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Martin J. Shea, Ph.D. 3/1/00

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Final Scientific Report

Martin J. Shea, Ph.D. DAMD17-96-1-6020

Introduction / Background work (1997-1998)

Isolation of a putative Wnt receptor

The first *Wnt* family member, *int-1*, was isolated over a decade ago by its ability to induce mammary tumors [1]. Since then, many *Wnt* family members have been discovered, comprising what is currently termed the Wnt family of growth factors [2,3].

Wnt family members are responsible for many diverse developmental functions in a wide variety of organisms. Yet despite extensive functional studies on Wnt growth factors, and their signaling pathway components, little is known about the receptor, or reception mechanism(s), for Wnt ligands.

As stated previously in my past annual report, my goal had been to isolate and functionally characterize a Wnt receptor. To achieve this goal, I had employed the yeast two-hybrid system [4,5] in an effort identify proteins that bind Wnt growth factors. Four full-length Wnt "baits" were constructed; Wnt-1, Wnt-3a, Wnt-4, and Wnt-7b. Activation libraries ("preys") were obtained and amplified. I had focused on two libraries of embryonic (9.5-10.5 day) and virgin mammary gland origin. Both of these tissues show an aberrant phenotype for either loss of and/or ectopic expression of various *Wnt* genes. Presumably, proteins responsible for receiving Wnt signals are found in these tissues. I had performed extensive two-hybrid screens with both activation libraries and the four Wnt baits. One clone, a serine threonine phosphatase termed *PPT* [6], was isolated.

Initial PPT analysis

As stated in my previous annual report (1997-1998), truncated murine forms of the receptor were tested using the *Drosophila* GAL4 activation system [7]. I felt confident the murine forms could be tested in flies because *Wnt /wingless* pathway components have been shown by several groups to be functionally conserved between species [8,9]. Resultant phenotypes observed were consistent with PPT having a role in Wnt / wingless signaling. Because of the encouraging transgenic results, I proceeded to more thoroughly analyze PPT's role in Wnt / wingless signaling by utilizing two systems: *Drosophila* and mouse.

Drosophila- The fly homolog was isolated by performing a low stringency screen with a *Drosophila* embryonic cDNA library. The homolog, termed *dPPT* , has overall amino acid identity of 59%, similarity of 75%.

Mouse- The murine *mPPT* genomic locus was cloned and mapped. Intron/exon boundaries were delineated and a genomic map created. A targeting vector was constructed that replaced the presumptive second exon of *mPPT* with an hprt cassette. This will create a truncated, out of frame protein product and, therefore, should be a null mutant for this locus. A *mPPT* null allele was generated in embryonic stem cells by homologous recombination and introduced into the germ line of chimerae mice. Heterozygous mice were generated and *mPPT* mutant mice obtained in the following generation.

Body (1998-1999)

Drosophila dPPT studies

With the *Drosophila* homolog in hand, it is possible to directly analyze dPPT's function in the fly and determine its role, if any, in wingless signaling. The following experiments were performed during the final year of funding.

Polytene localization and region analysis

As a first step to isolate *dPPT* mutants, it was necessary to determine where in the *Drosophila* genome the gene is located. Polytene chromosome *in situ* hybridization with Biotin-labeled *dPPT* cDNA was performed [10]. The localization of *dPPT* was found to be at 85E.

An analysis of the region was then undertaken to determine what tools were available to aid in the analysis of *dPPT* function. There were no existing complementation groups / alleles that could have been a *dPPT* mutant. All mutations in the region were of known genes and all other mutations isolated from previous screens were lost (A. Shearns, pers. comm.). Multiple deletions that apparently span the region do exist and were obtained for possible F2 screens. A search for transposable elements in the region revealed approximately 12, allowing the possibility of a "transposon hop" mutagenesis.

"Transposon hop" mutagenesis

A powerful tool available to *Drosophila* genticists is the ability to create mutants by local "hopping" of pre-existing transposable elements [11]. One creates a fly that has your choice transposable element (one residing in close proximity to your target gene) with transposase expressed ubiquitously. Local hopping of the element occurs in which the element duplicates and reinserts within close proximity of the original element. The resultant flies, scored for by a change in eye color, are expanded and analyzed.

All 12 pre-existing transposon lines in the region were first tested for possible insertion into *dPPT* itself. PCR was performed with multiple primers to the *dPPT* gene and the inverted repeat found at both ends of the element. The results came up negative for any fortuitous insertion into *dPPT*. Next, polytene chromosome *in situ* hybridization with Biotin-labeled transposable element DNA was performed on the 12 lines. One line precisely overlapped the region previously shown positive for *dPPT*, allowing for the possibility of *dPPT* mutant creation by local transposon hopping.

A "transposon hop" mutagenesis was undertaken. The key transposon line was crossed with a transposase expressing fly line and offspring generated. These "F1" flies contain both somatic and germ line hopping events. "F1" flies were then crossed to a balancer stock and the progeny, representing original germ line hopping events, were analyzed. All flies exhibiting a darker eye color change (because of the additional *white* gene present in the newly created transposable element) were isolated and expanded. It is the darker eyed flies that presumably represent a duplication of the original element and a new insertion event nearby.

The mutagenesis gave rise to 162 new fly lines. All were subsequently analyzed for insertion into the *dPPT* locus by PCR (described above). Of the 162 lines, one tested positive for a direct insertion into the *dPPT* gene.

The new insertion site was cloned and analyzed. Sequencing revealed the insertion to be 145 base pairs upstream of the presumptive start methionine.

This new fly line, while offering a direct opportunity for mutant *dPPT* analysis, still needed to be "cleaned up". The original transposon insertion still remained and needed to be removed, otherwise *dPPT* mutant analysis would be complicated by the presence of a second mutation. This problem can be solved by placing the mutant

chromosome over a non-balance chromosome. If a large enough number of flies are tested, and the distance between the two loci is not too small, natural crossovers will occur between both elements, thus separating the two mutations.

Approximately 3000 backcross matings to a "wild type" *yw* line were performed en masse for two generations and eye color change was scored (red to yellow/orange). One fly isolated from the experiment had a clear color change to a light orange (from a deep red). Presumably, there was a separation of the two elements, via natural crossing over, and only one element remained. PCR analysis subsequently revealed that the element remaining in this line was that in the *dPPT* locus and not that of the original insertion.

Creating (im)precise dPPT *deletions*

Curiously, the flies homozygous for the *dPPT* insertion are viable and fertile. This is not too surprising considering the fact that most transposable element insertions in the *Drosophila* genome tend not to confer lethality when homozygous (H. Bellen, pers. comm.). To create a *dPPT* mutant, an additional step was required.

When transposable elements don't duplicate but "hop out" of their location, an imprecise excision sometimes is created at the original site, deleting endogenous sequence. Depending on the extent of the deleted region, this could have severe consequences on the endogenous gene's function. The *dPPT* insertion line was crossed with the transposase line and the resultant flies in the second generation were scored for eye color. Two hundred individual white eyed fly lines were obtained. These lines presumably have the *white* gene within the transposable element removed, along with flanking sequence.

Are these new lines still homozygous viable? Recently, all 200 lines were self-crossed and the progeny analyzed. Of the 200, 14 lines exhibited lethality when scoring the test class, mutant chromosome over mutant chromosome class (the non-balancer class). This new lethal allele frequency is within the range one would expect for imprecise excisions leading to gene inactivation (J. Botas, pers. comm.).

Generation of a dPPT polyclonal antibody

An antibody to dPPT would prove invaluable. First, it would allow one to both test where in the developing fly dPPT protein is expressed in addition to protein subcellular localization (*e.g.* nuclear vs. cytoplasmic). Information obtained from such an analysis could help provide clues to protein function. Secondly, a dPPT antibody would provide an important tool for testing putative *dPPT* mutants. Possible null alleles can be tested for the presence or absence of dPPT protein, once the wild type pattern of expression has been established. Lastly, future studies involving co-immunoprecipitation approaches could be employed to help discover what other proteins dPPT interacts with. Such a study would complement the yeast two-hybrid approach and may help shed light on dPPT's role in Wnt/wingless signaling.

A possible dPPT antibody has been produced and awaits testing. A plasmid was constructed that expresses the C-terminal phosphatase region of dPPT under an inducible promoter. Protein expression in bacteria was obtained, and the specific dPPT protein isolated. dPPT protein samples were injected into pre-tested rats; individual rats showing little or no background when their pre-immune sera was tested against 0-24 hour *Drosophila* embryos. First and second bleeds have been performed, the final bleed to be taken soon.

Mouse *mPPT* studies

As stated previously, *mPPT* mutant mice were obtained and, on first account, appeared normal. Yet over time and further analysis, two interesting phenotypes were uncovered.

Female reproductive defect

mPPT mutant females have greatly reduced fertility compared to that of their wildtype littermates. When young mice of differing genetic background reached breeding age, multiple mating were set up with differing genetic combinations among the adults. What was consistently observed was that whenever homozygous mutant females (-/-) were bred, no litters of any respectable size were obtained. To date, only three times among dozens of mutant female matings was there any litter and, all the three times, only one pup was birthed. This phenomenon was consistent, irrespective of the genetic makeup of the male (+/+, +/-, -/-). Curiously, male mice appeared to have no breeding defects, though that is currently being examined properly with a fertility assay (see below).

Preliminary histological examinations of mutant ovaries revealed defects in ovary integrity. Ovaries were isolated from both wild type and *mPPT* mutant females at both 7 weeks and 6 months of age. Histology sections were prepared and analyzed. What is obvious from the sections is a marked reduction (approximately 50%) in size of the mutant ovary compared to that of the wild type. The size differential was already obvious at 7 weeks of age. Microscopic examination of the sections revealed the mutant ovaries to be highly vacuolarized, indicating tissue degradation (more evident in the 6 month old ovaries). Different staged follicles appeared to be present in mutants (as in wild type) but appeared to be reduced in number.

Does this relate to *Wnt* signaling? Perhaps. Recent studies on two Wnt ligands, Wnt-4 and Wnt-7a, have shown a need for these two proteins in the development of the murine reproductive tract [12,13]. Additionally, multiple *Wnts* have been shown to be differentially expressed in the murine female reproductive tract during development and the estrous cycle [14].

Hematopoietic defect

While female infertility represents a relatively early phenotype, other changes in *mPPT* mutant mice were observed over a longer time period. Most notable was a consistent weight reduction in mutant mice compared to wild type littermates. Also notable was a proneness of the mutants towards infection compared to wild type mice. This latter observation prompted us to examine the femur bone marrow of the mutant mice, the source in mice for infection fighting nucleated blood cells.

mPPT mutant mice have a marked reduction in bone marrow cells late in adult life. Though the numbers need to be collected properly, on early accounts the reduction of nucleated bone marrow cells is approximately 30-50 % at one year of age. The phenotype is observed in both sexes and appears to be progressive in nature; cell loss increasing with age.

How might the hematopoietic defect observed in *mPPT* mutants be related to Wnt signaling? Obviously much has to be determined with regard to cell type and developmental stage affected in the nucleated bone marrow cells. Yet independent evidence does suggest a role for the Wnt family of oncogenes in human and mouse hematopoiesis [15-17].

Ongoing / Future studies (2000-)

Drosophila

The 14 presumptive excision alleles of *dPPT* are currently being analyzed on both the molecular and phenotypic level. On the molecular level, PCR analysis is being employed to roughly determine what molecular lesions / rearrangements have occurred at each of the 14 loci. Subsequently, the region will be cloned and sequenced to determine what precisely is altered, at the DNA level, at each *dPPT* locus.

On the phenotypic level, a subset of the 14 alleles will be examined for a possible role in wingless signaling. Proof of *dPPT's* role in wingless signaling would be obtained if the new *dPPT* alleles produced a morphological (and molecular) phenotype similar to either a wingless null or gain of function mutation. Several approaches will be tried:

- Mitotic clones - By creating mutant clones in *Drosophila* imaginal discs, one can look at *dPPT* 's function late in *Drosophila* development. wingless, and pathway components, are known to have key functions in adult structure development.(*i.e.* wing and leg).

- Straight embryo cuticle preparations – To determine dPPT's role, if any, in the developing embryo. wingless, and pathway components, are crucial for proper patterning of the *Drosophila* embryo. Denticle belt patterns will be analyzed to observe if *dPPT* mutants phenocopy that of any known component of the wingless pathway.

- Ovo D embryo cuticle preparations – To analyze embryonic function as above but with this approach, eliminate the maternal contribution. Approximately half of wingless pathway components to date need their maternal contribution of protein removed for the full mutant phenotype to be revealed.

All of the above approaches have been exhaustively utilized on wingless and its pathway components, molecular and phenotypic analysis of the mutant phenotypes fully documented. It will be interesting to see if the same approaches with the presumptive *dPPT* mutants yield comparable phenotype(s), supporting a *dPPT* role in wingless signaling.

Are any of the 14 fly lines a complete loss of function allele? Sequence information will help determine which of the 14 lines are null and which are partial loss of function (hypomorph) alleles. In addition, dPPT antibody staining of each line will further address this issue. If no dPPT protein is detected in a particular line, this line would be a strong candidate for being a null mutation. Finally, direct side by side phenotypic comparison of the 14 lines will test the severity of each mutation relative to one another.

Mouse

The two interesting phenotypes observed in *mPPT* mutants are currently being analyzed in the following manner:

Female reproductive defect

- Fertility assay – Multiple matings of different genotype pairings have been set up. These include wild type, heterozygous, and homozygous *mPPT* mutant backgrounds of both males and females. The purpose of this study is to determine quantitatively the difference in breeding capability for both sexes of all three genotypes.

- Histological analysis – As noted earlier, *mPPT* mutant ovaries are smaller and highly vacuolarized. To better demonstrate this phenotype, additional sections will be examined with multiple mice covering a broad range of ages (3 weeks - 2 years).

- *mPPT in situ* hybridization – To determine where in the ovary *mPPT* RNA is expressed. *mPPT* RNA localization will help shed light on mPPT's function in the developing ovary.

- Hormone analysis – A hormone analysis of the blood will help determine if the ovarian defect is caused by the ovary itself or an extra-ovarian phenomenon.

- Ovary transplantation – Mutant *mPPT* ovaries will be transplanted to a wild type recipient females. This will test if the cause of the phenotype is extra-ovarian and, if so, will mutant ovaries in a wild type background be "rescued" and begin to produce viable litters. Conversely, wild type ovaries will be placed in a mutant recipient to test if only the ovaries are responsible for the phenotype observed. In this case, wild type ovaries may produce litters, irrespective of recipient genotype.

Hematopoietic defect

- Cell count assay – Determine quantitatively the decline of all nucleated bone marrow cells as a function of time.

- Flow cytometry – To determine, with the appropriate antibodies, which cell type(s), and at what developmental stage(s), are affected in mutant *mPPT* mice bone marrow / spleen (*i.e.* myloid vs. lymphoid cell lineage).

- Colony forming assay – A complementary assay to that of flow cytometry. By culturing bone marrow cells in methylcellulose media with the appropriate cytokines, one can determine which hematopoietic progenitor cells are affected in the *mPPT* mutants.

Conclusion

Growth factors transmit their signal by binding a receptor and activating a cascade of downstream events that eventually leads to cellular proliferation; normal or abberant. With regard to the Wnt / wingless family of growth factors / oncogenes, little is known about the receptor(s) or reception mechanism(s) involved. The present study may shed light on a key piece of this puzzle and unravel how Wnts cause cellular growth, often leading to tumorigenesis.

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