PHAGE DISPLAY TO IDENTIFY PEPTIDES BINDING TO OR PENETRATING THE MOUSE ZONA PELLUCIDA

By
Jeanette Lowe

Thesis submitted to the faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

Master of Science
in
Dairy Science
Molecular Cell Biology and Biotechnology Program

Francis C. Gwazdauskas, Chair
Christopher G. Russell
Eric A. Wong

November 19, 1999
Blacksburg, Virginia

Keywords: phage display, zona pellucida, mouse embryology, transgenesis
PHAGE DISPLAY TO IDENTIFY PEPTIDES BINDING TO OR PENETRATING THE MOUSE ZONA PELLUCIDA

Jeanette Lowe

(ABSTRACT)

The objective of this study was to identify peptide ligands, using phage display techniques, which bind sites on mouse embryos, ovaries, cytoplasmic membranes and/or intracytoplasmic components. Specifically, M13 coliphage 7-mer, 12-mer and 15-mer random peptide libraries were used separately for biopanning. Peptides derived from the amplified pools were sequenced and studied. The phage display for in vivo ovary experiments yielded no pool of peptides after two cycles of biopanning and re-amplification. With the same initial concentration of a random 7-mer or 12-mer library, there were repeating sequences derived after three and four biopanning cycles on mouse embryos and unfertilized ova. The sequences were not distinguishable from a control group. Subsequent experimentation using a random 15-mer library to select for internalized phage-peptides yielded two apparent consensus sequences, RNVPPIFNDVYWIAF (9/32 or 28%) and HGRFILPWWYAFSPS (11/32 or 34%). The 15-mer control group yielded no clones. The deduced peptide sequences were compared to known sequences to ascertain their uniqueness. No significant similarities were found, yielding two possible novel motifs. Through this adapted process of phage display and further research, the phage display technology may be used as a tool in the recognition of specific mouse gamete sites. By identifying binding sites of mouse gametes, the peptides might be exploited as a means of studying the embryo cell surface or cytoplasmic components and mouse sperm-egg interactions. Such peptides may also be used for macromolecule delivery in transfection or transgenesis.
ACKNOWLEDGEMENTS

I would like to express my deepest gratitude and appreciation to everyone that has helped me in this walk of my life. There are so many of you that have been there, in Virginia and in Huntsville, Alabama. Most of all, I want to thank God for blessing me with so many wonderful opportunities. My wealth is through Him and all the friends and experiences that have been brought into my life. I certainly could not have done this alone.

I would like to sincerely thank all of my committee members: My committee chairman, Dr. Frank Gwazdauskas, for all of his support and guidance. You were great to spend so much time corresponding with me and doing my legwork while I was away. Dr. Eric Wong for all of his time and guidance, and especially for listening when I needed it. A special thanks to Dr. Chris Russell who was there for the whole process. I appreciate the many hours you spent teaching and guiding me, even when you didn’t have a minute to spare. You and Tracy were my bridge to Huntsville; thank you both for helping me to settle into this new town and home.

I would like to extend my utmost appreciation and regards to Jim Hudson, the owner of Research Genetics. I have never known an owner and boss to be such a kind and caring person. Each one of us here is made to feel like true family. Jim, you gave me that light of hope when I wasn’t sure that one was left; many thanks to you for having faith in me. I really do mean it when I say that you are the best boss that was ever made. Please accept that compliment without condition; you deserve it many times over.

To all of my colleagues and friends at Research Genetics: I am truly grateful for all of your assistance and encouragement. I don’t have space here to acknowledge each by name, but you know who you are! A special thanks to Jennifer Rice for all of her time and attention with the mice, and for rushing to my rescue when schedules unexpectedly changed. A special mention to Greg Bell, Mei Lu, and Harold Zappe for their technical support and calming advice in the final hours. I am also grateful to Dr. Perry Kirkham at UAB for sharing his vast knowledge of phage display. To Jeff Brockman, you finally got acknowledged in someone’s paper … thanks for the “sanity checks”.

Last, but not least, thanks to my family for teaching me what is valuable and important in life. Those that are closest to me know that my grandparents are my idols; if I could pick any 3 new grandparents, I would be more than honored to still pick you! How fortunate I am.
# TABLE OF CONTENTS

**ACKNOWLEDGEMENTS** .................................................................................................................................................. iii
**LIST OF FIGURES** ....................................................................................................................................................... vi
**LIST OF TABLES** ......................................................................................................................................................... viii

## CHAPTER 1. INTRODUCTION ........................................................................................................................................... 1

## CHAPTER 2. LITERATURE REVIEW .......................................................................................................................... 3
- Phage Display ............................................................................................................................................................... 3
- Embryology .................................................................................................................................................................... 6
- Transgenesis .................................................................................................................................................................. 9

## CHAPTER 3. ....................................................................................................................................................................... 14
- ABSTRACT ...................................................................................................................................................................... 14
- INTRODUCTION .......................................................................................................................................................... 15
- MATERIALS AND METHODS ......................................................................................................................................... 16
  - Mice ......................................................................................................................................................................... 16
  - Embryo, Ova and Ovary Collection .......................................................................................................................... 16
  - Phage Display .......................................................................................................................................................... 17
  - A. 7-mer and 12-mer Phage: ................................................................................................................................... 18
    - I. Adaptation of Phage Display Techniques to Target Mouse Embryo Sites ......................................................... 19
    - II. Biopanning and Amplification of the Bound 7-mer and 12-mer Phage ............................................................... 22
    - Sequence, Analysis, and Characterization of 7-mer and 12-mer Bound Peptide Elution ................................. 23
    - Fluorescent Peptides for 7-mer and 12-mer Binding Results ................................................................................. 25
    - Non-denaturing Gel and Western .......................................................................................................................... 26
  - B. 15-mer Phage: .......................................................................................................................................................... 27
    - I. Adaptation of Phage Display Techniques to Target Mouse Embryo Sites ......................................................... 27
    - II. Biopanning and Amplification of the Bound 15-mer Phage ........................................................................... 28
    - Sequence, Analysis, and Characterization of 15-mer Bound Peptide Elution ................................................... 29
    - Immunofluorescence for 15-mer Binding Results ................................................................................................. 30

## RESULTS .......................................................................................................................................................................... 32
- Biopanning/Amplification and Sequencing .................................................................................................................. 32
  - Ph.D.™ 7 Results ....................................................................................................................................................... 34
  - Ph.D.™ 12 Results .................................................................................................................................................... 43
  - Random 15-mer Library Results .............................................................................................................................. 53
- Sequences Chosen for 7-mer and 12-mer Fluorescein Experiments ........................................................................... 58
- Westerns ....................................................................................................................................................................... 66
- Immunofluorescence for 15-mer Binding Results ......................................................................................................... 66
DISCUSSION ......................................................................................................................... 76
CONCLUSIONS ...................................................................................................................... 82
REFERENCES ....................................................................................................................... 85

CHAPTER 4. BIBLIOGRAPHY ................................................................................................. 88

CHAPTER 5. APPENDIX ........................................................................................................ 94

VITA .......................................................................................................................................... 97
LIST OF FIGURES

Figure 1A: Trial 1 results of phage concentration from biopanning and amplification at each cycle, using a Ph.D.7 phage display random library.............................. 35

Figure 1B: Trial 1 sequencing results derived from clones of Ph.D. 7 with mouse ova...... 36

Figure 1C: Trial 1 sequencing results derived from clones of Ph.D. 7 with mouse embryos ............................................................................................................................. 37

Figure 2A: Trial 2 results of phage concentration from biopanning and amplification at each cycle, using a Ph.D.7 phage display random library ...................................................... 39

Figure 2B: Trial 2 sequencing results derived from clones of Ph.D. 7 with mouse embryos ............................................................................................................................. 40

Figure 2C: Trial 2 sequencing results derived from clones of Ph.D. 7 with mouse ova...... 41

Figure 2D: Trial 2 sequencing results derived from clones of Ph.D. 7 control.............. 42

Figure 3: Trial 3 results of phage concentration from biopanning and amplification at each cycle, using a Ph.D.12 phage display random library......................... 43

Figure 4A: Trial 4 results of phage concentration from biopanning and amplification at each cycle, using a new Ph.D.12 phage display random library ............... 45

Figure 4B: Trial 4 sequencing results derived from clones of Ph.D. 12 with mouse embryos ................................................................................................................. 46

Figure 4C: Trial 4 sequencing results derived from clones of Ph.D. 12 with mouse ova..... 47

Figure 5A: Trial 5 results of phage concentration from biopanning and amplification at each cycle, using a new Ph.D.12 phage display random library ............... 49

Figure 5B: Trial 5 sequencing results derived from clones of new Ph.D. 12 with mouse embryos ................................................................................................................. 50

Figure 5C: Trial 5 sequencing results derived from clones of new Ph.D. 12 with mouse ova...................................................................................................................... 51
Figure 5D: Trial 5 sequencing results derived from clones of new Ph.D. 12 control ........... 52

Figure 6: Results of phage concentration from biopanning and amplification at each cycle, using a new 15-mer phage display random library ................................................................. 54

Figure 7A: Results of phage concentration from biopanning and amplification at each cycle, using a 15-mer phage display random library, at 37°C with 100 µM chloroquine ..... 56

Figure 7B: Sequencing results derived from clones of the random 15-mer phage library ... 57

Figure 8: Fluorescent peptide binding results of Fl- LPLTPLP with mouse ova ............ 60

Figure 9: Fluorescent peptide binding results of Fl-ATAYPNPFSPGA with mouse embryos ......................................................................................................................... 61

Figure 10: Fluorescent peptide binding results of Fl-ATAYPNPFSPGA with mouse ova .. 62

Figure 11: Fluorescent peptide binding results of mouse embryos, control with no Fl-peptide ......................................................................................................................... 63

Figure 12: Immunofluorescence results of the control 15-mer phage versus mouse zona-free embryos .................................................................................................................. 68

Figure 13: Immunofluorescence results of the control 15-mer phage versus mouse zona-free embryos .................................................................................................................. 69

Figure 14: Immunofluorescence results of #31 HGRFILPWWYAFSPS with mouse zona-free embryos ................................................................................................................. 70

Figure 15: Immunofluorescence results of #31 HGRFILPWWYAFSPS with mouse zona-free embryos ................................................................................................................. 71

Figure 16: Immunofluorescence results of #31 HGRFILPWWYAFSPS with mouse zona-free embryos ................................................................................................................. 72

Figure 17: Immunofluorescence results of #31 HGRFILPWWYAFSPS with mouse zona-free embryos ................................................................................................................. 73

Figure 18: Immunofluorescence results of #37 RNVPPIFNDVYWIIF with mouse zona-free embryos ................................................................................................................. 74

Figure 19: Immunofluorescence results of #37 RNVPPIFNDVYWIIF with mouse zona-free embryos ................................................................................................................. 75
LIST OF TABLES

Table 1a: Fluorescein labeled 7-mer and 12-mer peptides, qualitative and semi-quantitative results ................................................................. 94

Table 1b: Fluorescein labeled 7-mer and 12-mer peptides, qualitative and semi-quantitative repeat results ................................................................. 94

Table 2: Fluorescein labeled 7-mer and 12-mer peptides, carboxy-terminus; qualitative and semi-quantitative results ..................................................... 95

Table 3: Fluorescein labeled 7-mer and 12-mer peptides derived from the control groups, and a random 7-mer pool; qualitative and semi-quantitative results ........................................................................................................ 96
CHAPTER 1
INTRODUCTION

Since the development of phage display technology, applications of phage display techniques to the murine biotechnology field have been minimal. Phage display technology could be exploited as a useful tool in murine biotechnology research. Specifically, the end goal of this research is to find a more direct means of transgenesis through the manipulation of peptides identified by phage display. If peptides specific for the mouse embryo and/or sperm are identified through the use of phage display, then these peptides might be used to “attach” DNA of choice. Optimistically, the DNA would be delivered into a mouse embryo via the peptide attachment to its recognition site on the mouse embryo. In this manner, the peptide may carry the DNA into the embryo to allow integration of the foreign DNA into the mouse genome. Other manifestations of the technique might allow delivery of the exogenous DNA prior to the first round of DNA replication. Mouse embryos would then have a better chance of incorporating the exogenous DNA early enough in the cell cycle to increase the success rate of hemizygous transgenesis. Once successfully applied to murine technology, the technology might then be applied to larger mammals.

Phage display is a peptide ligand selection process that uses a combinatorial library of random peptides fused to a coat protein of a bacteriophage. Specifically 7-mer, 12-mer, 15-mer or N-mer peptides are genetically fused to the minor coat protein, gene III, of M13 filamentous coliphage and displayed on the phage’s surface exterior. The phage display can then be used to obtain a peptide ligand-receptor target molecule linkage. A physical linkage between the combinatorial library of random peptides and the DNA encoding each sequence allows for rapid identification of peptide ligands that bind a target domain. The peptide ligands that recognize a target domain are isolated and amplified by an in vitro process called biopanning. Biopanning is a selection process carried out by incubating a library of phage-displayed peptides on a plate or bead coated with a specific target. Bound phage remains after washing away the unbound phage. The specifically-bound phage is eluted, then amplified by E. coli infection, and passed through successive cycles of biopanning and amplification. These successive cycles are done in order to
enrich the pool of phage which specifically binds the target. A total of three to four cycles is carried out and individual clones are characterized by DNA sequencing.

The concept for this research is to substitute the plates or beads in the above process with mice or tissues in *in vivo* or *in vitro* studies. By using phage display to define binding sites on mouse embryos, the peptides might be exploited as a means of expanding current knowledge of mouse sperm-egg interactions. Once these sites are more clearly identified, the recognition peptides might be used to enhance studies of embryo cell surface molecules. It may be possible to further define known and identify yet unknown receptor sites. Other advantages might be to further clarify current knowledge of specific fertilization cycles. Ultimately, the peptides might be used as a means of delivering macromolecules for transfection or transgenic production. Transfection might occur by attaching a desired macromolecule to the defined peptide. The peptide would then be used as the macromolecule delivery tool by binding and entering through its recognition site on the oocyte.

In summary, the main purpose for the proposed research is to identify receptors in or on mouse eggs that might be used for transfection of oocytes. Foreign molecules, such as DNA, RNA, or peptides, may then be attached to the peptides identified through phage display. The peptide could be exploited as a delivery system for transfection. This delivery system would be especially useful in transgenic mouse production and possibly extend to the production of other transgenic animals.
CHAPTER 2

LITERATURE REVIEW

Phage Display

Phage Display (U.S. Patent #5,223,409, 1993; #5,403,484, 1995; #5,571,698, 1995), originally designed for basic peptide ligand-receptor target molecule linkage studies, has been adapted for use in many other areas of science research. Phage are very easy to select, manipulate, and clone. Since phage display is a powerful tool in expressing peptides in an exposed manner, hundreds of peptides can be displayed and tested at a time. There is a physical linkage between the peptide and the phage genome. The bound peptide sequence can be determined by sequencing the selected and amplified phage clone.

To name a few applications, phage display has been applied as an alternative to hybridoma work for antibody production and for intracellular targeting of recombinant antibodies (Cannon et al., 1996; Neri et al., 1995; Gargano and Cattaneo, 1997; Peterson, 1996), as a method for commercial purification of biotherapeutics through the generation of ligands (Maclennan, 1995), and as a tool for in vivo diagnostics (Dyax News, 1996). Antibodies, hormones, and DNA binding proteins have been displayed on phage, and variants with altered specificity or affinity have been isolated from libraries of random mutants (Barbas, 1993; Lowman et al., 1991). Phage display has been used to optimize the binding of catalytic antibodies to transition-state analogs. These methods can be used in conjunction with catalysis screening in order to identify variants with higher catalytic efficiencies (Baca et al., 1997). Phage display has also been used in the identification of peptide mimics of non-peptide ligands (Hoess et al., 1993). Another benefit of phage display is the construction, expression, and screening of comprehensive libraries for murine or human antibody Fab fragments displayed on the surface of filamentous phage (Engberg et al., 1996).
Phage display is a peptide ligand selection process that uses a combinatorial library of random peptides fused to a coat protein of a bacteriophage. Oligomers are genetically fused to the minor coat protein, gene III, of M13 filamentous coliphage for display on the phage’s surface exterior. The phage display can be used to obtain a peptide ligand-receptor target molecule linkage. A physical linkage between the combinatorial library of random peptides and the DNA encoding each sequence allows for rapid identification of peptide ligands that bind a target domain. The peptide ligands that recognize a target domain are then isolated and amplified by an \textit{in vitro} process called biopanning. Biopanning is a selection process carried out by incubating a library of phage-displayed peptides on a plate or bead coated with a specific target. Bound phage remains after washing away the unbound phage. The specifically-bound phage is eluted, then amplified by \textit{E. coli} infection, and passed through successive cycles of biopanning and amplification. These successive cycles are done in order to enrich the pool of phage specifically binding a target. A total of three to four cycles is carried out and individual clones are characterized by DNA sequencing protocols (U.S. Patent #5,223,409, 1993; #5,403,484, 1995; #5,571,698, 1995; Bonnycastle \textit{et al.}, 1996).

An M13 Ph.D.\textsuperscript{TM} 7 or Ph.D.\textsuperscript{TM} 12 from the Ph.D.\textsuperscript{TM} Phage Display Peptide Library Kit (New England BioLabs, Inc., Beverly, MA, USA) are useful for the application of phage display techniques. The Ph.D.\textsuperscript{TM} 7 peptide library consists of $2 \times 10^9$ electroporated sequences amplified once to achieve approximately 100 copies of each sequence in a 10 µl sample (U.S. Patent #5,223,409, 1993; #5,403,484, 1995; #5,571,698, 1995). This concentration compares to $20^7$, or $1.28 \times 10^9$ possible sequences using this 7-mer. The Ph.D.\textsuperscript{TM} 12 peptide library consists of $1.9 \times 10^9$ electroporated sequences amplified once to achieve approximately 70 copies of each sequence in a 10 µl sample (U.S. Patent #5,223,409, 1993; #5,403,484, 1995; #5,571,698, 1995). The 7-mer or 12-mer libraries are displayed at the N-terminus of pIII, with the first residue of the mature protein in the first randomized position. The peptide is followed by a short spacer, Gly-Gly-Gly-Ser, which in turn is followed by the wild-type pIII sequence (U.S. Patent #5,223,409, 1993; #5,403,484, 1995; #5,571,698, 1995; Bonnycastle \textit{et al.}, 1996).
The M13 phage is chosen in these phage display procedures for its f-1 origin. The ‘f’ refers to the filamentous appearance of the phage, and also refers to its dependence on the F-pilus for infection of its specific *E. coli* host. Collectively, M13, f1, and fd phage are referred to as Ff phage (Wilson and Finlay, 1998). The Ff virion consists of a single-stranded DNA genome, packaged into a tube that contains 2700 copies of a major coat protein, pVIII, closed at the ends by four to five copies each of four minor coat proteins (Wilson and Finlay, 1998). One of the minor coat proteins is pIII on the M13 coliphage, fused to oligomers for peptide display on the phage surface.

A recent development in the application of phage display is for the selection of organ-specific peptides. This process was accomplished *in vivo* by injections of a random peptide phage display library into mice. After the injection of a peptide library, the organs of interest were harvested, washed, and the bound phage eluted. This eluted phage was amplified and used in subsequent rounds of injection and selection (Pasqualini and Ruoslahti, 1996; Arap et al., 1998). Phage was found to transfect cells *in vivo* in a manner determined by blood flow (Pasqualini and Ruoslahti, 1996), and phage display libraries were used to isolate peptides that bind preferentially to tumor blood vessels (Arap et al., 1998). Peptides selected in this manner have been successfully used for the delivery of drugs targeted to tumor cells. When Arap *et al.* used phage display techniques to identify two peptides that bound tumor cells, these two peptides were coupled to an anticancer drug. One of these peptides, coupled to the anticancer drug doxorubicin, enhanced the efficacy of the drug against human breast cancer xenografts in nude mice. The peptide also reduced the toxicity of the drug (Arap *et al.*, 1998).

Since organ targeting was the focus of Pasqualini and Ruoslahti (1996), phage libraries were used to identify peptides that selectively delivered the phage to mouse brain and kidney blood vessels. One of these peptides was shown to target the brain with a particle attached, rather than the phage attached. Coupling this peptide to the surface of red blood cells aided the accumulation of the red blood cells to the brain in a greater magnitude than to the kidney. The peptide itself competitively blocked the peptide-red blood cell complex from localizing in the brain, yet had no effect on the kidney. As a result, these procedures may be adapted for grafting.
motifs to surface molecules of viruses, or to couple motifs to the surface of cells used in gene therapy (Pasqualini and Ruoslahti, 1996), thereby enhancing cell-specific targeting.

Another application for phage display libraries is to identify ligands for component antibodies in complex polyclonal antisera (Scott and Smith, 1990). The antigen does not need to be a previously known component. The peptides might aid in identifying and distinguishing differences among strains of viruses, bacteria or parasites when affinity purified with sera from infected patients. The peptides would then be used as a delivery tool for vaccines or immunogens (Scott and Smith, 1990). As stated by Scott and Smith, the value of the epitope library is that a large and important part of the epitope universe can be encompassed in a few microliters of solution. These few microliters can be effectively surveyed for specific affinity to an antibody, receptor, or other binding protein by simple recombinant DNA methods.

**Embryology**

To enhance research on sperm-ova interactions the identification and characterization of the receptors involved would be beneficial. Not only are zona pellucida or sperm receptors of interest, but investigation of factors or sites beyond the zona in an embryo (i.e., cytoplasmic membrane or cytoplasm) is important. The knowledge of specific sperm-ova interactions is still limited. Until recently, sperm receptors on the membrane of a mouse oocyte had not been identified (Cheng *et al.*, 1994). Further, some aspects of the sperm-egg interaction appear to be species-specific, while other aspects remain conserved among mammals. Zona pellucida glycoproteins, mZP3 and mZP2, and an integrin, α6β1, serve as sperm receptors on the murine ova membrane. Briefly, mZP3 is a primary sperm receptor, mZP2 is a secondary sperm receptor, and sp56 has been found as a possible sperm protein with affinity to ZP3 (Wassarman, 1992; Cheng *et al.*, 1994; Almeida *et al.*, 1995).

For natural oocyte fertilization to occur, mammalian sperm must bind to the zona pellucida of the ova. The zona pellucida is an extracellular coat that is formed around the mammalian oocyte
during ovarian follicular development. This coat is of uniform thickness, 5 to 10 µm in eutherian mammals (Green, 1997), and 7 µm thick in mice (Dietl, 1989). Once the zona pellucida is formed and the ovum has matured, ovulation occurs and the sperm binds to and penetrates the zona pellucida matrix during fertilization. Recent reports indicate that the sperm sp56 protein is responsible for the initial fertilization event. Numerous models have been proposed for the fertilization process, but a comprehensive and detailed process is yet to be completed (Dunbar et al., 1994).

The components of the mouse zona pellucida currently identified and defined are three sulfated glycoproteins. These glycoproteins which are synthesized by a membrane bound step to form a filamentous matrix are ZP1, ZP2 and ZP3. ZP1 is a dimer of two apparently identical subunits that cross-link the complexed co-polymers ZP2 and ZP3 (Hinsch et al., 1997; Green, 1997). The three proteins are found both in mice and humans and ZP3 occurs in various mammals as a highly conserved primary structure revealed by cDNA cloning (Hinsch and Hinsch, 1996). The mouse zona pellucida is composed of filaments ZP1, ZP2, and ZP3 constructed by association of globular proteins in a head-to-tail array; the ratio of ZP1 to ZP3 is approximately 1:1, and the ZP2-ZP3 heterodimers are the basic repeating units of the filamentous matrix cross-linked by dimeric ZP1 (Green, 1997).

Each component of the zona pellucida is important for successful ovum maturation and fertilization. While ZP1 is responsible for cross-linking ZP2 and ZP3, ZP3 is the ligand responsible for primary sperm association and binding. Once the sperm binds to the ZP3, the acrosome reaction is initiated. Glycoprotein ZP2 is also involved in fertilization by mediating secondary binding of spermatozoa. Proteases released during fertilization cause cleavage of ZP2, which in turn is responsible for the zona “hardening”, thus preventing polyspermy (Hinsch et al., 1997; Green, 1997).

In the past decade, murine ZP2 and ZP3 were the first components of the zona pellucida that were identified. Although there is extensive knowledge currently being gained on the ZP proteins, the structure remains unsolved (Green, 1997). ZP1 is a 623 amino acid polypeptide
chain. All zona proteins consist of a polypeptide chain with a signal peptide and a carboxyl-terminal transmembrane domain (Epifano et al., 1995). The murine glycoprotein ZP3 mRNA has 1317 nucleotides (nt) with an estimated poly(A) tail of 200-300 nt; murine ZP3 is an 83-85 kDa glycoprotein, while human ZP3 is 64 kDa (Ringuette et al., 1988; Rankin et al., 1998).

Rankin et al. (1998) manipulated the human and murine ZP3 glycoproteins. They tested the species-specificity and importance of ZP3. Mice were genetically altered to be deficient in the ZP3 protein. Without the ZP3 protein the mice could not form a zona pellucida, thus rendering them infertile. Transgenic mice were produced to express human ZP3 (67% identical to murine ZP3) and mated to ZP3 null mice in an attempt to rescue the ZP3 phenotype. The human ZP3-rescued female mice produced chimeric zona pellucidae with murine ZP1 and ZP2, but human ZP3. Human sperm did not bind to the chimeric zona, yet mouse sperm did bind to ovulated eggs in vitro and fertility was restored in vivo. Further experimentation would be beneficial to identify ZP3 and other ova-sperm interaction sites. Fertilization and 1-cell embryo maturation sites might even be identified beneath the zona pellucida membrane. Besides further characterization of ZP3 peptide sequences, ZP2 might be targeted and further characterized along with ZP1. Other sites to consider would be the cytoplasmic membrane and intracytoplasmic factors.

From a different perspective, there have been several domains on the spermatozoa implicated in the egg-sperm fertilization cascade. Several sperm proteins suggested to complement ZP3 are sp56 (sperm protein 56), β-1,4-galactosyltransferase and p95 (Shalgi and Raz, 1997). The first sperm surface protein to be identified and thought to have a role in sperm-egg recognition is sp56. This sp56 gains its name from its relative molecular mass of 56,000 Da and its specific affinity for mouse ova ZP3. The sp56 is a homomultimer, peripheral membrane protein located on the plasma membrane covering the acrosome where the sperm’s ZP3-binding sites are located. Differential extraction experiments indicate that sp56 is attached to the sperm surface via interactions sensitive to low pH and is not an integral protein (Cheng et al., 1994).
Another surface protein of the spermatozoa is PH-20, or Sperm Adhesion Molecule 1 (SPAM1), a single-chain protein anchored in the membrane (Zheng and Martin-Deleon, 1999). The N-terminus of the molecule possesses hyaluronidase activity. This protease activity of the sperm plasma membrane allows sperm to penetrate cumulus cells surrounding the oocyte. A secondary function of PH-20 is the binding of acrosome-reacted sperm to the zona pellucida (Myles and Primakoff, 1997).

Protein fertilin in varying structural forms is involved in sperm-egg fusion. Previously called PH-30, fertilin is a heterodimer with α and β subunits. This complex of sperm surface antigens is involved in fertilization; more specifically, fertilin is involved in the binding and fusion of sperm to the zona pellucida of the egg (Almeida et al., 1995). Even with this growing data on egg and sperm receptors, most interactions are still speculations rather than fully elucidated processes. The phage display technique may aid in identifying more receptors not yet targeted, and perhaps may help further characterize and validate the currently known receptors. From another aspect, phage display may aid in identifying peptides that bind mouse sperm at the sites that are responsible for egg recognition. Perhaps a peptide isolated through phage display techniques may be bound to the sperm to block ova recognition. The theory is to block either the sperm with a sperm-binding peptide or block the egg with an egg-binding peptide. In this manner, if the sperm ligand for an egg receptor is bound by a specific peptide, the sperm will not be able to bind to the egg thus preventing fertilization.

**Transgenesis**

Transgenic animals have been defined as animals that have integrated foreign DNA into their germline as a consequence of experimental introduction of DNA (Palmiter et al., 1985). Introduction of foreign DNA into mammals can be accomplished by microinjection of linear construct DNA into one pronucleus of fertilized eggs (Hamada et al., 1993; Wilmut et al., 1991). Many researchers use mice as disease models to study phenotypic and genotypic traits. Other scientists use mice to set the groundwork for transgenic animal production. If the process is
successful in mice for the DNA of choice, then larger domestic animals may be chosen for transgenic production of recombinant proteins. However, the production of transgenic livestock, especially cattle, has a painstakingly low efficiency due to a low transgene integration frequency (Gagne et al., 1995). To date, the only reported successful process for production of large animal transgenics is through microinjection or nuclear transfer methods. These methods are costly and very labor intensive.

In mice, a successful microinjectionist can expect only 5% of the injected eggs to develop into live transgenic animals. Usually, about 50 to 65% of the fertilized eggs survive the injection procedure; 20 to 25% of the implanted eggs develop into pups and 10 to 13% of the pups born can be hemizygous transgenic, containing one DNA integration site (Sedivy and Joyner, 1992). Since the production of transgenic mice is possible, the current procedures have become routine with few reports of new methods for improving the process. Furthermore, in farm animals, these processes may not be as efficient, resulting in increased costs and limited use of transgenic livestock (Burdon and Wall, 1992; Wall et al., 1990). Embryos are more difficult to recover from large animal species (Wilmut et al., 1991). The overall efficiency of transgenesis in large domestic animals varies between 0.5 and 1.5% as opposed to the reported 5% for mice (Gagne et al., 1995; Sedivy and Joyner, 1992). Despite these rates of transgenesis, there are few reports that investigate and elucidate the mechanisms of transgenesis (Hamada et al., 1993).

A drawback to using pronuclear microinjection for generating transgenic animals is the generation of a hemizygous or mosaic embryo, depending on when the injected DNA integrates into the genome, or even a nontransgenic embryo (Wilkie et al., 1986). The injected DNA integrates at random sites within the genome, and often multiple copies of the injected DNA are incorporated at one site. Due to the randomness of the integration site and copy number, transgene expression is highly variable. In some cases, the transgene copy number may be excessive and lead to overexpression, which disrupts the normal physiology of the animal. If integration occurs after the first round of DNA replication, mosaic or nontransgenic animals will be produced (Whitelaw et al., 1992).
Replication of DNA occurs in the pronuclear S-phase. It has been reported that the first pronuclear S-phase in the mouse occurs about 16 to 22 hours post insemination (hpi), average of 18 to 20 hpi, and continues for 4 to 5 h depending on the strain of mouse (Hogan et al., 1986; Howlett, 1986; Laurincik et al., 1994; Wilkie et al., 1986). In cattle, S-phase starts approximately 12 hpi and lasts from 7 to 9 h (Laurincik et al., 1994). Mouse zygotes have pronuclear visualization and S-phase within a tight window of each other. However, in cattle this tight window does not exist since pronuclear visualization occurs in mid to late S-phase (Laurincik et al., 1994). Therefore, the ability to perform DNA microinjection procedures during similar, early times within the pronuclear S-phase does not exist for cattle as it does for mice. Also, among embryos collected from the same animal at the same time, initiation and completion of DNA synthesis will be asynchronous (Luthardt and Donahue, 1973).

Other more recent techniques used in transgenesis may be slightly more efficient than the traditional pronuclear injection method, yet they are still cumbersome for arriving at successful transgenic animal production. The use of embryonic stem (ES) cell, somatic cell and nuclear transfer technology is being studied in vast detail in efforts to define the processes of DNA integration. If the right combination of events and materials can be determined and the DNA integration processes clearly defined, then more efficient use of animals and highly efficient transgenic numbers could be accomplished. In somatic cell technology and nuclear transfer, adult somatic cells have been used most recently for the production of cloned sheep from mammary tissue, cattle from muscle cells and mice from cumulus cells (Campbell, 1999; Heyman et al., 1998; Wakayama and Yanagimachi, 1999). There have been only a few of each of these species produced, and the technology is still new with specific processes remaining unrevealed.

Presently, ES cells are beneficial for site-directed mutagenesis by homologous recombination. This ability to specifically insert the exogenous DNA into a chosen site in the host genome is necessary for knock-out or knock-in mice. Knock-out and knock-in technology is a refinement of ES cell technology. Foreign DNA is targeted to a specific site in the host genome via homologous recombination in ES cells. Resultant chimeric mice, heterozygous for the knockout
mutation are then mated to produce homozygous knockout mice. These mice are used as models for development and disease studies by causing a loss-of-function of genes through knock-out, or function expression or overexpression by knock-in methods. These mice provide a useful model system for studying embryogenesis and behavior, and for studying certain human genetic diseases. One good example of knockout mice is in the study of cystic fibrosis. The gene causing an autosomal recessive mutation in the cystic fibrosis transmembrane receptor (CFTR) gene has been cloned and the biochemical function of its encoded protein studied. Using the human gene, the homologous mouse gene was isolated and mutations introduced. Gene-knockout techniques were applied to produce homozygous mutant mice that then showed the phenotype of cystic fibrosis in humans (Shastry, 1998; Bear et al., 1992; Collins, 1992).

Another method, intracytoplasmic sperm injection (ICSI), has been successfully applied to unfertilized mouse oocytes. Current technology allows the introduction of foreign DNA into mouse oocytes via DNA attachment to the spermatozoa followed by ICSI. The reason ICSI has been incorporated with basic sperm-mediated DNA transfer is that the basic sperm-mediated DNA transfer alone has not been repeated with the initial success (Robl, 1999). Mouse epididymal sperm cells have been shown to spontaneously uptake foreign DNA. The exogenous DNA is then internalized into the sperm nuclei where sperm endogenous nucleases are activated to mediate rearrangements of the exogenous DNA (Magnano et al., 1998). Further expansion of this process has been attempted by freeze-thawing, freeze-drying or detergent treating the sperm to disrupt their membranes. Foreign DNA is then added to coat the sperm, and the sperm-coated DNA injected into an unfertilized mouse oocyte. Exogenous DNA was reproducibly delivered via this procedure, with 20% of the offspring expressing the transgene (Perry et al., 1999). Even though these processes have been successful, sperm-mediated DNA transfer still has limitations. Sperm has to be injected using laborious ICSI techniques, and these techniques have not been widely practiced outside of human ICSI. There is no current evidence that the sperm-mediated DNA injection procedure in mice would be more efficient than traditional pronuclear microinjection (Robl, 1999).
Currently, the means of transgenic animal production are very costly and time consuming. Further application of the present work might allow the transgene, along with poly-L-lysine, to be linked to a peptide ligand. Poly-L-lysine has been shown to add protection to nonencapsidated DNA, shielding it against DNase activity. Poly-L-lysine also aids in increasing the efficiency of short-term expression in vitro and in vivo (Soeda et al., 1998). This polycation:DNA complex was found to act as a substrate for transgenesis at a lysine to phosphate ratio of 1:1, with DNA concentration at 50 µg per ml (Page et al., 1995). A proposed peptide-ligand plus poly-L-lysine and foreign DNA complex might be introduced upon fertilization into an oocyte. This may occur by the peptide-ligand binding the embryo recognition site, thus carrying the poly-L-lysine/exogenous DNA complex into the egg at fertilization. This process could ensure that the exogenous DNA is introduced prior to the first round of DNA replication known as S-phase, perhaps increasing the likelihood that the DNA will become incorporated into the animal’s genome.

If a peptide-ligand/poly-L-lysine/DNA complex method of transgenesis is possible, then microinjection and pronuclear visualization would no longer be an issue for introduction of DNA, and a new technology for making transgenics might be initiated. The labor-intensive problems and costs of current transgenic production could be drastically reduced, yielding a more efficient means of transgenic animal production. Finally, such a process would allow the treatment of many embryos en masse rather than the labor-intensive, one at a time process of pronuclear microinjection.
CHAPTER 3

PHAGE DISPLAY TO IDENTIFY PEPTIDES BINDING TO OR PENETRATING THE MOUSE ZONA PELLUCIDA

Jeanette Lowe

(ABSTRACT)

The objective of this study was to identify peptide ligands, using phage display techniques, which bind sites on mouse embryos, ovaries, cytoplasmic membranes and/or intracytoplasmic components. Specifically, M13 coliphage 7-mer, 12-mer and 15-mer random peptide libraries were used separately for biopanning. Peptides derived from the amplified pools were sequenced and studied. The phage display for in vivo ovary experiments yielded no pool of peptides after two cycles of biopanning and re-amplification. With the same initial concentration of a random 7-mer or 12-mer library, there were repeating sequences derived after three and four biopanning cycles on mouse embryos and unfertilized ova. The sequences were not distinguishable from a control group. Subsequent experimentation using a random 15-mer library to select for internalized phage-peptides yielded two apparent consensus sequences, RNVPPIFNDVYWIAF (9/32 or 28%) and HGRFILPWWYAFSPS (11/32 or 34%). The 15-mer control group yielded no clones. The deduced peptide sequences were compared to known sequences to ascertain their uniqueness. No significant similarities were found, yielding two possible novel motifs. Through this adapted process of phage display and further research, the phage display technology may be used as a tool in the recognition of specific mouse gamete sites. By identifying binding sites of mouse gametes, the peptides might be exploited as a means of studying the embryo cell surface or cytoplasmic components and mouse sperm-egg interactions. Such peptides may also be used for macromolecule delivery in transfection or transgenesis.
INTRODUCTION

Phage Display (U.S. Patent #5,223,409, 1993; #5,403,484, 1995; #5,571,698, 1995) was originally designed for basic peptide ligand-receptor target molecule linkage studies. Since its onset in the early 1990’s, phage display has been adapted for use in many other areas of research (Wilson and Finlay, 1998). To name a few adaptations, phage display is currently being applied as an alternative to hybridoma work for antibody production and for intracellular targeting of recombinant antibodies (Cannon et al., 1996; Neri et al., 1995; Gargano and Cattaneo, 1997; Peterson, 1996). It is also applied as a method for commercial purification of biotherapeutics through the generation of ligands (Maclennan, 1995), and as a tool for in vivo diagnostics (Dyax News, 1996).

Another benefit of phage display is the construction, expression, and screening of comprehensive libraries for murine or human antibody Fab fragments displayed on the surface of filamentous phage (Engberg et al., 1996). Phage are very easy to select, manipulate, and clone. Since phage display is a powerful tool in expressing peptides in an exposed manner, hundreds of peptides can be displayed and tested at a time due to the fact that a peptide and the phage’s genome are physically linked. The bound peptide sequence can be determined by sequencing the selected and amplified phage clone.

Normal phage display techniques involve biopanning a random peptide library against a known target and amplifying the phage-peptide(s) that bind. The process includes eluting and amplifying the phage that binds the target, plating to yield phage clones for isolating each clone’s DNA, and sequencing of that DNA for characterization of the peptide. The procedures in this paper expand upon the phage display techniques to determine whether a random peptide library might be used to identify a yet unknown target within mouse fertilization. In this manner, the phage clones that bind an unknown target can be sequenced to characterize peptides that the DNA encodes. Once these peptides are derived, they can be compared to other known peptide sequences listed through GenBank or other similar sequence databases.
The main purpose for this research is to identify receptors in or on mouse eggs that might be used for transfection of oocytes. Foreign molecules, such as DNA, RNA, and peptides may then be attached to the peptides identified through phage display. The peptide could be exploited as a delivery system for transfection. This delivery system would be especially useful in transgenic mouse production, and possibly extend to the production of other transgenic animals. Finally, the ligands of phage display are small peptides that could resemble naturally occurring oocyte or sperm ligands. This minimizes the possibility of complexes inducing an immune response in vivo (Hart et al., 1995).

MATERIALS AND METHODS

Mice

Female B6C3F1 mice (Harlan Sprague Dawley, Indianapolis, IN, USA), 3 to 4 wk of age were superovulated by intraperitoneal injection of 7.5 IU Pregnant Mare’s Serum Gonadotropin (PMSG, 367222; CalBiochem, San Diego, CA, USA), followed 46 to 48 h later with an intraperitoneal injection of 5 IU human chorionic gonadotropin (hCG, 230734; CalBiochem, San Diego, CA, USA). At the time of hCG administration, the females were placed with B6C3F1 male mice, 6 wk or older. The following morning only females with vaginal plugs were used to collect one-cell embryos, according to standard procedures (Hogan et al., 1986). Some of the mice after hCG injection were not mated in order to obtain unfertilized ova for experimental manipulation.

Embryo, Ova and Ovary Collection

The mated and unmated female mice were killed by cervical dislocation approximately 18 h post hCG injection. The ovaries and oviducts were removed and placed in a 35 mm petri
dish (Falcon 3001; Fisher, Norcross, GA, USA) containing 3 ml FHM (Flushing Holding Media, MR-025-D; Specialty Media, Lavalette, NJ, USA). Once separated from the oviducts, the ovaries were placed into a 15 ml conical tube (Fisher, Norcross, GA, USA) and plunged into liquid nitrogen until processed for phage introduction experiments. Eggs were removed from the ampulla region of the oviduct and placed into 3 ml of FHM containing approximately 0.2 mg/ml hyaluronidase (H 3884; Sigma, St. Louis, MO, USA). Once the cumulus cells were removed, the ova were placed into a fresh 35mm petri dish of FHM for immediate manipulation. If later manipulation or longer incubation was required, the embryos were rinsed through a fresh 35mm petri dish of KSOM (Embryo culture medium, MR-020-D; Specialty Media, Lavalette, NJ, USA). Once rinsed, approximately 20 embryos were placed per 20 µl drop of KSOM overlaid with mineral oil (embryo tested, M 8410; Sigma, St. Louis, MO, USA) in 35mm petri dishes and then placed in a 37.0°C, 5.0% CO₂ humidified air incubator (Model BB16 EC/CO₂; Heraeus Instruments, South Plainfield, NJ, USA) until further experimental manipulation.

For removal of zona pellucidae, a batch of oocytes was placed into a 500 µl drop of Acid Tyrode’s solution (T 1788; Sigma Chemical Co., St. Louis, MO, USA) in a 35 mm dish at 37.0°C. The oocytes were incubated approximately 1 min before the zona pellucida was digested. Immediately after the zona pellucida disappeared, the plate was flooded with FHM to stop the digestion. The oocytes were then allowed to incubate in FHM at 37.0°C for at least a 30 min recovery period. These oocytes with the zona pellucida removed will be referred to as zona-free embryos or zona-free ova. Embryos and/or ova collectively will be referred to as oocytes; unfertilized oocytes as unfertilized ova, and fertilized oocytes as embryos.

**Phage Display**

The phage used for the initial phage display experiments was M13 Ph.D.™ 7 or Ph.D.™ 12 from the Ph.D.™ Phage Display Peptide Library Kit (New England BioLabs, Inc., Beverly,
MA, USA). The *E. coli* for amplification was *E. coli* ER2537 provided in the kit. The phage library was introduced into the mice via tail vein injections or by specific incubation with embryos and ova. Later experiments included only a 15-mer random peptide library provided by Syncomm (Syncomm, Philadelphia, PA, USA). A K91 host was used for amplifying the 15-mer phage, and this 15-mer phage was applied to zona-free embryos.

The materials/solutions used for biopanning and amplification were made by standard recipes and procedures listed in the Ph.D.™ Phage Display Peptide Library Kit instruction manual (Version 1.01 provided in the kit) and in Sambrook *et al.*, 1989.

A. **7-mer and 12-mer Phage:**

Phage introduction was evaluated by assessing *in vivo* and *in vitro* systems. Groups 1, 2 and 3 were used to investigate intravenous introduction of phage. Groups 4 and 5 investigated *in vitro* phage introduction.

*Group 1:* Approximately 4 h prior to hCG administration, 0.1 cc of 4.5 x 10^{11} pfu Ph.D.™ 7 per mouse was injected into the tail vein of 5 mice. The 5 mice were mated and on the following day, the ovaries were harvested and placed into liquid nitrogen.

*Group 2:* The embryos from mice in Group 1 were collected from the oviducts at 18 h post hCG injection.

*Group 3a:* Group 3a consisted of 5 mice receiving PMSG. No hCG was administered 46 to 48 h later; instead 0.1 cc of 4.5 x 10^{11} pfu phage Ph.D.™ 7 was injected into the tail vein of each mouse. The mice were killed 5 min later, and the ovaries harvested and placed into liquid nitrogen.

*Group 3b:* Group 3b received the regular injection of PMSG and hCG, then the phage was injected and ovaries collected 5 min later and stored in liquid nitrogen.
**Group 4:** Embryos were collected from 3 mice that received the PMSG/hCG superovulation protocol. These mice were mated. The embryos were divided into three sets: embryos, zona-free embryos, and 2-cell embryos.

**Group 5:** Unfertilized ova were collected from 3 mice receiving the PMSG/hCG superovulation protocol, and not mated.

After collection, embryos/ova from groups 4 and 5, were placed separately into a 100 µl drop of FHM with a specified phage concentration (*Day 1*, cycle 1, below). These drops were overlaid with oil and left undisturbed for 15 min at room temperature. Each group was then washed through three consecutive plates of fresh FHM.

**I. Adaptation of Phage Display Techniques to Target Mouse Embryo Sites**

The phage display/biopanning methods used in these experiments were adapted for mice from existing protocols (U.S. Patent #5,223,409, 1993; #5,403,484, 1995; #5,571,698, 1995; Bonnycastle et al., 1996; Instruction manual, New England BioLabs, Inc., Beverly, MA, USA). Generally, phage were presented to ovaries or oocytes (either fertilized or unfertilized) and incubated for a specified time. This incubation is called “biopanning”. After biopanning, the phage were eluted with a glycine/BSA solution and neutralized with 1.0 M Tris-HCl, pH 8.0. Titers and plates were performed on the phage eluates to determine concentration in plaque forming units (pfu). The 7-mer and 12-mer eluates were amplified in *E. coli* ER2537, the 15-mer eluates in K91, then each was precipitated in a PEG/NaCl solution (20% polyethylene glycol and 2.5M sodium chloride). Another group of titers and plates was conducted to determine the amplified concentration in pfu. These amplified eluates were then introduced to a new set of ovaries or oocytes and incubated for a specified time. A total of three to four cycles of biopanning and amplification were
conducted using the eluates from the previous cycle. Selected eluates were prepared for sequencing and analysis.

Detailed protocols:

*Day 1*
For the first step in the first cycle of biopanning, the desired phage concentration was prepared for each trial. There were five trials of varying phage and concentration; phage and concentration for each trial were as follows.

*Trial 1:* 6.25 µl of Ph.D.™ 7 neat (~$10^{11}$ pfu/µl) was added to each 100 µl drop and mixed well with a pipet.

*Trial 2:* 6.25 µl of Ph.D.™ 7 neat (~$2 \times 10^{10}$ pfu/µl) was added to each 100 µl drop.

*Trial 3:* 6.25 µl of Ph.D.™ 12 neat (~$1.3 \times 10^{10}$ pfu/µl) was added to each 100 µl drop.

*Trial 4:* 50 µl of Ph.D.™ 12 amplified (~$2.24 \times 10^{10}$ pfu/µl) was added to 50 µl of FHM to make a 100 µl drop.

*Trial 5:* 6.25 µl of Ph.D.™ 12 neat (~$4 \times 10^{9}$ pfu/µl) was added to each 100 µl drop.

For the second cycle of biopanning, the phage eluates were prepared as follows. *In vivo* introduction of phage was accomplished by tail vein injections for Groups 1 and 3: 0.1cc of eluate was injected, and mice were separated for each group. For the third cycle, 0.05cc of eluate was injected into the tail vein. For Groups 4 and 5: in Trial 1, 5 µl of eluate was mixed in 100 µl FHM; Trial 3, 10 µl of eluate was mixed in 100 µl FHM; Trials 2, 4 and 5, 50 µl of eluate was mixed in 50 µl FHM. Phage was introduced into superovulated mice or onto collected embryos or ova.

Each group of collected embryos and ova were incubated with phage for 15 minutes at room temperature. Each group was then washed through three consecutive 35 mm petri
dishes containing 3 ml of FHM. Separately, each group was placed into 100 µl of 0.2M Glycine/1.0mg per ml BSA, pH 2.2, for 10 min at room temperature. This mixture acted as a non-specific competitor for the bound phage, eluting it from the binding molecule. Each group was then neutralized with 100 µl 1.0M Tris-HCl, pH 8.0, mixed well and stored at 4°C. Each sample was labeled as an eluate with the group specification.

Ovaries were harvested and plunged into liquid nitrogen. After freezing, each treatment group was crushed separately in 1.0 ml saline, transferred to a microfuge tube, and centrifuged at 10,600 x g for 5 minutes to pellet the ovaries. The groups were rinsed two times with 1.0 ml TBS (Tris-buffered saline: 50 mM Tris-HCl, pH7.5, and 150 mM NaCl). The pellets were resuspended in 100 µl 0.2M Glycine/1.0mg per ml BSA, pH 2.2, and allowed to incubate for 10 min at room temperature. The samples were then neutralized with 100 µl 1.0M Tris, pH 8.0, mixed well and stored at 4°C. Each sample was labeled as an eluate with the group specification.

**Day 2**

A 20 ml volume of LB (Luria-Bertani Medium) per sample to be amplified, was inoculated with a single colony of ER2537. Each culture was placed into a shaker incubator at 37.0°C and shaken until mid-log phase growth (approximately 2 h to 4 h). Top agar (agarose top medium) for LB plate overlay was melted in a microwave, and 3 ml aliquoted into each sterile culture tube, one for each titer to be plated. These tubes of top agar were cooled to 45.0°C until ready for the addition of the titered eluates. While preparing the eluate titers for plate overlay, one 100 mm plate (#0875713; Fisher, Norcross, GA, USA) of LB agar per expected titer was equilibrated to 37.0°C in an incubator.

A small amount of the eluate for each respective group was titered by adding 2 µl eluate to the first tube of 18 µl LB broth in microfuge tubes. Serial dilutions, or titers, were continued by transferring 2 µl of a dilution into the next microfuge tube of 18 µl LB broth (usually five titers was sufficient for unamplified eluates; five to fifteen titers was adequate.
for amplified eluates). For the control titers, 2 μl M13 phage was used instead of an eluate (control dilutions are usually sufficient in the eight to twelve titer range).

To each titer, 180 μl mid-log phase ER2537 was added and mixed gently. The culture was allowed to stand at room temperature for 5 min. Each 200 μl titer was then transferred to a 3 ml tube of the top agar, vortexed quickly, and immediately overlaid on an LB plate previously equilibrated at 37.0°C. The overlay was spread evenly and allowed to cool on the benchtop. Plates were inverted and incubated in a 37.0°C incubator overnight.

To prepare for Day 3 experiments, a 500 ml volume of LB in a 2 L flask was inoculated with ER2537. This culture was shaken in a shaker incubator at 37.0°C overnight.

II. Biopanning and Amplification of the Bound 7-mer and 12-mer Phage

Day 3

Plates from Day 2 experiments were evaluated and the plaques counted. The phage titer for each plate was recorded in pfu (plaque forming units).

The overnight E. coli culture was diluted to a mid-log phase density. For amplification of the eluate samples, 20 ml of this diluted ER2537 was aliquoted into a 100 ml flask per an eluate. Separately, an eluate from each group was added into one 20 ml ER2537 culture flask. All of the cultures were incubated in a shaker incubator at 37.0°C with vigorous shaking for 4.5 h. After the 4.5 h incubation, each culture was transferred to a centrifuge tube and centrifuged at 10,600 x g for 10 min at 4.0°C. The supernatant was placed into a new tube and the centrifugation was repeated. The upper 80% of the supernatant was pipetted into a fresh tube and 1/6 volume of PEG/NaCl solution (20% polyethylene glycol and 2.5M sodium chloride) was added. The phage was left to precipitate 60 min to overnight at 4.0°C.

A single colony of ER2537 was inoculated into 20 ml of LB, and grown to mid-log phase in a shaker incubator at 37.0°C with vigorous shaking. This culture was used for titers of
the amplified eluates. The overnight PEG precipitate was centrifuged for 15 min at 10,600 x g, 4.0°C. The supernatant was decanted and the tube re-centrifuged 30 s to 1 min. Any residual supernatant was removed with a pipette. The pellet was resuspended in 1.0 ml TBS (Tris-buffered saline). This suspension was transferred to a microfuge tube and centrifuged for 5 min at 4.0°C to pellet residual cells. The supernatant was transferred into a fresh microfuge tube and again precipitated with 1/6 volume of PEG/NaCl. This tube was incubated on ice for 15 to 60 min, then centrifuged for 10 min in a microfuge at 4.0°C. The supernatant was discarded and the tube was re-centrifuged for 30 s to 1 min. Any residual supernatant was removed with a micropipet. The pellet was resuspended in 200 µl TBS with 0.02% NaN₃ then microfuged for 1 min. The supernatant was transferred to a fresh microfuge tube. The supernatant was labeled as an amplified eluate for the appropriate group and stored at 4.0°C until titers and plates were performed.

Titors were performed and plates evaluated as in the procedures previously discussed. Another cycle of biopanning and amplification was carried out, starting again with the procedures in cycle one. The only exception to the repeat cycle was the use of the eluates derived after the amplification steps in place of the original phage introduction. A total of three to four cycles of amplification was performed, and titers and plates were evaluated at the end of each cycle.

Sequence, Analysis, and Characterization of 7-mer and 12-mer Bound Peptide Elution

Phage that bound the mouse oocytes were eluted and amplified, and then sequenced for characterization and analysis. Selected plaques were picked from titered plates containing preferably no more than 100 plaques. An overnight culture of LB inoculated with ER2537 was used as follows: 1 ml of diluted culture (1:100 dilution of overnight culture) was dispensed into culture tubes, one for each clone to be amplified and characterized. Ten to 20 tubes were set up for each group. Each plaque picked was stabbed with a sterile pipet tip and placed into one tube of the 1 ml diluted culture. Each plaque selected should
contain a single DNA sequence. These culture tubes were incubated for 4.5 h at 37.0°C with vigorous shaking.

Each culture was transferred separately to a microcentrifuge tube and centrifuged 30 s. The supernatant was then transferred to a fresh tube and re-centrifuged. With a pipet, the upper 80% of the supernatant was placed into a fresh tube. Each supernatant was labeled as an amplified phage stock, including the appropriate group name. A 500 µl volume of the amplified phage stock was placed into a fresh microfuge tube. A solution of 200 µl PEG/NaCl was added to the 500 µl phage and inverted to mix. The tube was left at room temperature for 10 min, then centrifuged 10 min, and the supernatant discarded. The tube was re-centrifuge for 1 min, and any remaining supernatant carefully pipetted away. The pellet was resuspended in 100 µl iodide buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 4 M NaCl) followed by the addition of 250 µl 100% ethanol. This mixture was incubated for 10 min at room temperature in order to precipitate mostly single-stranded phage DNA, leaving most phage protein in solution. The precipitate was centrifuged 10 min and the supernatant was discarded. The pellet was washed in 70% ethanol and dried briefly under vacuum at room temperature. The dried pellet was suspended in 30 µl TE buffer (10 mM Tris-HCl + 1 mM EDTA, pH 8.0). OD readings (an optical density value) of each sample were obtained using a spectrophotometer set at OD260. The concentration of each sample was calculated in µg/ml.

Each clone was sequenced using the ABI 377 sequencer machine with the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit and Protocol (Perkin Elmer, Foster City, CA, USA). Briefly, 100 ng of phage DNA and 3.2 pmol of -96 gIII sequencing primer provided in the phage display kit were added, then 8 µl ABI rhodamine dye terminator premix was added to equal a 20 µl total volume. On a thermal cycler: each sample was heated at 96°C for 1 min, followed by 25 cycles of 96°C for 30 s, 50°C for 15 s, and 60°C for 4 min, then held at 4.0°C. Centriflex Gel Filtration Cartridges (Edge Biosystems, Gaithersburg, MD, USA) were used to remove any unincorporated nucleotides, then dried by vacuum. Dried pellets were resuspended in 3 µl of 5:1 formamide:EDTA
blue dextran, and 1.6 µl of each sample was loaded on a 5% Long Ranger gel (SMC, Rockland, ME, USA) on the ABI 377 machine for a 9 h run. Sequence comparisons and analysis were performed by use of the BLAST search algorithm (Zhang and Madden, 1997) at GenBank (see ref.).

**Fluorescent Peptides for 7-mer and 12-mer Binding Results**

Peptides amplified by biopanning that have three or more repeats after sequencing and analysis were fluorescent dye labeled in Research Genetics’ peptide department. Synthesis of peptides was via Fmoc (Carpino, 1957; Chang and Meienhofer, 1978; Atherton et al., 1978). The solid phase synthesis (Merrifield, 1963) was on chlorotrityl resins (Novabiochem, La Jolla, CA, USA) using DIC/HBTU/HOBt (Di-Isopropyl carbodiimide/H 2-(H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/N-hydroxy benzotriazole, H2O) activation on Advanced Chemtech 396-5000 multiple peptide synthesizers (ACT, Louisville, KY, USA). Protected amino acids (Sygena-Genzyme, Cambridge, MA, USA) were double coupled at 8 fold excess for 1 h. Resins were DMF (Dimethyl formamide; EM Science, Gibbstown, NJ, USA) washed and coupled overnight with NHS-Fluorescein (n-hydroxy succinimydyl ester of fluorescein; Pierce, Rockford, IL, USA). Resins were MeOH (Methanol; Fisher, Pittsburgh, PA, USA) washed and cleaved in Reagent R:TFA (trifluoroacetic acid), EDT (ethanedithiol), Thioanisole, Anisole (Albericio et al., 1990). The TFA mixture containing the peptide in solution was precipitated in ether and washed extensively. Preparative HPLC of peptides (Mant and Hodges, 1996) was accomplished by a gradient of 0 to 80% acetonitrile (EM Science, Gibbstown, NJ, USA) in 0.1% TFA. Lyophilization of the various fractions and verification by MALDI-TOF using a Voyager mass spectrophotometer (PerSeptive, Foster City, CA, USA) yielded the synthetic peptides as a TFA salt. The peptides were then reconstituted to 2 mM in DEPC (Diethyl pyrocarbonate) treated water (750024; Research Genetics, Huntsville, AL, USA) and 1 drop of concentrated ammonium hydroxide was added if the salts were not completely solubilized.
Once the peptides were fluorescent dye labeled, each peptide was reconstituted and diluted to 20 µM in FHM. Each peptide was then introduced to a series of unfertilized ova, 1-cell zygotes, both zona-free ova and zona-free zygotes, and 2-cell embryos. The process included incubation of each of these groups with 20 µM fluorescent peptide, one peptide per test group. Each group of 10 to 15 selected ova or embryos was placed into 5 µl drops of the fluorescent peptide/FHM medium mix overlaid with oil, in a 35 mm petri dish. The groups were incubated in the dark at room temperature for 30 min and 1.5-h intervals. At the end of incubation, each group was washed separately through three consecutive dishes of FHM. Each group was then placed into 2 µl drops of FHM overlaid with oil in a 60 mm petri dish lid (Falcon 3004; Fisher, Norcross, GA, USA). The groups were viewed under fluorescence on an Olympus BH2-RFCA fluorescent microscope (Olympus America, Inc, Lake Success, NY, USA) equipped with a green single bandpass filter, excitation peak = 509 nm and FWHM = 31 (full width of the spectral band at half of the peak intensity), emission peak = 538 nm and FWHM = 44 (Vysis set 30-150291, 12534 Green 705; Olympus America, Inc, Lake Success, NY, USA).

**Non-denaturing Gel and Western**

Three groups of 700 to 800 embryos were collected from superovulated B6C3F1 mice. Each group was stored in approximately 2 to 5 µl FHM in a microfuge tube (#C-3260-BX; Gene Mate, Kaysville, VT, USA) at -20°C until ready to run on gels.

Each sample was thawed at room temperature. A 5X sample buffer solution (310 mM Tris-HCl, pH6.8, and 50% glycerol in water; Walker, 1994) and a 10% SDS solution (75008; Research Genetics, Huntsville, AL, USA) were added to each sample to produce a final concentration of 1X sample buffer and 1% SDS in 20 to 30 µl total volume. The sample was mixed gently while incubating at room temperature for 15 min. The full sample was then loaded into one well of a 4 to 15% acrylamide Tris-HCl minigel (161-1104; BIO-RAD Laboratories, Hercules, CA, USA). With one well as an empty space before and after the
sample, 5 µl of a prestained SDS-PAGE standard (low range #161-0305; BIO-RAD Laboratories, Hercules, CA, USA) was loaded into one well on each side. The gel was run for 30 to 45 min at 200V (Power Pac 300; BIO-RAD Laboratories, Hercules, CA, USA) in a Mini-Protean II Cell (165-2940; BIO-RAD Laboratories, Hercules, CA, USA) with 1X Tris/Tricine/SDS buffer (10X, 161-0744; BIO-RAD Laboratories, Hercules, CA, USA) in the chamber. The gel was then removed from the chamber and equilibrated for 30 min at room temperature in transfer buffer (25 mM Trizma base, 190 mM glycine and 20% methanol in deionized water).

A Western transfer of the gel was done in a transfer chamber and Mini Gel Cassette Holder (170-3931; BIO-RAD Laboratories, Hercules, CA, USA) with transfer buffer onto Hybond™-N membrane (RPN 2222N; Amersham Life Science Inc, Arlington Heights, IL, USA). The transfer was at 100V for 3 h with cooling. The membrane was then blocked with 2% BSA in TBS, and gently shaken for 30 min at room temperature. The membrane was washed in TBS, then incubated with a 20 µM fluorescent peptide and rotated in the dark at room temperature for 4 h. The membrane was washed in several changes of TBS. The membrane was then scanned for fluorescence, using a Molecular Dynamic’s Storm 860 optical scanner with FluorImager and ImageQuaNT™ v4.2 program (Molecular Dynamics, Inc, Sunnyvale, CA, USA).

B. 15-mer Phage:

I. Adaptation of Phage Display Techniques to Target Mouse Embryo Sites

Zona-free embryos were used in this group of experiments. The control group was phage itself processed through the same steps as the zona-free embryos. Detailed protocols were the same as for the 7-mer and 12-mer previously outlined, except for the changes listed below.
All FHM steps included 100 µM chloroquine (C 6628; Sigma, St. Louis, MO, USA) added unless otherwise specified. K91 was used as the host for amplification of the 15-mer instead of ER2537.

Detailed protocols:

**Day 1**
All steps were performed at 37.0°C. In the first cycle of biopanning, 6.25 µl of 15-mer phage pool neat (~10^{14} pfu/µl) was added into each 100 µl drop of FHM + 100 µM chloroquine. For the second and third cycle of biopanning, 50 µl of eluate was mixed with 50 µl FHM + 100 µM chloroquine. Zona-free embryos were introduced to a 100 µl drop of the phage dilution overlaid with oil. The phage and zona-free embryo mixture was incubated at 37.0°C for 15 min. Each group was then washed through seven consecutive 35 mm petri dishes of FHM. The first six dishes for each group contained 3 ml of FHM + 100 µM chloroquine, the seventh dish contained 3 ml of FHM with no additives. Procedures were continued as before, except 1% Triton-X 100 was added to the glycine/BSA step of each group for 10 min at 4.0°C. This Triton-X 100 was to lyse the zygotes.

**Day 2**
Procedures were continued as before.

**II. Biopanning and amplification of the Bound 15-mer Phage**

**Day 3**
This section was continued the same as previous procedures, except for the amplification process. Instead of amplification in the LB broth, amplification was done on large plates. Large agar plates were made using Nunc 243 x 243 x 18mm Bio-Assay dishes (#12565224; Fisher, Norcross, GA, USA). A 300 ml volume of LB Agar with 100 µg/ml kanamycin (K0879; Sigma, St. Louis, MO, USA) and 15 µg/ml tetracycline (BP912-100; Fisher,
Norcross, GA, USA) was poured per plate. Agar was left undisturbed to set. To 1.8 ml mid-log phase K91 per eluate, all of the eluate was added, vortexed and allowed to stand for 5 min. Each K91-eluate culture was then added to a separate 30 ml aliquot of top agar containing 100 µg/ml kanamycin and 15 µg/ml tetracycline. Each top agar was vortexed quickly and overlaid on a separate LB agar plate. Once set, the plates were inverted and incubated at 37.0°C overnight until plaques were visible.

Next, 10 ml LB was added onto each plate. Only the top agar was harvested into a separate tube for each plate. Each plate needed two 50 ml conical tubes, with the top agar divided evenly between the two tubes. Each tube was brought to ~45 ml mark with LB, then rotated for approximately 1 h at room temperature. The tubes were centrifuged at 10,600 x g for 10 min at 4.0°C. This procedure was continued as in the previous procedures. The final pellet was resuspended in 200 µl to 2.0 ml TBS with 0.02% NaN₃, depending on size and solubility of the pellet. Again, this procedure was continued as before.

**Sequence, Analysis and Characterization of 15-mer Bound Peptide Elution**

Twenty-four plaques were selected per sequencing batch. The selected plaques were processed as before, and incubated at room temperature to 30°C for 1 h with shaking. For each plaque culture, the 1 ml of culture was added to 3 ml of top agar containing 100 µg/ml kanamycin and 15 µg/ml tetracycline. The top agar culture was vortexed and overlaid onto one 100mm LB agar plate per plaque. The plates were inverted and incubated in a 37.0°C incubator overnight until plaques formed and were visible. A 5 ml volume of LB was added onto each plate. Only the top agar was harvested into a separate 15 ml tube for each plate. Each tube was brought to the 10 ml mark with LB and rotated for approximately 1 h at room temperature. The tubes of culture were then centrifuged at 10,600 x g for 10 min at 4.0°C. The supernatant was carefully removed, and procedures continued as before.
**Immunofluorescence for 15-mer Binding Results**

(personal communication with Mark J. Hay via email; Barry *et al.*, 1996).

Large LB agar 243 x 243 x 18mm Bio-Assay dishes were made and processed as described in *Biopanning and amplification of the Bound 15-mer Phage, Day 3* section above. For amplification of eluates, one eluate corresponding to each consensus sequence derived from the third cycle of 15-mer biopanning was selected. For this set of experiments, #31 corresponded to the derived consensus sequence HGRFILPWY AFSPS and #37 corresponded to RNVPIFNDV YWIAF. Only 2 µl of each eluate was added, separately, into 1.8 ml K91 mid-log phase host. The control was 10 µl of the stock 15-mer random pool added to 1.8 ml K91 mid-log phase host.

Embryos were collected and the zona removed with Acid Tyrode’s solution (T-1788; Sigma Chemical Co., St. Louis, MO, USA). The embryos were allowed to recover in FHM medium for at least 30 min before continuing. A batch of 75 to 100 zona-free embryos was added to each of three groups: 50 µl of #31 mixed with 50 µl FHM + 100 µM chloroquine; 50 µl of #37 mixed with 50 µl FHM + 100 µM chloroquine; 50 µl of control 15-mer mixed with 50 µl FHM + 100 µM chloroquine. The 100 µl drops were overlaid with oil and incubated at 37.0°C for 15 min. Each group was then washed through 7 consecutive 35 mm petri dishes. The first six dishes for each group contained 3 ml of FHM + 100 µM chloroquine, the seventh dish contained 3 ml of FHM with no additives.

Chambers were made for processing the embryos through immunofluorescence procedures. The wells were made by sealing #10 Stainless Fender Washers (080358820161; Lowe’s, Huntsville, AL, USA) with clear nail polish to microscope slides (#12-550-43, 25 x 75 x 1mm; Fisher, Norcross, GA, USA). Lint free tissues were used to wash and drain the slides on. The zona-free embryos were fixed in 100 µl drops of 0.5% to 2% formaldehyde for 20 min at room temperature in a 35 mm petri dish overlaid with oil. During this 20 min incubation: 1 mg/ml poly-L-lysine (P 1524; Sigma, St. Louis, MO, USA) in PBS was
applied to the central well of each slide. The slides were incubated at room temperature for 15 min. The poly-L-lysine was removed with a syringe and needle, and the wells washed with PBS, 3 times for 15 min each. A rounded drop of PBS was left in each well.

The prepared embryos were added to the slide wells, and blown gently to stick the embryos down onto the slide surface. A glass coverslip (#12-542B, 22 x 22 x 1mm; Fisher, Norcross, GA, USA) was gently placed over each chamber. The slides were centrifuged for 10 min at 1500 x g, 4.0°C in Sorvall Super T21 centrifuge equipped with a swinging arm rotor and 96-well plate platforms. After centrifugation, the top coverslip was gently pushed away. Embryos were stored in the chambers at 4.0°C in PBS for up to one week.

A solution of 0.25% Triton X-100 (T 9284; Sigma, St. Louis, MO, USA) in PBS was added to the central wells for 15 min at room temperature to permeabilize membranes, then washed with PBS. A solution of 2.6 mg/ml NH₄Cl (A 9434; Sigma, St. Louis, MO, USA) in PBS was added for 10 min at room temperature, and washed with PBS. The primary antibody of sheep anti-M13 antibody (#7-916192; 5 Prime-3 Prime, Boulder, CO, USA) was diluted 1:100 in PBS/Tween-20 (concentration of 1 µg/ml, P 1379; Sigma, St. Louis, MO, USA) and centrifuged at 17,900 x g for 3 min in a tabletop microfuge. A volume of 20 µl of the primary antibody was very carefully added to each central well and chambers covered with a petri dish lid to avoid evaporation. These covered chambers were incubated at room temperature for 1 h. The wells were then washed three times, 15 min each wash, with PBS/Tween.

The secondary antibody of biotinylated rabbit anti-sheep IgG (B-A600; Vector Laboratories, Burlingame, CA, USA) was diluted 1:100 in PBS/Tween and centrifuged as before. Again, the wells were washed three times, 15 min each wash, with PBS/Tween. Next, 1:500 Neutralite avidin-fluorescein conjugate (A-2662; Molecular Probes, Eugene, OR, USA) in PBS/Tween was added to each well and incubated for 1 h at room temperature, 20 µl per central well. The wells were then washed three times for 15 min with PBS/Tween. The central wells were overlaid with Vectashield (H-1000; Vector
Laboratories, Burlingame, CA, USA) and viewed on an Olympus BH2-RFCA fluorescent microscope (Olympus America, Inc, Lake Success, NY, USA) equipped with a green single bandpass filter, excitation peak = 509 nm and FWHM = 31 (full width of the spectral band at half of the peak intensity), emission peak = 538 nm and FWHM = 44 (Vysis set 30-150291, 12534 Green 705; Olympus America, Inc, Lake Success, NY, USA).

RESULTS

Biopanning/Amplification and Sequencing

The results of biopanning with 7-mer, 12-mer and 15-mer random peptides on ovaries, embryos, unfertilized ova and zona-free embryos are reported below.

Key abbreviations:
phage conc. = phage concentration
pfu/ml = plaque forming units per ml
1 in = starting phage concentration for step 1 in biopanning
1out = phage concentration obtained after biopanning and elution in step 1
r.a. 1 out = repeat amplification of 1 out for 2 in
2 in = 1 out amplified eluate, diluted to specified concentration for step 2 of biopanning
rpt. 2 in = repeat of 2 in, same experiment, for a new 2 out
r.a. 2 in = repeat amplification of 2 in
2 out = phage concentration obtained after biopanning and elution in step 2
r.a. 2 out = repeat amplification of 2 out for 3 in
3 in = 2 out amplified eluate, diluted to specified concentration for step 3 of biopanning
3 out = phage concentration obtained after biopanning and elution in step 3
4 in = 3 out amplified eluate, diluted to specified concentration for step 4 of biopanning
5 in = 4 out amplified eluate
For sequencing:
The phage clone sequences were reverse complemented and translated via OMIGA program (v1.03; Oxford Molecular Group, Inc. Campbell, CA, USA). Sequences are presented in 5’ → 3’ orientation (for the sense strand).

Amino Acid reference

| A | Alanine         | K | Lysine           |
| R | Arginine        | M | Methionine       |
| N | Asparagine      | F | Phenylalanine    |
| D | Aspartic acid   | P | Proline          |
| C | Cysteine        | S | Serine           |
| Q | Glutamine       | T | Threonine        |
| E | Glutamic acid   | W | Tryptophan       |
| G | Glycine         | Y | Tyrosine         |
| H | Histidine       | V | Valine           |
| I | Isoleucine      | B | Asparagine or aspartic acid |
| L | Leucine         | Z | Glutamine or glutamic acid |
Ph.D.™ 7 Results:

The first trial with Ph.D. 7 included ovaries from mated mice, embryos, ovaries from unmated mice and unfertilized ova. The phage display was successful in cycle one for binding and amplification on embryos and unfertilized ova; cycle two also appeared successful. Adversely, the ovary experiments yielded no pool of peptides after three cycles of biopanning and re-amplification.

Cycle 3 did not yield amplification for the embryo or ova groups (Figure 1A). Titers and plates had no plaques. A repeat amplification from a previous cycle yielded plaques upon titering/plating, but resulted in a decline of recoverable material. Amplification was not significant enough to carry each sequential step, even in the embryo and ova groups. Several attempts at varying the procedure did not increase yield. Since there was no significant amplification and a constant decline in phage concentration from step to step, this particular procedure was not continued.

Sequencing was performed on the eluates from each group of 3 in. Consensus sequences were not apparent for the ovary groups. Only four similar sequences were observed by subjective evaluation of the sequences derived from the unfertilized ova, SLP_S_F (Figure 1B). The embryos, however, produced the majority of one apparent consensus sequence. The sequence bias observed was LPLTPLP at 80% (12/15), along with two matching sequences of KQTLPSA (Figure 1C).
**Figure 1A:** Trial 1 results of phage concentration from biopanning and amplification at each cycle, using a Ph.D.7 phage display random library. The phage were injected intravenously into mice. One set of mice was mated, another set was not. Ovaries were collected from each set, along with embryos and unfertilized ova. Three cycles of biopanning were carried out.

**Key abbreviations:**
- phage conc. = phage concentration
- pfu/ml = plaque forming units per ml
- 1 in = starting phage concentration for step 1 in biopanning
- 2 in = 1 out amplified eluate, diluted to specified concentration for step 2 of biopanning
- 3 in = 2 out amplified eluate, diluted to specified concentration for step 3 of biopanning
- r.a. 2 out = repeat amplification of 2 out for 3 in
- 4 in = 3 out amplified eluate, diluted to specified concentration for step 4 of biopanning

---

**Legend:**
- Phage conc. from ovaries of mated mice
- Phage conc. from embryos
- Phage conc. from ovaries of unmated mice
- Phage conc. from unfertilized ova
Figure 1B: Trial 1 sequencing results derived from clones of Ph.D. 7 with mouse ova. The Ph.D. 7 was incubated with unfertilized ova \textit{in vitro}, and biopanned through three cycles. Sequencing was performed on the eluates from 3 in. Only four similar sequences of SLP\_S\_F, derived from the unfertilized ova, were observed by subjective evaluation of these sequences.
**Figure 1C:** Trial 1 sequencing results derived from clones of Ph.D. 7 with mouse embryos. The Ph.D. 7 was incubated with embryos *in vitro*, and biopanned through three cycles. Sequencing was performed on the eluates from 3 in. These embryos produced one apparent consensus sequence.
Trial 2 was conducted using a new random library of Ph.D.7 against embryos and unfertilized ova, and included a control group. This new random 7-mer library was ordered to compare to Trial 1 results, and a control group was added. There were four cycles of biopanning completed (Figure 2A).

Sequencing on cycle 3 and cycle 4 provided possible consensus sequences for all groups: embryos (Figure 2B), unfertilized ova (Figure 2C) and control (Figure 2D). The sequence biases observed for the embryos were NVQPTRP at 37%, IPVTYRT at 25% (4/16), SHQSPMF at 19% (3/16), and 19% (3/16) random sequences (Figure 2B). The consensus sequence observed for the ova was 100% LPHYIQN (Figure 2C). Note the frequency of repeated sequences in the control group (Figure 2D). The unexpected sequence biases observed were GNLNHLL (4/18), YAGPYQH (3/18), HAIYPRH (2/18), WPTLQWA (2/18), TNRLHPP (2/18), and 5/18 random sequences. At this point, the unbound phage appears not to have been completely washed away from each group. An experiment was carried out to test dilution of phage in successive wash plates. The data obtained indicated that at least three additional successive washes should be performed to wash away all unbound, non-specific phage (data not shown). Further experiments with the 7-mer and new wash techniques did not provide ample product after the second cycle of biopanning. The samples reached undetectable pfu in cycle 2.
Figure 2A: Trial 2 results of phage concentration from biopanning and amplification at each cycle, using a Ph.D.7 phage display random library. The phage were incubated with embryos and unfertilized ova separately, and compared to a control group of phage itself. Four cycles of biopanning were carried out.

Key abbreviations:
phage conc. = phage concentration
pfu/ml = plaque forming units per ml
1 in = starting phage concentration for step 1 in biopanning
2 in = 1 out amplified eluate, diluted to specified concentration for step 2 of biopanning
r.a. 2 in = repeat amplification of 2 in
3 in = 2 out amplified eluate, diluted to specified concentration for step 3 of biopanning
4 in = 3 out amplified eluate, diluted to specified concentration for step 4 of biopanning
5 in = 4 out amplified eluate
Figure 2B: Trial 2 sequencing results derived from clones of Ph.D. 7 with mouse embryos. The Ph.D. 7 was incubated with embryos in vitro, and biopanned through four cycles. Sequencing was performed on the eluates from 5 in.
Figure 2C: Trial 2 sequencing results derived from clones of Ph.D. 7 with mouse ova. The Ph.D. 7 was incubated with unfertilized ova *in vitro*, and biopanned through four cycles. Sequencing was performed on the eluates from 5 in.
Figure 2D: Trial 2 sequencing results derived from clones of Ph.D. 7 control. The Ph.D. 7 was incubated alone (no embryos or ova), and biopanned through four cycles. Sequencing was performed on the eluates from 5 in.
Ph.D. 12 Results:

Trial 3 was the first trial attempted with the phage random 12-mer library. The 12-mer experimentation was undertaken to test if the 7-mers derived from the random 7-mer library were within the amino acid sequences of the 12-mer. The 12-mer was also proposed to have a better binding affinity than the 7-mer. Experiments were conducted on embryos and unfertilized ova. Several attempts to retrieve a substantial product to continue were unyielding (Figure 3). A new Ph.D. 12 stock was ordered for further experiments.

Figure 3: Trial 3 results of phage concentration from biopanning and amplification at each cycle, using a Ph.D.12 phage display random library. The phage were incubated with embryos and unfertilized ova separately. Two cycles of biopanning were carried out. No phage were amplified from 2 out (= 3 in).

Key abbreviations:
phage conc. = phage concentration
pfu/ml = plaque forming units per ml
1 in = starting phage concentration for step 1 in biopanning
2 in = 1 out amplified eluate, diluted to specified concentration for step 2 of biopanning
r.a. 1 out = repeat amplification of 1 out for 2 in
rpt. 2 in = repeat of 2 in, same experiment, for a new 2 out
3 in = 2 out amplified eluate, diluted to specified concentration for step 3 of biopanning
Trial 4 was the second trial to test a phage random 12-mer library, but the first time with a newly received Ph.D. 12 stock. This new random 12-mer library was ordered to test if the last trial’s losses were due to an older library. The groups used were embryos and unfertilized ova. Both groups had sufficient eluates at the end of cycle 3 biopanning to isolate for sequencing (Figure 4A).

Sequencing was performed for both groups after cycle 2 was completed. Only the embryo group yielded four possible consensus sequences with two to four repeats each. Nevertheless, cycle 3 sequencing produced possible consensus sequences for both the embryo group (Figure 4B) and the unfertilized ova group (Figure 4C). The sequence biases observed for the embryos were SVSVGMKPSRP (6/21), YQLRPNASELRP (2/21), ATAYPNPFSPGA (2/16), and 11/21 random sequences (Figure 4B). The sequence bias observed for the ova was SVSVGMKPSRP (5/16), and 11/16 random sequences (Figure 4C).
**Figure 4A:** Trial 4 results of phage concentration from biopanning and amplification at each cycle, using a new Ph.D.12 phage display random library. The phage were incubated with embryos and unfertilized ova separately. Three successful cycles of biopanning were carried out.

**Key abbreviations:**
- **phage conc.** = phage concentration
- **pfu/ml** = plaque forming units per ml
- **1 in** = starting phage concentration for step 1 in biopanning
- **2 in** = 1 out amplified eluate, diluted to specified concentration for step 2 of biopanning
- **3 in** = 2 out amplified eluate, diluted to specified concentration for step 3 of biopanning
- **4 in** = 3 out amplified eluate, diluted to specified concentration for step 4 of biopanning
**Figure 4B:** Trial 4 sequencing results derived from clones of Ph.D. 12 with mouse embryos. The Ph.D. 12 was incubated with embryos *in vitro*, and biopanned through three cycles. Sequencing was performed on the eluates from 4 in.
**Figure 4C:** Trial 4 sequencing results derived from clones of Ph.D. 12 with mouse ova. The Ph.D. 12 was incubated with unfertilized ova *in vitro*, and biopanned through three cycles. Sequencing was performed on the eluates from 4 in.
Trial 5 was the third trial with the phage random 12-mer library. This trial was conducted to confirm previous 12-mer results and to add a control group. The groups tested were embryos and unfertilized ova, and a control group. All groups had sufficient eluates at the end of cycle 3 and cycle 4 biopanning to isolate for sequencing (Figure 5A).

Sequencing was completed for all groups after cycles 3 and 4. In cycle 3, the embryo group yielded many sequences that did not have the correct leader sequence before the inserted 12-mer. Therefore, many of the 12-mer sequences were not obtainable from the selected clones. Also in cycle 3, the unfertilized ova group had three repeats of one sequence and the control group had two to five repeats of one sequence. Cycle 4 sequencing yielded two YPTTSSSPFIHH and two random sequences for the embryo group (Figure 5B). The sequences observed for the ova were VPGSQNIFLPRS (4/16), SYYSTSGIPLGV (3/16), DLYTGSKGRMAE (3/16), ANXKTVSSPLRQ (3/16), and 3/16 random sequences (Figure 5C). The control group sequencing yielded two similar repeats of one sequence. The only possible repeat sequence observed for the control group was FYSHSSXXXX (2/8), (Figure 5D).

Since this trial was run simultaneously with the Ph.D.7 Trial 2, we thought that the unbound phage was not completely washed away from each group. An additional experiment was carried out (data not shown) to test dilution of phage in successive wash plates. Data obtained indicated that at least 3 additional successive washes should be performed to wash away all unbound, non-specific phage. Further experiments with the 12-mer and new wash techniques did not provide ample products. The samples reached undetectable pfu in cycle 1, amplified marginally to continue into cycle 2, then nothing out at the end of cycle 2. Based on these results, a 15-mer random library was received for further experiments and new wash procedures were implemented.
Figure 5A: Trial 5 results of phage concentration from biopanning and amplification at each cycle, using a new Ph.D.12 phage display random library. The phage were incubated with embryos and unfertilized ova separately, compared to a control group of phage itself. Four cycles of biopanning were carried out.

Key abbreviations:
- phage conc. = phage concentration
- pfu/ml = plaque forming units per ml
- 1 in = starting phage concentration for step 1 in biopanning
- 2 in = 1 out amplified eluate, diluted to specified concentration for step 2 of biopanning
- r.a. 2 in = repeat amplification of 2 in
- 3 in = 2 out amplified eluate, diluted to specified concentration for step 3 of biopanning
- 4 in = 3 out amplified eluate, diluted to specified concentration for step 4 of biopanning
- 5 in = 4 out amplified eluate
Figure 5B: Trial 5 sequencing results derived from clones of new Ph.D. 12 with mouse embryos. The Ph.D. 12 was incubated with embryos in vitro, and biopanned through four cycles. Sequencing was performed on the eluates from 5 in.
Figure 5C: Trial 5 sequencing results derived from clones of new Ph.D. 12 with mouse ova. The Ph.D. 12 was incubated with unfertilized ova *in vitro*, and biopanned through four cycles. Sequencing was performed on the eluates from 5 in.
**Figure 5D:** Trial 5 sequencing results derived from clones of new Ph.D. 12 control. The Ph.D. 12 was incubated alone (no embryos or ova), and biopanned through four cycles. Sequencing was performed on the eluates of 5 in.
**Random 15-mer Library Results:**

The newly adapted protocol for the phage random 15-mer library included amplification in LB broth with the addition of 100 µg/ml kanamycin and tetracycline at 20 µg/ml for liquid media culture and 40 µg/ml for solid media culture. Also included were new wash procedures adapted and resolved in the final 7-mer and 12-mer experiments.

The first attempt at biopanning and amplifying the phage random 15-mer library was terminated since no amplification was possible with the 100 µg/ml kanamycin and 20 µg/ml tetracycline for liquid media culture (Figure 6). The 15-mer phage library and K91 host are newer technology, and the applied procedure of amplification in liquid media did not adapt as expected. After testing different procedures, amplification could be attained with 100 µg/ml kanamycin and no tetracycline, but correct phage-peptide (tetracycline resistant insert) selection was not possible without the tetracycline. Tests were conducted on varying concentrations of tetracycline to find the optimal conditions.
Figure 6: Results of phage concentration from biopanning and amplification at each cycle, using a new 15-mer phage display random library. The phage were incubated with zona-free embryos and regular embryos, separately, compared to control phage itself. Three cycles of biopanning were carried out, without sufficient amplification to continue. Amplification was performed using LB broth.

Key abbreviations:
phage conc. = phage concentration
pfu/ml = plaque forming units per ml
1 in = starting phage concentration for step 1 in biopanning
1 out = phage concentration obtained after biopanning and elution in step 1
2 in = 1 out amplified eluate, diluted to specified concentration for step 2 of biopanning
2 out = phage concentration obtained after biopanning and elution in step 2
3 in = 2 out amplified eluate, diluted to specified concentration for step 3 of biopanning

The next few trials were done at 4.0°C, then changed to 37.0°C with 100 µM chloroquine. The chloroquine preserves the phage from rapid degeneration in incubation steps. Unfortunately, there was not sufficient amplification by the second biopanning cycle for each trial. To circumvent this problem, amplification was changed from LB broth with 100 µg/ml kanamycin and 20 µg/ml tetracycline to solid plate LB and top agar containing 100 µg/ml kanamycin and 40 µg/ml tetracycline. Again, amplification was minimal. This amplification dilemma was solved by lowering the concentration of tetracycline to 15 µg/ml in solid culture.
The final trial, using 37.0°C with 100 µM chloroquine for biopanning and plates with 100 µg/ml kanamycin + 15 µg/ml tetracycline for plate amplification, was successful. The control group had no phage carried after wash steps. The zona-free embryos maintained good phage titers from biopanning and amplification through a third cycle (Figure 7A).

Sequencing results on the third cycle (3 out) of zona-free embryo eluate showed two possible consensus sequences. HGRFILPWWYAFSPS was represented at 34% (11/32) of the total sequences obtained, and RNVPPIFNDVYWIAF at 28% (~9/32). The remaining 38% (12/32) of the sequences were random. There was no control group for this sequencing since no phage were obtained in the control at 3 out (Figure 7B).
Figure 7A: Results of phage concentration from biopanning and amplification at each cycle, using a 15-mer phage display random library, at 37°C with 100 μM chloroquine. The phage were incubated with zona-free embryos and compared to control phage itself. Three successful cycles of biopanning were carried out, all at 37°C with 100 μM chloroquine added to the media. Amplification was performed using LB plates and top agar with 100 μg/ml kanamycin and 15 μg/ml tetracycline.

Key abbreviations:
phage conc. = phage concentration
pfu/ml = plaque forming units per ml
1 in = starting phage concentration for step 1 in biopanning
1 out = phage concentration obtained after biopanning and elution in step 1
2 in = 1 out amplified eluate, diluted to specified concentration for step 2 of biopanning
2 out = phage concentration obtained after biopanning and elution in step 2
3 in = 2 out amplified eluate, diluted to specified concentration for step 3 of biopanning
3 out = phage concentration obtained after biopanning and elution in step 3
Figure 7B: Sequencing results derived from clones of the random 15-mer phage library. The 15-mer library was incubated with zona free embryos in vitro, and biopanned through three cycles. Sequencing was performed on the eluates from 3 out.
Sequences Chosen for 7-mer and 12-mer Fluorescein Experiments

Based upon the results obtained from sequencing of the 7-mer and 12-mer trials, any sequence that was represented two or more times within a sequence set was chosen for fluorescent studies. The purpose for fluorescent dye labeled peptides was to ascertain whether the labeled free peptides would bind to ova or embryos.

The sequences were fluorescent dye labeled and incubated for varying times with different groups of zona or zona-free embryos, zona or zona-free unfertilized ova, or 2-cell embryos. Fluorescent sequences were diluted to 20 nM, 200 nM, 2 µM or 20 µM for incubations at varying times of 30 min to 1.5 h with the different groups. Briefly, any fluorescent-peptide binding observed varied from faint background to good fluorescence in one or all of the polar bodies, the cytoplasmic membrane or within the cytoplasm. Some eggs had full cytoplasm fluorescence while others had a spotted cytoplasm appearance. Specific fluorescent peptide sets are indicated below.

First Sequence Set:
Fluorescein-LPLTPLP-OH
Fluorescein-KQTLPSA-OH
Fluorescein-ATAYPNPFSPGA-OH
Fluorescein-LGLQVEGPRQLP-OH
Fluorescein-YYPASSTIQSRP-OH
Fluorescein-SVSVGMKPSRP-OH
Fluorescein-YQLRPNAESLRF-OH

The controls for all sets were embryos and unfertilized ova, no peptide added to FHM.
A one h incubation with 20 nM concentration of each peptide in the first sequencing set on 1-cell embryos and unfertilized ova yielded minimal background fluorescence. Background fluorescence is defined as a very faint fluorescence throughout the embryo or ova. There was no obvious fluorescence due to the peptides, since fluorescence would be very bright and mostly at specific locations. As a result 1 h incubations of 20 nM and 20 µM concentrations on 1-cell embryos and unfertilized ova were compared. For 20 nM groups there was no significant fluorescence. For 20 µM groups, varying intensities of fluorescence were observed (Appendix Table 1a; Figure 8, Figure 9, and Figure 10).

Bright fluorescence was observed around the cytoplasmic membrane in 7/12 of the Fl-LPLTPLP ova viewed, with a faint to moderate cytoplasmic fluorescence (Figure 8). A few polar body structures also fluoresced. Fluorescence for the Fl-ATAYPNPFSPGA embryos was observed in the polar body structures, along with overall faint background fluorescence (Figure 9). The cytoplasm also appeared to have a few sections of slightly brighter fluorescence. Again, Fl-ATAYPNPFSPGA with ova rather than embryos fluoresced in the polar body structures, along with cytoplasmic membrane fluorescence (Figure 10). A faint background fluorescence was observed in some ova, with slightly brighter cytoplasmic fluorescence in other ova. The control for this set, embryos or ova without any fluorescein-peptide incubation, yielded a faint background (Figure 11).

Next, 30 min incubations of 200 nM, 2 µM, and 20 µM concentrations on 1-cell embryos and unfertilized ova were examined. Then a 30 min incubation with 20 µM concentration of fluorescein-LPLTPLP-OH and fluorescein-KQTLPSA-OH was performed on 2-cell embryos, 1-cell embryos, 1-cell zona-free embryos, unfertilized ova, and zona-free unfertilized ova. No visible fluorescence beyond a very faint background was observed.
Figure 8:
Fluorescent peptide binding results of Fl-LPLTLP with mouse ova. The peptide LPLTLP, derived with the 7-mer random phage library against a group of embryos and a group of ova, was fluorescein labeled as Fl-LPLTLP. A 20 μM concentration of Fl-LPLTLP was introduced to unfertilized ova, washed to remove any unbound peptide, then visualized on an Olympus BH2-RFCA fluorescence microscope equipped with a green single bandpass filter.
Figure 9:
Fluorescent peptide binding results of Fl-ATAYPNPFSPGA with mouse embryos. The peptide ATAYPNPFSPGA, derived with the 12-mer random phage library against a group of embryos, was fluorescein labeled as Fl-ATAYPNPFSPGA. A 20 µM concentration of Fl-ATAYPNPFSPGA was introduced to embryos, washed to remove any unbound peptide, then visualized on an Olympus BH2-RFCA fluorescence microscope equipped with a green single bandpass filter.
Figure 10:
Fluorescent peptide binding results of Fl-ATAYPNPFSPGA with mouse ova. The peptide ATAYPNPFSPGA, derived with the 12-mer random phage library against a group of embryos, was fluorescein labeled as Fl-ATAYPNPFSPGA. A 20 µM concentration of Fl-ATAYPNPFSPGA was introduced to unfertilized ova, washed to remove any unbound peptide, then visualized on an Olympus BH2-RFCA fluorescence microscope equipped with a green single bandpass filter. This ova group was compared to the embryo group in Figure 9.
Figure 11:
Fluorescent peptide binding results of mouse embryos, control with no Fl-peptide. This control group contained embryos with no peptide incubation. The embryos were treated and washed in the same manner as the peptide groups, then visualized on an Olympus BH2-RFCA fluorescence microscope equipped with a green single bandpass filter. This embryo group was compared to the embryo groups with phage incubation, and compared to Figure 9.
Second Sequence Set:
H-LPLTPLP(K-fluorescein)-CONH$_2$
H-KQTLPSA(K-fluorescein)-CONH$_2$
H-ATAYPNPFSPGA(K-fluorescein)-CONH$_2$
H-LGLQVEGPRQLP(K-fluorescein)-CONH$_2$
H-YYPASSTIQSRP(K-fluorescein)-CONH$_2$
H-SVSVGMKPSPRP(K-fluorescein)-CONH$_2$
H-YQLRPNAESLRF(K-fluorescein)-CONH$_2$

This second set of fluorescent labeled peptides was constructed to test whether fluorescein placement (amino- versus carboxy- terminus) had an effect on peptide binding.

Some fluorescence was observed in the 20 µM groups from 30 min incubations of 20 µM and 2 µM concentrations on 1-cell embryos and unfertilized ova. A repeat of the 30 min incubation with 20 µM concentration on 1-cell embryos and unfertilized ova gave similar results (Appendix Table 2).

To test the binding ability or non-ability due to the placement of the fluorescent marker, fluorescein-LPLTPLP-OH and fluorescein-KQTLPSA-OH versus H-LPLTPLP(K-fluorescein)-CONH$_2$ and H-KQTLPSA(K-fluorescein)-CONH$_2$ were compared. A 20 µM concentration, 1.5 h incubation, on 1-cell embryos and unfertilized ova yielded no fluorescence.

Third Sequence Set:
Fluorescein-LPLTPLP-COOH
Fluorescein-KQTLPSA-COOH
Fluorescein-LPHYIQN-COOH
Fluorescein-NVQPTRP-COOH
Fluorescein-DLYTGSKGRMAE-COOH
Fluorescein-VPGSQNIFLPRS-COOH
The third set of fluorescent labeled peptides was to confirm previous results of LPLTPLP and KQTLPSA, and to test more sequences derived from the 7-mer and 12-mer phage incubated with embryos and ova groups. Experiments with this third set were as follows.

A 1.5 h incubation with 20 µM concentrations of each fluorescent peptide on 1-cell embryos and unfertilized ova was performed with positive results (Appendix Table 1b). A repeat of these concentrations and timing adding in 1-cell zona-free embryos and zona-free unfertilized ova did not result in any fluorescence, only background.

For comparison, 2-cell embryos were processed to determine if there would be a similarity or difference in binding capacity due to the stage of the cell cycle. A 1.5 h incubation was performed on 2-cell embryos, with 20 µM concentrations of fluorescein-KQTLPSA-COOH and fluorescein-VPGSQNFLPRS-COOH. The only difference observed was fluorescence of the perivitelline space for fluorescein-VPGSQNFLPRS-COOH.

*Fourth Sequence Set:*

Random pool, fluorescein-XXXXXXXX
STVSSWTNDNL(K-fluorescein)-CONH₂
APARPPQLPYA(K-fluorescein)-CONH₂
GNLNHLL(K-fluorescein)-CONH₂
YAGPYQH(K-fluorescein)-CONH₂
HAIYPRH(K-fluorescein)-CONH₂
WPTLQWA(K-fluorescein)-CONH₂
TNRLHPP(K-fluorescein)-CONH₂
AMYSVYT(K-fluorescein)-CONH₂
SSHTISF(K-fluorescein)-CONH₂

This was a fourth set chosen for fluorescent studies of the repeat sequences derived from control groups. Since these apparent consensus sequences were derived from the control groups, they must be compared to the derived consensus sequences from the embryo and
ova groups, and compared to a complete random pool. Experiments with this fourth set were as follows.

One-cell embryos and unfertilized ova were incubated for 1.5 h at 20 µM concentrations of fluorescent oligomers (Appendix Table 3). Since this group consists of a random 7-mer and oligomers obtained from the controls, we expected that there would be little to no specific binding with the ova or embryos, yielding little or no fluorescence. Varying intensities of fluorescence were observed. The random 7-mer-fluorescein yielded very faint fluorescence on apparent polar body structures, with a faint cytoplasmic background. Perhaps there was enough free fluorescein in the peptide groups to non-selectively bind the polar body region of the eggs. Selected sequences were repurified to test this hypothesis and longer wash times were added. Similar results were observed.

**Westerns**

There was no visible binding of the fluorescent peptides: H-ATAYPNPFSPGA(K-fluorescein)-CONH₂, Fluorescein-LPLTPLP-COOH, Fluorescein-KQTLPSA-COOH, after Western transfer and scanning. Coomassie staining of a 10-20% SDS-PAGE gel after Western transfer showed that the embryo protein successfully migrated on the gel.

**Immunofluorescence for 15-mer Binding Results**

Experiments are currently being performed using the two 15-mer sequences, #31 HGRFILPWYAFSPS and #37 RNVPPIFNDVYWIAF, and a control random 15-mer pool versus zona-free embryos. Two complete sets of repeatable results have been obtained.
The control embryos had a ‘flattened’, spread out appearance with only one embryo remaining completely intact. Regardless, both types of control embryos produced similar results. There was a slight, speckled fluorescence within the cytoplasm with no other obvious or specific binding observed (Figure 12 and Figure 13).

The #31 and #37 embryos yielded results very different than the control group. Embryos with #31 showed several thick fluorescent areas of binding in the cytoplasm with some lighter fluorescence within the ‘folds’ of the cytoplasm (Figures 14 - 17). Some cytoplasmic membrane fluorescence was observed and a few presumed polar body structures fluoresced (Figure 16 and Figure 17). For #37, the outer cytoplasmic membrane region fluoresced much brighter and more consistently than in the #31 group (Figure 18). Another polar body appearing structure fluoresced in the #37 group (Figure 19).
Figure 12:
Immunofluorescence results of the control 15-mer phage versus mouse zona-free embryos. A control random 15-mer pool was incubated with zona-free embryos. This control is for comparison with the results obtained using the two 15-mer sequences, #31 HGRFILPWWYAFSPS (Figs. 14 through 17) and #37 RNVPPIFDVYWIAF (Figs. 18 and 19), derived from the phage display of a random 15-mer library with zona-free embryos. Visualization was accomplished on an Olympus BH2-RFCA fluorescence microscope equipped with a green single bandpass filter.
Figure 13:
Immunofluorescence results of the control 15-mer phage versus mouse zona-free embryos. A control random 15-mer pool was incubated with zona-free embryos. This control is for comparison with the results obtained using the two 15-mer sequences, #31 HGRFILPWWYAFSPS (Figs. 14 through 17) and #37 RNVPPIFNDVYWIIF (Figs. 18 and 19), derived from the phage display of a random 15-mer library with zona-free embryos. Visualization was accomplished on an Olympus BH2-RFCA fluorescence microscope equipped with a green single bandpass filter.
Peptide #31, HGRFILPWWYAFSPS (Figs. 14 through 17), derived from the phage display of a random 15-mer library with zona-free embryos, was incubated with zona-free embryos. Visualization was accomplished on an Olympus BH2-RFCA fluorescence microscope equipped with a green single bandpass filter.

**Figure 14:**
Immunofluorescence results of #31 HGRFILPWWYAFSPS with mouse zona-free embryos.
Figure 15:
Immunofluorescence results of #31 HGRFILPWWYAFSPS with mouse zona-free embryos. Peptide #31, HGRFILPWWYAFSPS (Figs. 14 through 17), derived from the phage display of a random 15-mer library with zona-free embryos, was incubated with zona-free embryos. Visualization was accomplished on an Olympus BH2-RFCA fluorescence microscope equipped with a green single bandpass filter.
Figure 16:
Immunofluorescence results of #31 HGRFILPWWYAFSPS with mouse zona-free embryos. Peptide #31, HGRFILPWWYAFSPS (Figs. 14 through 17), derived from the phage display of a random 15-mer library with zona-free embryos, was incubated with zona-free embryos. Visualization was accomplished on an Olympus BH2-RFCA fluorescence microscope equipped with a green single bandpass filter.
Figure 17:
Immunofluorescence results of #31 HGRFILPWWYAFSPS with mouse zona-free embryos. Peptide #31, HGRFILPWWYAFSPS (Figs. 14 through 17), derived from the phage display of a random 15-mer library with zona-free embryos, was incubated with zona-free embryos. Visualization was accomplished on an Olympus BH2-RFCA fluorescence microscope equipped with a green single bandpass filter.
Figure 18:
Immunofluorescence results of #37 RNVPPIFNDVYWIAF with mouse zona-free embryos. Peptide #37, RNVPPIFNDVYWIAF (Figs. 18 and 19), derived from the phage display of a random 15-mer library with zona-free embryos, was incubated with zona-free embryos. Visualization was accomplished on an Olympus BH2-RFCA fluorescence microscope equipped with a green single bandpass filter.
Figure 19:
Immunofluorescence results of #37 RNVPPIFNDVYWIAF with mouse zona-free embryos. Peptide #37, RNVPPIFNDVYWIAF (Figs. 18 and 19), derived from the phage display of a random 15-mer library with zona-free embryos, was incubated with zona-free embryos. Visualization was accomplished on an Olympus BH2-RFCA fluorescence microscope equipped with a green single bandpass filter.
DISCUSSION

Since the onset of publications describing phage display, phage display technology has been successfully adapted in many areas of research (Wilson and Finlay, 1998). Nonetheless, only within the past year has the display technology been successfully applied to an \textit{in vivo} selection procedure. In retrospect, not all ventures in phage display technology have been entirely successful; it appears that phage and host biology play important roles in this success (Wilson and Finlay, 1998; personal communication with Stephen Albert Johnston). The procedures here focus on the phage display techniques to determine whether a random peptide library can be used to identify a known or yet unknown mouse oocyte or sperm receptor. From earlier experimental results, not all aspects of the original phage display procedures appear to be completely or clearly definable. In spite of that, more experiments were implemented using different revisions and varying oligomer random peptide libraries. Promising results have been obtained from final and continuing work. As was anticipated, the continued adaptation and application for this research has been quite challenging yet rewarding.

Results from the phage random 15-mer library experiments show promise for the application of phage display techniques to mouse zona-free embryos. Originally, a random 7-mer phage library was necessary for initial experiments. New England BioLabs suggests that the 7-mer should be used in tests prior to the use of an n-mer greater than the 7-mer. On the other hand, literature (Wilson and Finlay, 1998; Barry, \textit{et. al,} 1996) and personal communications (Perry Kirkham) suggest that a longer peptide may be necessary when attempting to identify binding sites on complex structures. Mouse embryos without a doubt fall into the complex structure category. Libraries of relatively long peptides may have advantages; for example it is feasible for a 20-mer library to have increased efficiency over a hexapeptide, due to stronger binding. A 20-residue library may increase the effective library diversity; for each 20-mer peptide there is a theoretical diversity of $20^{20}$, or $\sim 1 \times 10^{26}$, unique peptide sequences (Wilson and Finlay, 1998). For a 7-mer peptide there is a theoretical diversity of $20^7$, or $\sim 1.3 \times 10^9$, different peptide sequences. Since the mouse embryo is so complex, it is likely that not all residues will bind a target site. Increasing the length of a peptide-ligand may aid in increasing the likelihood that some residue or residues may bind. Large peptides are also advantageous in presenting a variety of secondary
structures in addition to linear segments of amino acids (Barry et al., 1996). The smaller peptides may be present only in linear fragments with very little or no secondary structure.

Experiments at the outset of applying phage display to this mouse embryo work included intravenous injection of Ph.D.™ 7 into mice. We proposed that the phage could be delivered into the ovaries and onto ova in vivo. Recently published research has shown that vector transfection of cells in vivo is determined by the site of introduction and the areas of blood flow (Arap, et al., 1998; Barry et al., 1996). Capitalizing on blood flow, peptides that ‘home’ specifically to tumor blood vessels have been isolated through in vivo selection of phage peptide libraries. This isolation was accomplished when phage were injected intravenously into nude mice bearing human breast cancer carcinomas. Three main peptide motifs that targeted the phage into the tumors were identified. These results are cited as the first time that an in vivo selection procedure has been applied to a random library (Arap et al., 1998).

For our work, intravenous phage injections into mice for ovarian incorporation unfortunately yielded no significant consensus sequence, and no significant amplification of eluate was possible. No apparent sequence biases were observed at any point for any of the ovary phage clones. A second trial of this experiment produced no plaques implying that there was no bound phage. It is possible that the phage was not concentrated enough to yield successful results at the end of cycle two. Injection of an increased concentration of phage did not solve this dilemma. Another possibility is that the phage-peptide may be too complex to traverse the ovarian vasculature in order to incorporate or anchor there, or may not bind based on the tissue or cell types present in the ovaries (Pasqualini and Ruoslahti, 1996). With this pattern of results, the ovary segment of experiments was discontinued.

The next step included experiments with Ph.D.™ 7 introduced to unfertilized ova and embryos in vitro. Binding and amplification were successful with the phage library on embryos and unfertilized ova, with signs of possible bias towards a few sequences. The fertilized 1-cell embryo group showed a definite bias towards a consensus sequence LPLTLP, along with two matching sequences of KQTLPSA. Further biopanning and amplification did not produce any
recoverable material. Since the earlier Ph.D.™ 7 result yielded a bias in sequence that ran the full extent of the 7-mer, a 12-mer library was tested to see if the sequence bias continued beyond the 7-mer window. Also, the 12-mer was considered to have a greater and more consistent ability to bind such complex targets since it is a longer peptide (Wilson and Finlay, 1998; Barry et al., 1996). Therefore, Ph.D.™ 12 was used in further experiments to observe binding results with mouse oocytes.

The Ph.D.™ 12 library yielded several possible consensus sequences in the embryo group, but not in the unfertilized ova group. Several of the consensus sequences observed in the embryo group were ATAYPNPFSP, LGLQVEGPRQ, SVSVGMKPSP, and YYPASSTIQS. The control groups were sequenced shortly after these sequencing results were obtained. Control groups consisted of phage itself, no embryo or ova incubation, treated with the same washes and procedures as the phage that was incubated with the embryo or ova groups. Unexpectedly, the control groups for the Ph.D.™ 12 and the last Ph.D.™ 7 trials produced a few repeating sequences after three cycles of biopanning and amplification. This observation could be due to many aspects. Upon analyzing the full procedure, the wash process may not have been sufficient in clearing away all unbound phage. Later experiments did suggest that more wash steps were needed, yet even newly incorporated wash steps still allowed the control group to produce a few repeat sequences. A plausible explanation for the remaining phage after washes might be that the peptides have attached by nonionic or electrostatic interaction, or were incorporated by passive diffusion rather than by site specific binding. Ardhammer et al. (1999) concluded that in vitro membrane penetration of modified peptide nucleic acids could occur by passive diffusion. For our experiments, the diffusion and membrane domains might allow the phage to adsorb non-specifically during the wash steps. Without a good assay or control system, there was no way to sort the sequences obtained in the 7-mer or 12-mer trials based on the control results. The control results neither prove nor disprove that there was specific binding of the phage library to the embryos or unfertilized ova in the Ph.D.™ 12 and Ph.D.™ 7 trials. Also, there was no repeat of the 7-mer sequences within the 12-mer sequences derived.

In an effort to ‘visually’ prove or disprove the binding theories for the derived peptides, experiments were implemented to label the peptide ligands characterized in the Ph.D.™ 12 and
Ph.D.™ 7 trials with a fluorescent tag. The labeled peptides were used in incubation studies with unfertilized ova or embryos in expectation that the peptide would bind a target site. Theoretically, the fluorescent-peptide binding could be viewed by fluorescence microscopy, providing a visual assessment of the oocyte target. The initial results were not indicative of significant, specific binding sites among the groups since results varied between experiments (Tables 1a and 1b). One possibility is that the fluorescein label may have been attached to the inappropriate terminus of the peptide. Fusion proteins, constructed using the minor coat proteins pIII of the fd filamentous bacteriophage, display peptides on or near the amino-terminus (Scott and Craig et al., 1994). If the derived peptide ligand was not in the original orientation as displayed in respect to the amino-terminus, then the peptide might not bind the target as it had before, since the fluorescein tag could block the binding if the tag were attached to the amino end. With this in mind, the peptides were relabeled with the fluorescent tag on the carboxy-terminus of the sequence. The newly labeled group appeared to have more specific and brighter results in fluorescing than the previous groups (Appendix Tables 1a, 1b, and 2).

Control groups for these fluorescent dye labeled peptides were ambiguous since there was a slight background cytoplasmic fluorescence for most control groups (Figure 11). These control groups were embryos or ova incubated alone, with no fluorescent peptides added. Background fluorescence was also observed in many of the regular, non-control groups. Lysed oocytes fluoresced regardless of test group. Polar body, cytoplasmic membrane and varying cytoplasmic fluorescence was observed in many of the ova (Figures 8 and 10) or embryo groups (Figure 9). The polar body attachment seems to be a more specific binding, yet there was no definitive test to prove this. The fluorescing cytoplasmic membrane and other components could be due to non-specific affinity, passive diffusion or electrostatic interactions. The hydrophobicity and or chemical properties on the fluorescein tag could cause a high, non-specific affinity (Chang et al., 1993; Kroesen et al., 1992). Finally, the cytoplasmic membrane could appear to fluoresce if the fluorescent peptides, or free fluorescent label, were to passively diffuse into or through the zona pellucida. The fluorescent peptide or free fluorescein could then potentially become grouped, bound and unable to traverse the cytoplasmic membrane, or simply unable to be released from the oocyte in wash steps.
At this point, the results still show some promise for the adapted phage display procedures to work with the mouse embryos. Since this is new technology (i.e., phage display applied to oocytes) the results lack only a good control or test to prove or disprove the data. Perhaps the 7-mer and the 12-mer are not long enough or complex enough to bind to an embryo component. Or, the binding may or may not be specific. Upon further study, it was found that some laboratories have successfully applied techniques of immunofluorescence to embryo research (personal correspondence, Mark J. Hay). This immunofluorescence procedure will be used in place of the fluorescent labeling.

Even though the 7-mer and 12-mer libraries yielded results that were ambiguous, these results implied that the phage display procedures could work with modified protocols. The 7-mer and 12-mer results were valuable in adjusting the protocols and procedures for the phage display techniques with mouse oocytes. As previously mentioned, the results may imply that the peptide-ligand target sites are very complex, thus requiring a more complex peptide. A more complex peptide would present a structure with folding and linear segments, unlike the 7-mer or 12-mer. In this manner, the complex oligomer would have a better chance of binding at least one amino acid on a target site (Wilson and Finlay, 1998; Barry et al., 1996). The possibility of this higher affinity binding prompted the acquisition of a peptide longer than the 12-mer. The recent location and acquisition of a 15-mer random library from Syncomm Laboratories (Syncomm, Philadelphia, PA, USA), has proven to be beneficial to this research.

Also incorporated, based on the 12-mer and 7-mer results, were new tests for washing away any loosely bound or unbound phage. Figure 7A shows that the newly incorporated wash procedures were successful, made possible by tests with the previous protocols. Proof of the successful washes is provided by the fact that the 15-mer phage concentration versus zona-free embryos remained high, while the same phage lost any concentration in the control group at the end of each biopanning round. With the newly acquired information and materials, our procedures have currently been, and continue to be, adapted for the 15-mer and immunofluorescence experiments.
Results with the 15-mer library have been successful in providing two consensus sequences, HGRFILPWWYAFSPS and RNVPPIFNDVYWIAF. The sequence HGRFILPWWYAFSPS was present for 34% (11/32) of the total sequences obtained, and RNVPPIFNDVYWIAF for 28% (~9/32). The remaining 38% (12/32) of the sequences was random. There was no control group for this sequencing since no phage clones were obtained from the control at cycle three of biopanning. This lack of recovered phage indicates that the new washing procedures effectively rinsed the embryos free of the unbound phage (Figure 7A). No phage concentration was obtainable for the control at the end of each biopanning cycle, even though there was phage amplified from each biopanned eluate. This indicates that there may be one, or a few, phage present in the biopanned control group not detectable by plated titers. However, this amplification was still significantly lower in concentration than the zona-free amplification (~$10^4$ control versus ~$10^{15}$ zona-free). The zona-free groups still maintained high titers after biopanning and even higher titers from amplification, while the control reached essentially zero after biopanning and significantly low titers from amplification.

The 15-mer at 37.0°C plus 100 µM chloroquine yields different products and results (Figure 7A) than that obtained with the earlier 15-mer (Figures 5A and 6), or even the 7-mer (Figures 1A and 2A) and 12-mer experiments (Figures 3 and 4A). Chloroquine was added to preserve the phage from rapid degradation during the procedures (Barry et al., 1996). The phage titers out of each biopanning cycle were at expected levels for binding, and the amplification steps were as predicted. The control group indicated that there were sufficient washes for each group and that the sequences obtained for the zona-free embryo group were unique to that group in comparison to the control. The sequences have been searched through GenBank with no known sequence comparisons revealed. Based on a lack of correlation of significant similarities to these two sequences, HGRFILPWWYAFSPS and RNVPPIFNDVYWIAF may be novel and/or non-natural motifs. Furthermore, these two 15-mer sequences did not contain the same sequences derived in the 7-mer and 12-mer experiments.

The theorized success of this 15-mer library and new procedures over the earlier work is several-fold. First of all, the embryos were zona-free allowing direct access to the cytoplasmic
membrane and potentially the cytoplasm. Second, the embryos were lysed to release internal contents. Lysing was accomplished through Triton-X 100 addition at the glycine/BSA elution step. The lysing is beneficial for releasing any possible internalized, specifically bound peptides (Barry et al., 1996). Further, the amplification was more successful for this research by the plate method rather than standard broth methods. The 37.0°C incubation may have increased the stringency of the phage-peptide for binding to targets of the embryo. Also, chloroquine may have aided in preventing degeneration of internalized phage (Barry et al., 1996).

Immunofluorescence is currently being tested with the two 15-mer sequences, #31 HGRFILPWWYAFSPS and #37 RNVPPIFNDVYWIAF compared to a control random 15-mer pool, each with zona-free embryos. It appears that #31 and #37 have specific binding in the cytoplasmic material (Figures 14, 15 and 19) and around the cytoplasmic membrane (Figures 16, 17 and 18). Even though this fluorescence was not seen in the control group (Figures 12 and 13), results are still inconclusive. Future work will focus on refining this protocol in efforts of clearly defining the role and/or binding sites of #31 and #37 sequences in zona-free embryos.

CONCLUSIONS

In order to adapt phage display experiments to mouse research, phage display techniques were applied as follows. The phage display libraries used for these experiments were 7-mer and 12-mer random peptide libraries provided in a Ph.D.™ Phage Display Peptide Library Kit (New England BioLabs, Inc., Beverly, MA, USA), and a 15-mer random peptide library provided by Syncomm (Syncomm, Philadelphia, PA, USA). E. coli 2547 was the host for the 7-mer and 12-mer; a K91 host was used for the 15-mer. In earlier experiments 7-mer phage were introduced intravenously into prepubescent female mice; embryos and ovaries were collected separately after a specified time period. In other experiments 7-mer or 12-mer phage were introduced separately, either to collected embryos versus unfertilized ova or to embryos versus fertilized zona-free embryos. The latter, most successful experiment was a 15-mer phage incubated with zona-free embryos.
For each group, the bound phage was eluted and amplified in *E. coli* where specified. Following amplification, another two cycles of binding, elution and amplification were carried out per group. Three cycles produced a pool of specific peptide sequences that bound to the mouse embryos. From each cycle, a number of phage clones were purified and sequenced for characterization. The peptide sequences of the final elutions were then compared for possible sequence homologies through BLAST and GenBank (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov).

The results of this experiment have shown that phage display did provide a pool of specific peptides that bound the mouse oocytes. The peptides have been biopanned, amplified, and sequenced. The 7-mer and 12-mer experiments did give results encouraging for this new process even though clear, definitive results were not obtained with further testing. The control group for the 7-mer and 12-mer experiments is the same phage with the same procedures as for the embryo and ova experiments, only the phage is treated alone with no embryos or ova. The control should not have yielded any possible consensus sequences. Unfortunately these control groups did provide a few apparent consensus sequences. Therefore, in comparison with the control group for the 7-mer and 12-mer there does not appear to be a group specific consensus sequence forming after three cycles of biopanning. Specifically, there was not a clear difference between the results of the control group and the 7-mer or 12-mer, based on both the consensus sequences observed and the labeled peptide assay. Other revisions were made to these groups before deciding to expand testing to a longer peptide-phage. A random 15-mer phage library was then acquired and display experiments were revised in order to find an embryo specific consensus sequence. The 15-mer results were successful in providing two separate, apparent consensus sequences to zona-free embryos after three cycles of biopanning. The sequences appear to be group specific since there were no plaques from amplification or sequences derived for the 15-mer control group. These sequences are currently being tested and analyzed.

Through this adapted process of phage display and further research, phage display technology may be used as an *in vivo* tool in the detailed recognition of specific mouse gamete sites. Current experiments will be continued to conduct more extensive investigations into the targeting and
internalization of the specific peptide(s) acquired through the phage display. Once concluded, tests will be performed on the ability of the peptide(s) to internalize macromolecules such as exogenous DNA constructs.

If a peptide specific for cellular entry into mouse embryos is found, then the peptide might be used as a method of stable transfection. Subsequently, experiments can be designed to test the addition of poly-L-lysine and the peptide-DNA complex to promote transgenesis. Alternatively, in the future, a recognition site may be identified through phage display techniques on the sperm. The peptide-macromolecule might bind to the sperm and the peptide deliver the macromolecule into the egg at the time of fertilization. Another potential use of sperm-binding or egg-binding proteins is for blocking sperm-egg recognition and interaction. The recognition site, in this case, could be on the sperm or the egg. If a peptide can bind a sperm receptor used for egg recognition and fertilization, this binding may block the sperm’s ability to bind to the egg. Conversely, if a peptide can bind an egg receptor used for sperm recognition, the egg may not bind to the sperm. Without the sperm-egg recognition and binding, fertilization may be impeded or completely prevented. When a peptide can bind sperm sites sufficient to block fertilization in the mouse, the process may be tested on other mammals as a form of contraception.

In conclusion, with the current data and future research, phage display technology may be successfully adapted as a useful tool in mouse biotechnology. As a result, this research may provide new insights into: 1) chemistry of the egg cell surface, 2) interactions of sperm-egg binding in order to investigate a block to fertilization, and 3) new processes of targeting macromolecules to the embryo as a new technology for transgenesis. Current and future work will include more tests on the targeting and internalization of the specific-peptide phage, and tests on the ability of the peptides to internalize macromolecules such as DNA constructs for transgenesis.
REFERENCES


Hay, M.J. (personal communication) Imperial College of Science, Technology and Medicine Department of Biology. Sir Alexander Fleming Building, Imperial College Road, London SW7 2AZ, UK.


Johnston, S.A. (personal communication) Departments of Internal Medicine and Biochemistry, University of Texas Southwestern Medical Center. 5323 Harry Hines Boulevard, Dallas, TX 75235-8573.

Kirkham, P.M., Ph.D. (personal communication) Phage Display Core Facility, The University of Alabama at Birmingham Department of Medicine. THT 413, Birmingham, AL 35294-0006.


CHAPTER 4

BIBLIOGRAPHY


Hay, M.J. (personal communication) Imperial College of Science, Technology and Medicine Department of Biology. Sir Alexander Fleming Building, Imperial College Road, London SW7 2AZ, UK.


Johnston, S.A. (personal communication) Departments of Internal Medicine and Biochemistry, University of Texas Southwestern Medical Center. 5323 Harry Hines Boulevard, Dallas, TX 75235-8573.
Kirkham, P.M., Ph.D. (personal communication) Phage Display Core Facility, The University of Alabama at Birmingham Department of Medicine. THT 413, Birmingham, AL 35294-0006.


CHAPTER 5
APPENDIX

Table 1a: Fluorescein labeled 7-mer and 12-mer peptides

<table>
<thead>
<tr>
<th></th>
<th>20 µM, 1-cell Embryos</th>
<th>20 µM, Unfertilized ova</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bgd</td>
<td>PB</td>
</tr>
<tr>
<td>FI-LPLTPLP</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FI-KQTLPSA</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>FI-ATAYPNPFSPGA</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>FI-LGLQVQGPRQLP</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FI-VYPAESTQSRP</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>FI-LSVSGKPSPRP</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FI-YQLRPNAESLRF</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Fig. 8

Table 1b: Fluorescein labeled 7-mer and 12-mer peptides

<table>
<thead>
<tr>
<th></th>
<th>20 µM, 1-cell Embryos</th>
<th>20 µM, Unfertilized ova</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bgd</td>
<td>PB</td>
</tr>
<tr>
<td>FI-LPLTPLP</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FI-KQTLPSA</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>FI-LPHYIQN</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>FI-NVQPTRP</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FI-DLYTGSKVGRMAE</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>FI-VPGSQNIFLPRS</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Control</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Fig. 11

Tables 1a and 1b: Qualitative and semi-quantitative fluorescent peptide binding results. A select set of peptides, derived with the 7-mer and 12-mer random phage libraries against a group of embryos and a group of ova, were fluorescein labeled. Next, a 20 µM concentration of each fluorescent peptide was introduced to 1-cell embryos, and compared to unfertilized ova. Table 1a shows the results obtained from the first set of fluorescent-labeled peptides; Table 1b is a replicate set for clarification. The control groups were embryos or ova alone, with no fluorescent peptides.

Key:
- : no visible fluorescence
Bgd: background fluorescing
PB: polar body(ies) fluorescing
CM: cytoplasmic membrane fluorescing
Cyto: cytoplasm fluorescing
PS: perivitelline space between cells fluorescing
++ : medium fluorescence and/or several
+++ : bright fluorescence and/or several
++++ : very bright fluorescence and/or majority
g: grainy appearance to fluorescence
Table 2: Fluorescein labeled 7-mer and 12-mer peptides, carboxy-terminus

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Bgd</th>
<th>PB</th>
<th>CM</th>
<th>Cyto</th>
<th>Bgd</th>
<th>PB</th>
<th>CM</th>
<th>Cyto</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPLTPLP(K-fl)</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KQTLPSA(K-fl)</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ATAYPNFFSPGA(K-fl)</td>
<td>+++</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>LGLQVEGPRQLP(K-fl)</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>YYPASSTSQRSP(K-fl)</td>
<td>+++</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>SYSVGMKPSRP(K-fl)</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>YQLRPNAESLRF(K-fl)</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2: Qualitative and semi-quantitative fluorescent peptide binding results. A select set of peptides, derived with the 7-mer and 12-mer random phage libraries against a group of embryos and a group of ova, were fluorescein labeled. This set varies from Tables 1a and 1b in that the fluorescein tag is present on the carboxy-terminus. A 20 µM concentration of each fluorescent peptide was introduced to 1-cell embryos, and compared to unfertilized ova. The control groups were embryos or ova alone, with no fluorescent peptides.

Key:

- Bgd: background fluorescing
- PB: polar body(ies) fluorescing
- CM: cytoplasmic membrane fluorescing
- Cyto: cytoplasm fluorescing
- PS: perivitelline space between cells fluorescing
- : no visible fluorescence
+ : faint fluorescence and/or very few
++ : medium fluorescence and/or several
+++ : bright fluorescence and/or several
++++ : very bright fluorescence and/or majority
Table 3: Qualitative and semi-quantitative fluorescent peptide binding results. A select set of peptides, derived with the 7-mer and 12-mer random phage libraries control groups, were fluorescein labeled. Since these apparent consensus sequences were derived from the control groups, they must be compared to the previously derived consensus sequences, and compared to a complete random pool (represented by Fl-XXXXXX). A 20 µM concentration of each fluorescent peptide was introduced to 1-cell embryos, and compared to unfertilized ova. The control groups were embryos or ova alone, with no fluorescent peptides.

Key:

- Bgd: background fluorescing
- PB: polar body(ies) fluorescing
- CM: cytoplasmic membrane fluorescing
- Cyto: cytoplasm fluorescing
- PS: perivitelline space between cells fluorescing

- : no visible fluorescence
+ : faint fluorescence and/or very few
++ : medium fluorescence and/or several
+++ : bright fluorescence and/or several
++++ : very bright fluorescence and/or majority

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Bgd</th>
<th>PB</th>
<th>CM</th>
<th>Cyto</th>
<th>Bgd</th>
<th>PB</th>
<th>CM</th>
<th>Cyto</th>
</tr>
</thead>
<tbody>
<tr>
<td>Random pool: Fl-XXXXXXX</td>
<td>-</td>
<td>+++</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>STVSSWTNDNLS(K-fl)</td>
<td>+</td>
<td>+++</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>APARPPQLPY(K-fl)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GNLNHLI(K-fl)</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>YAGPYQH(K-fl)</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HAIYPRH(K-fl)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AMYSVYT(K-fl)</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>SSHTISF(K-fl)</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LGLQVEGPRQLP(K-fl)</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
VITA

Jeanette Lowe was born in Roanoke, VA on October 8, 1964. After graduating from Patrick Henry High School in 1983 with honors and a place in Whose Who Among American High School Students, she entered Virginia Polytechnic Institute and State University. There she received a Bachelor of Science degree in Biology in 1988 and received the honor of being among Most Valuable Women of America, 1988. Employment followed at Virginia Polytechnic Institute and State University in the Immunology Department, then in 1992 at TechLab, Inc. In 1993 she transferred to PPL Therapeutics, Inc as a Manager of Small Animal Technology in transgenic production. While working for PPL, Jeanette enrolled in graduate school at Virginia Polytechnic Institute and State University to pursue a Master of Science degree in Molecular Cell Biology and Biotechnology through the Dairy Science Department. During the pursuit of this degree, she accepted a Research Associate position in Transgenic Technology at Research Genetics, Inc in Huntsville, Alabama.

PUBLICATIONS

Cu/Zn Superoxide Dismutase Deficient Brucella abortus: Construction and Characterization. 

Brucella abortus Deficient in Copper/ Zinc Superoxide Dismutase is Virulent in Balb/C Mice. 
Microbial Pathogenesis