Adhesion G-protein-coupled receptor, GPR56, is required for Müllerian duct development in the chick

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Abstract

The embryonic Müllerian ducts give rise to the female reproductive tract (fallopian tubes, uterus and upper vagina in humans, the oviducts in birds). Embryonic Müllerian ducts initially develop in both sexes, but later regress in males under the influence of anti-Müllerian hormone. While the molecular and endocrine control of duct regression in males has been well studied, early development of the ducts in both sexes is less well understood. Here, we describe a novel role for the adhesion G protein-coupled receptor, GPR56, in development of the Müllerian ducts in the chicken embryo. GPR56 is expressed in the ducts of both sexes from early stages. The mRNA is present during the elongation phase of duct formation, and it is restricted to the inner Müllerian duct epithelium. The putative ligand, Collagen III, is abundantly expressed in the Müllerian duct at the same developmental stages. Knockdown of GPR56 expression using in ovo electroporation results in variably truncated ducts, with a loss of expression of both epithelial and mesenchymal markers of duct development. Over-expression of GPR56 in vitro results in enhanced cell proliferation and cell migration. These results show that GPR56 plays an essential role in avian Müllerian duct development through the regulation of duct elongation.

Introduction

In amniotic vertebrates, two pairs of ducts develop in close association with the gonads and mesonephric kidney; the Müllerian ducts and Wolffian ducts. In male embryos, the Wolffian ducts differentiate into the vas deferens, epididymis and seminal vesicles, under the influence of testosterone. The Müllerian ducts regress due to the action of testis-derived anti-Müllerian hormone (AMH) (reviewed in Josso et al. 2001, Klattig & Englert 2007, Mullen & Behringer 2014). In female embryos, the converse pattern arises. The Wolffian ducts regress due to the absence of testosterone, while the Müllerian ducts differentiate into female structures in the absence of foetal AMH (Cunha et al. 2017). In female mammals, Müllerian ducts give rise to the fallopian tubes, uterus, cervix and upper part of the vagina. In birds, the homologous structure is the oviduct, the site of albumen and egg shell deposition (reviewed in Roly et al. 2018).

Proper Müllerian duct formation is crucial for female reproductive tract development and function. In humans, disruption of Müllerian duct formation during embryonic development can lead to Mayer–Rokitansky–Küster–Hauser (MRKH) syndrome in 46,XX females (Morcel et al. 2008).
This rare condition is characterised by congenital agenesis or dysplasia of Müllerian-derived structures. The incidence is estimated at around 1 in 4500 live births (Londra et al. 2015, Patnaik et al. 2015). The molecular basis of MRKH is likely to be varied, with HOXA cluster, WNT, WT1 and PAX2 genes having been implicated, although only WNT4 has so far to been clearly linked to the condition (Lalwani et al. 2008, Philibert et al. 2011, Fontana et al. 2017) (reviewed in Patnaik et al. 2015). However, some studies have identified potential pathogenic mutations in the transcription factor genes, LIM1 (also known as LHX1) and TBX6 associated with MRKH syndrome (Ledig et al. 2012, Sandbacka et al. 2013). Meanwhile, a MRKH case was reported in which there was a partial deletion in the gene encoding the extra-cellular matrix protein, inter-trypsin inhibitor # 5 (ITIH5) (Morcel et al. 2012). However, most cases of MRKH do not have a molecular diagnosis. Defining the molecular mechanisms controlling embryonic Müllerian duct formation may enhance our understanding of MRKH.

There are three cellular components of the embryonic Müllerian duct: a canalised epithelial tube called the Müllerian duct epithelium (MDE), surrounding Müllerian duct mesenchyme (MM) and, externally, the Müllerian coelomic epithelium (MCE). The coelomic epithelial cells give rise to Müllerian epithelial tube and the mesenchymal layers. Animal models have been instrumental in identifying genes involved in embryonic Müllerian duct formation (Giuoli et al. 2007, Klattig & Englert 2007, Chiga et al. 2014, Cutting et al. 2014, Ayers et al. 2015a). Studies using chicken and mouse embryos have shown that there are three conserved phases of Müllerian duct development: specification, invagination and duct elongation (reviewed in Kobayashi & Behringer 2003, Mullen & Behringer 2014, Roly et al. 2018). These phases involve interaction between epithelial and underlying mesenchyme cells and epithelial-to-mesenchyme transition (EMT). During the first phase, a subset of coelomic epithelial cells at the anterior pole of the mesonephric kidney is specified as Müllerian precursors (Jacob et al. 1999, Guioli et al. 2007). This process involves BMP signalling and the expression of PAX2 (Atsuta & Takahashi 2016). PAX2 initiates FGF signalling from the coelomic epithelial cells, which then activates expression of the LIM1 (LHX1) homeodomain transcription factor (Orvis & Behringer 2007, Atsuta & Takahashi 2016). Both PAX2 and LIM1 are required for proper duct formation (Torres et al. 1995, Bouchard et al. 2002, Kobayashi et al. 2004, Huang et al. 2014, Atsuta & Takahashi 2016). Müllerian precursor cells are considered specified when they express LIM1. During phase two, the specified precursor cells invaginate, penetrating the underlying mesenchyme. Wnt4 produced by the mesenchyme is required for invagination. Invagination is impaired in Wnt4-null mice, leading to a lack of Müllerian duct derivatives (Vainio et al. 1999). In both mouse and chicken, Wnt4 is a marker of the Müllerian duct mesenchyme. Wnt4 also plays a role in the third phase of duct development: caudal elongation (Orvis & Behringer 2007, Prunskaitė-Hyyrylainen et al. 2016). During this phase, the invaginating epithelial cells form a canalised tube and migrate posteriorly. This involves cell proliferation and migration of the epithelia through the underlying mesenchyme, between the surface coelomic epithelium and the adjacent Wolffian duct (Fujino et al. 2009). An intact Wolffian duct is required for proper Müllerian duct formation, probably via the secretion of Wnt signalling molecules (Didier 1971, Carroll et al. 2005). Other factors required for duct development include Wnt7a, Wnt5a and retinoic acid signalling (Mendelsohn et al. 1994, Kastner et al. 1997, Miller & Sassoon 1998, Parr & McMahon 1998, St-Jean et al. 2019). Despite these molecular details, there must be other undiscovered factors involved duct formation, as evidenced by the frequent lack of molecular diagnoses in humans with Müllerian duct anomalies.

We previously conducted a screen for genes expressed in the embryonic urogenital system (Ayers et al. 2015b). One gene derived from this screen was G protein-coupled receptor 56 (GPR56), and it was found to be expressed in the embryonic Müllerian ducts in addition to female gonads. Also known as ADGRG1, GPR56 is a member of a relatively novel family of adhesion G protein-coupled receptors (Liu et al. 1999, Zendman et al. 1999). These proteins are thought to mediate both adhesion and G protein-coupled intracellular signalling (Yona et al. 2008, Mizuno & Itoh 2010, Hamann et al. 2015, Ji et al. 2018). GPR56 is located at the cell surface, where it can interact with the extracellular matrix (Xu 2010). Structurally, it contains a seven-pass transmembrane domain similar to those in conventional GPCRs, but with a large extracellular domain. GPR56 can undergo autoproteolytic cleavage, generating N- and C-terminal fragments (Shashidhar et al. 2005, Jin et al. 2007). The N terminal fragment can associate with extracellular ligands while remaining physically linked to the C terminal fragment, which can initiate intracellular RhoA signalling (Luo et al. 2014). Major sites of GPR56 expression include the brain, thyroid gland and heart (Iguchi et al. 2008, Ackerman et al. 2015). In mammals, GPR56 has an important role in neurogenesis, namely frontal cortex development. Mutations in the
human GPR56 gene result a severe developmental brain disease called bilateral frontoparietal polymicrogyria (BFPF) (Piao et al. 2004). In mice, targeted deletion of Grp56 results in neuronal malformations of the cerebral cortex, a developmental phenotype that recapitulates that seen in humans (Li et al. 2008). GPR56 is also implicated in cancer progression. In carcinomas, it can promote cell proliferation and metastasis (Ji et al. 2018). Furthermore, splice variants of GPR56 in humans can activate several transcription factors associated with tumorigenesis (Kim et al. 2010). In the brain, the apparent ligand of GPR56 is collagen type III (Luo et al. 2011). Ligands in other tissues can include heparin, transglutaminase 2 and, in the gut, progastrin (reviewed in Huang & Lin 2018). In the current study, we report novel expression of GPR56 in the developing Müllerian ducts of the chicken embryo. Using in ovo RNA interference, we find that GPR56 is required for Müllerian duct development during the elongation phase, consistent with its role in cell adhesion and cell migration in other developmental contexts. In vitro, over-expression of GPR56 can stimulate cell proliferation. GPR56 is a novel candidate gene for Müllerian-derived disorders.

Materials and methods

Eggs and embryos

Fertilized eggs were obtained from HyLine Brown chickens (Gallus gallus domesticus) and incubated under humid conditions at 37.8°C. Embryo urogenital systems including the mesonephric kidneys, gonads and Müllerian ducts were dissected at various time points during development (E4.5, E5.5, E6.5 and E8.5) and staged according to the criteria of Hamburger and Hamilton (HH) (Hamburger & Hamilton 1992). These time points span Müllerian duct specification (E4.5; up to HH stage 25), invagination (E4.5–5.5; HH stages 25–28) and elongation phases (E5.5–E6.5; HH stages 28–30) (Ayers et al. 2015a, Atsuta & Takahashi 2016). Whole urogenital tissues were processed for either whole mount in situ hybridisation or immunofluorescence as described below. By Australian law, animal ethics approval is not required for chicken embryos up to mid-gestation (E10.5).

PCR sexing

For genetic sexing of embryos, a small limb piece of limb bud was digested at 55°C for 20 min in PCR compatible digestion buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3, 0.1 mg/mL gelatin, 0.45% Nonidet P-40, 0.45% Tween 20 containing 0.2 mg/mL proteinase K). The genomic DNA was then used for a rapid PCR sexing reaction using the W-linked (female-specific) XhoI repeat sequence and 18S rRNA genomic sequence as internal control, as described (Clinton et al. 2001). The primers were XhoI-For: 5′-CCCCAAATATAACACGCTTCA-3′ and XhoI-Rev: 5′-GAAATGATTTTTGCGGAC-3′; 18S-For: 5′-AGCTCTTTTCTCGATTCGTCG-3′; 18S-Rev; 5′-GGGTAGACAACAGCTGAGCC-3′. By this method, females show two bands, while males show one.

RT-PCR and qRT-PCR

RNA was extracted from pooled E6.5 and E8.5 embryonic Müllerian ducts or chicken DF1 cells using TRIzol, according to the manufacturer’s instructions. Triplicate samples of pooled ducts were extracted. Five micrograms of total RNA per sample was treated with DNase I to remove contaminating genomic DNA (Ambion DNase-free kit) and 1 µg total RNA per sample was reversed transcribed into cDNA, using random hexamers together with AMV reverse transcriptase. For qRT-PCR and RT-PCR, the following chicken-specific primers were used: cGPR56.For: 5′-GTTGGGACATCTCTTGGTGA-3′; cGPR56.Rev: 5′-GACACACGTTGGGTCACAT-3′, β-actin.For: 5′-GCTACAGCTTCCAACACACA-3′, β-actin.Rev: 5′-TCTCTGCTGAAATCCGAT-3′, cCol3a1.For: 5′-AGAACACATCTCCGTGTCG-3′ and cCol3a.Rev: 5′-CTGTGGTCATTCCGTACATG-3′. RT-PCR was performed using GoTaq Flexi DNA polymerase according to the manufacturer’s instructions (Promega M8291). PCR cycling conditions were 95°C × 10 min, (95°C × 30 s; 5°C × 30 s; 60°C × 30 s) × 32, 72°C × 5 min, 4°C hold. Quantitative RT-PCR was carried out on triplicate samples using QuantNova SYBR® Green PCR Kit, as described previously (Hirst et al. 2017). For each primer pair, standard curves were first generated to confirm amplification efficiency (90–110%). Data were normalised against β-actin expression using the comparative cycle threshold method (2−ΔΔCT). For both RT-PCR and qRT-PCR, control cDNA samples were not incubated with reverse transcriptase. Data were analyzed using unpaired t-tests and two-tailed P values (two groups) or one-way ANOVA followed by Tukey HSD post hoc test (more than two groups) (GraphPad Prism).

Whole-mount in situ hybridisation

Whole-mount in situ hybridisation (WISH) was carried out as previously described (Andrews et al. 1997).
Urogenital systems were dissected from embryos at E4.5 (HH25), 5.5 (HH28), 6.5 (HH31) and 8.5 (HH34). At least three urogenital systems were used at each time point. Tissues were fixed overnight at 4°C in 4% paraformaldehyde in diethyl pyrocarbonate-treated PBS (DEPC-PBS), pooled by sex, washed in DEPC-PBS containing 0.1% Triton-X 100 (PBTX), and dehydrated through a methanol series to 100% methanol. Following rehydration through a descending methanol series, tissues were permeablised with proteinase K (10 µg/mL) for 40–80 min at room temperature depending on tissue size. Tissues were then washed briefly with PBTX, briefly re-fixed in 0.2% glutaraldehyde/4% paraformaldehyde in PBTX for 20 min, and then incubated overnight at 65°C in prehybridisation solution (53 SSC, 50% formamide, 2% Boehringer blocking reagent, 0.1% Triton X-100, 0.5% CHAPS, 1 mg/mL yeast RNA, 5 mM EDTA, 50 µg/mL heparin). RNA probes were generated from PCR products encoded by the gene, the primers we used previously described. For Collagen III, primers were used to amplify a 584 bp region; for antisense and sense RNA polymerase-binding sites. For antisense and sense probes, riboprobes were precipitated at 20°C and overnight and then subjected to antigen retrieval using a citrate-based buffer (pH 6.0; Dako # S1699) at 98°C for 30 min on a DEKO PT Link machine. Slides were then sectioned at 10, 12 or 18 µm to examine the localisation of mRNA within Müllerian ducts. For post-WISH immunostaining, 10 µm frozen sections of over-stained tissues were cut on a cryostat, thaw mounted onto glass slides and collected in PBS. Sections were air dried overnight and then subjected to antigen retrieval using a citrate-based buffer (pH 6.0; Dako # S1699) at 98°C for 30 min on a DEKO PT Link machine. Slides were then washed in DAKO wash buffer (#K8002) and equilibrated in a citrate-based buffer (pH 6.0; Dako # S1699) at 98°C for 30 min on a DEKO PT Link machine. Slides were then washed in DAKO wash buffer (#K8002) and equilibrated in PBS for 10 min at room temperatures. Sections were then permeablised in 1% Triton X-100/PBS (10 min), washed in PBS and then blocked for 1 h at room temperature in 2% BSA/PBS. Following washes in PBS, sections were incubated with primary antibody overnight at 4°C. The following antibodies were used: rabbit anti-chicken DMRT1 @ 1:5000 (in house) or mouse anti-pan cytokeratin @ 1:200 (Novus Biologicals, # NP2-29429). Sections were washed in PBS and secondary antibodies were applied. Detection was carried out using the Vector ABC immunoperoxidase staining kit, according to the manufacturer’s instructions (for DMRT1) or using immunofluorescence as described below (for cytokeratin).

**Immunofluorescence**

For immunofluorescence, frozen sections were used. Urogenital tissues of chicken embryos were dissected and briefly fixed in 4% paraformaldehyde/PBS at room temperature for 15 min and then cryoprotected by immersion in 30% sucrose/PBS overnight at 4°C and embedded in OCT compound. Frozen sections (10 µm) were then mounted onto Super-frost Plus slides, washed following rehydration through a descending methanol series, tissues were permeablised with proteinase K (10 µg/mL) for 40–80 min at room temperature depending on tissue size. Tissues were then washed briefly with PBTX, briefly re-fixed in 0.2% glutaraldehyde/4% paraformaldehyde in PBTX for 20 min, and then incubated overnight at 65°C in prehybridisation solution (53 SSC, 50% formamide, 2% Boehringer blocking reagent, 0.1% Triton X-100, 0.5% CHAPS, 1 mg/mL yeast RNA, 5 mM EDTA, 50 µg/mL heparin). RNA probes were generated from PCR products cloned into pGEM.T.Easy and confirmed by sequencing. For post-WISH immunostaining, 10 µm frozen sections of over-stained tissues were cut on a cryostat, thaw mounted onto glass slides and collected in PBS. Sections were air dried overnight and then subjected to antigen retrieval using a citrate-based buffer (pH 6.0; Dako # S1699) at 98°C for 30 min on a DEKO PT Link machine. Slides were then washed in DAKO wash buffer (#K8002) and equilibrated in PBS for 10 min at room temperatures. Sections were then permeablised in 1% Triton X-100/PBS (10 min), washed in PBS and then blocked for 1 h at room temperature in 2% BSA/PBS. Following washes in PBS, sections were incubated with primary antibody overnight at 4°C. The following antibodies were used: rabbit anti-chicken DMRT1 @ 1:5000 (in house) or mouse anti-pan cytokeratin @ 1:200 (Novus Biologicals, # NP2-29429). Sections were washed in PBS and secondary antibodies were applied. Detection was carried out using the Vector ABC immunoperoxidase staining kit, according to the manufacturer’s instructions (for DMRT1) or using immunofluorescence as described below (for cytokeratin).

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in PBS and permeabilised in 1% Triton X-100/PBS for 10 min at room temperature, as previously described (Smith et al. 2008). Sections were blocked in 2% BSA/PBS for 1 h at room temperature and incubated with primary antibodies in 1% BSA/PBS overnight at 4°C. Antibodies include rabbit anti-GFP (1:500; Rockland) and mouse anti-cytokeratin (1:1000; Novus). Sections were then washed in PBS and incubated for 1 h at room temperature with Alexa Fluor donkey anti-goat IgG-488 (1:1000) or AlexaFluor donkey-anti-mouse IgG-594 (1:1500) diluted in 1% BSA/PBS. Sections were washed in PBS then counterstained in DAPI/PBS and mounted in Fluorosave (Calbiochem). Images were taken on a Zeiss Axiocam MRc5.

Knockdown of GPR56 expression using RNA interference

To analyse the role of GPR56 in embryonic chicken Müllerian duct development, TOL2 plasmids expressing short hairpin RNAs were used. In the presence of transposase, TOL2 integrates into the genome, delivering stable gene (or shRNA) expression (Kawakami 2007, Sato et al. 2007). To test knockdown constructs, a TOL2-GPR56 overexpression plasmid was firstly generated. The chicken GPR56 open reading frame under the control of the CAGS promoter was cloned into TOL2 with a GFP reporter and a T2A linker (TOL2[CAGS→GFP-T2A-GPR56]). Knockdown constructs were generated by cloning several different candidate shRNAs, driven by the chicken U6-4 Pol III promoter (Wise et al. 2007), into a TOL2 plasmid expressing a BFP reporter (TOL2[(cU6.4→GPR56shRNAs)-CAGS→BFP]). Several candidate shRNAs were identified, ranked for likely effectiveness using a pre-established method (Clarke et al. 2017), cloned and tested in vitro for their ability to knockdown GFP-T2A-GPR56 expression using the fibroblastic chicken cell line, DF1. The efficiency of GPR56 knockdown by candidate shRNAs was tested by co-transfection of each shRNA plasmid with the GFP-T2A-GPR56 overexpression construct into DF1 cells according to the Lipofectamine 2000 protocol (Life Technologies).

Briefly, 500 ng of each GPR56 shRNA construct was diluted in a master mix of plasmids in Optimem (Life Technologies), such that each transfection also received 500 ng of TOL2(CAGS→GFP-T2A-GPR56) plasmid, 500 ng of a TOL2(CMV→mCherry) plasmid and 250 ng of a CAGS→Transposase plasmid in a uniform manner. After incubating 5 min with 1.5 µL of Lipofectamine diluted in OptiMem medium, transfection complexes were added to DF-1 cells in a 24-well plate. Cells were fixed 48 h post transfection, stained with DAPI and imaged using a Leica AF600LX microscope. GFP-T2A-GPR56 intensity was determined on a per cell basis using an established image analysis pipeline (Major et al. 2017). Background subtraction was performed using the mean of each image’s median pixel GFP intensity as a representative of background. Cell nuclei were identified using the DAPI stain and transfected cells gated for analysis using the mCherry marker. The short hairpin RNA showing the most robust loss of GFP-T2A-GPR56 in vitro, shRNA813, was chosen for in vivo experiments.

In vivo electroporation was used to deliver plasmids to the Müllerian duct. Day 2.5 (HH14-17) embryos were accessed by making a small hole in the side of the egg, contrasting with Indian ink, and the left coelom injected with TOL2 plasmids expressing GFP (1 µg/µL) and Transposase (0.6 µg/µL). Electroporation was carried out using a Harvard Apparatus BTX ElectroPorator (ECM 830; Fisher Biotech). Electroporation parameters were two pulses of 40 V for 10 ms, with a pulse interval of 150 ms. Eggs were sealed with tape and incubation allowed to proceed until E6.5 (HH30-31). Experiments were firstly conducted to assess the efficacy of Müllerian duct electroporation. Tissues were then processed for GFP expression, using immunofluorescence with a goat anti-GFP antibody (Rockland # 600-101-215; Antibody Registry number: AB_218182). The extent of GFP expression in the different Müllerian duct compartments was scored (MDM vs MDE vs MCE). For GPR56 knockdown, U-6 driven shRNA plasmids were used. TOL2((cU6.4→GPR56shRNAs)-CAGS→BFP) (1 µg/µL) was co-electroporated with transposase plasmid (0.6 µg/µL) into the left coelom of embryos at E2.5 (HH14-17). Embryonic development was allowed to proceed to day 6.5 (HH31). Tissues were then processed for PCR sexing and gene expression analysis, as described earlier. Control embryos were electroporated with TOL2 expressing a non-silencing shRNA (against firefly luciferase). Over 100 embryos were electroporated for each treatment, with approximately 20–30% embryo survival to E6.5.

Effect of GPR56 on cell proliferation and apoptosis

Immunostaining was carried out to study the effect of GPR56 knockdown on cell proliferation and apoptosis in vivo. Antibodies against the proliferation and apoptosis markers, phospho-histone 3 (PH3) and cleaved caspase-3 (CC3) were used, respectively. Firstly, whole mount in situ hybridisation was conducted following electroporation with non-silencing control shRNA or GPR56 shRNA, as described earlier. E6.5 urogenital tissues with high level BFP reporter were processed for in situ hybridisation.
Specimens with truncated ducts and loss of GPR56 mRNA were identified following knockdown. These specimens, and non-silencing controls, were over-stained and 6 µm frozen sections were cut. Sections were then subjected to antigen retrieval (1 h at 98°C in citrate buffer, pH 6.0) and immunoperoxidase was performed, using the Vector-stain ABC kit, as per the manufacturer’s instructions. For cell proliferation, rabbit anti-PH3 was used at a dilution of 1:500. To assess apoptosis, rabbit anti-cleaved caspase-3 was used @ 1:600. We established in pilot studies that these antigens remain preserved after subjecting embryonic chicken tissues to mRNA in situ hybridisation. Sections were taken at the anterior, mid and posterior regions of the Müllerian duct. Each group had six samples and all of the GPR56 knockdown samples had left ducts that were truncated at the mid-to-posterior pole, but were intact anteriorly. To quantify cell proliferation, the number of positive cells (brown cells) were counted in representative sections across the area of the Müllerian duct in the same field of view at the same magnification across samples. Data were analysed using unpaired t-tests (GraphPad).

To further study the effects of GPR56 on cell behaviour, the open reading frame was over-expressed in chicken DF1 embryonic fibroblasts. Cells were seeded onto wells of a six-well plate at 40% confluency (1.5 × 10^4 cells/well). The DF1 culture medium was DMEM + 1mM HEPES + 10% FBS+ penicillin/streptomycin (=DF1 medium). The next day, cells were transfected for 48 h with TOL2(CAGS→GFP-T2A-GPR56) (the same vector used for mis-expression in ovo) together with transposase. The presence of transposase results in stable genomic integration on the gene. Control wells were transfected with TOL2(CAGS→GFP-T2A-FLAG). Over-expression of GPR56 after 48 h was confirmed by RT-qPCR, as described previously (Hirst et al. 2017). RT-PCR was also used to verify whether DF1 cells express the putative GPR56 ligand, Collagen III. Transfections were conducted in triplicate and the experiment was carried out twice, and successful transfection was confirmed by extensive GFP expression. After 48 h, cell proliferation was assessed using EdU incorporation during S phase followed by flow cytometry. The Click-IT AlexFluor-647 kit (Life Technologies) was used as per the manufacturer’s instructions, as described (Wakeling et al. 2013). Briefly, 48 h post transfection, cells were treated with 20 µM EdU in culture medium for 1 h, washed in PBS, trypsinised to yield a single cell suspension and collected by centrifugation (1000 g× 1.5 min). Cells were fixed in 4% paraformaldehyde (15 min in the dark at room temperature), centrifuged and washed in PBS, then in Permwash buffer. Cells were then incubated in the EdU Click-IT reaction cocktail containing Alex Fluor 647 azide fluorescent dye for 30 min in the dark. Cells were centrifuged and resuspended in Permwash buffer, washed and then in resuspended in buffer containing propidium iodide (50 µg/µL). Samples were then analysed by flow cytometry on a BD Canto II analyser (BD Biosciences). In addition to GFP only transfected cells, controls included DF1 cells not exposed to EdU. Cells were gated for GFP expression and Alex Fluor 647 fluorescence. Approximately 60% of cells were GFP+ and this subset of cells was assessed for those in S phase of cell proliferation via the EdU incorporation. For all samples, at least 15,000 events (cells) were analysed. The percentage of GFP+ cells in S phase was compared for cells expressing GFP (control) and those expressing GPR56. Data were analysed using unpaired t tests.

**Effect of GPR56 on cell migration using the scratch assay and live cell imaging**

DF1 cells were plated onto six-well plates at a density of 2 × 10^4 cells/well in DF1 medium. After 24 h, cells were transfected with TOL2(CAGS→GFP-T2A-GPR56) or TOL2(CAGS→GFP-T2A-FLAG) control as described earlier. After 48 h, the scratch assay was performed on confluent cells to assess cell migration. For each well, a gap was made across the cell monolayer using a fixed head rubber policeman. Wells were then washed with PBS to remove dislodged cells and fresh warmed medium was added. Plates were then placed into a 37°C/5% CO₂ chamber mounted onto a Leica DMi8 microscope with 10× phase objective with appropriate annulus aperture engaged. Cell migration across the cell-free area was tracked over 24 h with images captured every 30 min. This assay was conducted in triplicate, where each well was independently transfected and five different areas of each well were analysed for cell migration (A total of 15 replicates for each condition). The migration of the cells in each field of view were analysed for the percentage of cell-free area compared to the whole image over time using FIJI (ImageJ 1.52n) software. Specifically, the data were processed in FIJI using the variance filter (set to 8), then Gaussian blur filter (set to 20) then the default threshold was applied unless a manual threshold was required. The analyse particles command was applied to the highlighted threshold area to generate a.csv file with the percentage of cell-free area over time. The data were collected in Excel to normalise all the time point to the 0 h images, which was set to 100%. The normalised results were imported into GraphPad Prism 7 to generate regression plots and perform statistical analysis.

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Results

GPR56 mRNA expression in embryonic chicken Müllerian duct development

Whole mount in situ hybridisation and qRT-PCR analysis showed that G-protein-coupled receptor, GPR56 was expressed developing chicken Müllerian ducts (Fig. 1). Expression was assessed at E4.5, E5.5, E6.5 and E8.5 (HH stages 25–34; n=3 for each sex, at each stage). These time points span the period of duct specification through elongation in chicken. In both sexes, GPR56 was specifically expressed in the Müllerian ducts of the developing urogenital system (Fig. 1A, B, C, D, E, F, G, H, I and J). At the earliest stage examined, E4.5 (HH25), GPR56 mRNA was localised at the cranial pole of early-forming duct, corresponding to the invagination and early elongation phases of development (Fig. 1A, B, C and D). Expression was maintained throughout the duct during caudal elongation at E5.5 (HH28) and E6.5 (HH30) (Fig. 1E, F, G and H). At E8.5 (HH34), GPR56 was strongly expressed in the developing female ducts and in the regressing male ducts. Expression was also detected in metanephric tubules (Fig. 1I and J). Quantitative RT-PCR analysis confirmed expression over E5.5–6.5 (stages 28–30). Sectioning of whole mount tissues showed that GPR56 mRNA was localized in the coelomic epithelium during duct invagination (Fig. 2). Subsequently, during duct elongation, GPR56 mRNA was specifically localized to the Müllerian duct epithelium (MDE). (Fig. 2C, D, E, F, G and H). GPR56 mRNA was also weakly expressed in the Wolffian duct (Fig. 2C and D).

Complementary expression of COL3A1 and GPR56 in the Müllerian duct

A number of potential ligands have been proposed or demonstrated for GPR56, including collagen 3, extracellular matrix-associated transglutaminase 2 (TG2), heparin, and the tetraspanin, CD81 (Xu et al. 2006, Luo et al. 2011, Huang & Lin 2018). We checked expression of these potential ligands in a Müllerian duct RNA-seq study and found only Collagen 3 to be highly expressed. Expression of the gene encoding collagen type 3 (COL3A1) was therefore examined by whole mount in situ hybridisation. COL3A1 transcripts were detected throughout the urogenital system during development (Fig. 3A). Sectioned whole mounts showed that COL3A1 was located in Müllerian coelomic epithelium (MCE) and Müllerian duct mesenchyme (MDM), but not in the Müllerian duct epithelium (MDE) (Fig. 3B). COL3A1 expression in the MDM was therefore complementary to that of GPR56 mRNA in MDE. RT-PCR confirmed expression of the COL3A1 ligand in E5.5–8.5 Müllerian ducts (Fig. 3C).

Knockdown of GPR56 expression in embryonic Müllerian ducts

RNA interference was used to assess the role of GPR56 in chicken Müllerian duct development. Several short
hairpin RNAs were designed against the GPR56 open reading frame and cloned into a TOL2 plasmid that uses the chicken U6-4 Pol III promoter to express the shRNA and BFP marker (Wise et al. 2007). These shRNA constructs were first tested in chicken DF1 cells for their ability to reduce expression of GPR56 when co-transfected with TOL2.CAGS→GFP-T2A-GPR56 plasmid. The T2A viral motif results in two independent proteins (GFP and GPR56 with no covalent bond) from a single mRNA transcript, allowing knockdown effectiveness to be assessed by reduction in GFP fluorescence levels (Fig. 4). DF1 cells transfected with TOL2.cU6.4→GPR56sh813-CAGS→BFP construct producing the most robust knockdown. This shRNA was therefore chosen for subsequent in ovo experiments using transposase-mediated genomic integration in embryos. The BFP served as an electroporation marker.

The developing chicken Müllerian duct was targeted by in ovo electroporation. By this method, integrating plasmids such as TOL2 can be used to deliver exogenous genes (for over-expression) or shRNAs (for knockdown) (Sato et al. 2007). We first tested the efficacy of Müllerian duct electroporation in ovo, using TOL2(CAGS→GFP) co-electroporated with transposase plasmid. DNA was injected into the left coelom and then electroporated into the intermediate mesoderm of E2.5 embryos, in the region of the presumptive Müllerian duct. By this method, robust GFP expression could be detected in the left duct when examined 4 days after electroporation (E6.5; HH30) (Fig. 5A). Sectioning followed by GFP immunofluorescence showed that the different compartments of the developing duct were targeted with different frequencies (Fig. 5B). In all cases the Müllerian coelomic epithelium could be targeted, but the underlying MDE and MDM were variably positive for GFP. Three broad categories were recognised, based on 12 samples examined. Type I: the MCE+MDM only were
targeted (67% of cases); Type II: primarily MCE + MDE (25% of cases) and type III: all three compartments targeted (8% of cases). As electroporation only targets the MCE (which is epithelial), and the MDM and MDE are derived from the MCE, it was not surprising that all compartments could be targeted. However, GPR56 was expressed exclusively in the MDE, which was only targeted by electroporation in approximately 33% of cases. A large number of embryos were therefore electroporated (100) to ensure targeting of the MDE in a sufficient number of samples. TOL2 in ovo electroporation was firstly used to assess the effects of over-expressing GPR56 in the left Müllerian duct. In situ hybridisation 4 days following electroporation confirmed over-expression, resulting in ectopic GPR56 expression in the left Müllerian duct mesenchyme. However, over-expression of the gene did not have any overt effect upon Müllerian duct structure or development to day 6.5, although this approach primarily lead to over-expression in the mesenchyme, not the epithelium where endogenous GPR56 was expressed (Supplementary Fig. 1A, see section on supplementary materials given at the end of this article).

Knockdown of GPR56 expression arrests Müllerian duct development

TOL2 vector expressing GPR56sh813 was electroporated into the left coelomic epithelium of E2.5 (HH14–17) and tracked by BFP reporter expression (Fig. 6A). Embryos were then examined at E6.5 (HH30). Electroporation efficiency and knockdown varied across embryos, and the BFP reporter varied in its intensity. This was partly due to the fact that the BFP protein was nuclear in its localisation and proved not as strong a marker as GFP. Nevertheless, unilateral knockdown of GPR56 was achieved, as assayed by whole mount in situ hybridisation (Fig. 6B). Knockdown resulted in variably arrested duct elongation. In most cases, normal duct development was noted at the anterior pole, with robust endogenous GPR56 expression and bilateral ducts comprising well-developed Müllerian duct epithelium and surrounding duct mesenchyme (Fig. 6Bii). This was correlated with a lack of BFP anteriorly. Expression of BFP reporter was more frequent posteriorly, indicating that this region of the duct was
more frequently targeted. Loss of GPR56 expression in the posterior region resulted in truncated duct elongation. From middle portion to the posterior end of left electroporated ducts, GPR56 expression was lost (Fig. 6Bii). In these regions completely lacking GPR56 expression, MDM and MDE were absent. However, on the right (non-electroporated) side, the duct developed normally, where endogenous GPR56 expression was not affected (Fig. 6Bii). The Wolffian ducts were not affected. Electroporation of non-silencing hairpin (firefly shRNA) had no effect on endogenous GPR56 expression or duct formation (Fig. 6C and D).

GPR56 knockdown abolishes Müllerian duct epithelium and duct mesenchyme

To further assess the effects of GPR56 knockdown on Müllerian duct development, marker gene expression was analysed. PAX2 is a marker of duct epithelium, (MDE), while DMRT1 is a marker of duct mesenchyme (Ayers et al. 2015a, Atsuta & Takahashi 2016). GPR56 knockdown resulted in failed caudal elongation by the MDE, as reflected by PAX2 staining, a marker of the MDE. In areas at or posterior to BFP reporter expression (and hence
GPR56 knockdown), the MDM, MDE and lumen were absent. In cases where delivery of the GPR56 hairpin was widespread (high BFP expression), PAX2 expression was absent along the entire length of the urogenital system, and the duct was absent at both anterior and posterior regions (Fig. 7A, B, C and D). PAX2 expression was present in the underlying Wolffian duct on both sides, showing that abnormalities in Müllerian duct formation were not a result of Wolffian duct defects. No effect on PAX2 expression was observed when the TOL2 non-silencing control construct was electroporated (Fig. 7E, F and G). These results suggest that GPR56 reduction inhibits MDE formation and MDE is essential for the caudal extension of the Müllerian duct. Consequently, loss of MDE formation due to GPR56 disruption arrests this process.

Müllerian duct mesenchyme was directly examined in samples showing unilateral loss of GPR56 expression following shRNA electroporation. In these experiments, GFP was used as the electroporation reporter rather than BFP. In urogenital systems electroporated on the left side, GPR56 expression was lost, coincident with the shRNA expression (GFP reporter) (Fig. 8A). In control tissues electroporated with non-silencing shRNA, normal bilateral GPR56 mRNA expression was observed, together with robust GFP reporter expression on the left duct (Fig. 8A). In sectioned whole mounts that were immunostained, there was a loss of DMRT1 mesenchymal marker expression on the left side (GPR56 knockdown) compared to the right (un-electroporated) (Fig. 8B). In control tissues (non-silencing shRNA), bilateral DMRT1 expression was observed. We also post-stained in situ tissues for pan cytokeratin, a marker of epithelia and cells undergoing epithelial-to-mesenchyme transition. The MDE was cytokeratin negative, supporting the notion that it is meso-epithelial in nature, rather than purely epithelial. Instead, cytokeratin demarcated the coelomic epithelium (MCE) on the left side of GPR56-knockdown tissues, with a lack of staining beneath. In contrast, the right side (un-electroporated) showed coelomic and underlying mesenchymal expression of cytokeratin (Fig. 8C). This result indicated that the coelomic epithelium was intact following GPR56 knockdown, but there was a failure of mesenchymal development. Control tissues showed cytokeratin protein expression in both left and right coelomic (surface) epithelium and underlying mesenchyme (Fig. 8C).

**GPR56 knockdown reduces cell proliferation in vivo**

To assess the effects of GPR56 on cell proliferation and cell death, immunostaining was carried out following electroporation of control and knockdown shRNAs. Urogenital tissues electroporated at E2.5 were processed for in situ hybridisation at E6.5 (stage 30). Six knockdown specimens were chosen that had truncated ducts and loss of GPR56 mRNA from the mid to posterior regions. Anteriorly, left electroporated ducts were still intact, but posteriorly, ducts were absent after mRNA knockdown These tissues were compared to six control tissues electroporated with non-silencing shRNA, normal bilateral DMRT1 expression (electroporated) side and normal duct on the right (arrows). Higher magnification shows loss of PAX2+ Müllerian duct (the Wolffian duct is intact). (D) Posterior (caudal) region, showing loss of the Müllerian duct on the left side (arrows). Higher magnification shows intact PAX2+ Wolffian duct on the left but no PAX2+ Müllerian duct. The right duct is intact, with PAX2 expression in the duct (arrow). (E) BFP expression in the left duct following electroporation of non-silencing control plasmid. (F) Normal expression of PAX2 in left and right Müllerian ducts of the same urogenital system. (G) Sections show normal duct development (arrows) and higher magnification images show normal PAX2 expression in both left and right ducts (arrows).
**GPR56 expression and sectioning.** The number of mitotic cells was similar between left (electroporated) and right (non-electroporated) control ducts and in the anterior and mid regions of GPR56-knockdown left and right ducts (Fig. 9A, B and C). In more posterior sections, we noted fewer mitotic cells in both control and GPR56-knockdown ducts. However, there were markedly fewer PH3+ cells in the posterior region of GPR56-knockdown samples (where the duct failed to form) compared to controls. This was quantified through cell counts (Fig. 9E). PH3 staining did appear reduced in the mid region, where the left duct was arresting in knockdown specimens (Fig. 9C), but this was not evident in the cell counts (Fig. 9D, mid region).

**GPR56 stimulates cell proliferation and cell migration in vitro**

To assess the potential function of GPR56, the gene was over-expressed in DF1 cells, a chicken fibroblastic cell line. Cells were transfected for 48 h with TOL2.GFP. T2A.GPR56+ transposase to facilitate stable integration. Quantitative RT-PCR confirmed robust over-expression of GPR56 relative to TOL2.GFP-T2A-FLAG transfected control cells. (Fig. 10A). We also confirmed expression of the ligand, COL3A1, in these DF1 cells (Fig. 10B).

After 48 h in culture, GPR56 significantly enhanced cell proliferation, as assessed by Edu incorporation and flow cytometry. In GFP+ control cells, 35% were S phase of cell proliferation. In comparison, an average of 47% of GPR56-expressing GFP+ cells were in S phase (unpaired t test; P<0.001) (Fig. 10C). To examine the effects of GPR56 on cell migration, naïve chicken DF1 cells were transfected for 48 h with GFP-GPR56 versus GFP-FLAG control. GPR56 significantly enhanced cell migration, as determined by the scratch assay. As shown in Fig. 11A, more cells migrated across the scratched area in the presence of GPR56 vs GFP control after 8, 16 and 24 h of live imaging. After 24 h, the GPR56 transfected cells had almost completely covered the scratch, while the GFP-transfected cells had not (Fig. 11A). This is also shown in Supplementary Video 1, where live cell imaging shows active cell migration rather than simply cell proliferation alone. These data were quantified and plotted, using Graphpad Prism. Cells expressing GPR56 migrated more rapidly over time (Fig. 11B). Linear regression analysis over the first 12 h revealed a significantly more rapid migration in the GPR56-transfected cells compared to GFP controls from 4 h (P<0.001) (Fig. 11C).
This study identifies *GPR56* as a novel gene involved in chicken Müllerian duct development. *GPR56* mRNA is expressed in the Müllerian duct epithelium during the elongation phase and knockdown causes loss of *GPR56* expression and variably truncated ducts. Ducts show arrested caudal migration correlated with the degree of knockdown. The expression of duct epithelial and mesenchymal marker, PAX2 and DMRT1, is lost in areas lacking *GPR56* expression and where ducts are absent. Loss of marker expression may be due to a direct effect of *GPR56* on gene expression, but more is likely due to failure of the cells to proliferate or migrate caudally.

Previous lineage tracing experiments in chicken embryos have shown that the lateral coelomic epithelium overlying the intermediate mesoderm gives rise to the Müllerian duct (Guioli et al. 2007, Atsuta & Takahashi 2016). There is initially a localised thickening of coelomic epithelium to form a placode at the cranial pole of the urogenital system at E3–E4.0 (HH19–23).
variable, and this resulted in varying degrees of targeting (Fig. 5). In some cases, extensive DNA delivery was achieved and in other cases, more restricted delivery. Some specimens showed BFP reporter expression along the entire Müllerian duct, but there was no knockdown of GPR56 expression. 32% of electroporated embryos showed loss of GPR56 mRNA expression and this resulted in truncated ducts. This low percentage is most likely due to the fact that in most cases (67%) the MDM and not the MDE was targeted, and GPR56 is expressed in the MDE. Nevertheless, effectively targeted embryos showed unilaterally arrested ducts following electroporation of the specific GPR56 shRNA. Ducts were truncated at various points along the cranio-caudal axis, reflecting varying degrees of targeting (BFP expression) and GPR56 knockdown. In all of these cases, the ducts were arrested in their caudal migration, not at the early specification stage. We first detected GPR56 expression at the onset of caudal duct extension at E4.5 (Fig. 1). GPR56 is likely to be required for proper migration of the MDE during the duct elongation phase. Duct mesenchyme was absent in those ducts lacking GPR56 expression and lacking the inner ducetal epithelium. This was evidenced by a lack of mesenchyme in over-stained tissue sections, and in the loss of the mesenchymal marker, DMRT1 (Fig. 8B). The fact that the MDM was also absent in such arrested ducts following GPR56 knockdown, despite an intact coelomic epithelium (Fig. 8C), indicates that the migrating MDE is required for MDM specification. It is thought that the MDM derives from the adjacent coelomic epithelium via an EMT, not from the MDE (Ayers et al. 2015a, Roly et al. 2018). The current study suggests that the migrating MDE is important for proper development of the mesenchymal compartment, either directly by providing a source of MDM cells via an EMT, or via interactive signal with the MCE to generate the MDM.

Mechanistically, GPR56 is likely to be stimulating both cell proliferation and cell migration in the Müllerian duct. These are both essential processes for proper duct formation. Knockdown of GPR56 expression in the developing Müllerian duct was associated with fewer PH3+ (mitotic) cells (Fig. 9), while we saw no apoptosis, as assessed by Cleaved caspase staining. Conversely, over-expression of GPR56 in chicken embryonic fibroblasts enhanced both cell proliferation and migration capacity (Figs 10 and 11). These data suggest that GPR56 in the chicken embryo functions to regulate duct development during the elongation phase. Cell proliferation is an important aspect of duct formation,

Figure 11
Cell migration is enhanced in DF1 cells expressing the GPR56 compared to cells expressing GFP control. 2 × 10^4 cells/ well were transfected with TOL2. GFP.T2A.GPR56 or TOL2.GFP.T2A.FLAG. (A) Representative phase-contrast images showing more rapid closure of the wound by cells expressing GPR56 vs GFP control over a 24-h period. (B and C) Wound closure expressed as the cell-free area normalised to time point 0 h (set to 100%). Data were collected over three independent wells (n = 15) and plotted using (B) a one-phase decay curve over 24 h (4-h intervals), half-life (T1/2) is shown, and (C) a straight line with nonlinear regression curve for the first 12 h at 2-h intervals, slope of line is indicated. Fits for B and C are significantly different (P < 0.0001). Multiple t tests of the time points were conducted (* indicates P < 0.00001) with significant differences (P < 0.0001) for all time points from 4 h onward. Green = GFP control; black = GPR56.

(Jacob et al. 1999). This is followed by invagination of these cells to form the MDE at E4.5 (HH24–25) (Atsuta & Takahashi 2016). The MDE then extends caudally through mesenchyme (the MDM), which is thought to derive from the coelomic epithelium via an epithelial-to-mesenchyme transition (EMT). Caudal extension occurs during E5.5–E6.5 (HH26–30) (Ayers et al. 2015a, Roly et al. 2018). In the experiments reported here, we electroporated integrating TOL2 plasmids at E2.5 (HH14-17), prior to the specification and invagination stages of duct development. Our preliminary studies using TOL2(CAGS→GFP) showed that all three compartments of the ducts could be targeted when electroporation was carried out prior to duct formation (Fig. 5). As electroporation can only target epithelia, not mesenchyme, our GFP data confirm that the MDE (and MDM) must ultimately derive from the coelomic epithelium, as proposed previously (Guioli et al. 2007). However, the electroporation method is innately

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as the MDE must actively proliferate. This is likely mediated by the ligand, Collagen III, which is abundantly expressed in the chicken Müllerian duct mesenchyme and is also expressed in chicken fibroblasts in vitro (Fig. 4). It would be of interest to test the effects of GPR56 knockdown on cell proliferation in primary cultures of Müllerian duct cells. We have attempted to establish primary culture of chicken embryonic Müllerian duct cells, dissociated with collagenase or trypsin. At present, we can culture and transfec mesenchyme derived from embryonic chicken Müllerian ducts, but PAX2+ epithelial cells (MDE cells) are challenging to culture and transfec (Z Y Roly et al., unpublished observations). As the MDE is the site of GPR56 expression, this assay cannot as yet be successfully conducted. In addition to stimulating cell proliferation, chicken GPR56 is likely to also have a role in MDE cell migration. Fibroblasts that express Collagen 3 and transfected with GPR56 showed enhanced cell migration relative to control cells (Fig. 10). It has been shown in rodent models that caudal extension of the Müllerian duct involves both active MDE cell proliferation and migration, influenced by endogenous factors such as PI3K/AKT signalling and diffusible factors such as WNT9B from the adjacent Wolfian duct (Carroll et al. 2005, Fujino et al. 2009). In other contexts, GPR56 can stimulate cell proliferation and migration via promoting PI3K/AKT signal transduction and epithelial to mesenchyme transition (Ji et al. 2018). A role in cell proliferation has also been reported for GPR56 in zebrafish and mouse neuronal models (Ackerman et al. 2015). Our data point to a novel role for GPR56 in developing Müllerian ducts via a similar mechanism. As contact with its ligand can activate intracellular Rho-mediated pathways, it would be of interest to examine the downstream effectors of GPR56 signalling in the Müllerian duct. Although we consider that GPR56 has a key role in duct elongation in the chicken, it may also play an earlier role, in duct specification or invagination. In some cases where the entire left side of the urogenital system could be targeted by electroporation, a complete absence of duct was noted (example, Fig. 7, showing loss of PAX2 marker expression). This may reflect either a failure of very early duct elongation, or failure of the preceding duct specification or invagination stages.

In the Müllerian duct, GPR56 glycoprotein localised on the surface of Müllerian epithelial cells may facilitate interaction with Collagen III in the extracellular matrix domain of the MDM. This interaction may be required for caudal extension of the duct via cell proliferation and migration. We currently do not have a suitable antibody that detects chicken GPR56 to test this interaction, but an epitope-tagged protein could be used to verify the location of the protein in ovo, using targeted delivery to the Müllerian duct. In the CNS, GPR56 is expressed in neural progenitor cells and can interact with Collagen III (Luo et al. 2011). GPR56 is implicated diverse neurological functions, such as oligodendrocyte formation, synapse development and myelination (Li et al. 2008, Ackerman et al. 2015). In humans, a loss-of-function mutation in GPR56 causes bilateral frontoparietal polymicrogyria (BFPP), characterised by disorganised cerebral cortical patterning (Piao & Walsh 2004, Piao et al. 2004, Li et al. 2008, Koira et al. 2009, Parrini et al. 2009). At least some of these roles involved interaction with collagen III in the extra cellular matrix. It would be of interest to examine the effects of deleting or knocking down Collagen III expression the chicken Müllerian duct mesenchyme. This might be predicted to phenocopy the effect of GPR56 knockdown, with arrested caudal extension of the Müllerian duct. However, extracellular ligands other than collagen III have also been reported for GPR56. These include transglutaminase 2 (TG2), heparin, progastrin and the tetraspanins CD9 and CD81 (Little et al. 2004, Xu et al. 2006, Huang & Lin 2018). These other putative ligands for GPR56 may be expressed in the chicken Mullerian duct. Given its robust expression, Collagen III is the most likely ligand in the chick Müllerian duct. The other aspect of GPR56 function is signalling. The signalling mechanism of GPR56 is not well understood, although it involves G protein-mediated Rho-dependent transcription. In neural progenitors, GPR56 inhibits rather than facilitates cell migration via Rho GTPase modulation of actin fibres (Shashidhar et al. 2005, Iguchi et al. 2008). The exact role of GPR56 on actin cytoskeletal remodelling in MDE cells requires further investigation.

GPR56 is considered to have both cell adhesive and intracellular signalling functions (Hamann et al. 2015). As a cell membrane localised glycoprotein, GPR56 comprises a large extracellular domain, interacting with extracellular matrix factors, and a seven-pass transmembrane component that can activate cytosolic G proteins (Salzman et al. 2016). In other cellular contexts, GPR56 has been implicated in cell adhesion, tumorigenesis and cell migration (reviewed in Jin et al. 2009). Upregulation of GPR56 has been reported in various cancers, including melanomas, ovarian, pancreatic and lung carcinomas, and in gliomas (Zendman et al. 1999, Shashidhar et al. 2005, Huang et al. 2008, Xu et al. 2010), while experimental inhibition of GPR56 expression is correlated with changes in cell adhesion and migration.
In the Müllerian duct, such interactions would involve the MDE and extracellular matrix in the MDM, as might be expected for an extending epithelial tube, or intracellular signalling, or both. GPR56 may also be necessary for the survival of MDE cells or MDM cells, or both.

While most previous studies have explored the role of GPR56 in neuronal or cancer cells, one study has demonstrated a role in the urogenital system. Chen and colleagues (Chen et al. 2010) reported that Gpr56 is required for proper testis development and fertility in mice. At embryonic stages, Gpr56 is expressed in Sertoli and germ cells, and possibly peritubular myoid cells. In Gpr56−/− mice, backcrossed onto a C57BL/6 background over several generations, seminiferous tubules were partly defective, with fewer SOX9+ Sertoli cells and a loss of germ cells. Interestingly, the testicular defect is asymmetric, with impaired testis cords on that side of the male gonad in direct contact with the mesonephric kidney. It was noted that the basement membrane of testis cords was defective in Gpr56-null mice (Chen et al. 2010). The authors hypothesised that Gpr56 is required for re-modelling of the testis cords that occurs during testicular development in mice (Combes et al. 2009).

Chen et al. noted that Gpr56 plays a role in planar cell polarity in various tissues and proposed that this may also apply to the testis cords. It is noteworthy that the testis tubule is similar in structure to the Müllerian duct, and so an effect of GPR56 on planar cell polarity might be a common mechanism of action in the two tissues. In the mouse studies, female fertility was not affected, and no expression was reported in murine Müllerian ducts. This suggests that Gpr56 alone is not required for proper duct development in mice. In contrast to the mouse studies, we have previously reported that GPR56 is expressed female-specifically, not male-specifically, in embryonic chicken gonads (Ayers et al. 2015b). Hence, the role of this gene in the urogenital system may be divergent between mouse and chicken. Expression of GPR56 in embryonic human Müllerian ducts has not been reported, but its function, if any, could also be divergent.

In summary, this study identifies a novel factor required for Müllerian duct formation in the avian model. This is the first report of a role for the adhesion G-protein coupled receptor, GPR56, in embryonic Müllerian duct development. Knockdown in vivo reduces cell proliferation. Conversely, GPR56 can stimulate both cell proliferation and cell migration in vitro, pointing to its likely mechanism of action in vivo. Future research should now focus on the downstream targets of GPR56 in the Müllerian duct epithelium. We found that PAX2 expression was lost in GPR56-knockdown Müllerian ducts. PAX2 could be a target of GPR56-mediated signalling, or the loss of PAX2 may reflect the loss of the MDE. Other experiments could address the regulation of GPR56 in the Müllerian duct. Lastly, GPR56, its ligands or its targets could be novel factors in the etiology of human disorders of Müllerian duct development.

**Supplementary materials**
This is linked to the online version of the paper at [https://doi.org/10.1530/JOE-19-0419](https://doi.org/10.1530/JOE-19-0419).

**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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