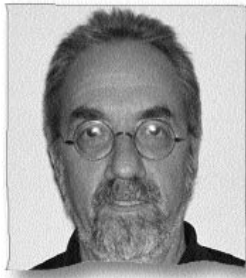


## Article

# Combinatorial peptide library binding of mammalian spermatozoa identifies a ligand (HIPRT) in the axin protein: putative identification of a sperm surface axin binding protein and intriguing developmental implications



Dr Pieczenik is a notable graduate of Andover Academy, and studied at Harvard University. He worked with Fred Sanger, Francis Crick, Sydney Brenner, Bruce Merrifield, Norton Zinder and other early founders of molecular biology at the MRC Laboratory of Molecular Biology in Cambridge, UK and the Rockefeller Institute in New York, USA. He is considered the father of combinatorial chemistry and made the first combinatorial libraries at the MRC Laboratory of Molecular Biology. For the past several years, Dr Pieczenik has been involved, with his co-authors and others, in studies related to whole-cell screening of gametes and early embryos. This work has led to several intriguing findings some of which are presented in the current manuscript.

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## Abstract

The identification of components in cell–cell interactions is an important research goal in reproductive and developmental biology. Such interactions are critical to gamete development, fertilization, implantation and basic development. Several proteins involved with sperm–oocyte interaction and other developmentally important phenomena have been identified. However, these are obviously only a subset of the molecular components involved in such complex cell–cell interactions. One method that has been used to identify binding partners for particular molecular targets is the use of combinatorial libraries accessible on phage surfaces. For the most part, this technique has mainly been applied to screen specific target moieties. However, in some cases whole-cell screening has been attempted. This study describes the first report of screening intact, living mammalian gametes using a proprietary whole-cell combinatorial library binding and analysis protocol. Results from the first screening protocol of mouse spermatozoa strongly identified a putative sperm-binding ligand using proprietary bioinformatic analysis. This amino acid sequence (HIPRT) precisely corresponds with a previously characterized highly conserved protein–protein interaction site in the axin protein. This sequence is found within the binding site for a known sperm surface protein, glycogen synthase kinase-3. This result not only provides proof of the utility of this technique to identify cell surface ligands in mammalian gametes, but it also suggests a potential role for spermatozoa in facilitating developmental axis formation in mammalian embryos.

**Keywords:** axin, combinatorial libraries, GSK-3, HIPTRT, peptides, spermatozoa

## Introduction

Understanding cell–cell interactions is a key goal in many areas of cell and developmental biology. This is particularly so in the case of early development. Despite years of basic research into such interaction, much remains mysterious (Myles *et al.*, 1994; Evans *et al.*, 1995; McElhinny and Warner, 2000; Neganova *et al.*, 2000; Ensslin and Shur, 2003).

Elucidating the key components involved with cell–cell interactions in fertilization, cleavage, implantation and other phenomena will not only provide great insights into the basic developmental process, but will also be of great value in the development of interventional techniques in assisted reproduction and contraception. A variety of techniques have been used in analysing the components involved with these cell–cell phenomena. For the most part, these strategies have

attempted to define the cell surface proteome and determine the function and importance of specific isolated proteins via genetic or biochemical analysis. Ideally, the vital cell surface could be screened *in situ*, allowing for a more natural and complete understanding of the components involved in cell-cell interactions

One approach is provided through the use of combinatorial libraries. (Pasqualini and Ruoslahti, 1996; Szardenings *et al.*, 1997; Pieczenik, 1999, 2003a,b; Merrifield, 2001; Cao *et al.*, 2003). With the completion of the human genome sequence, the information for all the possible sequences involved in all interactions in the human is theoretically available. The question is how to sort out all the interactions. A peptide combinatorial library can contain all possible sequences in the range of four to seven amino acids. The great preponderance of non-covalent binding interactions at the molecular level are made of interactions between small number of amino acids in the range of four to seven or at extremes, multiples of such sizes. Another constraint is the number of unique peptide sequences in the genome. Even if the whole genome were coding, e.g. 3 billion nucleotides coding for a unique 1 billion amino acids, then any given sequence could be uniquely specified by six to seven amino acids. This can be seen in that 20 to the power 6–7 has a range of 64 million to 1.28 billion. In reality, there are only about 20,000 coding sequences in the genome and even if each coding is generously assigned 500 amino acid lengths, this makes about 10 million unique sequences. This database can therefore be uniquely specified or searched by five or six amino acids. That is, one would expect to see any given six amino acid sequence appear once in the human genome. The figure of five amino acids is considered the unique determinant for almost all known protein sequences. Therefore, both the physical chemistry of interactions and the global statistic of the genome codings suggest that five amino acids constitutes a unique peptide and protein identifier (Pieczenik, 2003b).

Therefore, the genome is effectively a closed informational circle for sequences in the range of four to seven amino acids. The universe of antibody-antigen interactions is also not infinite, but defined in size (Pieczenik, 2003b). This is a strategy that was used in sequencing the  $\Phi$ X174 (Sanger and Coulson, 1975). The  $\Phi$ X174 genome is actually a physical circle and therefore continuous random sequencing will effectively 'close the circle'. A combinatorial library containing all possible four-to-seven amino acid sequences essentially creates a 'virtual' closed circle of all possible interacting ligands for the expressed genome or protein phenotype. This aspect of the closed and limited size of both the genome codings and the combinatorial libraries guarantees that if a binding ligand is identified in the range of four to seven amino acids, then chances are that it is unique to the function of that specific binding.

Intact live mouse spermatozoa have been screened using a proprietary combinatorial peptide library selection protocol. Following the initial four rounds of binding, amplification and selection, a consistent set of peptide sequences emerged as a putative ligand interacting with sperm surface component. Upon further analysis, the convergent peptide sequence (HIPRT) was identified and shown to be present in several proteins, including a well characterized, completely conserved region of the axin protein mediating interaction w/glycogen synthase kinase-3

(GSK-3) (Zeng *et al.*, 1997; Ikeda *et al.*, 1998). GSK-3 has been recently identified as a mammalian sperm surface protein with a potential role in the regulation of motility (Vijayaraghavan *et al.*, 1996, 2000; Smith *et al.*, 1999; Somanath *et al.*, 2004). This result demonstrates that whole cell combinatorial peptide library selection is a valid approach for the identification of sperm surface proteins. Furthermore, it provides intriguing molecular evidence supporting the theory that spermatozoa may play a role in the determination of polarity in mouse embryos.

## Materials and methods

A mouse sperm suspension was isolated from cauda epididymides of fertile male CB6F1 mice. Briefly, the epididymides and vas deferens were dissected in a 35 mm dish containing 5 ml of CZB medium (Chatot *et al.*, 1989) (pre-equilibrated at 37°C and 5% CO<sub>2</sub>) and gently compressed to release spermatozoa. Following a 10-min incubation period, the epididymides were removed and the sperm suspension incubated for a further 30-min period. A 500  $\mu$ l aliquot of the sperm suspension was removed from the top layer of media and placed in a 1.5 ml centrifuge tube. A 3  $\mu$ l volume of phage combinatorial peptide library (see below) was added to this tube and the suspension incubated for 1 h at 37°C. Following incubation, the sperm suspension was washed as follows. The suspension was centrifuged in a tabletop microcentrifuge at maximum speed for 1 min and the majority of the supernatant removed. The remaining volume was re-suspended in 1 ml of media via pipetting and separated into two 500  $\mu$ l aliquots in two 15 ml centrifuge tubes. A further 14 ml of CZB wash media was added to these tubes followed by 15 min incubation at room temperature. The tubes were centrifuged at 1000 g for 5 min and the supernatant removed down to an approximate 50  $\mu$ l volume. A further 14 ml of CZB wash media was added, followed by a 15 min incubation period at room temperature and identical centrifugation step. The supernatant was removed from each tube down to an approximate 50  $\mu$ l volume and this remaining volume was combined with a further volume of CZB media to a final volume of 500  $\mu$ l for library isolation. At each step in the protocol, an aliquot was examined and shown to contain motile sperm cells. In addition, aliquots were taken from the original suspension and wash volumes for assessment of the efficiency of the screening and washing protocol. This process was repeated three times using the screened and amplified library created by prior screens. However, in the second to fourth screening protocols, a 50  $\mu$ l volume of library was added to the 500  $\mu$ l sperm suspension.

## Combinatorial peptide library handling

The original combinatorial peptide library used in these experiments was derived from the 'PhD' Phage Display Peptide library (New England Biolabs, Beverly, MA, USA). General procedures and handling were done according to the protocols provided with these libraries. Bound phage were eluted at several different pH values in the range of 2–5 and several concentrations in the range of 0.1–1 mol/l Tris glycine at different times in the binding cycle. There was no panning, as described in the New England Biolab protocols.

Phage DNA was isolated by two different methods. One procedure used Qiagen protocols (Qiagen Inc., Valencia, CA,

USA) and another procedure used AmpliTemp protocols (Amersham Biosciences Inc., Piscataway, NJ, USA). Both gave equivalent results. DNA sequences were obtained using two different primers as available in the New England Biolab protocols. Sequencing was done by the Columbia University Sequencing facilities on an ABI DNA sequencer (Applied Biosystems Inc., Foster City, CA, USA) using both gel and capillary flow fractionation. DNA quality and quantity was determined via agarose gel electrophoresis on vertical minigels with novel illumination and filters. DNA concentrations were obtained from the agarose gel by a continuous web camera with special filters on a PC computer. Sequence analysis was done using ABI software (Applied Biosystems Inc.).

Peptide sequences were deduced from the phage DNA sequence using proprietary algorithms developed via the Nussinov, Pieczenik, Griggs and Kleitman algorithm homology variations (Nussinov *et al.*, 1978) and the renormalization functions developed by Pieczenik (unpublished). A diagrammatic representation of the library screening procedures is presented in **Figure 1**.

## Sperm staining

A peptide corresponding to the identified ligand sequence was directly synthesized by New England Peptide (Gardner, MA, USA) with a terminal rhodamine fluorescent label. Lyophilized peptide was originally diluted in PBS at a concentration of 2 mg/ml. A small volume of this peptide solution (3  $\mu$ l) was added to 60  $\mu$ l of a standard suspension of washed human spermatozoa in human tubal fluid (HTF) culture medium (Quinn *et al.*, 1985) (an approximately 1:20 dilution). Following a 15-min incubation at room temperature, a further 60  $\mu$ l volume of sperm suspension was added to this tube to dilute the peptide concentration and reduce background signal. A small volume of this suspension was used to make an air-dried smear on a standard microscope slide. When dry, a small volume of anti-fade mounting solution was placed on the slide and a coverslip mounted. Slides were imaged on an Olympus AX70 epi-fluorescent microscope (Tokyo, Japan). Images were captured using the Metasystems Isis 3 imaging system (MetaSystems Inc., Belmont, MA, USA).

## Results

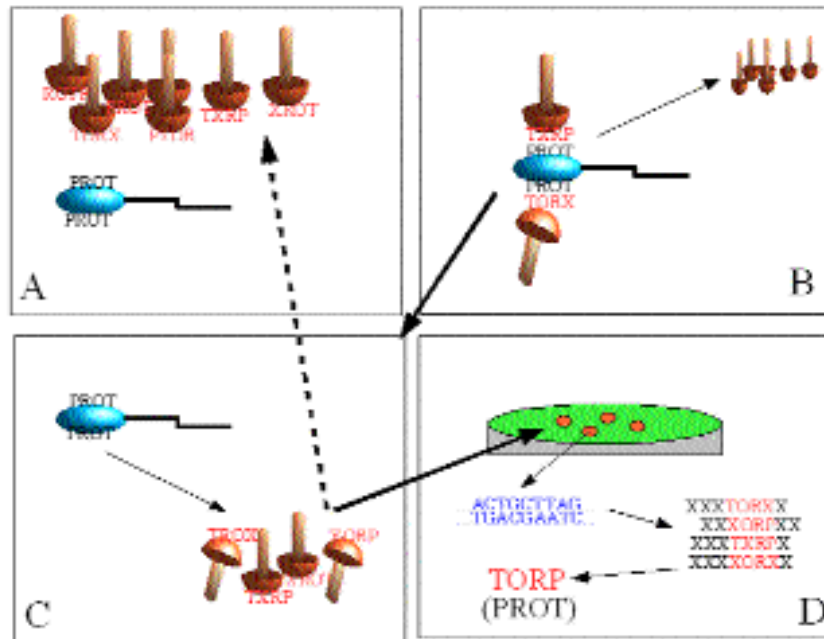
This analysis gave a binding sequence of mouse sperm with the combinatorial libraries containing a unique sequence of five amino acids: HIPRT. Analysis of the database using BLAST (basic local alignment search tool) revealed that the consensus sperm peptide ligand sequence was found in several proteins including axin 1 (Axis inhibition protein 1), pyruvate kinase, apolipoprotein B, and several viral and bacterial proteins (Altschul *et al.*, 1990, 1997). Reanalysis of the binding ligand sequences showed that several binding ligands had also contained an adjacent Tyrosine residue identical with the axin sequence: HIPRTY. The fluorescently labelled peptide corresponding to the identified sperm ligand, bound to human spermatozoa (**Figure 2**). The binding pattern corresponded to the posterior region of the sperm head and the flagellum. This pattern was consistent over multiple spermatozoa. Previous experiments using identically labelled peptides with similar

through different sequences (one differing from HIPRTY at only three amino acid positions) have demonstrated unique sperm binding patterns, indicating that such patterns are related to peptide sequence (data not shown, and Pieczenik, 2003a,b).

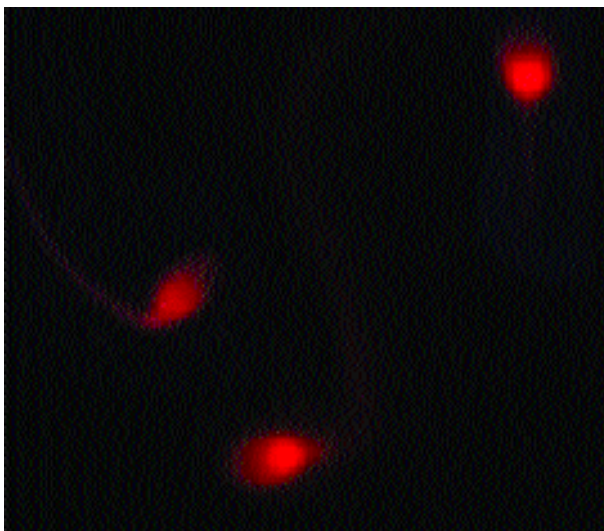
## Discussion

Peptide library screening of intact, living mouse spermatozoa revealed a robust binding ligand. This peptide sequence (HIPRT) was shown to be present in several proteins. However, the majority of these are related to bacterial or viral metabolism. This sequence is also a 100% match for residues 499–503 of the mouse axin protein. Reanalysis of binding ligand clones revealed that a further terminal tyrosine residue, present only in the sequence of axin, was also present in several clones but was not included in the original convergent sequence due to the stringency of the analysis. This strongly suggests that HIPRTY, a sequence unique to axin, could be considered as the true ligand. The residues comprising this ligand are completely conserved between the mouse, rat, chicken and human axin sequences (Zeng *et al.*, 1997; Ikeda *et al.*, 1998). These residues fall within a region identified by mutagenesis experiments in the rat as a binding site for glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) (rat sequence AA 353–437 corresponding to the mouse sequence AA 477–561) (Ikeda *et al.*, 1998). This area defines an extremely strong protein–protein interaction site. The interaction between axin and GSK-3 is so robust that an affinity chromatography protocol specifically isolating GSK-3 has been reported using immobilized axin (Primot *et al.*, 2000). Therefore, the sperm binding ligand identified by this blind screen not only identified a known protein, it also falls within a previously characterized, highly conserved site of protein–protein interaction with a known sperm surface protein. This result would indicate that the binding activity observed in this screen was related to GSK-3 present on the spermatozoa. However, this remains to be confirmed by analysis (via immunoprecipitation or other direct technique) of the binding behaviour of these specific proteins in the sperm–oocyte system. A fluorescently labelled peptide version of this ligand bound to human spermatozoa at the posterior portion of the sperm head and along the flagellum. This pattern is essentially identical to immunohistochemical staining for GSK-3 $\beta$  in bovine spermatozoa as discussed below and was different from previously observed sperm binding patterns for similar labelled peptides (Vijayaraghavan *et al.*, 2000; Pieczenik, 2003a,b).

GSK-3 is a serine/threonine kinase known to be regulatory component in a multitude of diverse cellular signalling pathways (Wodgett, 1990; Doble and Wodgett, 2003; Jope and Johnson, 2004). GSK-3 has two common isoforms,  $\alpha$  and  $\beta$ , encoded by unique genes. The two isoforms have essentially identical kinase domains and differ in an extended N-terminus region in the  $\alpha$  isoform. Both isoforms have been identified in spermatozoa and both exhibit axin-binding behaviour (Doble and Woodgett, 2003; Somanath *et al.*, 2004). GSK-3 has been characterized in the mature spermatozoa of the human, bovine, and rhesus macaque and during spermatogenesis in the mouse and rat (Vijayaraghavan *et al.*, 1996, 2000; Smith *et al.*, 1999; Guo *et al.*, 2003). In the bovine, immunohistochemistry for GSK-3 $\alpha$  shows a localization pattern in the posterior portion



**Figure 1.** Library screening diagram. (A) The cell of interest (blue) with a surface protein ('PROT') is exposed to a viral library. Viral particles (orange) display a surface protein with a section of random sequence indicated in red. Some viral particles bearing complementary peptide sequences will bind to the cell surface protein(s). (B) The cell is isolated from the remainder of the unbound library by dilution, washing and centrifugation. (C) Those viral particles binding to the cell are isolated via manipulation of pH. (D) This viral population forms the basis of a new library that is used to re-screen the cell (dotted arrow). Following the desired number of screening procedures, the final viral population is cultured and individual colonies are isolated. The DNA sequence (in blue) of these clones is determined and the corresponding protein sequences (in red/black) identified. Sequences of the binding viral clones are analysed to identify any convergent (i.e. common) sequence pattern indicating a specific binding ligand ('TORP', a complementary binding sequence to the original cell protein sequence 'PROT').



**Figure 2.** Human spermatozoa exposed to rhodamine-labelled axin peptide.

of the sperm head and along the flagellum (Vijayaraghavan *et al.*, 2000). GSK-3 is believed to play a primary role in the regulation of sperm motility possibly by activation of protein phosphatase II (Vijayaraghavan *et al.*, 1996).

It is unclear if this result, the unambiguous identification of a ligand protein from a vital whole cell screen, is a consistent characteristic of this library screening protocol. Prior screens using spermatozoa isolated and prepared by different methodologies did result in the convergent identification of true binding ligands. However, database analysis could not unambiguously associate these with known proteins. A screen of the mouse zona pellucida conducted in similar fashion and in parallel with the current study failed to identify a complete convergent ligand sequence following four rounds of selection. However, it did identify a unique amino acid (threonine) at the 2 position of the seven-amino-acid stretch (data not shown). This suggests a microheterogeneity of zona target complexity. This might be expected as the zona screens were performed with four individual zona pellucidae as opposed to the large population of cells present in the spermatozoa screens. Further repetition of the identical and modified screening protocol in

the spermatozoa of mouse and other species will help to clarify this question. In any case, this result demonstrates that such intact, vital, whole cell screens can identify native proteins.

## Developmental indications

The identification of axin as a protein containing a binding ligand for mammalian spermatozoa was initially a surprising result. The identification of this specific ligand as a site of association with a known sperm surface protein clarified this issue to some extent. It is certainly possible that the identification of sperm-associated GSK-3 via its axin-binding behaviour in this screen is coincidental. GSK-3 is a relatively ubiquitous enzyme with multiple regulatory roles and the identified ligand is an area of strong protein–protein interaction. However, an alternative concept is that the identification of the GSK-3-specific binding region of axin as a robust sperm ligand in this screen suggests that spermatozoa may be involved with an axin/GSK-3 $\beta$ -mediated phenomenon. Since it has been proposed that mammalian spermatozoa may play a role in embryonic axis determination; a function associated with axin and GSK-3, this becomes an intriguing possibility.

Axin is a scaffolding protein and part of a multi-protein complex involved with the regulation of  $\beta$ -catenin via the Wnt signalling pathway (Zeng *et al.*, 1997). The axin/GSK-3 association (mediated via the binding site identified in this screen) is a critical component of this complex developmental pathway involved in determining the anterior–posterior developmental axis in various species including *Xenopus* and rodents (Zeng *et al.*, 1997; Itoh *et al.*, 1998). Components of this system may even be conserved in ‘pre-bilaterians’ such as the *Cnidaria* (Hobmayer *et al.*, 2000; Wikramanayake *et al.*, 2003). In the mouse, lack of axin results in a duplication of the anterior–posterior embryonic axis due to mis-regulation of  $\beta$ -catenin signalling (Zeng *et al.*, 1997). GSK-3 $\beta$  acts as a negative regulator in this system by phosphorylating  $\beta$ -catenin and thereby stimulating its degradation. The binding of extracellular ligands such as Wnt activates this system by ultimately inhibiting GSK-3 $\beta$  and allowing the accumulation and nuclear translocation of  $\beta$ -catenin that in turn activates downstream genes (Ikeda *et al.*, 1998; Fagotto *et al.*, 1999). The regulation of the axin–GSK-3 $\beta$  complex formation and the activity of GSK-3 $\beta$  and other associated proteins is complicated and not fully understood. GSK-3 $\beta$  also phosphorylates axin itself, apparently stabilizing the complex (Jho *et al.*, 1999). In addition, other proteins such as GSK-3-binding protein (GBP) interact with the same region of GSK-3 $\beta$  involved with axin association and modulate its phosphorylation of both axin and  $\beta$ -catenin (Thomas *et al.*, 1999; Ferkey and Kimelman, 2002).

It is not clear how the actions of Wnt and downstream components are spatially regulated in the mouse embryo to bring about axis formation. The first morphological manifestation of anterior–posterior axis formation in the mouse is the generation of the primitive streak around day 6.5 of development (although existing asymmetries may underlie this process) (Smith, 1985; Gardner *et al.*, 1992). Cells in the anterior end of the primitive streak may respond to signals from the adjacent extra-embryonic or visceral endoderm cells

setting up axis formation mediated via the Wnt pathway. The physical and molecular events responsible for the initial generation of these developmental asymmetries remain to be determined. Analysis of axin mutants indicates that the very earliest manifestations of axis determination are perturbed. Wnt mutants do not exhibit perturbations in the early events in axis formation, and Wnt has not been shown to be expressed prior to primitive streak formation (Moon *et al.*, 1997).

In the best studied system, *Xenopus*, axis determination can be traced to events generating asymmetry in the fertilized egg. Cortical rotation in the fertilized *Xenopus* egg relative to the sperm entry point brings about a redistribution of maternal determinants, resulting in the generation of an axis determining region on what will become the ventral side of the developing embryo opposite to the sperm entry point (Kofron *et al.*, 2001). Current evidence suggests that oriented microtubules resulting from the sperm aster are used for the kinesin-mediated transport of GBP and other proteins to this region of the cytoplasm. Asymmetrically localized GBP then mediates the localized suppression of GSK-3 activity by destabilizing the GSK-3-axin complex, leading to an increase in  $\beta$ -catenin in this region (Weaver *et al.*, 2003). The cortical rotation process is apparently unique to *Xenopus*, although evidence suggests a similar microtubule-transport-dependent process in zebrafish (Sumoy *et al.*, 1999).

Early development in the mouse is known to be highly regulative. However, a variety of studies have suggested that asymmetries in the fertilized mouse egg and events in the developing preimplantation embryo may at least play a role in determining the positioning of subsequent embryonic axes, and the sperm entry point has been implicated in this phenomenon (Gardner, 1997; Piotrowska *et al.*, 2001). Mammalian oocytes are clearly polarized, with a variety of maternal proteins differentially distributed across the animal–vegetal axis (Antczak and Van Blerkom, 1997, 1999; Edwards, 2001). In the mouse, some studies have suggested that the sperm entry point is associated with the position of the axis of symmetry in the blastocyst and thus in subsequent downstream developmental axes (Piotrowska and Zernika-Goetz, 2001; Plusa and Zernika-Goetz, 2002). Also, there is evidence that the first cleavage plane in mouse embryos does bisect the zygote passing near the sperm entry point and that the blastomere inheriting the sperm entry point exhibits more rapid second cleavage (Pitrowska and Zernicka-Goetz, 2001). There is also good evidence of a cytoplasmic rotational process in human oocytes with a possible connection to the fertilizing spermatozoon (Edwards and Beard, 1997; Bioiso *et al.*, 2002). A rotational ‘wave’ of cytoplasmic movement, distinct from the metaphase spindle, was observed in human oocytes following ICSI (Payne *et al.*, 1997). This wave seemed to be related to the timing and formation of the second polar body did not exhibit such a wave. However, overtly identical cytoplasmic movement was also observed in unfertilized oocytes. The fertilizing spermatozoa, via aster-associated microtubules, is clearly involved with the creation of the early cytoplasmic architecture, including polar body position (Van Blerkom *et al.*, 1995; Edwards and Beard, 1997). However, the role of sperm entry in determining early and downstream asymmetries has been strongly debated, and it is possible that such asymmetries are simply pre-existing in the oocyte

(Gardner and Davies, 2003; Johnson, 2003; Zernika-Goetz, 2003). Evidence from a variety of studies demonstrates that the animal-vegetal axis of the egg and polar body position can be traced forward to the asymmetric positioning of cells within the blastocyst inner cell mass (ICM) and regions of the visceral endoderm that will define the anterior–posterior embryonic axis (Gardner 1997; Beddington and Robertson, 1999; Weber *et al.*, 1999; Ciemerych *et al.*, 2000; Piotrowska *et al.*, 2001). The recent birth of normal mice from true oocyte parthenogenesis provides final proof that spermatozoa (and positional information derived from the sperm entry point) are not absolutely required for the generation of downstream asymmetries and correct mammalian development (Kono *et al.*, 2004). However, this does not rule out the possibility that such sperm-dependent positional information may have a dispensable, redundant, or ancestral role. Duplication of function with alternate structures is a common evolutionary protective mechanism.

The ‘rediscovery’ via a novel binding assay of a component of the axin/Wnt pathway associated with spermatozoa provides the first specific potential molecular basis of a role for spermatozoa in the generation of early asymmetries. GSK-3 associated with the internalized spermatozoa could bind to axin, GBP, and/or other components of this system creating an asymmetric distribution of a unique protein complex in the fertilized egg and in downstream blastomeres and embryonic regions. While a study examining the distribution of directly labelled sperm components during early cleavage failed to discern any relevant asymmetries, this does not rule out the concept that transient interaction with the spermatozoa could set up such asymmetries in as yet uncharacterized cytoplasmic components (Gardner and Davies, 2003). For instance, a sperm-associated GSK-3/axin system could be involved with regulating the cytoplasmic rotation process process re-orienting the early zygote following fertilization, and this in turn could lead to a determination of downstream asymmetries (Edwards and Beard, 1997).

A sperm-associated protein has not been directly implicated in axis formation in any species, and it is difficult to envision a mammalian role for axin-GSK-3 similar to the situation in *Xenopus*. Furthermore, evidence indicates that a Wnt gradient acting on an essentially uniform axin-GSK-3 distribution determines the subsequent  $\beta$ -catenin gradient and axis development in the day E6.5 mouse embryo. However, the axin-GSK-3 complex is known to be subject to a variety of subtle regulative controls and these may be involved with setting up and ‘fine-tuning’ of this process. Upstream asymmetries derived from sperm-associated GSK-3 could conceivably play at least a dispensable role in this regulation. It is also possible that sperm-associated GSK-3 may be involved with mediating other cryptic events in early development (unrelated to axis orientation *per se*) through an association with cytoplasmic axin, GBP or other proteins.

Due to the regulatory nature of early development in the mouse, the clearly dispensable role of spermatozoa in this process, and the prevalence of GSK-3 in other cellular processes it is difficult to conceive of experimental manipulations that might reveal a role for sperm-associated GSK-3 in development. The labelling experiments discussed above have demonstrated a connection between specific

cellular distributions in post-implantation embryos and earlier physical asymmetries such as the egg axis and first cleavage planes (Gardner and Davies, 2003; Zernika-Goetz, 2003). Possibly sperm or early cleavage-specific interference with GSK-3 function (or axin binding) might be applied in combination with labelling studies to reveal a potential effect. The synthesized peptide ligand preparation might be a useful reagent in such studies.

This peptide might also have an effect via GSK-3 on sperm function (motility, binding behaviour, etc.) and studies to examine this phenomenon are currently ongoing. Furthermore, GSK-3 is a common enzyme thought to play a role in several human diseases including type 2 diabetes, Alzheimer’s disease and bipolar disorder (Jope and Johnson, 2004). Pharmacological manipulation of GSK-3, such as by the non-specific inhibitor; lithium, is an important issue in human medicine (Doble and Woodgett, 2003). Several studies have examined the effect of axin protein-based reagents on GSK-3 function (Hedgepeth *et al.*, 1999; Zhang *et al.*, 2003). It is possible that the axin HIPRTY peptide could also have utility in this area.

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