

Award Number: W81XWH-05-1-0289

TITLE: Harnessing Novel Secreted Inhibitors of EGF Receptor Signaling for Breast Cancer Treatment

PRINCIPAL INVESTIGATOR: Mark A. Lemmon, Ph.D.

CONTRACTING ORGANIZATION: University of Pennsylvania  
Pennsylvania 19104-3246

REPORT DATE: April 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

# REPORT DOCUMENTATION PAGE

*Form Approved*  
*OMB No. 0704-0188*

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

<b>1. REPORT DATE (DD-MM-YYYY)</b> 01-04-2006			<b>2. REPORT TYPE</b> Annual		<b>3. DATES COVERED (From - To)</b> 7 Mar 2005 - 6 Mar 2006	
<b>4. TITLE AND SUBTITLE</b> Harnessing Novel Secreted Inhibitors of EGF Receptor Signaling for Breast Cancer Treatment					<b>5a. CONTRACT NUMBER</b>	
					<b>5b. GRANT NUMBER</b> W81XWH-05-1-0289	
					<b>5c. PROGRAM ELEMENT NUMBER</b>	
<b>6. AUTHOR(S)</b> Mark A. Lemmon, Ph.D.  E-Mail: <a href="mailto:mlemmon@mail.med.upenn.edu">mlemmon@mail.med.upenn.edu</a>					<b>5d. PROJECT NUMBER</b>	
					<b>5e. TASK NUMBER</b>	
					<b>5f. WORK UNIT NUMBER</b>	
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b>  University of Pennsylvania Pennsylvania 19104-3246					<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012					<b>10. SPONSOR/MONITOR'S ACRONYM(S)</b>	
					<b>11. SPONSOR/MONITOR'S REPORT NUMBER(S)</b>	
<b>12. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited						
<b>13. SUPPLEMENTARY NOTES</b>						
<b>14. ABSTRACT:</b> The aim of our research is to develop protein therapeutics that can neutralize the growthfactors that activate members of the EGF receptor family in breast cancer. Whereas otheragents that target this receptor system target the receptor itself (eg. Herceptin, Iressa),we propose to target the activating ligand. Our model is the protein Argos from Drosophila,which we discovered inhibits EGF receptor signaling naturally in the fruit fly byinactivating the ligand. Our goal, in effect, is to 'humanize' Argos by making it bind humanEGF receptor ligands and to use a human protein scaffold to do so. In the past year, we haveidentified the minimum fragment of Argos (218 amino acids) required for its function, andhave identified two loops that contribute to its ligand binding site. We have also growncrystals, and anticipate determining a high resolution structure in the next year. We havefound that our human structural scaffolds (Dickkopf proteins) do not bind human EGFs. Ournext step is to take the important structure/function information obtained in the past year,and apply it as we screen in phage display for Argos and Dkk variants that bind human EGFs.						
<b>15. SUBJECT TERMS</b> EGF Receptor, Inhibitor, Antagonist, Argos, Ligand Sink, Signaling						
<b>16. SECURITY CLASSIFICATION OF:</b>				<b>17. LIMITATION OF ABSTRACT</b>	<b>18. NUMBER OF PAGES</b>	<b>19a. NAME OF RESPONSIBLE PERSON</b> USAMRMC
<b>a. REPORT</b> U	<b>b. ABSTRACT</b> U	<b>c. THIS PAGE</b> U	<b>19b. TELEPHONE NUMBER (include area code)</b>			

## Table of Contents

Cover.....	
SF 298.....	2
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	11
Reportable Outcomes.....	12
Conclusions.....	13
References.....	14
Appendices.....	none

# Harnessing Novel Secreted Inhibitors of EGF Receptor Signaling for Breast Cancer Treatment

## INTRODUCTION

The aim of this research project is to develop novel inhibitors of signaling through receptors from the epidermal growth factor (EGF) receptor family by targeting (and neutralizing) the growth factor ligands rather than by targeting the receptors themselves. Apart from the success of Herceptin®, approaches that target ErbB receptors have yielded disappointing results in clinical trials, and this can be rationalized in retrospect. In a *Nature* letter [1] published in late 2004, we described our recent discovery that an inhibitor of EGF receptor signaling in *Drosophila* (named Argos) functions as a specific 'ligand-sink', thus blocking signaling through this system. We proposed to develop a detailed understanding of how Argos achieves this 'ligand-sink' activity, and to analyze possible orthologs of these inhibitory molecules in humans and elsewhere. Our ultimate goal is to be able to modify the growth factor-neutralizing abilities of human orthologs of Argos (or structurally related proteins), thus providing the essential groundwork for developing an innovative approach for breast cancer treatment that will attack the activating ligands in this signaling system rather than the receptors (which are targeted by current therapies). Blocking the activity of EGFR/ErbB ligands in cancer would provide a novel approach to the inhibition of signaling through all 4 members of the EGF receptor family when it goes awry in breast cancer, and would complement existing (receptor-targeted) therapies very well.

Receptor tyrosine kinases (RTKs) from the EGF receptor family are (in some cases) well-validated therapeutic targets in breast and other cancers. The success of the ErbB2/HER2-targeted Herceptin® antibody in breast cancer treatment [2] has spurred efforts to achieve similar results with agents targeted to other receptors in this family. ErbB2/HER2/Neu is overexpressed to high levels in approximately 30% of breast cancer cases [3]. The EGF receptor has been reported to be overexpressed in 14% - 91% of breast cancer patients [4]. It was therefore anticipated that therapeutic agents targeting the EGF receptor might show similar efficacy to Herceptin® in breast and other cancers [5]. However, although they looked very promising in preclinical studies [6, 7], EGF receptor inhibitors such as the cetuximab/Erbitux® antibody and tyrosine kinase inhibitors such as Iressa® and Tarceva® have yielded rather disappointing results in clinical trials [8-10], raising questions about whether the EGF receptor itself is really a good therapeutic target.

One of the key differences between ErbB2/HER2/Neu and the EGF receptor is that ErbB2 can be activated simply by over expression (as occurs in breast cancer), whereas *EGF receptor still needs activating ligand even when over-expressed*. It follows from this, and from the biology of ErbB receptors (outlined below), that for clinical responses similar to those with Herceptin®, we should target the ligands of other receptors in the family rather than the receptors themselves. Developing a novel set of agents with this capability is the aim of our current research.

## BODY OF PROGRESS REPORT

We proposed three central strategies for identifying or generating Argos-like molecules that will function as 'ligand-sinks' for the growth factors that activate human EGF receptor family members:

- analyzing the ability of distant Argos homologs in humans to bind human EGF-related ligands
- adaptation of human proteins that are structurally related to Argos for action as ligand sinks in signaling by the human EGF receptor family
- adaptation of *Drosophila* Argos to bind (and neutralize) human EGF-related ligands

Our conviction is that this combination of strategies, supported by structural work on Argos plus *in vitro* biophysical and cell biological studies, will provide starting points for developing drugs that can sequester the growth factors that activate EGF receptor family members in cancer (rather than targeting the receptors themselves). We believe that such agents will have significant advantages over other drugs that are currently in (disappointing) trials for EGF receptor inhibition in breast and other cancers.

### **Task 1.**

**To test the hypothesis that hDkks bind the EGF domains of EGF-related growth factors, and can act as inhibitory ligand 'sinks' for signaling by ErbB receptors**

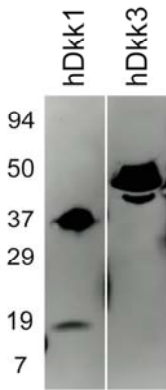
We have made significant progress in this aim in the past year (with hDkk1 and hDkk3), and our results so far suggest that these molecules do not interact with human EGF-related ligands. As described in detail in the initial proposal, identification of the hDkks as human orthologs of Argos would have been the most straightforward (and ideal) route to development of molecules that can be used as therapeutic 'anti-EGFs'. In the initial application, we stated that:

*"It is quite possible (if not likely) that hDkk proteins will not bind to any of the human ErbB ligands, although the clear demonstration that hDkk1 and hDkk2 bind to EGF domains in LRP-6 makes this a reasonable hypothesis to test."*

The fact that our results so far indicate that this simplest route may not be productive focuses our attention more squarely on Aim 2 of the proposed research – to use hDkk proteins and Argos as 'scaffolds' for developing 'anti-EGF' proteins targeted against human ErbB ligands. Indeed, hDkk and Argos are structurally-related proteins that both bind EGF domains through their C-termini, and it does not seem unreasonable to suppose that we can alter their specificity. Progress towards this aim will be described in a later section.

Task 1a      *Establish expression systems for the 4 human Dkk proteins in insect cells, and develop strategies for purification of near milligram quantities (months 1 to 4)*

We have succeeded in expressing hDkk1 and hDkk3 by secretion from transfected *Drosophila* Schneider-2 (S2) cells as proposed (using the Invitrogen *Drosophila* Expression System). The proteins have their native signal sequences, and are tagged at their C-termini with a hexahistidine tag. The proteins are secreted into the S2 cell medium, and can be purified by passing dialyzed medium over a Ni-NTA column, followed by elution of bound protein with 100 mM imidazole, and subsequent gel filtration on a Superose-12 column. This purification procedure is identical to that we have used for Argos [1]. The purified protein is homogeneous, as illustrated by the Western blot shown in Figure 1, and we have succeeded in producing several hundred micrograms of material that appears quite pure when analyzed by SDS-PAGE. We are in the process of optimizing similar studies with hDkk2 and hDkk4, which have expressed less well than hDkk1 or hDkk3 in our studies so far.



**Figure 1**

Western blot analysis of purified hDkk1 and hDkk3 purified from the conditioned medium of transfected S2 cells. Protein was detected using an antibody directed towards the C-terminal hexahistidine tag of the protein. hDkk1 has a predicted mass of 27kDa (without carbohydrate), and contains one N-linked glycosylation site. hDkk3 has a predicted mass of 36kDa (without carbohydrate), and contains four potential N-linked glycosylation sites.

Task 1b      *Using surface plasmon resonance, analyze binding of each hDkk protein to the 11 immobilized human ErbB ligands (months 4 to 6)*

Biacore studies have been performed in two 'formats'. In one, we have generated biosensor surfaces on which hDkk1 or hDkk3 have been immobilized. We have not detected binding of any human EGF-related growth factor to these surfaces. In the second approach, we have generated biosensor surfaces on which any one of the 11 human EGF-related growth factors has been immobilized, and passed purified hDkk1 or hDkk3 across these surfaces to assess binding. Here, we have straightforward positive control experiments, since binding of the extracellular regions of EGFR or ErbB4 can be demonstrated for each ligand. In no case have we obtained convincing evidence for interaction of the hDkk proteins with human EGF-related growth factors, suggesting that the hypothesis tested in Aim 1 of the original proposal is incorrect.

Task 1c      *Analyze the ability of each human Dkk protein to antagonize ErbB receptor activation by each ErbB ligand in a cellular context, and determine IC50 values for any positive cases (months 4 to 8)*

As anticipated given the outcome of Task 1b, in the proposed cellular studies, we have seen no inhibition of growth factor-induced EGF receptor family member signaling upon addition of excess hDkk1 or hDkk3.

Task 1d      *Perform additional biophysical and structural characterization of any positive interactions between hDkk proteins and human ErbB ligands, using analytical ultracentrifugation and X-ray crystallography (months 8 to 24)*

As described in the proposal, we aim to use hDkk proteins and Argos as 'scaffolds' for developing novel antagonists for human ErbB ligands. To achieve this goal, a structural understanding of Argos itself, and its interactions with *Drosophila* ligands, is required. We have made significant progress in this (see Task 2b), and hope to determine the structure of Argos very soon. We are also in the process of scaling up hDkk1 and hDkk3 production for crystallization trials with these proteins. Comparison of the structures of human Dkk proteins and *Drosophila* Argos will be a very important goal in the next project period, serving to determine whether it is feasible to use human Dkk proteins as scaffolds for Argos-like functions.

## Task 2.

### To adapt human Dkk proteins and *Drosophila* Argos to bind and neutralize human ErbB ligands

Since our studies so far indicate that the human Dkk proteins do not bind ErbB ligands, we are focusing our attention on engineering or evolving Dkk and Argos proteins to interact with the human EGF-related growth factors, following the strategy described in our initial proposal for this (likely) eventuality. Our plan is to display the basic scaffolds as pIII fusions on M13 phage, and to select from limited randomized libraries to identify forms of hDkk proteins and Argos that can bind human EGF (and other ErbB ligands). As with all phage display projects, one of the first challenges is to display the protein on the phage surface while maintaining function. This is usually best achieved with the smallest fragment (or domain) of the protein that retains function.

Since our preparations of the 423aa (mature) full-length secreted form of Argos suffer from significant degradation, and appeared quite heterogeneous – probably because of significant glycosylation, we chose to complete Task 2b before Task 2a as listed in the Statement of Work.

**Task 2a**      *Establish procedures for displaying the C-terminal cysteine-rich regions of hDkk1, hDkk2 and Argos as pIII fusions on M13 phage, and ensure that they are correctly presented by using phage ELISA assays to check binding to positive controls (LRP6 for hDkk1 and hDkk2; Spitz for Argos) (months 1 to 4)*

In order to maximize success of this task, and to display the most ‘well-behaved’ protein species on phage, we chose to complete Task 2b before Task 2a. As described below, we now have very high quality protein for an Argos fragment (Argos<sub>218</sub>) that maintains full functionality, but has minimal glycosylation. This (and Dkk of equivalent size) should serve as an ideal target for phage display. Having reached this point, we have recently generated clones for phage display of Argos<sub>218</sub>, and are now evaluating them.

**Task 2b**      *Generate a set of nested deletions to determine what are the smallest C-terminal cysteine-rich fragments of hDkk1/2 and Argos that will bind to the positive control targets, and test binding by phage ELISA (months 4 to 8)*

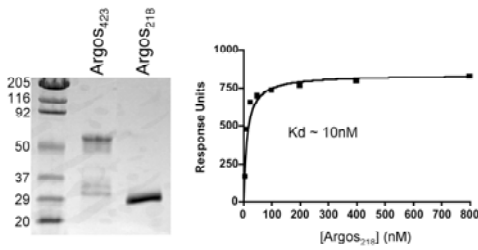
By comparing the *D. melanogaster* Argos sequence with those from *M. domestica* and *A. mellifera*, we chose – after much trial-and-error – to delete residues 22-111 and 165-280 from the pre-protein (1-21 constitute the signal sequence). These deletions remove the blue parts of the primary structure as schematized in Figure 2, which are largely unconserved regions that have little predicted secondary structure and contain multiple potential O-glycosylation sites.



**Figure 2**

Schematic of Argos primary structure. Mutants identified in Task 2d are noted.

Residues 111-165 of Argos (N1 in Figure 2) appear to constitute an N-terminal domain with four cysteines, while residues 280-444 (N2 in Figure 2) constitute a C-terminal domain with 12 cysteines. Our deletion mutant contains only these two domains, fused directly to one another to yield a 218aa protein (which we term Argos<sub>218</sub>). Argos<sub>218</sub> expresses well, has excellent chromatographic properties, and the purified protein appears homogeneous by SDS-PAGE (Figure 3). Argos<sub>218</sub> and binds strongly to Spitz and the Spitz EGF-like.  $K_D$  for Spitz binding is ~10nM (Figure 3), which is very similar (in fact perhaps slightly stronger) to what we have previously published for Spitz binding by full-length Argos [1]. Thus, Argos<sub>218</sub> appears to have the same functional properties as the full-length secreted protein, but is much more robust – as required for phage display. Further deletion from Argos<sub>218</sub> impairs function.

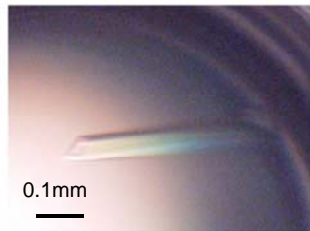


**Figure 3**

In the left-hand panel, a Coomassie-stained SDS-PAGE gel of full-length Argos<sub>423</sub> and the Argos<sub>218</sub> deletion mutant is shown, illustrating the dramatic improvement in homogeneity in the deletion mutant. In the right-hand panel, binding of Argos<sub>218</sub> to the immobilized Spitz EGF-like domain is shown as a representative saturation-binding curve. The Spitz-binding properties have not been affected by the deletions.

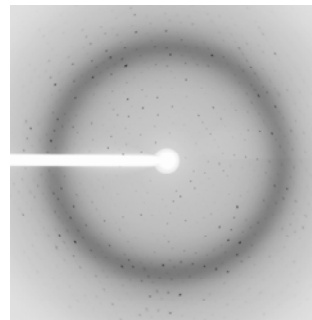
These *in vitro* studies have clearly identified the smallest cysteine-rich fragment of Argos that will bind to the positive control target (Spitz) – using purified protein rather than phage display as initially proposed. We plan to generate analogous fragments of the hDkk proteins for analysis in the next project period.

Since it would be very valuable to know the Argos (and Dkk) structures when designing randomization strategies for our phage display approach to generating novel anti-EGFs, we have also generated crystal of Argos<sub>218</sub>. Purified Argos<sub>218</sub> forms single crystals from 15% PEG 3400, 0.2M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1 M KCl, 0.1M Acetate pH 4.6. The crystals are needle-shaped, with dimensions of up to 1 x 0.05 x 0.05 mm. An example is shown in Figure 4. We can cryostabilize these crystals by growing in the presence of 7% glycerol, or passing through paraffin oil. The crystals diffract to 2.6Å resolution at ALS (Berkeley, CA) or APS (Chicago, IL), and have space group C2 with a = 112Å, b = 62Å, c = 73Å and β = 101°. There are two Argos<sub>218</sub> monomers in the asymmetric unit. A representative image from an APS native dataset, is shown in Figure 5. We will determine the structure of 'apo' Argos from these crystals during the next year, and work towards the structure of an Argos/Spitz complex. We are also working towards crystallizing hDkk1 and hDkk3.



**Figure 4**

Example of a single crystals of Argos<sub>218</sub> grown as described in the text.



**Figure 5**

Representative image from a single Argos<sub>218</sub> crystal taken at APS beamline 23IDin, with 0.5° oscillation angle and a 3 second exposure. This crystal was grown in the presence of 7% glycerol, and frozen directly from the drop.

**Task 2c**

*Define conditions under which the observed weak binding of Argos-bearing phage to human EGF can be readily detected - to ensure that some signal can be obtained in f (below), and that this can be 'tuned' by altering stringency.*

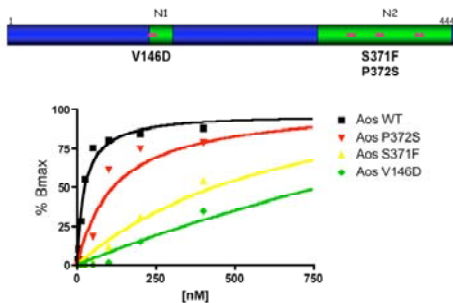
This task will be initiated once phage are produced – early in the next project period.



## Task 2d

Generate mutants for display on M13 to determine which regions of *hDkk1/2* and *Argos* are primarily responsible for defining the specificity of EGF domain recognition, and test these mutants by phage ELISA (months 8 to 12)

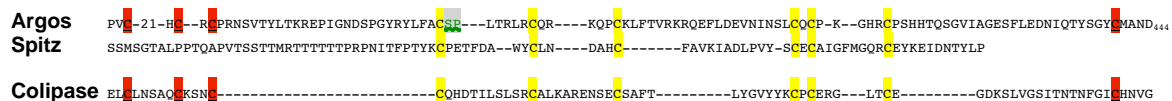
Rather than using phage display to address this question, we have taken advantage of a recent collaboration in our studies of *Argos* to identify critical parts of the EGF domain binding site on *Argos in vivo*. We have collaborated with Joseph Duffy at Indiana University, who is currently analyzing data from an ethyl-methane sulfonate (EMS) mutagenesis screen designed to recover modifiers of an *Argos* misexpression phenotype in the developing *Drosophila* eye [11-13]. This screen has generated suppressors (loss-of-function alleles) and enhancers (gain-of-function) of the *Argos* misexpression phenotype, which can correspond to mutations in the *argos* transgene (intragenic) or in other genes (extragenic). Following mapping of these modifiers (suppressors or enhancers) to a single gene, the responsible lesions are being identified by sequencing, utilizing non-mutagenized parental flies as a wild-type reference. This screen has led to the identification of an allelic series of mutations in the *argos* transgene that decrease its activity.



**Figure 6**

In the upper panel, a schematic representation of *Argos* sequence is shown. Pink circles represent sites at which mutations cause loss-of-function in the Duffy lab misexpression screen. In the lower panel, Biacore-derived curves are shown for binding of wild-type, P372S, S371F, and V146D *Argos* to immobilized Spitz. The S371F and V146D mutations significantly impair Spitz binding, and the P372S mutation has a moderate effect. Thus, loss-of-function in this *in vivo* screen so far correlates with impaired Spitz binding *in vitro*.

Critically, in addition to mutations at conserved cysteines (which probably cause misfolding – and thus loss of function – of *Argos*), two mutations in the C-terminal region of *Argos* (S371F and P372S), and one within the N-terminal cysteine-rich cluster domain (V146D) have already been identified. We have produced recombinant protein corresponding to the V146D, S371F, and P372S mutants of *Argos* by secretion from baculovirus-infected Sf9 cells, and used Biacore to assess the binding of these mutated proteins to immobilized Spitz (Figure 6). None of the mutations adversely affected protein production, arguing that they do not substantially affect folding or stability.



**Figure 7**

Alignment of the C-terminal cysteine-rich region of *Argos* (residues 313-444 of pre-*Argos*: the region initially suggested to resemble EGF [14]) with the EGF-like domain from Spitz. Cysteines that define the EGF domain fold are shaded yellow. Note that an additional unpaired cysteine (red) would be left at the C-terminus of *Argos* if an EGF-like disulfide bonding pattern is assumed. The pattern of cysteines in *Argos* aligns better with that in porcine colipase, as shown. Porcine colipase includes a region that also suggests an EGF-like domain (yellow cysteines), but the disulfide bonding pattern is different [15]. S371 and P372 in *Argos* – in the loop following the fourth cysteine shown – are shaded gray (over green text).

Mutation of V146 or S371 had a very significant effect on Spitz binding, reducing affinity by 46-fold and 37-fold respectively (Figure 6). The P372S mutation, which also resulted in a loss of function in the *Argos* misexpression screen, reduced Spitz binding by around 2.5-fold, from a  $K_D$  of 15nM for wild-type *Argos* to 32nM for P372S *Argos*. These results actually represent the first

correlation between Argos function *in vivo* and its ability to bind and sequester Spitz. They also identify the loop containing S371 and P372 (see Figure 7) and that containing V146 as regions to target in our first round of sequence randomization for phage display in the coming year.

## KEY RESEARCH ACCOMPLISHMENTS

- Produced recombinant protein for hDkk1 and hDkk3
- Determined that hDkk1 and hDkk3 do not bind human ErbB ligands – arguing that the first hypothesis to be tested in our proposed research is incorrect. This defines the direction of our studies to generate novel anti-EGFs based on Dkk and Argos structural scaffolds
- Identified smallest fragment of Argos (Argos<sub>218</sub>) that maintains function
- Crystallized Argos<sub>218</sub> for X-ray crystallographic studies
- Identified two inter-cysteine loops in Argos that are critical for EGF domain binding, allowing us to focus on these regions for randomization in phage display studies

## REPORTABLE OUTCOMES

1. Results presented in invited talks at ASBMB Meeting in San Diego, CA (April 2005) and FASEB Meeting on Receptors and Signal Transduction, Snowmass, CO (August 2005)
2. A manuscript describing identification of mutated forms of Argos that no longer bind Spitz has been prepared for submission to *J. Biol. Chem.*: “*Argos mutants define an affinity-based threshold for Spitz inhibition in vivo*”, by D. Alvarado, T.A. Evans, R. Sharma, M.A. Lemmon, and J.B. Duffy.

## CONCLUSIONS

Our central aim is to develop novel proteins with the ability to bind to and inactivate the growth factors that activate receptors from the human EGF receptor family. This aim follows from our discovery that Argos, an EGFR inhibitor in *Drosophila melanogaster*, functions by sequestering the activating ligands in *Drosophila*. We had identified human Dickkopf (Dkk) proteins as potential Argos equivalents, based on their predicted pattern of disulfide bonding (and copurification with neuregulin in one reported study), and hypothesize that they will share a similar overall structure to Argos. In the past year, we have established that the Dkk proteins do not bind human ErbB ligands, indicating that the simplest possible route to discovering a human 'anti-EGF' will not work. We have therefore turned to the alternative (and most likely) strategy described in detail in the original proposal - to use Dkk proteins and Argos as structural 'scaffolds' that will be subjected to selection through phage display for molecules that can neutralize human EGF-related molecules.

We have identified the smallest fragment of Argos that is capable of neutralizing *Drosophila* EGFR ligands, and are well on our way to determining the structure of the protein. We have also identified two key loops in Argos (and by alignment in the Dkk proteins) that are critically involved in interactions with EGF ligands. Thus, in the past year we have completed important characterization steps that define the precise scaffold in which we can 'evolve' Argos to bind human instead of *Drosophila* EGF ligands, and have made important progress towards identifying the binding site. Our next step is to display the minimal regions of Argos and Dkk proteins on phage, randomize the short loops in which we have identified ligand-binding mutations, and screen for interactors with human EGFR ligands.

### So What ?

Argos is an inhibitor of EGF receptor signaling used by Nature to controlling signaling through this receptor in *D. melanogaster*. Aberrant signaling through the EGF receptor and its relatives is well known to play an important role in breast and other cancers. We propose to follow Nature's example, and design (or discover) a molecule that can function like Argos in breast cancer patients where EGF receptor family signaling is over-active. We have made significant strides in understanding the way in which Argos binds to and neutralizes the growth factors that activate the EGF receptor, and have determined the minimum protein required for this – as well as identifying key parts of its interaction site. The findings over the past year place important constraints on our design requirements, and set the stage for design and selection of novel anti-EGFs in the next project period.

## References

- 1 Klein, D. E., Nappi, V. M., Reeves, G. T., Shvartsman, S. Y. and Lemmon, M. A. (2004) Argos inhibits epidermal growth factor receptor signalling by ligand sequestration. *Nature* **430**, 1040-1044
- 2 Slamon, D. J., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., Fleming, T., Eiermann, W., Wolter, J., Pegram, M., Baselga, J. and Norton, L. (2001) Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *New Engl. J. Med.* **344**, 783-792
- 3 Slamon, D. J., Goldolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A. and Press, M. F. (1989) Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* **244**, 707-712
- 4 Klijn, J. G., Berns, P. M., Schmitz, P. I. and Foekens, J. A. (1992) The clinical significance of epidermal growth factor receptor (EGF-R) in human breast cancer: a review on 5232 patients. *Endocr. Rev.* **13**, 3-17
- 5 Mendelsohn, J. and Baselga, J. (2000) The EGF receptor family as targets for cancer therapy. *Oncogene.* **19**, 6550-6565
- 6 Fry, D. W. (2003) Mechanism of action of erbB tyrosine kinase inhibitors. *Exp. Cell Res.* **284**, 131-139
- 7 Ranson, M. and Sliwkowski, M. X. (2002) Perspectives on anti-HER monoclonal antibodies. *Oncology* **63**, 17-24
- 8 Mendelsohn, J. and Baselga, J. (2003) Status of epidermal growth factor receptor antagonists in the biology and treatment of cancer. *J. Clin. Oncol.* **21**, 2787-2799
- 9 Arteaga, C. L. and Truica, C. I. (2004) Challenges in the development of anti-epidermal growth factor receptor therapies in breast cancer. *Semin. Oncol.* **31(1 Suppl 3)**, 3-8
- 10 Dancey, J. E. and Freidlin, B. (2003) Targeting epidermal growth factor receptor - are we missing the mark ? *Lancet* **362**, 62-64
- 11 Alvarado, D., Rice, A. H. and Duffy, J. B. (2004) Bipartite inhibition of *Drosophila* epidermal growth factor receptor by the extracellular and transmembrane domains of Kekkon1. *Genetics* **167**, 187-202
- 12 Guichard, A., Srinivasan, S., Zimm, G. and Bier, E. (2002) A screen for dominant mutations applied to components in the *Drosophila* EGF-R pathway. *Proc. Natl. Acad. Sci. U S A* **99**, 3752-3757
- 13 Penton, A., Wodarz, A. and Nusse, R. (2002) A mutational analysis of dishevelled in *Drosophila* defines novel domains in the dishevelled protein as well as novel suppressing alleles of axin. *Genetics* **161**, 747-762
- 14 Freeman, M., Klambt, C., Goodman, C. S. and Rubin, G. M. (1992) The *argos* gene encodes a diffusible factor that regulates cell fate decisions in the *Drosophila* eye. *Cell* **69**, 963-975
- 15 van Tilbeurgh, H., Bezzine, S., Cambillau, C., Verger, R. and Carriere, F. (1999) Colipase: structure and interaction with pancreatic lipase. *Biochem. Biophys. Acta* **1441**, 173-184