical role in the development of suncus organs, at least teeth and the GT.

As gene expression in mesenchymal cells was not analysed in this study, the genes associated with mesenchymal differentiation in the suncus GT remain unknown. Mesenchymal differentiation is an indispensable process for sexual dimorphism in the mouse GT (Miyagawa *et al.* 2009b). The developmental process of external genitalia has been elucidated from studies in rodents, mainly mice (Haraguchi *et al.* 2000, Suzuki *et al.* 2003, Yamada *et al.* 2006, Georgas *et al.* 2015, Ipulan *et al.* 2016). Bone is present in the penis (os penis) and clitoris (os clitoris) of mice, whereas only male rats possess this bone (Glucksmann *et al.* 1976, Murakami 1987, Yamada *et al.* 2003, Rodriguez *et al.* 2012, Weiss *et al.* 2012, Sinclair *et al.* 2016c). The os penis plays a role in maintaining erection during copulation, because hypoplasia of the os penis are observed in infertile *Hoxa13*- and *Hoxd13*-deficient mice (Dollé *et al.* 1993, Post & Innis 1999) and males with a wider os penis achieve higher reproductive success than males with a narrower one in mice (Stockley *et al.* 2013, Schultz *et al.* 2016). In contrast, there is no corresponding structure in the suncus and humans (Yamada *et al.* 2003, Kamikawa-Miyado *et al.* 2005). As the corpus cavernosum penis, which is covered with an extremely thick tunica albuginea, exists

in the male external genitalia of the suncus and humans (Yamada *et al.* 2003, Kamikawa-Miyado *et al.* 2005), we presumed that its function may be comparable to that of the os penis, indicating the unique utility of this species in reproductive/copulation biology. In addition, the external genitalia of the male suncus possesses a muscular structure, which we proposed as the musculus ischiocavernosus dorsalis (Kamikawa-Miyado *et al.* 2005); this structure is absent in humans and mice. As described, the internal inner structures within of the external genitalia of adult animals have interspecies differences, indicating that the differentiation of the GT is regulated in a species-specific manner. In other words, the genes expressed during the development of external genitalia, especially in GT mesenchymal cells, and the molecular mechanisms driving this process may be species specific. Thus, phylogenetically distinct species, especially non-rodent animals such as the suncus, would be potentially useful models for studying morphological diversity in the mammalian external genitalia. Several genes, including those encoding molecules in the BMP signalling pathway, *Wnt/β-catenin*, *Fgf10-Fgfr2*, *EphB2-EphrinB2* and *Hoxa/Hoxd* are involved in the control of mouse GT development (Cohn 2011, Blaschko *et al.* 2012, Ipulan *et al.* 2014, Gredler *et al.* 2015). Furthermore, the time window of androgen actions during the mouse GT masculinisation is from E15.5 to 16.5, and the sexually dimorphic processes are controlled by cross-talk between androgen signalling and growth factors in mice (Miyagawa *et al.* 2009b, Ipulan *et al.* 2014), implying that cross-talk between androgen signalling and growth factors presumably plays a role in the morphological diversity. However, some experimental limitations existed in the present study, and further morphological and molecular studies regarding the suncus GT development are being performed by our group and others. Further studies are necessary to clarify what genes and mechanisms such as androgen-triggered mechanism are associated with the development of the suncus GT.

In summary, we demonstrated here that the expression patterns of growth factors *Fgf8* and *Shh* in the suncus GT were similar to those in the mouse GT and that there were species-specific morphological characteristics in the suncus GT, although the two genes examined in this study exhibited parallel expression patterns in both species. Our findings provide insights into mammalian GT development. To fully understand the mechanism of GT morphogenesis, further examination of the expression patterns of other genes that contribute to the development of the suncus GT is needed.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-16-0231>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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