Natriuretic peptide type C induces sperm attraction for fertilization in mouse

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Mammalian spermatozoa undergo selective movement along the isthmus of the oviduct to the ampulla during ovulation, which is a prerequisite for fertilization. The factor(s) that involves in selective spermatozoa movement is still unknown. In this study, we found that the oviductal epithelium in mouse ampulla expressed high levels of natriuretic peptide type C (NPPC) in the presence of ovulated oocyte-cumulus complexes (OCCs). Spermatozoa expressed NPPC receptor natriuretic peptide receptor 2 (NPR2, a guanylyl cyclase) on the midpiece of flagellum. NPPC increased intracellular levels of cGMP and Ca2+ of spermatozoa, and induced sperm accumulation in the capillary by attraction. Importantly, spermatozoa from Npr2 mutant mice were not attracted by NPPC, preventing fertilization in vivo. Oocyte-derived paracrine factors promoted the expression of Nppc mRNA in the ampulla. Therefore, NPPC secreted by oviducal ampulla attracts spermatozoa towards oocytes, which is essential for fertilization.

Oocytes attract spermatozoa by secreting chemical factors to promote fertilization. In animals with external fertilization, species-specific sperm chemoattractant proteins bind to membrane guanylyl cyclase receptors15, or the receptors associated with guanylyl cyclases3 on the sperm flagellum, and stimulate rapid synthesis of cyclic guanosine monophosphate (cGMP)4. Ca2+ entering through a K+-selective cGMP-gated ion channel5,6 increases flagellar asymmetry, resulting in chemotactic movement7,8 along the gradient of chemoattraction4,10,11. Currently, chemotaxis has not been definitively established in mammalian sperm.

In mammals, a considerable fraction of the spermatozoa inseminated rapidly reaches the storage site in the isthmus of the oviduct with reduced motility12, but only a few spermatozoa recover their motility and swim from the storage to the fertilization site in the ampulla when ovulation occurs13,14. Experimental data suggest chemical attraction for spermatozoa near the oocyte in the ampulla, to trigger fertilization15. The signal originates in the oocyte microenvironment16,17, including follicular fluid18–20, oviducal fluid19 and oocyte-conditioned media21, which is most likely conducive to capacitated spermatozoa22,23 and is correlated with fertilization success24.

Amino acid sequence analysis suggests that mouse natriuretic peptides (NPs), including type A (NPPA, also known as ANP), type B (NPPB, also known as BNP) and type C (NPPC, also known as CNP), exhibit features similar to the chemoattractant peptides in marine invertebrates (see Supplementary Fig. S1). Further, NPPA attracts mammalian spermatozoa in vitro25,26, and NPPC increases human sperm motility27. However, the chemoattraction mechanism of naturally occurring attractant(s) after ovulation remains unclear. The aim of this study was to investigate the expression pattern of NPPC in mouse oviduct and its cognate receptor natriuretic peptide receptor 2 (NPR2) on spermatozoa, and their role in sperm attraction and fertilization.

Results

Nppc mRNA expression in mouse ampulla depends on stimulation of ovulated oocyte-cumulus complexes (OCCs). In general, species-specific chemoattractant proteins are secreted to attract the spermatozoa during ovulation25,26. Therefore, the gene expression of natriuretic peptides in mouse oviduct was analyzed using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Nppc mRNA was expressed predominantly in the ampulla of estrous mice, and its levels were dramatically higher than those of Nppa and Nppb.
mRNAs (see Supplementary Fig. S2a,b). Induction of ovulation also resulted in high levels of Nppc mRNA in the ampulla (Fig. 1a). Expression of Nppc mRNA in the ampulla was determined by in situ hybridization showing Nppc mRNA expression in epithelium (arrows) of oviductal ampulla. Frozen sections of ampullae were hybridized with DIG-labeled antisense probe detecting Nppc mRNA (left panels), and the sense probe detecting control group (right panels). Sense probes yielded only background staining. Asterisks (*) indicate the cumulus cell. Scale bars, 50 μm.

During ovulation, the released oocyte-cumulus complexes (OCCs) reside in the ampullae awaiting fertilization.19 Higher levels of Nppc mRNA were detected in the ampullae after ovulation (Fig. 1d). Therefore, we determined the possible role of ovulated OCCs in regulating Nppc mRNA levels. Co-culture of ampullae with OCCs dramatically promoted Nppc mRNA expression (Fig. 1e). However, OCCs expressed low levels of Nppc mRNA (Fig. 1a) as reported previously.30 Thus, the levels of Nppc mRNA in ampulla are regulated by ovulated OCCs. The effects of oocytes and cumulus cells derived from OCCs were further determined. Microsurgical extirpation of oocytes from complexes (OOX cumulus cells) only partially promoted Nppc mRNA expression by ampullae in vitro (Fig. 1e). However, co-culture of ampullae with denuded oocytes (three oocytes/μL) restored Nppc mRNA

Figure 1. Expression of Nppc mRNA by epithelium of oviductal ampulla. (a) Comparison of steady-state levels of Nppc mRNA in the ampulla, isthmus, uterotubal junction (junction) and ovulated oocyte-cumulus complexes (OCCs) isolated from mice at diestrus or ovulation. The mean value in the uterotubal junction from mice at diestrus was set as 1. Bars indicate the mean ± SEM of three experiments. *P < 0.05; ***P < 0.001. (b) In situ hybridization showing Nppc mRNA expression in epithelium (arrows) of oviductal ampulla. Frozen sections of ampullae were hybridized with DIG-labeled antisense probe detecting Nppc mRNA (left panels), and the sense probe detecting control group (right panels). Sense probes yielded only background staining. Asterisks (*) indicate the cumulus cell. Scale bars, 50 μm. (c) Comparison of NPPC levels in the ampulla, isthmus, and uterotubal junction of mice at ovulation. Bars indicate the mean ± SEM of three experiments. **P < 0.01. (d) Effect of ovulation on Nppc expression in ampulla. Nppc mRNA levels in the ampullae were increased only in the presence of the ovulated OCCs. In the left panel, the mean value of the ampullae isolated at 0 h post hCG was set as 1. Bars indicate the mean ± SEM of three experiments. **P < 0.01. (e) The effects of OCCs, cumulus cells, oocytes and oocyte-derived paracrine factors on Nppc expression in ampullae. Ampullae isolated from preovulatory mice (at 11 h post hCG) were cocultured with OCCs, oocytectomized (OOX) cumulus cells, denuded oocytes (oocyte; three oocytes/μL), or oocyte-derived paracrine factors human GDF9 (500 ng/mL), human BMP15 (500 ng/mL) and human FGF8B (FGF8, 100 ng/mL), or the combination of the three proteins for 3 h and levels of Nppc mRNA were determined. The mean value in the control (no treatment) group was set as 1. Bars indicate the mean ± SEM; n, number of independent replicates. *P < 0.05; **P < 0.01.
levels equivalent to those promoted by co-culture with OCCs, suggesting that the levels of Nppc mRNA in the ampulla are regulated by oocyte-derived paracrine factors. Growth differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15) and fibroblast growth factor 8B (FGF8) are paracrine growth factors secreted by oocytes. Each of these growth factors only slightly promoted the expression of Nppc mRNA by ampulla (Fig. 1e). However, the combination of the three proteins restored the levels of Nppc mRNA equivalent to those promoted by co-culture with oocytes or OCCs (Fig. 1e).

NPR2 is located on the flagellar midpiece of spermatozoa. Typically, chemoattractants bind to sperm surface receptors. NPPC acts locally as an autocrine and paracrine regulator via natriuretic peptide receptor 2 (NPR2), a guanylyl cyclase-coupled receptor. The levels of NPPC receptor Npr2 mRNA were detected in spermatozoa (see Supplementary Fig. S3a). To test the location of NPR2 on the sperm, we used a fluorescent-labeled ligand NPPC (FAM-NPPC) to bind with the receptor. This method is widely used for location of chemoattractant receptor on non-mammalian sperm. The green fluorescence, representing FAM-NPPC binding site, was observed on the midpiece of flagellum, especially on the distal midpiece (Fig. 2a, see Supplementary Fig. S3c–e). The positive signal was seen in 23.6 ± 4.4% of capacitated sperm (Fig. 2b). Few spermatozoa (2.6 ± 0.5%) showed fluorescence staining in the samples and competitive binding in the presence of excess unlabeled peptide (1 μM NPPC) (Fig. 2b). Furthermore, few capacitated spermatozoa derived from Npr2 mutant mice showed FAM-NPPC binding (see Supplementary Fig. S4). All these results indicate that NPR2 was the binding site of FAM-NPPC on mouse sperm. However, the positive staining in fresh spermatozoa was only 2.3 ± 0.3% (see Supplementary Fig. S3f). The endogenous levels of NPR2 were further detected using immunoprecipitation followed by Western blot, which confirmed that NPR2 was expressed predominantly in sperm flagellum (Fig. 2c). Two immunoreactive bands (~130 kDa and ~117 kDa) correspond to NPR2 with different amounts of glycosylation as observed in previous studies of mouse and rat granulosa cell.

Figure 2. Expression of NPR2 on sperm flagellum. (a) Capacitated spermatozoa were incubated with FAM-NPPC (100 nM) for 30 min to ensure fully reaction. The binding of FAM-NPPC to the spermatozoon as visualized by fluorescence (up panel) and merge (down panel) fields. Scale bar, 20 μm. (b) Comparison of FAM-NPPC binding in spermatozoa without or with NPPC (1 μM) competition. Bars indicate the mean ± SEM of three experiments. n = 500 for each group. **P < 0.01. (c) A representative western blot of NPR2 from sperm heads and flagella. NPR2 antibody recognized two immunoreactive bands that were obvious in the fraction of sperm flagella.

NPPC induces sperm accumulation in the capillary. The intriguing juxtaposition of the cell types expressing the ligand NPPC and its cognate receptor NPR2 suggests a functional relationship. We found that NPPC enhanced straight-line velocity of the sperm (Table 1). NPPC significantly increased sperm accumulation in the capillaries, and the concentration (to form optimal chemoattractant gradient) is 0.1 nM (Fig. 3a). Furthermore, the number of spermatozoa in the capillaries under descending gradient (Fig. 3b, right panel, NPPC+/− group) was significantly decreased compared with that in the absence of any gradient at all (NPPC+/+ group).
Thus, NPPC induces sperm accumulation by attraction, which is most likely attributed to chemokine- sis and chemotaxis. The rate of FAM-NPPC binding was further increased in the attracted spermatozoa (see Supplementary Fig. S3f).

NPPC elevates intracellular cGMP and Ca^{2+} levels in spermatozoa. Stimulation of spermatozoa by chemoattractants increases intracellular cGMP levels, and triggers a rapid and transient elevation of intracellular Ca^{2+}, which is essential for attraction. In the present study, NPPC significantly increased the intracellular levels of cGMP in spermatozoa (Fig. 3c). Next, we examined the effect of NPPC on Ca^{2+} levels in spermatozoa.

Table 1. Effect of NPPC on sperm motility. VAP, average path velocity; VSL, straight line velocity; VCL, curvilinear velocity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; STR, percentage of straightness; LIN, percentage of linearity; progressive motility (% of motile spermatozoa with VAP \(\geq 50\) μm/s and STR \(\geq 80\%\)). Bars indicate the mean ± SEM of three experiments; \(n = 500\) for each sample. * \(P < 0.05\).

| Treatment | Control  | NPPC     |
|-----------|----------|----------|
| Progressive motility (%) | 20.6 ± 0.9 | 34.7 ± 0.9* |
| VAP (μm/s) | 110.4 ± 1.9 | 130.1 ± 1.2* |
| VSL (μm/s) | 73.9 ± 1.3 | 93.1 ± 1.4* |
| VCL (μm/s) | 216.8 ± 3.9 | 221.2 ± 1.9 |
| ALH (μm)  | 9.2 ± 0.2  | 9.0 ± 0.2  |
| BCF (HZ)  | 24.4 ± 0.5 | 23.6 ± 0.4 |
| STR (%)   | 61.0 ± 0.8 | 69.6 ± 0.5 |
| LIN (%)   | 34.3 ± 0.6 | 44.0 ± 1.0 |

Figure 3. NPPC induces sperm accumulation in the capillary. (a) Effect of NPPC on sperm accumulation in the capillaries at different concentrations. After capacitation, spermatozoa were added to a 150 mL droplet at a final concentration of \(1 \times 10^6\) cells/mL, and capillaries were exposed to various doses of NPPC. The number of spermatozoa in the capillaries was counted after incubation for 20 min. Bars indicate the mean ± SEM of three experiments with three repeats per group in each experiment. * \(P < 0.05\); ** \(P < 0.01\). (b) Effect of NPPC on sperm accumulation in the capillaries with ascending and descending gradients. For the ascending gradient, the droplet contained 0.1 nM NPPC, and the capillary was supplemented without (−/−) or with 0.1 nM NPPC (−/+). For the descending gradient, the droplet contained 0.1 nM NPPC, and the capillary was supplemented without (+/−) or with 0.1 nM NPPC (+/+). Bars indicate the mean ± SEM of three experiments with two repeats per group in each experiment. * \(P < 0.05\); ** \(P < 0.01\). (c) Effect of NPPC on cGMP levels in spermatozoa. Capacitated spermatozoa were incubated without or with 1 nM NPPC for 20 min, and the amounts of cGMP were evaluated using enzyme immunoassay kits. Bars indicate the mean ± SEM of four experiments. ** \(P < 0.01\).
by measuring the changes in fluorescence intensity. The fluorescence intensity was dramatically increased in the head and flagellar midpiece in response to NPPC (Fig. 4a,d). As reported before, NPPC induced a transient response (7.9 ± 1.6%), where Ca\(^{2+}\) levels returned to baseline within 140 sec (Fig. 4b,c), and a prolonged response (18.6 ± 4.5%), where Ca\(^{2+}\) levels remained high and returned to baseline after 140 sec. NPPC induced Ca\(^{2+}\) response in 26.5% of the sperm population, consistent with the FAM-NPPC binding rate in capacitated spermatozoa. In addition, we observed a time lag of 2–3 sec in the response between the head and the flagellar midpiece (see Supplementary Fig. S5), consistent with other studies that a tail to head propagation of a calcium wave is found in response to several agents including 8-Br-cGMP. This finding indicates that Ca\(^{2+}\) elevation starts in the flagellar midpiece, and immediately propagates toward the head. The initial site of Ca\(^{2+}\) elevation in spermatozoon is consistent with NPR2 distribution.

Ca\(^{2+}\) entering via cGMP-sensitive cyclic nucleotide-gate (CNG) channels plays an important role in sperm attraction in marine invertebrates. Subsequently, we used l-cis-Diltiazem (l-cis-D, a CNG-channel inhibitor) to determine the role of CNG channels in NPPC-induced Ca\(^{2+}\) elevation. The addition of 0.1 nM NPPC increased the maximum fluorescence intensity three-fold (Fig. 4d,e), whereas pre-incubation of spermatozoa with 50 μM l-cis-Diltiazem (l-cis-D; middle panel) for 1 min, or without or with NPPC incubation for 1 min in Ca\(^{2+}\)-free medium (right panel). Scale bars, 20 μm. (e) Comparison of normalized mean fluorescence intensity response (ΔF/F₀) in the sperm head and flagellar midpiece after different treatments. Bars indicate the mean ± SEM of five experiments. n, number of spermatozoa for each bar. **P < 0.01.

Figure 4. NPPC induces Ca\(^{2+}\) influx in mouse spermatozoa. (a) Micrographs of mouse spermatozoa before (left panel) and after (right panel) stimulation with 0.1 nM NPPC. Images were presented in a pseudocolor format. Scale bars, 20 μm. (b) Representative fluorescence intensity-time relationship showing transient and prolonged responses. The traces showed the maximal fluorescence intensity (F\(_{\text{max}}\)) in sperm head and flagellar midpiece after the addition of NPPC (NPPC add) in two spermatozoa (1 and 2, in a). F₀ represents the baseline fluorescence before application of stimulus. (c) Percentage of each type of response induced by NPPC. Bars indicate the mean ± SEM of four experiments. n = 31 to spermatozoa responding. (d) Representative micrographs of spermatozoa without or with 0.1 nM NPPC incubation (left panel) or supplemented with 50 μM l-cis-Diltiazem (l-cis-D; middle panel) for 1 min, or without or with NPPC incubation for 1 min in Ca\(^{2+}\)-free medium (right panel). Scale bars, 20 μm. (e) Comparison of normalized mean fluorescence intensity response (ΔF/F₀) in the sperm head and flagellar midpiece after different treatments. Bars indicate the mean ± SEM of five experiments. n, number of spermatozoa for each bar. **P < 0.01.
to induce accumulation (Fig. 5a) and Ca$^{2+}$ response (Fig. 5c,d) of spermatozoa from Npr2 mutant mice. Npr2 mutant males show normal spermatogenesis 44, and the spermatozoa exhibited normal motility (see Supplementary Table S1) and the ability for fertilization in vitro 45. However, these mutant males fail to mate naturally due to erectile dysfunction (ED) accompanying a trapped foreskin 44. Therefore, we investigated their ability for in vivo fertilization via artificial insemination (AI). After 4 h, the spermatozoa arriving at the ampullae were counted. The number of spermatozoa from Npr2 heterozygous males was 23.8 ± 3.2 in each ampulla. However, few spermatozoa (2.9 ± 0.5) were observed in Npr2 mutant males (Fig. 5b). After 1.5 days, the rate of two-cell embryos was 77.7 ± 3.4% with spermatozoa derived from Npr2 heterozygous males, and was only 5.4 ± 1.7% in Npr2 mutant males (Fig. 5f). Therefore, NPPC/NPR2 plays an essential role in attracting spermatozoa for fertilization.

Discussion

In the present study, we showed that ovulation stimulated NPPC, which bound to NPR2 on spermatozoa, to increase attraction for oocytes that reside in the ampulla, and ensure normal fertilization. Unlike marine invertebrates, the mammalian spermatozoa are placed inside the female genital tract without interspecific sperm competition, and species specificity for sperm attraction is not observed 46. Interestingly, the sequence of the chemoattractant peptide NPPC in mammals is highly conserved 47.
A stable long-lasting attraction is a prerequisite for fertilization. After the LH surge, mammalian OCCs are ovulated into the oviductal ampullae, awaiting fertilization. It is reported that oocyte arrival in the oviduct results in a change in the oviductal gene expression, which plays important roles in gamete transport, fertilization, and embryo development. In our study, Nppc mRNA levels were dramatically increased locally. In vitro studies further showed that oocytes and oocyte-derived paracrine factors stimulated the transcription of Nppc in the ampullae, which resulted in high levels of NPPC protein. These results are consistent with previous studies suggesting that oocyte-stimulated SMAD signaling promoted Nppc mRNA expression in granulosa cells. Thus, the timing of the availability of NPPC in oviductal ampulla is programmed to the availability of oocytes at this site. It promoted a stable attractant gradient in the oviduct, which is consistent with previous studies showing that spermatozoa exhibit an attractive response and swim from the storage area to the fertilization site of ampulla during ovulation.

Progratone has been a candidate as the attractant mammalian follicular fluid. However, sperm attraction is not correlated with progesterone levels in the follicular fluid. Progesterone induces sperm accumulation probably by inducing hyperactivation. Interestingly, the follicular fluid of pre-ovulatory follicles contains a high concentration of NPPC produced by mural granulosa cells to maintain oocyte meiotic arrest. However, NPPC concentration in the follicular fluid dramatically decreases during ovulation. Furthermore, tiny amounts of follicular fluid are released at the onset of ovulation, which prevent formation of a stable and persistent attractive gradient along the oviduct lumen. Recently, it is reported that coitus induces oviductal flow that clears the oviduct of cellular debris and provides rheotactic guidance for sperm. Anyway, the oviductal flow is beneficial for the establishment of a chemoattractant gradient in the oviduct. On the other hand, myosalpinx contractions play important roles in the formation of sperm assemblage in the isthmus, and in the transport of the assemblage to the middle region of the oviduct. Thus, the range of sperm chemotaxis in the oviduct may be relatively short. In the present study, the ovulated oocytes without cumulus matrix are unfertilized, leading to severe female infertility.

In the present study, cumulus cells slightly expressed Nppc mRNA and also stimulated the transcription of Nppc mRNA in the ampullae. It needs further study whether cumulus cells also participate in this attractive response.

FAM-NPPC bound to NPR2 at the midpiece of sperm. While few spermatozoa showed NPR2 positive staining (FAM-NPPC binding), the staining was increased in capacitated spermatozoa (see Supplementary Fig. S3f), consistent with previous studies. The increase in NPR2-positive spermatozoa may be related to unmasking of NPR2, since Npr2 mRNA levels were not increased by capacitation (see Supplementary Fig. S3b) and the sperm nuclear transcription is in a dormant state. A quarter of spermatozoa showed NPR2-positive staining, probably due to the fact that only a small fraction of spermatozoa is capacitated at a given time. The number of spermatozoa with NPR2-positive staining was further increased after treatment with NPPC, suggesting that only the spermatozoa with functional NPR2 are attracted by NPPC. This is consistent with recent studies that only a few spermatozoa migrate from the isthmus to the ampulla during the progression of fertilization.

Thus, the capacitated spermatozoa show attraction by exposing the functional receptor NPR2, and travel from the isthmus of the oviduct to the ampulla during ovulation.

Chemoattractant binding results in a transient cGMP-induced influx of extracellular Ca²⁺ via Ca²⁺ channels in marine invertebrates. It plays a crucial role in the regulation of sperm attraction. Cyclic GMP-sensitive CNG channels have been detected in mammalian sperm, which control the Ca²⁺ entry into spermatozoa. In the present study, NPPC increased cGMP levels, and induced Ca²⁺ influx via CNG channels, suggesting that NPPC elevated Ca²⁺ also increases the swimming speed of spermatozoa along the chemoattractant gradient. NPPC-induced attraction is accompanied by enhanced sperm motility. Spermatozoa swim along the gradient of NPPC with enhanced speed, which is required for movement along the long oviduct to the oocytes. It is suggested that hyperactivation enables spermatozoa to reach the oocyte by assisting escape from the oviductal sperm reservoir. CatSper-null mutant spermatozoa that cannot undergo hyperactivation are not able to migrate to the site of fertilization. In the present study, very few Npr2 mutant sperm arrived at the ampulla after 4 h post mating. Although Npr2 mutant had no effect on sperm motility, it needs further study whether there is a problem in the percentage of hyperactivated spermatozoa in Npr2 mutant mice.

This study demonstrates a complex regulatory network involving oocytes, spermatozoa and the oviductal epithelium in ampulla (Fig. 6). The oviductal epithelium in ampulla secretes NPPC that provides a stable and persistent gradient of attraction in the oviduct. Spermatozoa are attracted to oocytes in response to NPPC. Oocytes promote NPPC expression, which is critical for fertilization. Our findings may have potential clinical implications for the treatment of infertility and for contraception.

**Materials and Methods**

**Animals.** ICR (CD1) female (8 weeks old) and male (3–6 months old) mice were purchased from the Laboratory Animal Center of the Institute of Genetics and Developmental Biology (Beijing, China). Mice were housed under controlled temperature (23 ± 2°C) and light (12 h light/12 h darkness) with food and water ad libitum in an air-conditioned room. Ovulation was induced by treating the diestrous female mice with 5 IU equine chorionic gonadotropin (eCG), followed by 5 IU of human chorionic gonadotropin (hCG) 46 h to 48 h later. CBA/Ca A/Npr2cn-2J/A-Nppcn-2J (Npr2cn-2J/Npr2cn-2J) mice were produced by crossing heterozygous males and females obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Npr2cn-2J is a loss-of-function mutation at the Npr2 locus. The homozygous mutant mice exhibit an achondroplastic phenotype readily distinguishable from wild-type counterparts as early as 6 days of age. Mice were maintained according to the Guide for the Care and Use of Laboratory Animals (Institute for Learning and Animal Research at China Agricultural University). All the methods were approved by the Institutional Animal Care and Use Committee of China Agricultural University.

**Isolation of ampulla, isthmus, and uterotubal junction.** The ampulla, isthmus, and uterotubal junction were isolated from the oviducts of mice at diestrous or estrus using a pair of 26 gauge needles under a...
stereomicroscope as reported previously\textsuperscript{14,26}. In some experiments, the ampulla, isthmus, and uterotubal junction were isolated from ovulating mice, or from unilaterally ovariectomized mice. The unilaterally ovariectomized mice were used after two weeks of recovery. All the samples were immediately frozen in liquid nitrogen and stored at \(-80{^\circ}\text{C}\) until mRNA expression was analyzed as described below.

**Co-culture of oviductal ampullae with oocyte-cumulus complexes (OCCs) and oocytectomized (OOX) cumulus cells and denuded oocytes.** Ampullae were cultured in a medium of bicarbonate-buffered MEM\textsubscript{\(\alpha\)} with Earle's salts, supplemented with 75 \(\mu\text{g/mL}\) penicillin G, 50 \(\mu\text{g/mL}\) streptomycin sulfate, 0.23 mM pyruvate, and 3 mg/mL BSA. All the reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. The ampullae were isolated from pre-ovulatory mice (at 11 h post hCG), and OCCs were collected from the ampullae of ovulating mice (at 13 h post hCG). Groups of four ampullae with or without 100 OCCs were transferred into 50 \(\mu\text{L}\) drops. In some experiments, denuded oocytes (DOs) were obtained by removing the cumulus cells with 1 mg/mL hyaluronidase. OOX cumulus cells were produced by microsurgically removing oocytes, but not the zona pellucida, from the OCCs. OOX cumulus cells or oocytes obtained from 150 OCCs were co-cultured with four ampullae in a 50 \(\mu\text{L}\) drop. Occasionally, the ampullae were cultured with a recombinant human growth differentiation factor 9 from \textit{E. coli} (GDF9, 500 ng/mL), recombinant human bone morphogenetic protein 15 from \textit{CHO} cells (BMP15, R&D Systems, Minneapolis, MN, USA. 500 ng/mL) and/or recombinant human fibroblast growth factor 8B from HEK 293 cells (FGF8, 100 ng/mL). All the cultures were incubated at 37 \(^\circ\text{C}\) under 5\% CO\(_2\). Each experiment was repeated at least three times. At the end of 3 h, the ampullae were frozen immediately in liquid nitrogen and stored at \(-80{^\circ}\text{C}\) until analysis of mRNA expression as described below.

**Quantitative RT-PCR.** Total RNA was isolated and purified using the RNeasy micro-RNA isolation kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. Reverse transcription was performed directly after RNA isolation using the QuantiTect reverse transcription system (Qiagen). Real-time PCR was conducted to quantify steady-state mRNA levels using an ABI 7500 real-time PCR instrument (Applied Biosystems, Foster City, CA, USA). The levels of mRNAs were first normalized to the expression levels of a housekeeping gene, ribosomal protein L19 (\textit{Rpl19}), and expressed relative to a control group whose expression level was set at 1. PCR primers for \textit{Nppc}, \textit{Npr2} and \textit{Rpl19} were reported previously\textsuperscript{30,72}. \textit{Nppa} and \textit{Nppb} primer sequences were indicated in Supplementary Table S2. The specificity of the primers was confirmed by sequencing of the band after electrophoresis.

**Measurement of NPPC levels.** Samples were prepared using a modified method reported previously\textsuperscript{56}. The ampulla, isthmus, and uterotubal junction were separated from ovulating mice (at 13 h post hCG). Each segment from 10 mice in a single experiment was transferred into a 1.5 mL centrifuge tube and stored at \(-80{^\circ}\text{C}\). Prior to protein analysis, the tissues in 100 \(\mu\text{L}\) of 1.0 M acetic acid were boiled for 5 min, and homogenized on ice using a tissue homogenizer (T10 basic, IKA, Germany) and lysed with ultrasonic cell disruptor (Scientz-IID, Ningbo, China). We added 500 \(\mu\text{L}\) of MeOH to solubilize the lipids. The samples were centrifuged at 20,000 \(\times\) g at 4 \(^\circ\text{C}\) for 30 min, and the supernatant contained 1.0–5.0 mg of protein were frozen. For each sample, 100, 50 and 10 \(\mu\text{g}\) of the protein extracts were lyophilized and assayed by fluorescent enzyme immunoassay kits (Phoenix Pharmaceuticals, Belmont, CA, USA) according to the manufacturer’s instructions.

**In situ hybridization.** The ampullae were isolated from ovulation-induced mice, embedded rapidly in optimal cutting temperature compound (OCT; Sakura, Tokyo, Japan), and frozen at \(-80{^\circ}\text{C}\) prior to analysis. Frozen sections (10 \(\mu\text{m}\)) were prepared using a CM 1950 cryostat microtome (Leica, Wetzlar, Germany) and mounted on Super-Frost Plus slides (Thermo Scientific, Waltham, MA, USA) for \textit{in situ} hybridization using DIG-labeled riboprobes as reported previously\textsuperscript{73}. Briefly, sections were hybridized with DIG-cRNAs (1.5\(\mu\text{g/mL}\)) overnight after pre-hybridization, and incubated with anti-digoxigenin-AP. The sections were incubated with NBT/BCIP (nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate, toluidine salt; Roche Diagnostics, IN,
USA) to detect bound digoxigenin-AP. Sequences of PCR primers used to amplify cDNA templates for preparation of in situ probes were reported previously30.

**Sperm preparation.** Sperm collection and incubation were carried out in Tyrode (T6) medium comprising: 114.0 mM NaCl, 3.2 mM KCl, 0.5 mM MgCl2, 2.0 mM CaCl2, 0.4 mM NaH2PO4, 24.9 mM NaHCO3, 5.6 mM glucose, 0.5 mM sodium pyruvate, 10 mM sodium DL-lactate (60% wt/vol), 10 mM hepes, 0.01 mg/mL phenol red and 10 mg/mL bovine serum albumin (BSA). A T6 medium without BSA was used for the measurement of Ca2+ levels. Caudal epididymal sperm cells were obtained from male mice. Excised caudal epididymis was rinsed and fragmented in a 400 μL drop of T6 medium. Immediately following dispersion of spermatozoa into the medium, the whole sperm suspension was transferred to a 1.5 mL plastic tube. For in vitro capacitation, the spermatozoa were incubated for 1.5 h in T6 medium in a 37 °C incubator under 5% CO2.

**Identification and localization of NPPC receptor.** The NPPC receptor sites on spermatozoa were identified by FAM-labeled (the Mono-5-(and 6)-carboxyfluorescein label) NPPC (FAM-NPPC, Phoenix Pharmaceuticals, Belmont, CA, USA). Fresh spermatozoa and capacitated or chemoattractant-treated spermatozoa were incubated with 100 nM FAM-NPPC for 30 min to ensure full reaction. The spermatozoa were fixed with 2% paraformaldehyde for 5 min, and washed three times with PBS, 5 min each time at 300 × g to remove unbound ligand. Fluorescent-labeled ligand binding was assessed using a confocal laser-scanning microscope (Nikon A1R, Tokyo, Japan). Cells with uneven dye loading were excluded from the analysis. Competition experiments were performed by incubating the spermatozoa for 30 min in the presence of 100 nM FAM-NPPC and 1 μM of unlabeled NPPC.

**Sperm motility.** Capacitated spermatozoa were incubated in T6 medium without (control) or with 1 nM NPPC for 30 min. The sperm motion was determined using a CASA system (Version.12 CEROS, Hamilton Thorne Research, Beverly, MA, USA). The parameters included curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), percentage of linearity (LIN; VSL/VCL × 100%), and percentage of straightness (STR; VSL/VAP × 100%). Progressive motility (% of motile spermatozoa with average path velocity (VAP) ≥ 50 μm/s, and straightness ratio (STR) ≥ 80%) were determined. Cells were detected with a minimal contrast of 50 and minimal cell size of 4 pixels. Sixty frames were acquired at a frequency of 60 Hz. Almost 10 random fields were evaluated for each sample, accounting for a total of 500 spermatozoa.

**Western blot.** A total of 2 × 108 spermatozoa were collected from the caudal epididymis of adult male mice as described above. The sperm heads and tails were separated according to the previous study3. Briefly, the spermatozoa were snap-frozen in liquid nitrogen and diluted with 600 μL of PBS. The sperm suspension was passed thirty times through a 26-gauge needle on ice and centrifuged at 1000 × g for 5 min at 4 °C to precipitate the sperm heads. Sperm flagella were recovered from the supernatant by centrifugation at 10,000 × g for 20 min at 4 °C. NPR2 was immunoprecipitated as reported previously36. Briefly, the samples were diluted to 600 μL in an immunoprecipitation buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 50 mM NaF, 10 mM NaH2PO4, 2 mM EDTA, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 μM microcystin-LR, and protease inhibitors. The samples were incubated with 0.6 μL of rabbit polyclonal NPR2 antiserum provided by Lincoln R. Potter36 for 1 h at 4 °C, and then with 25 μL of washed protein A magnetic beads (Thermo Fisher Scientific, Rockford, IL, USA) overnight at 4 °C. The beads were washed with Tris-buffered saline with Tween 20 (TBST) and resuspended in Laemmli sample buffer. The proteins were eluted by heating at 100 °C for 10 min.

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Imaging analysis of the intracellular Ca\(^{2+}\) levels. Ca\(^{2+}\) levels were detected by a method modified from the previous study\(^{43}\). Capacitated spermatozoa were loaded with 5\(\mu\)M Fluo 3-AM (Dojindo Laboratories, Kumamoto, Japan) and 0.06% pluronic F-127 for 30 min at 37°C in the dark. The sperm suspension (3.5 × 10\(^5\) cells/mL) was placed on glass coverslips treated with polylysine at 0.01% in the recording chamber and after 5 min the external solution was infused to wash the spermatozoan. Imaging analysis of Ca\(^{2+}\) response in motile spermatozoa was monitored using the confocal laser-scanning microscope (Nikon, A1R). We used a sample frequency of 1 Hz, and recorded for 3–4 min after stimulus. The elevation of Ca\(^{2+}\) in the sperm head and flagellar midpiece was expressed as \(F_{\text{max}}/F_0\) ratios after background subtraction, where \(F_{\text{max}}\) represented the maximum peak of fluorescence signal intensity, and \(F_0\) denoted the baseline, calculated as the average of the first 15 sec prior to stimulus application. All Ca\(^{2+}\) imaging experiments were carried out at 37°C. Cells with uneven dye loading were excluded from the analysis. NPPC was used at 0.1 nM, and the inhibitor l-cis-Diltiazem was used at 50\(\mu\)M with a pre-incubation of 15 sec. NPPC and the inhibitor were dropped into the recording chamber using pipette tips and the recordings were conducted in the continuous presence of stimuli. Ca\(^{2+}\)-free experiments were conducted using Ca\(^{2+}\)-free T6 medium without BSA, obtained by omitting Ca\(^{2+}\) and adding 1 mM EGTA.

Artificial insemination. Eight-week-old female mice were induced to ovulate using a slight modification of the procedure described above. The doses of both eCG and hCG were 2 IU. Artificial insemination was conducted at 13 h post hCG by depositing capacitated spermatozoa of \(Npr2^{+/-}\)/\(Npr2^{-/-}\) heterozygote or \(Npr2^{-/-}/Npr2^{-/-}\) mutant male mice (approximately 3 months old) into the female uterus via cervix. The volume of spermatozoa inseminated per female was 50\(\mu\)L containing 3–5 × 10\(^5\) spermatozoa. Subsequently, the female mice were mated with vasoligated male immediately to develop a vaginal plug. In some experiments, capacitated spermatozoa from CD1 males were pre-incubated with 50\(\mu\)M l-cis-D for 10 min before artificial insemination. After 4 h, the ampulla was separated, and the spermatozoa and OCCs were flushed out of the ampulla. The oocytes and cumulus cells were diffused with 1 mg/ml hyaluronidase. The spermatozoa in the medium were counted. 36 h later, embryos and presumptive zygotes in the oviduct were collected and captured on a 20 × 20 mm\(^2\) matrix. Imaging analysis of the intracellular Ca\(^{2+}\) levels was performed using the confocal laser-scanning microscope (Carl Zeiss, LSM 710). Imaging experiments were carried out at 37°C. Cells with uneven dye loading were excluded from the analysis. NPPC was used at 0.1 nM, and the inhibitor l-cis-Diltiazem was used at 50\(\mu\)M with a pre-incubation of 15 sec. NPPC and the inhibitor were dropped into the recording chamber using pipette tips and the recordings were conducted in the continuous presence of stimuli. Ca\(^{2+}\)-free experiments were conducted using Ca\(^{2+}\)-free T6 medium without BSA, obtained by omitting Ca\(^{2+}\) and adding 1 mM EGTA.

Statistical analysis. All the experiments were repeated a minimum of three times. Results were expressed as mean ± SEM. Significant differences between experimental and control groups were analyzed using Student's t-test. A P value of less than 0.05 was considered statistically significant.

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Author Contributions
N.K. and X.X. performed the most of the experiments. Y. Zhang and Y. Zhao performed Ca\(^{2+}\) assay. Y.W. and X.H. performed the cGMP assay. M.Z., G.X. and J.Q. designed the study and performed data analysis. M.Z. and N. K. interpreted the results and wrote the manuscript.

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