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14. ABSTRACT The original Statement of Work called for analysis of proteins and organelles involved in autophagic cell death (Task 1); for analysis of expression profiles and identification of pathways affected by tamoxifen treatment in parental cells and in resistant derivatives (Task 2); and for functional testing of identified differentially regulated genes as potential components of regulatory pathways for autophagy-related cell death (Task 3). It is important to recognize that Tasks 1 and 2 are parallel lines of investigation, so that Task 2 does not depend on completion of Task 1, and reversal of the timing of their execution does not affect execution of the overall project. By its nature Task 1 requires significant commitment of time (18 months projected) and is best performed by the same individual; on the contrary, Task 2 is associated mostly with analysis of data generated by expression profiling. Due to unforeseen delays, task 2 was begun before task 1.					
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Introduction.

In June 2007 we started the project “Lysosome-mediated cell death and autophagy-dependent multidrug resistance in breast cancer.” with the goal of defining signal transduction pathways involved in autophagic/lysosomal type of cell death. The project is based on detection of changes in signaling pathways induced by four different and seemingly unrelated genes. These genes have been initially identified by their protective effects against cell death induced by tamoxifen in MCF7 cells. We have shown that overexpression of these genes protects MCF7 cells against a number of different drugs, so that a nearly identical profile of drug resistance is observed for all four genes. Considering that tamoxifen is a well-established inducer of autophagic/lysosomal cell death in MCF7 cells, our system is well-suited for analysis of molecular events associated with this phenomenon. In addition, autophagic cell death-related events have never been implicated in multidrug resistance before, so our system allows detailed analysis of this new type of resistance designated “autophagy-dependent multidrug resistance” or AD-MDR.

The original Statement of Work called for analysis of proteins and organelles involved in autophagic cell death (Task 1); for analysis of expression profiles and identification of pathways affected by tamoxifen treatment in parental cells and in resistant derivatives (Task 2); and for functional testing of identified differentially regulated genes as potential components of regulatory pathways for autophagy-related cell death (Task 3). It is important to recognize that Tasks 1 and 2 are parallel lines of investigation, so that Task 2 does not depend on completion of Task 1, and reversal of the timing of their execution does not affect execution of the overall project. By its nature Task 1 requires significant commitment of time (18 months projected) and is best performed by the same individual; on the contrary, Task 2 is associated mostly with analysis of data generated by expression profiling. Due to these differences and taking into account unforeseen delays with beginning of the project, pending move to another institution, and unavoidable loss of the postdoctoral associate involved in the project at its beginning, we concentrated on Task 2, reversing the order of execution of Tasks 1 and 2.

Body.

As proposed in the original submission, six cell lines were prepared for expression profiling: parental MCF7, MCF7 transduced with GFP-expressing vector, B6 (clone 9), B6 (clone b21), D10, and E5. Two clones for the B6 insert were chosen to compare potential effects of GFP expression on the expression profiles; B6 (clone 9) does not express GFP, while B6 (clone B 21) does.

These cells were plated in 100 mm dishes 24 hr before the beginning of the experiment. At time 0 media was changed in all plates; control plates (designated “0”) received the same drug-free media, while experimental plates (designated “10”) received media with 10 mkM of 4-hydroxytamoxifen. This design was adopted to exclude potential effects of media change. Twenty four hours later cells were lyzed on the plate with RNazol, and total RNA was isolated using standard techniques. As the result, six sample sets were obtained, each with untreated and treated sample pairs.

The samples were provided to the microarray facility for hybridization to Illumina bead microarrays (over 46,000 features). To monitor processing-related variability, three repeats for each pair were analyzed; hence, 36 samples were processed (6 cell lines x 2 conditions x 3 repeats). This design allows multiple types of analysis; e.g. identification of changes due to expression of GFP (pairs MCF7 vs MCF7-GFP and B6-9 vs B6-b21), identification of gene-specific changes in expression (B6 vs D10 vs E5), identification of 4-hydroxytamoxifen-related changes (pairs of untreated vs treated cells), etc. For this project, however, common changes in resistant clones have to be compared against common changes in MCF7 and MCF7-GFP following treatment with 4-hydroxytamoxifen.

Initially, expression profiles were compared for triplicates to confirm good correlation of results. At this stage distribution of signals for one of the triplicates for E5 was dramatically different from the rest of the group apparently reflecting some variability in microarray processing. This sample was removed from further consideration.

Next, coefficient of variability for each feature was determined, and raw expression data were modified to reflect up-regulation (+1), down-regulation (-1), or no change (0) of expression. This step allowed identification of genes that were similarly affected in both controls (MCF7 and MCF7-GFP) on one hand, and in all resistant clones (B6-9, B6-b21, D10, and E5), on the other. Six categories of genes with differential regulation were possible (Table 1):

Table 1. Groups of differentially expressed genes

Group	Parental	Progeny	RESULTS
1	0	(+1)	37
2	0	(-1)	23
3	(+1)	0	8
4	(-1)	0	31
5	(+1)	(-1)	NONE
6	(-1)	(+1)	NONE

Importantly, no genes were recovered in two groups with the most extreme differences of expression (groups 5 and 6); this observation correlated well with the observed resistance to tested drugs, which was considerable but not extreme. It was also significant that the two largest gene groups contained genes that were either activated in resistant clones in response to tamoxifen (group 1; 37 genes) or have not been repressed by the drug (group 4; 31 genes). This observation correlated well with increased metabolic activity observed in resistant clones after treatment with tamoxifen.

Considerable problems were encountered when differentially expressed genes were subjected to analysis by Bioconductor, as was planned in the original proposal. The most useful information obtained from the analysis reflected assignment of genes to different GO processes but did not indicate potential pathways differentially affected by tamoxifen in parental cells and in resistant clones. An alternative approach was required.

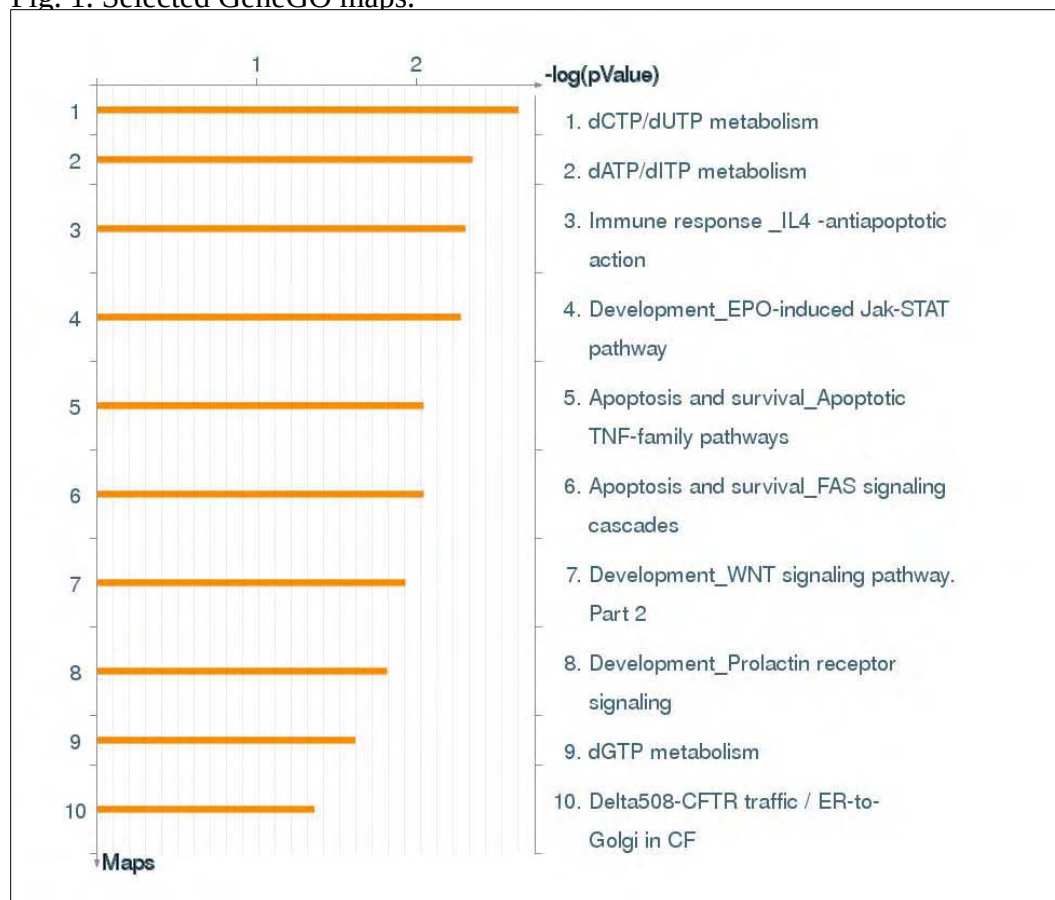
Three alternatives were identified; all three were pursued in order to avoid undue reliance on any single one as it happened with Bioconductor. Moreover, potential redundancy of the analysis would produce the most probable pathways involved in resistance; these pathways would be

recognized by more than one approach and would then be the first to be examined. The first approach was provided by Pathway Workflow analysis of GeneGO Company; the second – by PathwayAssist of Ariadna Genomics, and the third – by Ingenuity Pathways Analysis of Ingenuity Systems.

GeneGO analysis. Differentially regulated genes (table 1) were analyzed by the Pathway Workflow analysis, which included *Enrichment analysis* and construction of *GeneGo maps*. Enrichment analysis consists of matching gene IDs for the common, similar and unique sets of the uploaded files with gene IDs in functional ontologies in MetaCore. The ontologies include canonical pathway maps, GeneGo cellular processes, GO cellular processes and diseases categories. The degree of "relevance" to different categories for the uploaded datasets is defined by p-values, so that the lower p-value gets higher priority. Canonical pathway maps represent a set of about 500 signaling and metabolic maps covering human biology in a comprehensive way. All maps are drawn from scratch by GeneGo annotators and manually curated. Experimental data is visualized on the maps as blue (for downregulation) and red (upregulation) histograms. The height of the histogram corresponds to the relative expression value for a particular gene/protein.

As the result, the following maps were selected:

Fig. 1. Selected GeneGO maps.



Involvement of dNTP metabolic pathways (3 out of 10 selected) is surprising; potential explanation may be linked to changes in mitochondrial metabolism and production of energy; indeed, maps of these processes contain almost exclusively mitochondrial enzymes. Interestingly, the most upregulated transcript in these pathways is POLG, which encodes the catalytic subunit of mitochondrial DNA polymerase gamma.

Activation of antiapoptotic pathways (4 out of 10 selected) suggests that cell death mechanisms are indeed involved, and indirectly confirms selection of genes for analysis and analysis itself. Interestingly, the most downregulated gene for these set of maps is Bcl2; considering that this gene links mitochondria and cell death, the data suggests that Bcl2 may be involved in autophagic cell death and AD-MDR.

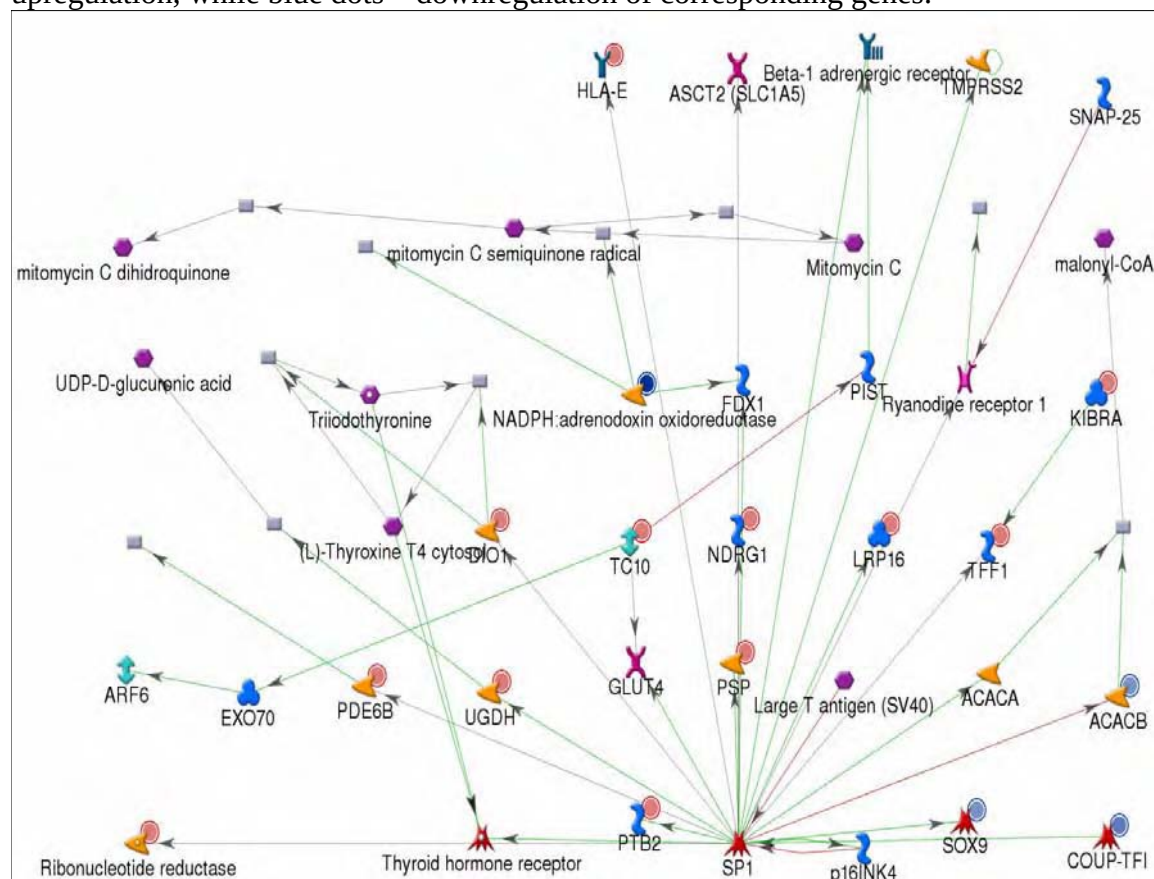
GeneGo analysis also identified the most relevant processes that include selected genes (Fig. 2). The gene content of the uploaded files was used as the input list for generation of biological networks using Analyze Networks algorithm with default settings. This was a variant of the shortest paths algorithm with main parameters of (1) relative enrichment with the uploaded data, and (2) relative saturation of networks with canonical pathways. In this workflow the networks were prioritized based on the number of fragments of canonical pathways on the network.

Fig. 2 Processes potentially involved in AD-MDR.

No	Processes	Size	Target	Pathways	p-Value	zScore
1	deoxyribonucleotide metabolic process (12.0%), deoxyribonucleotide biosynthetic process (8.0%), cartilage condensation (8.0%)	50	16	0	2.67e-35	62.66
2	regulation of biological quality (38.9%), wound healing (16.7%), blood coagulation (13.9%)	50	13	0	6.90e-26	45.32
3	cell cycle (40.0%), cell cycle process (37.5%), mitotic cell cycle (25.0%)	50	11	5	3.72e-21	38.31
4	cell differentiation (68.3%), cellular developmental process (68.3%), regulation of biological process (85.4%)	50	12	0	9.67e-24	42.71
5	apoptosis (41.0%), programmed cell death (41.0%), signal transduction (69.2%)	50	10	2	7.16e-19	34.80

An example of the highly scored network is presented in Fig. 3 (next page). Thick cyan lines indicate the fragments of canonical pathways. Up-regulated genes are marked with red circles; down-regulated with blue circles. The 'checkerboard' color indicates mixed expression for the gene between files or between multiple tags for the same gene. The value of this analysis is difficult to assess at this point, but the adaptability of the system is encouraging: it may identify pathways that are completely novel and are not included into consensus maps of canonical pathways. It is also significant that the analysis identified concordant regulation of multiple factors with established links (Fig. 3); this result opens the possibility of hypothesis generation. For example, upregulation of NDRG1 in hypoxia [1] may be tested in both parental cells and in resistant clones and may involve differential regulation by MYC and PTEN [2, 3] while downregulation of NDRG1 by estradiol is in good agreement with expected effects [4].

Fig. 3. An example of a highly scored network developed *de novo*. Red dots indicate upregulation, while blue dots – downregulation of corresponding genes.



Finally, the GeneGo analysis has identified nodal genes that describe the most important networks. It is illuminating that a number of components of stress response pathway (JAK, JNK1, STAT5, RelA, etc) are recognized as nodal genes alongside with nuclear transcription factors (Sp1, ESR1, RARA, p53, E2F1, etc). It is also encouraging that a significant number of genes identified by GeneGo analysis is located in membranes (Integrin, KLRK1, VLDLR, LRP6, LPL, etc) and at least some of them are involved in endocytosis (VLDLR, LRP6, APP, LPL) or regulation of lysosomes (SNAPIN). Membranes are an essential component of autophagic cell death, while endocytosis and especially lysosomal regulation are directly related to autophagocytosis. While analysis of the data is still incomplete, it appears that pathways identified by GeneGo already produced several potential leads that can be experimentally tested.

Importantly, GenGo analysis is only the first of three approaches that is employed to analyze expression data. It is obvious at this point that the approach proposed in the initial project (Bioconductor analysis) was inadequate for the task at hand; one of the reasons was an overly optimistic projection provided by the bioinformatician. However, the first step of alternative data analysis has already produced important results, suggesting that the goal of pathway analysis will be successfully completed.

Fig. 4 List of most relevant network objects based on network analysis.

Unique gene list in 5 ntwrks	Hubs				Transcription factors		Receptors		Secreted proteins	
	SP1	SP1	SP1	SP1	SOX9	COUP-TFI	HLA-E	Amyloid beta42	APOE	Thrombospondin 1
	ESR1 (nuclear)	ESR1 (nuclear)	ESR1 (nuclear)	ESR1 (nuclear)	SP1	SP1	Amyloid beta42	LRP6	NGF	PAI1
	p53	p53	Neuroserpin	Neuroserpin	Thyroid hormone receptor	ESR1 (nuclear)	PAR2	alpha-IIb/beta-3 integrin	Thrombospondin 1	VEGF-A
	SP1	ESR1 (nuclear)	Snapin	Snapin	ELF3	GATA-3	alpha-IIb/beta-3 integrin	epsilon RI	Thrombospondin 1	APOE
	SP1	p21	RARalpha	RARalpha	ESR1 (nuclear)	RARalpha	APP	KLRK1 (NKG2D)	PAI1	MICB
		PLC-gamma 1	Thrombin	Thrombin	GATA-3	RBB2	LRP6	MAFA-L	VEGF-A	
			p53	p53	NK31	AML1 (RUNX1)	KLRK1 (NKG2D)	VLDLR	WNT4	
			CDK1 (p34)	CDK1 (p34)	RARalpha	p53	VLDLR		APOE	
			HSC70	HSC70	AML1 (RUNX1)	AML1 (RUNX1)			MICB	
			CHIP	CHIP	NHLH2	Androgen receptor				
			ESR1 (nuclear)	ESR1 (nuclear)	p53	COUP-TFI				
			E2F1	E2F1	AML1 (RUNX1)	E2A				
			SP1	SP1	Androgen	E2F1				
			p53	p53	COUP-TFI	ESR1 (nuclear)				
			GSK3 beta	GSK3 beta	E2A	p53				
			p21	p21	E2F1	PPAR-gamma				
			JNK1(MAPK8)	JNK1(MAPK8)	ESR1 (nuclear)	RARalpha				
			Androgen receptor	Androgen receptor	p53	RelA (p65 NF-kB subunit)				
			Erk (MAPK1/3)	Erk (MAPK1/3)	PPAR-gamma	SOX9				
			Bcl-2	Bcl-2	RARalpha	SP1				
			EGFR	EGFR	(p65 NF-kB subunit)	RARalpha				
			RelA (p65 NF-kB subunit)	RelA (p65 NF-kB subunit)	SOX9	SP1				
			CDK1 (p34)	CDK1 (p34)	SP1	STAT5				
			SP1	SP1	RARalpha	ZIC2				
			PtdIns(3,4,5)P3	PtdIns(3,4,5)P3	SP1					
			Syk	Syk	STAT5					
			PLC-gamma 1	JAK2						
			JAK2	LPL						
			LPL	Polycystin						
			Polycystin	STAT5						
			STAT5	VLDLR						
			VLDLR	Annexin V						
			Annexin V	Heparin						
			Heparin							

Ariadna Genomics analysis has been completed, and the data are under study. Even cursory examination of top 20 components affected in resistant cells as compared to parental MCF7 indicates that processes related to nucleotide metabolism, cell biogenesis, cell death, and intracellular organelles are among the most affected (sorted by the number of Entities, Table 3).

Table 3. Twenty most profoundly affected biological processes selected after comparison between resistant derivatives and parental cells.

Name	
biological_process	
cellular_process	
cellular_component	
cellular physiological process	
cell	
cellular metabolism	
intracellular	
cell communication	
nucleobase, nucleoside, nu	
and nucleic acid metabolis	
physiological process	
cell organization and bioger	
signal transduction	
development	
membrane	
cell death	
cytoplasm	
cell differentiation	
intracellular organelle	
intracellular membrane-bou	
nucleus	

Table 4. Pathways and processes affected in tamoxifen-resistant derivatives.

activation of jnk
EPHA4 -> STAT3 signaling pathway
mitotic spindle checkpoint
PTEN signaling
FAS signaling
induction of apoptosis by extracellular signals
filopodium
homophilic cell adhesion
integrin alpha5beta1
integrin alpha6beta1
integrin alpha8beta1
integrin alpha9beta1
integrin alphaVbeta3
late endosome
post-golgi vesicle-mediated transport
rough endoplasmic reticulum membrane
sarcolemma
signal recognition particle (sensu eukaryota)
vesicle budding
vesicle coating

When individual processes are compared between parental cells and resistant derivatives several pathways appear to be selectively affected in resistant cells. In particular, GPCR q/11 (GNA11) and multiple components of the RhoA pathway are intersecting with Toll-like receptor (TLR) – AP1 and EPHA4-STAT pathways, PTEN signaling, integrin-mediated signaling, and vesicle budding/coating. Some of the pathways affected in all derivatives are listed in Table 4. It is encouraging that activation of STAT pathway is independently selected by two different approaches (GeneGO and Ariadna), which used different strategies for analysis. It is also remarkable that

multiple integrins have been identified as critical players by both systems, suggesting that integrins are associated with critical changes that accompany AD-MDR. Another significant finding is involvement of endosomal/vesicle formation and transport, including post-Golgi transport, also detected by GeneGO and Ariadna. These findings correlate well with the hypothetical involvement of vesicle formation and transport in autophagic cell death.

A significant amount of work remains to be done to test the model; however, the outlines of novel signal transduction pathways that regulate AD-MDR begin to appear.

A new postdoctoral associate has joined the project (see CV in Appendix). Dr. Shah has significant experience in sophisticated molecular techniques, is well-versed in cell biology, and has both practical and theoretical knowledge of steroid receptor biology. There is little doubt that he will be able to successfully complete the project.

Key Research Accomplishments.

Expression profiling of 36 samples has been completed.
Statistical analysis has been finished and informative genes have been identified.
Pathway modeling by GO processes has been accomplished.
Analysis of pathways by Ariadna Genomics algorithm has been done.
Agreement with Ingenuity for pathway analysis has been reached.

Reportable Outcomes.

A number of pathways potentially involved in AD-MDR has been identified.

Conclusion.

Difficulties encountered with the project due to the late start, transfer to a new institution, and loss of the postdoc involved have been resolved. Task 2 is proceeding successfully, and a testable hypothesis is being developed within the expected timeframe. A new postdoc (Dr. Chirag Shah, see CV attached) has started on the project.

Initial assumptions regarding analysis of expression profiles have been proven incorrect; alternative analysis has been designed, tested, and implemented. While analysis of the data is still in progress, the outline of new transduction pathways that regulate a major unknown cell death process becomes visible.

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Appendix.

CV of Dr. Chirag Shah..

DR. CHIRAG ASHWINKUMAR SHAH

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Chicago, IL 60612
USA
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Email: chishah76@yahoo.com

Education:

Ph. D. Mumbai - 2006 Biochemistry (**Science**) (Reproductive Biology)

M. Sc. MUMBAI 2000 Biochemistry

B. Sc. MUMBAI 1997 Microbiology

Awards/Honors:

Recipient of **Senior Research Fellowship** from Council for Scientific and Industrial Research (CSIR, New Delhi) from April 2003.

Recipient of the **Best Performance Award (Student category) for the year 2000-2001** at the Institute for Research in Reproduction, Parel, Mumbai.

Recipient of **Junior Research Fellowship** from Lady Tata Memorial Trust from August 2001.

Recipient of the **Best Poster Award** for the paper "Identification, Localization and Characterization of Progesterone Binding Sites on Human Spermatozoa" at 17th National Conference of Society for Reproductive Biology and Comparative Endocrinology, Surat, India Jan 24 – 26, 1999.

Second prize winner for the Science Project Competition at Royal College, Mumbai, 1997.

Membership:

Life member of the Indian Society for the Study in Reproduction and Fertility

Extra Curricular Activities:

Diploma in Junior Computer Management from TIIT System Institute.

Expert in Windows 2000 operating System.

Expert in MS Office, PageMaker, Internet Explorer, Netscape etc.

Research Experience:

Involved in following projects at the institute

- *Characterization of Progesterone Receptors and Binding Sites in Human Testis and Spermatozoa (for M.Sc. and Ph.D. degree)*
- *Exploring the Roles of SRY Gene in Human Testis and Spermatozoa*

Research Interest:

- Cancer biology
- Reproductive biology
- Proteomics
- Stem cell biology

Techniques:

- **Molecular Biology**

- RT-PCR
- PCR
- Cloning & Transformation
- Plasmid isolation
- Non radioactive *in situ* hybridization
- Radioactive and non-radioactive Northern blot
- Southern blot
- cDNA library construction and screening
- Basic *in silico* analysis (BLAST, FASTA, MASCOT, ExPASy)

- **Biochemistry**

- 2-Dimensional electrophoresis
- Tryptic digestion and MALDI-tof
- SDS-PAGE
- Western Blot
- Ligand Blot
- Immunoprecipitation
- *In-vitro* kinase assays
- Radioimmunoassays & Radio receptor assays
- Gel chromatography

Cell Biology

- Stem cell culturing and differentiating
- X gal staining
- Confocal Fluorescence microscopy
- Flow cytometry
- Immunohistochemistry/Cytochemistry/Immunofluorescence
- Electron microscopy including tissue processing & block preparations
- Ultrastructural immunogold localization
- Cryosectioning

Publications:

1. Lee T, **Shah CA** and Xu EY Gene Trap Mutagenesis: A Functional Genomics Approach towards Reproductive Research ***Molecular Human Reproduction***, 11: 771-779 (2007)
2. **Shah CA**, Sachdeva G and Puri CP. (2007) Role of progesterone and its putative receptor in spermatozoa. ***Embryo Talk***
3. Modi D, **Shah CA**, and Puri CP (2007) Non Genomic membrane progesterone receptors on human spermatozoa. In Proceedings of the International Congress on Gamete Biology: Emerging Frontiers on Fertility and Contraceptive Development eds. Gupta SK, Koyama K and Murray J Society of Reproduction and Fertility Supplement 63 Nottingham University Press pp 515-530

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IN PREPARATION:

- 1) Localization of highly conserved stem cell factor-Pumilio in mouse spermatozoa. **Chirag Shah** and Eugene Y. Xu
- 2) Functional conservation of a reproductive regulator from fly to mouse **Chirag Shah**, Villian Naeem, Yanmei Chen, Yin Wang, Terrance Lee, Mike VanGompel, Renee Reijo Pera and Eugene Yujun Xu

Conferences Attended:

1. Presented a poster "Intracellular and membrane bound receptors for progesterone in human testes and spermatozoa", at International Symposium on advances and challenges in reproductive health research in the post genomic era, held at **Mumbai, India** from January 9-12, 2005
2. Presented a paper "Progesterone Receptor in Human Testis and Spermatozoa: Molecular and Cellular Characterization". At Workshop in Reproduction, held at **Caen, France** from October 19-21, 2004. (Oral presentation)

3. Presented a poster "Spatio-temporal expression of progesterone receptors in human spermatozoa". Rapid Response to Steroid Hormones, **Florence, Italy** from September 12-14, 2003.
4. Presented a poster "Spatio-temporal expression of progesterone receptors in human testis". Fourteenth Annual Meeting of Indian Society for the Study of Reproduction and Fertility, Department of MRDG, Indian Institute of Science, **Bangalore, India** from January 23-25, 2004.
5. Presented a poster "Molecular Characterization of Progesterone Receptor on human spermatozoa" at National Conference on Recent Advances in Reproductive Health', held at Department of Zoology, University of Rajasthan, **Jaipur, India** from February 6-8, 2003
6. Presented a poster x"Studies on Functional Significance of Progesterone Receptor on Human Spermatozoa" at International Congress on Fertilization, Embryo Development and Implantation, held at National Institute of Immunology, **New Delhi, India** from November 6-9, 2000.
7. Presented a poster "Molecular Characterization of Progesterone Receptor on Human Spermatozoa" at 10th Annual Meeting of 'Indian Society for the Study of Reproduction and Fertility' held at Rajiv Gandhi Centre for Biotechnology, **Thiruvananthapuram, India**, from September 8-10, 1999.
8. Presented a poster "Identification, Localization and Characterization of Progesterone Binding Sites on Human Spermatozoa", at 17th National Conference of Society for Reproductive Biology and Comparative Endocrinology held at **Surat, India** from January 24-26, 1999. (***Received best poster award***)