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13. SUPPLEMENTARY NOTES

14. ABSTRACT: The transcriptional programs cooperatively required for the malignant progression of breasttumors are largely unknown. In this project we propose the isolation of ArtificialTranscription Factors (TFs) for the discovery of gene panels, which cooperate during thegeneration of metastatic behavior. An ATF is made by linkage of a DNA-binding domain (DBD)with a transcriptional effector domain, which mediates activation or repression of endogenousgenes. ATFs are typically made of arrays of Cys2-His2 zinc finger (ZF) domains. The objectiveof this proposal is to apply large ATF libraries to identify and regulate genes thatcooperate during the process induction of breast cancer cell invasion and progression. Wehave delivered ATF libraries into non-invasive breast cancer cell lines. We have selectedATFs able to induce or enhance breast cell invasion. The ATF-selections were performed invitro using matrigel invasion assays (Boyden chambers). In the future, ATFs modulating cellinvasion will be profiled using DNA microarrays to determine genes differentially regulatedby the ATF that are responsible for the phenotype change.

15. SUBJECT TERMS
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INTRODUCTION

Metastatic spread, and not primary tumor burden, is the leading cause of breast cancer deaths. The development of metastatic behavior during breast cancer progression is a dynamic process thought to require the concerted action of multiple genes (1). The transcriptional programs cooperatively required for the malignant progression of breast tumors are largely unknown. In addition, novel therapeutic strategies should be able to target multiple targets dysregulated during disease progression. In this project we propose the isolation of Artificial Transcription Factors (TFs) for the discovery of gene panels, which cooperate during the generation of metastatic behavior. An ATF is made by linkage of a DNA-binding domain (DBD) with a transcriptional effector domain, which mediates activation or repression of endogenous genes (2-3). ATFs are typically made of arrays of Cys2-His2 zinc finger (ZF) domains (4-6). Importantly, ATFs these ZF domains can be linked to both activator and repressors of transcription, facilitating both up- and down-regulation of tumor cell phenotypes (2-3). We have developed a novel genome-wide approach for the functional identification and regulation of genes involved tumor progression. We have generated libraries of ATFs by recombination of large repertoires of sequence-specific zinc finger (ZF) domains (7-10). ZF domains were linked to an activator (VP64) or repressor (SKD) of transcription (2-3). When delivered into tumor cell populations, ATF libraries have the potential to activate or repress virtually any gene. The objective of this proposal is to apply ATF libraries to identify and regulate genes that cooperate during the process induction of breast cancer cell invasion and progression. We have delivered ATF libraries into non-invasive breast cancer cell lines. We have selected ATFs able to induce or enhance breast cell invasion. The ATF-selections were performed in vitro using matrigel invasion assays (Boyden chambers, Figure 1). In the future, ATFs modulating cell invasion will be profiled using DNA microarrays to determine genes differentially regulated by the ATF that are responsible for the phenotype change. The regulation of these markers will be validated by real time PCR and other analyses. Finally, we will compare the transcriptional profiles of ATF-expressing cells with available microarray profiles of highly metastatic breast tumors. We expect this proposal will lead to the functional identification of novel markers of breast cancer disease progression that could be used as early predictors of malignant behavior. In the future, these ATFs could be used as master genetic switches to modulate malignant behavior in *in vivo* models of breast cancer.

BODY

In this section we will describe the main results and conclusions for the tasks outlined in the statement of work for year 1 of this proposal.

List of Tasks and expected outcomes:

Task 1. To *isolate artificial transcription factors (ATF) from combinatorial libraries capable of regulating in vitro cell invasion in breast cell lines* (months 1-12)

a) *Select ATFs able to re-program non-invasive (hMEC-hTERT, MCF10A and MCF7) breast cell lines towards highly invasive cell lines (months 1-10)*

a1. First round of selection: -Infection of host cells by ATF libraries (2 weeks)
-Matrigel selection and propagation of selected clones, Genomic DNA extraction of selected pools, PCR of retrovirally integrated DNA, Sfi-I digest of PCRs and re-cloning in the pMX-IRES-GFP vector, library amplification (3 weeks)

a2. Second round of selection: As described in A1: 5 weeks.
a3. Third round of selection: As described in A1: 5 weeks.
a4. Fourth round of selection: As described in A1: 5 weeks.
a5. If necessary, fifth round of invasion selection: 5 weeks.

Final product expected after month number 6: At the end of the first 6 months we expected to obtain the **enriched pools** of ATFs inducing cell invasion in some of the non-invasive breast cell backgrounds.

a6. Establish single clone retroviral DNAs (single ATF monoclonal populations) from enriched pools in the linear phase of the selection (we will pick one of the enriched rounds described above), 10 clones per selection pool. We will determine the ATF sequence by plasmid DNA sequencing. (2 weeks)
a7. Infect host cells with selected ATFs, perform matrigel invasion assays to determine single ATF clone activity, which will be expressed as fold enhancement of cell invasion. (months 8-10).

Final product after month number 10: We expect to have isolated and sequenced **individual ATFs** (single clones) able to enhance cell invasion in non-invasive breast cells. We will also obtain invasion activity measurements of each of these clones.

b) *Study the capability of these selected ATF, linked to other transcriptional domains (i.e. repressor domains instead of activator domains) to down-regulate cell invasion in highly invasive cell lines MDA-MB231, MDA-MB435 and MDA-MB-361 (months 10-12,* tasks as described in a7). In these experiments, DNA binding domains (made of ZF domains) will be linked to a different effector domain. If the initial selection was done with an activator, then we will exchange the activator for the repressor. We expect that the exchange of the effector function will down-regulate invasion by repression of genes necessary for invasion.

Final product after month number 12: We expect to have enriched ATF pools and single ATF clones able to modulate cell invasion: to induce cell invasion in some of the non-invasive breast backgrounds and to down-regulate cell invasion in some of the highly invasive breast backgrounds. We will also obtain invasion activity measurements of each of these clones.

Obtained Results and Discussion:

Task 1a1-a5. In order to select for ATFs inducing invasive behavior we first transduced mammary cell lines with 3ZF retroviral libraries linked to either the VP64 activator domain or the SKD repressor domain. The retroviral vectors used for the transductions were the pMX-IRES-3ZF-library-VP64 and pMX-IRES-3ZF-library-SKD. The GFP marker expressed in the retroviral vector allowed the tracking of the ATF-transduced cells by flow cytometry. We focused our analysis in the MCF-7 cell line since upon retroviral transduction of the MCF-10A and hMEC cell lines these cells underwent morphological differentiation. This could be due to the media conditions used to grow the retroviral producing cells (293T gagpol cells), which contain fetal bovine serum (FBS). MCF-7 cells grow in presence of FBS and thus, these cells were not affected by the media used to harvest the retrovirus. These cells were subsequently used for the selection experiments described in **Figure 1**. We have recently generated in the lab a MCF-7 cell line expressing a luciferase marker (MCF-7-luc) which will allow us to follow invasive behavior in real time in a mouse model using Bioluminiscence Imaging, BLI (year 3, task 3 of our statement of work)

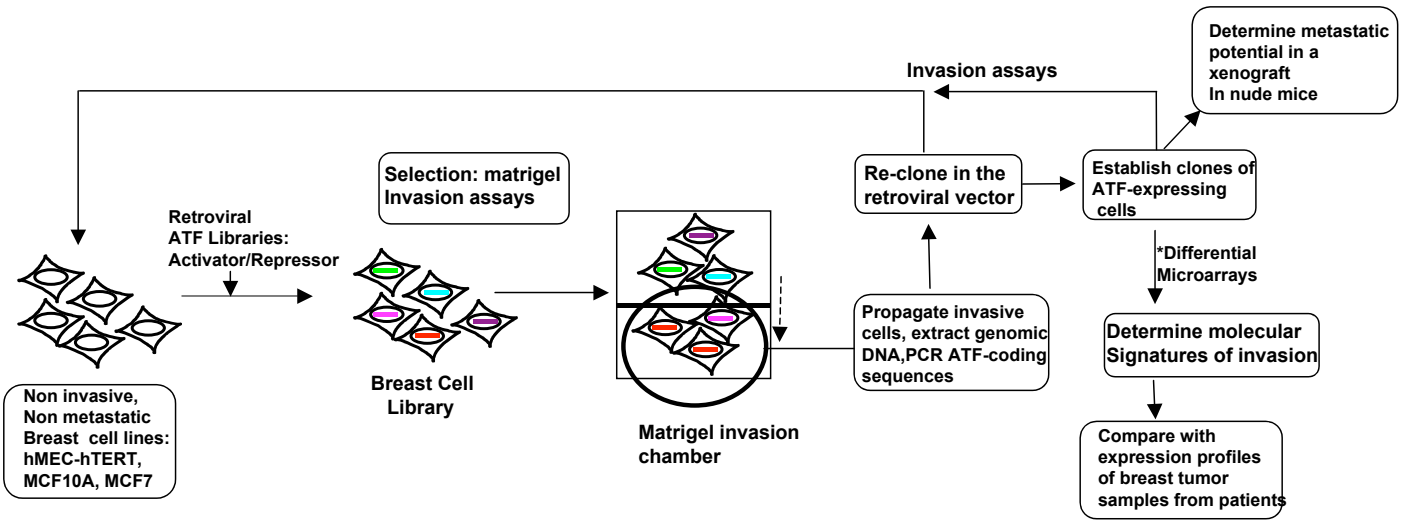
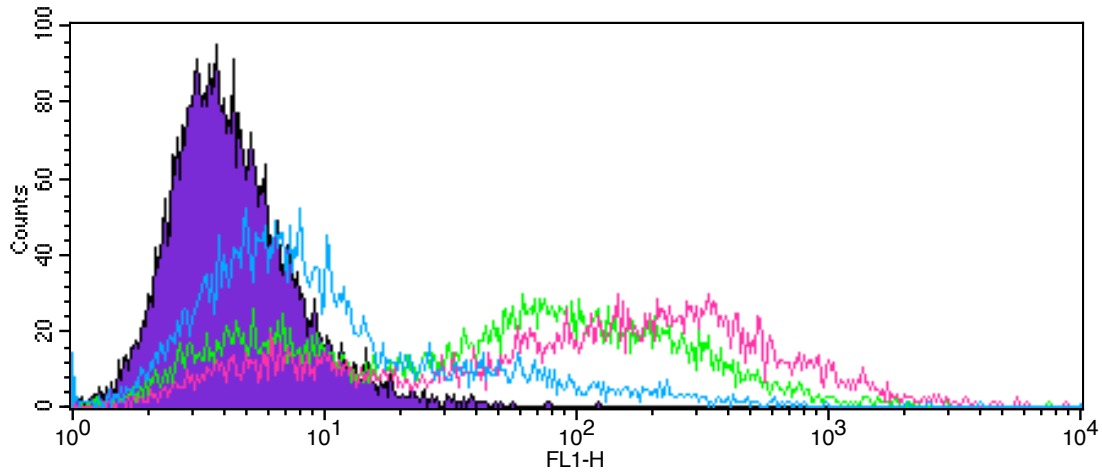


Figure. 1. Strategy to select ATFs able to regulate *in vitro* breast cancer cell invasion using matrigel-coated transwells. Cells are transduced with ATF libraries linked to activator or repressor domains. Transduced cells are genetically different and comprise a breast cell library. In the library the ATFs have the capability to up- or down-regulate any gene in the human genome. Thus, the transduced cells display phenotypic plasticity. In order to select for ATFs up-regulating cell invasion we used boyden chambers. Transduced cells are loaded in top compartment of the transwell in serum-free media and stimulated to invade by adding 0.1-2% serum in the bottom well. Non-invading cells will be removed from the upper well. Cells able to pass through the matrigel matrix are recovered and propagated. ATFs are re-cloned in the retroviral vector for next rounds of selections, as described above. Individual ATF expressing cells were isolated for invasion assays, gene expression analysis (year 2) and mouse-based studies (year 3).



(GFP)

Figure 2. Assessment of the efficiency of retroviral transduction of MCF-7 cells with ZF Libraries using flow cytometry for the GFP marker. Purple = MCF-7 cell line, Green = MCF-7 transduced with the 3ZF repressor library, Pink= MCF-7 transduced with the 3ZF activator library, Blue = MCF-7 cells transduced with empty vector expressing GFP

As shown in **Figure 2**, MCF-7 cells were efficiently transduced using our 3ZF activator and repressor libraries, with transduction efficiencies from 70-85%. These cells were next used for the first round of selection, as described in the research proposal. MCF-7 cells and control cells transduced with empty retroviral vector gave no-background in the assay, and an average of 50-100 cells from the library-transduced cells were collected from the bottom well of the transwell. Due to the low yield, we seeded 1-5 millions cells on the top well. Since the library size has 10,000 clones, each individual library member was represented 100-500 times. In these experiments we recovered approximately 0.01% of cells in the first round of selection. Cells from the first round of selection were maintained in a 6-well plate in serum-containing media for about 2-weeks, until the pate reached 65% of confluence. We then extracted genomic DNA using the Qiagen Kit and this DNA was used for the PCR reaction to isolate the ZF inserts using previously published conditions (7-9).

As shown in **Figure 3**, the PCR amplification of cells selected from matrigel invasion assays were successful and single bands were obtained. These bands were digested using the enzyme Sfi-1 and re-cloned in the initial retroviral vector. A total of 5 rounds of selections were performed as described in Tasks 1a1-a5. Upon 3 rounds of selection the yield was increased to 0.1-0.2% and this yield never increased upon round 4 and 5 indicating that clones were already amplified and the selections were saturated after round number 3.

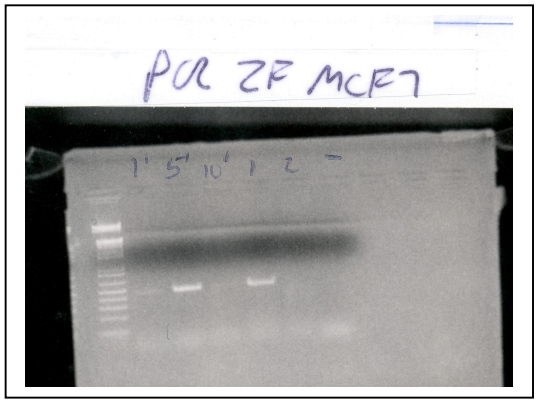


Figure 3. PCR-amplification of ZF-inserts from genomic DNA obtained from MCF-7 cells transduced with the 3ZF-VP64-library that underwent 1 selection round by matrigel invasion assays. Different PCR conditions were tested and optimized in the assay. Single PCR bands were digested and subcloned in the pMX-IRES-GFP-VP64 retroviral vector for additional rounds of selection.

Figure 4 shows the final product of task a5, which is a quantification of cell invasion from amplified pools (after 3 rounds of selection). As seen in this figure, the amplified VP64 pools gave a statistically significant enhancement in cell invasion compared to the MCF-7 cell line transduced with empty retroviral vector (control). However, we didn't observe a significant enhancement with the repressor library and consequently we focused our analysis in investigating single ATF clones from the activator pools. It could be several reasons why the repressor library didn't work in these assays. Perhaps enhancing invasion might be easier using gain-of-function, meaning direct activation of "pro-invasive" genes, which are found silenced or poorly expressed in a non-invasive cell line such as MCF-7. Another possibility could be that ATF-mediated repression of targets might not be efficient 100% to ensure a phenotype in a boyden chamber. Perhaps novel and more sensitive methods would have picked-up ATF repressors able to induce invasion. Boyden chambers have the limitation that the efficiency of invasion is not 100%. We observe that even for highly invasive breast cancer cell lines such as MDA-MB-231 only a fraction of cells are able to pass through the matrigel. Perhaps we have "missed" ATF-repressors due to the intrinsic low frequency of the invasive process in boyden chambers.

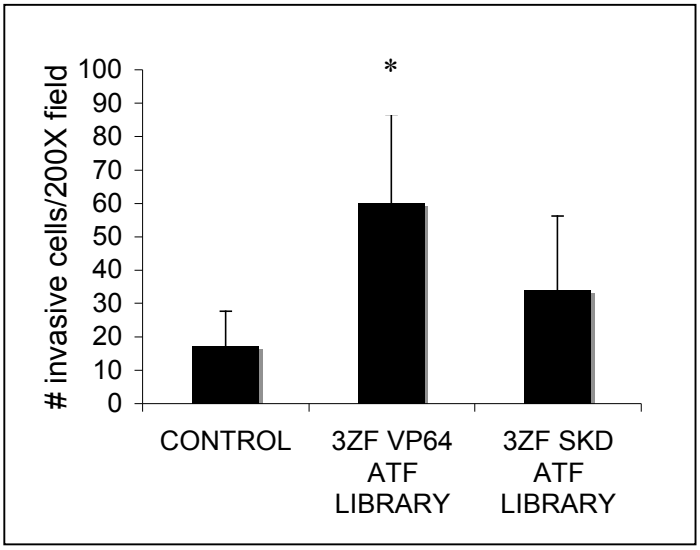


Figure 4. ATFs are able to induce an invasive phenotype in vitro. Matrigel invasion assays for cells that have undergone one round of selection for invasion. Cells that pass through the matrigel are fixed, stained, and counted. *P<0.05 for the 3ZF VP64 ATF library compared to the MCF-7 cell line transduced with empty GFP vector.

Tasks a6-a7

We subsequently took the activator (VP64) and repressor (SKD) pools re-cloned in the retroviral vectors, transformed *E.coli* and we picked single colonies for individual (single-clone) plasmid preparations. These single ATFs were individually transduced into the MCF-7 cell line and tested in 24-well format matrigel invasion assay. This was necessary in order to identify the invasion activity of single ATFs, as in the pools some ATFs could have a cooperative effect. Indeed, due to the process of retroviral transduction is expected that many ATF-expressing retroviruses are integrated in the same cell and these ATFs could cooperate in the phenotype. Thus, for an assessment of the effect of individual ATFs, it is necessary to prepare single ATF clones.

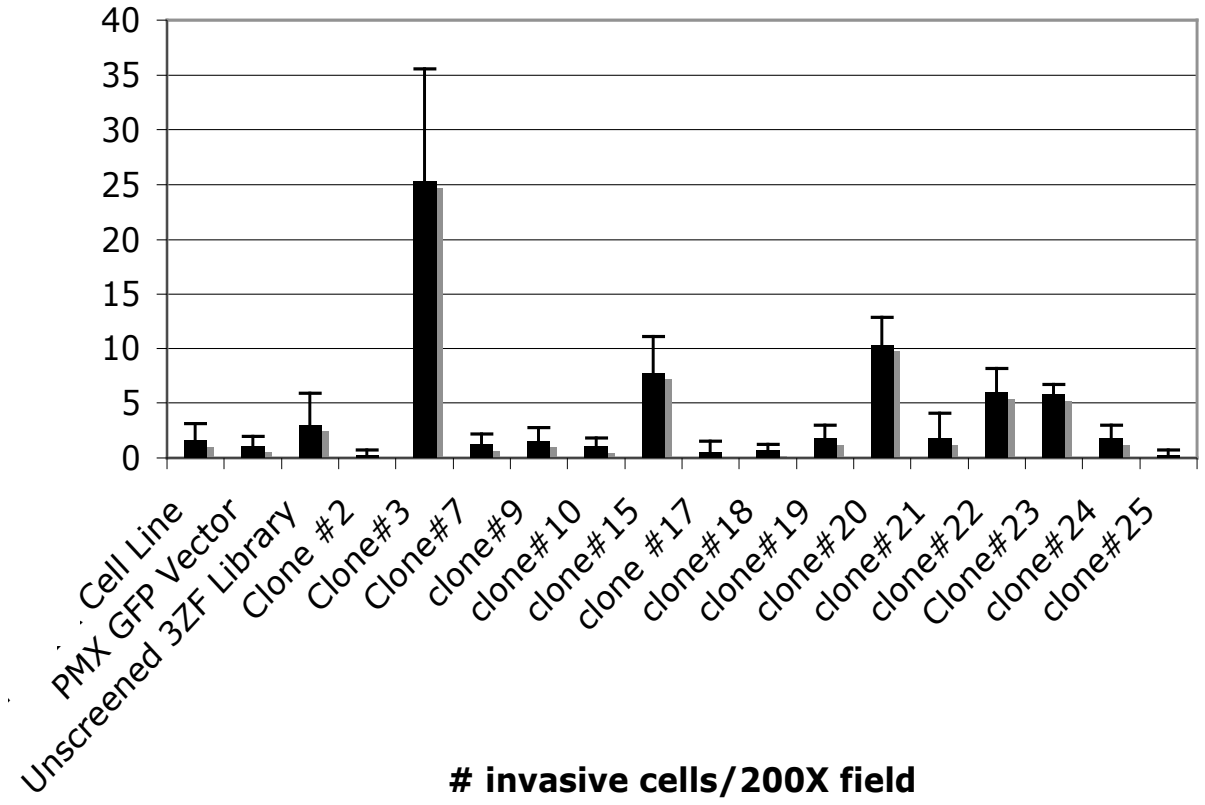


Figure 5. Monoclonal populations of ATFs induce cell invasion in matrigel invasion assays (boyden chambers) in the MCF-7 cell line. Matrigel invasion assays for MCF-7 cells transduced with activator 3ZF clones. Cells that pass through the matrigel are fixed, stained, and counted under an inverted tissue culture microscope. Three wells per clone were tested and we counted four fields per well. In the figure the average of 12 fields and standard deviations are indicated.

We subsequently sequenced the retroviral DNA from clones providing the highest invasion capability (**Figure 6**). We have determined the putative binding sites for the transcription factors based on the information available for the specificity of the ZF α-helices (5-6). As seen in **Figure 6** the clones shown high degree of diversity indicating that they could target different endogenous promoters or perhaps different 9-bp sites within

the same promoter. We are currently transferring the ZF inserts from the pMX-IRES-ZF-VP64 vector to a pMX-IRES-GFP-SKD to assess the capability of the ATFs to repress target genes in the MDA-MB-231 cell line. We will next study if the exchange of the effector function down-regulates cell invasion in this cell line (task 1b) and we should complete this in the next two weeks.

	ZF3	ZF2	ZF1	DNA Binding Site
Clone #2	DPGHLVR	RSDKLVR	RSDVLVR	5'-GGC GGG GTG -3'
Clone #3	QAGHLAS	QAGHLAS	QRANLRA	5'-TGA TGA AAA -3'
Clone #15	QAGHLAS	RRDALNV	QRANLRA	5'-TGA ATG AAA -3'
Clone #20	REDNLHT	RSDTLSN	TSGELVR	5'-TAG AAG GCT -3'
Clone #22	QRANLRA	DPGHLVR	RSDVLVR	5'-AAA GGC GTG -3'
Clone #23	DPGALVR	TSGSLVR	TTGNLTV	5'-GTC GTT AAT -3'
Clone #24	QAGHLAS	QSSSLVR	QSSNLVR	5'-TGA GTA GAA -3'

Figure 6. Predicted DNA binding sites for 3ZF activator clones. Individual 3ZF activator clones that were cloned from the pool of ZFs showed enhanced invasive behavior were sequenced. The ZF DNA binding sites were identified from the known SP1 binding sites repertoire. Aminoacids shown in red, blue and pink represent the ZF α-helical positions –1, +3 and +6, respectively, which contact nucleotide positions 3', middle, and 5', respectively.

KEY RESEARCH ACCOMPLISHMENTS

-Selection of pools of ATF activators able to induce invasive behavior in vitro using the MCF-7 host cell line for the selections

-Isolation of single ATF activators with capability to up-regulate cell invasion in the MCF-7 cell line

REPORTABLE OUTCOMES

ABSTRACTS AND MANUSCRIPTS:

-Abstract and poster from Adriana Beltran at the Pharmacology retreat, University of North Carolina at Chapel Hill- May 2007

-Beltran, A., Liu, Y., Parikh, S., Brenda, T., and Blancafort, P. Interrogating genomes with combinatorial transcription factor libraries: asking zinc finger questions. *Assay Drug Dev Technol*. 2006; 4:317-331

AWARDS

-V-Foundation award for breast cancer research to Pilar Blancafort, May 2005

-SPORE breast cancer award UNC-Chapel Hill

CELL LINES AND CLONES

-Retrovirally-transduced MCF-7 cells able to invade in vitro

-ATF-sequences and clones able to up-regulate cell invasion in the MCF-7 cell line

CONCLUSION

In this report we have shown that we were able to isolate ATFs made of 3ZF domains linked to the VP64 activator domain up-regulating cell invasion in the non-invasive breast cancer cell line MCF-7. Several clones of ATFs were isolated and their sequences were characterized. The most powerful ATFs will be processed by DNA-microarray in year 2 in order to understand which group of target genes are involved in activation of invasive programs.

"So What Section"
The molecular programs and genetic cascades responsible for the development of metastatic behavior remains largely unknown. Thus, there is a need to dissect genes that cooperate for the development of metastasis. The ATFs isolated in our work can provide clues about the target genes that generate metastatic behavior. In addition, the advantage of ATFs is that they can be used in the future as direct regulators of these genes. An ATF can be linked to different effector domains, which can repress or silence gene expression. Thus, we hope that the future modification of these ATFs could interfere with gene expression and function in metastatic cell lines. ATFs made of 3 ZF domains directed against the VEGF gene are presently in clinical trials (ref). Thus, we hope that the result of our work could lead to the future development of anti-metastatic reagents.

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(3) Beltran, A., Liu, Y., Parikh, S., Brenda, T., and Blancafort, P. Interrogating genomes with combinatorial transcription factor libraries: asking zinc finger questions. *Assay Drug Dev Technol*. 2006; 4:317-331

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BIOGRAPHICAL SKETCH

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NAME	POSITION TITLE
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eRA COMMONS USER NAME PILAR_BLANCAFORT	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Universitat de Barcelona	B.Sc.	1988-1993	Molecular Biology
Université de Montréal	Ph.D.	1995-1999	Biochemistry
The Scripps Research Institute	Res. Assoc	1999-2003	Biochemistry
The Scripps Research Institute	Senior Res. Assoc.	2003-2004	Biochemistry
The University of North Carolina at Chapel Hill	Ass. Prof.	2005-present	Pharmacology

A. Positions and Honors

Positions and Employment

1988-1993 -B.Sc., Molecular Biology and Biochemistry, Universitat de Barcelona (Spain)
Personal Project and last year of B.Sc (1992-1993)
Universite Libre de Bruxelles (Belgium) with Dr. Alain Ghysen
1994-1995 - M.Sc. Program, Biochemistry, Université de Montréal (Canada)
1995-1999 - Ph.D. Program, Biochemistry, Université de Montréal with Robert Cedergren
1999-2003 - Research Associate in Dr. Carlos Barbas, III laboratory, Department of Molecular Biology, The Scripps Research Institute
2003-2004 -Senior Research Associate in Dr. Carlos Barbas III laboratory, Department of Molecular Biology, The Scripps Research Institute
2005 -Assistant Professor, University of North Carolina at Chapel Hill, Department of Pharmacology
Member, Lineberger Comprehensive Cancer Center.
2007 Member, American Association for cancer research, AACR

Teaching Experience

1994-1997 -Teaching assistant, biochemistry undergraduate laboratory, Faculté des Arts et des Sciences, Université de Montréal
2002 -Supervised summer High school teacher Paul Messier, The Scripps Research Institute
2003 -Supervised College student Leanna Lagpacan, The Scripps Research Institute
2004 -Supervised Ph.D student Sharon Bergquist, The Scripps Research Institute
2005, 2006 -I taught the Pharmacology graduate studies courses: Techniques in gene manipulation, seminars in Pharmacology, and nuclear receptors

Scholarships and awards

1992-1993 -EEC ERASMUS fellowship to finish last year of B.Sc. in ULB (Université libre de Bruxelles Belgium), Fellowship from Fondation Jean Branchet (ULB, Université Libre de Bruxelles) to support foreign students at the ULB
1998 -Winner, Simon-Pierre Noel Price Department of Biochemistry, University of Montreal.
1994-1999 -FES (Faculté des Études Supérieures) fellowship to support foreign students in the University of Montreal

1999-2004 -Research supported by Novartis Pharma fellowship (Oncology)
2005 -University Research Council Award, UNC-CH
-Lung Cancer Discovery Award
-GI SPORE Reseach Award
-V Foundation Award in cancer research (breast cancer fellowship in honor of Julie Stewart)
2006 -Department of Defense Idea award (breast cancer).
2007 - Carolina Center of Cancer Nanotechnology Excellence (C-CCNE) pilot grant award
-Golfers Against Cancer Award

Patents: Blancafort, P. and Barbas III, C.F. "Zinc finger libraries". International Patent Application Serial No PCT/US03/03705 (2001).

B. Peer Reviewed Publications

(* most significant to proposed work)

Blancafort, P., Ferbeyre, G., Sariol, C., and Cedergren, R. Pol I-driven integrative expression vectors for yeast. *Journal of Biotechnology* 1997;56:41-7

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C. Research Support
Active Research Support

Lung Cancer Discovery Award (Blancafort) 1/1/2006 – 12/31/2007
American Lung Association
Discovery of novel artificial transcription factors regulating lung cancer cell invasion and progression The major goals of this project are the isolation transcription factors controlling motility and invasion in vitro in lung cancer cells. No primary cells are used, no mouse models are investigated in this study.

Idea Award (Blancafort) 12/01/05 – 11/30/06
Department of Defense
Zinc Finger Transcription factors as novel genetic switches to regulate malignant progression of breast tumors. The major goal of this project are to isolate ATFs modulating metastatic behavior of non-invasive breast cells using an orthotopic xenograft model of breast cancer in nude mice.