Identification and Characterization of Sertoli and Leydig Cell Homing Peptides

Introduction

The testis, being an immuno-privileged organ with the toughest blood-testis barrier (BTB), creates a major hurdle in the development of male contraceptive drugs and the treatment of testicular infections, infertility, and cancer. Limitations of conventional drug delivery to treat testicular pathology include adverse side effects of systemically administered drugs, high dosing frequency, inability to achieve sufficiently high intra-testicular concentrations for therapeutic activity, and failure of large molecules to cross the blood–testis barrier. These issues can be circumvented by specifically targeting Sertoli and Leydig cells with the help of targeting ligands.

Targeted drug delivery to treat testicular pathologies will be more advantageous than conventional drug delivery methods. Targeted drug delivery assists the drug molecules in accumulating at the desired site or tissue and reduces the systemic side effects. Targeted drug delivery involves passive targeting based on anatomic or physiological factors and active targeting using specific targeting ligands[1]. Depending upon the type of receptor expressed or overexpressed, the location and nature of the cell, and the metabolism profile of a specific cell or pathological condition, they have specific tags called zip codes[2]. These zip codes can be utilized to differentiate between target and non-target tissues or cells for identifying targeting ligands. Homing peptides are the potential molecules as ligands for targeted drug delivery and diagnosis as well as can act as therapeutic molecules. They have various advantages over other types of ligands, such as high specificity, low immunogenicity, ease of modification, low cost, and rapid clearance from non-target sites, etc[3]. Phage display peptide libraries provide an excellent platform for the selection of such peptide ligands using the procedure called bio-panning. The phage display technique was firstly described by George P Smith in 1985. In phage display, DNA encoding for a specific peptide sequence is incorporated into the the bacteriophage genome, leading to the expression of the peptide on the bacteriophage coat[4]. People have identified peptide ligands targeting various cancers and normal organs, including the brain, kidney, skin, pancreas, prostate, Breast tissue, human umbilical cords, adipose tissue and heart[2,5–13]. Peptides are being used as therapeutics and ligands for active targeting of drugs. RGD and NGR peptides recognizing integrin and aminopeptidase N are the tumour targeting peptides. Peptides identified using phage display libraries are being used to deliver drugs

across blood tissue barriers such as blood-brain barrier and gastrointestinal tract[14]. Peptides are highly selective and potent hence provides good efficacy, safety, and tolerability. They are cost-effective and easy to manufacture and modify[15]. Germ cells, Sertoli cells, and Leydig cells are essential components of the testis. Sperm production occurs in seminiferous tubules with Sertoli cells, and androgen production occurs between seminiferous tubules by Leydig cells. Various types of disorders are associated with these cells, including infertility, testicular cancer, and oxidative stress. Novel peptides targeting Sertoli cells can open new avenues for targeted drug delivery to testis for the treatment of various testicular pathologies.

Objectives

1. Identification of Sertoli and Leydig cell homing peptides using phage display peptide library
2. Characterization of identified Sertoli and Leydig cell homing peptides
3. Identification of the target/receptors of Sertoli and Leydig cell homing peptide

Materials and Methods

Phage display library

Ph.D.-12 library was purchased from New England Biolabs. This is a combinatorial library of random 12-mer peptides fused to a minor coat protein (pIII) of the M13 phage. The displayed peptide is expressed at the N-terminus of pIII. The library consists of approximately 10^9 unique sequences. 10µl of the library was used to start the experiment, which contained approximately 100 copies of each phage clone displaying a unique peptide.

Cell Culture

TM3 (Mouse Leydig cells) (ATCC CRL-1714) and TM4 (Mouse Sertoli cells) (ATCC CRL 1715) were obtained from an American-type culture collection, H9C2 from NCCS Pune, and HEK293 was gifted by Dr. Susane Thomas of Bioinformatics Center ICMR-NIRRCH.

TM3 and TM4 cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) Nutrient Mixture F-12 supplemented with 5% horse serum and 2.5% fetal bovine serum, and H9C2 and HEK293 cells were cultured in DMEM Nutrient Mixture F-12 (Ham) (1:1) supplemented with 10% fetal bovine serum. All the cell lines were maintained at 37° C with 5% CO2.

Animal use and care in the study

All the procedures performed on adult Balb/C male mice (6 to 8 weeks old) were approved by the Institutional Animal Ethics Committee of ICMR-National Institute for Research in Reproductive and Child Health Mumbai (IAEC-03/20).

In-vitro biopanning

Mouse Sertoli cells (TM4)/ Mouse Leydig cells (TM3) were seeded at the density of 0.5 x 10^6 cells/well in 6 well plates before 48 hours of biopanning. The spent medium was removed on the day of biopanning, and DMEM/F12 w/o serum was added to the wells. After 1-hour incubation at 37^0C with 5%, CO_2, a biopanning solution containing 1×10^{11} phages in 10µl of Ph.D. 12 library, protease inhibitor, and chloroquine in PBS with 0.1% BSA was added and incubated for 1 hour, followed by removing unbound phages with washing four times for 5 minutes with 0.1%BSA. The cell surface homing phages were eluted with two washes of 1 mL 0.1 M HCl-Glycine, pH 2.2 + 0.9% NaCl for 5 minutes. Again, the cells were washed with 0.1% BSA twice, and 1 mL of 30 mM Tris –HCl, pH 8.0, was added and incubated on ice for 30 min, and cells were frozen at -20^0 C overnight. The next day cell-penetrating phages were collected from the disrupted cells. Enrichment of the Sertoli/Leydig cell surface homing and Sertoli/Leydig cell-penetrating phages was achieved with three rounds of biopanning[16].

In-vivo biopanning

Amplified Sertoli/Leydig cell surface homing phage pool and Sertoli/Leydig cell-penetrating phage pool from 3rd round of in-vitro biopanning were used to perform in-vivo biopanning. Briefly, 1×10^{11} Phages in 100 µl of DMEM medium were injected into Balb/C mice intravenously and allowed to circulate for 30 min. Mice were sacrificed, and all vital organs were collected and stored at -80^0C. The next day, testis was minced in DMEM medium, and phages were recovered and amplified for the next round of biopanning. Enrichment was achieved through three rounds of such in-vivo biopanning[17].

Phage titration and amplification during biopanning

Phage titration was performed with the qPCR standard curve method described earlier by Peng et al.[18], ten-fold concentrations of M13 ssDNA from 0.1fg/µl to10^6 fg/µl (2.689×10^1 gc/µl to 2.689×10^8 gc/µl) are used as standards. Samples were precipitated with PEG/NaCl for M13 phage isolation and treated with DNase I at 37^0C for 10 min, followed by heat denaturation at 100^0C; these samples were used as a template for qPCR. The reaction mixture of 10µl consists of 5µl SYBr green Power Up 2x MM, 0.5 µl (10 µM stock) forward primer and reverse primer each, 2µl nuclease-free water, and 2µl template ssDNA (standard/sample). Primers used are forward primer 5′-CAC CGT TCA TCT GTC CTC TTT-3′ and reverse primer 5′-CGA CCT GCT CCA TGT TAC TTA G-3′. Reaction conditions were 50^0C for 2 min, 95^0C for 2 min, 40 cycles of 95^0C for 15sec and 60^0C for 1 min, melt curve at 95^0C for 15 sec, 60^0C for 1 min and 95^0C for 15 sec. The reaction was performed on Applied Biosystem's QuantStudio5 Real-time PCR and analyzed with QuantStudio™ Design and Analysis software.

Plaque assay and sanger sequencing

Plaque assay was performed according to the New England Biolab's instruction manual. Blue-colored plaques were picked and amplified in E. coli ER2738 host cells. Amplified phage clones were isolated with PEG precipitation, and ssDNA was isolated according to the protocol mentioned by Green et al[19]. PCR was performed to amplify the product using forward primer: 5'-TGG TTG TTG TCA TTG TCG GC-3' and reverse primer: 5'-GCA AGC CCA ATA GGA ACC CA-3'. PCR reaction mixture of 50µl was consisted of 25µl 2x DreamTaq Green PCR Master Mix, 2.5µl each of forward and reverse primer (10µM stock), 15µl nuclease-free water, and 5µl template. PCR conditions were Initial denaturation at 95^0C for 5 min, 40 Cycles of denaturation at 95^0C for 30 sec, annealing at 55^0C for 30 sec, extension at 72^0C for 30 sec and Final extension at 72^0C for 5 min. The reaction was performed on Agilent's Sure Cycler 8800. The PCR product was run on 1.8% agarose gel, extracted using PureLink Quick gel extraction kit; Sanger sequencing was performed with -96gIII reverse sequencing primer provided in PhD 12 phage library kit.

Next-generation sequencing of phage pool

Phage pool from the 3rd round (last round of in-vitro panning) and the 6th round (last round of in-vivo panning) were subjected to NGS. Phage DNA (ssDNA) was isolated from the phage pools as described by Green et al. [19], and PCR was performed to prepare 77 bp amplicon for Illumina sequencing; the primers and barcodes were used were picked from Matochko et al.[20]. forward primer 5'CCT TTC TAT TCT CAC TCT3', reverse primer 1 with barcode 5'TTC CGA TAA CCC GAA CCT CCA CC3' and reverse primer 2 with barcode 5'CTG ACC GAA CCC GAA CCT CCA CC3'. The reaction mixture consisted of 25 µl OneTaq® Hot Start Quick-Load® 2X Master Mix, 5µl of each forward and reverse primer and 5µl of template ssDNA. The reaction was performed at 95^0C for 3 min, 35 cycles of denaturation at 94^0C for 10 sec, annealing at 60^0C for 20sec, elongation at 72^0C for 30sec, and final elongation at 72^0C for 5 min. The PCR product was run on 2% agarose gel and was extracted using GeneJet gel extraction kit. Polyacrylamide gel electrophoresis (10%) was performed with 2µl of final product to be subjected to sequencing along with low range DNA ladder. The final 77bp PCR product was further submitted for Illumina sequencing at Bioxplore labs, Chennai, India.

Peptide synthesis

Biological grade SCHP1, SCHP2, LCHP1 and LCHP2 peptides with >95% purity were commercially obtained from AsianBioChem, Thrissur, Kerala, India. FITC tagged peptides i.e. SCHP1-Ahx-FITC, SCHP2-Ahx-FITC, LCHP1-Ahx-FITC, and LCHP2-Ahx-FITC and Cy5.5

tagged peptides i.e. {C(Cy5.5)}-SCHP1, {C(Cy5.5)}-SCHP2, {C(Cy5.5)}-LCHP1, and {C(Cy5.5)}-LCHP2 with 99% purity were obtained from Lifetein, LLC, USA.

Confocal microscopy of peptides

Briefly, 0.1×10^6 cells were seeded on a pre-sterilised coverslip in 12 well plates and allowed to attach overnight. The next day FITC labeled SCHP1, SCHP2, LCHP1, and LCHP2 peptides were added per ml per well and incubated for 1 hr. Cells were washed three times with DPBS w/Ca Mg, fixed with 4% PFA, and counterstained with DAPI for the nucleus and rhodamine-phalloidin (Invitrogen Cat#R415) for the cytoskeleton. Slides were prepared and analyzed with confocal microscopy.

Flow cytometry analysis of peptides

Briefly, 0.5×10^6 cells were seeded per well in 6 well plates and allowed to attach overnight. The next day, cells were treated with different concentrations of the FITC labeled SCHP1, SCHP2, LCHP1, and LCHP2 for 1 hr. After the incubation, cells were detached with 1Mm EDTA solution, washed three times with DPBS, and analyzed with the flow cytometer.

MTT assay

Cytotoxicity of the SCHPs and LCHPs was assessed with MTT assay. Briefly, 0.05×10^6 TM4/ TM3 cells were seeded per well of 96 well plates and allowed to attach overnight. The next day, the cells were treated with two different two-fold increasing concentrations of SCHP1, SCHP2, LCHP1 and LCHP2 peptides for 48 hrs. After 48 hr incubation, spent medium was removed, and MTT solution was added at 50µg/well and incubated for 4 hr at 37^0C. MTT solution was removed, and 100µl of DMSO was added to each well and incubated for 30 min at room temperature. OD was taken at 570 nm, and the percent cell viability was calculated.

Circular Dichroism Spectroscopy

Circular dichroism (CD) spectroscopy was performed on a Jasco J-810 spectropolarimeter using a 1-mm cuvette. The peptides were dissolved in different concentrations i.e 10%, 25%, 50%, 75% and 90% of trifluoroethanol (TFE) in water at a concentration of 0.5 mg/mL. Spectra were collected every 2 nm from 260 to 190 nm. Spectra were collected at every 2 nm from 260 to 190 nm. The CD spectra are reported as CD [mdeg] and the secondary structure were determined using Reed's reference.

In-vivo imaging

SCHP1, SCHP2, LCHP1, LCHP2 and CTP conjugated to Cy5.5 NIR dye were obtained from LifeTein LLC for in vivo imaging using IVIS lumina III in-vivo imaging system. Adult male Balb/C mice (6-8 weeks old were randomly distributed in groups (n=3), i.e., SCHP1, SCHP2, and free Cy5.5 dye. Cy5.5 labeled SCHP1 and SCHP2 were injected into mice intravenously

20µg in 100µl PBS. Mice were dissected, and vital organs, including testis, were collected after 1hr,6hrs, and 24 hrs. The organs imaging was performed using Perkin Elmer's IVIS lumina III small in-vivo imaging system, and images were analysed with Living Image software.

Results:

Screening and Identification of Sertoli cell homing peptides

SCHPs/ LCHPs were identified using the PhD12 phage display peptide library. Three rounds of in-vitro bio-panning with TM3 (mouse Leydig cell line)/ TM4 (mouse Sertoli cells line) were performed to select Sertoli/Leydig cell surface homing peptides (LCSHPs) and Sertoli/ Leydig cell-penetrating peptides (LCPPs). Phages obtained after the third cycle of the TM3 bio-panning were used for in-vivo bio-panning with the Balb/C mouse model, wherein again three rounds of the bio-panning procedure were performed to select testis homing peptides from the SCSHPs/LCSHPs and LCPPs/LCPPs (Illustration 1).

After the 6^{th} round of bio-panning, i.e., the 3^{rd} round in the mouse model, we got enriched SCSHP/LCHP and SCPP/LCPP pools from the testis. M13 bacteriophage titration after each round indicated enrichment of phage pool in 3^{rd} and 6^{th} rounds of bio-panning for screening SCSHPs/LCSHPs and SCPPs/LCPPs (Fig.1).

Sertoli/Leydig cell homing peptide identification was performed using next-generation amplicon sequencing. Phage pools from the 3^{rd} round (last round of in-vitro panning) and 6^{th} round (last round of in-vivo panning) from the SCSHP and SCPP biopanning were subjected to high throughput sequencing. The number of unique peptides normalised with a total number of good reads for both the SCSHP/LCSHP and SCPP/LCPP panning experiments was decreased in the 6^{th} round compared to the 3^{rd} round (Fig. 1), which indicates enrichment of a few peptides from 3^{rd} round to 6^{th} round. The overall comparison of the unique peptides found in all four sequencing runs is shown in Figure.2). Only 1551 and 127 peptides were found common in all the four phage pools sequenced for Sertoli cell and Leydig cell respectively. Peptides GSWNTFRAQPTI and YSLRLTSVTAPT were selected for further validation and named SCHP1 and SCHP2, respectively. Only 103 common peptides were found in all the SCHPs and LCHPs and only 1 in the selected SCHPs and LCHPs. The top 10 selected peptides and their frequency in last round of each bio-panning is shown in Table 1a. and 1b. for SCHPs and LCHPs.

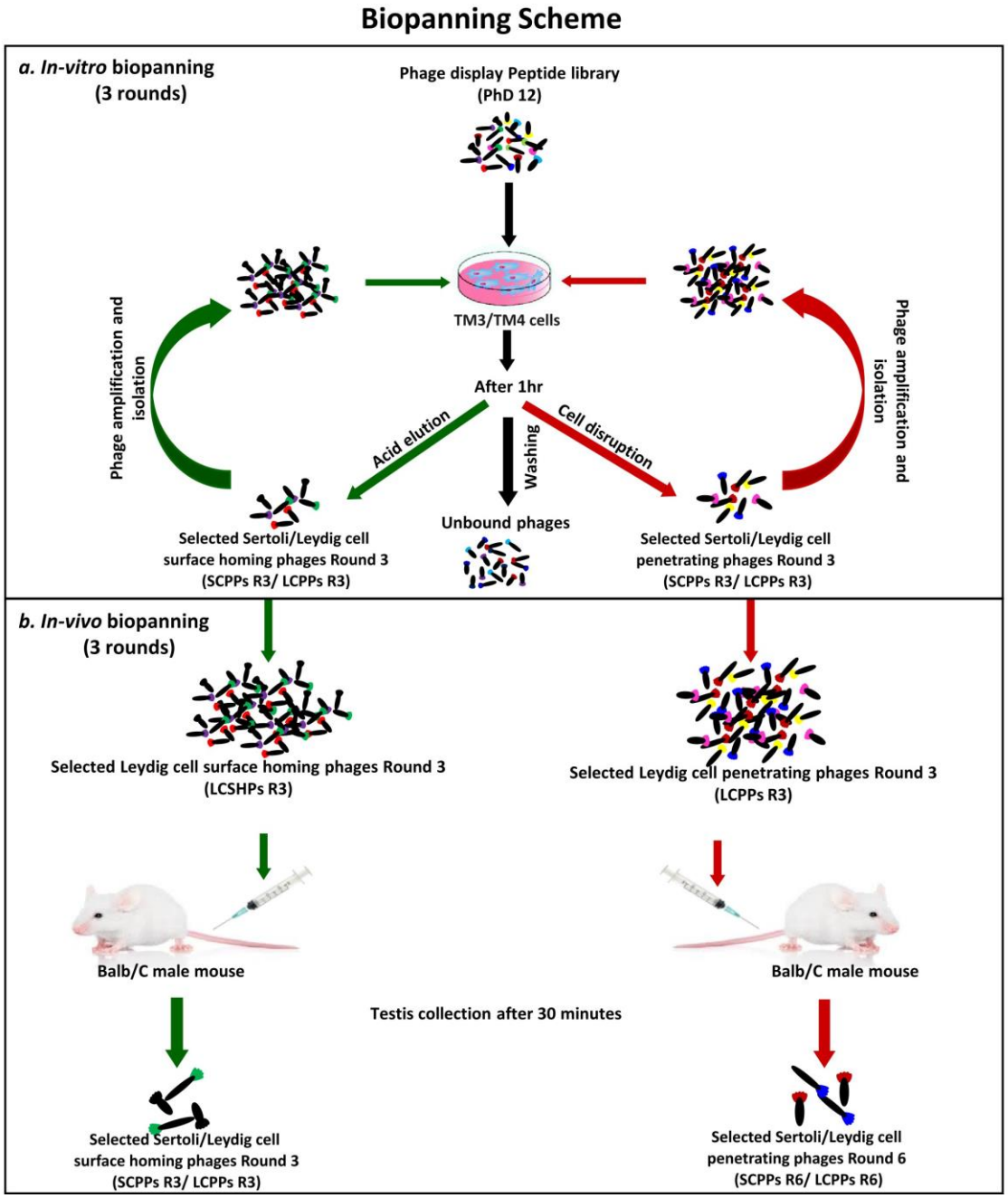

Illustration 1: Scheme of the biopanning experiment used for the identification of the Sertoli and Leydig cell homing peptides.

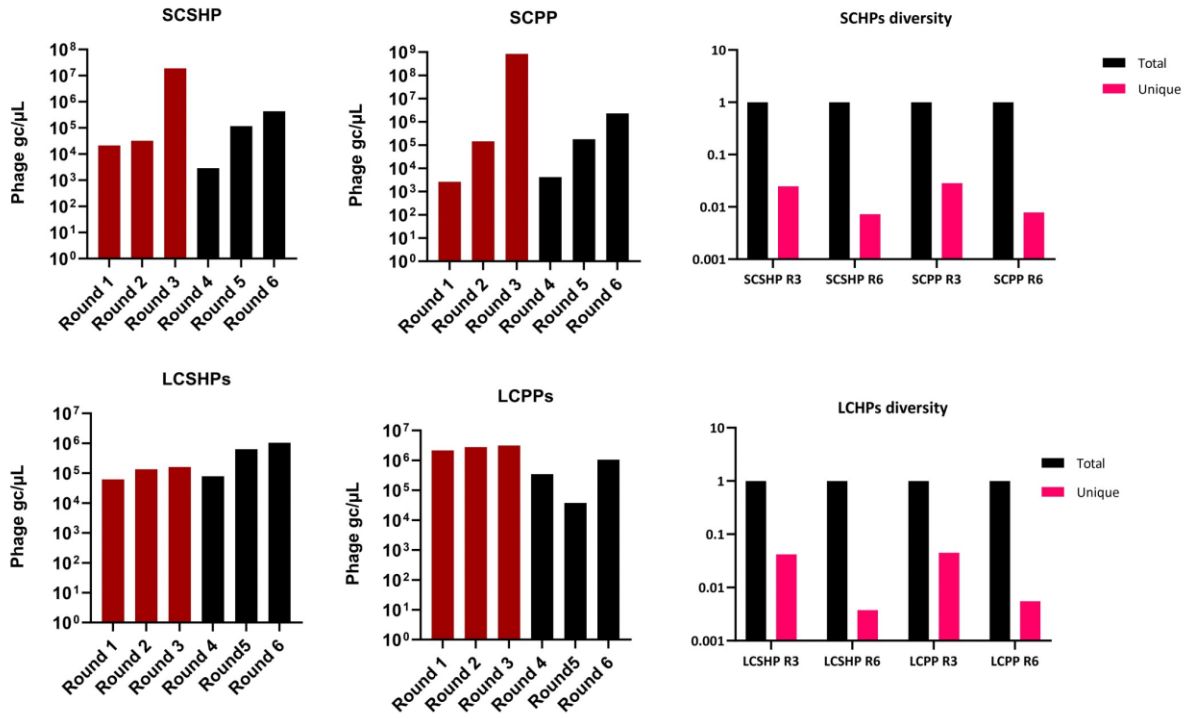

Figure 1: Titration of the phages after each rounds of biopanning and the unique peptide diversity for the experiment for SCSHP, SCPP, LCSHP, and LCPP.

Table 1: Top 10 Sertoli cell homing peptides (SCHPs)

SCHPs	R3 SCSHP Frequency	R6 SCSHP Frequency	% SCSHP enrichment	R3 SCPP Frequency	R6 SCPP Frequency	% SCPP enrichment
GSWNTFRAQPTI	25921 (3.170%)	493948 (64.966%)	61.796	24557 (4.761%)	39328 (5.424%)	0.663
YSLRLTSVTAPT	68959 (8.435%)	166731 (21.929%)	13.494	23668 (4.588%)	570722 (78.713%)	74.124
GSWNTLRAQPTI	118 (0.014%)	2329 (0.306%)	0.292	288 (0.055%)	414 (0.057%)	0.001
GPWNTFRAQPTI	160 (0.019%)	2256 (0.296%)	0.277	212 (0.041%)	406 (0.055%)	0.015
GSRNTFRAQPTI	80 (0.009%)	1596 (0.209%)	0.200	140 (0.027%)	203 (0.027%)	0.001
GSWSTFRAQPTI	84 (0.010%)	1559 (0.205%)	0.195	183 (0.035%)	298 (0.041%)	0.006
GSWNTFRVQPTI	71 (0.008%)	1149 (0.151%)	0.142	98 (0.019%)	160 (0.022%)	0.003
GSWNTSRAQPTI	45 (0.005%)	1115(0.146%)	0.141	109 (0.021%)	205 (0.028%)	0.007
GSWDTFRAQPTI	56	1056	0.132	145	231	0.004

	(0.006%)	(0.138%)		(0.028%)	(0.031%)	
GSWNTFRAQLTI	36	859	0.109	65	100	0.001
	(0.004%)	(0.112%)		(0.012%)	(0.013%)	

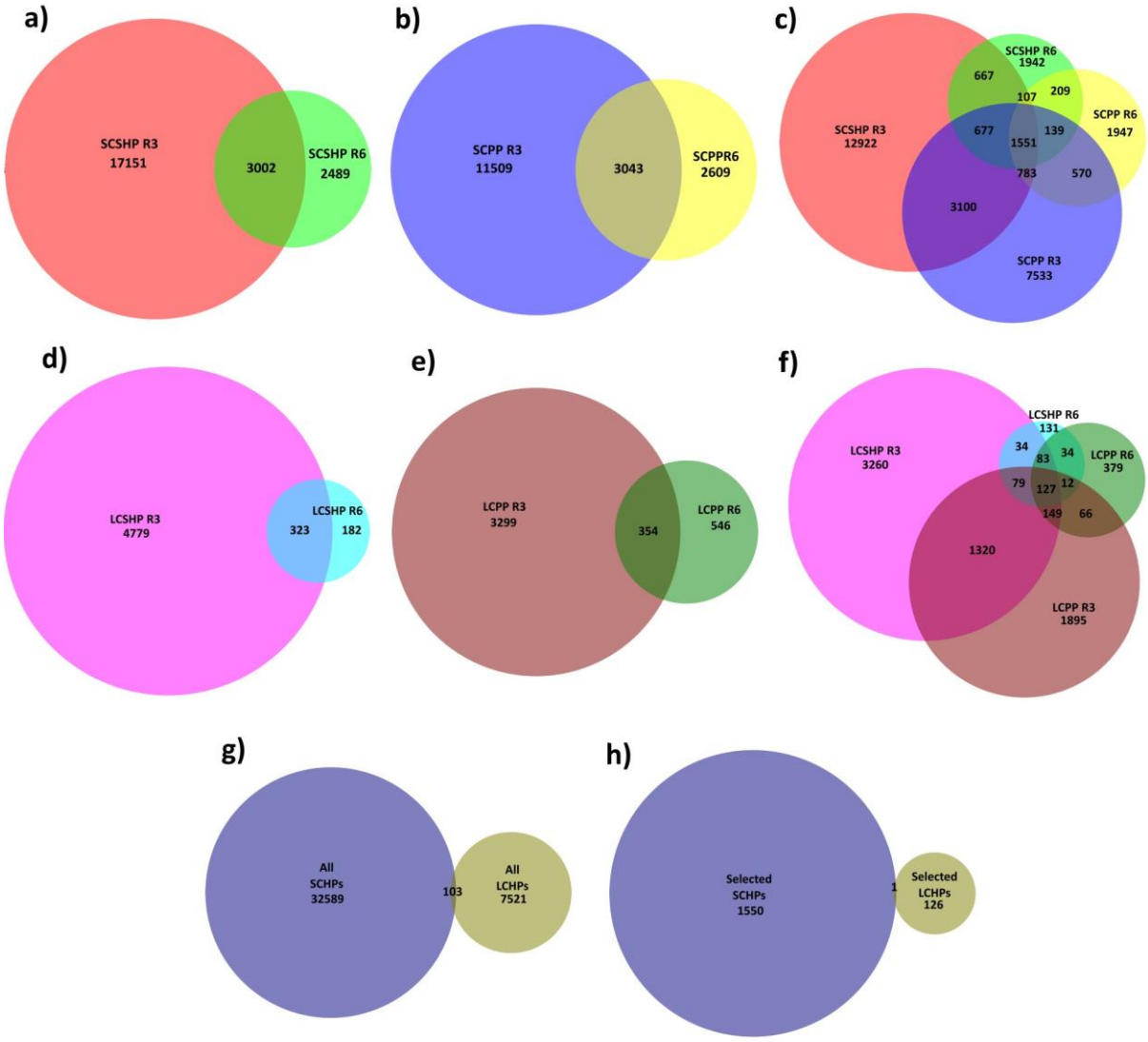

Figure 2: Summary of the number of unique peptides identified via next generation sequencing in round 3 (R3) and round 6 (R6) of the of the SCHPS, SCPP, LCSHP, and LCPP biopanning experiments and comparison between the SCHPs and LCHPs.

Table 2: Top 10 Leydig cell homing peptides (LCHPs)

LCHPs	R3 LCSHP Frequency	R6 LCSHP Frequency	LCSHP enrichment (%)	R3 LCPP Frequency	R6 LCPP Frequency	LCPP enrichment (%)
HHGANSLGLVQS	25805 (21.150%)	117522 (87.368%)	66.218	220 (0.271%)	60058 (36.938%)	36.667
YALGRPSLQGPN	2125 (1.742%)	2657 (1.975%)	0.234	585 (0.720%)	19786 (12.170%)	11.448
NQCKECFIRAGD	1093 (0.896%)	1642 (1.220%)	0.325	626 (0.771%)	13249 (8.1497%)	7.377
NESGITRIALQD	7 (0.005%)	62 (0.047%)	0.040	33 (0.040%)	598 (0.368%)	0.327
HHGANSLGLVRS	37 (0.030%)	203 (0.150%)	0.121	2 (0.002%)	152 (0.093%)	0.091
HHGANSLGLMQS	30 (0.024%)	135 (0.100%)	0.076	1 (0.001%)	78 (0.0480%)	0.047
HHGENSLGLVQS	23 (0.019%)	100 (0.074%)	0.055	1 (0.001%)	67 (0.041%)	0.040
HHGADSLGLVQS	21 (0.018%)	93 (0.070%)	0.052	1 (0.001%)	60 (0.037%)	0.036
YALGRPSLQGPS	3 (0.002%)	11 (0.009%)	0.006	3 (0.003%)	36 (0.022%)	0.018
NQRKECFIRAGD	3 (0.002%)	4 (0.002%)	0.001	2 (0.002%)	28 (0.017%)	0.015

Motif analysis of the selected SCHPs and LCHPs:

Motif discovery analysis of common 1551 SCHPs using STREME (Sensitive, Thorough, Rapid, Enriched Motif Elicitation)[21] (https://doi.org/10.1093/bioinformatics/btab203) resulted in six enriched ungapped motifs with p-value <0.05. GSAK motif is enriched in 203 (13.1%) sequences followed by FRAQPTI, YSLRLT, SVTAPT, RDTH, and MKA (Figure 3 A to F). GRPS, YAL, GPN, and KQD are the four identified motifs from the 127 common LCHPs shown in Figures 3G, 3H, and 3I. GRPS, YAL, and GPN motifs are present in the LCHP2 which indicates these are the motifs probably involved in the homing of the LCHP2 to the Leydig cells.

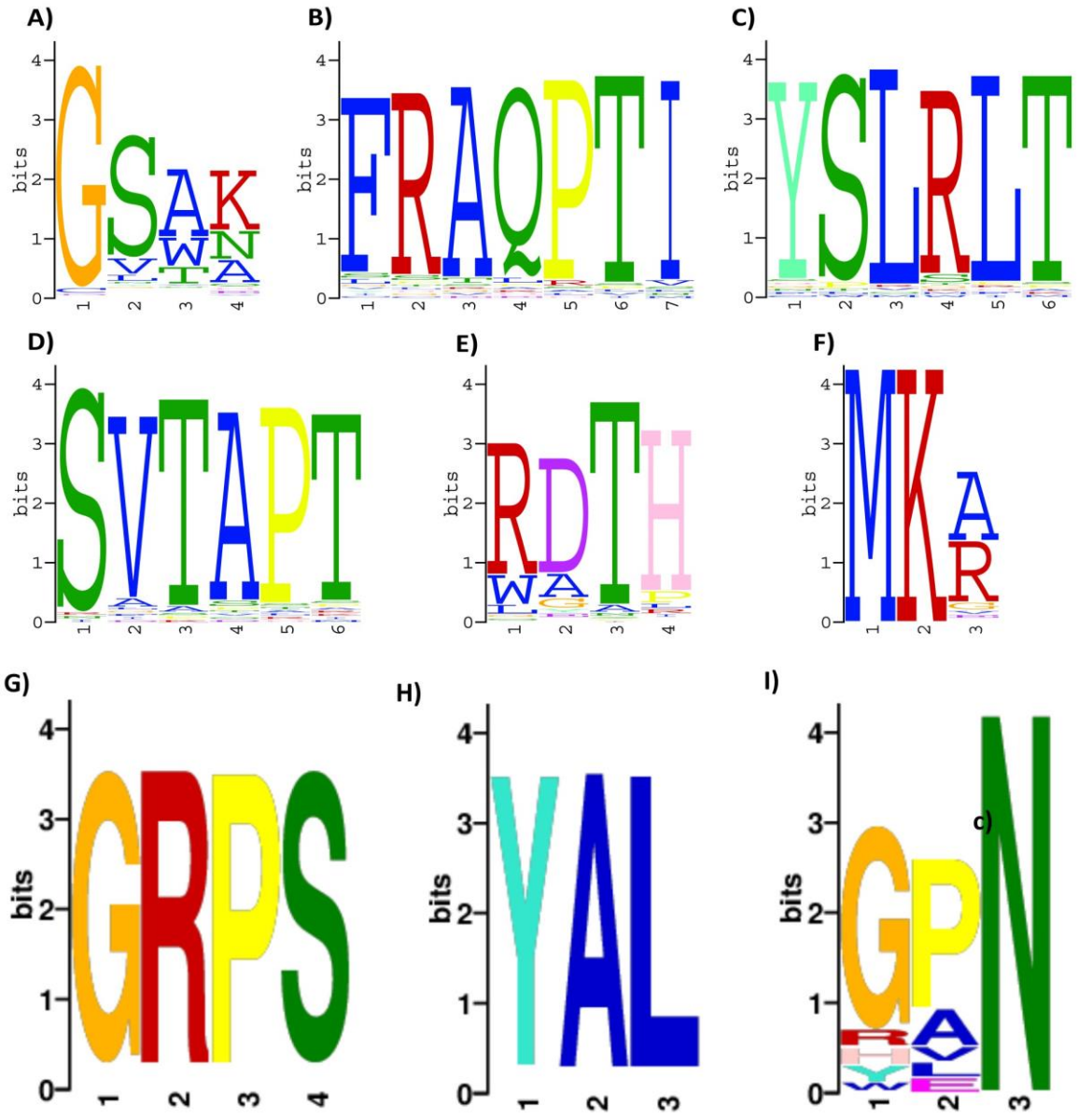

Figure 3: Motifs discovered with STREME A-F are the motifs discovered from common 1551 SCHPs and G,H and I are the motifs from 127 common LCHPs.

Specific homing of SCHPs and LCHPs to the TM4 mouse Sertoli cells and TM3 mouse Leydig cells

Uptake of FITC-ahx-SCHP1, FITC-Ahx-SCHP2, FITC-ahx-LCHP1 and FITC-Ahx-LCH2 by TM4 and TM3 cells were studied with confocal microscopy and micrographs of the TM4 and TM3 show that the peptides SCHP1 and SCHP2 can bind to TM4 cells but not TM3 and LCPH1 and LCHP2 can bind to TM3 but not TM4 cells (Fig. 4a and b).

The uptake of SCHP1 and SCHP2 by TM4 cells was significantly higher than control TM4 cells at all three concentrations tested. Further, both the peptides' uptake was increased significantly at 200µM compared to 50µM and 100µM. SCHP1 uptake by TM3, H9C2 and HEK293 cells was significantly lower than TM4 cells at all three concentrations. SCHP2 uptake by TM4 cells was also significantly higher than TM3, H9C2 and HEK293 cells at all three concentrations (Fig 4c). The results indicate that the SCHP1 and SCHP2 peptide can efficiently target mouse Sertoli cells (TM4) in-vitro even at lower concentrations. Quantitative analysis of uptake of LCHP1 and LCHP2 by TM3, TM4, and H9C2 cells was performed using flow cytometry (Figures 4d and 4e). LCHP1 showed significantly higher uptake in TM3 cells at 100µM and 200 µM concentrations. Whereas, LCHP2 showed significantly higher uptake in TM3 than TM4 at 50 µM, 100µM, and 200 µM concentrations and significantly higher uptake compared to H9C2 at only 50 µM. The H9C2 cells show equivalent uptake to TM3 of LCHP2 at 200 µM and higher uptake at 50 µM and 100µM, which could be due to autofluorescence of the cells because the unstained H9C2 also showed higher fluorescence intensity. Moreover, the *in-vivo* biodistribution of the LCHP2 did not show significant uptake in the heart which suggests the higher uptake by H9C2 which are rat cardiomyocytes may not be the concern for targeting Leydig cells in the in-vivo system.

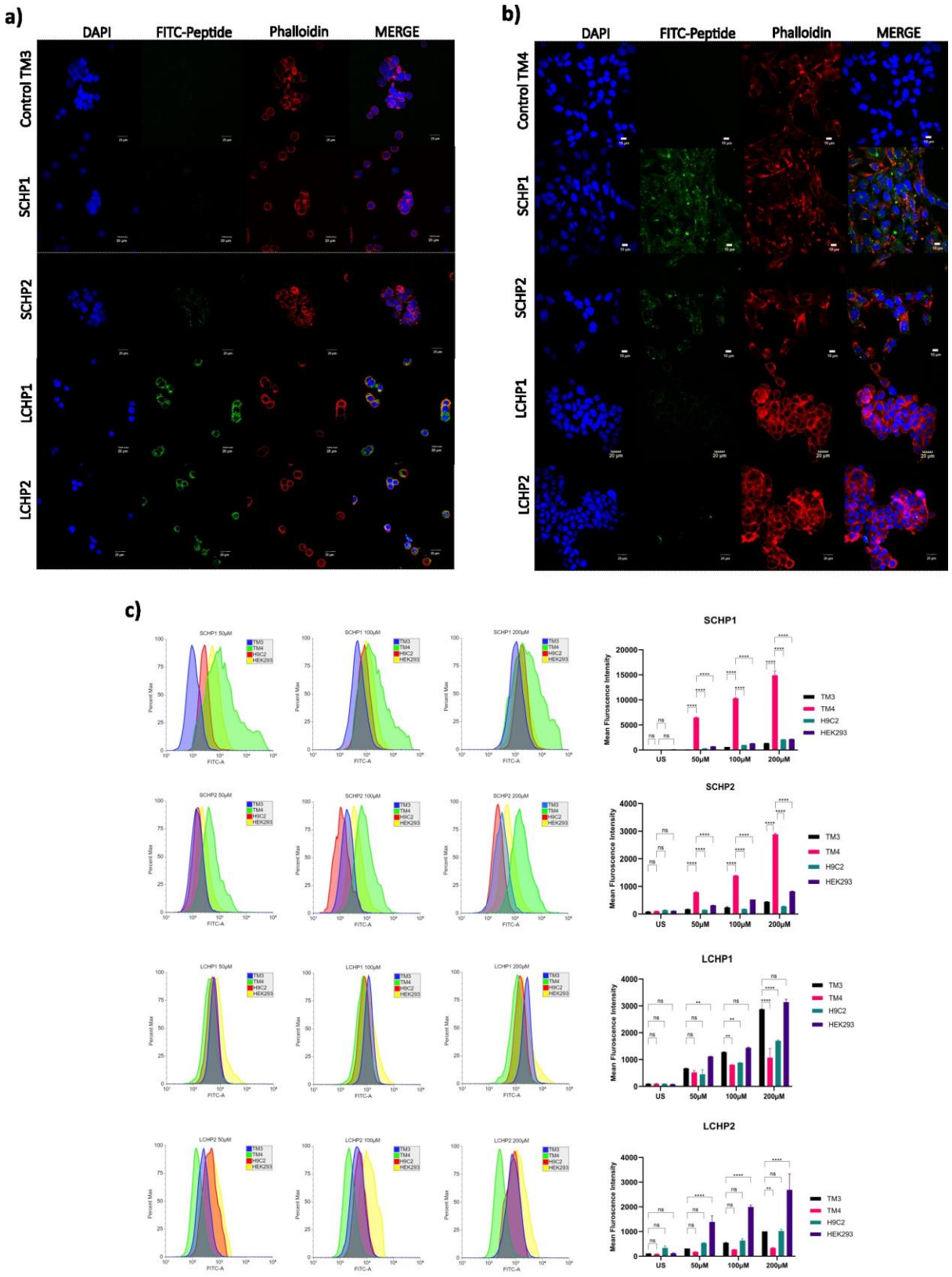

Figure 4: Uptake of SCHP1, SCHP2, LCHP1 and LCHP2 by TM3 and TM4 cell lines with confocal microscopy a and b, and with flow cytometry c.

Circular Dichroism Spectroscopy analysis for Secondary structure selected SCHPs and LCHPs

The secondary structure formation of the SCHP1, SCHP2, LCHP1 and LCHP2 in the environment with different polarities was analysed with Circular Dichroism spectroscopy. Peptides were dissolved in different concentrations of trifluroethanol (TFE) in water. Circular dichroism spectra of the SCHP1 (Fig. 4a), SCHP2 (Fig. 4b), LCHP1 (Fig. 4c) and LCHP2 (Fig. 4d) indicate, as the concentration of TFE increases, there is a decrease in the random structure and increase in the beta sheets and helix structures, which indicated that in the peptide may form more specific beta sheets and helix structures in the intra-testicular environment which is similar to a higher percentage of TFE.

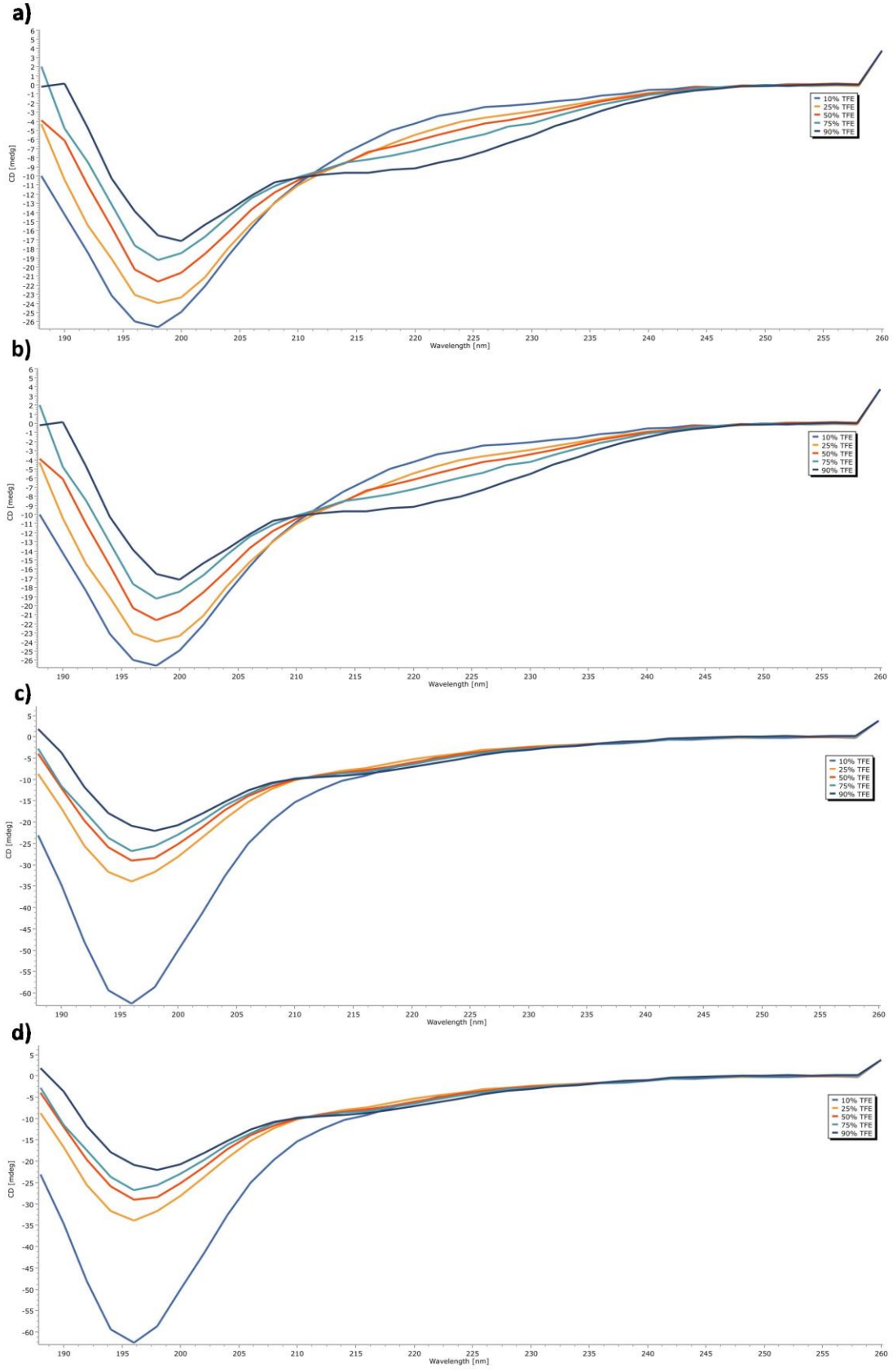

Figure 5: Circular Dichroism analysis of SCHP1 (a), SCHP2 (b), LCHP1 (c) and LCHP2 (d) in different concentrations of TFE.

Targeting testis with SCHP1 and SCHP2 peptides in a mouse model

In-vivo bio-distribution and testicular targeting potential of Cy5.5 tagged SCHP1, SCHP2, LCHP1, LCHP2 and CTP peptides were analyzed with in-vivo imaging, wherein 20µg of each peptides were injected intravenously to the mice and mice were sacrificed after 1hr, 6hrs and 24hrs to realise the bio-distribution of the peptides. Representative images of the organs after 1hr, 6hrs, and 24 hrs of in-vivo bio-distribution of SCHP1, SCHP2, LCHP1, LCHP2, Cy5.5 dye and CTP are shown in figure 6a and 6b.

Statistical analysis of the Avg Radiant Efficiency [p/s/cm²/sr] / [µW/cm²] showed that SCHP1 has significantly higher uptake in testis compared to Heart, brain, and epididymis at 6hrs time point and uptake in liver is not significantly higher (Fig. 6c), SCHP2 has significantly higher uptake in testis compared to heart spleen and brain at 24 hrs time point whereas uptake in lungs and kidney is not significantly higher (Fig. 6d). Testis showed significantly higher accumulation of LCHP1-Cy5.5 compared with heart at all the three time points i.e. 1hr, 6hrs, and 24hrs. The accumulation of LCHP1-Cy5.5 was significantly higher in testis compared with spleen at 1hr and compared with spleen, brain, and epididymis at 6hrs time point. Moreover, the signal because of LCHP1-Cy5.5 rapidly decreased with time in liver and kidneys (Figure 6f). LCHP2-Cy5.5 showed better uptake in testis than LCHP1-Cy5.5 with the significantly higher accumulation in testis compared with heart and spleen at 1hr, 6hrs, and 24hrs. LCHP2-Cy5.5 also showed significantly higher accumulation in testis compared with brain, epididymis, and seminal vesicles at 6hrs and 24hrs time points (Figure 6g). However in the case of free Cy5.5 dye, there is no significant uptake in testis at all the time points and uptake by lungs was significantly higher compared to testis at 1hr and 24 hrs time point, uptake in the liver was also significantly higher at all the three-time points (Fig. 6e). The distribution of CTP-Cy5.5 was more uniform in all the organs and there was no significant difference in accumulation of peptide in all analysed organs except liver and kidneys where the uptake was significantly higher in in later two compared with testis (Figure 6h).

The results of in-vivo bio-distribution suggest that Cy5.5 tagged SCHP1, SCHP2, LCHP1 and LCHP2 have a different pattern of bio-distribution compared to free Cy5.5 dye and CTP, distribution of SCHP1, SCHP2, LCHP1, LCHP2, Cy5.5 and CTP is similar in organs involved in the elimination of the drug from the body such as liver kidney and lungs, but compared to other organs uptake of SCHPs and LCHPs is higher in testis which indicated its targeting efficacy towards testis.

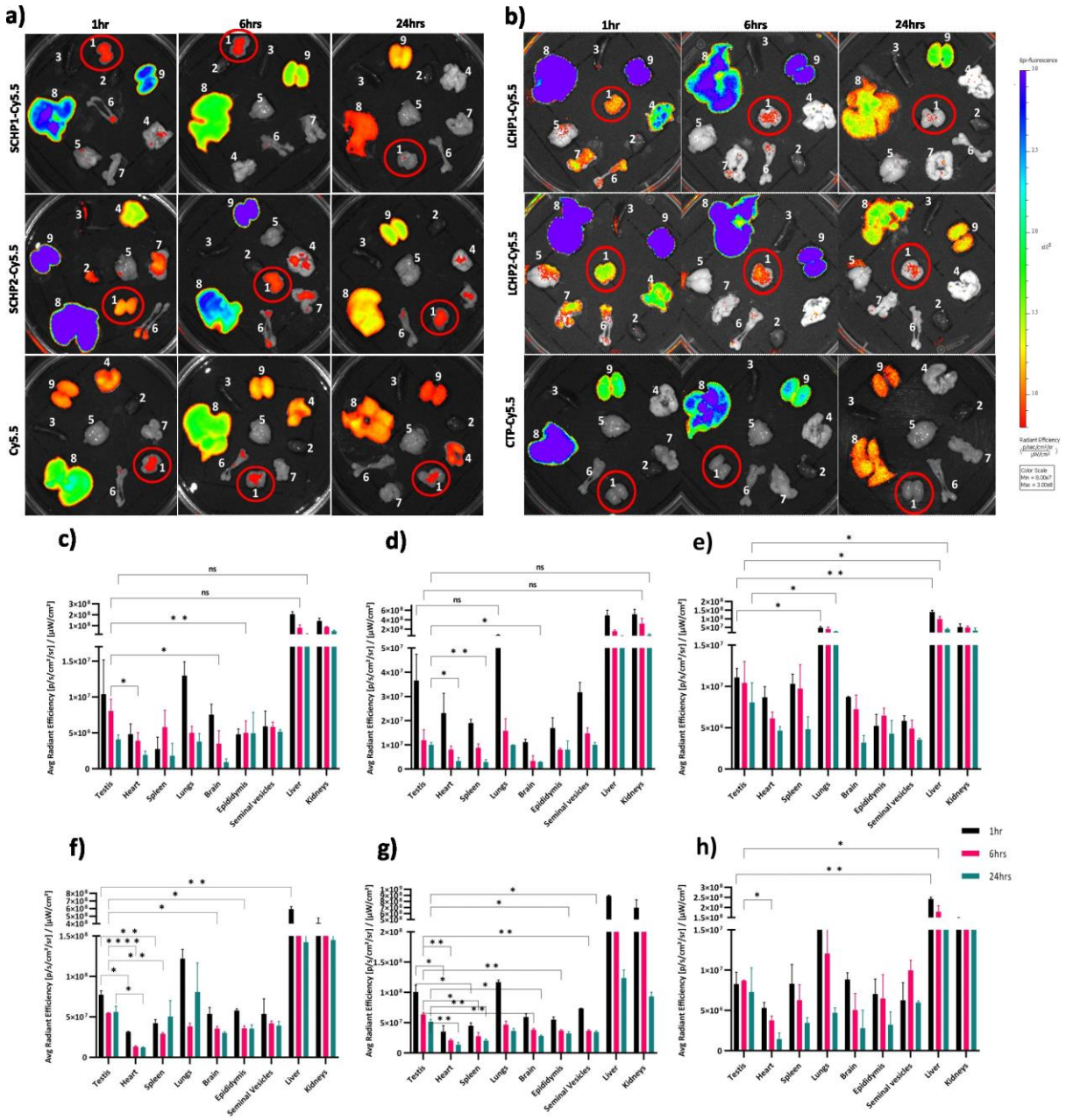

Figure 6: In vivo bio-distribution representative images of SCHPs (a) and LCHPs (b), and statistical analysis of SCHP1, SCHP2, LCHP1, LCHP2, Cy5.5, and CTP at 1hr, 6hrs, and 24hrs time point.

Statistical Analysis:

All the statistical analysis was done on GraphPad Prism version 9. Two way anova with tukey's multiple comparisons was performed for the in vitro uptake study and in vivo bio distribution.

Discussion

Conventional drug delivery to treat testicular pathologies requires high dosing frequency, resulting in adverse side effects and systemic toxicity. These issues can be circumvented by specifically targeting testicular cells with the help of specific ligands. Cell homing peptides are potential candidates as a targeted drug delivery agents and are in clinical trials for various cancer targeting. This is the first study to date demonstrating the identification of Sertoli cell and testis homing peptides using a phage display peptide library. SCHP, SCHP2, LCHP1 and LCHP2 were the highly enriched topmost peptides during in-vitro and in-vivo rounds of biopanning. Sertoli and Leydig cell homing potential of the SCHPs and LCHPs respectively was established with the in-vitro studies. Further, SCHPs/ LCHPs can specifically accumulate in the testis better than Cy5.5 dye and CTP and hence can serve as an ideal targeting ligand for the smart drug delivery to the testis. The efficacy of the male non-hormonal contraceptive drug like Adjudin, can be increased while decreasing their side effects on other organs by the use of SCHPs to develop targeted delivery system. Antioxidants and drugs for treating male infertility whose site of action is inside the testis can also be targeted using these SCHPs/ LCHPs. Targeted drug delivery strategies can be developed with use of identified LCHPs for the treatment of various testicular disorders including male infertility, testicular cancer and testicular infections. Many of the peptides face the problems of stability, and in this study, we have not studied any aspect related to the stability of SCHPs/ LCHPs; in the future, this could be studied, and effects could be made by the development of peptidomimetics to increase their stability.

Impact of the research in the advancement of knowledge or benefit to mankind

The testicle, an organ privileged with immunity due to Blood Testis Barrier (BTB), pose a major obstacle to the development and delivery of drugs to the testes. Existing therapies for the treatment of testicular pathologies are hampered by negative side effects of systemically administered drug, high dosing frequency, inability to achieve sufficiently high intra-testicular concentrations for therapeutic activity, and failure of large molecules to cross the blood–testis barrier. These problems can be prevented by targeting testicular cells using specific ligands such as homing peptides. There is very little progress in the field of drug discovery and delivery for the treatment of testicular pathologies and Currently there is no any testicular cell targeting ligand available for the development of the targeted drug delivery systems. This is the first study to demonstrate the successful selection of testis targeting ligands, Sertoli cell homing

peptides (SCHPs) i.e. SCHP1 and SCHP2, and Leydig cell homing peptides (LCHPs) i.e. LCHP1 and LCHP2. The identified novel Sertoli cell homing peptides (SCHPs) i.e. SCHP1 and SCHP2, and Leydig cell homing peptides (LCHPs) i.e. LCHP1 and LCHP2, have the potential to use as peptide ligand of the development of the active targeted drug delivery systems for the delivery of male contraceptive drugs and for the delivery of the drugs for the treatment of male infertility due to hypogonadotropic hypogonadism and testicular oxidative stress, testicular cancers, and testicular infections etc. development of targeted drug delivery systems using these peptides as targeting agent could open new avenues for the treatment of different testicular pathologies.

Literature reference

1. Vasir, J., Reddy, M., and Labhasetwar, V. (2006). Nanosystems in Drug Targeting: Opportunities and Challenges. Curr. Nanosci. *1*, 47–64. 10.2174/1573413052953110.

2. Pasqualini, R., and Ruoslahti, E. (1996). Organ targeting in vivo using phage display peptide libraries. Nature *380*, 364–366. 10.1038/380364a0.

3. Majumdar, S., and Siahaan, T.J. (2012). Peptide-mediated targeted drug delivery. Med. Res. Rev. *32*, 637–658. 10.1002/med.20225.

4. Smith, G.P. (1985). Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science *228*, 1315–1317. 10.1126/science.4001944.

5. Maruta*, F., Parker, A.L., Fisher, K.D., Murray, P.G., Kerr†, D.J., and Seymour, L.W. (2003). Use of a Phage Display Library to Identify Oligopeptides Binding to the Lumenal Surface of Polarized Endothelium by Ex Vivo Perfusion of Human Umbilical Veins. J. Drug Target. *11*, 53–59. 10.1080/1061186031000086063.

6. Lee, N.K., Kim, H.S., Kim, K.H., Kim, E.-B., Cho, C.S., Kang, S.K., and Choi, Y.J. (2011). Identification of a novel peptide ligand targeting visceral adipose tissue via transdermal route by in vivo phage display. J. Drug Target. *19*, 805–813. 10.3109/1061186X.2011.572974.

7. Andrieu, J., Re, F., Russo, L., and Nicotra, F. (2019). Phage-displayed peptides targeting specific tissues and organs. J. Drug Target. *27*, 555–565. 10.1080/1061186X.2018.1531419.

8. Arap, W., Kolonin, M.G., Trepel, M., Lahdenranta, J., Cardó-Vila, M., Giordano, R.J., Mintz, P.J., Ardelt, P.U., Yao, V.J., Vidal, C.I., et al. (2002). Steps toward mapping the human vasculature by phage display. Nat. Med. *8*, 121–127. 10.1038/nm0202-121.

9. Kolonin, M.G., Saha, P.K., Chan, L., Pasqualini, R., and Arap, W. (2004). Reversal of obesity by targeted ablation of adipose tissue. Nat. Med. *10*, 625–632. 10.1038/nm1048.

10. Rajotte, D., and Ruoslahti, E. (1999). Membrane dipeptidase is the receptor for a lung-targeting peptide identified by in vivo phage display. J. Biol. Chem. *274*, 11593–11598. 10.1074/jbc.274.17.11593.

11. Essler, M., and Ruoslahti, E. (2002). Molecular specialization of breast vasculature: a breast-homing phage-displayed peptide binds to aminopeptidase P in breast vasculature. Proc. Natl. Acad. Sci. U. S. A. *99*, 2252–2257. 10.1073/pnas.251687998.

12. Zhang, L., Hoffman, J.A., and Ruoslahti, E. (2005). Molecular profiling of heart endothelial cells. Circulation *112*, 1601–1611. 10.1161/CIRCULATIONAHA.104.529537.

13. Zahid, M., Phillips, B.E., Albers, S.M., Giannoukakis, N., Watkins, S.C., and Robbins, P.D. (2010). Identification of a Cardiac Specific Protein Transduction Domain by In Vivo Biopanning Using a M13 Phage Peptide Display Library in Mice. PLoS One *5*, e12252.

14. Ghosh, D., Peng, X., Leal, J., and Mohanty, R.P. (2018). Peptides as drug delivery vehicles across biological barriers. J. Pharm. Investig. *48*, 89–111. 10.1007/s40005-017-0374-0.

15. Fosgerau, K., and Hoffmann, T. (2015). Peptide therapeutics: Current status and future directions. Drug Discov. Today *20*, 122–128. 10.1016/j.drudis.2014.10.003.

16. McGuire, M.J., Li, S., and Brown, K.C. (2009). Biopanning of phage displayed peptide libraries for the isolation of cell-specific ligands. Methods Mol. Biol. *504*, 291–321. 10.1007/978-1-60327-569-9_18.

17. Work, L.M., Nicol, C.G., Denby, L., and Baker, A.H. In Vivo Biopanning. Methods *108*, 395–413. 10.1038/380364a0.

18. Peng, X., Nguyen, A., and Ghosh, D. (2018). Quantification of M13 and T7

bacteriophages by TaqMan and SYBR green qPCR. J. Virol. Methods *252*, 100–107. 10.1016/j.jviromet.2017.11.012.

19. Green, M.R., and Sambrook, J. (2017). Preparation of Single-Stranded Bacteriophage M13 DNA by Precipitation with Polyethylene Glycol. Cold Spring Harb. Protoc. *2017*, pdb.prot093419. 10.1101/pdb.prot093419.

20. Matochko, W.L., and Derda, R. (2015). Next-generation sequencing of phage-displayed peptide libraries. Methods Mol. Biol. *1248*, 249–266. 10.1007/978-1-4939-2020-4_17.

21. Bailey, T.L. (2021). STREME: accurate and versatile sequence motif discovery. Bioinformatics *37*, 2834–2840. 10.1093/bioinformatics/btab203.