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Research Article

Adhesion G protein-coupled receptor G2 is dispensable for lumicrine signaling regulating epididymal initial segment differentiation and gene expression†

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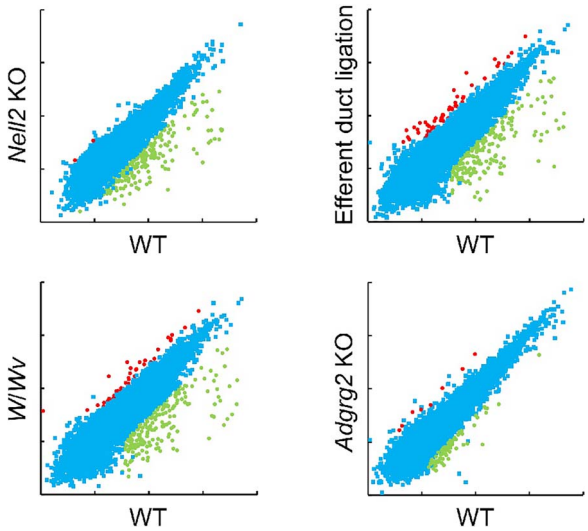
Abstract

The mammalian epididymis is the organ for functional sperm maturation. In rodents, the initial segment, the most proximal region of the epididymis, plays a critical role in sperm maturation. The luminal epithelial differentiation and the following gene expression of the initial segment are regulated by the lumicrine signaling, a testis-epididymis transluminal secreted signaling. Adhesion G protein-coupled receptor G2 (ADGRG2) is expressed in the efferent duct and the initial segment epididymis. In the preceding study, *Adgrg2* ablation decreased the expression of several genes expressed in the initial segment. Such downregulated genes include those known to be regulated by lumicrine signaling, suggesting the involvement of ADGRG2 in lumicrine signaling. The present study examined whether ADGRG2 is associated with the lumicrine signaling regulating epididymal initial segment differentiation and gene expression. *Adgrg2*-null mice were generated by CRISPR/CAS9-mediated genome editing. The postnatal differentiation of the *Adgrg2*-null male epididymal initial segment was histologically comparable with that of control wild-type animals. The RNA-seq of *Adgrg2*-null mice was performed together with those of efferent duct-ligated and *W*/*Wv* mice in both of which lumicrine signaling is defective. The comparative transcriptome analyses clarified that the expressions of genes expressed in the initial segment and regulated by lumicrine signaling were decreased by *Adgrg2* nullification. However, the extent of such downregulations observed in *Adgrg2*-null epididymis was not so prominent compared with those of lumicrine signaling deficient *Nell2*$^{-/-}$, efferent duct-ligated, or *W*/*Wv* mice. Collectively, these findings indicate that ADGRG2 is dispensable for the lumicrine regulation of epididymal initial segment differentiation.

Summary Sentence

The association of ADGRG2 with the lumicrine-mediated epididymal initial segment differentiation and gene expression was examined by generating *Adgrg2*-null mice and concluded to be dispensable.

Graphical Abstract



Keywords: lumicrine, epididymis, initial segment, G protein-coupled receptor, sperm maturation

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Introduction

The epididymis, a highly coiled epithelial duct, constitutes a sperm transport route; testicular spermatozoa enter into the epididymis from the efferent duct and go out through the vas deferens. The spermatozoa become functionally mature and fully fertile in the epididymis [1, 2]. In rodents, the initial segment (IS) is the most proximal region of the epididymis which is characterized by the pseudostratified tall epithelial cells. The IS plays a critical role in sperm maturation by expressing specific proteins; disrupting the expression of such proteins causes male infertility [3, 4].

One of such regulatory mechanisms recently characterized is lumicrine signaling, in which testis secreted factors released into the lumen of seminiferous tubules go through luminal space via an efferent duct and act on the IS epithelium to influence its development and function [5–7]. There are several molecules identified to be included in lumicrine signaling. Neural epidermal growth factor–like like 2 (NELL2) and NELL2-interacting cofactor for lumicrine signaling (NICOL) are the molecular entities of testis-derived lumicrine factors [4, 8]. NELL2 and NICOL constitute together a molecular complex and activate cell surface receptor tyrosine kinase ROS1 which is expressed in the IS of epididymis. Mice lacking *NEL-like 2* (*Nell2*), *predicted gene 1673* (*Gm1673* or *Nicol*), or *Ros1 proto-oncogene* (*Ros1*) exhibit IS differentiation failures and are completely male infertile because the epididymal spermatozoa remain immature and the ejaculated spermatozoa are unable to migrate from the uterus into the oviduct in the female reproductive tract [4, 8, 9]. Upon these findings, the increasing significance is to ask how the lumicrine regulation of IS differentiation is characterized at the molecular level and whether there are more factors associated with lumicrine signaling.

Adhesion G protein-coupled receptor G2 (ADGRG2) is expressed in the male reproductive tract including efferent duct and caput epididymis [10]. Structurally, ADGRG2 belongs to the adhesion G protein-coupled receptor (GPCR) family [11]. The extracellular region of adhesion GPCRs is exceptionally large and contains the membrane-proximal GPCR autoproteolysis-inducing domain. Dysfunction of ADGRG2 results in male infertility. In humans, a hemizygous loss-of-function mutation in ADGRG2 causes congenital bilateral absence of the vas deferens [12]. In mice, targeted inactivation of *adhesion G protein-coupled receptor G2* (*Adgrg2*) caused a dysregulated fluid reabsorption within the efferent ducts leading to a backup of fluid accumulation in the testis and a subsequent stasis of spermatozoa within the efferent ducts [13].

It would be beneficial to understand the function of ADGRG2 to examine how ADGRG2 transmits ligand-induced signals. ADGRG2 protein is abundantly localized on the apical surface of the ducts [10], implying that ADGRG2 binds its ligands coming from the male reproductive tract luminal space as in lumicrine signaling. The association of ADGRG2 with lumicrine signaling is also implied from its regulation of gene expression in the epididymis; upon *Adgrg2* ablation, the expressions of many genes are downregulated in the caput epididymis [14] and several of such genes are known to be regulated by lumicrine signaling [4]. These findings raise a possibility that ADGRG2 is included in lumicrine signaling and lumicrine-mediated gene expression, although it has never been fully explored yet. In the present study, the association of ADGRG2 in lumicrine signaling was investigated based on the histological and transcriptomic studies of *Adgrg2*-null mice generated by CRISPR/CAS9-mediated genome editing.

Materials and methods

Animals

B6D2F1 mice and *W/Wv* mice were purchased from Japan SLC, Inc. *Nell2* (or *Ros1*) knockout (KO) mice were obtained previously [4]. For efferent duct ligation, the efferent ducts of 10-week-old B6D2F1 males were unilaterally ligated and the ipsilateral epididymis was excised 4 weeks after ligation. *Adgrg2* KO mice (B6D2-Adgrg2<em1Osb>) were generated on a B6D2F1 background using CRISPR/CAS9-mediated genome editing. Briefly, crispr (cr) RNA#1 and crRNA#2 (Sigma, custom synthesis), SygRNA SpCas9 tracrRNA (Sigma, #TRACRRNA05N-5NMOL), and TrueCut Cas9 Protein v2 (ThermoFisher, #A36496) were injected into fertilized eggs obtained by mating C57BL/6J females with DBA males. The treated eggs were implanted into pseudopregnant ICR females to obtain F0 individuals. The wild-type B6D2F1 females were mated with the F0 males to obtain F1 *Adgrg2*$^{+/-}$ females. The resulting F1 *Adgrg2*$^{+/-}$ females were mated with B6D2F1 wild-type males to obtain *Adgrg2*$^{-}$/Y KO F2 males and *Adgrg2*$^{+}$/Y wild-type control littermate males. This F2 generation individuals were used for the experiment. The crRNA sequences and genotyping primer sequences and the number of target sites of 12mer + PAM and 8mer + PAM are summarized in Supplementary Table S1. The number of 20mer + PAM target sites for both crRNAs in mouse genome is 1; there are no potential off-target sites. The potential 12mer + PAM off-target sites for each crRNA are summarized in Supplementary Table S2. The genotyping primer sequences are listed in Supplementary Table S3. The mouse line generated in this study will be deposited as frozen sperm at the RIKEN BioResource Research Center (BRC) and Center for Animal Resources and Development (CARD) at Kumatomo University. The BRC and CARD repository IDs for B6D2-Adgrg2<em1Osb> will be assigned soon and then the mouse line will be made available to all researchers. All experiments involving animals were approved by the Institutional Animal Care and Use Committees of Osaka University (Osaka, Japan) and were conducted in compliance with the university guidelines and regulations for animal experimentation.

Epididymal anatomy

In this study, the epididymal anatomy followed Johnston et al. [15] in which the IS is included in the caput epididymis. Because of the difficulty in dissecting IS separately from caput epididymis especially in mice in which IS differentiation is ablated, the IS was dissected together with the caput and such a tissue dissection was indicated by the description "IS-caput."

Antibodies

The following commercially available antibodies were used: rabbit monoclonal anti-ERK1/2 (#4695) and anti-phospho-ERK1/2 (#4370) (Cell Signaling Technology), rabbit polyclonal anti-ADGRG2 (#HPA050029, Atlas Antibodies), rabbit polyclonal anti-ADAM28 (#22234-1-AP, Proteintech), mouse monoclonal anti-ACTIN (#sc-53014, Santa Cruz), mouse monoclonal anti-GAPDH (#sc-32233, Santa Cruz), mouse monoclonal anti-ADAM3 (#sc-365288, Santa Cruz), mouse monoclonal anti-ADAM2 (#MAB19292, Merck

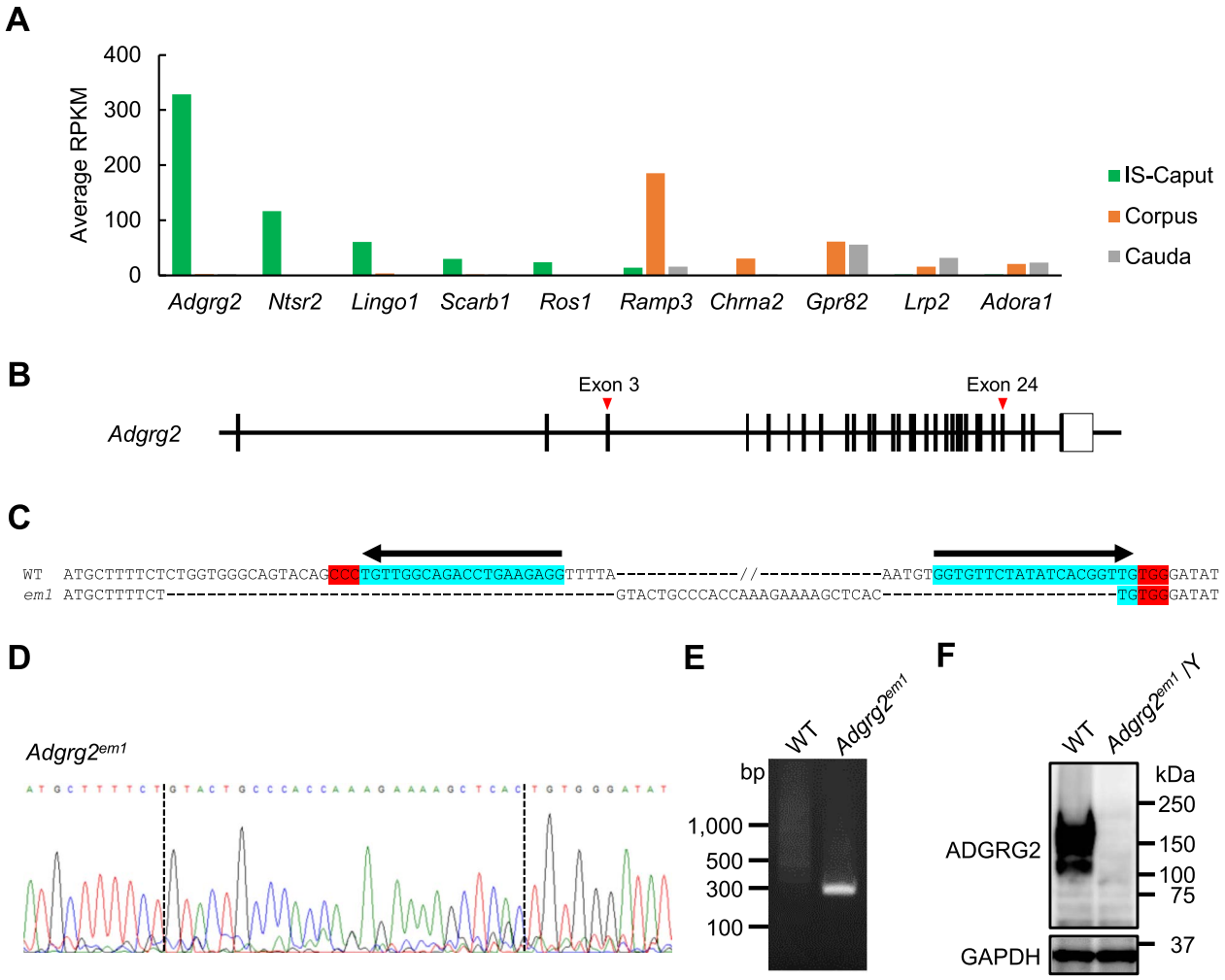


Figure 1. Generation of *Adgrg2*-null mice. (A) The expression of genes encoding cell surface receptors expressed in IS-caput, corpus, and cauda epididymis. The expression levels are represented in RPKM. (B) A schematic representation of mouse *Adgrg2* gene. (C) The DNA sequence of *Adgrg2* WT and *em1* alleles. Locations of guide RNA target sites PAM sequences are also shown. (D) The electropherogram of *Adgrg2*^{em1} allele DNA sequencing. (E) Genomic PCR to detect *Adgrg2*^{em1} allele. (F) Immunoblot analysis of ADGRG2 protein expression in the IS epididymis of WT and *Adgrg2*^{em1}/Y mice. Immunoblot detection of GAPDH is also shown as an internal control.

Millipore), peroxidase-conjugated goat polyclonal anti-rabbit IgG (#111-036-045, Jackson Immunoresearch), and goat polyclonal anti-mouse IgG (#115-036-062, Jackson Immunoresearch). Rabbit polyclonal antibody against OVCH2 was obtained as described previously [4]. The dilution conditions of antibodies are summarized in Supplementary Table S4.

Transcript analyses

The epididymis was dissected into three parts, i.e., IS-caput, corpus, and cauda. Total RNA was isolated from mouse tissues using an RNeasy mini kit (Qiagen, #74104). The ages of mice at tissue sampling were as follows: 8-week-olds for *Adgrg2*$^{-}$/Y and its control WT, and 14-week-olds for *W*/*Wv*, efferent duct-ligated WT, and their control non-treated WT. RNA-seq of IS-caput epididymal transcripts was performed as follows: libraries for sequencing were prepared from isolated RNAs using a TruSeq stranded mRNA sample prep kit (Illumina, #20020594) and sequenced on a NovaSeq6000 (Illumina) using 101 bp single-ended mode. The obtained sequence reads were mapped onto a mouse reference genome (mm10) using TopHat ver. 2.1.1 [16]. Fragments per kilobase of exon per million mapped reads (FPKM) values were calculated for each gene using Cufflinks ver. 2.2.1 [17]. The obtained RNA-seq data have been deposited in the Gene Expression Omnibus (GEO) database under the accession code GSE232896 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE232896) for *Adgrg2*$^{-}$/Y mice and GSE232898 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE232898) for EDL and *W*/*Wv* mice. The RNA-seq data of *Nell2* KO IS-caput epididymis used for the comparative transcriptome study was obtained from NCBI GEO: GSE133920 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE133920). The RNA-seq data of WT IS-caput, corpus, and cauda epididymis used for the comparative transcriptome study was also obtained from NCBI GEO: GSE138517 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE138517).

Protein analyses

The 8-week-old tissues were homogenized in lysis buffer (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Triton X-100) containing protease inhibitor cocktail (Nacalai Tesque, Japan, #25955-24) and phosphatase inhibitor cocktail (Nacalai Tesque, #07575-51) and centrifuged at 12,000×*g* at 4°C

for 15 min. The resulting supernatants were then recovered as crude tissue protein extracts. The extracted proteins were separated by SDS-PAGE under reducing conditions using e-PAGEL precast gel (Atto, Japan, #E-T/R/D520L). As a molecular weight standard, Precision Plus Protein Dual Color Standards (Bio-Rad, #1610374) was used. The separated proteins were electrotransferred onto Immobilon-P polyvinylidene difluoride membranes (Merck, #IPVH00010) and the membranes were blocked with 3% bovine serum albumin (BSA)/TBST. The membranes were incubated with primary antibodies overnight. The bound antibodies were detected by following incubation with peroxidase-conjugated secondary antibodies and chemiluminescent reaction using Chemi-Lumi One Super (Nacalai Tesque, #02230). The chemiluminescent signals were detected using Amersham ImageQuant 800 (Cytiva).

Histology

The epididymides fixed with 4% formaldehyde/PBS overnight were immersed in paraffin and sectioned at 5 μm using a microtome. The sections were stained with hematoxylin and eosin (HE) and photographed using a system microscope (BX53; Olympus).

Statistical analysis

All experiments were repeated biologically at least three times and similar results were obtained. Statistical analyses were performed using Student's *t*-tests with Microsoft Excel 2019 (Microsoft).

Results

Targeted deletion of *Adgrg2* gene in mice

The epididymal transcriptome confirmed that *Adgrg2* is most abundantly and exclusively expressed in the caput epididymis including the IS among genes encoding cell surface receptors including *Ros1* (Figure 1A). *Adgrg2* gene was targeted by CRISPR/CAS9-mediated genome editing; two crRNAs targeting around exons 3 and 24 of the *Adgrg2* gene on chromosome X were introduced together with tracrRNA and CAS9 protein into fertilized eggs by electroporation to expect the deletion of the sandwiched region (Figure 1B). Finally, a mutant allele *Adgrg2*^{em1} consisting of 50 518 bp deletion and 26 bp insertion was obtained (Figure 1C–E). After confirmation of inheritance of the mutant allele, *Adgrg2* ablation was also examined at the protein level. When the IS-caput protein lysates were analyzed by immunoblotting, anti-ADGRG2 immunoreactivity was detected at around 150 kDa in WT protein extract but it completely disappeared in *Adgrg2*^{em1}/Y one (Figure 1F). There results indicate that the generated *Adgrg2*^{em1} allele is an *Adgrg2*-null allele and hereafter described as *Adgrg2*$^{-}$.

Histological and biochemical characterization of *Adgrg2* knockout IS-caput epididymis

The postnatal development of *Adgrg2*$^{-}$/Y (*Adgrg2* KO) male IS was examined histologically. The postnatal differentiation of the epididymal initial segment starts at around postnatal day 19 under the influence of testicular lumicrine factor [18]. In the control WT animals, the thickened luminal epithelium characteristic of differentiated epididymal IS was not observed at 2 weeks old but was apparent at after 4 weeks old (Figure 2A–C) The differentiation of the IS epithelium was also observed in *Adgrg2* KO epididymis and there was no prominent histological abnormality observed (Figure 2D–F).

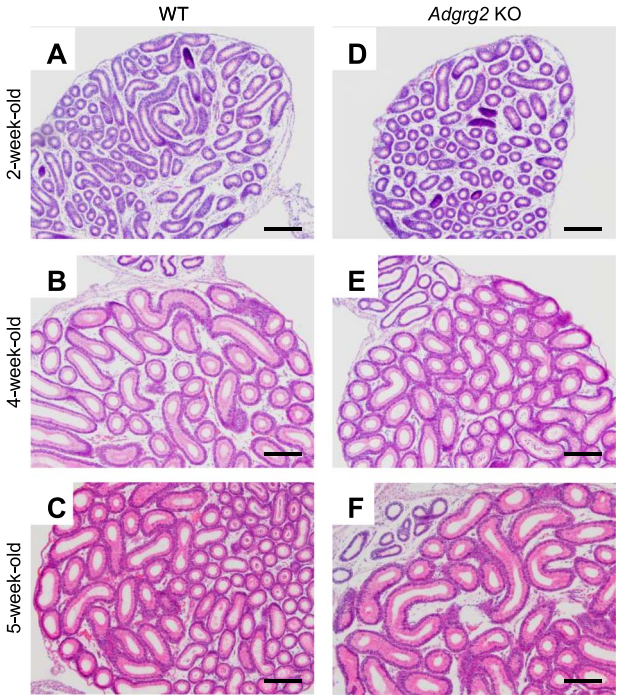


Figure 2. Histology of *Adgrg2* KO IS-caput epididymides. (A–F) HE-stained sections of WT (A–C) and *Adgrg2* KO (D–F) IS-caput epididymides at 2 weeks (A, D), 4 weeks (B, E), and 5 weeks (C, F) old. Bars, 200 μm.

Differentiation of the IS epithelium is regulated by NELL2/NICOL-ROS1-mediated lumicrine signaling [4, 8, 19]; lumicrine factors NELL2 and NICOL are secreted from testicular germ cells along spermatogenesis and act on epididymis to trigger IS differentiation. The 8-week-old epididymal IS of *Nell2*$^{-/-}$ (*Nell2* KO) males significantly degenerated when compared with that of WT (Figure 3A–D). The IS luminal epithelium of 8-week-old *Adgrg2* KO males was not critically affected (Figure 3E and F).

Along with the IS differentiation, the ROS1 kinase is activated and the phosphorylation level of extracellular signal-regulated kinase (ERK) increases [20]. The differentiating epididymal IS specifically expresses various proteins in a lumicrine-dependent manner [4, 21]. Therefore, the expression and phosphorylation levels of specific proteins are indicative of lumicrine-mediated IS differentiation. When examined by immunoblotting, the level of phosphorylated ERK was significantly decreased in the *Nell2* KO IS-caput epididymis but not critically affected in *Adgrg2* KO one (Figure 3G). The secreted proteins ovochymase 2 (OVCH2) and a disintegrin and metalloproteinase domain-containing protein 28 (ADAM28) are abundantly expressed in a lumicrine-dependent manner in the epididymal IS [4, 22]. The expression levels of these secreted proteins were significantly diminished in the *Nell2* KO IS-caput epididymis mice but not in *Adgrg2* KO ones (Figure 3G). The amount of processed form of ADAM3, which is initially expressed as a precursor in testis, decreased in the spermatozoa isolated from *Adgrg2* KO cauda epididymis (Figure 3H and I). Collectively, these results indicate that lumicrine-mediated IS differentiation is not prominently affected in *Adgrg2* KO epididymis at both the histological and protein expression levels.

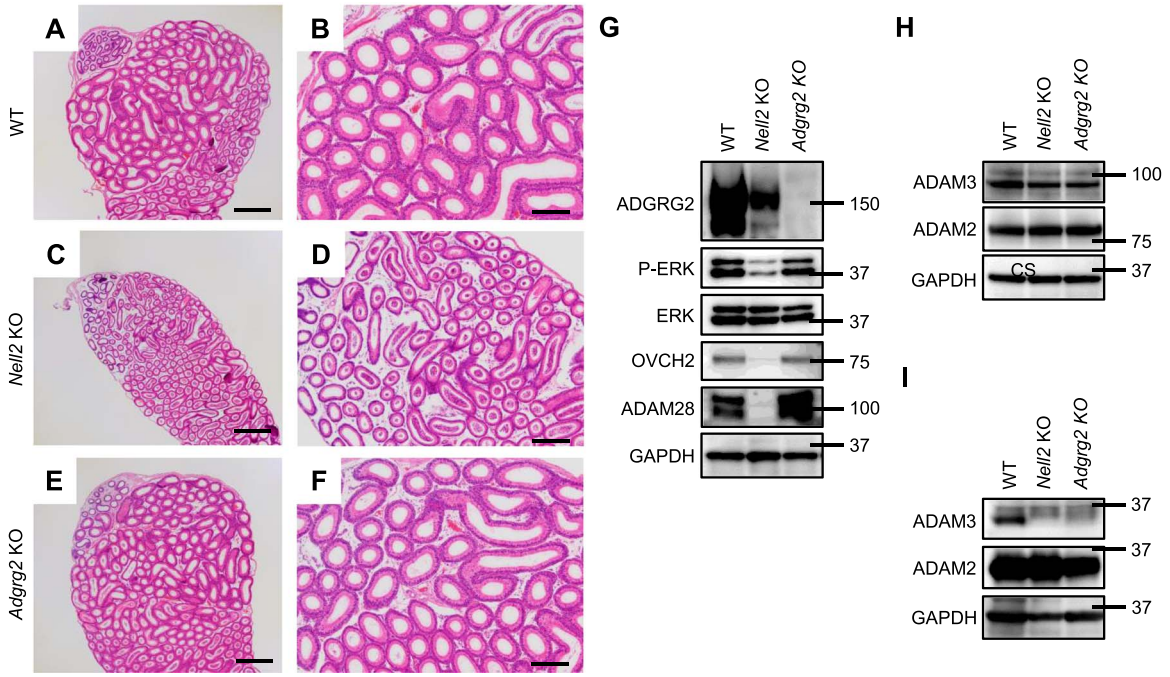


Figure 3. Comparative histology of *Adgrg2* KO and *Nell2* KO IS-caput epididymides. (A–F) HE-stained sections of WT (A, B), *Nell2* KO (C, D), and *Adgrg2* KO (E, F) IS-caput epididymides at 8 weeks old. Bars, 200 μm (A, C, E). (G) Immunoblot analyses of IS differentiation-associated proteins (P-ERK, OVCH2, and ADAM28) in 8-week-old WT, *Nell2* KO, and *Adgrg2* KO IS-caput epididymal protein lysates. ERK and GAPDH immunoblots are also shown as internal controls. (H, I) Immunoblot analyses of sperm proteins (ADAM3 and ADAM2) in 8-week-old WT, *Nell2* KO, and *Adgrg2* KO testis (H) and cauda epididymal sperm (I) protein lysates. GAPDH immunoblots are also shown as internal controls.

Transcriptome analysis of *Adgrg2* KO epididymis

The association of ADGRG2 in lumicrine signaling was investigated further by epididymal RNA-seq analysis. The transcriptome analysis of the *Nell2* KO mouse IS-caput epididymis identified the downregulated expression of a group of genes [4]. The lumicrine signaling can also be interfered with by efferent duct ligation (EDL) which disconnects the luminal communication between the testis and epididymis or by depleting testicular germ cells as in *Kit* mutant *W*/*Wv* mice [4–6]. To evaluate the transcriptome of *Adgrg2* KO IS-caput epididymis from the viewpoint of lumicrine signaling, RNA-seq was also performed for the IS-caput epididymides of EDL and *W*/*Wv* mice. The obtained RNA-seq results are summarized in Supplementary Data File S1.

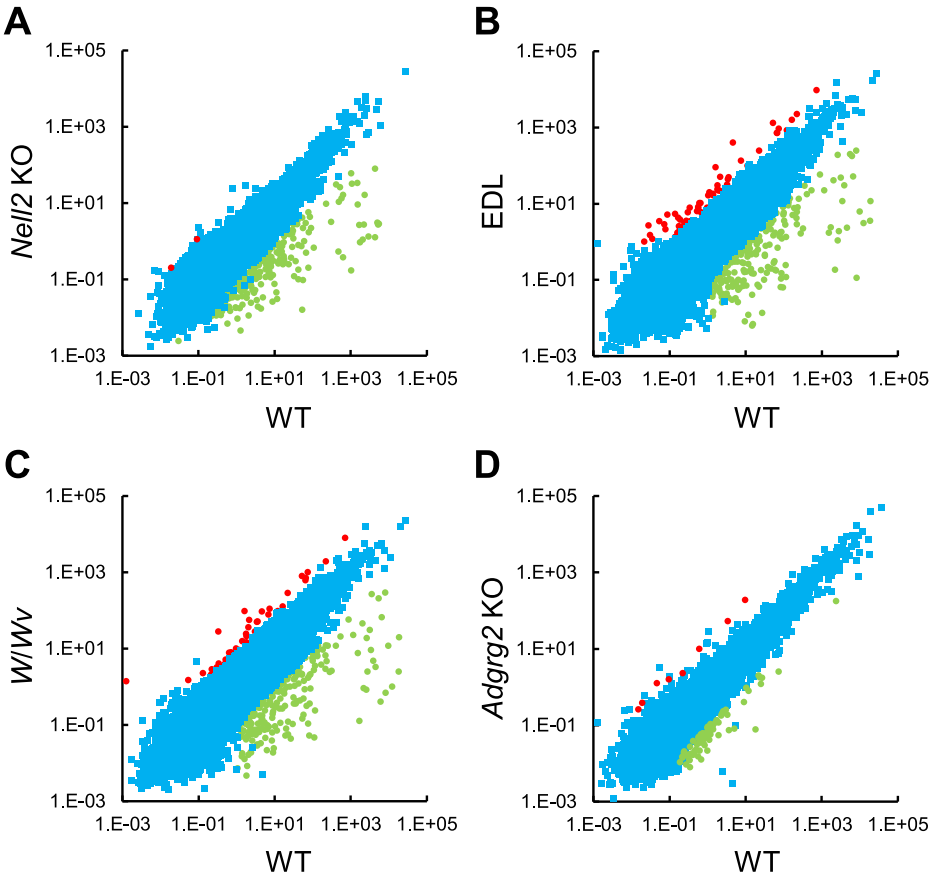
As observed in *Nell2* KO mice, the expressions of many genes were statistically significantly downregulated in the EDL and *W*/*Wv* IS-caput epididymides compared with their control WT ones (Figure 4A–C). There were also statistically significant decreases in gene expression in *Adgrg2* KO IS-caput epididymis (Figure 4D), but the number of such affected genes was apparently small compared with those observed in *Nell2* KO, EDL, and *W*/*Wv* IS-caput epididymides (Figure 4E).

The transcriptome of *Adgrg2* KO epididymis was examined further. Eighty-one genes significantly downregulated (1/10 > downregulation, the read number in WT > 10, and *t*-test *P* value < 0.05) in *Nell2* KO IS-caput epididymis were selected and compared between *Nell2* KO, EDL, *W*/*Wv*, and *Adgrg2* KO IS-caput epididymides. When the expressions of these 81 genes were compared, they were similarly downregulated in *Nell2* KO, EDL, and *W*/*Wv* IS-caput epididymides (Figure 5A and B). Most of these genes were also downregulated in *Adgrg2* KO IS-caput epididymis, but the extent of such downregulations was quite moderate in *Adgrg2* KO IS-caput epididymis compared with those in *Nell2* KO, EDL, and *W*/*Wv* ones (Figure 5A and B). Collectively, although *Adgrg2* ablation affected the expression of lumicrine-regulated genes, the IS-caput transcriptome of *Adgrg2* KO mice did not reproduce those characteristics of lumicrine-deficient mice.

Discussion

Adgrg2 is expressed in both efferent duct and epididymal IS. As major defects of *Adgrg2* ablation appear only in the efferent duct [11], the function of ADGRG2 is less characterized in IS epididymis than in the efferent duct. The epididymis IS is often evaluated by histology because it is characterized by the highly differentiated tall luminal epithelial cells and its defective differentiation is characterized by a decrease in the height of epithelial cells. However, histological analysis is not sufficiently informative to assess the function of proteins in lumicrine signaling at the molecular level. In the preceding study, the IS gene expression of *Adgrg2* KO mice was investigated by using a microarray platform and finally, approximately 3300 genes were detected [14]. In the present study, the RNA-seq of *Adgrg2* KO IS-caput epididymis was performed and the read numbers were determined on 24 346 genes. To fully characterize ADGRG2 association with lumicrine signaling, the IS-caput RNA-seq was also performed on lumicrine-deficient EDL and *W*/*Wv* mice. The total number of genes whose expressions were characterized is seven times larger in the present study than that in the previous microarray study and seems sufficient to characterize the regulatory mechanism of epididymal IS-caput gene expression by ADGRG2.

A possibility that ADGRG2 function as a lumicrine receptor has been implicated by its abundant expression in the IS and subcellular localization on the apical surface of the luminal



	Fold change	*P* value	Number of genes			
			Nell2 KO	EDL	*W*/*Wv*	*Adgrg2* KO
●	0.1>	0.05>	253	236	232	38
●	≥10	0.05>	9	63	42	10
■	Others		23,798	24,047	24,072	24,298

Figure 4. RNA-seq analyses of lumicrine-deficient and *Adgrg2* KO IS-caput epididymis. (A–D), RNA-seq of WT vs *Nell2* KO (A), WT vs EDL (B), WT vs *W*/*Wv* (C), and WT vs *Adgrg2* KO IS-caput epididymis (D). RPKM (A) or FPKM (B–D) values are plotted. (E) Summary of comparative RNA-seq analyses of *Nell2* KO, EDL, *W*/*Wv*, and *Adgrg2* KO IS-caput epididymides. Graphic symbols correspond to those in panels A–D.

epithelial cells. In lumicrine-deficient mice, the amount of processed ADAM3 decreases in the cauda epididymal spermatozoa [4, 8]. Decreased amount of processed ADAM3 in the spermatozoa of *Adgrg2* KO cauda epididymis observed in the present study also implies the association of ADGRG2 in lumicrine signaling. However, unlike the lumicrine-deficient *Nell2*, *Nicol*, and *Ros1* KO mice [13], apparent histological abnormalities were not observed in the IS of *Adgrg2* KO mice generated in the present study, as in another *Adgrg2* mutant mouse line [11]. In addition, the expression levels of proteins regulated by lumicrine signaling did not alter significantly in *Adgrg2* KO IS epididymis. *Adgrg2* association with the lumicrine signaling was also examined by transcriptome analyses. The IS-caput epididymal transcriptome of the EDL and *W*/*Wv* mice was similar to that *Nell2* KO mice, indicating that the *Nell2* KO epididymal transcriptome reflects that characteristic of defective lumicrine signaling. The RNA-seq of *Adgrg2* KO IS-caput epididymis also detected the downregulation of genes whose expression was decreased in *Nell2* KO IS-caput epididymis. However, the extent of such downregulation in *Adgrg2* KO mice appeared considerably moderate compared with those observed in lumicrine-deficient animals such as *Nell2* KO, EDL, or *W*/*Wv* mice. Collectively, these results indicate that ADGRG2 is dispensable for lumicrine signaling. ADGRG2 may indirectly regulate, if any, lumicrine signaling. One possibility is that ADGRG2 function in the efferent duct secondary affects the IS epididymis gene expression as *Adgrg2* ablation causes defects in the efferent duct, which is located upstream of the epididymis in the male reproductive tract. Another possibility is that a crosstalk between the signaling pathways mediated by ROS1 and ADGRG2 occurs in the IS epididymis, as such a signaling crosstalk between receptor tyrosine kinases and GPCRs is known [23]. It is reported that in the efferent duct the pharmacological activation of angiotensin II receptor type 2, a GPCR expressed, can rescue fluid reabsorption defect caused by *Adgrg2* ablation [24]. Since in IS-caput region of epididymis neurotensin receptor type 2 (NTSR2),

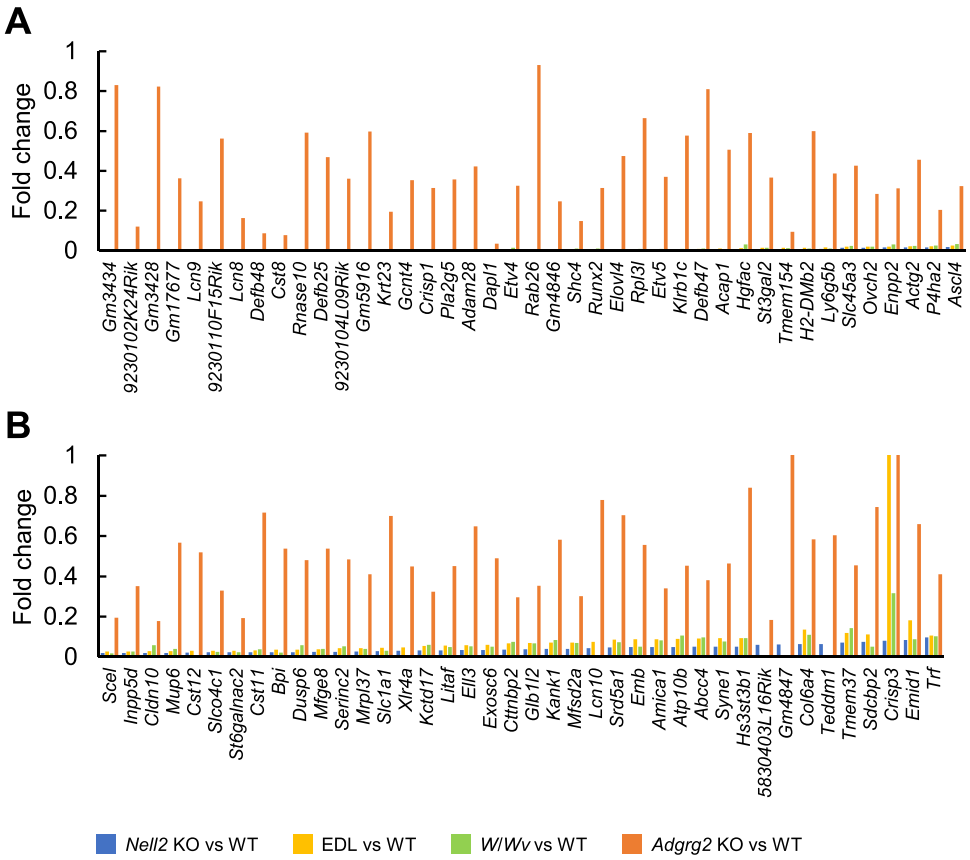


Figure 5. Comparative representation of genes downregulated in lumicrine-deficient mouse IS-caput epididymides. (A, B) Fold change of gene expressions in *Nell2* KO, EDL, *W/Wv*, and *Adgrg2* KO IS-caput epididymides compared with their control WT ones. Only genes downregulated in *Nell2* KO IS-caput epididymis (>10 RPKM in WT, fold change < 0.1, and *t*-test $P < 0.05$) are selected.

another GPCR encoded by *neurotensin receptor 2* (*Ntsr2*), is also expressed (Figure 1A), it might be interesting to examine whether the activation of NTSR2 rescue IS-caput gene expression altered by *Adgrg2* ablation. Recently, steroids dehydroepiandrosterone, dehydroepiandrosterone sulfate, and deoxycorticosterone were identified as potential ligands for ADGRG2 [25]. It is of interest whether such steroid derivatives act on ADGRG2 in a manner similar to lumicrine as ADGRG2 is apically expressed.

Increasing evidences indicate the pivotal role of epididymal IS in sperm maturation. Further studies on the regulatory mechanism of IS differentiation and gene expression will contribute to a better understanding of sperm maturation at the molecular level and to the development of novel male contraceptives targeting sperm maturation.

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Supplementary material

Supplementary material is available at *BIOLRE* online.

Conflict of Interest: The authors declare no competing interests.

Author contributions

DK designed and performed the experiments. DK and MI wrote and revised the manuscript. All authors read, corrected, and approved the manuscript.

Data availability

The RNA-seq data underlying this article are available in NCBI GEO (https://www.ncbi.nlm.nih.gov/geo/) under accession codes GSE133920, GSE138517, GSE232896, and GSE232898.

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