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ORIGINAL ARTICLE

A novel approach to nonsurgical sterilization; application of menadione-modified gonocyte-targeting M13 bacteriophage for germ cell ablation *in utero*

Barbara A. Fraser[1,2] | **Kasey Miller**[1,2] | **Natalie A. Trigg**[1,2] | **Nathan D. Smith**[3] | **Patrick S. Western**[4,5] | **Brett Nixon**[1,2] | **Robert J. Aitken**[1,2,6]

[1]Priority Research Centre for Reproductive Science, The University of Newcastle, Callaghan, NSW, Australia

[2]Pregnancy and Reproduction Program, Hunter Medical Research Institute, New Lambton Heights, NSW, Australia

[3]Analytical and Biomolecular Research Facility, The University of Newcastle, Callaghan, NSW, Australia

[4]Centre for Reproductive Health, Hudson Institute of Medical Research, Clayton, Victoria, Australia

[5]Department of Molecular and Translational Science, Monash University, Clayton, Victoria, Australia

[6]Faculty of Health and Medicine, The University of Newcastle, Callaghan, NSW, Australia

Correspondence
Robert J. Aitken: Room BB2-06, Bowman Building, University Drive, Callaghan, NSW 2308, Australia.
Email: john.aitken@newcastle.edu.au

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Abstract

There remains a compelling need for the development of nonsurgical sterilizing agents to expand the fertility management options for both domestic and feral animal species. We hypothesize that an efficacious sterilization approach would be to selectively ablate nonrenewable cell types that are essential for reproduction, such as the undifferentiated gonocytes within the embryonic gonad. Here, we report a novel strategy to achieve this goal centered on the use of a chemically modified M13 bacteriophage to effect the targeted delivery of menadione, a redox-cycling naphthoquinone, to mouse gonocytes. Panning of the M13 random peptide 'phage display library proved effective in the isolation of gonocyte-specific targeting clones. One such clone was modified via N-succinimidyl-S-acetylthioacetate (SATA) linkage to the N-terminus of the major PVIII capsid protein. Subsequent deacetylation of the SATA was undertaken to expose a thiol group capable of reacting with menadione through Michael addition. This chemical modification was confirmed using UV spectrophotometry. In proof-of-concept experiments we applied the modified 'phage to primary cultures of fetal germ cells and induced, an approximately, 60% reduction in the viability of the target cell population. These studies pave the way for in vivo application of chemically modified M13 bacteriophage in order to achieve the selective ablation of nonrenewable cell types in the reproductive system, thereby providing a novel nonsurgical approach the regulation of fertility in target species.

KEYWORDS

(MeSH): 'phage display, bacteriophage, contraceptive, gonocytes, menadione, ROS

Abbreviations: ps, pachytene spermatocytes; RO water, distilled water purified by reverse osmosis; rs, round spermatids; SATA, N-succinimidyl-S-acetylthioacetate; spg, spermatogonia.

1 | INTRODUCTION

The development of nonsurgical approaches to animal sterilization has traditionally focused on the development of contraceptive vaccines that target key proteins regulating the reproductive process.[1,2] While such strategies have met with limited success, issues associated with efficacy, reliability, and undesirable off-target effects have hampered their widespread adoption.[2-4] An alternative approach would be to selectively ablate nonrenewable cell types critical for reproduction, including primordial follicles, spermatogonial stem cells, or their immediate precursors, undifferentiated fetal gonocytes.[5] The latter is of particular interest because if this cell type could be selectively destroyed *in utero*, it would mean that any offspring would be born sterile, thereby obviating the need for surgical castration of the juvenile offspring.

In order to induce selective gonocyte ablation, we have been guided by the rapid, efficient induction of sterility observed in both sexes following exposure to ionizing radiation.[6] The latter is an effective sterilant because it leads to the generation of oxygen free radicals via the homolytic fission of water and both the male and female germlines are inherently sensitive to oxidative attack.[7] The consequent overproduction of reactive oxygen species (ROS) overwhelms the germ cells' limited antioxidant defenses inducing lipid peroxidation[8,9] and the liberation of reactive lipid aldehydes that adduct and damage vulnerable biomolecules essential for cell survival.[10-14] The onset of this oxidative damage cascade propels affected cells, particularly germ cells, down an apoptotic pathway leading to cell death.[7,15-17] Moreover, when the germline is destroyed by oxidative stress the resulting infertility is known to be permanent.[6,18] On the basis of these data, we have sought to biochemically recapitulate the impact of ionizing radiation as a strategy for eliminating developing germ cells within the fetal gonad and achieving the nonsurgical induction of sterility.

In order to focus this oxidative stress, we have investigated the potential use of bacteriophage to selectively deliver redox-cycling toxicants to mouse fetal gonocytes. This population of germ cells possess extremely active plasma membrane redox systems that are capable of responding to the presence of quinones, such as menadione, at their exofacial surface by redox cycling these molecules and thus generating large quantities of ROS.[19-22] Thus, we have exploited the capacity of random peptide 'phage display libraries to identify peptides that can bind to selected cellular targets with great affinity and specificity.[23-26] While 'phage DNA can be extracted and sequenced to identify the peptide insert responsible for the binding activity, use of the latter in isolation can be problematic owing to low aqueous solubility and/or limited stability in an in vivo setting. Additionally, any modifications that alter the three-dimensional structure of a peptide can compromise, or result in the complete loss of, their binding efficiency.[27]

To avoid these potential limitations, it is possible to use 'phage particles themselves as carriers for the delivery of a specific payloads following modification in their capsid.[25,28,29] In the case of M13 bacteriophage, this protein coat comprises ~ 2700 copies of the major PVIII capsid protein.[25,30] The first three N-terminal residues of PVIII reside on the outer surface of the particle where they are readily accessible for the stable chemical linkage of reagents,[31,32] a property that has been exploited for a number of clinical applications.[32-34] This study was therefore designed to achieve three aims: (i) to identify bacteriophage clones capable of selectively binding to gonocytes within the fetal gonad, (ii) to chemically modify these 'phage clones via the covalent linkage of a redox-cycling quinone such as menadione to the PVIII capsid proteins and (iii) to examine the ability of such a construct to induce cell death within the gonocyte population.

2 | MATERIALS AND METHODS

2.1 | Materials

Ammonium chloride, sodium hydroxide, sodium chloride, sodium bicarbonate, *N*- hydroxylamine, ethylenediaminetetraacetate disodium salt (EDTA), menadione, succinimidyl S-acetylthioacetate (SATA), 4′,6-diamidino-2-phenylindole (DAPI), trypan blue, bovine serum albumin (BSA), phosphate-buffered saline solution (PBS), dimethylsulfoxide (DMSO), and Dulbecco's Modified Eagle's Medium/ Nutrient Mixture F-12 Ham (DMEM/F-12), 2-hydroxyethyl methacrylate (poly-HEMA) were all purchased from Merck (Darmstadt, Germany), 32% w/v AnalaR hydrochloric acid (HCl) was supplied by VWR International (Radnor, PA, USA), and polyethylene glycol – 8000 (PEG) was from Promega (Madison, WI, USA). Protein Lo-Bind Eppendorf tubes (1.5 ml) were supplied by Eppendorf (AG Hamburg, Germany), Amicon Ultra-4 Ultracentrifuge tubes 10 kDa NMWL and Mowiol 4-88 were from Merck. Hanks Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+}, Cell Dissociation Buffer (enzyme free), Hanks Balanced Salt Solution, Dulbecco's Modified Eagle's Medium (DMEM), L- glutamine, penicillin-streptomycin, sodium pyruvate, and 40/ 70 μM nylon cell strainers were each purchased from Thermo Fisher Scientific (Waltham, MA, USA). Glycine and tris(hydroxymethyl)aminomethane (Tris) were purchased from Astral Scientific, (Sydney, NSW, Australia). Fetal bovine serum was purchased from Bovogen (Keilor, Vic., Australia).

2.2 | Animals and cell culture

In this study, all mice were housed under a conventional controlled light and temperature regimen (12-h light: 12-h dark cycle, 21-22°C). All procedures involving mice were conducted in accordance by the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health. Additionally, mice were handled, monitored and killed with the approval of the University of Newcastle's Animal Care and Ethics Committee (approval number A-2015-507) in accordance by the NSW Animal Research Act 1998, NSW Animal Research Regulation 2010 and the Australian Code for the Care and Use of Animals for Scientific

Purposes 8th Ed. Both Swiss (CD1) and OG2 (OCT4-GFP; octamer-binding transcription factor 4, also known as POU5F1) transgenic mice were utilized for isolation of splenocytes, gonocytes, and germ cells from both fetal gonads and mature testes, respectively. OCT4-GFP mice were generously donated to the study by Associate Professor Patrick Western of the Germ Cell Development and Epigenetics Research Group and the Centre for Reproductive Health at the Hudson Institute of Medical Research, Melbourne, Australia. Breeding colonies were then held at Australian BioResources, Garvin Institute of Medical Research (Moss Vale NSW, Australia), and the University of Newcastle's Central Animal House. Immediately before tissue isolation, animals were killed by CO_2 asphyxiation.

All primary and established cell lines were maintained in a humidified atmosphere at 37°C and 5% CO_2. All media was supplemented with 10% fetal bovine serum, 6 mM L-glutamine, 30 μg/mL penicillin-streptomycin and 1 mM sodium pyruvate. Immortalized mouse proximal caput epididymal epithelial (mECap18) cultures were used as a nonspecific cell control for investigating the specificity of modified gonocyte 'phage clones (Sipilä et al, 2004). mECap18 cells obtained as a generous gift from Dr Petra Sipilä (Institute of Biomedicine, Department of Physiology, University of Turku, Turku, Finland) were maintained in DMEM and subcultured at a ratio of 1:10 into 12-well culture plates as previously described.[35] These cells were grown to 60% confluency before being subjected to an 18-hour incubation with modified 'phage. Approximately 3×10^4 isolated primary gonocytes expressing GFP were maintained for 18 hours in DMEM/F-12 in 12-well culture plates coated with 20 mg/mL poly-HEMA for low adhesion during modified 'phage incubation.

2.3 | Isolation and purification of fetal gonocytes

Fetal germ cells were isolated on day E13.5 of pregnancy, at which time the gonads have differentiated to the point that ovaries and testes can be easily distinguished with germ cells still inherently expressing the OCT4-GFP reporter. Isolated embryos were placed in PBS on ice and gonads located and carefully microdissected using a stereo microscope, before being placed in a droplet of HBSS without Ca^{2+} and Mg^{2+}, but supplemented with 1 mM EDTA, 0.5% BSA. In order to preserve proteinaceous binding sites on the surface of germ cells, mechanical isolation using BD Ultra-Fine II 30-gauge needles (BD Biosciences, San Jose, CA) in the absence of trypsin[36] were used to tease the gonads into fragments, facilitating release of germ cells into the supporting media. The media suspension containing germ cells and fragmented gonadal tissue was collected and centrifuged at 400 × g for 5 minutes. Gonadal liberation and single-cell release was further aided by resuspension of the remaining pellet in Cell Dissociation Buffer (enzyme free), HBSS and incubation at 32°C in the dark with slow rotation for 45 minutes; with gentle pipetting of the cell suspension undertaken every 10 minutes.[37] Finally, the single-cell suspension pellet was washed (400 × g for 5 minutes) and resuspended in HBSS without Ca^{2+} and Mg^{2+}, but supplemented with 1 mM EDTA, 0.5% BSA and gently filtered through a 40 μM nylon cell strainer into a precoated (HBSS without Ca^{2+} and Mg^{2+}, 1mM EDTA, 0.5% BSA) polystyrene round bottom flow cytometry tube (12 × 75 mm; BD Biosciences). GFP + cells were isolated by fluorescence-activated cell sorting (FACS) (BD FACSAria II; BD Biosciences) (70 μM nozzle and sample temperature maintenance of 4°C). FACSDiva Version 6.1.3 software (BD Biosciences) was used to gate off forward scatter (cell size) vs side scatter (cell complexity or granularity) (FSC-A vs SSC-A), where a plot of GFP vs. phycoerythrin (PE-A) using wild-type GFP- cells was then used to distinguish between auto fluorescing and true GFP + cells. The purified GFP + gonocyte population was centrifuged at 400 × g and resuspended into fresh HBSS without Ca^{2+} and Mg^{2+}, 1mM EDTA, 0.5% BSA media in preparation for bacteriophage biopanning.

2.4 | M13 Bacteriophage biopanning, sequencing, and preparation for chemical modification

The whole-cell surface 'phage panning procedure used to screen for peptides specifically binding to gonocytes, was conducted in vitro using the filamentous M13 bacteriophage sourced from a 'phage display peptide library kit (New England BioLabs, Ipswich, MA, USA). Figure 1 depicts the process of iterative purification of cell-specific M13 bacteriophage. Approximately 2×10^{11} PFU of the library was added to GFP + gonocyte suspension (~3×10^5 cells/100 μL) and incubated for 1 hour at 37°C with gentle rotation in the dark. Following 4 × 5 minute washes, all unbound 'phage were washed away with HBSS without Ca^{2+} and Mg^{2+}, 1 mM EDTA, 0.5% BSA to ensure elimination of nonspecific 'phage contamination. The 'phage that remained bound to germ cell surfaces were removed by incubating for 10 minutes at room temperature in a general elution buffer (0.2 M glycine–HCl, pH 2.2, 1 mg/mL BSA), for nonspecific disruption of binding interactions, followed by the addition of neutralizing solution buffer (1 M Tris - HCl, pH 9.1).

An early log phase culture of host strain *E coli* K12 ER2738 (F´proA + B+ lacIq Δ(lacZ)M15 zzf::Tn10(TetR)/ fhuA2 glnV Δ(lac-proAB) thi-1 Δ(hsdS-mcrB)5) provided and as described in PhD-12 'phage Display Peptide Library Kit was infected with eluted 'phage and cultured (5 hours at 37°C, 250 rpm) for amplification of first-round eluate. Round one 'phage eluate was recovered from amplified culture supernatant following centrifugation (10 minutes at 12 000 × g, 4°C) and purified by precipitation at 4°C overnight with 20% (w/v) polyethylene glycol 8000/ 2.5 M NaCl. Precipitated 'phage were then concentrated by centrifugation (15 minutes at 12 000 × g, 4°C) and resuspended in 1 mL sterile PBS in preparation for the next round of panning. For this study, the biopanning procedure was repeated a total of 4 rounds, using 2×10^{11} PFU of 'phage from the previously purified round for the consecutive rounds of in vitro selection against gonocytes.

After three rounds of biopanning, the final round eluate of enriched 'phage, with affinity for gonocytes, was prepared for DNA sequencing. Individual 'phage plaques were isolated from LB/IPTG/

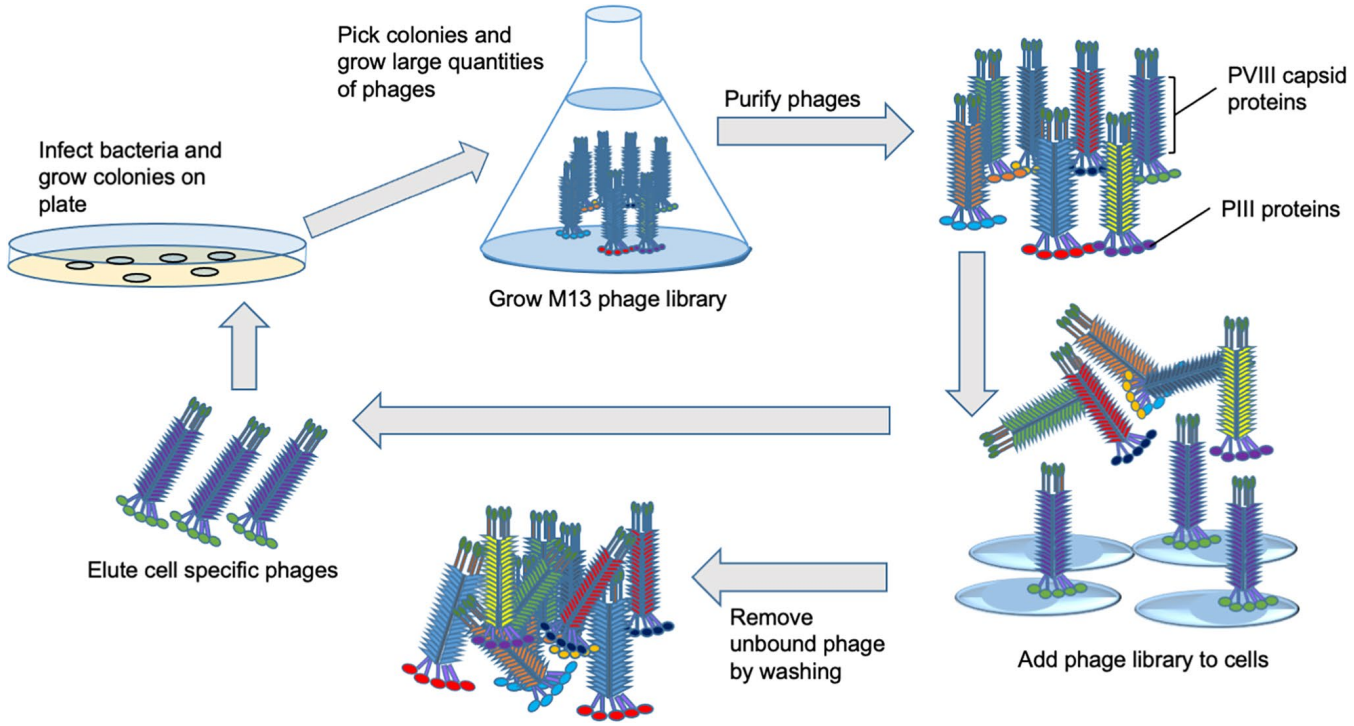

FIGURE 1 Phage panning for cell-specific peptides is an iterative process involving many rounds of incubation, washing and amplification. The major coat protein is PVIII (H2NAEGDDPAKAAFNSLQASATEYIGYAWAMVVVIVGATIGIKLFKKFTSKAS-Phage) and the phage cell-binding is facilitated by a 12 amino acid sequence within the PIII proteins

Xgal plates incubated overnight with *E coli* K12 ER2738 (as described in PhD-12 'phage Display Peptide Library Kit), where only blue plaques carrying the *lacZα* gene were selectedto avoid contamination with wild-type environmental filamentous 'phage. Selected individual 'phage plaques were amplified with an early log phase culture of *E coli* K12 ER2738 at 37°C, 250 rpm for 5 hours. A portion of each individual amplified 'phage culture was used isolate single-stranded DNA (ssDNA), with the remaining 'phage culture precipitated and purified as described earlier. QIAprep Spin M13 Kit (Qiagen, Valencia, CA, USA) was used to isolate 'phage clone ssDNA from 1-3 mL 'phage supernatant according to the manufacturer's instructions. Briefly, 'phage supernatant was precipitated, passed through a QIAprep spin column where the 'phage was retained on the silica membrane. Retained 'phages were then facilitated to bind and lyse, allowing M13 ssDNA to adsorb tightly to the membrane, where following elution, ssDNA was recovered.

Bacteriophage clone ssDNA was sequenced with two separate primers, −28 gIII sequencing primer (5´- HOGTA TGG GAT TTT GCT AAA CAA C −3´) (PhD-12 'phage display peptide library kit) and downstream sequencing primer (5' – ^{HO}AGT TTC GTC ACC AGT ACA– 3') (Merck). Sanger sequencing was performed by the Australian Genome Research Facility Ltd (St. Lucia, QLD, Australia). GENtle DNA sequencing data interpretation software was used to retrieve clone DNA sequences and to provide an interface with the ExPASy Bioinformatics Resource Portal for corresponding amino acid sequence translation to determine foreign peptide sequences. The clones were assessed using the TUPScan in SAROTUP suite, http://immunet.cn/sarotup/cgi-bin/TUPScan.pl, to confirm that they were not commonly known or possible propagation related target-unrelated peptides (PRTU).

2.5 | pBLAST NCBI and Smith-Waterman search for peptide/protein similarities

Basic Local Alignment Search Tool (BLAST) online database (http://blast.ncbi.nlm.nih.gov/Blast) was used to determine 12-mer clone peptide sequence homology with known mouse proteins using a blastp (protein-protein BLAST) algorithm. The database was set as nonredundant protein sequence (nr), the organism was restricted to *Mus musculus* (taxid: 10090). The general algorithm parameters for the search were set with an expect threshold of 10, with appropriate adjustments to improve the results for short queries. Sequences producing significant alignments were set to sort based on E value (lowest to highest). Additionally, a Smith-Waterman similarity search (SSEARCH) was conducted through PepBank (Massachusetts General Hospital) at https://fasta.bioch.virginia.edu/fasta_www2/fasta_www.cgi#sp|Q9QUN9 within the Mouse/Uniprot reference library. The algorithm used was the Smith-Waterman (SSE2, Michael Farrar 2006) (7.2 Nov 2010) and the parameters were BL50 matrix (15:-5)xS, open/ext: −10/−2.

Clustal Omega multiple sequence alignment software was used to align identified amino acid peptide sequences of selected clones, identifying commonly occurring motifs. Additionally, multiple sequence alignment software was used to align the resulting homologous protein sequences with the corresponding clone displayed peptides.

Additionally, a Smith-Waterman similarity search was conducted through PepBank (Massachusetts General Hospital) at https://fasta.bioch.virginia.edu/fasta_www2/fasta_www.cgi#sp|Q9QUN9 within the Mouse/Uniprot reference library. The algorithm used was the Smith-Waterman (SSE2, Michael Farrar 2006) (7.2 Nov 2010) and the parameters were BL50 matrix (15:-5)xS, open/ext: −10/-2.

2.6 | Immunocytochemical assessment of cell surface binding efficiency and specificity of gonocyte 'phage clone

To assess both the efficiency and specificity of the selected gonocyte binding clone, various cell types were isolated from both Swiss CD1 and OCT4-GFP mice and exposed to the 'phage clone of interest (2 x 10^{11} PFU). Following exposure in the dark at 37°C for 1 hour with gentle rotation, cells were washed three times with (PBS for splenocytes, HBSS for gonocytes, DMEM for spg, ps and rs) to remove unbound and loosely adherent 'phage were washed away. 'phage remaining bound to the cell surface were detected using rabbit anti-M13 + Fd bacteriophage coat proteins primary antibody (Abcam, Cambridge, England, UK. CN ab6188, Lot numbers GR3214127-1 and −2) (diluted 1:300) and goat anti-rabbit IgG H&L preadsorbed Alexa Fluor 568 (Abcam, ab175696, Lot numbers GR146340-4 and −5) (diluted 1:200) for 20 minutes and 10 minutes, respectively. Finally, cells were counterstained with 2 μg/mL DAPI for 10 minutes before mounting on poly-L-lysine coated slides with 10% Mowiol 4-88. 'phage binding was analyzed using Axio Imager A1 fluorescence microscope (Carl Zeiss Micro Imaging GmbH, Jena, Thuringia, Germany), with cells categorized on the basis of presence or absence of surface-bound 'phage. Isolation of the aforementioned cell types for assessment of gonocyte 'phage clone binding efficacy and specificity was achieved using published protocols. Briefly, GFP + gonocytes and type A spermatogonia were isolated from OCT4-GFP embryonic gonads and neonatal mouse testes, respectively, as described earlier. Enriched populations of pachytene spermatocytes and round spermatids were isolated from mature Swiss CD1 mouse testes with cell surface antigens being maintained through enzyme-free dissociation[37] and fractionated by unit-gravity sedimentation through linear BSA gradients for 3.5 hours.[38] Splenocytes were isolated from Swiss CD1 spleen tissue followed by homogenization and filtration through a 70 μM cell strainer. Red blood cells were lysed using red blood cell lysis buffer (155 mM ammonium chloride, 24 mM sodium bicarbonate, 0.137 mM EDTA, pH 7.35) for 5 minutes, with remaining splenocytes washed with PBS and diluted to 2 × 10^6 cells/ 100 μL.

2.7 | Chemical modification in 'phage PVIII coat proteins with menadione

To modify the 'phage clone of interest, PEG was avoided during precipitation. Instead, GONBC7 'phages were prepared for modification using isoelectric precipitation, as described by Dong et al[39] Briefly, 'phage clone suspensions were amplified as described earlier, in a 10-fold culture upscale. Following amplification, cell-free supernatant was adjusted to pH 4.2 with 5 M HCl, briefly vortexed and the 'phage pelleted, before residual culture medium and HCl was removed using a PBS wash. Rinsed 'phage pellets were stored at −20°C in PBS for chemical modification.

To covalently attach menadione to the N-terminal of the PVIII proteins of the'phage capsid, N-succinimidyl S-acetylthioacetate (SATA) was added to liberate free thiol groups. The C-3 of menadione is electrophilic and reacts quickly with the nucleophilic thiols[40] once the SATA is deacetylated. After amplification and precipitation, M13 'phage was resuspended in 10 mM PBS, pH 7.8, in a 1.5 ml Lo-Bind Eppendorf tube, and 2700 meq of a fresh solution of SATA in DMSO was added (Figure 2, Reaction 1). The reaction was then slowly rotated end-to-end overnight at room temperature before being subjected to ultracentrifugation using a 10 kDa cut-off membrane. SATA-modified 'phage were washed three times in PBS before resuspension in PBS for storage at −20°C. To deacetylate the SATA (Figure 2, Reaction 2), 100 μl of deacetylation solution (0.5 M hydroxylamine.HCl and 25 mM EDTA in PBS, pH 7.5) was added for every 1 ml of SATA-modified 'phage. The reaction was then incubated on slow end-to-end rotation at room temperature for 2 hours before being cleaned up with ultracentrifugation. For the addition of menadione, (Figure 2, Reaction 3), the 'phage were resuspended in PBS, pH 7.5, and 10 meq, with respect to free thiol, of menadione in DMSO was immediately added to make a final concentration of 10% DMSO. After overnight incubation at 37°C on a thermoshaker, the reaction was again cleaned up by ultracentrifugation. The 'phage was then resuspended in PBS and then twice precipitated by isoelectric focusing before UV-spectrophotometry.

2.8 | Confirmation of 'phage modification by UV-spectrophotometry

The unmodified 'phage were diluted with reverse osmosis (RO) water to match the titer of the menadione-modified 'phage (1 × 10^{10} PFU/mL). A 100-fold dilution in RO water of the two 'phage solutions was made along with a 45 nM menadione solution. These solutions were then used for UV spectrophotometry (UV-VIS Recording Spectrophotometer, UV-2501PC, Shimadzu System No. 2-01442) in the wavelength range of 350 nm to 240 nm and using RO water as a blank.

2.9 | Efficacy of menadione-modified 'phage applied to gonocytes and the negative control cell line, mECap18 cells

Unmodified and menadione-modified GONBC7'phage efficacy for targeted delivery of the redox cycling quinone, menadione, was assessed in vitro using primary and established cell culture techniques

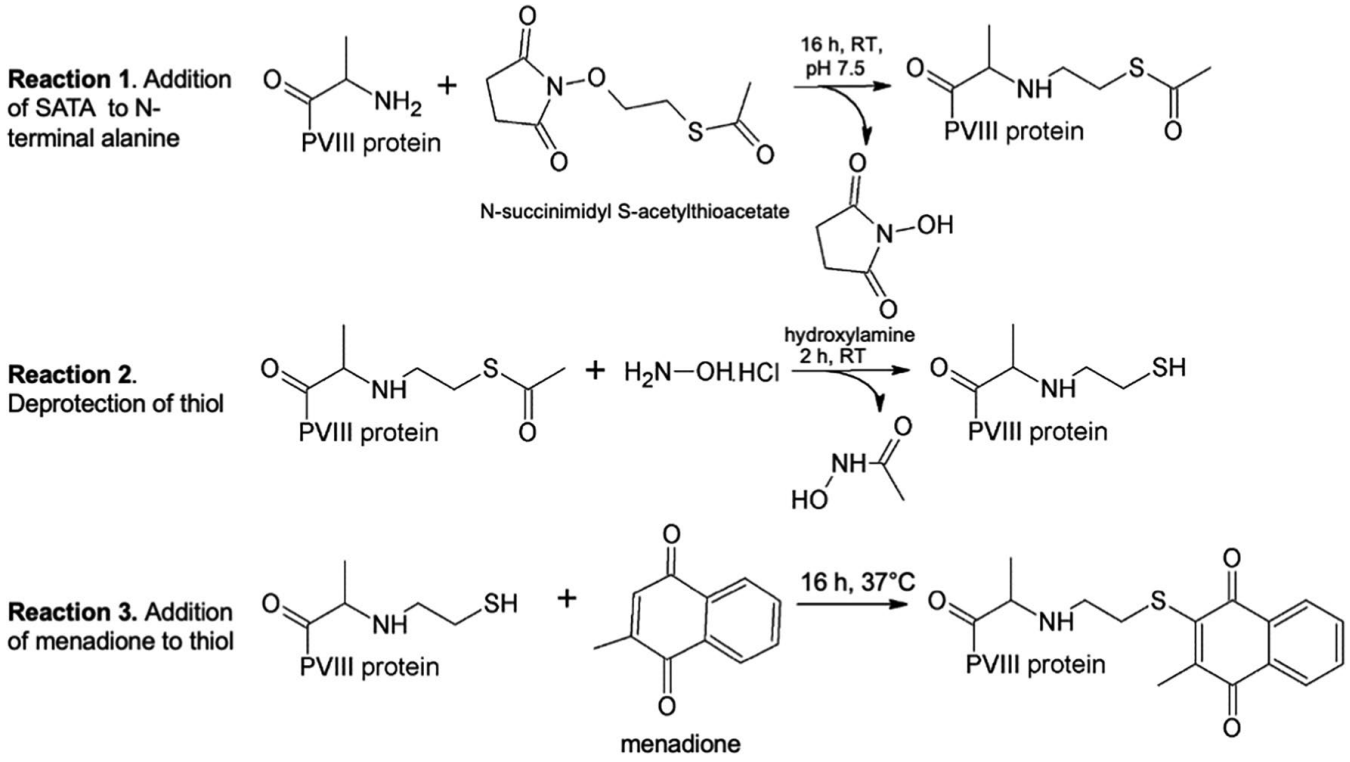

FIGURE 2 Reaction scheme for phage modification with menadione 80x44mm (300 x 300 DPI)

described earlier. Briefly, following 18 hours of culture with menadione-modified GONBC7 (1×10^{10} PFU), unmodified GONBC7 (1×10^{10} PFU) and free menadione (45 nM), cell media were aspirated from mECap18 and primary gonocytes and cells washed with PBS. Cells were detached using Cell Dissociation Buffer (enzyme free), HBSS with gentle agitation, followed by a 5-minute wash in respective culture medium. The dose of menadione was calculated to be equivalent to the dose delivered by the 'phage, assuming that all ~ 2700 PVIII proteins possessed a conjugated menadione molecule. Cell vitality was assessed using trypan blue (final concentration 0.4%) under phase microscopy.

2.10 | Statistical analysis

Statistical analysis of vitality data was conducted using Microsoft Excel (v16.0; Microsoft Corporation, Redmond, WA) as follows: a single-factor ANOVA was conducted on the data from all treatments. If the p-value was < 0.05, then pair-wise Student's *t* tests (two-sample assuming unequal variance) were conducted.

3 | RESULTS

3.1 | Bacteriophage panning and sequencing

Bacteriophage panning was conducted against a mixed population of GFP-expressing gonocytes from both male and female embryos

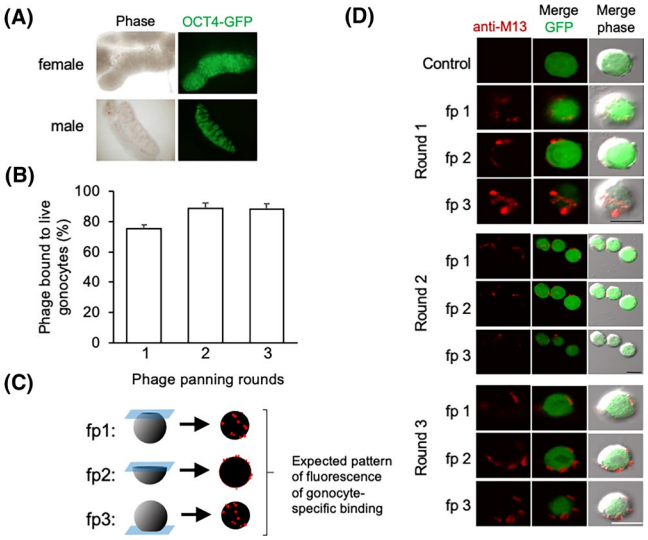

FIGURE 3 A. Male and female gonads at E13.5 showing GFP expressing gonocytes; B. The number of gonocytes displaying phage binding in iterative rounds of panning, n = 3 for each round; C.Explanation of focal plane (fp) imaging. D. Representative images of gonocytes-specific M13 phage binding to gonocytes for panning rounds 1-3 through three focal planes for each gonocyte. GFP is shown in green and the Alexa Fluor 568 tag of the secondary antibody in red (used to detect the anti-M13 primary antibody to the bacteriophage). Scale bar = 10 μm

(Figure 3A). As anticipated, iterative rounds of panning against the gonocyte cells increased the binding efficacy of the 'phage such that 75% and 88% of the gonocyte population had bound 'phage from the

first versus the third rounds, respectively (Figure 3B). Additionally, confocal images taken through several focal planes (Figure 3C) indicated that the surface coverage of the bound 'phage on the gonocytes appeared to decrease over the three rounds (Figure 3D), possibly reflecting an increase in 'phage binding specificity. After completion of the third round of panning, the DNA from eight randomly selected 'phage clones was sequenced and these sequences were translated to amino acid sequences. Five of the eight targeted clones (ie, 75%) harbored unique peptide sequences (Table 1) and, accordingly, additional criteria were applied to aid in the selection of the most appropriate targeting agent. Of the five individual clones, all were deemed to be related to the target and not to the artefactual amplification of the 'phage. We sought to avoid selection of encoded peptides with poor aqueous solubility, thus, the translated peptide sequences of the five unique 'phage clones were analyzed using the Innovagen peptide solubility calculator (https://pepcalc.com/peptide-solubility-calculator.php; Table 1) and clone 8 was predicted to have poor aqueous solubility. The peptides from the remaining four clones possessed very little sequence similarity (Figure 4A), but all were predicted to have good aqueous solubility and had either a neutral or basic nature with a similar overall amino acid composition (Figure 4B,C). Clones 4, 5, and 6 were discounted as candidates as they possessed one or more methionine residues. The thiol group of cysteine and methionine residues are particularly susceptible to oxidation, which could compromise the efficacy of downstream conjugation reactions and/or binding by the peptide to its target. On the basis of this assessment, two 'phage clones, hereafter referred to as GONBC3 and GONBC7, were selected and amplified for confirmation of their specificity of gonocyte binding.

3.2 | Binding of selected gonocyte 'phage clones—cell surface binding efficiency and specificity

Both of the selected clones appeared to have good surface coverage when bound to gonocytes (Figure 5A,B), but showed minimal to no binding to splenocytes (Figure 5C,D). Clones GONBC3 and GONBC7 displayed similar binding efficiency to OCT4-GFP gonocytes, with GONBC3 binding to 81% of the cells and GONBC7 binding to 84.3% of the cells. Similarly, their binding to the nonspecific cell type, splenocytes, was low with binding rates of 4% and 2.7%, respectively (Figure 5D).

3.3 | Physiochemical properties of peptide displayed by GONBC7

The peptide sequence displayed on the PIII capsid of GONBC7 was determined to be GNNPLHVHHDKR. Six of the eight amino acid residues comprising this peptide possess hydroxyl and amine functional groups, whilst only three have hydrophobic properties. The peptide displayed by GONBC3, RDYHPRDHTATW, had a very similar composition to that of GONBC7 with three hydrophobic and five amino acid residues possessing hydroxyl and amine functional groups. The overall hydrophilicity of the two peptides supports the likelihood of good aqueous solubility at physiological pH (Figure 4C). Having selected GONBC3 and GONBC7, their displayed peptides were subjected to sequence alignment using a combination of pBLAST and Smith-Waterman search algorithms (Table 2). This analysis demonstrated that GONBC3 had high similarity to serine-threonine protein kinase R (RIOK2). However, RIOK2 is a cytoplasmic protein involved in ribosomal biogenesis (https://www.uniprot.org/uniprot/Q9CQS5). Similarly, the other candidates with the highest shared highest sequence alignment to GONBC3; that is, U5 small nuclear ribonucleoprotein, procollagen galactosyltransferase and lipocalin 11, are each intracellular proteins. The best candidate for the identity of GONBC3 peptide may thus be the uncharacterized protein, MCG1046832, isoform CRA_c. This protein is ubiquitously expressed but has the highest expression within the embryo (https://www.uniprot.org/uniprot/D3YTU4). In terms of the other selected clone, GONBC7, both forms of alignment algorithm identified high similarity between the encoded peptide and mouse Dickkopf-related protein 3 (DKK3); a secreted protein with receptor antagonist activity. In the case of pBLAST, strong alignment (50% identity, 83% similarity) was recorded for amino acid residues 106-117 of the DKK3 protein. The Smith-Waterman search assessed 1,1745,449 residues in 22,277 sequences. The protein with the best Smith-Waterman ranking (score of 50) was again DKK3 (60% identity, 90% similarity across a 10 amino acid overlap; that is, residues 106-115) (Table 2).

On the basis of their binding efficiency and specificity, as well as their physicochemical characteristics, the translated peptide sequences for clones GONBC3 and GONBC7 were synthesized along with a C-terminus flanking sequence (GGGS) corresponding

TABLE 1 Sequencing results from eight of the clones that bound to OCT4-GFP gonocytes

Clone #	Sequence	Sequence readout quality	PRTU	Molecular weight ($g.mol^{-1}$)	Isolelectric point (pI)	Charge	Nature	Water sol
1-3	RDYHPRDHTATW	good	no	1554.62	7.66	0.2	basic	good
4,5	QVNGLGERSQQM	good	no	1346.47	6.58	0	neutral	good
6	SEELSMRAMKAL	good	no	1365.62	6.75	0	neutral	good
7	GNNPLHVHHDKR	good	no	1423.54	10.11	1.3	basic	good
8	SSVQAIALKQGS	good	no	1188.33	9.86	1	basic	poor

Note: PRTU = propogation related, target unrelated.

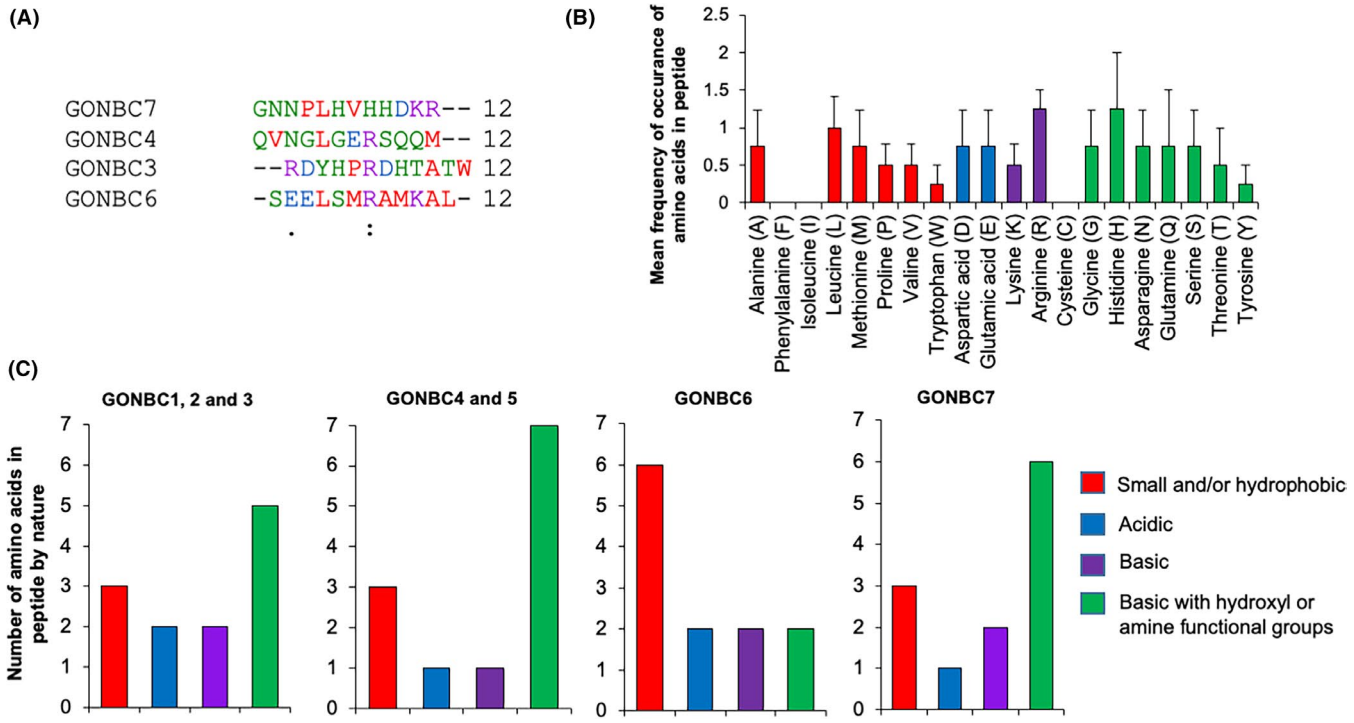

FIGURE 4 A. Sequence alignment of peptide sequences expressed by clones GONBC3, 4, 6, and 7. B. Occurrence of individual amino acids, colored by nature, composing the peptide sequences expressed by clones GONBC1-7. C. Nature of amino acids composing peptide sequences expressed by clones GONBC1-7

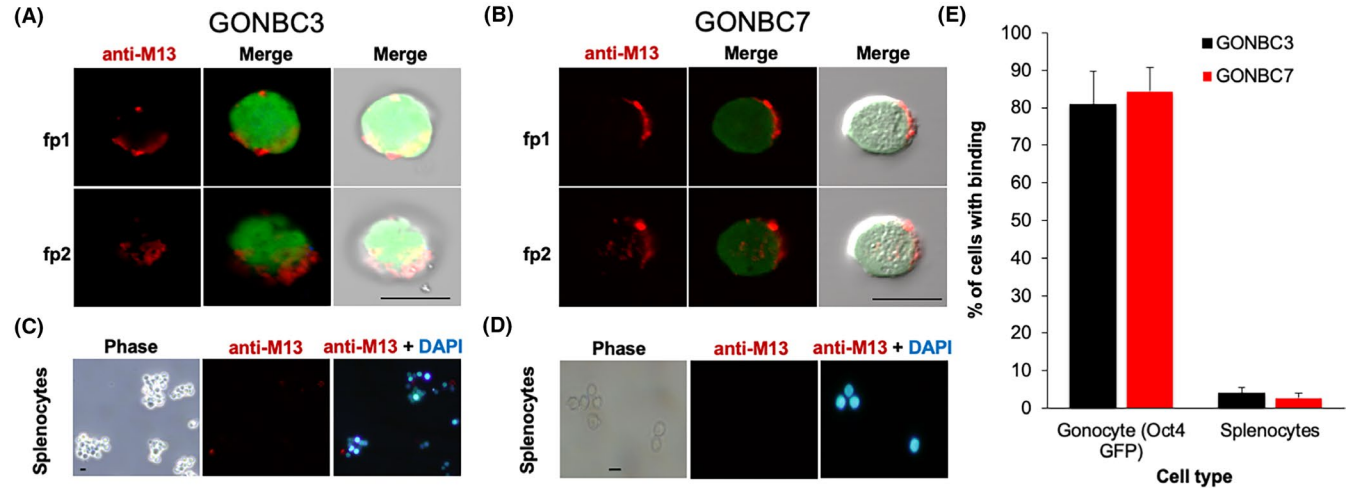

FIGURE 5 Representative images of gonocyte specific M13 phage clones GONBC3 and GONBC7 binding to (A and B) OCT4-GFP gonocytes (images captured at two focal planes (fp) for each cell), and (C and D) splenocytes. Bound phage were detected using anti-M13 antibody and a fluorescently-tagged secondary (red), the splenocyte nuclei were stained with DAPI (blue). Scale bar = 10 μm. E. Comparison of binding efficiency between GONBC3 and GONBC7

to the PIII capsid protein. Additional modifications included acetylation of the N-terminus and biotinylation of the C-terminus giving NH_2-GNNPLHVHHDKRGGGS(Lys(Biotin) and NH_2-RDYHPRDHTATW GGGS(Lys(Biotin)). Contrary to expectations, however, these synthetic peptides displayed minimal binding efficiency, with less than 1% of isolated gonocytes having detectable levels of bound peptide following an incubation of 1 hour (results not shown).

3.4 | Assessment of the binding efficiency and specificity of 'phage clone GONBC7

Owing to the lack of targeting efficacy displayed by the synthetic GONBC3 and GONBC7 peptides, we elected to instead pursue the development of a gonocyte targeting vector incorporating the entire 'phage clone. GONBC7 was selected for this purpose as it showed marginally greater binding to gonocytes isolated from

TABLE 2 Top five hits from Smith-Waterman similarity search, conducted through PepBank

		% identity	% similarity	Amino acid overlap	Alignment Same identity in red Similarity in blue
GONBC3					
1	Serine/threonine-protein kinase R	50	83.3	12	RDYHPRDHTATW 140 RDYHKHRHNVSW 151
2	U5 small nuclear ribonucleoprotein	58.3	66.7	12	RDYHPRDHTATW 257 KDLHPRDIDAFW 268
3	MCG1046832, isoform CRA_c	75	87.5	8	RDYHPRDHTATW 145 HPQDHQAT 152
4	Procollagen galactosyltransferase	66.7	88.9	9	RDYHPRDHTATW 74 HPRERTALW 83
5	Lipocalin 11	41.7	83.3	12	RDYHPRDHTATW 26 QDFHPEQVTGPW 37
GONBC7					
1	Dickkopf-related protein 3 (DKK3)	60	90	10	GNNPLHVHHDKR 106 GNNTVHVHQE 115
2	tRNA butosine-synthesizing protein (TWY3)	54.5	90.9	11	GNNPLHVHHDKR 213 NNPLCTHKNRR 222
3	26S proteasome non-ATPase regulatory subunit 10 (PSD10)	54.5	81.8	11	GNNPLHVHHDKR 173 GNTPLHLACDE 183
4	Fatty acid desaturase 1 (FADS1)	50	87.5	8	GNNPLHVHHDKR 348 PMHIDHDR 355
5	Predicted gene 10772 (F7AIG4)	55.6	77.8	9	GNNPLHVHHDKR 45 PAHLHRHKR 53

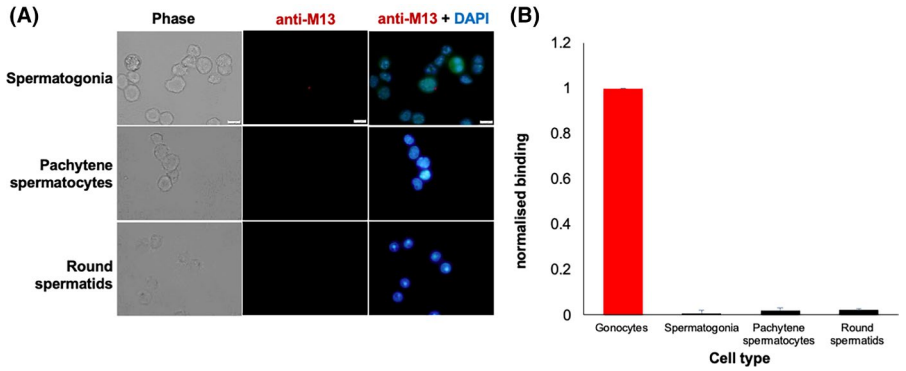

FIGURE 6 A. The gonocytes-binding specific phage, GONBC7, was incubated with, the male spermatogenic cells, spermatogonia (Swiss CD1 mice), pachytene spermatocytes and round spermatids (Oct4GFP mice), n = 3.The phage was then detected using anti-M13 antibody and a fluorescently-tagged secondary, red, the nuclei were stained with DAPI, blue, some autofluorescence appeared in the spermatogonia,green. Images taken using 40x objective. B. Percent binding data for GONBC7 was compared by normalizing to percent binding to gonocytes, n = 3. Scale bar = 10 μm

E13.5 OCT4-GFP gonads (ie, 84% vs 81%; Figure 5E). Moreover, the GONBC7 'phage displayed more uniform adherence across the plasma membrane of these cells (Figure 5B) and, importantly, GONBC7 displayed only minimal binding when incubated under equivalent conditions with unrelated populations of splenocytes (2.6%) or latter stage male germ cells including spermatogonia (0.7%), pachytene spermatocytes (1.7%), and round spermatids (1.7%) (Figure 6), thus confirming its affinity and specificity for gonocytes.

3.5 | Confirmation of modification in 'phage PVIII coat proteins of GONBC7 with menadione

The normal absorption spectra of menadione in RO water is shown in Figure 7 (yellow trace). Notably, unconjugated menadione has an absorption maximum at 262 nm and an additional absorption peak, albeit of lower magnitude, at 337 nm. By comparison, the absorption maximum of the unmodified 'phage is 258 nm (Figure 7, green trace). As anticipated on the basis of previous work,[41] the conjugation of

menadione to the 'phage produced a modest shift in the UV spectra such that a peak in absorption was recorded at 260 nm (Figure 7, blue trace); midway between that of unconjugated menadione and the unmodified 'phage. In addition, the modified 'phage displayed a notably higher absorption spectrum than that of the unmodified 'phage at wavelengths over 300 nm, coincident with that of the ancillary menadione peak.

3.6 | Efficacy of menadione-modified 'phage when applied to gonocytes and mECap18 cells

Having confirmed the menadione conjugation of the gonocyte targeting GONBC7 'phage, we next sought to assess the cytotoxicity of this vector. For this purpose, primary cultures of gonocytes were challenged with the menadione-modified GONBC7 'phage for 18 hours prior to the assessment of their vitality. This strategy led to a highly significant 3-fold decrease in gonocyte viability compared to equivalent populations of untreated cells ($P = .0007$) (Figure 7A). The selectivity of this response was demonstrated by the complete absence of any effect on the vitality of control gonocyte populations, comprising cells incubated with unmodified 'phage, free menadione, or the DMSO vehicle (Figure 8A). Moreover, the unrelated mECap18 cell line also proved recalcitrant to all applied treatments, including that of the modified GONBC7, with vitality remaining above 89% irrespective of the treatment (Figure 8B). Such data provide proof-of-concept that the specificity of 'phage adhesion can be harnessed to selectively ablate progenitors of both male and female germs cells in vitro.

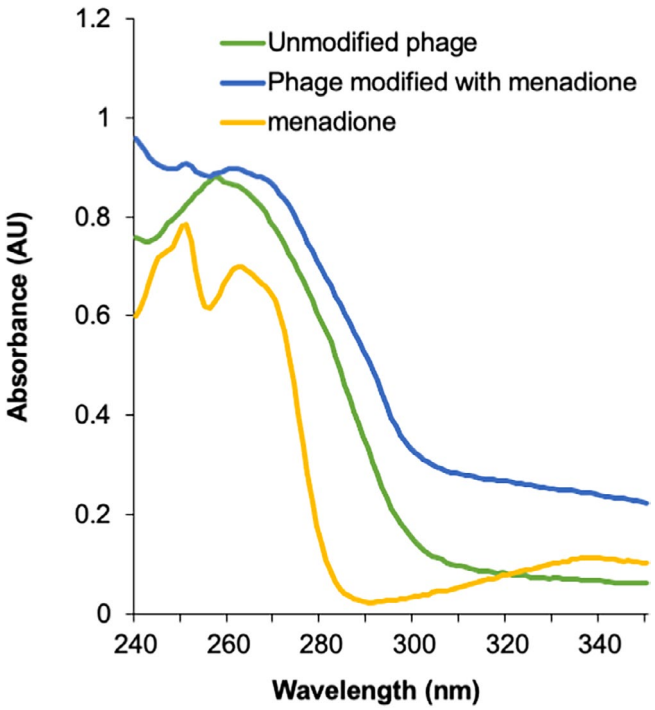

FIGURE 7 Confirmation of phage modification using UV spectrophotometry. UV-vis spectra showing the absorbance of solutions comprising unmodified M13 bacteriophage (green trace), menadione-modified M13 bacteriophage (blue trace), and free menadione (yellow trace). The viral pfu counts were comparable for M13 and M13-menadione. The concentration of free menadione was titrated to be comparable to that of M13-menadione based on the assumption of 100% saturation of the thiol linker (that is, 2700 menadione molecules per phage)

4 | DISCUSSION

The ultimate objective of this field of research is to develop novel reagents that can destroy the undifferentiated gonocyte population within the gonadal primordia of male and female embryos, thereby effectively sterilizing the offspring in utero and obviating the need for postnatal surgical castration. If it could be achieved, such a facility would revolutionize the archaic, unsophisticated practices that are currently being used to regulate the fertility of domestic and feral animals while significantly improving animal welfare. In this study, we describe one possible strategy by which such an objective might be achieved.

Using 'phage biopanning we were able to select a 'phage clone, GONBC7, that exhibited affinity to and specificity for embryonic gonocytes. The encoded peptide, GNNPLHVHHDKR, displayed by GONBC7 on its PIII capsid proteins, has similarity to amino acid residues 106-117 of Dickkopf-related protein 3 (DKK3). DKK3 is an extracellular, dual-function protein that has been shown to effect inhibition of the canonical Wnt signaling pathway in a cell-specific and context-dependent manner.[42] This activity appears to rest with the ability of DKK3 to bind promiscuously to a diverse group of cell surface expressed receptors and co-expressed ligands.[42] Whilst, the precise mechanism of DKK3 action has yet to be elucidated, the protein has been found to have a specific role in specifying cell fate during embryogenesis.[43] This finding aligns with the essential regulatory role that Wnt signaling fulfills during both ovarian[44,45] and testicular development.[45] It is possible, therefore, that the GONBC7 'phage peptide may mimic DKK3 by binding to an, as yet, elusive cell surface receptor on the gonocytes. Clinically, this inhibitory pathway is particularly important in the male because the unprecedented global increase in testicular cancer appears to have its origins in fetal life and to reflect the impaired ability of gonocytes to differentiate into spermatogonial stem cells during gonadal development.[46] These data, raising the possibility that DDK3 might play a key role in the regulation gonocyte differentiation with implications for the developmental origins of cancer, warrant further consideration.

Contrary to our expectation, the affinity of GONBC7 to gonocytes was not exhibited by the isolated GNNPLHVHHDKR peptide. Indeed, the almost complete lack of binding of the synthetic peptide displayed by the GONBC7 clone indicates that, for this peptide to retain its binding capacity and for the effective targeting of gonocytes, it may need to remain within the three-dimensional context of the native bacteriophage protein. Thus, to retain the absolute specificity and selectivity of GONBC7, modification in the 'phage itself appeared to be a superior strategy. Li and co-workers[33] have

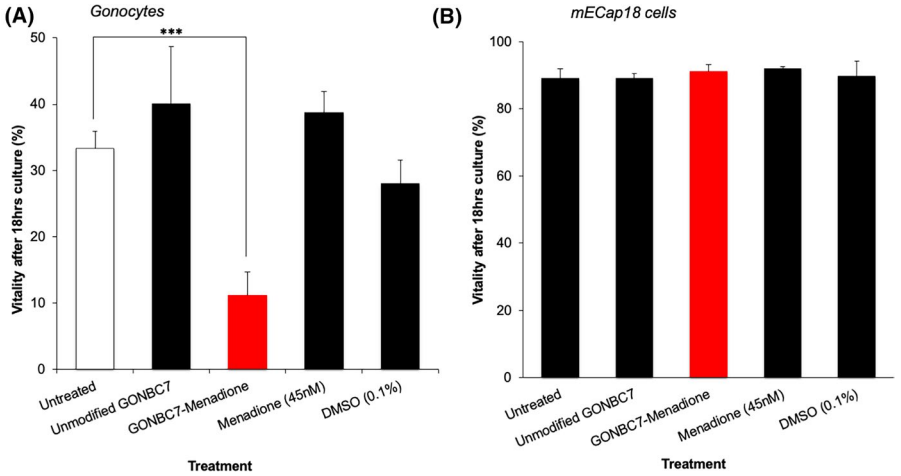

FIGURE 8 Assessment of the viability of A. OCT4-GFP-gonocyte primary cultures or B. mECAP18 cell cultures following 18 hours treatment with either unmodified GONBC7 phage, menadione-modified GONBC7 phage, menadione or DMSO vehicle. All experiments were replicated between 3-9 times and data are shown as mean vitality + SEM. *** $P = .0007$. The concentration of free menadione was titrated to be comparable to that of M13-menadione based on the assumption of 100% saturation of the thiol linker (that is, 2700 menadione molecules per phage)

previously reported the use of NHS conjugated to tetramethylrhodamine and UV-visible spectrophotometry to confirm the reactivity of the M13 bacteriophage amine groups; demonstrating that PVIII capsid proteins are readily modified using this conjugation strategy, with individual 'phage each accommodating up to 1600 molecules of dye. Therefore, this approach would also mean that the quantity of menadione delivered to the gonocytes would be at least a thousand-fold greater than that which could be delivered using menadione conjugated directly to the peptide. The success of our 'phage modification with menadione was qualitatively confirmed by UV spectrometry. However, the most compelling evidence for the success of the modification along with the selective targeting of the modified 'phage was the significant cell death precipitated by the menadione-modified 'phage in the cultured gonocytes, but not in the negative control mECap18 cell line. The use of redox chemistry is significant because it automatically achieves a natural amplification in the efficiency of the cell ablation system, each molecule of menadione conjugated to the 'phage generating many molecules of free radical toxicant. The net result of this strategy is to recapitulate the impact of ionizing irradiation on the reproductive system by selectively removing the germ cell component of male and female gonads while leaving the somatic cells intact.[47]

The modified 'phage, used to effect the oxidative attack on gonocytes illustrated in this study was chosen for its specificity for the target cell type and the incorporation of low doses of reagent that are nontoxic and readily metabolized. This was evidenced by the lack of impact on the vitality of gonocytes following the application of 45 nM unconjugated menadione. It is therefore likely that, if applied in vivo, the modified 'phage would elicit a highly targeted response with reduced risk of deleterious side effects. Intravenous administration has been the most commonly utilized method for in vivo 'phage delivery and has been shown to be both safe and effective.[29] Moreover, transplacental transfer of bacteriophage after intravenous administration has been observed in both guinea pig[48] and mouse.[49] We are proposing that a single injection of the intact modified phage, once administered intravenously, would cross the placenta to deliver the cytotoxic agent to the fetal gonads and thereby effect ablation of the gonocytes.

The most important future application of this biotechnology is for the intrinsically specific and humane nonsurgical control of fertility in pest and domestic animal species. The development of a novel, robust, single-administration sterilization technology has the potential to have a dramatic impact on the way in which we manage the fertility of domestic animals in veterinary practice. The future possibilities for this technology, however, go beyond the microcosm of reproductive science. Fundamentally, this article describes a cell ablation technology that could be used to remove any cell type exhibiting a sensitivity to oxidative stress, including several different types of cancer.[50]

In summary, selection of a bacteriophage clone capable of targeting the surface of gonocytes, following by direct modification in the 'phage coat proteins with the redox-cycling naphthoquinone, menadione, produced a reagent capable of inducing the rapid, effective depletion of gonocytes, in vitro. These data open the way for the use of this technology in vivo to effect the humane nonsurgical castration of domestic and feral animals. Such a technology should be of immense value to conservationists, veterinarians, biotechnologists, and agriculture.

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AUTHOR CONTRIBUTIONS

BAF performed the study, drafted, and revised the manuscript. RJA, PSW, and BN conceived and designed the study and revised the

manuscript. KM performed the study. NAT performed the study. NDS performed the spectrophotometry analyses. All authors approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The raw data underpinning this manuscript are available at the following location: http://cr8pubprd.priv/publish/A_novel_approach_to_non-surgical_sterilization_20200729141421.zip

ORCID

Robert J. Aitken https://orcid.org/0000-0002-9152-156X

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