Effects of androgen and oestrogen on IGF pathways controlling phallus growth

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Abstract

The development of the mammalian phallus involves hormone-dependent mesenchymal–epithelial signalling mechanisms that contribute to urethral closure and regulation of phallus elongation and growth. In marsupials, most differentiation and growth of the phallus occurs post-natally, making them amenable to direct hormone treatment. Expression of *IGFs*, *FGFs*, *EFNB2*, *MAFB*, *DLX5* and *AP-1* mRNAs in the phallus at day 50 post-partum (pp) were altered after treatment of tammar wallaby young from day 20 to 40 pp with androgen, oestrogen or after castration at day 25 pp. However, the most interesting changes occurred in the IGF pathway genes. Androgen treatment upregulated *IGF1* in female phalluses and oestrogen treatment upregulated *IGF1* in male phalluses, but it was downregulated by castration. *IGFBP3* was higher in female phalluses and downregulated by androgen. *IGF1* expression was higher in all untreated male than in female phalluses from day 50 to 150 pp, but *IGFBP3* had the reverse pattern. At day 90 pp, when urethral closure in males is progressing and male phallus growth is accelerating. IGF1 and PCNA protein were only detected in the male urorectal septum, suggesting for the first time that closure and elongation may involve IGF1 activation of cell proliferation specifically in male phalluses. These effects of sex steroids on gene expression and on the IGF1 signalling pathway in particular, suggest that the developing phallus may be especially susceptible to perturbation by exogenous hormones.

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Introduction

Hypospadias is one of the most common developmental defects and currently affects approximately 1/125 male births in Western countries (Sweet *et al.* 1974), increasing by 2% each year in Western Australia (Paulozzi *et al.* 1997, Paulozzi 1999, Nassar *et al.* 2007). At least a proportion of this increase appears to be due to environmental endocrine disruptors (EEDs), such as insecticides, herbicides and many plastics (Suk Kim *et al.* 2004, Willingham *et al.* 2006, Fernandez *et al.* 2007, Vilela *et al.* 2007, Hsieh *et al.* 2008). Some androgenic EEDs masculinise the female phallus, resulting in an enlarged clitoris (Sane & Pescovitz 1992, Wiig *et al.* 1998, Welsh *et al.* 2010). Hypospadias can also be induced in mammals by gene mutation or treatment with androgen inhibitors such as flutamide during development *in utero* (Wolf *et al.* 2000, Coveney *et al.* 2002, Leihy *et al.* 2002, 2004, Petiot *et al.* 2005, Seifert *et al.* 2008, Welsh *et al.* 2008, 2010, Chew *et al.* 2014, Zheng *et al.* 2015).

Urethral closure and differentiation of the phallus are under the regulation of complex gene networks. In mice, fibroblast growth factor 10 (FGF10) and its receptor

fibroblast growth factor receptor 2 isoform IIIb (FGFR2IIIb) positively regulate genital tubercle (GT) outgrowth (Haraguchi *et al.* 2000, Satoh *et al.* 2004, Petiot *et al.* 2005, Gredler *et al.* 2015, Harada *et al.* 2015). Deletion of *Fgf10* or *Fgfr2IIIb* results in abnormal urethra formation (Haraguchi *et al.* 2000, Satoh *et al.* 2004, Petiot *et al.* 2005, Gredler *et al.* 2015, Harada *et al.* 2015). FGF10, acting via the FGFR2IIIb receptor (Ornitz *et al.* 1996, Zhang *et al.* 2006), may direct epithelium–mesenchymal crosstalk and regulate GT patterning and urethral closure (Satoh *et al.* 2004, Gredler *et al.* 2015, Harada *et al.* 2015). EphrinB2 (EFNB2) is expressed in the ventral midline of the GT and cooperates with its receptor, tyrosine-protein kinase receptor EPH-3 (EPHB2) through a directional signalling pathway to maintain normal urorectal development in mice (Adams 2002, Dravis *et al.* 2004, Yucel *et al.* 2007). If any part of the pathway is missing, severe hypospadias and urorectal malformations occur (Dravis *et al.* 2004, Yucel *et al.* 2007). V-Maf Avian Musculoaponeurotic Fibrosarcoma Oncogene Homologue B (*Mafb*), an androgen-dependent gene, is predominantly expressed in mesenchymal cells of male phalluses (Suzuki *et al.* 2014, Matsushita *et al.* 2015). In mice, its expression is

upregulated in female GT mesenchyme after exogenous androgen treatment, but is downregulated in male GT mesenchyme of *androgen receptor* (*Ar*) knockouts (KO) (Suzuki *et al.* 2014). *Mafb* KOs in mice fail to form a urethra (Suzuki *et al.* 2014), suggesting a key role for *Mafb* in masculinization of the male phallus. *Distal-Less Homeobox 5* (*Dlx5*) is expressed in the developing urethral epithelium and GT mesenchyme in mice (Acampora *et al.* 1999, Merlo *et al.* 2004, Suzuki *et al.* 2008). In *Dlx5*-knockout mice, there is severe hypospadias and reduced testosterone during phallus development (Nishida *et al.* 2008, Suzuki *et al.* 2008).

The function of the activator protein-1 (AP-1) genes during phallus development has been less well studied. AP-1 proteins, including V-Jun Avian Sarcoma Virus 17 Oncogene Homolog (C-JUN), JunB Proto-Oncogene (JUNB), JunB Proto-Oncogene (JUND), FBJ Murine Osteosarcoma Viral Oncogene Homolog (C-FOS), FBJ Murine Osteosarcoma Viral Oncogene Homolog B (FOSB), MAFB and Activating Transcription Factor 3 (ATF3) sub-families, control downstream gene expression to regulate cell proliferation, transformation and apoptosis (Vogt & Bos 1990, Angel & Karin 1991).

Insulin growth factor 1 (IGF1) plays an important role in regulating somatic growth in mammals including humans in whom it can affect phallus growth (Laron *et al.* 1966). It associates with a high-affinity-binding protein, insulin growth factor-binding protein 1–6 (IGFBP1–6) (Hwa *et al.* 1999). In humans, abnormal penile development seen in Laron syndrome can be improved by treatment with IGF1 (Levy & Husmann 1996, Laron & Klinger 1998). Binding proteins of IGF1 (IGFBPs) increase the lifespan of IGF1 in all tissues (Stewart *et al.* 1993, Kalus *et al.* 1998). Interestingly, IGFBP3 negatively regulates cell growth and proliferation through IGF-independent mechanisms (Schmid *et al.* 1991, Valentinis *et al.* 1995, Duan & Xu 2005). In addition, *IGF1* and *IGFBP3* have differing responses towards androgen and oestrogen in human cell lines (Huynh *et al.* 1996, Le *et al.* 2006). Dihydrotestosterone and testosterone increase cell growth and *IGF1* expression, but decrease *IGFBP3* expression in human prostatic stromal cells (Le *et al.* 2006). Interestingly in the tammar wallaby *Macropus eugenii*, there is a significantly higher hepatic expression of IGFs in males than in females at day 70 pp, the time that the male phallus becomes sexually dimorphic, and IGF1 in the plasma rises steadily from day 50 to 250 pp (Menzies *et al.* 2012).

Phallus differentiation in the tammar is initiated during an androgen ‘window of sensitivity’ that we first described occurs between day 20 and 40 post-partum (pp) (Leihy *et al.* 2004), and now refined to an even shorter period between day 25 pp and day 30 pp in the developing tammar pouch young (Chew *et al.* 2014). This is the same as the ‘male programming window’ subsequently described in the rat (Welsh *et al.* 2008, 2010) and human (Welsh *et al.* 2014). We have shown

that phallus-regulating genes are not only sensitive to androgen but also to oestrogen during this programming (or androgen imprinting) window (Chew *et al.* 2014, Chen *et al.* 2018). Gonadal oestrogen in untreated young is below detection in both sexes between at least the day of birth and day 50 pp (Renfree *et al.* 1992). Urethral closure and elongation in the tammar phallus are both androgen dependent (Leihy *et al.* 2004, Chew *et al.* 2014, Renfree *et al.* 2014, Chen *et al.* 2018), requiring at least a short-term exposure to androgen, while maintaining phallus elongation requires a constant level of androgen (Leihy *et al.* 2004, Renfree *et al.* 2014, Chen *et al.* 2018). Altering androgen or oestrogen in males during the window of sensitivity leads to abnormal phallus phenotypes and failed urethral closure (Leihy *et al.* 2004, Renfree *et al.* 2014, Chen *et al.* 2018). However, after oestrogen treatment, there is no change in androgen levels in treated males (Chen *et al.* 2018). In the tammar, the phallus does not become sexually dimorphic until after day 50 pp and urethral closure is not complete until after day 150 pp (Leihy *et al.* 2011). The tammar wallaby therefore provides a unique model in which to study the direct effects of hormonal manipulation on gene expression during phallus development without the complicating effects of maternal physiology and placental transfer. We therefore treated female tammar with androgen and males with oestrogen or castration during the androgen imprinting/male programming window of sensitivity, then used transcriptomic analysis and bioinformatic tools to identify the effects at day 50 pp. We also sampled normal phalluses at day 90 and 150 pp when urethral closure is complete.

Materials and methods

Animals

Tammar wallabies (*Macropus eugenii*) of Kangaroo Island (South Australia) origin were held in our breeding colony. The sex of the pouch young was identified by the presence of scrotal buds (male) or mammary primordia (female) (Wai-Sum *et al.* 1988). The age of the pouch young was recorded from the day of birth or at later stages from head length using published growth curves (Poole *et al.* 1981). Phalluses were collected from pouch young tammar wallaby after anaesthesia with Zoletil 100 (Tiletamine HCl 50 mg/mL, Zolazepam HCl 50 mg/mL, Virbac, Cat# 1p6080-3, 1 mL/kg) and killed by overdose of sodium pentobarbitone (60 mg/mL, to effect). Samples were snap-frozen and stored at -80°C for RNA-Seq analysis or fixed in 4% (w/v) paraformaldehyde, embedded in paraffin and cut into 7 μm sections for histological analysis and section *in situ* hybridization. All experimental procedures complied with the Australian National Health and Medical Research Council (2013) guidelines and approved by the University of Melbourne Animal Experimentation Ethics Committees. Phalluses were collected as above from untreated young at day 20, day 30, day 50, day 90 and day 150 pp males and females for qPCR (each stage $n=5$).

Phalluses for immunohistochemical analysis ($n=3$) were collected as above from day 90pp males and females. Male pouch young were castrated ($n=5$) as previously described (Leihy *et al.* 2004, Renfree *et al.* 2009, Chew *et al.* 2014) or treated with oestrogen (2.5 mg/kg/day) orally from day 20pp to day 40pp ($n=5$) as previously described (Coveney *et al.* 2001). Female pouch young ($n=5$) were injected intra-peritoneally with 5 α -androstane-3 α ,17 β -diol (adiol, 10 mg/kg/week) from day 20pp to day 40pp as previously described (Leihy *et al.* 2004). Phalluses were collected at day 50pp ($n=5$) for RNASeq analysis and qPCR ($n=5$).

RNA extraction and cDNA preparation

RNA was extracted from developing phalluses with the RNeasy Mini kit (Cat# 74804 Qiagen) following on the manufacturer's recommendations. RNA concentration was calculated with a Qubit 2.0 fluorometer (ThermoFisher scientific). All RNA samples were treated with a DNA-free kit (Ambion, Cat #1906) to prevent genomic DNA contamination. Two hundred nanograms of total RNA was used for cDNA template synthesis by using the transcription high fidelity cDNA Synthesis kit (Cat# 05081963001, Invitrogen).

RNA-Seq assembly and analysis

RNA samples were further analysed using TapeStation (Agilent). Appropriate amounts of RNA were subjected to multiplex indexed-RNA-Seq analyses using TruSeq kit (Illumina) and HiSeq2500 analyzer (Illumina). Roughly 10–14 $\times 10^6$ reads (100bp) were obtained from each sample after Q.V. >30 filtering. The average quality score was ~39. The RNA-Seq data were assembled with Trimmomatic (Bolger *et al.* 2014) and Cufflinks-Cuffdiff (Trapnell *et al.* 2010) pipeline and analysed with CummeRbund R package (Goff *et al.* 2012). The average mapping rate from all samples was above 80% and the quality was good for differential expression analysis. The tamarin wallaby genome 3.0 (T Heider, A Fujiyama, MB Renfree, AJ Pask and R O'Neill, personal communication) was used as a guide for RNA-Seq assembly. The annotation was obtained by blasting against the UNIPROT protein database (Apweiler *et al.* 2004). Differentially expressed genes were extracted with CummeRbund R package and basic R scripts (Team 2014).

Quantitative PCR

FastStart Universal SYBR mix (Roche, Cat#04913914001) was used to detect gene expression levels. PCR primers (Supplementary Table 1, see section on [supplementary data](#) given at the end of this article) were designed with the online program Primer 3 (<http://primer3.ut.ee/>). The efficiency of each set of primers was within 95–105%. The amplification conditions for qPCR were 1 cycle of 95°C for 10 min; 45 cycles of 95°C for 30s, 58°C for 30s, 72°C for 1 min and 1 cycle of 72°C for 7 min. All genes were run with triplicates. Three house-keeping genes (*HMBS*, *18S* and *GAPDH*) were used to normalise the expression level according to MIQE guidelines (Bustin *et al.* 2009).

Section in situ hybridization

Phalluses for section *in situ* hybridization were collected from day 20pp and day 90pp pouch young ($n=3$). The paraffin-embedded phalluses were sectioned at 7 μ m. Primers (Supplementary Table 2) were designed with the program Primer 3 online (<http://primer3.ut.ee/>). The probes for *situ* hybridization (ISH) were labelled with DIG RNA labelling Mix (Roche, Cat#11277073910) and generated with T7/SP6 polymerase synthase kit (Promega, Cat #P1460). The sections were pre-hybridized for 2 h at 42°C and hybridized for 16–18 h at 42°C. Then, the sections were washed and incubated with anti-digoxigenin-AP (1:300 dilution, Roche, Cat #11093274910) for 16–18 h at 4°C and colour developed with NBT/BCIP (1:50 dilution, Roche, Cat #11681451001). The sections were counter-stained with Nuclear Fast Red solution (Sigma Aldrich, Cat #N3020). Negative controls were incubated with sense probe (Supplementary Fig. 1 and data not shown). Photos were taken with an Olympus BX51 Fluorescence Microscope and Olympus DP70 Camera System.

Immunofluorescence

The paraffin-embedded phalluses were sectioned at 7 μ m. The sections were de-paraffinized with histolene and rehydrated with serial dilutions with ethanol. Antigen retrieval was performed by heating in 30% power of a microwave (1000W) for 20 min in 0.01 mol/L sodium citrate buffer (pH 10) for IGF1. No antigen retrieval was performed for proliferating cell nuclear antigen (PCNA), a marker of cell proliferation. The primary antibody, IGF1 (rabbit polyclonal, 1:100 dilution, GroPep Bioreagents, Cat# PAA2) or PCNA (rabbit polyclonal, 1:30 dilution, Santa Cruz Biotechnology, Cat# sc-7907) were added to the section and incubated at 4°C overnight. The negative controls were incubated with rabbit immunoglobulin fraction (Dako, Cat# X0903) without primary antibody. The secondary antibody donkey anti-rabbit IgG (Supplementary Table 3) was performed at room temperature for 1 h. DAPI (1:10,000 5 mg/mL, Invitrogen, Cat# D3571) was used as a nuclear counterstain. Fluorescent mounting medium (Dako, Cat# S302380) was used for mounting. The edges of coverslips were sealed with nail polish and stored at 4°C in the dark.

Statistical analysis

Three biological replicates were used for immunofluorescence and section *in situ* hybridisation. Five biological replicates were used for qPCR and RNA-Seq analysis. FPKM (Fragments Per Kilobase of transcript per Million reads mapped) is a normalised estimation of gene expression based on RNA-Seq data. FPKM are calculated from the number of reads that mapped to each particular gene sequence taking into account the gene length and the sequencing depth. Three housekeeping genes were used for qPCR calculation based on MIQE guidelines (Bustin *et al.* 2009). Student's *t*-test was used to calculate the significance (* $P<0.05$, ** $P<0.05$, *** $P<0.001$) of normalized RNA-Seq data and qPCR data. All error bars represent the standard error of the mean (S.E.M.).

Results

Conserved mRNA localization of *FGF10*, *FGFR2-IIIb*, *EFNB2*, *MAFB* and *DLX5*

The mRNA distribution of *FGF10*, *FGFR2-IIIb*, *EFNB2*, *MAFB* and *DLX5* was conserved with those of eutherian mammals during phallus development. *FGF10* and its receptor *FGFR2-IIIb* were co-localised in the urethral epithelium and phallus epithelium in both male and female phalluses at day 20pp and day 90pp (Figs 1 and 2). *EFNB2* was detected in the urethral epithelium and phallus epithelium in the phallus at day 20pp and day 90pp (Figs 1 and 2). *EFNB2* mRNA was also found in mesenchymal cells around the urethral epithelium in phalluses at day 20pp (Fig. 1). *MAFB* was mainly found in the epithelium and corpora cavernosa (Figs 1 and 2). *DLX5* mRNA was detected primarily in the urethral epithelium (Figs 1 and 2). Mesenchymal staining of *DLX5* was found in male phalluses at day 20pp and day 90pp (Figs 1 and 2).

Hormonal responsiveness and expression pattern of *FGF10*, *FGFR2-IIIb*, *EFNB2*, *MAFB* and *DLX5* during phallus differentiation

RNA-Seq expression of *FGF10* and *EFNB2* was higher in male phalluses than female phalluses at day 50pp (Fig. 3). They were both upregulated after adiol treatment in female phalluses but downregulated by castration in males (Fig. 3). In males, *FGF10* was not affected by oestrogen treatment, but *EFNB2* was downregulated (Fig. 3). *MAFB* was higher in untreated female phalluses than male phalluses at day 50pp (Fig. 3) but was upregulated in male phalluses at day 50pp after castration. *MAFB* expression did not change after treatment with either adiol or oestrogen (Fig. 3) and *FGFR2-IIIb* and *DLX5* did not change after adiol

treatment, oestrogen treatment or castration (data not shown).

In untreated phalluses, there were no significant changes in expression of *FGF10*, *FGFR2-IIIb*, *EFNB2* and *DLX5* measured by qPCR between male and female phalluses before day 50pp (Fig. 4), but *MAFB* was higher in female phalluses than male phalluses at this stage. By day 90pp, *FGF10*, *FGFR2-IIIb*, *DLX5*, *EFNB2* and *MAFB* were all significantly higher in male than in female phalluses at day 90pp (Fig. 4).

Androgen dependent AP-1 genes

AP-1 genes, including *C-FOS*, *FOSB*, *C-JUN*, *JUNB*, *JUND* and *ATF3* mRNA were all significantly higher in male than in female phalluses at day 50pp and were all significantly upregulated after adiol treatment. Within the AP-1 genes, only *C-FOS* was downregulated after oestrogen treatment of males (Fig. 5).

Distribution of *IGF1* and *PCNA* during phallus elongation

In day 90pp male and female phalluses, *IGF1* was predominantly localised in the extracellular matrix and cell membranes of the urethral epithelium, phallus epithelium and corpora cavernosa (Fig. 6). Some staining also occurred in the cytoplasm of urethral epithelial cells and phallus epithelial cells (Fig. 6). Interestingly, *IGF1* was identified in the extracellular matrix and cytoplasm of mesenchymal cells of only the male urorectal septum (URS) at day 90pp (Fig. 6). Staining of day 90pp phalluses with PCNA antibody showed that the protein was localised in the nucleus of the basal urethral epithelial cells, the phallus epithelial cells and mesenchymal cells of the corpora cavernosa (Fig. 7), with a small amount of staining in the cytoplasm and

Figure 1 mRNA distribution of *FGF10*, *FGFR2IIIb*, *EFNB2*, *MAFB* and *DLX5*, in day 20pp male and female phalluses irrespective of sex, mRNA of *FGF10*, *FGFR2IIIb*, *EFNB2*, *MAFB* and *DLX5* were all detected in the urethral epithelial cells and phallus epithelial cells in the phalluses at day 20pp. *EFNB2*, *MAFB* and *DLX5* were also expressed in the mesenchyme in male and female phalluses. Section *in situ* hybridisation staining of *FGF10* (A1–A4), *FGFR2IIIb* (B1–B4), *EFNB2* (C1–C4), *MAFB* (D1–D4) and *DLX5* (E1–E4). E, epithelium; M, mesenchyme; UE, urethral epithelium; Dist, distal; Prox, proximal. Scale bar: 200 μm.

Figure 2 mRNA distribution of *FGF10*, *FGFR2IIIB*, *EFNB2*, *MAFB* and *DLX5*, in day 90pp male and female phalluses. Irrespective of sex, mRNA of *FGF10*, *FGFR2IIIB*, *EFNB2*, *MAFB* and *DLX5* were all detected in the urethral epithelial cells and phallus epithelial cells in the phalluses. *FGF10* and *DLX5* were expressed in the mesenchyme in male phalluses at day 90pp. The urethral closure is complete at the base of male phalluses at this stage. There is a slight variation in sectioning depth shown in the proximal sections of phalluses, so apparent differences in the degree of urethral closure are due to the level of sectioning used. Section *in situ* hybridisation staining of *FGF10* (A1–A4), *FGFR2IIIB* (B1–B4), *EFNB2* (C1–C4), *MAFB* (D1–D4) and *DLX5* (E1–E4). D, diverticulum; E, epithelium; M, mesenchyme; U, urethra; UE, urethral epithelium; Dist, distal; Prox, proximal. Scale bar: 200 μm.

extracellular matrix of urethral epithelial cells, phallus epithelial cells and mesenchymal cells (Fig. 7). Similarly, the nuclear PCNA was only found in mesenchymal cells of the male URS in phalluses at day 90pp but not in that of female phalluses (Fig. 7).

Hormonally responsive IGF1, IGFBP3 and IGFBP6

From the RNA-Seq and qPCR data, *IGF1* expression was higher in untreated male phalluses than female phalluses at day 50pp, at day 90pp and day 150pp (Fig. 8). It was upregulated after adiol treatment in females and oestrogen treatment in males, but downregulated after castration in males at day 50pp (Fig. 8). *IGFBP6* was also upregulated after adiol treatment in females and oestrogen treatment in males (Fig. 8). In contrast, *IGFBP3* was higher in untreated female phalluses than male phalluses at day 50pp, day 90pp and day 150pp

(Fig. 8) and downregulated by adiol treatment in females at day 50pp (Fig. 8).

Discussion

Differentiation and growth of the phallus depends on a suite of genes that have specific spatial and temporal expression patterns. Sex steroids are critical to this process and control gene expression. *FGF10*, *EFNB2* and *MAFB* were affected by both androgen and oestrogen treatments, while *AP-1* genes appear to be solely under the control of androgen. The complimentary expression patterns of *IGF1* and the receptor *IGFBP3* suggest that the balance of these two genes might be key to understand urethral closure and phallus elongation, which may be mediated by *IGF1*-induced rapid proliferation of cells in the urorectal septum and urethral epithelia.

Figure 3 Transcriptomic expression of *FGF10*, *EFNB2* and *MAFB* in normal and hormone treated phalluses at day 50pp. *FGF10* was higher in male phalluses than female phalluses at day 50pp, upregulated in female phalluses after androgen treatment, and downregulated in males after castration. *EFNB2* expression was higher in male phalluses than female phalluses at day 50pp, upregulated in female phalluses after androgen treatment and downregulated in males after oestrogen treatment or castration. *MAFB* expression was higher in female phalluses at day 50pp than that in male phalluses, upregulated after castration in males, but not affected by androgen treatment nor oestrogen treatment. A, androgen; E2, oestrogen; FPKM, Fragments Per Kilobase of transcript per Million mapped reads. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Error bar: S.E.M.

Figure 4 qPCR analysis on the expression of *FGF10*, *FGFR2IIIb*, *EFNB2*, *MAFB* and *DLX5* during postnatal phallus development. The expression level of *FGF10*, *FGFR2IIIb*, *EFNB2*, *MAFB* and *DLX5* was significantly higher in male phalluses than in female phalluses at day 90 pp. The expression of *FGF10* and *EFNB2* were similar from day 20 pp to day 90 pp. There was no differences in expression level between male phalluses and female phalluses until after day 50 pp. *MAFB* was significantly higher in female phalluses at day 50 pp. *DLX5* was significantly reduced in the female phalluses at day 90 pp. The expression of all genes was relative to *18S*, *GAPDH* and *HMBS*. * $P < 0.05$. Error bar: S.E.M.

FGF10, *FGFR2IIIb*, *EFNB2*, *MAFB* and *DLX5* were all expressed in the urethral epithelium as well as in the phallus epithelium in the tammar, as shown in mice (Haraguchi *et al.* 2001, Perriton *et al.* 2002, Dravis *et al.* 2004, Petiot *et al.* 2005, Gredler *et al.* 2015, Harada *et al.* 2015). In mice, these genes are all crucial for maintaining cell proliferation and cell survival in the phallus (Bhushan *et al.* 2001, Shaulian & Karin 2001, Bendall *et al.* 2003, Hart *et al.* 2003, Steinberg *et al.* 2005, Weiler *et al.* 2009, Yu *et al.* 2016). *FGF10* expression increased after androgen treatment in phalluses at day 50 pp in the tammar. A similar effect of androgen on *FGF10* expression also occurs in prostate cancer cells (Lu *et al.* 1999, Nakano *et al.* 1999, Donjacour *et al.* 2003) and in cultured mice phalluses in which *FGF10* expression is downregulated after adding the anti-androgen flutamide (Petiot *et al.* 2005). *EFNB2* expression was also increased after androgen treatment, but was downregulated by oestrogen and by castration in males. Interestingly, the opposite effect occurs in castrated mice in which *EFNB2* is downregulated in the epididymis after treating with testosterone propionate (Snyder *et al.* 2009). However, it is known that the hormonal responsiveness of *EFNB2* is different in

different tissues (Nikolova *et al.* 1998, Yucel *et al.* 2007, Snyder *et al.* 2009) so both androgen and oestrogen can influence its action.

MAFB was significantly higher in female phalluses at day 50 pp and upregulated after castration. However, *MAFB* subsequently increased in male phalluses and became higher than that in female phalluses by day 90 pp. This was not expected since the expression of *Mafb* is upregulated in male phalluses in mice, but these were sampled before E18.5, well before the male phallus growth and elongation takes place (Suzuki *et al.* 2014, Matsushita *et al.* 2015). Interestingly, from our previous studies in the tammar, *SHH* was significantly higher in female phalluses between day 30 and day 40 pp but lower in female phalluses between day 50 to day 60 pp (Chew *et al.* 2014). The expression pattern of *SHH* was very similar to that of *MAFB*, with one difference that the change of *SHH* expression occurred about 10 days earlier. It is possible that *MAFB* may be a downstream target of *SHH* signalling under the negative regulation of androgen. In addition, *Mafb* expression is also controlled by β -catenin, the transcription factor of Wntless-Type MMTV Integration Site Family, Member (WNT)/ β -catenin pathway (Matsushita *et al.* 2015).

Figure 5 RNA-Seq analysis on the expression pattern of AP-1 genes in phalluses at day 50 pp. The expression of *C-FOS*, *FOSB*, *ATF3*, *C-JUN*, *JUNB* and *JUND* were increased after androgen treatment. The expression level of those genes was significantly higher in male phalluses at day 50 pp than that in female phalluses. A, androgen; E2, oestrogen; FPKM, Fragments Per Kilobase of transcript per Million mapped reads.

* $P < 0.05$, ** $P < 0.05$, *** $P < 0.001$. Error bar: S.E.M.

Figure 6 IGF1 distribution in male and female phalluses at day 90pp. In both male and female phalluses, IGF1 was primarily found in the extracellular matrix and cytoplasm of epithelial cells and in the diverticulum. Some IGF1 was detected in the corpora cavernosa. In the URS, IGF1 was found only in male phalluses (see insets). CC, corpus cavernosum; D, diverticulum; E, epithelium; U, urethra; UE, urethral epithelium; UG, urethra groove; URS, urorectal septum. Red staining: IGF1. Blue staining: DAPI. Scale bar: 1.0mm.

SHH, upstream of WNT/ β -catenin signaling, regulates cell proliferation by controlling WNT/ β -catenin activity during GT outgrowth in mice (Miyagawa *et al.* 2009).

In the tammar, *AP-1* genes have higher expression in males and were upregulated in females after androgen treatment at day 50pp (Fig. 9). Similarly, androgen induces *c-Fos* expression in the rat hippocampus (Kerr *et al.* 1996) and upregulates *ATF3* expression in human prostate cancer cells (Pelzer *et al.* 2006). In addition, androgen induces the AP-1 DNA-binding and transcriptional activity in human prostate carcinoma cells (Church *et al.* 2005). *AP-1* genes regulate cell proliferation (reviewed in (Angel & Karin 1991), and since androgen treatment can induce phallus elongation (Leihy *et al.* 2004) and urethral closure (Chen *et al.* 2018) in the tammar, it is likely that *AP-1* genes contribute to this. *C-FOS* was affected by oestrogen treatment in males, suggesting a possible target of oestrogenic EEDs during phallus development, at least in the tammar.

The strong expression of the growth factor IGF1 in the URS, urethral and phallus epithelia, accompanying the rapid proliferation in those tissues in the male phallus was not expected. PCNA, a marker of proliferation known to regulate cell survival (Witko-Sarsat *et al.* 2010), was also strongly expressed in nuclei of male URS, urethral and phallus epithelia. IGF1 and nuclear PCNA were detected in the urorectal septum of only male, but not female phalluses, suggesting that IGF1 might induce urethral closure by regulating URS proliferation, between at least day 90 to day 150 when closure is complete (Fig. 9).

IGF1 was consistently more highly expressed in male than in female phalluses at day 50pp, day 90pp and day 150pp. In females, its expression was upregulated after androgen treatment. Clearly, *IGF1* is androgen

dependent in the tammar during phallus differentiation. Such androgenic dependency of *IGF1* is conserved with eutherian mammals, as *IGF1* mRNA increases after testosterone treatment in bovine muscle satellite cells (Kamanga-Sollo *et al.* 2004), rat uterine tissue (Sahlin *et al.* 1994) and human prostate cancer cell lines (Arnold *et al.* 2005, Le *et al.* 2006). However, IGF1 has not previously been reported in the URS.

After oestrogen treatment, *IGF1* was significantly upregulated in male phalluses (Fig. 9). Similarly, oestrogen also increases *IGF1* expression in the primate cerebral cortex (Cheng *et al.* 2001) and in mice uteri (Hewitt *et al.* 2010). *IGFBP6* had similar androgen and oestrogen responsiveness as *IGF1*.

This androgen and oestrogen responsiveness of *IGFBP6* is also seen in other species (Koike *et al.* 2005, Henderson *et al.* 2006), including in the rat epididymis after inhibiting of dihydrotestosterone (DHT) (Henderson *et al.* 2006), and in prostate cancer cells after treatment with the synthetic oestrogen diethylstilboestrol (DES) (Koike *et al.* 2005). In contrast,

Figure 7 PCNA localisation in male and female phalluses at day 90pp. In both male and female phalluses, PCNA staining was found in the nuclei of basal urethral epithelial cells, phallus epithelial cells and diverticulum. The nuclear PCNA was also found in mesenchymal cells in the corpora cavernosa. In the URS, nuclear PCNA was found only in male phalluses (see insets). Cytoplasmic PCNA was detected in the urethral epithelium, phallus epithelium and diverticulum in both sexes, and there was some mesenchymal cytoplasmic and extracellular matrix staining. A, anus; CC, corpus cavernosum; D, diverticulum; E, epithelium; M, mesenchyme; U, urethra; UE, urethral epithelium; UG, urethra groove; URS, urorectal septum. Red staining: PCNA. Blue staining: DAPI. Scale bar: 1.0mm.

Figure 8 Expression pattern of *IGF1*, *IGFBP3* and *IGFBP6* in the phalluses. In RNASeq data (left), *IGF1* was higher in male phalluses than female phalluses at day 50pp. It was upregulated after androgen treatment in females and oestrogen treatment in males but was downregulated after castration in males. *IGFBP3* was higher in female phalluses than male phalluses at day 50pp. It was downregulated after androgen treatment in females. *IGFBP6* was both upregulated in males after oestrogen treatment and in females after androgen treatment. In qPCR (right), *IGF1* was higher in male phalluses than female phalluses at day 50pp, day 90pp and day 150pp. It was upregulated after androgen treatment in females but was not affected by oestrogen treatment or castration at day 50pp. *IGFBP3* was higher in female phalluses than male phalluses at day 50pp, day 90pp and day 150pp. A, androgen; E2, oestrogen; FPKM, Fragments Per Kilobase of transcript per Million mapped reads. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Error bar: S.E.M.

IGFBP3 has the opposite expression pattern to that of *IGF1* in the tammar phalluses. It has higher expression in female phalluses than male phalluses at days 50, 90 and 150pp, and its downregulation after androgen treatment in females are consistent with ligand (*IGF1*) receptor (*IGFBP3*) interactions. A similar pattern occurs in prostate cancer cells after treatment with androgen (Kojima *et al.* 2006, Peng *et al.* 2008) or synthetic androgen (Peng *et al.* 2008).

The complimentary expression patterns of *IGF1* and *IGFBP3* suggest that *IGF1* might be a largely unrecognised

growth factor in phalluses that activates cell proliferation, while *IGFBP3* negatively regulates cell growth and proliferation in females, as seen in many other studies (Schmid *et al.* 1991, Valentinis *et al.* 1995, Grimberg & Cohen 2000, Pollak 2000, Duan & Xu 2005, Le *et al.* 2006). *IGFBP3* activates protein tyrosine phosphatase (PTPase), so reversing the autophosphorylation of insulin-like growth factor 1 receptor (IGF1R) and tyrosine phosphorylation of insulin receptor substrate 1 (IRS1) (Duan & Xu 2005). In addition, *IGF1* and *IGFBP3* have different responsiveness towards androgen

Figure 9 Summary of androgen and oestrogen effects on gene expression of *IGF1*, *IGFBPs*, *FGF10*, *EFNB2*, *MAFB* and *AP-1* in phalluses (top) and diagram of how *IGF1* regulates urethral closure (bottom). Adiol treatment upregulated gene expression of *IGF1*, *IGFBP6*, *FGF10*, *EFNB2* and *AP-1*, but downregulated *IGFBP3* expression. Oestrogen increased *IGF1* and *IGFBP6* expression but decreased *EFNB2* and *C-FOS* expression. Castrating males induced an upregulation of *MAFB* and a downregulation of *IGF1*, *FGF10* and *EFNB2* in phalluses. *IGF1* was higher in male phalluses from day 50pp to day 90pp, and induced URS proliferation and urethral closure. In contrast, *IGFBP3* was higher in female phalluses and inhibited urethral closure. Adiol treatment and oestrogen treatment both increased *IGF1* expression and induced urethral closure of day 150pp phalluses. Castrating males decreased *IGF1* expression in phalluses and prevented urethral closure. A, anus; D, diverticulum; E, epithelium; E2, oestrogen; M, mesenchyme; U, urethra; UG, urethral groove. Red indicates decreased levels; green indicates increased levels.

and oestrogen in human cell lines (Huynh *et al.* 1996, Le *et al.* 2006). Dihydrotestosterone and testosterone increase cell growth and the expression of *IGF1*, but decrease *IGFBP3* expression level in human prostatic stromal cells (Le *et al.* 2006). Oestrogen increases *IGF1* expression in primate cerebral cortex (Cheng *et al.* 2001), but downregulates *IGFBP3* expression in human breast cancer cells (Huynh *et al.* 1996). In addition, fulvestrant, an anti-oestrogen, increases *IGFBP3* mRNA abundance and its protein accumulation in human breast cancer cells (Huynh *et al.* 1996). Therefore, maintaining a balance of *IGF1* and *IGFBP3* might be important for normal phallus development in the tammar. Both *IGF1* and *IGFBP6* were upregulated in male phalluses at day 50pp after oestrogen treatment. However, gonadal oestrogen at this stage in untreated young is below detection in both sexes (Renfree *et al.* 1992), so while this is unlikely to be a physiological effect, it highlights the possibility that both *IGF1* and *IGFBP6* could be potential targets of oestrogenic EEDs, such as DES, atrazine and bisphenols.

Conclusions

All the genes examined were affected by exogenous steroids during phallus development in the tammar. The conserved localisation and increased expression of *FGF10*, *FGFR2IIIb*, *EFNB2*, *MAFB*, *DLX5* and *AP-1* genes suggest that androgen influences cell proliferation and apoptosis to maintain phallus growth and elongation in the tammar as in eutherian mammals. In particular, *IGF1* appears to induce urethral closure by activating cell proliferation in the urorectal septum of male phalluses, while *IGFBP3* may negatively regulate this process. Thus, complex gene regulatory systems under the control of both androgenic and oestrogenic steroid hormones during phallus development and the effects on the *IGF1* signalling pathway in particular suggest that the developing phallus may be especially susceptible to exogenous sex steroids.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-18-0416>.

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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