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**The mechanisms of
inhibition of fertilization and activation of development
by fertilin β derived oligopeptide polymers.**

A Dissertation Presented

by

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to

The Graduate School

in Partial Fulfillment of the

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Abstract of the Dissertation

The mechanisms of inhibition of fertilization and activation of development by fertilin β derived oligopeptide polymers.

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Doctor of Philosophy

in

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Stony Brook University

2008

The sperm protein fertilin β , a member of the ADAM family of proteins, is implicated in sperm-egg binding in all mammals studied to date. Multivalent inhibitors containing the three-amino acid binding sequence of sperm fertilin β disintegrin domain, Glu-Cys-Asp (ECD), are effective inhibitors of fertilization. We probed sperm-egg interactions by designing, synthesizing and testing a variety of molecular probes incorporating the ECD peptide in vitro. Evidence suggests that egg integrin $\alpha_6\beta_1$ functions in the fertilization pathway. We investigated the role of β_1 integrin in mammalian fertilization and the mode of inhibition of fertilin β derived polymers. We determined that polymers displaying the

ECD peptide from the fertilin β disintegrin domain mediate inhibition of mammalian fertilization through a β_1 integrin receptor on the egg surface. Inhibition of fertilization is a consequence of competition with sperm binding to the cell surface, not activation of an egg-signaling pathway. Sperm binding to β_1 integrin on the egg increases the rate of sperm attachment to the egg surface, but does not increase the number of sperm that attach or fuse.

Dedication

This is dedicated to my father Raymond John Baessler. His ways of acceptance, love, kindness, honesty, and forgiveness are something to be admired. I wish he were alive to see this. This is also dedicated to my mom, Anita Doris Baessler, for all her love and support over the years. I couldn't have done it without her. An extra-special dedication must be given to my girlfriend Elizabeth Sarah Ginsburg who I love and adore. She, like me, has followed a path to acquire a higher level of education. I pray that she will be successful in her endeavors. Over the years, she has given me much love, encouragement, and support to help me achieve this goal. I also dedicate this to my two sisters Karen Baessler and Kim Baessler, and all my nieces and nephews. I'd like to also give a special dedication Chris Cassidy, Richard Smyth, and to John Conroy who sponsored me, and inspired me throughout my education and up until his death. Lastly I dedicate this work to the people I have helped, and will continue to help, in my community service efforts at Cumulus House.

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LIST OF SYMBOLS

α	alpha
β	beta
μ	micro
ζ	zeta
♀	female
♂	male
ADAM	a disintegrin and metalloprotease
Ala, A	alanine
Arg, R	arginine
BAPTA-AM	1,2-bis(<i>o</i> -aminophenoxy)ethane- <i>N,N,N',N'</i> -tetra-acetic acid acetoxymethyl ester
BSA	bovine serum albumin
Ca^{2+}	calcium
CG	cortical granules
CRISP1	cysteine rich secretory protein
Cys, C	cysteine
ECD	glutamate-cysteine-aspartate
ECM	extracellular matrix
ER	endoplasmic reticulum
FITC	fluorescein isothiocyanate
FI	fertilization index
FR	fertilization rate
Glu, E	glutamate

h	hour
HET	heterozygote
hCG	human chorionic gonadotropin
ICSI	intra-cytoplasmic sperm injection
Ig	immunoglobulin
IP ₃	inositol trisphosphate
KO	knockout
MII	meiosis II
mAB	monoclonal antibody
min	minutes
PIP ₂	phosphatidyl inositol-4,5 bisphosphate
PBS	phosphate buffered saline
PLC	phospholipase C
PMSG	pregnant mare serum gonadotropin
RGD	arg-gly-asp
ROMP	ring opening metathesis polymerization
rt	room temperature
Ru	ruthenium
Ser, S	serine
SVMP	snake venom metalloprotease
WT	wild type
ZP	zona pellucida

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Studying the biological and biochemical pathways of fertilization has been fascinating, for within those pathways are the keys to life. I learned, observed, and experienced things so marvelously complicated and interesting in relation to this topic. The biochemical theories and methods used in this work have advanced me in ways that 5 years ago I could not have imagined. I will always have fond memories of working in her lab.

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Biotechnology (Proceedings of the II International Conference, Biomicroworld 07, University of Seville, Spain) Antonio Mendez-Vilas editor, Scientific World Publishing Co, Hackensack (New Jersey), London, Singapore. I would like to thank Dr. Joseph Kreit, his wife and daughter, Mona and Sarah Kreit, for their hospitality when I visited Morocco. Joseph has always been supportive of me, and provided me with guidance and insight in many areas. His scientific knowledge and strong spiritual values are truly admirable.

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CHAPTER 1

Introduction and Background

1. Mammalian Fertilization in vivo
2. Sperm Proteins Involved in Fertilization
3. Egg Proteins Involved in Fertilization
4. Mammalian Egg Activation
5. Probing the Fertilization Pathway in vitro with the Fertilin β Mimic
6. The Zona Pellucida (ZP-free) Free IVF Inhibition Assay
7. Specific Aims and Experimental Approach

1. Mammalian Fertilization In Vivo

Fertilization is an extremely complex and important event because it relates directly to quality of life and environmental issues. The lack of population control treatments and infertility are huge problems on a global scale. The National Center for Health Statistics (2008) reports that over 6.1 million women alone in the U.S. suffer from infertility (<http://www.cdc.gov/nchs/FASTATS/fertile.htm>). In addition, the U.S. Census Bureau reports (2008) that the world population has reached 6.7 billion persons and is projected to reach 9 billion by 2040 (<http://www.census.gov/ipc/www/idb/worldpopinfo.html>). These data really demonstrate the need for fertilization related research. This section describes fertilization in vivo.

Inspired by forces of nature and after copulation, sperm enter the female reproductive tract and start their journey towards the egg. Multiple and complex biochemical reactions must take place to allow for fusion of gamete membranes and subsequent development (2, 3). Prior to reaching the egg, sperm cells undergo the capacitation reaction that is induced by secretions in the female reproductive tract (4). Only capacitated sperm can penetrate and cross barriers which surround the egg (Figure 1-1).

The first barrier is a perimeter of approximately 3000 ovarian granulosa cells, or the cumulus cells matrix. Sperm penetrate the cumulus matrix using the flagellar motion of their tail and a cell surface hyaluronidase PH-20 (5-7). The second barrier surrounding the egg, is the zona pellucida (ZP), which is a glycoprotein network comprised of ZP1, ZP2 and ZP3 proteins (8-10). The ZP consists of long filaments of ZP2 and ZP3 that are cross-linked by ZP1. Sperm binding to glycoprotein ZP3 triggers the acrosome reaction, which exposes key proteins on the sperm head for the binding and fusion steps to follow

(11-13). During the acrosome reaction, enzymes that are exocytosed from the sperm head (14, 15) enable sperm to penetrate the zona pellucida.

After penetrating the ZP, sperm enter the perivitelline space and bind to the egg surface. Finally the sperm head and tail are incorporated into the egg cytosol via membrane fusion. A series of complex signaling events known as egg activation is triggered upon fusion (16). The egg blocks polyspermy, fertilization by more than one sperm, at the zona pellucida and the egg plasma membrane (17-21). Events which follow include the resumption of meiosis, extrusion of the second polar body, fusion of the male pronucleus with the female pronucleus, DNA replication, mitosis and cytokinesis. The precise regulation mechanisms and timing of these events all contribute to form the zygote (16).

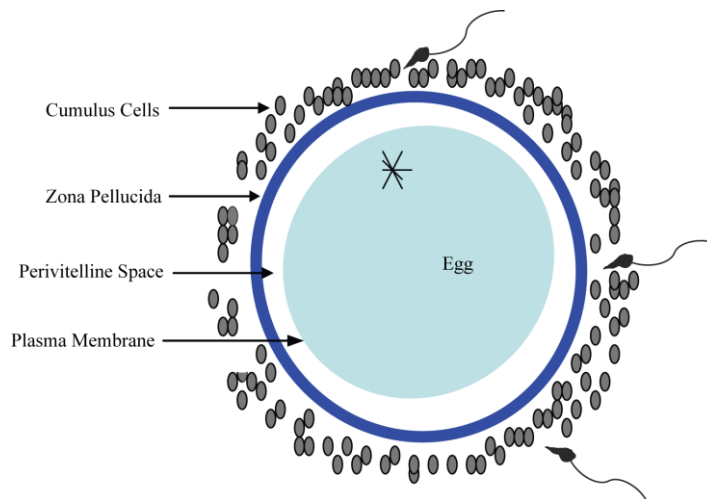


Figure 1-1. Overview of Fertilization. Sperm penetrate the cumulus cells and bind to the zona pellucida to undergo the acrosome reaction. The acrosome reaction releases proteases enzymes which allow sperm to cross into the perivitelline space. Gamete binding and fusion occurs at the plasma membrane.

2. Sperm Proteins Involved in Fertilization

Sperm are required to go through a series of important maturation steps prior to attaining the capacity to fertilize an egg. Upon ejaculation and migration through the female reproductive tract, sperm undergo a series of biochemical and membranous changes called capacitation. During this process, a wide variety of modifications occur in the head and flagellum, membrane, cytosol, and cytoskeleton. Membrane lipids and proteins are reorganized, complex signal transduction mechanisms are initiated, and sperm develop hyperactivated motility. The main purpose of capacitation is to ensure that sperm reach the oviduct at the appropriate time, and express the proper proteins to undergo the acrosome reaction (22).

Sperm are uniquely designed to accomplish their goal, which is penetrating the egg. Each sperm head is equipped with a cap-like membrane bound vesicle, derived from the Golgi apparatus called the “acrosomal vesicle”. The contents of the acrosome, enzymes like hyaluronidase and acrosin, are secreted when sperm bind zona pellucida protein ZP3 (14, 15). The identity of the sperm receptor that binds ZP3 is still unknown and so are many of the signal transduction components in the pathway. Suggested candidates are signal transduction proteins such as G-proteins, phospholipase C (PLC), inositol-3, 4, 5-trisphosphate (IP3) and IP3 receptors, voltage sensitive calcium channels, and glycosyl transferases (23-27). The acrosome reaction changes the morphology of the sperm head, and exposes key proteins such as fertilin α , fertilin β , cyritestin, Izumo, and CRISP1 (cysteine- rich secretory protein 1 also known as DE) (13, 28-30). All of these have been implicated in binding to the egg membrane (after sperm pass through the ZP), and will be the focus of the discussion to follow.

ADAMs

Fertilin α , fertilin β , and cyritestin, or ADAM 1, 2 and 3 respectively, are members of the ADAM (A Disintegrin and A Metalloprotease domain) family of proteins (31, 32). ADAM proteins are multifunctional transmembrane proteins (33). They play roles in proteolytic processing of other transmembrane proteins, in cell adhesion, and in cell fusion events (34). Fertilin α , fertilin β , and cyritestin are proteolytically processed from precursor to mature forms by a cleavage between the metalloprotease domain and the disintegrin domain (35). There have been 40 ADAM proteins identified to date. ADAMs were originally discovered when a heterodimer named fertilin was identified in guinea pig sperm as being the antigen of antibody PH-30, an antibody that inhibits fertilization 75% (29). One subunit of the fertilin heterodimer is composed of ADAM1 (fertilin α) and the other ADAM2 (fertilin β).

ADAM proteins are homologous to soluble snake venom metalloproteases (SVMPs) and share approximately 30% sequence similarity and similar domain organizations (Table 1-1 & Figure 1-2) (36, 37). ADAM domains include a signal sequence, a prodomain, a metalloprotease domain, a disintegrin domain, a cysteine-rich domain, an EGF-like (epidermal growth factor) repeat, and a transmembrane domain. Most SVMPs are soluble except for class P-IV SVMPs that are membrane bound. The domain structure of the soluble SVMPs does not include an EGF-like repeat, or a transmembrane domain. The disintegrin domain structure and function of SVMPs were characterized prior to the discovery of ADAMs (38). Class P-II SVMPs contain a highly conserved Arg-Gly-Asp (RGD) motif within their disintegrin domain. Small peptides and peptidomimetic sequences containing RGD mimic extracellular ligands such as fibronectin, and bind to

integrin receptors $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ (40-41). The closest relative to ADAMs are class P-III snake venom metalloproteases (SVMP III's) (Table 1-1) (32). The disintegrin domain of class III SVMPs inhibits platelet aggregation by binding to integrin $\alpha_2\beta_1$ (42). It was therefore proposed and later confirmed that the disintegrin domain of ADAM family member fertilin β also binds to an integrin.

The ADAM2 (Fertilin β) subunit of the fertilin heterodimer lacks the RGD disintegrin loop and instead has an ECD motif, which is key to its function. The disintegrin loop of fertilin β is approximately 30 amino acids in length. Examination of various mutated forms of the disintegrin loop revealed that the ECD sequence mediates fertilin β 's interaction at the egg plasma membrane (43, 44). The terminal aspartate residue of the ECD sequence is important. Mutating the sequence to ECA or ECK results in nearly complete loss of function (43, 44). Replacing the aspartate residue with glutamate, ECD to ECE, to conserve the charge, results in a partial loss of function. Mutating the glutamate, ECD to ACD also results in partial loss of adhesive function. Taken together these data point to the ECD tripeptide being the adhesion mediating motif of fertilin β .

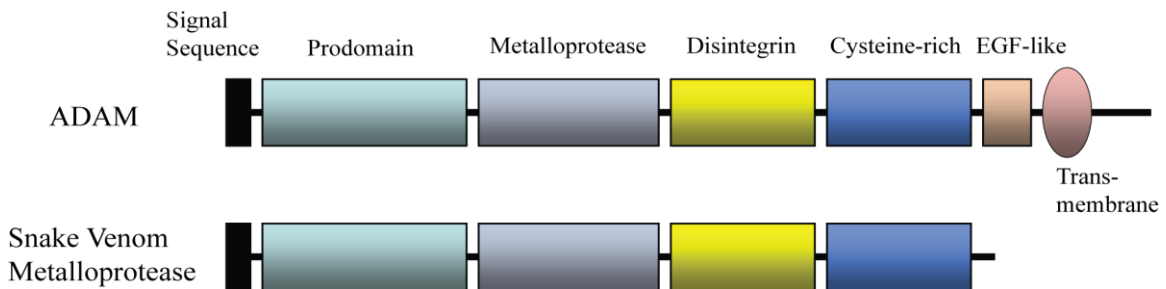


Figure 1-2. Domain Organization of ADAMs and Soluble SVMPs.

SVMP P-II disintegrins	
Kistrin	CKFSRAGKICRIP <u>RGD</u> -MPDDRCTGQSADC
Echistatin	CKFLKEGTICKRA <u>RGD</u> -DMDDYCNGKTCDC
Barbourin	RFMKKGTVCRVA <u>KGD</u> -WNDDTCTGQSADC
SVMP P-III disintegrins	
HR1B	CRFRTAGTECRAA <u>ES</u> <u>ECD</u> IPESCTGQSADC
Jararhagin	CKFSKSGTECRAS <u>MS</u> <u>ECD</u> PAEHCTGQSSEC
Atrolysin E	CKFTSAGNVCR <u>PAR</u> <u>SECD</u> IAESCTGQSADC
ADAM family disintegrin domains	
Fertilin α (mouse)	CTFKKKGSLCRPAED <u>VCD</u> LPEYCDGSTQEC
Fertilin β (mouse)	KLKRKGEVCR <u>LAQD</u> <u>ECD</u> VTEYCNGTSEVC
Cyritestin (mouse)	CTIAERGRLCRKSKD <u>QCD</u> FPEFCNGETEGC
Fertilin β (guinea pig)	CEFKTKGEVCRE <u>STD</u> <u>ECD</u> LPEYCNGSSGAC
Fertilin β (monkey)	CLFMSQERVCR <u>PSFD</u> <u>ECD</u> LPEYCNGTSASC
Fertilin β (rabbit)	CTFKEKGQSCR <u>PPVG</u> <u>ECD</u> LFEYCNGTSALC
Fertilin β (human)	CLFMSKERMCR <u>PSFE</u> <u>ECD</u> LPEYCNGSSASC
Fertilin β (bovine)	CAFIPKGHICRG <u>STD</u> <u>ECD</u> LHEYCNGSSAAC
Fertilin β (rat)	NLKAKGELCRPA <u>NO</u> <u>ECD</u> VTEYCNGTSEVC

Table 1-1. Sequence Alignment of Class P-II, P-III and the Disintegrin Domain of ADAMs. The disintegrin domain is approximately 30 amino acids in length. The proposed binding loop is underlined.

Genetic knockout studies on mouse ADAMs involved in fertilization have been performed (45-50). As mentioned previously, fertilin is a heterodimeric protein complex expressed on the sperm surface. Its two subunits are composed of ADAM1 (fertilin α) and ADAM2 (fertilin β). During mouse sperm maturation two different isoforms of ADAM1 are synthesized, ADAM1a, and ADAM1b. Only ADAM1b is present on the plasma membrane of epididymal sperm (46); ADAM1a resides in the ER. The ADAM1a KO mice are infertile because KO sperm are incapable of migrating from the uterus into the oviduct and binding to the egg ZP. The inability of ADAM1a KO sperm to bind the ZP may be explained by the low level of ADAM3 (cyritestin) found on the knockout sperm surface (48, 49, 51). ADAM3 (cyritestin) deficient mice are infertile due to an inability of sperm to bind and penetrate the zona pellucida (51). Male mice lacking ADAM1b are fertile, and ADAM1b deficient sperm do not have abnormal migration patterns, nor do they have difficulty with ZP binding and membrane fusion. These results seem counterintuitive since only ADAM1b is expressed on the sperm surface, and not ADAM1a. Sperm from ADAM2 (fertilin β) null mice are deficient in sperm-egg membrane adhesion, sperm-egg fusion, migration from the uterus into the oviduct, and binding to the egg zona pellucida (45).

Protein expression levels on the sperm membrane change in ADAM knockout sperm. Knocking out genes for ADAM2 or cyritestin results in a strong decrease of ADAM1 levels. Knocking out the cyritestin gene results in a 50% reduction of ADAM2 on mature sperm, and knocking out ADAM2 results in a reduction of cyritestin expression on the sperm surface (49, 52). Interestingly the ADAM proteins that were reduced or missing from mature knockout sperm were translated at normal levels (159). It is proposed that

ADAMs have chaperone-like activity, and that ADAM protein passage through the secretory pathway (during sperm cell maturation) needs to be cooperative to bring about normal surface expression. This cooperation may be part of a posttranslational control system that regulates the correct expression of sperm membrane proteins (53).

Izumo

Izumo, an immunoglobulin superfamily protein, was originally identified with antibodies that inhibited sperm-egg fusion. The protein was further characterized by biochemical methods, and gene cloning techniques (13). Izumo is specifically expressed in the testis and detectable only on acrosome-reacted sperm.

Izumo was found to be essential for sperm-egg fusion. Prior to Izumo's discovery, no fusion related proteins on sperm had been identified. Disrupting the gene for Izumo causes sterility in male mice. Izumo KO sperm are capable of migrating from the uterus into the oviduct, and penetrating the zona pellucida. However, they are incapable of fusing with the egg plasma membrane. Izumo deficient females had a normal fertility when crossed with wild-type sperm. The fusion defect was further confirmed by using intra cytoplasmic sperm injection (ICSI), which bypasses the binding and fusion steps of fertilization. Izumo deficient female mice successfully reproduce after ICSI injection of Izumo KO sperm. Western blot confirmed that knocking out the gene for Izumo does not affect expression levels of related proteins, ADAM2, CD147, and sp56 (13).

CRISP-1

CRISP-1 (cysteine-rich secretory proteins) protein, also known as DE, was identified as a protein synthesized in the epididymis which localizes to the dorsal region of the sperm head during epididymal transit. After the acrosome reaction occurs, CRISP-1 migrates to the equatorial region of the sperm head (30, 54). CRISP-1 is a good candidate fertilization protein since the equatorial region of the sperm head is the site of sperm-egg membrane fusion.

Genetic studies were performed to determine the protein's role in fertilization. Male and female CRISP-1 KO mice were fertile. *In vivo* sperm motility and the ability to undergo the acrosome reaction were not affected in CRISP-1 deficient mice. *In vitro* fertilization assays showed that CRISP-1 KO sperm exhibited a significantly reduced ability to penetrate both ZP-intact and ZP-free eggs (30, 54).

A role for CRISP-1 in gamete fusion was suggested by studies using polyclonal antibodies against CRISP-1 which significantly inhibited sperm-egg fusion and not binding (55). Moreover, data from IVF inhibition assays using purified CRISP-1 showed a significantly reduced percentage of sperm fused to eggs but no effect on the number of sperm which bound to eggs (56).

Thus far we have discussed several sperm proteins believed to be involved in the fertilization pathway. It is clear that multiple sperm proteins are involved in gamete adhesion and fusion events. It is likely that sperm have evolved these mechanisms to retain a genetic advantage.

3. Egg Proteins Involved in Fertilization

Integrins

Integrins are a super-family of heterodimeric transmembrane surface receptors composed of two non-covalently associated α and β subunits. Integrins are involved in fundamental processes such as cell attachment, cell migration, cellular differentiation, the immune response, the maintenance of tissue architecture, and inside out signaling (57, 58).

Each subunit consists of a single transmembrane domain, a large extracellular domain of several hundred amino acids (composed of multiple structural domains), and typically, a small cytoplasmic domain (59). The extracellular domain binds to many ligands, whereas the intracellular cytoplasmic domain anchors to cytoskeletal proteins. Integrins mediate cell adhesion by recognizing consensus sequences within ligands of the extracellular matrix such as laminin and fibronectin, and/or specific proteins exposed on the surface of adjacent cells. Integrins bind to their ligands in a cation (Ca^{2+} or Mg^{2+}) dependent manner. In mammalian cells, there are genes that encode for at least 18 α subunits and 8 β subunits (57). Of the various possible combination of α and β subunits that can exist, only 24 have been identified thus far.

Integrin receptors have been implicated in fertilization. Many integrins are expressed on mammalian oocytes such as $\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_6\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_v\beta_8$, $\alpha_{IIb}\beta_3$ (60-63). Integrins are known to bind ADAM protein family members (Table 1-2). ADAMs are homologous to SVMPs and SVMPs inhibit platelet aggregation by binding to integrins (42, 64). The sequence homology between the disintegrin domain of Class III SVMPs and ADAMs led to the proposal that integrins mediate gamete adhesion (32, 36, 37).

Subsequently, it was found that ADAM2 (fertilin β) disintegrin domain peptides containing ECD, were able to bind to the egg plasma membrane and inhibit sperm-egg binding and fusion (43, 60, 65-67). Chen *et al.* used a linear peptide derived from fertilin β 's disintegrin domain and *p*-benzoylphenylalanine to photoaffinity label integrin $\alpha_6\beta_1$ (68). Function-blocking monoclonal antibody (mAb) GoH3, against the α_6 integrin subunit, inhibits sperm-egg binding and fusion under specific conditions (60). Antibodies against the β_1 integrin subunit also inhibit sperm-egg binding and fusion, or inhibit recombinant fertilin β peptide binding (69, 70).

Integrin $\alpha_6\beta_1$ is the predominant egg surface protein, and is known to cluster at the site of sperm contact (71, 72). Integrins are known to be associated with tetraspanin CD9 (73-77) and eggs from CD9 null mice do not fuse with sperm (78-80). The role of egg $\alpha_6\beta_1$ integrin, or any integrin, came into question when genetic studies were performed. *In vitro*, eggs in which either the α_6 integrin gene or the α_3 integrin gene is disrupted are fertilized (81). Moreover, mice with a conditional knockout of the β_1 integrin in their eggs are fertile *in vivo* (62).

Integrin	ADAM
$\alpha_9\beta_1$	1, 2, 3, 9, 12, 15
$\alpha_6\beta_1$	2, 3, 9
$\alpha_4\beta_1$	28
$\alpha_5\beta_1$	15
$\alpha_v\beta_3$	15
$\alpha_v\beta_5$	9

Table 1-2. Integrin Receptors that Bind ADAMs (60-63).

Tetraspanins

Tetraspanins are a protein superfamily comprised of 34 proteins, 33 of which have been identified in humans (82, 83). Tetraspanins have four transmembrane domains, intracellular N and C-termini, and two extracellular domains, one short, and one long. What makes these molecules distinct is the conservation of key residues (CCG), and the helical folding structure of two large extracellular loops (84). Tetraspanins are highly important physiologically. They have function in motility, activation, proliferation, differentiation, co-stimulation, migration, and signal transduction. A major characteristic of tetraspanins is they tend to interact and localize in large protein networks known as the “tetraspanin web”.

Tetraspanin CD9’s role in fertilization was confirmed by genetic knockout studies. The major defect of CD9 deficient mice was a severely reduced fertility in females (78-80). Sperm migrated normally up the female reproductive tract and crossed the zona

pellucida, but could not fuse with the egg membrane. When sperm were injected into the egg cytosol by ICSI in CD9 KO eggs (to bypass the membrane fusion step), the fertilized eggs developed normally. These results indicate that CD9 has an essential role in sperm-egg fusion.

Tetraspanin CD9 was found to associate with the egg β_1 integrin. Recently Ziyat *et al.* (72) demonstrated that CD9 controls the formation of clusters that contain other tetraspanins and integrin $\alpha_6\beta_1$ in mouse and in human eggs. During fertilization, the CD9/integrin $\alpha_6\beta_1$ complex undergoes major relocalization at the site of sperm contact. Antibody cross-linking of integrin $\alpha_6\beta_1$ in CD9 KO eggs yields large disperse patches which are very different than wild-type controls (72). Therefore, CD9 may control the mobility of integrin $\alpha_6\beta_1$ in the membrane. In other studies, anti-CD9 mAb, JF9, potently inhibited sperm-egg binding and fusion in vitro. JF9 also disrupted binding of fluorescent beads coated with native fertilin or a recombinant fertilin β disintegrin domain (73).

GPI-anchored Proteins

GPI-anchored proteins have also been implicated in fertilization. GPI-anchored proteins are a diverse family of molecules that includes membrane-associated enzymes, adhesion molecules, activation antigens, differentiation markers, protozoan coat components, and other glycoproteins (85). GPI-anchored proteins possess a covalently linked glycosylated phosphatidylinositol moiety, which attaches the protein portion of the molecule exclusively on the extracellular side of the cell surface. GPI-anchored proteins can be removed from the cell surface by treatment with phosphatidylinositol specific phospholipase C (PI-PLC) (86).

Treatment of mouse eggs with PI-PLC significantly reduces gamete binding and fusion, indicating a role for GPI-anchored proteins in fertilization (87). Genetic studies targeting the first steps of GPI anchor bio-synthesis were performed using the *Cre/loxP* recombination system. The *pig-a* gene was specifically deleted in the egg to prevent the delivery of GPI-anchored proteins to the plasma membrane (88, 89). *Pig-a* deficient female mice were infertile, and sperm were able to bind but not fuse to the egg plasma membrane. The identity of the GPI-anchored protein is unknown. However loss of all GPI-anchored proteins in the egg could lead to disruption of lipid domains and of normal protein to protein interactions within them. As of yet, no studies have been published that suggest there is any interaction between tetraspanin CD9 and GPI-anchored proteins on oocytes.

4. Mammalian Egg Activation

For fertilization to occur in vivo, sperm that have penetrated the zona pellucida and entered the perivitelline space must bind and fuse with the egg. A sequence of complicated events then ensues which are collectively referred to as egg activation. Egg activation begins when sperm trigger release of free intracellular Ca^{2+} (16, 90, 91). Calcium release occurs via enzyme phospholipase C (PLC) that hydrolyzes PIP_2 (phosphatidyl inositol-4,5 bisphosphate) on the cytosolic leaflet of the egg membrane to produce second messenger IP_3 (inositol trisphosphate). IP_3 binds to its receptor InsP_3R , a major intracellular Ca^{2+} channel on the endoplasmic reticulum (92-95). Upon binding of IP_3 the receptor undergoes a major conformational change to allow Ca^{2+} release. Calcium release occurs in pulses or oscillations from the point of sperm fusion across the egg. Subsequent events such as blocks to polyspermy (i.e, cortical granule exocytosis)

resumption of the meiotic cell cycle, pronuclei formation, the initiation of DNA synthesis are all dependent on the Ca^{2+} response. In all species studied to date, the initial rise in Ca^{2+} induced by sperm is essential for development.

There are at least two mechanisms to explain how egg activation occurs. The first mechanism postulates that sperm bind an egg membrane receptor to initiate the event, and the second hypothesis postulates that sperm provide a soluble factor PLC ζ (phospholipase C zeta) that is released into the egg's cytosol upon gamete membrane fusion. The latter became widely accepted after it was found that sperm extract induced egg activation, and that removing PLC ζ from sperm extract abolishes Ca^{2+} release in eggs. In addition, it was shown that in certain species PLC ζ artificially introduced into the egg is sufficient to produce Ca^{2+} oscillations as well as normal embryo development to blastocyst stage (96-98). Thus far, genetic experiments have not confirmed these results.

Blocks to polyspermy at the zona pellucida and egg plasma membrane are essential for an organism to survive. (17-20, 99, 100). For the egg, the cytosolic rise in calcium induces exocytosis of cortical granules. Cortical granules are secretory vesicles that reside just below the egg plasma membrane. The granules contain proteases, polysaccharides, and peroxidases. When exocytosed, the cortical granule contents modify the zona pellucida so that it no longer supports sperm binding (17, 94, 101). Much less is known about the block to polyspermy that occurs at the egg plasma membrane. However, it is believed that Ca^{2+} plays a role in facilitating the membrane block establishment by regulating the timing of the event. The membrane block to polyspermy is also dependent upon rearrangement of the actin cytoskeleton. Treating eggs with inhibitors of calcium

signaling or actin microfilament-disrupting drugs decreases the oocyte's ability to mount the membrane block (19).

When mammalian eggs are ovulated, they are arrested at metaphase II of their second meiotic division until fertilized or activated by artificial means. Calcium oscillations induced by sperm last for several hours and end at the time of pronuclei formation (91, 92, 102). Pronuclei are the haploid nuclei of either sperm or egg that are present in the egg cytosol. The appearance of two pronuclei is the first sign of successful fertilization as observed during in vitro fertilization (IVF). Both Ca^{2+} oscillations, and subsequent events such as pronuclei formation can be induced by a variety of parthenogenetic reagents, and ICSI (93, 96, 103, 104). Arg-Gly-Asp (RGD) analogues that mimic the disintegrin domain of Class II SVMPs and extracellular matrix proteins such as fibronectin, also induce egg activation in *Xenopus* and bovine oocytes (105, 106). Sperm induce Ca^{2+} oscillations of distinct frequencies, amplitudes and spatial organization. Thus far, any oscillations observed in ICSI fertilized eggs or eggs activated by artificial means do not appear at all similar to those induced by sperm (107).

5. Probing the Fertilization Pathway in vitro with the Fertilin β Mimic

A variety of receptor-ligand contacts occur during sperm-egg binding. Their organization, localization, and binding properties still need to be explored. We have designed and synthesized molecular probes to interrogate the fertilization system. Our probes are designed to have high affinity and avidity for their target receptors. The bases for their design are discussed below.

In many biological systems increasing the valency of a ligand (i.e., presenting multiple copies of a ligand) will increase a biological response. For example: sperm having multiple copies of proteins involved in egg adhesion would give them a multivalent binding capacity, which in turn would increase their affinity/avidity for the egg membrane receptors. Multivalent cell to cell interactions such as this occur regularly and are the basis for many biological events that occur.

Synthetic multivalent probes which mimic the effect just described are an effective means for investigating biological systems. Many of these are potent inhibitors and effectors of biological functions. The potency of multivalent inhibitors over monovalent inhibitors may be explained by several factors. For example: a monovalent inhibitor/effector has the capacity of binding and blocking one receptor, whereas a multivalent probe has the capacity to cluster multiple receptors and block multiple active sites (Figure 1-3). Also, multivalent probes localized and bound to a receptor might result in steric stabilization or a statistical rebinding effect (108, 109) .

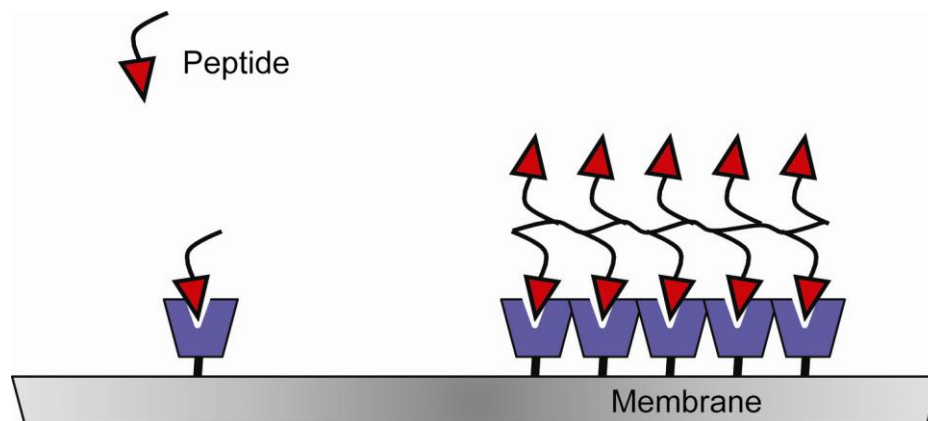


Figure 1-3. Monovalency and Multivalency. A monovalent peptide can only bind one receptor, a multivalent peptide can cluster multiple receptors.

The proposed binding loop of sperm protein fertilin β contains a highly conserved ECD peptide. Data from many labs, including ours, found that linear peptides incorporating the ECD disintegrin domain peptide inhibit fertilization in many species (44, 67, 110-112). It was established that small monomers containing the ECD motif modestly inhibit fertilization in vitro with an IC₅₀ of approximately 500 μ M. The ECD monomer lacked potency due to its monovalent nature and low affinity for egg receptors. However, because it was an inhibitor, the ECD motif became central to designing more potent inhibitors of fertilization.

We designed multivalent inhibitors containing multiple copies of ECD on a molecular scaffold by ruthenium-catalyzed ring-opening metathesis polymerization (ROMP) (113-116). Placing multiple copies of the biologically active peptide on a scaffold would increase a ligand's affinity for its target. By the ROMP method, functionalized monomers are polymerized using a ruthenium-catalyst (Figure 1-4). The functionalized monomers can be designed to incorporate any peptide of choice. The functional group can also vary in amino acid sequence. For example, to find the most potent inhibitors of fertilization, we varied the amino acid sequence of the disintegrin domain; created functionalized monomers based on the sequence of our choice, and polymerized them into a variety of structures by ROMP (1, 116-118). These polymers were then tested by IVF assays in mouse eggs.

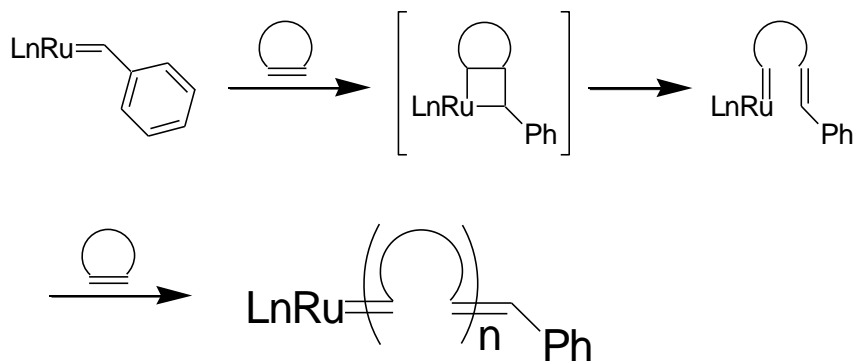


Figure 1-4. Mechanism of ROMP (113-114). Ruthenium catalysis is initiated by mixing the metal alkylidene complex with a highly strained cyclical olefin based monomer (e.g. norbornene ECD peptide). A metalla-cyclobutene intermediate is formed. After electron transfer, and ring opening an additional highly strained cyclical olefin monomer can react. As the reaction proceeds, monomers are added sequentially until termination.

ROMP has many attractive features for its application in making fertilization inhibitors. First the length of a polymer can be easily controlled by varying the monomer to catalyst ratio. In addition, co-block polymers containing biologically active ligands can be separated by spacer regions in well defined ratios. The polydispersities are also typically very low for ruthenium-catalyzed ROMP polymers.

Lab members Dr. Kenny S. Roberts, and Dr. Younjoo Lee prepared a family of ROMP-derived polymers containing multiple copies of ECD. Multivalency improved inhibition of fertilization 50-to 70-fold over the monomer (1, 119). High affinity probes such as this may prove useful for tagging cell surface molecules and identifying receptors in the egg membrane in the future.

6. The Zona Pellucida (ZP-free) Free IVF Inhibition Assay

ZP-free IVF assays are commonly used to establish the potency of inhibitors that target the egg plasma membrane. Zona pellucida removal can be accomplished by a variety of methods both enzymatic and chemical (Figure 1-5). Removing the zona pellucida with a brief acidic treatment is most common. Using ZP-intact eggs is not practical for assessing the strength of inhibitors that target the egg plasma membrane since sperm-egg membrane binding cannot be accurately assessed. Inseminating ZP-free eggs with a low concentration of sperm is essential to reveal subtle inhibitory effects of inhibitors.

Several parameters can be measured during an IVF assay: the average number of sperm bound per egg, the mean number of fused sperm per egg (fertilization index, FI) and the percentage of eggs fused with at least one sperm (fertilization rate, FR). Membrane fusion is scored as the fluorescent labeling of sperm nuclei with Hoechst dye present in the preloaded eggs.

For multivalent inhibitors, concentrations can be considered both in peptide ligand and in polymer. For example, a 10 μM “in polymer” solution containing 10 copies of the ECD peptide, contains 100 μM ECD ligand. Peptide ligand concentrations are useful for comparing the inhibition efficiencies of polymers containing different numbers of ligands. However the concentrations considered in this work are “in polymer” unless otherwise noted.

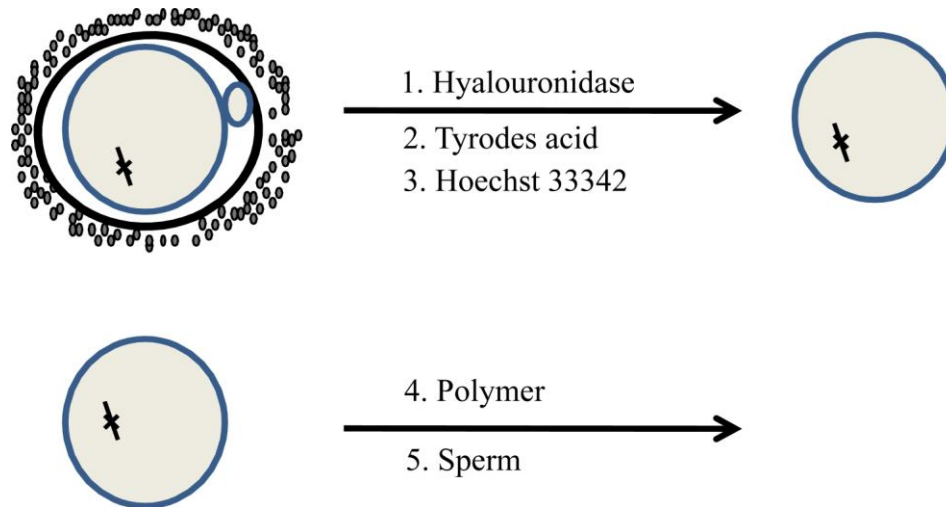


Figure 1-5. The in vitro Fertilization Assay. Outer layer of cumulus cells is removed with hyaluronidase. The zona pellucida is removed with Tyrodes acid pH~2. Eggs are loaded with fluorescent indicator Hoechst 33342.

7. Specific Aims and Experimental Approach

In previous work, our lab synthesized an ^{125}I -labeled peptide incorporating the consensus sequence Asp-Glu-Cys-Asp (DECD) of fertilin β 's disintegrin domain. The peptide contained a benzophenone photoaffinity probe and inhibited sperm-egg fusion. Upon photoactivation in the presence of whole mouse eggs, a single polypeptide was covalently labeled and identified by immunoprecipitation as the $\alpha_6\beta_1$ integrin complex (68). Based on these photoaffinity labeling experiments and inhibition and binding experiments with fertilin β derived analogues, it was widely accepted that fertilin β and integrin $\alpha_6\beta_1$ were binding partners. However, recent knockout experiments have suggested that none of the integrin receptors present on mouse eggs or known to be ADAM receptors are essential for fertilization (62, 81). Based on these results the mechanism of fertilization inhibition by ECD polymers came into question. We sought

to understand the mechanism of inhibition of fertilin β derived polymers. The unanswered questions are described below.

Is polymer inhibition really mediated through integrin $\alpha_6\beta_1$? To answer this question we tested a variety of fertilin β derived peptides, and polymers to define the most potent inhibitor in wild-type eggs. After determining the most potent inhibitor, we asked if the inhibitor loses its potency in the integrin knock out. By using the Cre-loxP method, we generated a colony of β_1 integrin KO mice (62). β_1 integrin KO mice do not express the α_6 integrin subunit on the egg membrane and thus our question could be thoroughly addressed. We performed IVF polymer inhibition assays using integrin β_1 KO eggs.

Is there another means by which polymers can inhibit fertilization? Do polymers inhibit fertilization via an egg signaling pathway which induces a membrane block, or do they inhibit by directly blocking sperm? Several strategies were used to answer these questions. First, we tested the reversibility of polymer inhibition potency. Eggs were treated with fertilin β derived polymers and washed prior to insemination to determine if polymer inhibition is reversible. Secondly, we tested fertilin β polymers to see if they could induce events associated with egg activation such as calcium release and pronuclei formation. Lastly we investigated the ability of fertilin β polymers to induce the egg membrane's block to polyspermy, since the membrane block could account for the observed inhibition.

What is the role of integrin $\alpha_6\beta_1$ on the egg membrane, if it is not necessary for fertilization? Does the integrin play any role at all in mediating adhesion of sperm in vitro? Do sperm-egg binding kinetics differ between WT and β_1 KO eggs? Since integrins are classically known to be involved in mediating cell adhesion events and bidirectional

signaling, we investigated the kinetics of sperm binding and fusion in WT and integrin β_1 KO eggs under IVF assay conditions.

How do sperm-egg binding kinetics differ between WT eggs treated with inhibitor and untreated WT eggs? How do the sperm binding kinetics compare between WT eggs that have the β_1 integrin blocked by biochemical means and eggs that have the β_1 integrin knocked out? WT eggs were treated with inhibitor and inseminated. The rates of sperm binding and fusion were measured over time.

Does the polymer inhibitor bind within the $\alpha_6\beta_1/CD9$ complex? To answer this question we used a fluorescent polymer in conjunction with immunofluorescence to determine if the polymer colocalizes in the complex.

CHAPTER 2

Fertilin β Derived Polymers Inhibit Fertilization via Integrin $\alpha_6\beta_1$

1. Introduction
2. Results and discussion
3. Conclusion

1. Introduction

The molecular mechanism of sperm–egg membrane binding and fusion is yet to be elucidated. Molecular probes have been designed in our lab to further our knowledge of the pathway. In previous work, multivalent inhibitors containing the three-amino acid binding sequence of fertilin β , ECD, proved to be effective inhibitors of fertilization (*1, 116*). Sperm-egg interactions were probed by examining the potency of fertilization inhibition by polymers that contained from 3 to 70 ECD ligands in densities ranging from 10-100%. These polymers were synthesized by ruthenium-catalyzed ring opening metathesis polymerization (ROMP) (*1,116*). Evaluation of the polymer potencies, and synthesis of a tri-block copolymer from 2 building blocks, revealed that two multivalent contacts are sufficient for maximal inhibition. The initial work suggested that the distance between ECD ligands requires 7-9 monomers spanning 4-5 nm. However later work revealed that the optimum bivalent inhibition model required the ECD ligands to be spaced by approximately 96 monomers (Figure 2-1) (*128*). It was concluded that inhibition requires recruitment of two receptors on the egg surface into an inhibitory complex. The candidate receptor for mediating inhibition is proposed to be integrin $\alpha_6\beta_1$.

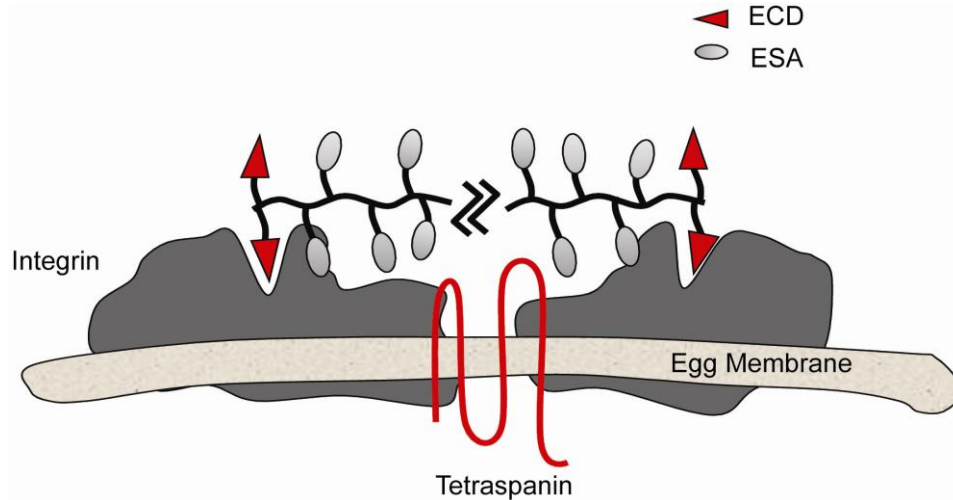


Figure 2-1. The Proposed Bivalent Model. The polymer-receptor binding complex. The inhibitor binds integrin $\alpha_6\beta_1$. The $\alpha_6\beta_1$ integrin is believed to be associated with tetraspanin CD9 in mammalian eggs.

The role of integrin $\alpha_6\beta_1$ or any other integrin in fertilization is controversial. Mouse eggs null for the α_6 or β_1 genes were fertile *in vivo* and *in vitro*. The ability of other known egg integrins to function in the fertilization pathway were ruled out when it was determined that neither anti-mouse β_3 integrin function-blocking monoclonal antibody (mAb) nor α_v integrin function-blocking mAb inhibited sperm binding to or fusion with β_1 integrin null eggs (62, 81). These results led to the conclusion that none of the known integrins reported on the egg surface are essential for fertilization.

Several ideas exist to explain the fertility of the β_1 KO mice. The fertility of β_1 KO mice could arise from the existence of naturally occurring overlaps in protein function, or by β_1 KO eggs compensating for genetic alterations by upregulating expression of another protein. It is more than likely that multiple egg surface proteins are involved in the adhesion step between sperm and egg.

Currently, the egg surface proteomics in the β_1 KO is unclear. Aside from the lack of cell surface expression of the α_6 subunit on the β_1 KO eggs, the expression patterns of other egg surface proteins in the KO have not been investigated.

If sperm ADAMs are active in the fertilization pathway as evidence suggests, then surely there could be other egg receptors, integrin and non-integrin that function as ADAM receptors in the β_1 KO eggs. In fact any combination of the known α and β integrin subunits which are still expressed on the egg surface (α_2 , α_5 , α_v , β_3 , and β_5) may compensate for the loss of $\alpha_6\beta_1$. He et al. (62) ruled this hypothesis unlikely by using function blocking antibodies against other integrin subunits in β_1 KO eggs. These results, are interesting, however the conclusions may not be completely valid. The function blocking antibodies used in their IVF assay, were selected for blocking extracellular matrix (ECM) proteins such as fibronectin which bind to integrins via an RGD motif (120). The predicted disintegrin domain of ADAM2 contains an ECD binding loop. Unfortunately no atomic resolution structures exist for either fertilin β , or integrin $\alpha_6\beta_1$, and the binding interface for the ligand-receptor pair has not been identified. Therefore, it is hard to predict the specificity of the antibody blocking disintegrin binding.

The proposed integrin-fertilin β , receptor-ligand partnership needs to be verified. The main pieces of evidence to support the hypothesis that integrin $\alpha_6\beta_1$ is fertilin β 's binding partner are: the photoaffinity labeling of integrin $\alpha_6\beta_1$ by fertilin β disintegrin domain ECD mimics, the ability of the ECD analogues to inhibit fertilization, and the in vitro binding adhesion studies with recombinant fertilin β (44, 67, 110-112).

Extensive design and development of angiogenesis inhibitors for use in therapy of cancer, and retinal angiogenesis have been undertaken. In endothelial cells, ECM-protein

analogues of collagen and fibronectin were found to inhibit angiogenesis by binding to cell surface integrins $\alpha_1\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (Table 2-1) (121-124). Based on these data, one would predict that deleting the integrin would impair angiogenesis. However generation of knockout mice lacking one or more α_v integrins, showed that α_v integrins are not always essential for angiogenesis (125). The genetic deletions of the integrins failed to block angiogenesis and in some cases even enhanced it (123). Additionally, genetic deletion blocks the anti-angiogenic effect of the ECM-analogue inhibitors in vitro (Table 2-1). This disparity suggests the need for further evaluation of the mechanism of α_v integrin antagonists in anti-angiogenic therapeutics.

Proposed inhibitor	Source	Inhibition of angiogenesis in vitro	Genetic deletion blocks anti-angiogenic effect in vitro	Proposed integrin receptor	Dependence on receptor shown in vitro	Dependence on receptor shown in vivo
Endostatin	Collagen α (XVIII)	+	+	$\alpha_5\beta_1$		
Arresten	Collagen α_1 (IV)	+	+	$\alpha_1\beta_1$	+	KO
Tumstatin	Collagen α_3 (IV)	+	+	$\alpha_v\beta_3$	+	KO

Table 2-1: ECM-derived Inhibitors of Angiogenesis in Endothelial Cells Lose Inhibition Potency in Integrin KO's (121-124).

The investigations into integrins $\alpha_1\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ and angiogenesis inhibition suggest that a related phenomenon may occur in fertilization. Thus, we asked if fertilin β analogues will inhibit fertilization when their proposed binding partner, integrin $\alpha_6\beta_1$, is genetically deleted?

The inhibitive potency of fertilin β derived polymers may or may not be abrogated in β_1 KO eggs. Moreover, inhibition observed may be due to activation of a pathway. In light of the conflicting results between genetic and biochemical methods; we investigated if fertilin β disintegrin polymers (Figure 2-2) still inhibit fertilization in the β_1 integrin knockout system. Our strategy was to generate a mutant colony of β_1 integrin knockout mice and to test polymers **1₁₀**, **1₂2₁₃**, and **2₁₀** as inhibitors. Our goal was to verify observations made in our lab and others which suggest that egg integrin $\alpha_6\beta_1$ is sperm protein fertilin β 's binding partner.

Polymers Used in These Studies.

Three norbornenyl-derived polymers were used in this work: **1**₁₀, **2**₁₀, and **1**₂**2**₁₃ (Figure 2-2). Polymers like **1**₁₀ that can span multiple receptor binding sites are more potent inhibitors of fertilization than polymers containing 2-3 copies of the ECD peptide in close proximity (3 Å along the backbone) or inhibitors that incorporate only a single copy of ECD (*1*). Multivalent polymer **1**₁₀ contains on average 10 copies of the ECD peptide, whereas control polymer **2**₁₀ contains 10 copies of a mutated sequence Glu-Ser-Ala (ESA) that does not inhibit fertilization. We mutated the cysteine and aspartate residues because they are critical for binding (*43, 44, 112*). We used a mutated sequence rather than a scrambled sequence because tripeptide polymers are more synthetically accessible and the position of the two charges in the ECD tripeptide cannot be truly scrambled. Previously, we demonstrated inhibition is sequence dependent because a scrambled pentapeptide (Cys-Thr-Glu-Val-Asp) incorporated into a polymer does not inhibit fertilization, whereas, the native sequence (Glu-Cys-Asp-Val-Thr) does (*118*). Polymer **1**₂**2**₁₃, contains on average two ECD peptides at one terminus of the polymer, the remainder of the polymer is ESA peptides.

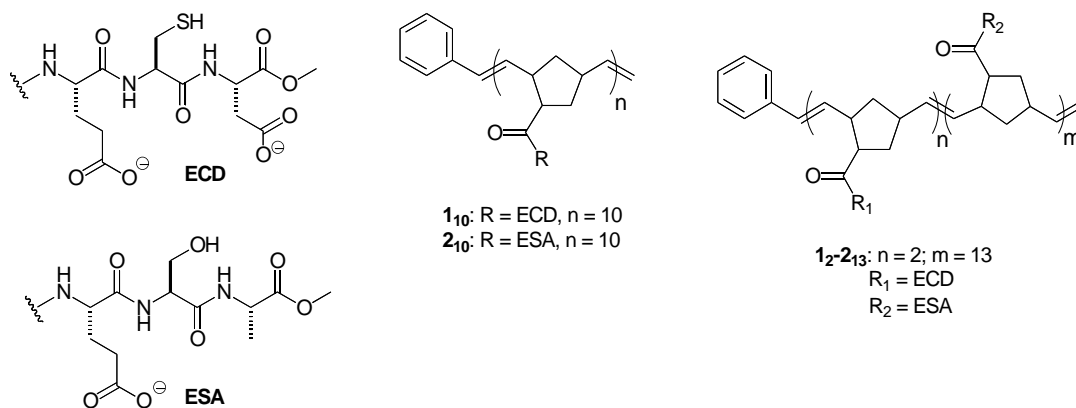


Figure 2-2. Structures of Polymers Used in this Study. Multiple copies of glutamate-cysteine-aspartate (ECD) and/or glutamate-serine-alanine (ESA) peptides are displayed along a norbornene scaffold using ring opening metathesis polymerization (ROMP) (1).

2. Results and Discussion

Generation of β_1 Integrin Conditional Knockout Mice

Oocyte-specific β_1 integrin conditional KO mice were generated by the *Cre/loxP* recombinant system. The floxed β_1 integrin gene mice were previously described (152). Transgenic male mice expressing the Cre recombinase under the control of the ZP3 promoter and homozygous for the floxed β_1 integrin gene (σ , Cre+ β_1 f/f), were mated with female floxed β_1 integrin mice (ϕ , Cre- β_1 f/f) (62). Offspring were genotyped by PCR to detect the ZP3 Cre transgene and the floxed β_1 integrin gene. Female mice with both the ZP3 Cre transgene and homozygous floxed β_1 integrin gene (Cre+ β_1 f/f) were putative β_1 integrin conditional knockout mice. These mice were used for in vivo mating, and eggs collected from these mice were used for IVF assays, and immunofluorescent staining, which confirmed the β_1 integrin knockout (Figure 2-3).

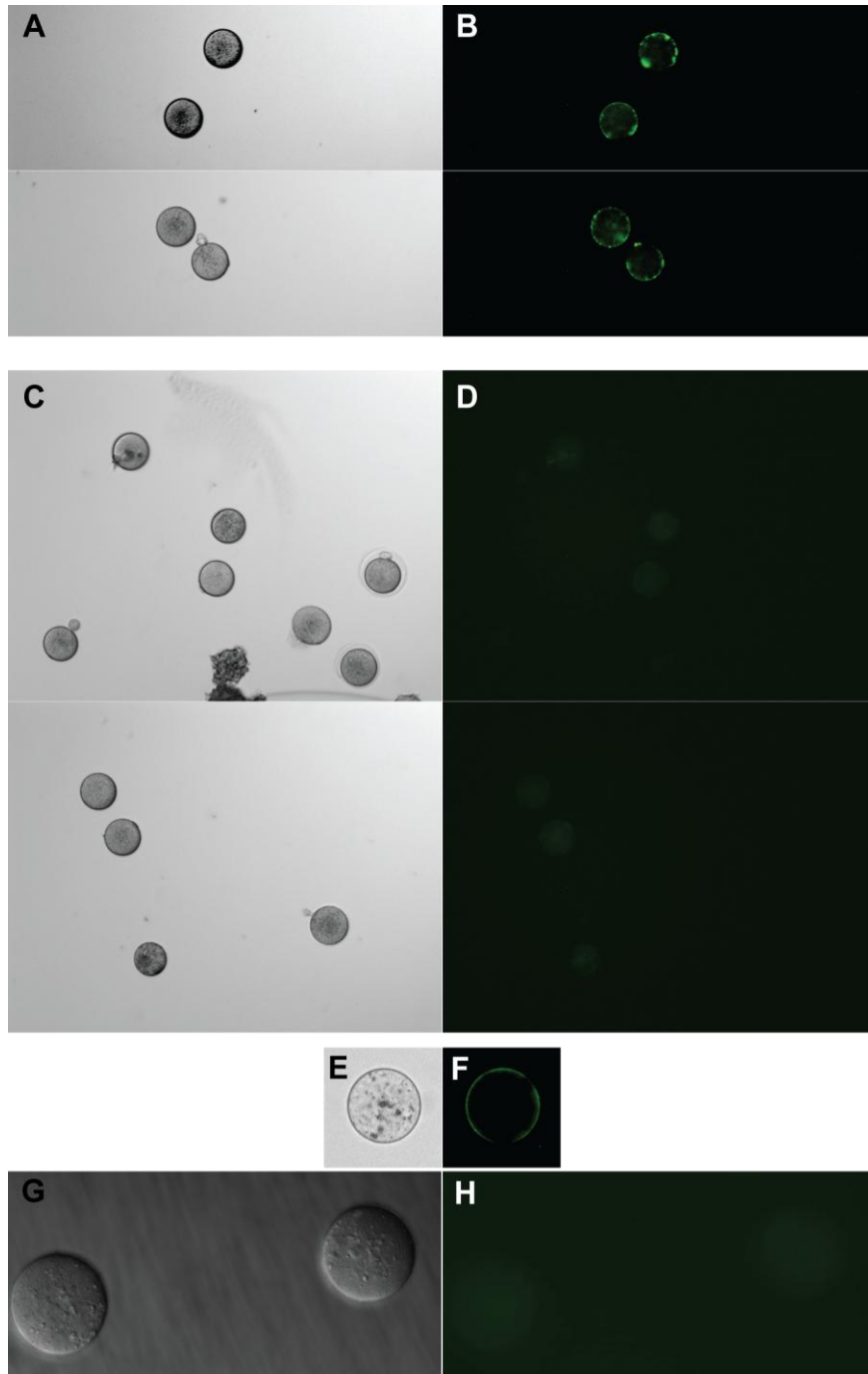


Figure 2-3. Immunofluorescence Images. Immunofluorescent staining of ZP-free eggs with a-d) anti- β_1 integrin mAb CD29, 20x objective and e-h) anti- α_6 integrin mAb GoH3, 20X objective with magnification increased by zoom function on Axiocam software. a, e) DIC and b, f) epi-fluorescence images of WT eggs. c, g) DIC and d, h) epi-fluorescence images of KO eggs.

We tested whether the β_1 integrin is required for inhibition of fertilization by ECD polymers. Eggs homozygous for the β_1 integrin knockout allele ($\text{Cre}^+\beta_1\text{f/f}$, KO), eggs heterozygous for the β_1 integrin knockout allele ($\text{Cre}^+\beta_1\text{+/f}$, HET) and wild-type ($\text{Cre}^-\beta_1\text{+/+}$, WT) eggs were harvested (62). Immunofluorescence microscopy with anti- β_1 and anti- α_6 integrin antibodies confirmed that the β_1 integrin knockout eggs had no β_1 integrin or α_6 integrin on the plasma membrane (Figure 2-3).

Zona pellucida (ZP)-free KO, HET, and WT eggs were assayed with varying concentrations of **1₁₀**. In these assays, the cumulus cells and the zona pellucida layers surrounding the egg were removed in order to test interactions at the egg plasma membrane. The number of eggs fertilized (Figure 2-4) and the average number of sperm fused per egg (Figure 2-5) were determined. As previously observed, polymer **1₁₀** inhibited fertilization of wild-type eggs, and inhibition was concentration dependent. The approximate IC_{50} was 2.5 μM polymer. This IC_{50} is different than previously reported (1) because the polymer length and stereochemistry were different due to the use of a newer ROMP precatalyst (126-128). Polymer **1₁₀** inhibited fertilization of KO eggs $19 \pm 6\%$ at the highest concentration used (50 μM in polymer, 500 μM in peptide), as compared with $73 \pm 4\%$ inhibition of WT egg fertilization at the same concentration. In experiments described in chapter 3 (Figure 3-5b), when 1% DMSO was included in the assay buffer with polymer **1₁₀**, no inhibition of KO fertilization was observed, whereas, the inhibition of WT fertilization was unchanged. This result suggests that the small amount of inhibition detected in KO fertilization is due to non-specific hydrophobic binding. Negative control polymer **2₁₀** containing ESA peptides was assayed and did not inhibit fertilization even at 500 μM polymer (5 mM peptide, Figure 2-4c). These data

indicate that inhibition of fertilization by 1_{10} is mediated by the β_1 integrin on the egg membrane.

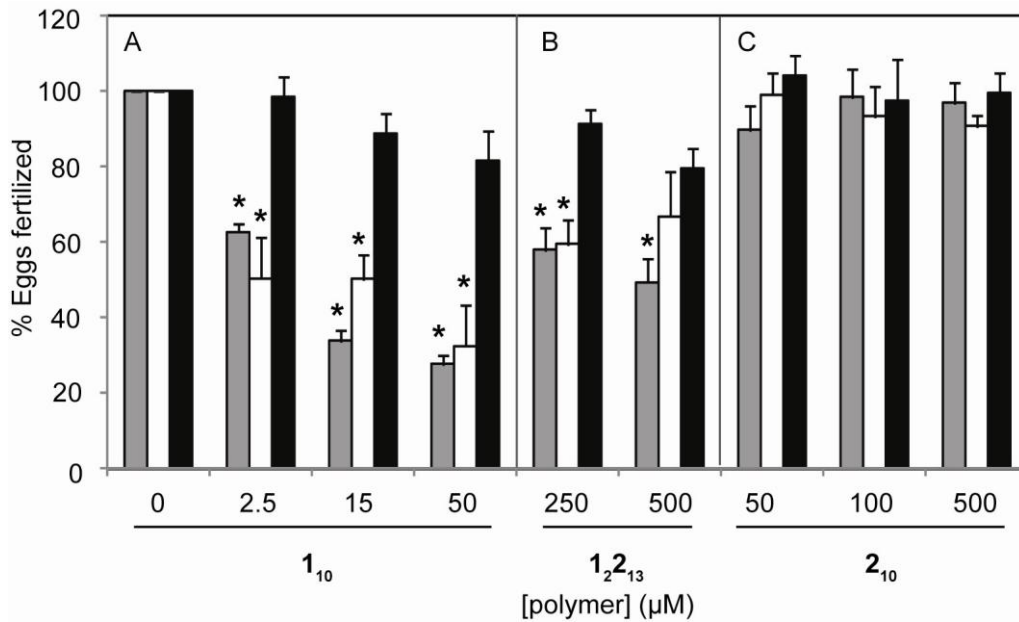


Figure 2-4. Multivalent and Monovalent ECD polymers Inhibit ZP-free Fertilization via an Egg β_1 Integrin. Gray bars: WT ($Cre^{-}\beta_1^{+/+}$) eggs. White bars: HET ($Cre^{+}\beta_1^{+/f}$) eggs. Black bars: KO ($Cre^{+}\beta_1^{f/f}$) eggs. Experiments were performed with an average of 50 eggs (range 25-90) at each condition in 3–6 independent experiments. The percentage of eggs fertilized in the presence of inhibitor are shown relative to the untreated control for each condition. In the untreated controls, $96 \pm 4\%$ of WT, and $94 \pm 6\%$ of HET eggs, and $92 \pm 6\%$ of KO eggs were fertilized. The average number of sperm fused per egg was 1.7 ± 0.1 , and 1.8 ± 0.3 , and 1.6 ± 0.2 , respectively. The concentrations of polymer are shown, not the concentrations of peptides. Errors are the s.e.m. * $p < 0.05$

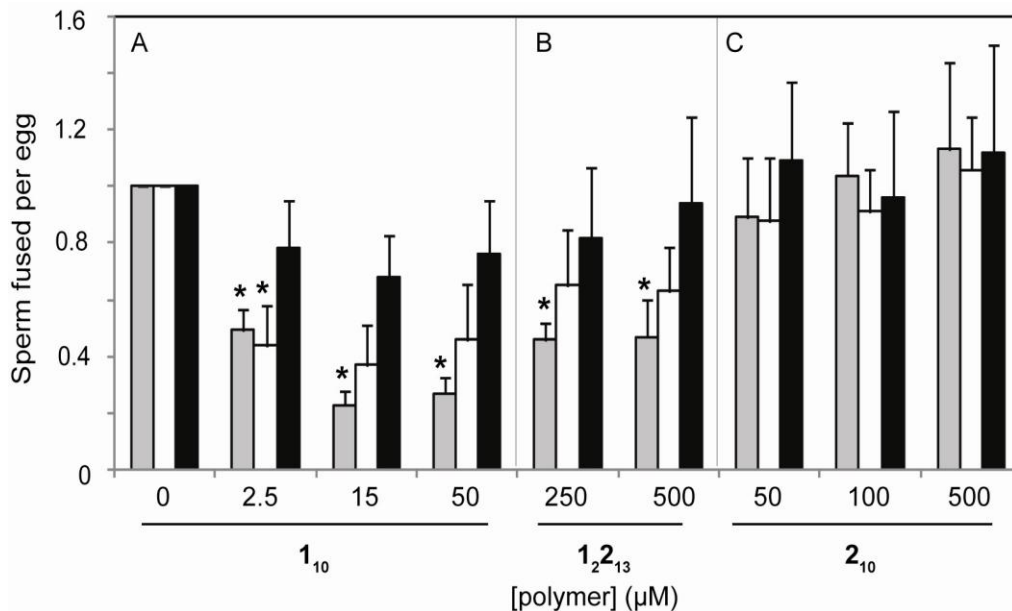


Figure 2-5. Multivalent and Monovalent ECD polymers Inhibit ZP-free Fertilization via an Egg β_1 Integrin. Gray bars: WT (Cre- β_1 +/+) eggs. White bars: HET (Cre⁺ β_1 +/f) eggs. Black bars: KO (Cre⁺ β_1 f/f) eggs. Experiments were performed with an average of 50 eggs (range 25-90) at each condition in 3–6 independent experiments. The average number of sperm fused per egg in the presence of inhibitor are shown relative to the untreated control for each condition. In the untreated controls, 96 ± 4 % of WT eggs, and 94 ± 6 % of HET eggs, and 92 ± 6 % of KO eggs were fertilized. The average number of sperm fused per egg was 1.7 ± 0.1 , and 1.8 ± 0.3 , and 1.6 ± 0.2 , respectively. The concentrations of polymer are shown, not the concentrations of peptides. Errors are the s.e.m. * $p < .05$ for WT versus KO eggs treated with equal concentrations of polymer **1₁₀** or **1₂₁₃**.

Inhibition of Fertilization by 1₂2₁₃.

We considered the possibility that the β_1 integrin is required to mediate fertilization inhibition by **1₁₀**, but not for sperm to bind the egg. If the higher abundance β_1 integrin were to act as an anchor for **1₁₀** and tether it to the egg surface, the avidity of terminal ECD ligands binding to a second, lower abundance sperm receptor would increase. In this scenario, inhibition by a non-avid, monovalent or low valency inhibitor that blocked the second unknown receptor used by sperm would be equipotent in WT and KO fertilization. Therefore we tested **1₂2₁₃**, a low valency polymer, as an inhibitor of fertilization in WT and KO eggs (Figure 2-4). Polymer **1₂2₁₃** inhibited fertilization of WT eggs 100-fold less potently than polymer **1₁₀** as expected based on previous work. Polymer **1₂2₁₃** inhibits WT fertilization 51% at 500 μ M, whereas only 20% inhibition is observed in KO fertilization. This difference is statistically significant ($p < 0.05$) and indicates that ECD peptide binding to β_1 integrin on the WT egg blocks sperm binding. We observed that inhibition of HET fertilization by polymer **1₁₀** is equipotent to inhibition of WT fertilization (Figure 2-4, 2-5). Therefore, the loss of inhibition is not due to differences in the genetic backgrounds of the mice. Thus, β_1 integrin-mediated avidity for a second sperm receptor is not responsible for inhibition of WT fertilization. Polymer **1₂2₁₃** was used as a low valency control as well as an aggregation control. If supramolecular structures form in solution, the valency of a polymer would be higher than designed and inhibition might be due to the supramolecular structure. We found that polymer **1₂2₁₃** was no more effective an inhibitor than a monomeric ECD peptide (Figure 2-6). Thus, supramolecular aggregates are not responsible for the inhibition observed in the experiments.

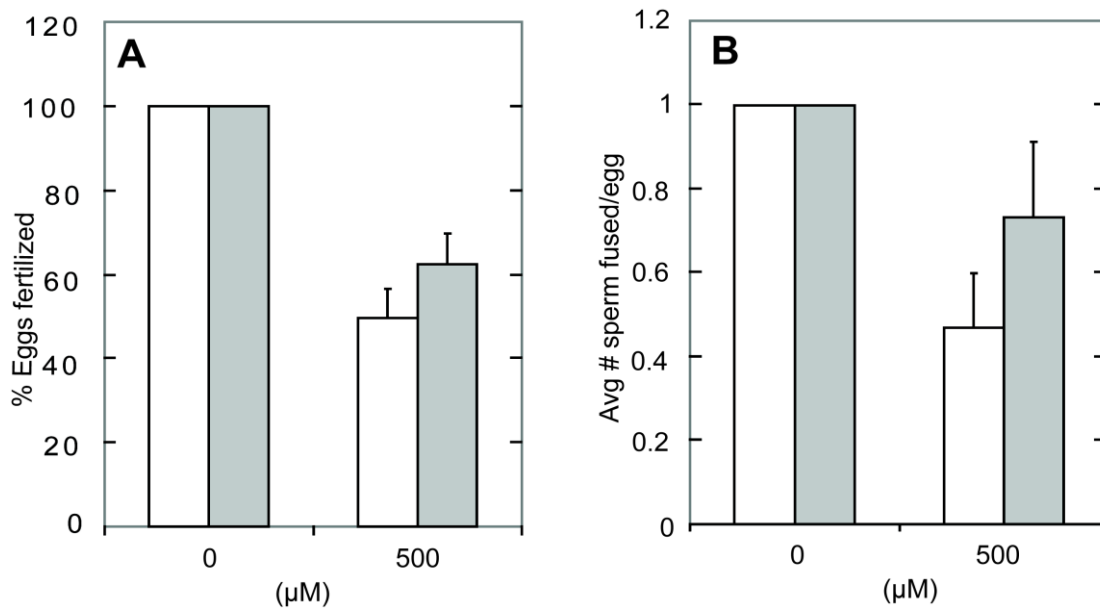


Figure 2-6. Monovalent ECD Polymer and Monovalent ECD Peptide Inhibit ZP-free WT Fertilization. White bars: **12213**. Gray bars: Ac-Glu-Cys-Asp-OMe. Experiments were performed with an average of 30 eggs (range 20-50) at each condition in 2-3 independent experiments. The average number of eggs fertilized or sperm fused per egg in the presence of inhibitor are shown relative to the untreated control for each condition. In the untreated controls, $96 \pm 4\%$ of WT eggs were fertilized. The average number of sperm fused per egg was 1.7 and 1.4 ± 0.1 for **12213** and Ac-Glu-Cys-Asp-OMe, respectively. The concentrations of peptide are shown. Errors are the s.e.m.

3. Conclusion

ECD polymers inhibit fertilization by binding to the egg surface β_1 integrin, most likely present as the $\alpha_6\beta_1$ complex. So far the complex is known to be composed of the β_1 integrin and tetraspanins CD9 and CD81 (77, 80). Inclusion of the integrin within the tetraspanin cluster in wild-type eggs may improve sperm avidity for the egg surface. Inhibition by ECD peptides and polymers saturates at 70% inhibition. The incomplete blockage of sperm binding and fusion is consistent with the role of β_1 integrin as a non-essential adhesion receptor. The combined evidence demonstrating that mouse eggs lacking the β_1 integrin are fertile, and that CD9 is essential for sperm-egg fusion would appear to indicate that a CD9 partner other than an integrin could function as sperm adhesion receptor to initiate the gamete fusion process. β_1 integrin KO eggs can bypass this sperm-integrin adhesion step via an unknown receptor. As a consequence, sperm may attach and fuse to the egg plasma membrane more slowly.

How fertilization inhibition is mediated by ECD polymers is still in question. Several possibilities exist. First, ECD polymers might directly block sperm from binding to its receptor or another receptor within the β_1 complex. Second, ECD polymers may bind to the eggs and change the egg membrane in a non-physiological way so that it no longer can function normally in sperm binding or fusion. For example, ECD polymer to egg receptor binding, might lead to signaling which induces the egg membrane block to polyspermy.

CHAPTER 3

Activation versus Competition Model for Inhibition

1. Introduction
2. Egg Activation by Polymer 1₁₀
3. Investigating the Mechanism of Inhibition by 1₁₀: Competition vs Activation

1. Introduction

Sperm induced egg activation is an absolute requirement for fertilization to proceed normally. The focus of this chapter is to determine if there is any relation between egg activation, and the inhibition mediated by polymers like $\mathbf{1}_{10}$.

A sperm's unique task is to travel through the female reproductive tract to reach the ovulated egg in the oviduct. Ovulated mammalian eggs are arrested at the second meiotic metaphase (MII) until fertilization. At fertilization, the sperm-egg interaction initiates a sequence of biochemical events that leads to embryonic development. These events are known as egg activation (*16, 90-92*).

Several models have been established to explain how egg activation occurs. The first postulates that egg activation occurs by extracellular binding between sperm and egg membrane receptors. In this model, the receptors are believed to be coupled to a trimeric GTP-binding protein (G-protein), or to have tyrosine kinase activity which activates a phospholipase C (PLC) in the oocyte. The second model posits that eggs are activated upon sperm-egg membrane fusion, when the soluble sperm membrane component phospholipase C zeta ($\text{PLC}\zeta$) is released into the eggs cytosol (*96-98*). Both models support the involvement of PLC in the process, either derived from sperm or native to the egg. Phospholipase C hydrolyzes phosphatidylinositol (PIP_2) in the egg membrane to produce second messenger inositol 1,4,5-triphosphate (IP_3). IP_3 induces calcium release by binding its receptor in the ER, and is required for egg activation to occur (*92, 93*).

The events triggered within the egg can be classified as early and late events of activation (*129*). Early events include a transient rise of intracellular calcium which leads to blockages to polyspermy and to the resumption of meiosis. Later events of egg

activation include decondensation of the sperm head, maternal RNA recruitment, formation of pronuclei, initiation of DNA synthesis and cleavage. The initial increase of intracellular calcium is critical for the initiation of both early and late events of egg activation. Figure 3-1 summarizes a series of events which can be measured by microscopy.

The initial rise of intracellular calcium is responsible for blocks to polyspermy that occur at the zona pellucida, and the egg plasma membrane (18, 19, 21, 99). Blocks to polyspermy prevent multiple fertilization events, which are lethal. The block to polyspermy at the zona pellucida is one of the primary egg activation events induced by sperm and involves cortical granule (CG) exocytosis. Cortical granules modify the zona pellucida and block sperm penetration and activation. Calcium oscillations are essential for cortical granule exocytosis to occur. In order to get complete CG release and to induce exit from MII arrest, the egg needs to have experienced multiple calcium spikes (130, 131). Suppressing calcium oscillations with the intracellular calcium chelator BAPTA-AM will prevent cortical granule exocytosis, the primary block to polyspermy at the ZP, and exit from MII arrest (101).

The secondary block to polyspermy, at the egg plasma membrane, is poorly understood. Evidence for its existence comes from several ZP-free studies which show that sperm-egg binding and membrane fusion are reduced in previously fertilized eggs (18, 132, 133). The sperm blockage which is mounted at the egg plasma membrane can also be suppressed by both BAPTA-AM and cytochalasin D (19). Cytochalasin D binds to the fast-growing end of microfilaments and blocks the addition of monomeric actin which prevents actin polymerization (134). ZP-free mouse eggs treated with either

reagent and then inseminated become significantly polyspermic in comparison to controls.

A downstream target of calcium is maturation promoting factor (MPF) (135). MPF activity is high in unfertilized eggs. Maturation promoting factor is a dimer containing both catalytic and regulatory subunits. Its catalytic subunit, CDK1, is inactive when bound to its regulatory partner cyclin B. Calcium transients drive the resumption of the cell cycle by decreasing the activity of MPF and increasing the activity of calmodulin dependent protein kinase II (CaMKII) (135, 136). MPF activity rapidly decreases at fertilization when degradation of the cyclin B subunit is induced by CaMKII (137). If degradation of cyclin B is prevented, eggs will remain arrested at MII, even though sperm have fused and calcium oscillations have been initiated (138).

Parthenogenesis is defined as the initiation of development without the contribution of sperm. It occurs naturally in a variety of species such as birds, reptiles, amphibians and fish. During the process, an egg can develop to term without having any male genetic components and without meiotic chromosome reduction (139). A variety of naturally occurring internal and external stimuli and environmental conditions can trigger the effect. It insures proliferation of species if a male is not present or not needed. Parthenogenesis is not a form of natural reproduction in mammals. In mammalian eggs the early events of parthenogenesis and embryonic development can be induced by artificial means in vitro. Parthenogenetically activated mammalian eggs do not develop to term. Artificial, parthenogenetic activation can be triggered by injecting calcium into the eggs cytosol or exposing the egg to reagents agents such as ethanol, thimerosal, and

strontium (102-104, 106, 107). Calcium oscillations induced by parthenogenetic reagents do not mimic the dynamics of those induced by sperm.

Integrin receptors are known to mediate a wide range of biological activities. Their two main functions are attachment of cells to the extracellular matrix, and mediating signal transduction. A number of integrins are known to do both simultaneously (140, 141). Integrins link the extracellular matrix with the cellular cytoskeleton. Aggregation and clustering of integrins can initiate the assembly of cytoskeletal components, and focal adhesions. Focal adhesion complexes contain cytoplasmic cytoskeletal components such as talin, vinculin and α actinin, and can act as a scaffold for assembly of catalytic signaling proteins (142-144). They provide a structural link between the actin cytoskeleton and the extracellular matrix and are regions in which signal transduction is initiated. The aggregation of integrins activates focal adhesion kinases and leads to the assembly of multicomponent signaling complexes (145)

Some integrins that are known to mediate calcium signaling are: the $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ integrins in platelets (146), the α_v integrins in endothelial cells, the $\alpha_v\beta_3$ integrin in osteoclasts, and β_2 integrins in leukocytes (140). Integrin mediated calcium signaling is believed to be linked to extracellular adhesion events.

Integrins can bind a variety of extracellular ligands, and several recognize the RGD peptide sequence found in ECM proteins and snake venom metalloproteases (SVMPs) (38, 39, 147). Some common integrin ligands which contain the RGD peptide are fibronectin, laminin, type IV collagen, and Class II SVMP's. Integrins that recognize and bind to the RGD sequence include platelet integrin $\alpha_{IIb}\beta_3$ and ubiquitous integrins $\alpha_5\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$ (140, 141, 148, 149). Treatment of bovine oocytes with a soluble RGD

peptide blocks fertilization, induces intracellular calcium transients, and initiates parthenogenetic development (105). So far, the role integrins play in the signal transduction pathway leading to embryogenesis in mammals is unclear.

ECD peptide probes can be used to determine if there is a correlation between the mechanisms of egg activation and fertilization inhibition in mouse eggs. Thus far our evidence has proved that integrin $\alpha_6\beta_1$ is the ECD peptide binding partner. We hypothesized two possible mechanisms of inhibition by ECD polymers. In the first mechanism, ECD polymers may directly compete with or block sperm binding sites on the egg plasma membrane. A second possible mechanism is that the multivalent polymers trigger an intracellular signal, which activates the egg membrane block to polyspermy.

Our first goal was to investigate if inhibitor **1₁₀** (10 copies of ECD), and control polymer **2₁₀** (10 copies of ESA), induced parthenogenetic events such as those summarized in Figure 3-1. **1₁₀** and **2₁₀** were tested for their ability to induce calcium oscillations and pronuclear formation in both the WT and β_1 KO system. The next goal was to determine how ECD polymers like **1₁₀** inhibit fertilization. Is the polymer to receptor binding reversible? Do polymers actuate the membrane block to polyspermy? We used two strategies to answer these questions. The first was to wash the polymer from the egg surface prior to insemination, and the second was to inhibit the egg activation pathway with inhibitors cytochalasin D and BAPTA-AM in polymer **1₁₀** treated eggs.

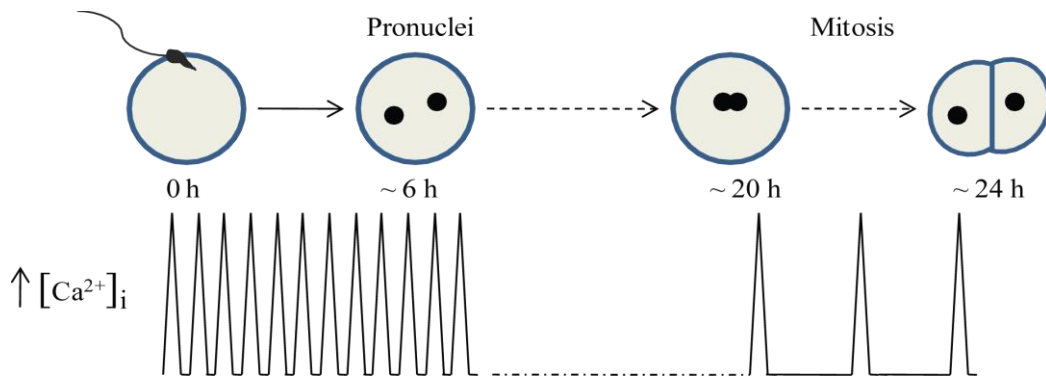


Figure 3-1: Intracellular Calcium $[Ca^{2+}]_i$ Oscillations in Fertilized Mammalian Eggs. Metaphase II arrested mammalian eggs show a series of $[Ca^{2+}]_i$ oscillations lasting several hours, until pronuclei formation in the one-cell embryo.

2. Egg Activation by Polymer 1_{10}

Pronuclei Formation

We tested if 1_{10} could induce pronuclei formation in ZP-intact WT and KO eggs. Sperm were allowed to capacitate, but not acrosome react, prior to insemination so that they could bind and penetrate the ZP. After insemination or polymer treatment, ZP-intact eggs were scored for pronuclei formation (Figure 3-2). WT and KO eggs were both activated by 1_{10} , but less efficiently than sperm activate eggs. Polymer 1_{10} appeared to activate more WT eggs than KO eggs, but the difference was not statistically reliable. No significant activation was observed with control polymer 2_{10} .

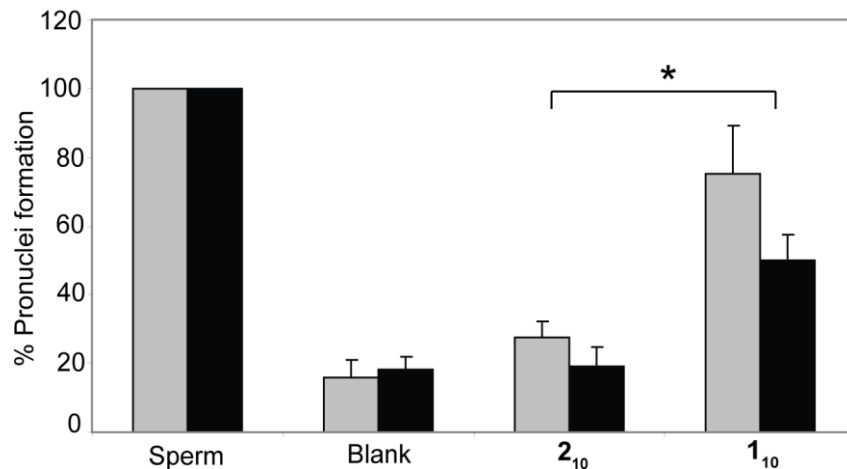


Figure 3-2: Polymer 1_{10} is a Parthenogenetic Agent. The percentage of pronuclei formed at each condition is shown relative to the sperm-treated control. After 2 hours of polymer treatment (50 μ M 1_{10} or 500 μ M 2_{10}) or insemination, ZP-free eggs were washed and 6 hours later were scored for pronuclei formation. Gray bars: WT, Black bars: KO. In the sperm-treated controls, $51 \pm 7\%$ of WT eggs and $63 \pm 4\%$ of KO eggs were activated. 50-100 eggs were assayed at each condition in 3–5 independent experiments. The concentrations of polymer, not peptide, are shown. Errors are the s.e.m. * $p < 0.05$.

Calcium Oscillations

We tested whether polymer **1**₁₀ could induce calcium oscillations. WT and KO eggs that were harvested no later than 12 hours after superovulation with hCG, were treated with polymer **1**₁₀. Polymer **1**₁₀ induced calcium oscillations in both WT and KO eggs and the peak frequencies, durations, and intensities were similar (Figure 3-3). The control polymer **2**₁₀ did not induce oscillations in either egg type.

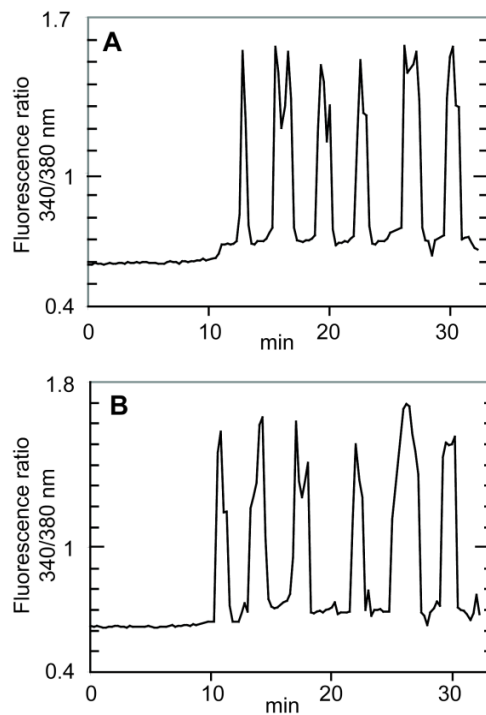


Figure 3-3: Calcium Oscillations. Representative $[Ca^{2+}]_i$ oscillations in ZP-free eggs treated with **1**₁₀ (30 μ M). Eggs were loaded with 10 μ M Fura-2-AM, washed, and placed on a microscope stage thermostatted at 37 °C. The ratio of emitted fluorescence intensities at 510 nm with excitation at 340 nm and 380 nm were recorded using Carl Zeiss Axiovision CD28 Software. Approximately 10 eggs were imaged for each experiment. A) WT eggs, 70% had calcium oscillations, and B) KO eggs 63 % had calcium oscillations.

	Peak freq. (sec)	Avg duration (peak width)	Avg intensity	# of peaks
WT	240 ± 27.4	75 ± 10.7	153459 ± 10069	6
KO	258 ± 41.3	72 ± 9.6	154862 ± 3869	6

Table 3-1: The Peak Frequencies, Durations, and Intensities of Calcium Oscillations Induced by **1₁₀**.

Polymer **1₁₀** is a parthenogenetic agent as measured by the formation of pronuclei and intracellular calcium increase in both WT and β_1 KO systems. The peak frequencies, durations, and intensities of the calcium oscillations observed in both genotypes were similar (Table 3-1). The calcium rise observed in both cases are not comparable to those induced by a fertilizing sperm. This is not surprising because sperm extracts, and various chemical reagents such as ethanol, ionomycin, and thimerosal can also initiate the calcium response and these do not resemble those triggered by sperm either (103, 104, 107). The blend of proteins, phospholipids and carbohydrates which interact as the gamete membranes fuse, cannot be duplicated in a laboratory setting. The mechanisms of activation mediated by **1₁₀** are unclear, and may be non-specific. It is clear that **1₁₀** mediates inhibition by binding a β_1 integrin receptor, however the egg activation response does not occur by the same means. **1₁₀** may bind to a receptor other than a β_1 integrin to initiate the response.

Egg activation could be linked to the inhibition of fertilization mediated by our polymers. Two possible hypotheses exist. The first is that the inhibition of sperm-egg

binding might be occurring via an extracellular polymer to receptor binding event that activates a signal transduction pathway, which in turn actuates the membrane block to polyspermy. The second is that the polymer directly blocks sperm from binding to an egg receptor.

3. Investigating the Mechanism of Inhibition by **1**₁₀: Competition vs Activation

If inhibition is due to activation of the egg membrane block to polyspermy, the block is not expected to be reversible. Therefore, we tested whether inhibition by polymer **1**₁₀ was reversible. A multivalent ECD fluorescently-tagged polymer is not internalized into eggs, and after three washes the polymer is not detected on the surface of the egg. Moreover, washing eggs six times does not affect egg penetrability (Figure 3-4).

Eggs were treated with **1**₁₀ and inseminated immediately after washing or three hours after washing to allow the egg membrane block to reach a maximum (19, 21). Washing completely eliminated inhibition regardless of insemination time. Reversible inhibition is consistent with a competitive binding mechanism and not an activation mechanism.

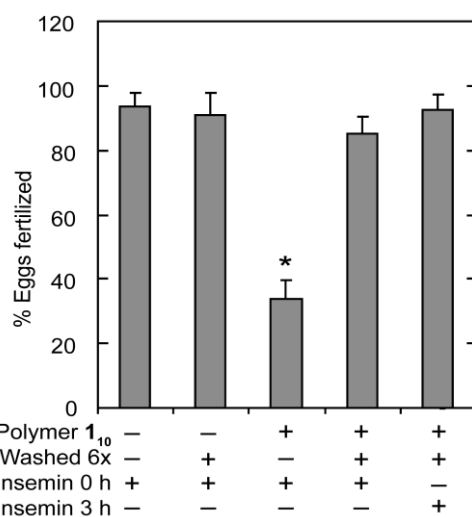


Figure 3-4: Inhibition by Polymer **1**₁₀ is Reversible. ZP-free eggs were treated with polymer **1**₁₀ (10 μ M) for 45 minutes and then were inseminated after 0 or 3 hours with 1×10^5 /mL sperm or they were washed 6 times in M16 3% BSA prior to insemination. Approximately 40-50 eggs per condition were tested in three independent experiments. In the unwashed control, $94\% \pm 4\%$ WT eggs were fertilized. The average number of sperm fused per egg was 1.8 ± 0.3 . Error is sem. * $p < 0.05$ versus other four conditions.

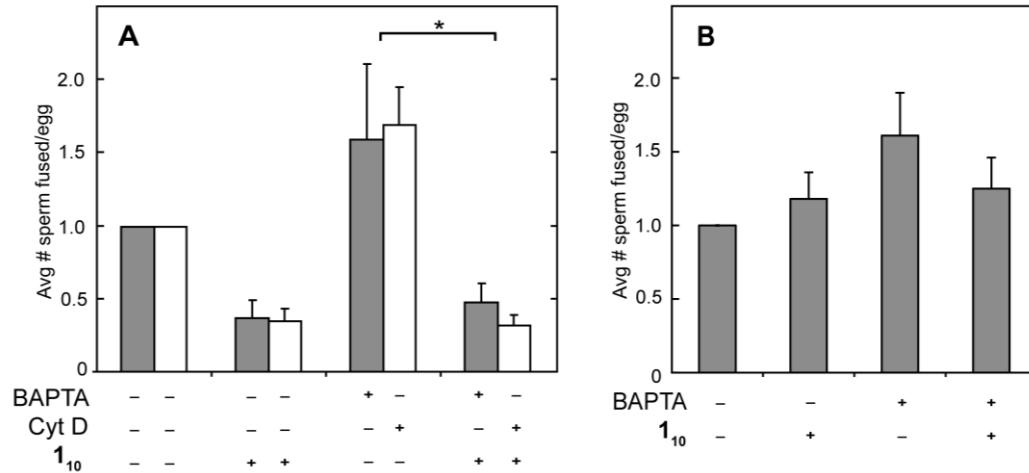


Figure 3-5: Inhibition by Polymer **1**₁₀ does not require Egg Activation. ZP-free eggs were treated with BAPTA-AM (10 μ M, gray bars) or cytochalasin D (40 μ M, white bars), followed by by polymer **1**₁₀ (10 μ M) for 45 min, then inseminated with 1×10^5 /mL sperm. Error is sem. * $p < 0.05$. Approximately 60 eggs were tested at each condition in 5 independent experiments. The average number of sperm fused per egg is normalized to the untreated control. In the untreated controls, the average number of fused sperm was 1.3 ± 0.2 (BAPTA-AM) and 1.8 ± 0.3 (cytochalasin D). a) WT eggs. b) KO eggs.

To further test whether egg activation was responsible for inhibition by polymer, we blocked the egg activation pathway. Calcium signaling and cytoskeletal rearrangement are required for establishing the membrane block to polyspermy (19, 21). ZP-free eggs were treated with BAPTA-AM or cytochalasin D. As expected, WT eggs treated with BAPTA-AM or cytochalasin D fused with nearly twice as many sperm as untreated eggs (Figure 3-5, A) (19). Treatment of KO eggs with BAPTA-AM resulted in the same increase of sperm fusion (Figure 3-5, B). Thus, β_1 integrin is not required for sperm initiation of the membrane block. Importantly, blocking the egg activation pathway in WT eggs did not reduce the inhibition potency of **1**₁₀ (Figure 3-5, A). These data indicate that **1**₁₀ directly blocks sperm from binding to the β_1 integrin. Inhibition of fertilization is not caused by polymer initiating the egg's membrane block to polyspermy.

The egg signaling events and the inhibition of fertilization observed when eggs are treated with **1₁₀** are not related. Egg activation by **1₁₀** does not appear to be sufficient to induce an egg-membrane block to polyspermy. To date, no parthenogenetic reagents, nor sperm introduced by ICSI, have been able to actuate the membrane block (*18, 99, 100*). The molecular players that lead to the membrane block remain to be identified.

CHAPTER 4

Proposed Role for $\alpha_6\beta_1$ Integrin in Mammalian Fertilization

1. Introduction
2. Kinetics of Sperm Binding and Fusion : WT vs β_1 integrin KO
3. Kinetics of Sperm Binding and Fusion in 1_{10} treated eggs
4. Probing the $\alpha_6\beta_1$ Integrin Complex by Immunofluorescent Detection
5. Conclusion

1. Introduction

The adhesive properties of sperm and egg are crucial in the fertilization pathway. Researchers have attempted to elucidate the molecular basis of these adhesive events but many questions remain unanswered. A combination of both genetic and biochemical methods is useful for studying cell to cell interactions of different steps in sperm-egg adhesion. Thus far many labs have used both methods to identify molecules in the pathway.

The biochemical evidence for direct binding of integrin $\alpha_6\beta_1$ to intact sperm has been complemented by immunohistochemical methods. Both experimental strategies strongly support the idea that integrin $\alpha_6\beta_1$ can function as a sperm receptor in mice. To analyze the role of integrins in the fertilization pathway, Takashi et al. pre-incubated sperm with biotin-labeled egg surface proteins (71). The solubilized proteins from eggs inhibited sperm-egg fusion. A western blot analysis of the proteins under reducing conditions indicated that a major-labeled band of 135 kDa bound to sperm. When the proteins were removed from the mixture by immunodepletion methods using the anti-integrin α_6 antibody GoH3, it was found that the 135 kDa egg surface fragment was the α_6 integrin subunit.

In the same study, fluorescent microscopy was used to investigate the localization of integrin $\alpha_6\beta_1$ at the site of sperm contact. At the early stages of sperm-egg fusion, the integrin α_6 and β_1 subunits clustered at the sperm binding site (71, 72). The frequency of cluster formation was closely related to that of sperm-egg fusion, indicating that integrin receptors are accumulated by sperm destined for fusion (71). The $\alpha_6\beta_1$ integrin disappeared from the site of sperm penetration at about 60 min post-insemination or later.

These results strongly suggest that the $\alpha_6\beta_1$ integrin plays an integral role at an early stage of sperm-egg binding and fusion.

Prior to the discovery of the ZP3-Cre-loxP method (150, 151) the direct study of adhesion events between sperm and mouse eggs null for the β_1 integrin was impossible. This is because the β_1 null mutation is lethal due to the deformation of the inner cell mass at the early blastocyst stage (152). However, the advent of the ZP3-cre-loxP method has made it possible to specifically delete the β_1 gene in the oocyte and in no other cell. Deleting the β_1 integrin gene also blocks the expression of the α_6 integrin subunit on the egg membrane (62). It is likely that the α_6 integrin subunit is translated but degraded at some point. The egg proteins that function to compensate for the loss of the β_1 integrin in the KO, are sufficient for fertilization to occur. However it is doubtful that the majority egg surface protein, the β_1 integrin, is there for no purpose. If it does have a purpose, then what is it? Having the β_1 KO mice allows for a direct visual observation and comparison of the early adhesion events which occur between sperm and egg in vitro.

2. Kinetics of Sperm Binding and Fusion: WT vs β_1 Integrin KO

We hypothesized that if β_1 integrin was important for sperm adhesion, the kinetics of sperm binding to KO eggs would be altered. The average number of sperm bound and fused to WT and KO ZP-free eggs in a single focal plane was monitored over 20 minutes. Sperm binding to KO eggs was delayed 1–2 minutes ($p < 0.05$) compared with sperm binding to WT eggs and a concomitant 1–2 minute delay in sperm fusion was observed (Figure 4-1). After 5.5 minutes, no significant difference in the number of sperm bound and fused was detected. These data suggest that β_1 integrin aids sperm adhesion to the

egg and imply that this adhesion step may be bypassed by attachment to other proteins in a binding-fusion complex.

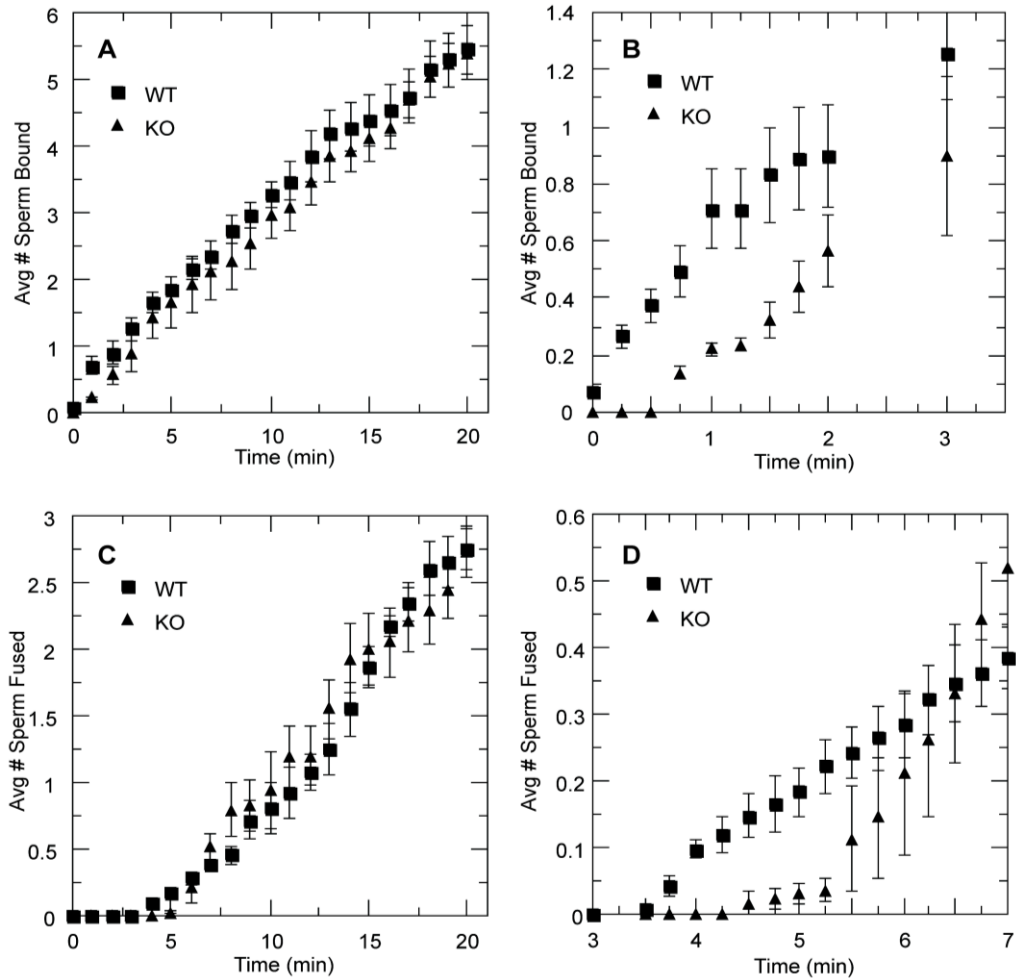


Figure 4-1 The Presence of β_1 Integrin on the Egg Increases the Rate of Sperm Binding and Fusion. Approximately 100 eggs were tested for each genotype in 6 independent experiments. Errors are s.e.m. a) Kinetic comparison of sperm binding to ZP-free WT and KO eggs. Data are shown for 1-minute intervals. b) Magnification of first 3 minutes from panel (a). Data are shown for 0.25-minute intervals. $p < 0.05$ for 0 – 2 minutes between WT and KO eggs. c) Kinetic comparison of sperm fusion kinetics to WT and KO eggs. Data are shown for 1-minute intervals. d) Magnification of first 7 minutes from panel (c). Data are shown for 0.25-minute intervals. $p < 0.05$ for 4 – 5 minutes between WT and KO eggs.

3. Kinetics of Sperm Binding and Fusion in $\mathbf{1}_{10}$ treated eggs

We knew that contacts between sperm and ZP-free eggs are non-adhesive when eggs are pre-treated with polymer $\mathbf{1}_{10}$ since the polymer inhibited fertilization. Sperm reach the egg surface, to find their receptor binding sites blocked by polymer $\mathbf{1}_{10}$. Sperm, being unable to bind, move off into solution.

We measured the kinetics of sperm binding and fusion in WT ZP-free eggs, pretreated with 50 μM inhibitor $\mathbf{1}_{10}$. The average number of sperm bound and sperm fused per egg was measured over 20 minutes. The sperm-egg binding trajectory shows a gradual increase in binding along the curve over 20 min (Figure 4-2). The comparison of binding events between $\mathbf{1}_{10}$ treated WT eggs (Figure 4-2), and untreated WT eggs) shows that approximately 75% inhibition of sperm binding occurs from competition at 20 min. Polymer $\mathbf{1}_{10}$ can never completely overcome sperm binding. The comparison of sperm-egg fusion events between $\mathbf{1}_{10}$ treated WT eggs (Figure 4-2), and untreated WT eggs (Figure 4-1C) shows that approximately 80% of sperm fusion is inhibited by $\mathbf{1}_{10}$. The first sperm-egg membrane fusion event is delayed by approximately 2.5 min in $\mathbf{1}_{10}$ treated WT eggs versus non-treated eggs. Therefore sperm, although inhibited, can bypass the polymer blocking step in $\mathbf{1}_{10}$ treated eggs, enter into the fusion pathway which is not reversible at that point.

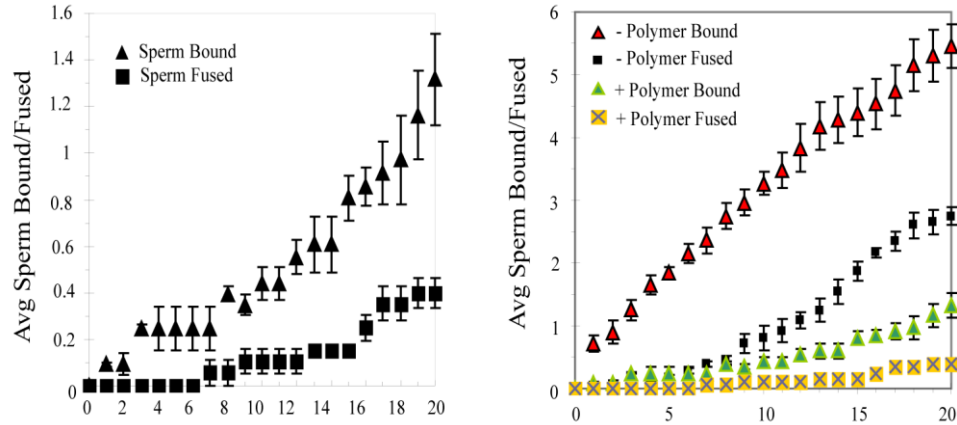


Figure 4-2. The Sperm Binding and Fusion Kinetics of WT Eggs treated with **1**₁₀. Approximately 20 eggs were treated with 50 μ M of polymer **1**₁₀ in 3 independent experiments. Errors are s.e.m. Measurements were taken at 1-min intervals.

4. Probing the $\alpha_6\beta_1$ Integrin Complex by Immunofluorescent Detection

The $\alpha_6\beta_1$ integrin and CD9 are co-localized on the egg surface. Since the fertilin β polymer binds directly to the β_1 integrin, the polymer might be used to block immunofluorescent detection of either integrin subunit, or possibly tetraspanin CD9 that is complexed with the integrin in the membrane. This would provide evidence that the polymer binds to the integrin/CD9 complex. Since the egg washing step removes inhibition by the polymer (Chapter 3), and our current immunofluorescent detection protocols have multiple wash steps, we chose to use polymer **1**₂**2****7****1**₂-**OG**₂, a fluorophore linked co-block polymer (153), that can remain bound even after washing (Figure 4-3).

Polymer **1**₂**2****7****1**₂-**OG**₂ was designed and synthesized by a previous lab member Dr. J.C. Lee for the purpose of photoaffinity labeling and mapping the egg integrin binding site of fertilin β . Polymer **1**₂**2****7****1**₂-**OG**₂ incorporates 2 copies of the fertilin β ECD motif on either end. The ECD peptides are separated by a spacer region containing ESA peptides. The

polymer was designed to contain photoreactive amino acids (β Ala-BpaECDOMe) and Oregon Green fluorophore for photoaffinity labeling and effective detection, The novelty of this polymer is that it can be permanently crosslinked to its receptor on the egg membrane by using UV light ($\lambda_{\text{max}} = 350 \text{ nm}$) and thus it cannot be washed off the egg surface (153). (Note: Polymer **1₂2₇1₂-OG₂** was successfully used to fluorescently label the egg surface. However the experiment for the identification of the labeled egg receptors was not successful (153)).

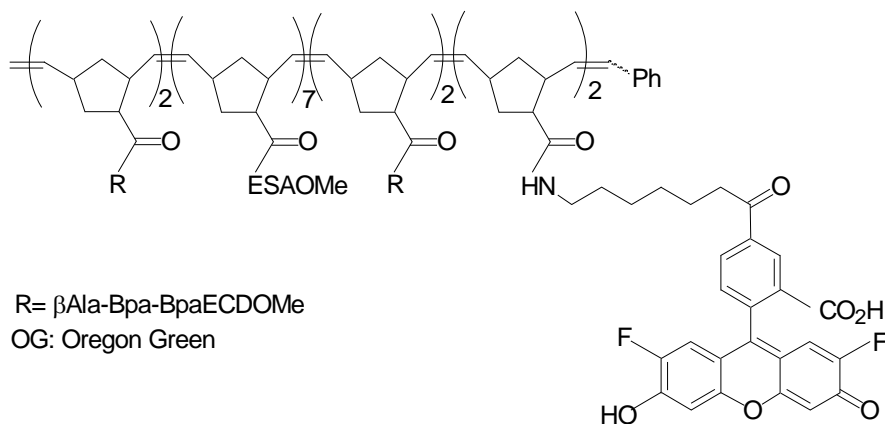


Figure 4-3. Fluorophore-linked Polymer **1₂2₇1₂-OG₂**.

In this experiment, WT ZP-free eggs were treated with **1₂2₇1₂-OG₂** for 45 min and irradiated to crosslink the polymer to egg membrane receptors. Following the polymer treatment, the eggs were incubated with primary antibodies against the α_6 , the β_1 integrins subunits, or tetraspanin CD9. We hypothesized that if the polymer were blocking

primary antibody from binding to its epitope, that it would be evidenced by fluorescent labeling inefficiency of the secondary antibody Alexa568.

As seen in Figure 4-4, polymer **1₂2₇1₂-OG₂** binds to the surface of the egg but does not inhibit detection of either integrin subunit, or tetraspanin CD9 that is associated with the integrin in the membrane. The disintegrin domain ECD peptides in polymer **1₂2₇1₂-OG₂** do not block primary antibodies from binding to their epitopes. Polymer **1₂2₇1₂-OG₂** is not sufficient in size to sterically hinder primary antibody binding from where it binds on the integrin.

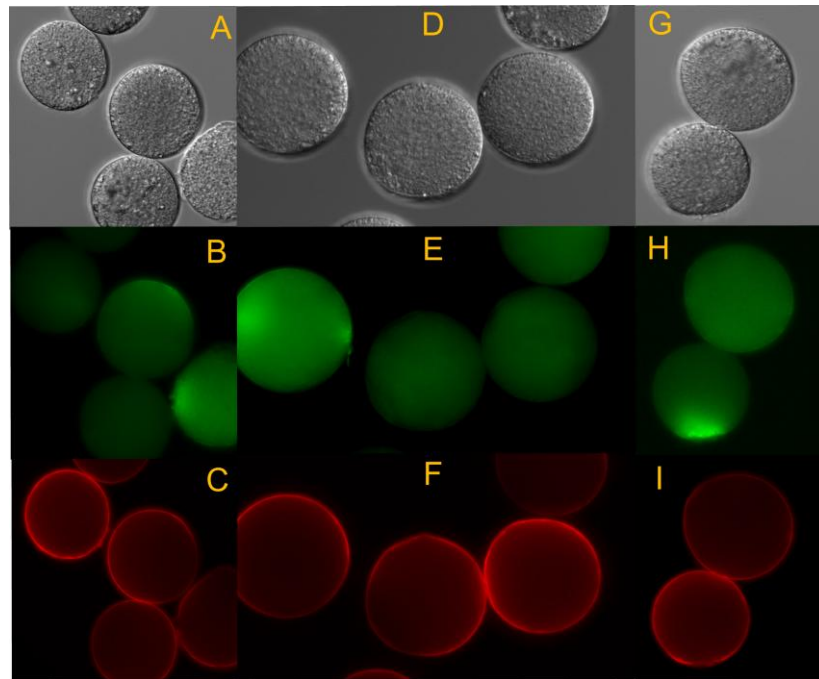


Figure 4-4. Immunofluorescence Images. Immunofluorescent staining of WT ZP-free eggs (B,E,H) with polymer **1₂2₇1₂-OG₂**, (C) anti- α_6 integrin mAb GoH3, (F) anti- β_1 integrin mAb CD29, and (H) anti-CD9 mAb. Eggs were viewed with 20x objective. (A, D, G) DIC images and (C, F, I) epi-fluorescence images.

Recently Ziyayat *et al.* (72) demonstrated in ZP-free WT eggs, that CD9 is evenly distributed around the egg plasma membrane, and that the protein associates with integrin $\alpha_6\beta_1$. In addition, the CD9/integrin $\alpha_6\beta_1$ complex undergoes major relocalization at the site of sperm contact. Therefore, we sought to determine if the distribution of CD9, was evenly distributed in β_1 integrin KO eggs. We treated β_1 integrin KO eggs with primary anti- β_1 integrin mAb CD29, and anti-CD9 mAB. Results show that CD9 is expressed evenly on the egg plasma membrane even when the β_1 integrin is deleted (Figure 4-5).

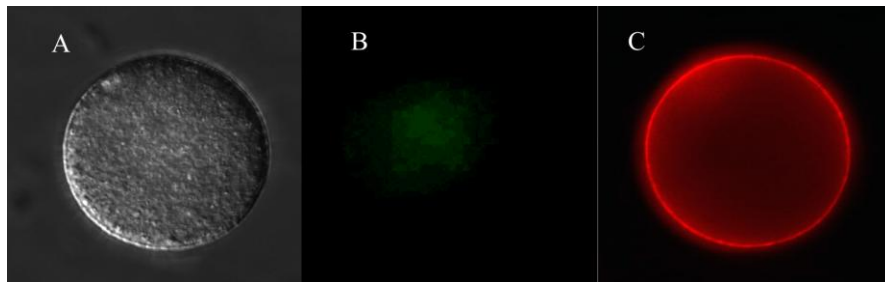


Figure 4-5. Immunofluorescent Staining of CD9 in β_1 integrin ZP-free KO eggs. A) DIC image, B) anti- β_1 integrin mAb CD29, and C) anti-CD9 mAB.

5. Conclusion

The overall trajectories of the rate of sperm binding and fusion between β_1 KO and WT eggs are similar with the exception of the first 2 minutes. The lag time for early sperm-egg adhesion is statistically significant between KO and WT. This suggests that early adhesion events between sperm and egg are partially mediated by integrin $\alpha_6\beta_1$. Integrin $\alpha_6\beta_1$ is not essential for fertilization, and there are other egg surface proteins involved in the adhesion step to compensate for the loss of the integrin. β_1 integrin KO

eggs can bypass the sperm-integrin adhesion step. As a consequence, sperm attach and fuse to the egg plasma membrane more slowly. The reduced rate of binding does not impair fertility under laboratory mating conditions, but may confer an evolutionary advantage in the wild.

In Figure 4-2, sperm binding WT eggs pretreated with inhibitor **1₁₀** reflect the transient nature of sperm binding when the β_1 integrin is blocked. Sperm do meet the egg in solution, and do not bind because adhesion is perturbed by the polymer. However, some sperm do bind and enter the fusion pathway being committed to fertilization. Sperm binding and fusion is inhibited by approximately 75-80% by **1₁₀**. This is consistent with all inhibition data we have gathered thus far. No polymer inhibitor has ever been potent enough to completely overcome sperm binding. This is most likely due to additional and unknown egg receptors that function to bind sperm.

When we compare the inhibition of sperm binding to WT eggs, which have the β_1 integrin blocked by biochemical means (Figure 4-2), to the kinetics of the β_1 integrin KO (Figure 4-1), we would have expected the trajectory of the KO binding curve to have been shifted far to the right. However this is not the case. The reason for this is unclear and unknown. One can only speculate that there is a natural overlap of protein function that exists in the microdomain which compensates for the loss of the β_1 integrin. There may be another integrin or an additional receptor that is upregulated and expressed on the membrane.

Recently it has been proposed that the presence of the $\alpha_6\beta_1$ integrin on sperm can compensate for the loss of the egg integrin by membrane exchange (154). If the $\alpha_6\beta_1$ integrin is indeed present on the sperm head, then there is a chance that ADAMs on the

egg surface might act as their binding partner. Thus far, both fertilin α , and ADAM5 have been found to be expressed in ovary tissue (160).

The proteins involved in gamete binding are not fully understood. The fertilization proteins identified on the egg surface are tetraspanins CD9, CD81, integrin $\alpha_6\beta_1$, and GPI-anchored proteins (60, 78, 80, 86). On the sperm membrane, ADAMs, Izumo, and CRISP-1 have been recognized (13, 30, 45) (Figure 4-6). The egg and sperm proteins at the sperm-egg membrane interface continue to be elusive.

Several questions that need to be answered are: What is/are the sperm adhesion protein(s) in **1₁₀** treated eggs? In **1₁₀** treated eggs, do sperm bind and fuse to the egg membrane via the same complex containing tetraspanin CD9, or a totally different complex altogether? What is the sperm adhesion protein in β_1 KO eggs? Is there another egg-sperm adhesion complex on the egg surface?

Since CD9 is necessary for sperm fusion (73), it probably is localized near sperm adhesion proteins involved in fertilization. The identity of this adhesion protein is unknown. The model: Polymers like **1₁₀** bind within the β_1 integrin/CD9 complex and block fertilization by competition. Sperm somehow overcome the competition and bind to the unknown sperm adhesion receptor, just long enough to enter the membrane fusion pathway via CD9.

The biochemical strategies presented here may eventually be better developed for use in the pharmaceutical industry to solve complex biological problems pertaining to fertility and overpopulation of species. In the future, it may be possible to develop a molecular probe with higher affinity and avidity for an egg receptor. That probe may

then be used to extract receptors from the egg membrane. Thus far numerous attempts to do so have been unsuccessful and further experiments are necessary.

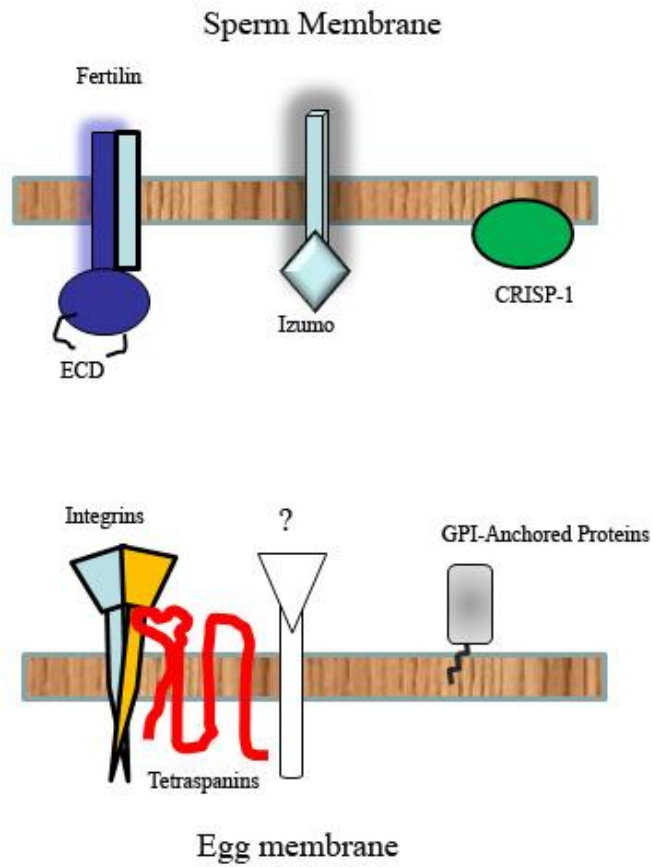


Figure 4-6. Molecular Players at the Sperm-Egg Interface.

CHAPTER 5

Experimental Methods

General Materials and Methods

Generating the β_1 Conditional KO Mice.

Isolation of Spermatozoa and Oocytes for IVF Assay

Immunofluorescence

Polymer Labeling Method and Immunofluorescent Detection

Induction of Calcium Oscillations

Measurement of Pronuclei Formation

Reversibility of Inhibition by Polymers

Inhibition of Egg Block to Polyspermy

Sperm Binding Kinetics in β_1 KO versus WT ZP-free Eggs

Kinetics of Sperm Binding and Fusion in 1_{10} treated WT Eggs

Experimental Methods

General Materials and Methods. All experiments performed with mice were in accordance with the National Institutes of Health and United States Department of Agriculture guidelines, and the specific procedures performed were approved by the Stony Brook University IACUC (protocol #0616). Mice containing the floxed β_1 integrin gene (*155*, *156*) were provided by Ruth Globus (NASA Ames Research Center) with permission from Reinhardt Fässler (MPI, Martinsried). Transgenic mice expressing the Cre recombinase under the control of the ZP3 promoter were obtained from Paul Primakoff (UC Davis) with permission from Jamie Marth (UC San Diego). Some mouse genotyping was performed by Transnetyx. Male and female ICR wild-type mice were purchased from Taconic Inc.

M16 Assay Buffer. Stock solutions were prepared which contained the following: 94.6 mM NaCl, 4.78 mM KCl, 2 mM CaCl₂•2H₂O, 1.19 mM KH₂PO₄, 1.19 mM MgSO₄•7H₂O, 20.0 mM NaHCO₃, 23.28 mM sodium lactate, 0.33 mM sodium pyruvate, 5.56 mM glucose, 0.006% penicillin G potassium salt, and 0.005% streptomycin sulfate. All stock solutions were kept at 4 °C. Stocks A and D were stored for up to 3 months, and stocks B and C were changed every 2 weeks. IVF buffer was prepared fresh from stock solutions just prior to performing the assay. The IVF buffer contained the following components: 2 mL stock A + 2 mL stock B + 0.2 mL stock C + 0.2 mL stock D + ddH₂O to 20 mL. Prior to the addition of ddH₂O, 100 mg of BSA was added for a 0.5% solution, and 600 mg of BSA was added for the 3% solution. IVF buffers were filtered through a 0.2 μ m filter and kept on ice.

Stock solutions	Component	Quantity in g	Concentration in assay buffer
Stock A (100 mL) 10x conc.	NaCl	5.534	95 mM
	KCl	0.356	4.7 mM
	KH ₂ PO ₄	0.162	1.2 mM
	MgSO ₄ •7H ₂ O	0.293	1.2 mM
	60% w/v sodium lactate	4.543	23.28 mM
	Glucose	1.0	5.6 mM
	Penicillin K ⁺ salt	0.06	
	streptomycin sulfate	0.05	
Stock B (10 mL) 10x conc	NaHCO ₃	0.201	20 mM
	Phenol red	0.001	
Stock C (10 mL) 100x conc.	Sodium pyruvate	0.036	0.3 mM
Stock D (10 mL) 100x conc.	CaCl ₂	0.216	2 mM

Table 5-1: Stock Solutions for IVF Buffer.

Tyrodes Solution. The solution was prepared, filtered through a 0.2 μm filter and the pH adjusted to 2. Aliquots were stored for future use at $-20\text{ }^{\circ}\text{C}$.

Component	Quantity in g/100 mL
NaCl	0.8
KCl	0.02
CaCl ₂	0.02
MgCl ₂	0.01
Na ₂ HPO ₄	0.005
Glucose	0.1
Polyvinylpyrrolidone	0.4

Table 5-2: Composition of Tyrodes Acid.

Preparation of Polymers. Polymers were prepared with the [(H₂IMes)(3-Br-pyridine)₂Cl₂Ru=CHPh] precatalyst (157) instead of [(H₂IMes)(PCy₃)Cl₂Ru=CHPh] as previously described (1,128). Before use, the polymers were fully reduced with 10 mM TCEP for 1 to 2 h, precipitated with 1N HCl and washed with ddH₂O, and then re-dissolved in ddH₂O adjusted to pH 7 with NH₄OH.

Generating the β_1 Conditional KO Mice. Mice with the floxed β_1 integrin gene and mice expressing Cre recombinase were used to generate progeny for the assays as previously described by He et al. (62). The schematic below describes the breeding plan used to generate the C57 β_1 integrin mutants.

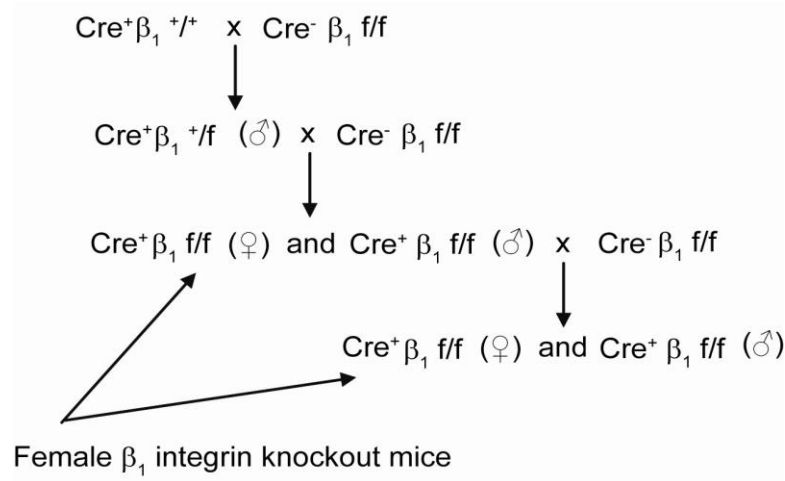


Figure 5-1: Breeding Schematic to Produce the β_1 Integrin KO Mice.

Tail Digestion, DNA Purification, and Genotyping. Spermidine and proteinase K were purchased from Sigma. *Taq* polymerase was purchased from Roche Pharmaceuticals, and dNTP mix (10 mM each) was purchased from Strategene (La Jolla, CA). Primers for PCR were purchased from IdtDNA (Coralville, IA).

To detect the presence of the floxed β_1 integrin gene the following primers were used:

T56: AGG TGC CCT TCC CTC TAG A

L1: GTG AAG TAG GTG AAA GGT AAC

L26: TAA AAA GAC AGA ATA AAA CGCAC

For ZP3 detection the following primers were used:

Cre12: GGA CAT GTT CAG GGA TCG CCA GGC G

Cre13: GCA TAA CCA GTG AAA CAG CAT TGC TG

For genotyping, tail snips obtained from DLAR were sent to Transnetyx Inc. (Cordoba, TN) or digested to isolate DNA. The lysis buffer used to digest the tails consisted of 0.3% SDS, 50 mM Tris. HCl, pH 7.5, 50mM EDTA, 100 mM NaCl, 5mM DTT, and 0.5 mM spermidine (Sigma) stored at rt. Tail snips were placed in 0.5 mL of lysis buffer and 15 uL of proteinase K 20 mg/mL (Sigma) was added. Tails were digested overnight at 56 °C in a water bath, and cooled to rt. Then 0.5 mL phenol/chloroform/isoamyl alcohol (Roche) was added directly to the sample and vortexed for 3 min. The sample was centrifuged at 15,000x g at 4 °C for 10 min and the supernatant was transferred to a fresh tube and 0.5 mL isopropanol was added to the supernatant. The sample was again centrifuged at 15,000 xg, 4 °C for 10 min. The supernatant was removed and the pellet was washed in 70% ethanol and centrifuged at 15,000 xg for 5 min. After repeating the previous step and removing the supernatant, the pellet was air dried for 1 h and dissolved in 50 µL 10 mM Tris. HCl pH 8.0, at 50 °C overnight. 1.2 µL was added for each PCR reaction described below. PCR product was analyzed on a 1.5% agarose gel containing ethidium bromide for the following bands: β_1 floxed allele: 400 bp, wild-type: 320 bp. ZP3 cre recombinase: 260 bp fragment.

PCR Reaction Mix

Components	1 reaction (30 μ L)
10X PCR Buffer	3 μ L
dNTP mix (10 mM each)	0.6 μ L
Mouse tail DNA	1.2 μ L
Primers (10 μ M each)	1.2 μ L
Taq DNA polymerase	1.2 μ L
DMSO	1.5 μ L
ddH ₂ O	To 30 μ L

Table 5-3: PCR Reaction Mix.

PCR protocols

Integrin β_1 conditional knockout:

94 °C	4 min				1 Cycle
94 °C	30 s,	63 °C	30 s,	72 °C	30 s 1 Cycle
94 °C	30 s,	62 °C	30 s,	72 °C	30 s 1 Cycle
94 °C	30 s,	61 °C	30 s,	72 °C	30 s 1 Cycle
94 °C	30 s,	60 °C	30 s,	72 °C	30 s 1 Cycle
94 °C	30 s,	59 °C	30 s,	72 °C	30 s 1 Cycle
94 °C	30 s,	58 °C	30 s,	72 °C	30 s 1 Cycle
94 °C	30 s,	57 °C	30 s,	72 °C	30 s 1 Cycle
94 °C	30 s,	56 °C	30 s,	72 °C	30 s 1 Cycle
94 °C	30 s,	55 °C	30 s,	72 °C	30 s 1 Cycle
94 °C	30 s,	54 °C	30 s,	72 °C	30 s 1 Cycle
94 °C	30 s,	53 °C	30 s,	72 °C	30 s 32 Cycles
72 °C	10 min				1 Cycle

Product was stored at 4 °C

ZP3 Cre transgene:

94 °C	3 min		1 Cycle
94 °C	30 s	}	32 Cycles
65 °C	45 s		
72 °C	45 s		
72 °C	5 min		1 Cycle

Product was stored at 4 °C

Isolation of Spermatozoa and Oocytes for IVF Assay. Sperm were isolated from the cauda epididymis and vas deferens of 8-month-old ICR retired male breeders. Sperm were released from dissected cauda and vas deferens into 3% BSA M16-modified Krebs-Ringer medium. Released sperm were incubated at 37 °C, 5% CO₂ for 3 h in the same medium to allow them to capacitate and acrosome react. Eggs were collected from the oviducts of 8 to 10 week old superovulated females. Mice were superovulated by

injecting 5 IU PMSG (obtained through NHPP, NIDDK and Dr. A. F. Parlow), followed 48–52 h later by an injection of 10 IU hCG. 14–16 h after hCG injection, oviducts were removed from euthanized mice and were incubated in prewarmed M16 medium with 0.5% BSA. Cumulus-egg complexes were collected and transferred to 500 μ L drops of 0.5% BSA medium containing 30 μ g/mL hyaluronidase surrounded by mineral oil. After 5 min incubation, cumulus-free metaphase II eggs (eggs with one polar body) were collected, transferred first to an 80- μ L drop of 0.5% BSA medium, and then washed through six 50- μ L drops of the same medium. Eggs were recovered for 1 h before treating with Tyrodes acid. Zona pellucidae (ZP) of metaphase II eggs were removed by incubating eggs in a 100 μ L Tyrodes acid drop for 1 min at rt followed by mechanical removal of the ZP through a pipette. ZP-free eggs were washed six times with 0.5% BSA medium, were recovered for 2 h in 0.5% BSA/M16, and then were loaded with Hoechst 33342 at 10 μ g/mL for 30 min. Eggs were washed again 6 times with pre-warmed 3% BSA medium and placed in 100 μ L drops of 3% BSA/M16. Polymer solution was added to the egg drop (no more than 5 μ L of stock solution) and the eggs incubated for 45 min prior to sperm addition. Eggs were inseminated with 1×10^5 sperm/mL for 45 min, were washed six times in 3% BSA/M16, and were mounted onto glass microscope slides. Sperm binding and fusion were scored by epi-fluorescence microscopy and DIC microscopy (NIKON Eclipse 400, 40 \times , 0.75 NA objective). Fusion was scored as the fluorescent labeling of sperm nuclei with Hoechst 33342 present in the loaded eggs. The mean number of sperm fused per egg (fertilization index, FI) and percentage of eggs fertilized (fertilization rate, FR) were measured.

In previous work (1) IC₅₀ curves were calculated by a 3 parameter fit (GRAFIT software) to the equation:

$$y = (100-b) / \{1 + ([I]/ IC_{50})\}^s.$$

Where y is the percent FR or FI, b is the remaining percent fertilization after saturation with inhibitor, and s is the slope of the fit. Errors were reported as s.e.m.

Immunofluorescence. Cumulus and ZP layers were removed as described above and allowed to recover in 0.5% BSA in M16 at 37 °C 5% CO₂ for 2 h. Eggs were then treated with 30 µg/mL rat anti-α₆ integrin mAb GoH3 (isotype IgG2a, Molecular Probes) or with 5 µg/mL rat anti-β₁ mAb CD29 (isotype IgG2a, BD Pharmingen), or with 30 µg/mL rat-anti-CD9 (Pharmingen) for 45 min, washed in a 300 µL drop of M16 by slow agitation for 10 min, and fixed with 4% paraformaldehyde. Then the eggs were stained with FITC (30 µg/mL), Alexa488-conjugated or Alexa568 (5-10 µg/mL) IgG2a goat anti-rat secondary antibody (Molecular Probes) for 45 min, washed again in 300 µL of M16 by gentle agitation, and mounted in the same buffer on slides pretreated with Cell-Tak (Sigma). Eggs were imaged on a Zeiss Axiovert with a GFP/FITC filter and 0.55 NA, 20x air objective.

Polymer Labeling Method and Immunofluorescent Detection. Cumulus and ZP layers were removed. Eggs were placed in a 100 µL drop of M16/1% PVP containing 125 µM (in polymer concentration) of **1₂2₇1₂-OG₂** and allowed to incubate at RT for 45 min. Following polymer treatment, the eggs were irradiated with UV light (λ_{max}=350nm) at

4 °C for 15 min and washed by gentle agitation for 10 min in a 300 µL drop of M16/1% PVP on a shaker. Eggs were then treated with 30 µg/mL rat anti- α_6 integrin mAb GoH3 (isotype IgG2a, Molecular Probes) or with 5 µg/mL rat anti- β_1 mAb CD29 (isotype IgG2a, BD Pharmingen), or 30 µg/mL rat-anti-CD9 (Pharmingen) for 45 min, washed in a 300 µL drop of M16 by slow agitation for 10 min, and fixed with 4% paraformaldehyde. Then the eggs were stained with or Alexa568 conjugated (10 µg/mL) IgG oat anti-rat secondary antibody (Molecular Probes) for 45 min, washed again in 300 µL of M16 by gentle agitation, and mounted in the same buffer on slides pretreated with Cell-Tak (Sigma). Eggs were imaged on a Zeiss Axiovert with GFP/FITC, and rhodamine filters 0.55 NA, 20X Or 40x (no oil) objective.

Induction of Calcium Oscillations. Eggs were harvested no later than 12 h after hCG injection. After ZP removal with Tyrodes acid and recovery for 1.5 h-2 h, the ZP-free eggs were incubated for 30-40 min in 10 µM Fura-2AM, 0.025% Pluronic F-127/0.05% BSA/M16. Eggs were washed six times in 50 µL drops of 0.05% BSA/M16, and transferred to dishes with glass cover slip bottoms (MatTek Corp.) pretreated with Cell-Tak (Sigma) and allowed to adhere for 10 min. Samples were placed on a microscope stage thermostatted at 37 °C. Polymer (30 µM) was added to egg samples directly on the microscope stage. The ratio of fluorescence emission at 510 nm with excitation at 340 nm and 380 nm was recorded using Carl Zeiss Axiovision CD28 Software.

Measurement of Pronuclei Formation. Eggs were harvested and their cumulus cells were removed with hyaluronidase and allowed to recover for 1 h in 1.5% BSA/M16. Eggs were placed in 100 μ L drops of the same buffer (covered with mineral oil), which contained either of the following: buffer only, capacitated sperm (1×10^5 sperm/mL), or polymers (**1**₁₀, 50 μ M; **2**₁₀, 500 μ M). After 2 h, all eggs were washed six times in 50 μ L drops of 1.5% BSA/M16 in parallel and incubated for another 6 h at which time they were scored for pronuclei formation by inspection under DIC optics. Prior to insemination and after isolation from the cauda epididymis, sperm were incubated in 1.5% BSA M16-modified Krebs-Ringer medium 37 °C, 5% CO₂ for 1.5 h to allow capacitation without acrosome reaction.

Reversibility of Inhibition by Polymers. IVF inhibition assays were performed as described above with the exception that the ZP-free, polymer-treated (10 μ M) eggs were either inseminated without washing away polymer or were washed 6 times in 50 μ L drops of M16 3% BSA prior to insemination. Capacitated and acrosome-reacted sperm were added to eggs at 2 time points: immediately after washing the eggs, or 3 h after the wash. The final concentration of sperm was $1-5 \times 10^5$ sperm/mL. Eggs were inseminated for 45 min, then they were washed 6 times in 50 μ L drops of 3% BSA/M16. Eggs were mounted onto glass microscope slides, and FR and FI were scored as described above.

Inhibition of Egg Block to Polyspermy. Stock solutions of

1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid acetoxymethyl ester (BAPTA-AM, Sigma) and cytochalasin D (Sigma) were prepared in dimethylsulfoxide (DMSO).

ZP-free eggs were loaded with Hoechst 33342 in 0.5% BSA/M16 as described above. Eggs were then treated with 10 μ M BAPTA-AM or 40 μ M cytochalasin D for 60 min in M16 0.5% BSA, 0.025% pluronic F-127. Control eggs were incubated in 1% DMSO. After 60 min of incubation with drug, BAPTA-AM treated eggs were washed 6 times in 50 μ L drops of 0.5% BSA/M16, cytochalasin D treated eggs were not washed because actin perturbation induced by cytochalasin D is reversible, and the drug can be washed out (158). Drug-loaded eggs were treated with polymer (10 μ M) as described above for 45 min. Eggs were inseminated with 1×10^5 sperm/mL for 45 min, washed 6 times in 50 μ L drops of 3% BSA/M16, and the fertilization index (FI) was measured.

Sperm Binding Kinetics in β_1 KO versus WT ZP-free Eggs. After ZP removal with Tyrodes acid and 1.5-2 h recovery in 0.5% BSA/M16, eggs were transferred to glass bottom dishes which were pretreated with Cell-Tak and the eggs were allowed to adhere for 10 min. Samples were placed on a microscope stage thermostatted at 37 °C and inseminated. The plane of focus was centered on the equator of the egg. DIC and Hoechst 33342 images were recorded every 2 sec for 20 min using Carl Zeiss Axiovision CD28 Software. Images were scored for sperm bound and sperm fused.

Kinetics of Sperm Binding and Fusion in 1_{10} treated WT Eggs. After ZP removal, eggs were washed six times with 0.5% BSA medium and recovered for 2 h in 0.5% BSA/M16. Eggs were then loaded with Hoechst 33342 at 10 $\mu\text{g}/\text{mL}$ for 30 min. Eggs were washed 6 times with pre-warmed 3% BSA medium and placed in the incubator for 10 min. Glass bottom dishes, pre-treated with Cell-Tak, were prepared to contain 100 μL drops of 3% BSA/M16 with a final concentration of 50 μM polymer 1_{10} . Eggs were added to the 50 μM polymer drop and the samples were placed on a microscope stage thermostatted at 37 $^{\circ}\text{C}$ and inseminated. Sperm binding and fusion was scored using Carl Zeiss Axiovision CD28 Software.

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