Identifying the Targets of Progesterone in Human Breast Cancer

By

Mukul Sacchit Godbole

LIFE09201204009

Tata Memorial Centre

Mumbai

A thesis submitted to the

Board of Studies in Life Sciences

In partial fulfillment of requirements

for the Degree of

DOCTOR OF PHILOSOPHY

of

HOMI BHABHA NATIONAL INSTITUTE

June, 2018

Homi Bhabha National Institute

Recommendations of the Viva Voce Committee

As members of the Viva Voce Committee, we certify that we have read the dissertation prepared by Mr. Mukul Sacchit Godbole entitled "**Identifying the targets of progesterone in human breast cancer**" and recommend that it may be accepted as fulfilling the thesis requirement for the award of Degree of Doctor of Philosophy.

S. N. Dalal — 26/6/18

Chairperson – Dr. Sorab N Dalal — Date

A Dutt — 26/6/18

Guide/Convener – Dr. Amit Dutt — Date

R. Annapoorni — 26/6/18

External Examiner – Dr. Annapoorni Rangarajan — Date

V. Prasanna — 26/6/18

Member – Dr. Prasanna Venkatraman — Date

Dibyendu Bhattacharyya — 26/06/2018

Member – Dr. Dibyendu Bhattacharyya — Date

Left the host institute and relocated abroad in Oct 2017 — A Dutt — 26/6/18

Technical Advisor – Dr. Harsha Gowda — Date

Final approval and acceptance of this thesis is contingent upon the candidate's submission of the final copies of the thesis to HBNI.

I hereby certify that I have read this thesis prepared under my direction and recommend that it may be accepted as fulfilling the thesis requirement.

Date: 26/06/18
Place: Navi Mumbai

A Dutt

Dr. Amit Dutt
(Guide)

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Navi Mumbai Mukul Sacchit Godbole

Date: 26th June 2018

DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

Navi Mumbai Mukul Sacchit Godbole

List of Publications arising from the thesis

Journal:

1. "Progesterone suppresses the invasion and migration of breast cancer cells irrespective of their progesterone receptor status – a short report.", **Godbole M**, Tiwary K, Badwe R, Gupta S, Dutt A, *Cellular Oncology (Dordr)*, **2017**, *40(4)*: 411-417. PMID: 28653288 **(Thesis work)**.

2. "*miR-129-2* mediates down-regulation of progesterone receptor in response to progesterone in breast cancer cells", **Godbole M**, Chandrani P, Gardi N, Dhamne H, Patel K, Yadav N, Gupta S, Badwe R, Dutt A, *Cancer Biology & Therapy*, **2017**, *18(10)*: 801-805. PMID: 28876975 **(Thesis work)**.

Chapters in books and lectures notes: None

Conferences:

1. **M Godbole,** K Tiwary, N Gardi, K Patel, R Chaubal, K Karve, V Parmar, N Nair, S Gupta, R Badwe, A Dutt. Progesterone Suppresses Breast Cancer Invasion and Migration by Up-regulating a Serine/Threonine Protein Kinase *SGK1*; ENZYMES Conference, ACTREC (2017) (Poster presentation).
2. **M Godbole,** K Tiwary, N Gardi, K Patel, R Chaubal, K Karve, V Parmar, N Nair, S Gupta, R Badwe, A Dutt. Progesterone up-regulates and activates a tumor metastasis suppressor gene *NDRG1* in human breast cancer cells; All India Cell Biology Conference (AICBC), Gwalior (2016) (Poster presentation)

3. **M Godbole**, R Badwe, N Gardi, K Patel, K Tiwary, R Chaubal, K Karve, V Parmar, N Nair, S Gupta, A Dutt. Progesterone up-regulates and activates a tumor metastasis suppressor gene *NDRG1* in human breast cancer cells; Tata Memorial Centre Platinum Jubilee, A conference of new ideas in cancer—challenging dogmas (2016) (Poster presentation).
4. **M Godbole**, P Chandrani, H Dhamne, K Patel, N Gardi, K Tiwary, S Gupta, R Badwe, A Dutt. Dual Regulatory Model for Regulation of *SGK1* by Progesterone in Human Breast Cancer Cells; Tata Memorial Centre Platinum Jubilee, A conference of new ideas in cancer—challenging dogmas (2016) (Poster presentation).
5. **M Godbole**, K Patel, N Gardi, R Chaubal, K Karve, V Trivedi, V Parmar, N Nair, S Gupta, R Badwe, A Dutt. Progesterone up-regulates *NDRG1*, a tumor suppressor gene, in human breast cancer cells; MOSCon Pune (2016) (Poster presentation).
6. **M Godbole**, K Patel, N Gardi, R Chaubal, K Karve, V Trivedi, V Parmar, N Nair, S Gupta, R Badwe, A Dutt. Progesterone up-regulates *NDRG1*, a tumor suppressor gene, in human breast cancer cells; 34th Annual Convention of Indian Association for Cancer Research (IACR), Jaipur (2015) (Poster presentation).
7. K Patel, **M Godbole**, V Trivedi, K Karve, S Gupta, R Badwe, A Dutt. Identify the transcriptional targets of progesterone in human breast cancer; Second Global Cancer Genomics Consortium Symposium at ACTREC (2012) (Poster presentation).

Others:

1. “Notch pathway activation is essential for maintenance of stem-like cells in early tongue cancer”, Upadhyay P*, Nair S*, Kaur E, Aich J, Dani P, Sethunath V, Gardi N, Chandrani P, **Godbole M**, Sonawane K, Prasad R, Kannan S, Agarwal A, Kane S, Gupta S, Dutt S, Dutt A, *Oncotarget*, **2016**, *7(31)*:50437-50449. PMID: 27391340.
2. “CRE: a cost effective and rapid approach for PCR-mediated concatenation of KRAS and EGFR exons”, Ramteke MP, Patel KJ, **Godbole M**, Vyas M, Karve K, Choughule A, Prabhash K, Dutt A, *F1000Research*, **2016**, *4:160*. doi: 10.12688/f1000research.6663.2. PMID: 27127615.

Mukul Sacchit Godbole

ACKNOWLEDGEMENTS

From reveille to retreat of my PhD tenure, I have had the honor and privilege to seek support, guidance, help, suggestions and best wishes from a countless souls. I take this opportunity to thank them all for being a part of this journey.

At the very outset, I wish to thank my PhD mentor Dr. Amit Dutt with my deepest gratitude. He inculcated the thought in me that PhD is not just another degree; rather it's an informed step towards creation of a thoughtful and philosophical human being who can stand for himself with his wisdom and training. Dr. Dutt has been the source of inspiration since the beginning of my PhD and shall stay so always. He has encouraged and guided me throughout and I thank him with all my heart for his belief in me and his support. He is a far-sighted mentor, who has played an important role in shaping my scientific and overall personality. He has always inspired me for the planning and execution of my research work. I take this opportunity to thank him for encouraging me in developing my presentation and writing skills as well, with the lab meet presentations, manuscripts, discussions and a unique methodology termed 'pick-of-the-day', all of which I have thoroughly enjoyed. Dr. Dutt has expanded my scientific vision and I am thankful to him for providing me the opportunity to collaborate with students in the lab and with research groups outside ACTREC. I also thank him for raising my limits and making an environment conducive to work in the lab through all the phases of my PhD tenure.

I have had the great honor and privilege to work under the guidance of two of the finest human beings and clinician-scientists in the field of Cancer, Dr. Rajendra Badwe (Director, TMC) and Dr. Sudeep Gupta (Dy. Director, CRC, ACTREC). They have been a role model for me and I have been immensely benefited from all the meetings and discussions with them. I take this opportunity to express my heartfelt gratitude to Dr. Badwe and Dr. Gupta for their constant support, suggestions and insights in my project, which was initiated as an approach to unravel the mechanisms of progesterone in breast cancer in the Dutt laboratory.

I would like to thank my doctoral committee members Dr. Girish Maru (Ex-chairperson), Dr. Sorab N Dalal (Chairperson), Dr. Prasanna Venkatraman, Dr. Dibyendu Bhattacharyya and Dr. Harsha Gowda (IOB, Bangalore) for their guidance and suggestions in my work. Their critical outlook has helped me in shaping my project and me as a student in all these years at ACTREC. Also I would like to thank Dr. Shilpee Dutt for her insights, suggestions and support in my work. I have always enjoyed discussing my work with her and obtaining her critical comments that have been of immense benefit to me. She has always encouraged me to strive for more and I am grateful to her for her best wishes.

I would like to express my special thanks to Dr. Shubhada Chiplunkar (Director, ACTREC), Dr. Rajiv Sarin (Ex-Director, ACTREC) and Dr. Surekha Zingade (Ex-Dy.Director, CRI, ACTREC), for providing an excellent infrastructure and facilities at ACTREC and their constant support to budding researchers like me. I would

also like to thank the ACTREC-HBNI for providing PhD fellowship, Tata Memorial Centre (TMC) Project-2712 for funding the project, and Wellcome Trust/DBT India Alliance for their financial support.

I would like to thank Dr. Milind Vaidya and Mrs. Sharada Sawant (Vaidya Lab, ACTREC) for providing me the opportunity to work as a master's dissertation trainee in their lab in the year 2010. The five months spent in their laboratory motivated me to apply and pursue my doctoral degree at ACTREC. Also I would like to thank Dr. Shaida Andrabi (University of Kashmir) for providing me the pWZL-Myr-Neo-SGK1 construct which I have used in my thesis work. I wish to thank Prof. Tapas Kumar Kundu and his team (JNCASR, Bangalore) for giving me the opportunity to collaborate with them for their work. I extend my sincere thanks to Mr. Uday Dandekar (Incharge, CIR) and Mr. Durgadas Kulkarni for all their help and efforts in maintaining the common instrument rooms and facilities which I have used during my thesis work. I thank the ACTREC security and fire-fighting team for maintaining a safe environment for performing research and the canteen and retreat and hostel facilities. I specially thank the digital imaging facility members Vaishali madam, Tanuja madam and Mr. Jayraj for their help and support in all the microscopy related experiments, and also the Flow cytometry facility team, the Proteomics and the Genomics facility team at ACTREC. I am equally thankful to the Administrative department for their constant support and help in my tenure, especially in the last phase during my applications for International conference and the Passport. I also thank the IT team, the Accounts, Stores, Dispatch and Purchase departments for all the help and support. I would like to extend my special thanks to Mr. Mote, Mrs. Sharma, Mr. V. K. Singh, SCoPE cell and the HBNI-Academic office, especially Maya madam, for their guidance and support in all academic matters. I thank Ojaswini madam and Prerna madam for their best wishes and blessings. I also thank the Wellcome trust/DBT India Alliance and The Hindu newspaper for highlighting two of my research publications describing my PhD work and for encouraging me and the entire Dutt lab team for our work.

In my opinion, research lab is like a microenvironment that nurtures the thoughts and work process of each student who is an integral component of the niche. Dutt laboratory is more than a microenvironment for me and this family has provided me with the most crucial support system for my PhD tenure and I am thankful to each member of this family. Firstly, I would like to thank the entire Progesterone-Breast cancer group for all the help and support throughout my tenure. I am thankful to Dr. Kuldeep, Kunal, Kanishka, Dr. Pratik, Rohan, Nilesh, Dr. Hemant, Dr. Manoj, Sharan, Prachi, Ratnam, Mallika, Vaishakhi and Neelima who have been the strong pillars of this team and I have thoroughly enjoyed working alongside them and all the brain-storming sessions. I owe a great debt to Dr. Kuldeep for helping me become an independent researcher and always supporting and guiding me in my work. I had the great privilege of having friends in the Dutt lab like Prajish, Dhananjay Sir, Dr. Pawan, Dr. Jyoti, Trupti, Asim, Sanket, Vaibhav, Bikram, Deepak Iyer, Bhasker, Hitesh, and Suhail and I thank them for their suggestions in my work, for being the support system in the

Up's and Down's of my tenure and maintaining a joyful atmosphere in the lab. We have shared great moments together including the scientific work presentations, conferences, and workshops, and the fun-filled events like birthdays, lab outings, parties, and celebrations for special moments. I would like to thank Prachi, Kanishka, Dr. Pratik, Asim, Sanket, Neelima, Ratnam, Prajish, Bhasker, Trupti, and Dhananjay Sir for helping me in my work during the down phase of my health. A special thanks to Dr. Pratik for guiding and helping me in the preparation of manuscript drafts, academic presentations and work reports and to Prachi for all her help in the most crucial phase of my tenure. I take this opportunity to thank Asim and Sanket for their timely help, for tolerating me in my down phases and being close friends since their entry in the lab! Also, I thank Ratnam for being the undeterred pillar of the Dutt lab and her help in lab management and support in my manuscripts and experiments. I am also thankful to my fellow mates from the Shilpee laboratory Sameer, Shraddha, Smita madam, Shailesh, Ekjot, and Jyothi for providing a friendly atmosphere and all their help and suggestions in my work. Working in the lab would not have been possible without the most important support system provided by Mr. Deepak Amburle, Mr. Shailesh Parvate, Mr. Deepak Chavan and Mr. Rane. I extend a heartfelt thanks to all of them for their technical support in my work. It has been delightful experience to have worked alongside Deepak, Dhananjay Sir and Shailesh Sir and all the fun-filled chats with them.

I would like to specially thank Prajish, who was not only been a lab mate and a batch mate, but also a close friend. Since the beginning we have enjoyed working alongside, laughed our hearts out, helped in work and academic matters, discussed, criticized and at the same time stood for each other in all the Up's and Down's! Music is an eternal part of my life and I wish to thank the Barefeet Project members and my fellow ACTREC mates with whom I thoroughly enjoyed practicing and performing on stage for various concerts. The time spent with all the friends at ACTREC shall always stay close to my heart.

Everyone needs those special friends who share all your happy and sad moments. I have been extremely privileged to have close friends and I would like to thank all of them for their constant support and care.

This journey has been possible with the love, care and support of all my family members. I am deeply thankful to my Aai-Baba, Ajji-Ajoba, Maushi-Kaka, Mama-Mami, Kaka-Kaku, and my cousins Shruti, Anu tai, Kaushik dada and Harsh. I also thank them all for understanding me and joyfully accepting my absence on several family occasions! In these years I have had my down phases but my family has been my rock-solid support throughout and has stood by me mentally and physically. A special thanks to my aunt Sujata maushi for being my mother away from home! I take this opportunity to thank Dr. Anita, Dr. Manoj and Dr. Vidya, who are not only my doctors but also my family members, for helping me maintain my health, for their correct diagnosis and timely medication, and being my constant support throughout!

My dream to pursue PhD has come true all thanks to my parents. They have guided me through the path, nurtured my admiration for biology and have always boosted me to ask questions and seek answers to them. Their constant support, love, care and teachings has helped me throughout all the journeys. I thank them whole-heartedly for their encouragement and blessings! I proudly and lovingly dedicate my PhD thesis to my parents.

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ABBREVIATIONS

ACTREC	Advanced Centre for Treatment, Research and Education in Cancer
HBNI	Homi Bhabha National Institute
PR	Progesterone receptor
ER	Estrogen receptor
PRE	Progesterone response element
SGK1	Serum- and glucocorticoid-regulated kinase 1
NDRG1	N-Myc downstream regulated gene 1
Her2	Human epidermal growth factor receptor 2
RNA	Ribose nucleic acid
DNA	Deoxy-ribose nucleic acid
miR	microRNA
UTR	Untranslated region
EGFR	Epidermal growth factor receptor
ERK1/2	Extracellular signal-regulated kinase 1/2
AKT1	RAC-alpha serine/threonine-protein kinase
GR	Glucocorticoid receptor
MR	Mineralocorticoid receptor
AR	Androgen receptor
mPR	Membrane progesterone receptor
PGRMC1	Progesterone Receptor Membrane Component 1
SERBP1	Serpine1 mRNA binding protein 1
AP-1	Activating protein-1
EGR1	Early growth response gene 1
DUSP1	Dual-specificity phosphatase 1
MPA	Medroxy-progesterone acetate
TNBC	Triple negative breast cancer
STAT5A	Signal transducer and activator of transcription 5A
EZF	Epithelial zinc finger protein
F3	Tissue factor
MAPK	Mitogen-activated protein kinase
CUEDC2	CUE Domain-Containing Protein
KDa	Kilodalton
Src	SRC proto-oncogene, non-receptor tyrosine kinase
Hrs	Hours
ml	Millilitre
µl	Microlitre
nM	Nanomolar
µg	Microgram
FBS	Fetal bovine serum
BCA	Bicinchoninic acid
DMEM	Dulbecco’s Modified Eagle’s Medium
RPMI 1640 Medium	Roswell Park Memorial Institute Medium

SUMMARY OF PHARMACOLOGICAL INHIBITORS/MICRORNA INHIBITORS USED IN THE THESIS

Sr. No.	Inhibitor/ pharmacological agent	Function(s)	Concentration used	Purpose of usage
1.	Mifepristone	Progesterone receptor (PR) antagonist [1]	100nM	To block the activity of PR in cells to identify the PR-independent modes of action of progesterone
2.	SGK1-inhibitor (GSK650394A)	Inhibition of SGK1 kinase activity [2]	1μM	To check whether SGK1 kinase regulates the expression of NDRG1 and cell migration and invasion
3.	anti-*miR-129-2*	Inhibition of *miR-129-2*	50nM, for transfection in breast cancer cells	Inhibit the activity of *miR-129-2* to confirm its role in regulation of PR
4.	anti-*miR-29a* anti-*miR-101-1*	Inhibition of *miR-29a* and *miR-101-1*	50nM, for transfection in breast cancer cells	Inhibit the activity of *miR-29a* and *miR-101-1* to confirm their role in regulation of *SGK1*
5.	Puromycin	Selection of transduction positive clones	1μg/ml	Selection of stable transduction clones derived after over-expression or knockdown of *SGK1*, *NDRG1*, and *EGR1*
6.	Neomycin/G418	Selection of transduction positive clones	1400μg/ml	Selection of stable transduction clones derived after over-expression of *SGK1*
7.	Mitomycin-C	Inhibiting cell proliferation [3]	5mg/ml	Inhibition of cell proliferation prior to wound scratch assay

Homi Bhabha National Institute

SYNOPSIS OF Ph.D. THESIS

1. **Name of the Student:** Mukul Sacchit Godbole
2. **Name of the Constituent Institution:** Tata Memorial Centre, ACTREC
3. **Enrolment No.:** LIFE09201204009
4. **Title of the Thesis:** Identifying the Targets of Progesterone in Human Breast Cancer
5. **Board of Studies:** Life Sciences

SYNOPSIS

1. Introduction:

Breast cancer is leading cause of death in women [1], with increasing incidences globally [2]. The heterogeneity of this cancer type makes it challenging to diagnose and treat patients and the five year survival rate is about 52% [3]. Breast cancer is classified into five major sub-types—luminal A, luminal B, Her2-overexpressing, basal-like and normal-like subtypes, as evidenced from various genomic characterization studies [4]. Along with the conventional mode of treatment, patients are subjected to endocrine therapy (like tamoxifen or trastuzumab) based on the IHC analysis for the classical estrogen receptor (ER), progesterone receptor (PR) and epidermal growth factor receptor-2 (Her2). Despite the initial response to these endocrine therapies and the advances in early diagnosis, disease relapse remains a major problem, especially for node-positive patients [5]. However, pre-operative progesterone treatment of breast cancer patients with node-positive disease has shown

better survival outcome irrespective of the PR status of patients [6]. These results corroborate early reports where surgery performed in the luteal phase (progestogenic phase) of menstrual cycle provided survival benefit to breast cancer patients [7, 8]. Moreover, in vitro observations about the effect of progesterone support the clinical findings [9, 10]. However the mechanistic role of progesterone in conferring survival benefit to breast cancer patients independent of the PR status remains to be understood. On the other hand, progesterone has been shown to decrease the expression of its own receptor (PR) [11-13]. Also the utility of progestogens (progesterone-like compounds) in clinical settings has been topic of immense debate [14]. Thus it is important to understand the role played by progesterone and PR in breast cancer.

Along with resistance to therapy, another causal factor for relapse of breast cancer is the metastasis of the disease [15]. Of note, previous reports suggest that progesterone decreases the migration and invasion of only the PR-positive breast cancer cells in vitro [9], indicating requirement of PR expression to mediate this effect [16]. However, whether progesterone affects metastasis of cancer cells, independent of PR status, remains elusive. Mechanistically, metastasis of breast cancer cells is known to be affected by multiple molecular factors including activation of protein kinases [17]. For instance, protein kinases like EGFR, AKT or FAK are known to activate the processes of migration and invasion of breast cancer cells [18, 19]. Additionally, these kinases act synergistically and it has been shown that the invasive capacity of breast cancer cells can be suppressed by abrogating their activation [20]. Also, pathways downstream to these kinases may serve to either promote or restrain processes of cell invasion and migration. Thus suppressing activation of these kinases and downstream

pathways could potentially mitigate migration and invasion of cancer cells. Interestingly, steroid hormones and their receptors have been shown to affect the activity of such kinases [16, 21]. However, whether other receptors like glucocorticoid receptor or membrane progesterone receptor could mediate responses to progesterone in breast cancer cells remains to be studied.

Additionally, progesterone has been shown to affect the transcriptional activation of genes in breast cancer cells in a PR-isoform dependent manner [9, 22]. However, molecular targets of progesterone in breast cancer independent of the PR status of cells have not been characterized and an in-depth genomic analysis in PR-positive and PR-negative breast cancer cells needs to be performed. Such analysis would help in identifying coding and non-coding targets of progesterone to aid in understanding effect of progesterone in breast cancer.

2. Specific Objectives:

To understand the role of progesterone in breast cancer, we have taken a functional genomics and proteomics approach. We intend to identify the targets of progesterone in breast cancer, independent of the PR status of cells, with the following specific objectives:

Specific objective 1: Genomic approach to identify targets of progesterone in breast cell lines.

Specific objective 2: Proteomic analysis of breast cell lines upon progesterone treatment

Specific objective 3: Functional validation of progesterone candidate genes in breast cancer cell line by rescuing phenotype.

Specific objective 1: Genomic approach to identify targets of progesterone in breast cell lines.

Work plan:

a. Selection of breast cell lines with varying receptor expression

b. Standardization of progesterone treatment

c. Genome-wide expression array and microRNA profiling

d. Real time based validation of candidate genes and microRNAs

- To begin with, we selected a panel of seven breast cell lines with different PR/ER/Her2 receptor statuses for identifying the targets of progesterone and the identity of cell lines was confirmed by STR profiling. The progesterone treatment conditions, 10nM concentration and treatment for 6h, were standardized based on consistent up-regulation of three known progesterone target genes [22] in real time PCR analysis.
- Next we performed expression array analysis of the breast cell lines treated with progesterone and combination of mifepristone and progesterone to identify targets of progesterone. Differential gene expression analysis identified Serum- and glucocorticoid-regulated kinase 1, SGK1, as the top up-regulated gene while N-Myc Downstream-regulated gene 1, NDRG1, as one of the recurrently up-regulated genes in breast cell lines upon treatment with progesterone. Both these genes were interesting candidates for our study owing their biological role reported previously [9, 23] and their genetic link

whereby SGK1 has been shown to phosphorylate NDRG1 [24]. Moreover SGK1 and NDRG1 were found to be co-expressed in our expression array analysis. We could validate the expression of both these genes using real-time PCR and western blot analysis in response to progesterone.

- Furthermore, we studied the expression of AP-1 network genes which are known to regulate the transcription of NDRG1 [25, 26]. Real-time PCR analysis of FOS, JUN, EGR1 and DUSP1 suggested an increase in expression of these AP-1 network genes in response to progesterone in breast cell lines independent of the PR-status of cells. This suggests that progesterone could potentially regulate expression of the tumor suppressor gene NDRG1 via the AP-1 network genes in breast cancer cells.

- Apart from the coding targets of progesterone, we also studied the non-coding microRNA gene targets of progesterone in breast cancer cells. We performed small RNA sequencing of four breast cancer cell lines (T47D, BT474, MCF7 and MDA-MB-231) treated with progesterone, using Illumina HiSeq 1000 with eight multiplex libraries. Differential expression analysis of microRNAs suggested 20 microRNAs to be commonly up-regulated and 19 microRNAs to be commonly down-regulated in breast cancer cells. Of note, our analysis predicted *SGK1* to be target of *miR-29a* and *miR-101-1*, while *PR* to be target of *miR-129-2*. *miR-29a* and *miR-101-1* were found to be down-regulated while *miR-129-2* was found to be up-regulated in response to progesterone treatment. These results were validated using real-time PCR analysis of breast cancer cells treated with progesterone.
- Next, we functionally validated the anti-correlation between expression of genes and their respective microRNA partners, by performing luciferase assay with co-

expression of the microRNAs and 3'UTR of *SGK1* and *PR*. Of note, our analysis suggests that inhibition of these microRNAs relieved the repression in luciferase activity *in vitro*.

- Functional characterization of the coding and non-coding microRNA targets of progesterone has been performed in the third objective.

<u>Specific objective 2:</u> Proteomic analysis of breast cell lines upon progesterone treatment

<u>Work plan:</u>

a. Quantitative proteomic analysis of breast cancer cells treated with progesterone

b. Western blot based validation of candidate proteins in breast cancer

- We initially performed 2D gel electrophoresis of proteins isolated from breast cancer cells treated with progesterone. Our analysis identified cathepsin-D and glutathione-S-transferase, which are known targets of progesterone, to be up-regulated in breast cancer cells. However we did not pursue 2D gel electrophoresis owing the low resolution. Next, we performed iTRAQ analysis of four breast cancer cell lines treated with progesterone to identify the differentially expressed proteins in response to progesterone. However, no conclusive set of proteins could be identified in this quantitative proteomic approach. Also the re-analysis of iTRAQ data was planned but could not be performed.
    - To study the effect of progesterone on activation of kinases, we performed proteome profiling of PR-positive and PR-negative breast cancer cells in response to progesterone using a phospho-kinase array platform. In both cell

lines together we observed 7 of 43 kinases to be de-phosphorylated in response to progesterone viz. Akt1/2/3, STAT3, p70 S6 Kinase, RSK1/2/3, PLC-γ1, FAK and Fgr. In addition we observed significant de-phosphorylation of ERK1/2, MSK1/2, EGFR, p27, TOR and p38α in response to progesterone.

- It is known that majority of these kinases are known regulators of cell migration and invasion and that blocking the activity of these kinases can attenuate these phenotypes [18, 19]. Thus our results suggest that progesterone suppresses the phosphorylation of 12 kinases out of 43 in a PR independent manner and that this could affect the cell migration and invasion of breast cancer cells. We performed western blot based validation of phosphorylation changes of candidate kinases in breast cancer cells upon treatment with progesterone. In T47D and MDA-MB-231 cells, our western blot analysis suggests significant reduction in phosphorylation of EGFR, AKT and ERK1/2 kinases in response to progesterone, consistent with earlier reports [27]. Interestingly, our real time PCR analysis suggests an increased expression of dual specificity phosphatase, DUSP1, in breast cancer cells upon treatment with progesterone. DUSP1 has been shown to de-phosphorylate these kinases in breast cancer cells [27]. Next we performed in vitro cell migration and invasion assay using panel of breast cancer cells to analyze the effect of progesterone on these cell phenotypes. In continuation to our earlier findings, our results suggest that blocking PR using mifepristone did not affect the activity of progesterone to suppress the cell migration or invasion of breast cancer cells. This suggests that progesterone suppresses these cellular phenotypes in a PR-independent manner. Our results corroborate the findings

of the clinical trial where progesterone was shown to reduce recurrence of node-positive breast cancer patients independent of their PR-status [6].

Specific objective 3: Functional validation of progesterone candidate genes in breast cancer cell line by rescuing phenotype.

Work plan:

a. Gain or loss of function study of candidate genes in breast cell lines.

b. Cell based assays for studying the function of candidate progesterone target genes

We have performed extensive functional characterization of genomic (coding and non-coding) and proteomic targets of progesterone in this objective.

- Functional validation of microRNA-gene interaction:

  In order to validate the physical interaction between *miR-129-2* and *PR* in breast cancer cells, we inhibited the expression of *miR-129-2* and performed western blot analysis of PR. The results obtained suggested that upon inhibition of *miR-129-2*, PR expression is stabilized and remains unaltered even upon progesterone treatment as compared to expression of PR in cells treated with negative control. Thus our analysis validates interaction of *miR-129-2* with *PR* in breast cancer. Next we studied the expression of PR in the TCGA cohort (n=359) in patients with high *miR-129-2* expression and in absence of *miR-129-2* expression. Our analysis suggests that PR expression was significantly elevated in patients with absence of *miR-129-2* expression as compared to patients with high expression of *miR-129-2*. Taken together, we show that PR expression is controlled by *miR-129-2* in response to progesterone in breast cancer. These observations are of biological and

clinical significance since use of microRNA inhibitors can help stabilize the target protein levels in patients and aid in the treatment under the adjuvant and neo-adjuvant settings along with the conventional mode of treatment. However the clinical efficacy of microRNA inhibitors needs to be tested for further usage.

- Establishing cell-based assays to study the effect of progesterone and candidate genes:

1. We performed cell invasion and migration assays in breast cancer cell lines treated with progesterone. Our *in vitro* analysis using a panel of breast cancer cells with different PR/ER/Her2 expression suggested that progesterone suppressed the invasion and migration of these cells irrespective of the hormone receptor status.

    - Next, treatment of breast cancer cells with combination of mifepristone (PR-antagonist) and progesterone also led to decrease in invasion and migration as compared to untreated cells. This suggests that blocking PR using mifepristone did not affect the activity of progesterone to suppress the cell migration or invasion of breast cancer cells. This suggests that progesterone suppresses these cellular phenotypes in a PR-independent manner. Our results corroborate the findings of the clinical trial where progesterone was shown to reduce recurrence of node-positive breast cancer patients independent of their PR-status [6].

    - Genetic and pharmacological perturbation of *SGK1* in breast cancer cells:

As shown in objective-1, up-regulation of SGK1 and NDRG1 was validated using western blot analysis. However the increase in phosphorylation of NDRG1 was not

significant in response to progesterone. Further, we have shown that progesterone up-regulates the expression of the AP-1 network genes in breast cancer cells. As reported previously, the expression and activity of AP-1 family members are regulated by various cellular kinases in response to stress and mitogenic stimulus [28]. In our cell line based expression array analysis, *SGK1*, a serine/threonine kinase, was found to be up-regulated in breast cancer cells in response to progesterone treatment. The members of AP-1 network (*EGR1* and *FOS*) regulate the expression of *NDRG1* via binding sites in the promoter region. Thus to understand whether *SGK1* can regulate the expression of the AP-1 network genes and hence *NDRG1*, we set to over-express and deplete or pharmacologically inhibit *SGK1* expression in breast cancer cell lines. These genetic and pharmacological perturbations of *SGK1* were performed in PR-positive (T47D) and PR-negative (MDA-MB-231) breast cancer cells.

1. Over-expression of *SGK1*:

   We have used constructs that over-express wild-type *SGK1* and myristoylated *SGK1* in cells. Upon over-expression of *SGK1*, expression and phosphorylation of NDRG1 was increased in both the cell lines. Thus, in addition to earlier reports where SGK1 was shown to phosphorylate NDRG1 [24, 29], SGK1 also up-regulated expression of *NDRG1*. Further, *SGK1* was found to up-regulate the expression of AP-1 network genes in breast cancer cells. Next wound migration and invasion assays performed with these cells suggested that *SGK1* decreased these cellular phenotypes in both the cell lines. Thus our results suggest that *SGK1* mimics the effect of progesterone in breast cancer cells.

2. Genetic depletion and pharmacological inhibition of *SGK1*:

a. Upon knockdown of *SGK1*, the expression and phosphorylation of NDRG1 was decreased. Also the expression of AP-1 network genes was decreased upon depletion of *SGK1*. Moreover, cell migration and invasion of breast cancer cells was increased in these cells as compared to sh-NT (non-targeting) clone.

b. Similar results were obtained upon pharmacological inhibition of SGK1 in both PR-positive and PR-negative cell lines.

c. Thus it can be concluded that *SGK1* mimics the effect of progesterone in regulating the expression of *NDRG1* which potentially regulates cell migration and invasion of breast cancer cells.

- To delineate the role played by *NDRG1* in breast cancer, we depleted the expression of *NDRG1* in breast cancer cells. Consistent with earlier reports [30, 31], our western blot analysis suggests that knockdown of NDRG1 increased the phosphorylation of EGFR, AKT and ERK1/2 kinases. Of note, breast cancer cells showed an increase in cell migration upon depletion of *NDRG1*. These results help strengthen our hypothesis that progesterone suppresses breast cancer cell migration via *NDRG1*.
    - Next, we depleted the expression of *EGR1*, member of the AP-1 network, in both T47D and MDA-MB-231 cells to study role of *EGR1* in regulating expression of *NDRG1*. Interestingly, our western blot analysis suggests that knockdown of *EGR1* led to depletion in expression of *NDRG1*. Moreover, the cells show increased cell migration, consistent with previous reports [25, 32]. Since we have earlier shown that *SGK1* regulates the expression of *EGR1* and *NDRG1* in breast cancer cells, our study suggests that progesterone potentially utilizes the *SGK1*/*AP-1 network*/*NDRG1* axis

to de-phosphorylate ERK1/2, AKT and EGFR kinases and suppress breast cancer cell invasion and migration in a PR-independent manner.

3. <u>Conclusion:</u>

We used genomic and proteomic approaches to identify the targets of progesterone in a panel of breast cancer cell lines followed by their functional validation. The finding of this study furthers our understanding on how progesterone functions *in vitro* in breast cancer cells. We show that progesterone suppresses cell invasion and cell migration in a panel of breast cancer cells independent of their PR statuses by inactivating kinases. Expression array analysis of breast cancer cells treated with progesterone led to an identification of *SGK1* and *NDRG1* as genomic targets of progesterone. Using genetic and pharmacological perturbation experiments, we show that *SGK1* regulates the expression of *NDRG1* affecting cell invasion and cell migration independent of the PR-status of breast cancer cells, detailing a mechanistic basis for pre-operative progesterone intervention in breast cancer patients.

Furthermore, we show that progesterone-mediated up-regulation of *miR-129-2* leads to down-regulation of *PR* and that inhibiting *miR-129-2* could stabilize expression of PR in breast cancer. Since absence of PR could make tumors resistant to endocrine therapy, our study suggests that stabilization of PR by inhibiting microRNAs such as *miR-129-2*, along with standard treatment modalities, could help in enhancing clinical response to endocrine therapies among breast cancer patients. This study also identifies dual-regulation of *SGK1* in response to progesterone. My work led to an understanding of an intricate

genetic interaction up-regulating the expression of *SGK1* by down-regulating the expression of two novel microRNA *miR-29a* and *miR-101-1* that target the 3'UTR of *SGK1*.

Taken together, our study provides the first lead to model a randomized clinical trial, by using an *in vitro* study, to systematically elucidate the role of pre-operative progesterone intervention in breast cancer patients by targeting novel coding and non-coding genes.

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- **Publications in Referred Journal:**

a. Published—

1) **Godbole M**, Tiwary K, Badwe R, Gupta S, Dutt A. *Progesterone suppresses the invasion and migration of breast cancer cells irrespective of their progesterone receptor status – a short report*. ***Cellular Oncology***, 40(4):411-417, 2017; PMID: 28653288; doi: 10.1007/s13402-017-0330-z (**Thesis work**).
2) Upadhyay P*, Nair S*, Kaur E, Aich J, Dani P, Sethunath V, Gardi N, Chandrani P, **Godbole M**, Sonawane K, Prasad R, Kannan S, Agarwal A, Kane S, Gupta S, Dutt S, Dutt A. *Notch pathway activation is essential for maintenance of stem-like cells in early tongue cancer*. ***Oncotarget***, 2016. doi: 10.18632/oncotarget.10419.
3) Ramteke MP, Patel KJ, **Godbole M** et al. *CRE: a cost effective and rapid approach for PCR-mediated concatenation of KRAS and EGFR exons*. ***F1000Research***, 2016, **4**:160. doi: 10.12688/f1000research.6663.2.

b. Accepted—

Godbole M, Chandrani P, Gardi N, Dhamne H, Patel K, Yadav N, Gupta S, Badwe R, Dutt A. *miR-129-2 mediates down-regulation of progesterone receptor in response to progesterone in breast cancer cells*. (Accepted for publication in ***Cancer Biology & Therapy***) (**Thesis work**).

c. Communicated/In preparation—

Godbole M et al, *Progesterone up-regulates and activates a tumor metastasis suppressor gene NDRG1 in human breast cancer cells* (In preparation) (Thesis work).

Other Publications—

a. Book/Book Chapter: N.A

b. Conference/Symposium (Oral/Poster presentation):

1. **M Godbole,** K Tiwary, N Gardi, K Patel, R Chaubal, K Karve, V Parmar, N Nair, S Gupta, R Badwe, A Dutt. Progesterone Suppresses Breast Cancer Invasion and Migration by Up-regulating a Serine/Threonine Protein Kinase *SGK1*; ENZYMES Conference, ACTREC (2017) (Poster presentation).

2. **M Godbole,** K Tiwary, N Gardi, K Patel, R Chaubal, K Karve, V Parmar, N Nair, S Gupta, R Badwe, A Dutt. Progesterone up-regulates and activates a tumor metastasis suppressor gene *NDRG1* in human breast cancer cells; All India Cell Biology Conference (AICBC), Gwalior (2016) (Poster presentation).

3. **M Godbole**, R Badwe, N Gardi, K Patel, K Tiwary, R Chaubal, K Karve, V Parmar, N Nair, S Gupta, A Dutt. Progesterone up-regulates and activates a tumor metastasis suppressor gene *NDRG1* in human breast cancer cells; Tata Memorial Centre Platinum Jubilee, A conference of new ideas in cancer—challenging dogmas (2016) (Poster presentation).

4. **M Godbole**, P Chandrani, H Dhamne, K Patel, N Gardi, K Tiwary, S Gupta, R Badwe, A Dutt. Dual Regulatory Model for Regulation of *SGK1* by Progesterone in Human Breast Cancer Cells; Tata Memorial Centre Platinum Jubilee, A conference of new ideas in cancer—challenging dogmas (2016) (Poster presentation).

5. **M Godbole**, K Patel, N Gardi, R Chaubal, K Karve, V Trivedi, V Parmar, N Nair, S Gupta, R Badwe, A Dutt. Progesterone up-regulates *NDRG1*, a tumor suppressor gene, in human breast cancer cells; MOSCon Pune (2016) (Poster presentation).

6. **M Godbole**, K Patel, N Gardi, R Chaubal, K Karve, V Trivedi, V Parmar, N Nair, S Gupta, R Badwe, A Dutt. Progesterone up-regulates *NDRG1*, a tumor suppressor gene, in human breast cancer cells; 34th Annual Convention of Indian Association for Cancer Research (IACR), Jaipur (2015) (Poster presentation).

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Signature of Student M.S. Ghatbandhe
Date 30/08/17

Doctoral Committee:

S. No.	Name	Designation	Signature	Date
1.	Dr. Sorab N Dalal	Chairman	S. N Dalal	30/08/17
2.	Dr. Amit Dutt	Guide & Convener	A Dutt	30/08/17
3.	Dr. Prasanna Venkatraman	Member	P Ramana	30/08/17
4.	Dr. Dibyendu Bhattacharyya	Member	D. Bh	04/09/17
5.	Dr. Harsha Gowda	Technical Advisor	Harsha	01/09/17

Forwarded Through:

S. V. Chiplunkar 4/9/17

Dr. S.V. Chiplunkar
Director, ACTREC
Chairperson,
Academic & training Program, ACTREC

Dr. S. V. Chiplunkar
Director
Advanced Centre for Treatment, Research & Education in Cancer (ACTREC)
Tata Memorial Centre
Kharghar, Navi Mumbai - 410 210.

Prof. K. Sharma
Director, Academics
T.M.C.

PROF. K. S. SHARMA
DIRECTOR (ACADEMICS)
TATA MEMORIAL CENTRE,
PAREL, MUMBAI

SUMMARY

Breast cancer is the most commonly occurring cancer in females worldwide and the outcome, amongst the other causal factors, is influenced by hormones. Surgery performed in the progestogenic luteal phase of menstrual cycle showed beneficial effect on the survival of pre-menopausal breast cancer patients. Based on these early observations, a clinical trial with pre-operative progesterone intervention was conducted in a cohort of breast cancer patients, which suggested an increase in overall and disease-free survival of patients independent of their menopausal and PR status. However, the molecular mechanism of action of progesterone remained to be explored. To understand the effect of progesterone in breast cancer, I performed a functional genomics and proteomics analysis of breast-derived cell lines representing different hormone receptor statuses. First, I studied the effect of progesterone on the cell migration and invasion of breast cancer cells by performing cell-based assays and proteome profiling for activation of kinases. I observed that progesterone suppresses the activation of multiple kinases and concomitantly inhibits the migratory properties of breast cancer cells. I also observed that PR plays a non-essential role in regulation of these phenotypes since blocking the activity of PR did not hamper the effect of progesterone.

Secondly, genomic analysis for identification of microRNAs de-regulated by progesterone led to the discovery of *miR-129-2* which regulates expression of *PR* in breast cancer cells. I found that *miR-129-2* and PR hold an inverse correlation in breast cancer patients, as observed in the TCGA analysis. Interestingly, upon

inhibition of *miR-129-2* activity in breast cancer, expression of PR was reinstated and such anti-microRNA strategies may hold promise to stabilize PR expression in breast cancer patients and potentially improve the response to endocrine therapies.

In the final part of my work, I performed a functional genomics analysis of seven breast-derived cell lines using microarray gene expression analysis. I observed an increased expression of Serum- and glucocorticoid-regulated kinase 1, *SGK1* and N-Myc downstream regulated gene 1, *NDRG1*, in response to progesterone treatment of breast cell lines irrespective of their PR statuses. Using genetic perturbation approaches, I observed that *SGK1* regulates the expression of *NDRG1* and cell migration and invasion phenotype in breast cancer. In an effort to identify mediators downstream to *SGK1*, I observed that members of the AP-1 network genes can respond to progesterone treatment and *SGK1* to regulate the expression of *NDRG1*. I also observed that *NDRG1* regulates the activation of kinases like EGFR, AKT1 and ERK1/2 and thus regulates cell migration and invasion of breast cancer cells independent of their PR status. Thus the regulation of these phenotypic properties of breast cancer cells could be mediated by increased expression of *SGK1* and *NDRG1* in response to progesterone. Furthermore, I also observed that progesterone suppresses the expression of two novel microRNAs *miR-29a* and *miR-101-1* that target 3'UTR of *SGK1*. Since progesterone is known to increase expression of *SGK1* via progesterone response elements in the promoter region, I postulate that progesterone maintains a sustained expression of *SGK1* in a dual-regulatory manner in breast cancer cells.

In overall, my study helps in identification of a tight network of genes regulated by progesterone which suppresses the cell invasion and migration of breast cancer, where the effects of progesterone are primarily mediated via the *SGK1* and *NDRG1* axis. Of note, I show that progesterone mediates its activity independent of the PR status of breast cancer cells. My study presents the first leads to model the randomized clinical trial studying the effect of progesterone in breast cancer.

I. INTRODUCTION AND REVIEW OF LITERATURE

1.1 An Introduction to Cancer:

Majority of the human cells possess the capacity to grow, divide, differentiate and become part of a complex system that makes a functional human body. Complex processes govern the smooth functioning of the cells, controlling derailment of the system [4]. However, sometimes the random alterations in the machinery, which otherwise is corrected by the cells, prevail over the normalcy and give rise to a diseased state [5]. This process can be rightly explained, in a dramatic fashion, as, "*You either die a hero, or you live long enough to see yourself become the villain…*" *(a quote from 'The Dark Knight' movie).* Cancer is one such old-fashioned villain that originates from own cells of the body, surpasses the immune system, and evade the neighboring tissue structure to colonize and expand its empire. It remains to be one of the toughest enemies to defeat even today, while we still are way behind in understanding the ever-evolving mechanisms utilized by cancer. Cancer is amongst the leading causes of death and accounts for one in eight deaths globally. Development of cancer is a complex multistep process, which involves acquisition of heritable genetic alterations and natural selection processes which transforms a seemingly normal cell in to cancerous [6, 7]. There are numerous types of cancers, with several different subtypes arising in a tissue-specific manner, each with own set of genetic and epigenetic abnormalities. This uniqueness of each cancer type demands development of more-so-unique treatment modalities and yet we are unable to catch-up with this recalcitrant disease. Though the percentage of incidence and mortality for each cancer type varies in a region- and economic-status specific manner, lung cancer

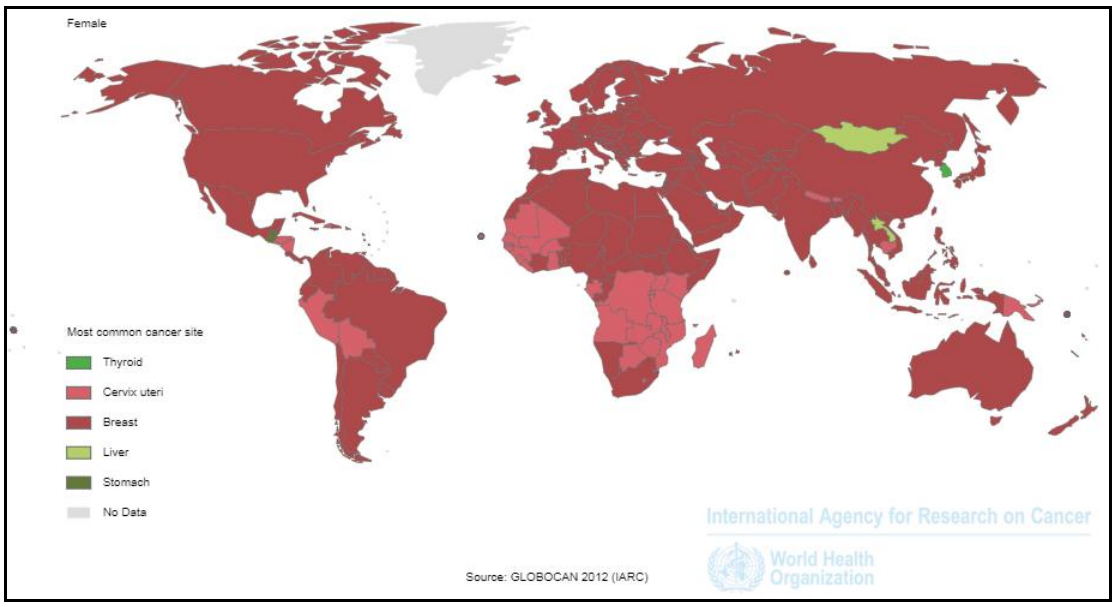
in males and breast cancer in females is the leading cause of death due to cancer worldwide [8]. A variety of causes have been associated for the initiation and progression of sporadic cancerous lesions for both the sexes, including environmental and lifestyle-related components like the use of tobacco in any form, exposure to occupational and environmental carcinogens, alcohol, diet and obesity, infection by cancer-associated pathogens like viruses and bacteria, and reproductive and hormonal factors that lead to development of cancer especially in women. Apart from these external causative agents, hereditary genomic alterations in certain genes have been found as causative factors for many familial cancer types [9].

The initiation and progression of cancers originating in organs like the breast, ovary, endometrium, prostate and testis are influenced by the endogenous and externally administered hormonal agents, apart from the genetic alterations associated with each of these cancers. Hormones are known to alter the cellular properties of hormone-responsive cells and influence normal physiological development of these organs. However, through complex interactions and increased exposure to hormones, women develop breast and endometrial cancers [10]. The properties of cancer development and progression in these two organs are different and many studies have been directed to understand the influence of hormones on female cancers.

1.2 Breast cancer:

Breast cancer is the second most common cancer and its incidence accounts for 25% of all cancer types globally [11, 12]. Specifically, in women, breast cancer is the leading cause of death and the death-rates are about 4-times higher than other cancer types (I-Figure-1). On an average, women are prone to develop breast cancer in the window period of 40-50 years of age and that second cancer may arise later in life,

showing a bimodal distribution of incidence [13, 14]. Some of the known causal factors include prolonged exposure to endogenous hormones, late age at first pregnancy, hereditary associations (mutations in *BRCA1/2* genes), alcohol consumption, obesity and physical inactivity [15, 16]. Like other cancer types, breast cancer harbors mutations in key driver genes like *TP53*, *AKT1*, *CDH1*, *GATA3*, *PIK3CA*, *PTEN* and *RB1*, along with *AKT2*, *APC*, *ARID1A and ARID1B*, *CASP8*, *CDKN1B*, *MLL3* and so on [17, 18]. In addition, hormone replacement therapy has long been debated as a causal factor for breast cancer risk and thus the treatment options for breast cancer patients vary based on their menopausal status [19, 20]. With the advent of better diagnostic and screening techniques, the incidence of breast cancer has reduced to a significant extent. The screening methods employed for breast cancer include mammography, breast examination, imaging methods (MRI, PET-scan, PET–CT and others), genetic testing and IHC [21, 22]. However, another school of thought prevails that these early diagnostic tools have led to an over-diagnosis and over-treatment of patients [23].



I-Figure- 1: Overall incidence of most common female cancers worldwide

[Adapted from GLOBOCAN 2012, (globocan.iacr.fr)]. The colors indicate the propensity of each cancer in different parts of the world.

1.2.1 Classification of breast cancer:

Breast cancer is amongst the most heterogeneous class of cancers and is considered to be a collection of different diseases, rather than just one disease with varying features [24]. Breast cancer has been classified in to five major sub-types based on gene expression signatures: luminal-A, luminal-B, Her2-enriched, basal-like and claudin-low subtypes [25, 26]. All of these subtypes have unique gene expression patterns and vary with respect to their hormone receptor statuses, such as the luminal class is usually ER-positive with varying PR expression, Her2-enriched show high expression of the Her2 receptor, while basal-like and claudin-low subtype is usually triple negative (PR-/ER-/Her2-), along with other molecular differences and metastatic preferences for these subtypes (I-Table-1) [27-30]. Classification of breast cancers into different subtypes has helped in an improved understanding of intrinsic molecular characteristics of tumors and stratification of patients to provide them with tailor-made treatments and thus aid in better survival by avoiding unnecessary exposures to chemotherapeutic agents. In general, patients with the luminal-A tumors have a better survival outcome, while those with the basal-like or Her2-enriched tumors show poor survival outcome [26].

I-Table- 1: Summary of intrinsic subtypes of breast cancer
[Adapted from Goldhirsch et al., 2011, Annals of Oncology] The classification is based on classical IHC staining for ER and PR, detection of over-expression or amplification of HER2, and Ki-67 labeling which is a marker for cell proliferation [30].

Sr. No.	Intrinsic breast cancer subtype	Clinico-pathologic definition
1.	Luminal A	ER and/or PR positive HER2 negative Ki-67 low
2.	Luminal B (HER2 negative)	ER and/or PR positive HER2 negative Ki-67 high
3.	Luminal B (HER2 positive)	ER and/or PR positive HER2 over-expressed or amplified Ki-67 varying
4.	HER2 over-expression	HER2 over-expressed or amplified ER and PR absent
5.	Basal-like (Triple-negative)	ER and PR absent HER2 negative

1.2.2 Endocrine therapy for breast cancer:

Like most cancer types, breast cancer patients are also treated using surgical removal of tumor mass, followed by radiotherapy and chemotherapy. However, since breast cancer is amongst the group of endocrine-responsive cancers, patients are also given endocrine/hormonal therapy in adjuvant and neo-adjuvant settings, comprising of selective estrogen receptor or progesterone receptor modulators (SERMs or SPRMs) or Her2-blocking monoclonal antibodies, based on the subtype or hormone receptor status of breast cancer [31-33]. The patient's age, tumor size, tumor grade, involvement of lymphnodes, and the hormone receptor status influence the treatment regimen [34]. Moreover, each breast cancer subtype needs systematic treatment—

a) Her2-enriched: As this tumor subtype shows an increased expression of HER2, patients are treated using monoclonal antibodies (trastuzumab, pertuzumab) and kinase inhibitors like lapatinib or afatinib, in combination with chemotherapeutic agents [35]. However a large proportion of patients develop resistance to these agents and hence a better targeted therapy is needed for treatment [36].

b) TNBC/Basal-like: Absence of hormone receptors, along with mutations in *BRCA1* and the metastatic nature of this subtype makes TNBC as the difficult-to-treat cancers, with only available options of radiotherapy and neoadjuvant chemotherapy [37]. Chemotherapeutic options like anthracycline/taxane-based agents are a gold standard, along with newer agents like the platinum-based drugs or those targeting PARP (synthetic lethality in *BRCA1* mutant patients) such as olaparib, are being considered for treatment of TNBC patients [38].

c) Luminal subtype: Almost two-thirds of breast cancer cases are ER-positive and of the luminal type. However, the subtypes vary in their hormone receptor status and luminal-B is mostly PR-negative [39]. Presence of hormone receptors makes these tumors suitable candidates for hormonal therapy and treatment with aromatase inhibitor and tamoxifen reduces the recurrence of this subtype [40].

However, nearly 70-80% of patients receiving chemotherapeutic or endocrine treatments are over-treated or undergo unnecessary surgical interventions [41]. In order to prevent the overtreatment, recent advances in gene expression analyses have helped in the development of newer prognostic tools like PAM50, 70 gene-based MammaPrint, and 21-gene Oncotype DX, that predict the response of patients to chemotherapy, metastatic potential of the primary tumour and patient outcome [42-45].

1.2.3 Recurrence of breast cancer:

Breast cancer shows metastatic behavior and is known to metastasize to adjacent lymph-nodes and distant locations like the lung, bone, liver and brain [46, 47]. Metastatic property of breast cancer causes more deaths than the primary tumor itself. Since predicting the metastasis of breast cancer is difficult, majority of the patients receive adjuvant chemotherapy of which only half of the patients relapse and the other half are over treated [48]. Even with the advances in diagnostic and treatment modalities, node-positive patients relapse with a metastatic disease [21]. The classical metastasis theory of breast cancer suggests that a subset of primary site tumor acquires properties to metastasize to distant locations and that such cells are selected over the period of progression of the disease. However, newer theories have challenged this belief and it is now believed that breast cancer cells acquire mutations in metastasis-causing genes at a much early stage of tumorigenesis [49].

Thus, the metastatic nature and resistance to therapy account for the relapse and recurrence of breast cancer. Generally, patients with luminal-type tumors have a lower risk of recurrence and those with the Her2-enriched and basal-like (TNBC) tumors have an increased risk for local and regional recurrence of the disease [50]. Moreover, breast cancer poses a unique threat that the disease can recur even after first 10 years after diagnosis and that after initial response to endocrine agents, a significant percentage of patients relapse with metastatic tumor. Thus patients may have to be kept on maintenance therapy for a prolonged period, even after remission of the tumor, to remove traces of the disease and reduce chances of recurrence [51].

1.2.4 Correlation of PR expression with prognosis of breast cancer:

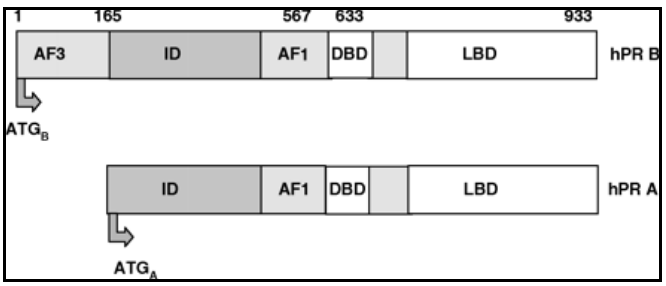
From a prognostic point-of-view, patients with PR positive tumors show better response to therapy and delayed metastasis [52]. For patients on endocrine therapy targeting ER, PR is considered an important predictor for clinical response to the therapy [53]. However, during progression of the disease, breast tumors lose expression of hormone receptors, while some show over-expression of growth factor receptors leading to resistance to endocrine therapy [54-56].

PR expression correlates with prognosis of breast cancer patients [53, 57, 58]; and, patients with PR/ER-positive tumors respond better to endocrine therapy as compared with patients with PR-negative/ER-positive or -negative tumors [59]. Of note, presence of PR has been shown to associate with lower metastasis of tumors to distant sites [52]. However, patients show resistance to endocrine therapy with influence by factors like loss of either of the hormone receptors (PR/ER), or by over-expression of Her2 receptor which circumvents the endocrine suppression or due to over-expression of proteins like cyclin-D1 [59, 60]. A slightly intriguing observation shows that hormones can down-regulate their own receptors. For instance, estrogen has been shown to decrease expression of ER via up-regulation of microRNAs [61]. Similarly, progesterone regulates the degradation of PR by phosphorylation regulated by MAPK and ubiquitination induced by CUEDC2. Apart from this, PR is down-regulated by methylation of promoter of PR and microRNA-mediated down-regulation in breast and endometrium cells [62-64]. However, microRNAs regulating PR expression in response to progesterone have not been studied in detail [65]. Thus, a better understanding of mechanisms leading to PR down-regulation may aid in maintaining the expression of PR in breast cancer. Thus these clinical observations mandate a

detailed investigation of hormone receptor status of patients receiving endocrine therapy.

1.2.5 Progesterone and Progesterone receptor (PR):

Progesterone is a membrane soluble steroid hormone, secreted by the ovary, that produces intracellular signals and aids in formation of epithelial cells, differentiation and side-branching of cells, maturation and alveologenesis of the mammary gland [66, 67]. Cyclical variations in the levels of estrogen and progesterone make the menstrual cycle, which comprises of an unopposed estrogenic phase and luteal progestogenic phase. These cyclical variations have varying effect on the hormone-responsive tissues like the uterus, ovary and the breast. At the breast tissue level, progesterone hypothetically acts in a paracrine manner by stimulating the PR-positive cells in the tissue to activate signaling in the PR-negative cells and shows differential effects on cells based on their PR status [68, 69].

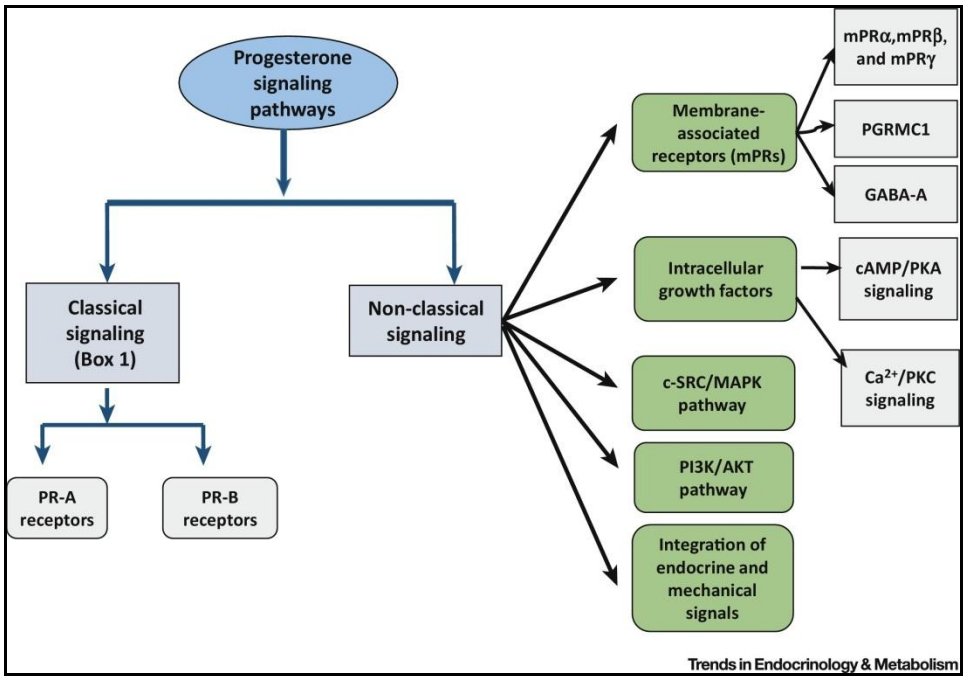


I-Figure- 2: Basic structure of the two isoforms of human progesterone receptor (hPR)
[Adapted from Chabbert-Buffet et al., 2005, Hum Reprod Update]. Different domains of the PR have been depicted where each abbreviation stands for— AF, activation function; ID, inhibitory domain; LBD, ligand binding domain; DBD, DNA binding domain; ATG, initiation of transcription codon [70].

PR is a core member of the superfamily of nuclear steroid hormone receptors [71]. *PR* is a single gene, located on chromosome position 11q21-11q23 and encodes two

isoforms— PR-A and PR-B, with PR-B being the complete protein (116KDa) synthesized from 933 amino acids, while PR-A lacks the first 164 amino acids at the N-terminal and is 769 amino acids in length (81KDa) (I-Figure-2) [72, 73]. PR is expressed in reproductive organs like the breast, endometrium, ovary and other organs like the brain where PR plays an important role in development, differentiation, and proliferation of target tissues and the pathological processes in endocrine-based cancers [74, 75]. The expression of PR is under tight regulation by estrogen and progesterone [63, 76]. PR is localized to the cytoplasm in a non-functional state, where it associates with Hsp90 chaperone. When progesterone enters the cell, it binds and dimerises PR and the complex enters the nucleus to function as a transcription factor by recognizing a consensus sequence GGT/AACAnnnTGTTCT, termed as progesterone response elements (PRE), in the promoter of target genes. This mode of activity is termed as the genomic pathway regulated by progesterone and PR. However, of the many potential PRE-binding sites in the genome, PR occupies only a small fraction upon progesterone stimulation, and that cooperation with transcription factors like FOXA1, ETS, STAT or SP1 are essential for binding PR cistromes [74, 75]. Other steroid hormone receptors like the glucocorticoid receptor (GR), mineralocorticoid receptor (MR) and androgen receptor (AR) recognize similar consensus sequences, and work synergistically through overlapping cistromes regulated by these hormone receptors [77-79]. Recently, progesterone-mediated alteration in ER cistrome by PR has been shown in breast cancer [58, 80]. Also, progesterone has been shown to associate with GR and AR to mediate its function in breast and other cancer types [77]. Thus essentially, progesterone may function even in the absence of PR by associating with other receptors; however, the pathways or

phenotypic changes activated in response to these events remain to be elucidated. Progesterone is also known to function via a non-genomic pathway by associating with other nuclear receptors (like ER or GR) or Src proteins in the cytoplasm and mediate immediate effects post hormonal stimulation [81]. Moreover, progesterone is known to associate with the membrane progesterone receptor (mPR) family, consisting of three members— mPRα, PGRMC1 and SERBP1, all of which interact with progesterone on the cell membrane and stimulate downstream signaling events in the cell, without entering the nucleus (I-Figure-3) [82, 83]. However, the clinical significance of these receptors is just emerging and needs large cohort validation [84, 85].



I-Figure- 3: The classical and non-classical signaling pathways regulated by progesterone

[Adapted from Garg et al., 2017, Trends Endocrinol Metab]. Non-classical pathway exerts rapid signaling events independent of the PR [86].

1.2.6 Phenotypic, genomic and proteomic effects of progesterone

Progesterone is primarily involved in the differentiation and proliferation of target cells in a context-dependent manner [87-89]. In the breast cells, progesterone induces cell proliferation in a biphasic manner, by increasing cell proliferation in the first 24-48hrs and then inhibiting proliferation during second cell cycle even after addition of fresh progesterone to the cells [90]. Under *in vitro* settings, progesterone functions as an anti-estrogenic moiety by reducing proliferation of breast cancer cells. However, the anti-proliferative activity was found to be dependent on the expression of PR in breast cancer cells [91, 92]. On the contrary, progesterone may induce proliferation of cancer cells under *in vivo* conditions [93]. Progesterone has also been shown to influence the growth of cancer cells, again in a PR-dependent manner [94, 95]. However these anti-proliferative and growth-repressing actions of progesterone are challenged by the model for development of breast cancer, where progesterone has been hypothesized to help in progression of the initial breast tumor lesions [69]. The pro-angiogenic property of progesterone has been observed in cancer cells by up-regulation of expression of vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) support this notion [96, 97]. However, progesterone may have a dual role in regulating the progression of cancer cells. It has been observed processes like migration and invasion of cancer cells are important for the progression and metastasis of cancer. To this end, progesterone has been shown to reduce the migration of PR-positive breast cancer cells *in vitro*. Not only does progesterone influence migration and cell morphology of breast-derived cells but also of cells derived from the lung and endometrium [58, 80, 98-101]. Whilst this

phenotypic influence, progestins have been shown to increase the migration of PR-positive breast cancer cells [102]. This disparity in the action of progesterone and progestin can be attributed to the differential effect imparted by these agents in breast cancer cells [103, 104]. However, it remains to be elucidated in a mechanistic manner whether progesterone can influence the migration and invasion of breast cancer cells independent of the PR status of cells.

These phenotypic effects of breast cancer cells derive their origin from the genomic and proteomic changes that progesterone induces in a context-dependent manner. The genomic effects are transient transcriptional changes triggered by change in chromatin architecture via recruitment of transcription factors [105]. Progesterone recruits chromatin modifiers and induces transcriptional changes via association with PR and other hormone receptors (like GR and ER) to either activate or repress the transcription of target genes [58, 106, 107]. It has been proposed that progesterone can regulate expression of gene sets in a similar manner in topologically associating domains (TADs) [108]. Interestingly, PR can alter the transcriptional activity of ER in breast cancer cells treated with estrogen and progesterone, suggestive of an anti-estrogenic activity of progesterone in breast cancer [58, 80]. Moreover, studies have been conducted to study genes regulated by progesterone receptor in an isoform-specific manner. Majority of these targets of progesterone are related to regulation of cell-cycle and proliferation [109, 110].

On the other hand, effect of progesterone on expression and activation of proteins has remained under-studied. Proteomic analysis of bovine uterus has been performed which suggests an essential role of progesterone in development of embryo during the

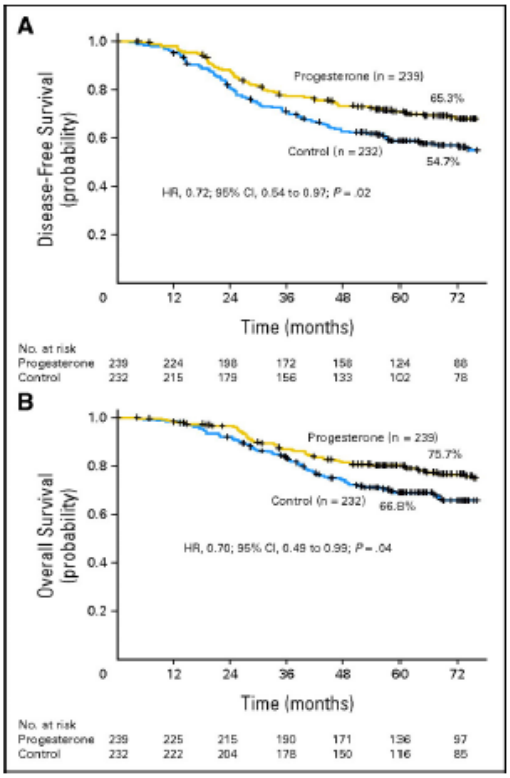
pre-implantation stage [111]. Recently, a LC-MS/MS-based analysis of the human endometrial cells treated with progesterone in combination with calcitriol has identified proteins involved in the anti-tumorigenic action of progesterone [112]. However, such global proteome profiling efforts have not been taken using the human breast tissue cells. Mostly, candidate-based approaches have characterized the effect of progesterone on proteins involved in regulation of cell-cycle or proliferation of cells. Reports suggest that progesterone inhibits estrogenic activity by up-regulating proteins that inhibit growth of the PR-positive breast cancer cells [95, 113]. Certain studies have also shown that progesterone alters the phosphorylation of certain kinases and increases migration of breast cancer cells [102, 114, 115]. A detailed characterization of effect of progesterone on the activity of kinases also remains to be explored.

1.3 Progesterone influences survival of breast cancer patients:

Physiologic concentrations of estrogen, progesterone, prolactin hormones influence processes of pregnancy and development of hormone-responsive organs in women. On the other hand, prolonged exposure to these hormones has also been associated with development of cancer, especially estrogen [116]. However, few studies have found no harm associated with endogenous progesterone or as hormone replacement therapy given to postmenopausal women [117-120]. And a lot of debate surrounds the use of progestin for hormone replacement therapy, the jury is still out [121].

Observational studies have been conducted to study the effect of the phases of menstrual cycle on the outcome of breast cancer. The analysis suggests that surgery performed in the progestogenic (luteal) phase associates with a favorable prognosis as compared to surgery performed in other phases of the menstrual cycle [122-124].

Moreover, it was found that elevated serum progesterone levels correlated with better survival of breast cancer patients [124, 125]. These early observational studies conceived a randomized clinical trial in Tata Memorial Hospital (TMH), India, where a cohort of operable breast cancer patients was subjected to single-depot progesterone intervention 5-14 days before surgery. Patients with different menopausal stages, PR expression and tumor grades were randomly grouped into the control group and the pre-operative progesterone group. Overall in the cohort of 1000 patients, progesterone did not influence the survival of patients; however, analysis in a selected cohort of patients with node-positive disease showed a 10% increase in disease-free and overall survival of patients receiving pre-operative progesterone treatment as compared to patients in the control group. Interestingly, the beneficial effect was observed independent of the PR status or the menopausal status of breast cancer patients (I-Figure-4). Though pre-/peri-operative endocrine intervention has shown beneficial effects in receptor-positive breast cancer patients [126, 127], the randomized clinical trial by Badwe et al is the first of its kind with pre-operative progesterone intervention and provides a hypothesis-generating outcome to be further tested on a larger cohort [128].



I-Figure- 4: Survival analysis with pre-operative progesterone treatment
[Adapted from Badwe et al., 2011, J Clin Oncol] The survival plots depict overall and disease free survival for node-positive breast cancer patients [128]

Moreover, recently it has been quoted that perioperative progesterone intervention may have beneficial effects on survival of obese postmenopausal women [129]. To be assigned to endocrine therapies, the patients are monitored for presence of hormone receptors, so that the patients can benefit from these agents. However, the randomized clinical trial did not enrich only receptor positive patients and the effect of progesterone was independent of the PR-status. Thus the findings are intriguing and mechanistic approaches to understand the role of progesterone independent of PR expression are required. These clinical findings are of immense value since a single injection of progesterone rendered patients a better prognosis [130].

1.4 Rationale of the study:

Vast numbers of studies have focused on identifying the PR-dependent gene expression profiles in breast cancer [109, 131]. Although many candidate-based studies have been performed, no global proteomic and genomic changes have been reported specifically in response to progesterone in breast cancer, independent of the PR status. Moreover, majority of the studies have been performed in presence of other hormones. Hence, whether progesterone can alter transcriptional signature of breast cancer cells irrespective of the PR expression remains to be explored.

Of note, to identify targets of progesterone independent of PR, one approach could be to perform transcriptional and proteomic profiling of breast cancer patient-derived tissue samples from the randomized clinical trial with pre-operative progesterone and compare the expression profiles with that of tumor samples derived from patients in the control group. Such a study can provide immense details about the long-term effects of progesterone on breast cancer and is currently being performed at Tata Memorial Centre. However, the unique targets of progesterone may not be discerned from this approach since it will be an additive effect of all hormones together with progesterone, together with the heterogeneity of tumor samples. It has already been suggested that progesterone can alter the transcriptional profile of breast cancer in the presence of estrogen using *in vitro* studies [58]. Moreover, the effect observed in samples derived from the clinical trial will be a delayed response to progesterone and it may not be possible to validate the results using orthologous methodologies. An alternative approach is to perform an *in vitro* study using a panel of breast-derived cell lines which represent different hormone receptor statuses and breast cancer subtypes and technically are a better system for performing functional genomic and proteomic

analysis. A large repertoire of breast-derived cell lines can be recruited for such a study [132-134]. These cell lines are a homogenous system and provide the opportunity for functional validation of candidate genes using genetic and pharmacologic manipulation, in this case in response to progesterone. Of note, the progesterone-specific effects can be monitored by regulating the progesterone treatment conditions and by depletion of most of the interfering hormones and growth factors from the conditioning medium and the cell lines provide an opportunity to block the activity of PR receptor to identify PR-independent targets of progesterone [70, 135, 136]. The effect of progesterone can be elucidated using short-term (from few minutes to few hours) or long-term (24-72hrs) treatment. Most of the studies to understand the genomic effects of progesterone have utilized short-term (3-6hrs) treatment conditions since hormones induce gene expression changes immediately after their addition to cells [58, 109]. Thus cell lines represent a better system to elucidate the molecular action of progesterone in breast cancer and also are of immense utility to study the role of candidate genes by genetic perturbation approaches.

II. OBJECTIVE OF THE STUDY

Specifically, few key questions need to be addressed to elucidate the targets of progesterone in breast cancer:

1. What are the genomic and proteomic targets of progesterone in breast cancer cells independent of the PR status?

2. Can progesterone abrogate the cell migration and invasion phenotype of breast cancer?

3. What is the possible mode of action of progesterone that helped improve the survival of breast cancer patients?

To provide a molecular basis for solving each of the above questions, it is imperative to adopt a functional genomics and proteomics approach using a panel of breast-derived cell lines to capture the short-term effects of progesterone, without bias to the hormone receptor status of the cells.

To fill the void in the understanding of targets of progesterone, we have the following specific objectives:

2.1 Genomic approach to identify targets of progesterone in breast cell lines

In this objective, we aim to identify the coding and non-coding targets, identified using microarray gene expression analysis and small RNA sequencing analysis respectively and performed in a panel of breast-derived cell lines, in response to progesterone treatment.

2.2 Proteomic analysis of breast cell lines upon progesterone treatment

In this second objective, we have focused on the proteomic changes induced by progesterone, independent of the PR status of cell lines.

2.3 Functional validation of progesterone candidate genes in breast cancer cell line by rescuing phenotype

In this final part of the study, we have performed functional validation of candidate genes and microRNAs obtained from both the earlier objectives. Specifically, we have performed genetic and pharmacological perturbations of the candidate genes or microRNAs, phenotypic assays to understand the global effects induced by progesterone and upon perturbation of candidate genes, irrespective of the hormone receptor statuses.

III. PROGESTERONE SUPPRESSES THE INVASION AND MIGRATION OF BREAST CANCER CELLS IRRESPECTIVE OF THEIR PROGESTERONE RECEPTOR STATUS – A SHORT REPORT

[An excerpt; as published in *Cellular Oncology* (2017); 40(4):411-417]

Abstract

Purpose Pre-operative progesterone treatment of breast cancer has been shown to confer survival benefits to patients independent of their progesterone receptor (PR) status. The underlying mechanism and the question whether such an effect can also be observed in PR negative breast cancer cells remain to be resolved.

Methods We performed proteome profiling of PR-positive and PR-negative breast cancer cells in response to progesterone using a phospho-kinase array platform. Western blotting was used to validate the results. Cell-based phenotypic assays were conducted using PR-positive and PR-negative breast cancer cells to assess the effect of progesterone.

Results We found that progesterone induces de-phosphorylation of 12 out of 43 kinases tested, which are mostly involved in cellular invasion and migration regulation. Consistent with this observation, we found through cell-based phenotypic assays that progesterone inhibits the invasion and migration of breast cancer cells independent of their PR status.

Conclusion Our results indicate that progesterone can inhibit breast cancer cell invasion and migration mediated by the de-phosphorylation of kinases. This inhibition appears to be independent of the PR status of the breast cancer cells. In a broader context, our study may provide a basis for an association between progesterone

treatment and recurrence reduction in breast cancer patients, thereby providing a lead for modelling a randomized *in vitro* study.

3.1 Introduction

Metastasis is a major cause of recurrence in breast cancer patients [48]. As a standard mode of treatment, patients with metastatic breast cancer are subjected to adjuvant hormonal therapy [137]. Also, pre-operative progesterone treatment has been shown to reduce the recurrence rate among node-positive patients, independent of their progesterone receptor (PR) status [128]. While the invasion and migration of breast cancer cells play major roles in establishing metastases [138, 139], *in vitro* cell-based observations on the effect of progesterone, restricted to PR positive cells, have corroborated the clinical observations [58, 80, 102]. As yet, however, the effect of progesterone on the invasion and migration of PR negative breast cancer cells remains to be systematically explored [140].

The metastatic nature of breast cancer cells is known to be affected by multiple molecular factors, including the activation of protein kinases [141]. The protein kinases EGFR, AKT or FAK have, for instance, been found to activate the processes of migration and invasion of breast cancer cells [142, 143]. In addition, it has been found that these kinases may act synergistically and that abrogating their activation may decrease the invasive capacity of breast cancer cells [144]. Also, pathways downstream of these kinases may serve to restrain cell invasion and migration [141]. Although these kinases have been found to be affected by steroid hormone receptors [145], it remains to be explored whether they mediate the responses to progesterone in breast cancer cells.

To address the question whether progesterone can regulate cellular migration and invasion of breast cancer cells independent of their PR status, we selected a panel of breast cancer-derived cell lines with different PR statuses. Next, we performed a phospho-proteomic screening of kinases associated with migration and invasion using a human proteome phospho-kinase array platform, and studied their phosphorylation status after treating the respective cells with progesterone. Our cell-based phenotypic and biochemical analysis results suggest that progesterone may mitigate the invasion and migration of breast cancer cells, irrespective of their PR status.

3.2 Materials and methods

3.2.1 Breast cancer-derived cells

The BT474, T47D, MCF7, ZR-75-1, MDA-MB-231 and BT-549 breast cancer-derived cell lines were obtained as a gift from Dr. Slamon's Laboratory (Department of Medicine, UCLA, USA). The cell lines were authenticated by DNA short tandem repeat (STR) profiling using the Promega GenePrint 10 system in conjunction with the GeneMarker HID software tool and the ATCC database. The cells were tested for mycoplasma and, if necessary, made mycoplasma-free using an EZKill Mycoplasma Removal reagent (HiMedia). BT474, T47D, MCF7 and MDA-MB-231 cells were cultured in DMEM medium (Gibco), whereas ZR-75-1 and BT-549 were cultured in RPMI-1640 medium (Gibco). BT-549 cells were supplemented with 0.023 IU/ml insulin. All culture media were supplemented with 10% (v/v) FBS (Gibco), 2.5 mg/ml Amphotericin-B (Abbott) and 1.25 µl/ml Gentamycin (Abbott). The cells were

cultured at 37°C in a 5% CO_2 incubator. The PR/ER/Her2 receptor statuses of all the cells as reported in [133] were validated by reverse transcriptase-PCR.

3.2.2 Progesterone treatment

Cells were grown to 70-80% confluence and then serum starved in DMEM low glucose medium (HiMedia) for a period of 24h. Next, the cells were treated with 10 nM 17-α hydroxy-progesterone caproate (progesterone) (MP Biomedicals) in the same medium for 6h. In case of mifepristone + progesterone (M+P) combination treatment, 100 nM RU486 (mifepristone) was added for 2h followed by 10 nM progesterone treatment for 6h in the same medium. An equal volume of alcohol was used as vehicle control.

3.2.3 Protein sample preparation

Cells were grown to a 70-80% confluence in a 100 mm culture dish and washed thoroughly with sterile 1x PBS. Next, the cells were subjected to 24h serum starvation (using DMEM low glucose phenol-red free medium) followed by progesterone treatment for 8h. Alcohol was used as vehicle control. After progesterone treatment, the cells were harvested using a sterile cell scraper and cell lysates were prepared in RIPA Buffer (Sigma-Aldrich) supplemented with a protease-inhibitor cocktail solution (Sigma-Aldrich) and 0.1 M DTT. After intermittently tapping and vortexing the samples on ice, cell debris was pelleted by centrifugation at 14000 rpm after which the protein concentrations were determined using BCA reagent (MP Biomedicals). Bovine serum albumin was used as a standard and the estimations were performed in triplicate.

3.2.4 Phospho-kinase activation profiling

Kinase activation profiling of T47D (PR-positive) and MDA-MB-231 (PR-negative) breast cancer-derived cells was performed using a Human Phospho-kinase array kit (ARY003B; R&D Systems) according to the manufacturer's instructions. Briefly, cells were grown in T75 flasks till 70-80% confluence was reached, serum-starved for another 24 h (in serum-free phenol-red-free DMEM medium) and treated with progesterone for 8h. Next, the cells were harvested, washed with 1x PBS and lysed, after which 400 μg protein from untreated and progesterone-treated samples was incubated overnight at 4°C with a pre-blocked antibody array nitrocellulose membrane. Subsequently, the membranes were incubated with detection antibodies and probed using streptavidin-HRP, after which signals were developed using chemi-reagents provided with the kit. Exposures to X-ray films were taken from 10 seconds to 10 minutes (till saturation was reached). Signal densities of reference spots on both membranes were compared between each pair of membranes used for the control and progesterone-treated samples. The pixel density of each spot, in duplicate, was calculated using ImageJ Array Analyzer plugin. The average pixel density for the duplicate spots for each of the kinases was subtracted from the negative control density. The average pixel densities for control and progesterone-treated samples were plotted as percent phosphorylation for each phospho-kinase. The differential phosphorylation cut-off value was set at 20% increase or decrease in phosphorylation of kinases in response to progesterone.

3.2.5 Western blotting

Equal amounts of cell lysate were resolved by 10% SDS-PAGE and transferred to PVDF membranes using a wet transfer method. Primary antibodies directed against p-EGFR (Y1068) (Cell Signaling, 3777S; Dilution 1:500), p-AKT (S473) (Cell Signaling, 4060S; Dilution 1:500), p-ERK1/2 (T202/Y204) (Cell Signaling, 9101S; Dilution 1:1000), total EGFR (1005) (Santa Cruz Biotechnology, sc-03; Dilution 1:1000), total AKT (11E7) (Cell Signaling, 4685S; Dilution 1:1000), total ERK2 (c-14) (Santa Cruz Biotechnology, sc-154; Dilution 1:1000) and β-actin (I-19)-R (Santa Cruz Biotechnology, sc-1616-R; Dilution 1:3000) were diluted in 3% BSA solution prepared in 1x TBST and incubated over-night at 4°C. A goat anti-rabbit IgG-HRP secondary antibody (Santa Cruz Biotechnology, sc-2004; Dilution 1:3000) was used for the detection of primary antibody binding. ECL Western Blotting Substrate (Pierce) and Takara Chemiluminescence substrate (ClonTech Takara) were used for visualization of the protein bands on X-ray films (Fuji Films).

3.2.6 RNA extraction and real-time PCR

For RNA extraction from alcohol and progesterone-treated breast cancer-derived cells, the respective cells were treated with progesterone for 6h. Next, TRIzol reagent (Invitrogen) was used to lyse the cells after which RNA was isolated according to the manufacturer's protocol. RNA concentrations were measured using NanoDrop. For assessment of the *DUSP1* transcript levels, cDNA was synthesized using a High capacity cDNA reverse transcription kit (Applied Biosystems) and subjected to quantitative real-time PCR using a Roche Light-Cycler-II 480 instrument in conjunction with a Roche real-time master mix (Roche). Expression changes were

calculated using the $2^{-\Delta\Delta CT}$ method. *GAPDH* was used as internal control for normalization. The primer sequences used for *DUSP1* were Forward primer OAD-571: CCTGCAGTACCCCACTCTACG; Reverse primer OAD-572: CCCAAGGCATCCAGCATGTCC and for *GAPDH* Forward primer OAD-328: AATCCCATCACCATCTTCCA; Reverse primer OAD-329: TGGACTCCACGACGTACTCA.

3.2.7 Cell invasion assay

A Matrigel invasion assay was performed using 24-well Transwell inserts (Corning) coated with 100 µg matrigel and allowed to settle for 24hr at 37ºC. Next, 35000 cells suspended in 350 µl serum-free medium were seeded into the upper chamber and 600 µl of 10% serum-containing medium was added to the lower chamber. After this, the cells were allowed to invade for 16-18h at 37ºC, followed by fixation of the invaded cells and staining by crystal violet. After mounting the membrane using DPX on a slide, the cells were observed under an upright microscope. Ten random fields were chosen after which the number of cells in each field was counted and plotted as percentage cell invasion.

3.2.8 Scratch wound healing assay

Confluent cell monolayers in 6-well plates, grown in phenol-red free DMEM for 18hrs without serum, were subjected to a scratch with a sterile pipette tip. After this, the cells were briefly rinsed using 1X PBS to remove debris and subsequently incubated with low-glucose phenol-red free DMEM medium containing 10% charcoal-stripped FBS (Gibco). The cells were treated with 10 nM progesterone or

100 nM mifepristone or a combination of both. Alcohol was used as a vehicle control. Cell migration at the wound surface was measured during a period of 20 h under an inverted microscope. Quantification was performed using the ImageJ wound healing plugin tool by measuring the distance of the wound edge of the migrating cells from the start point to the migrated point in three separate wounds in three independent experiments.

3.3 Results and discussion

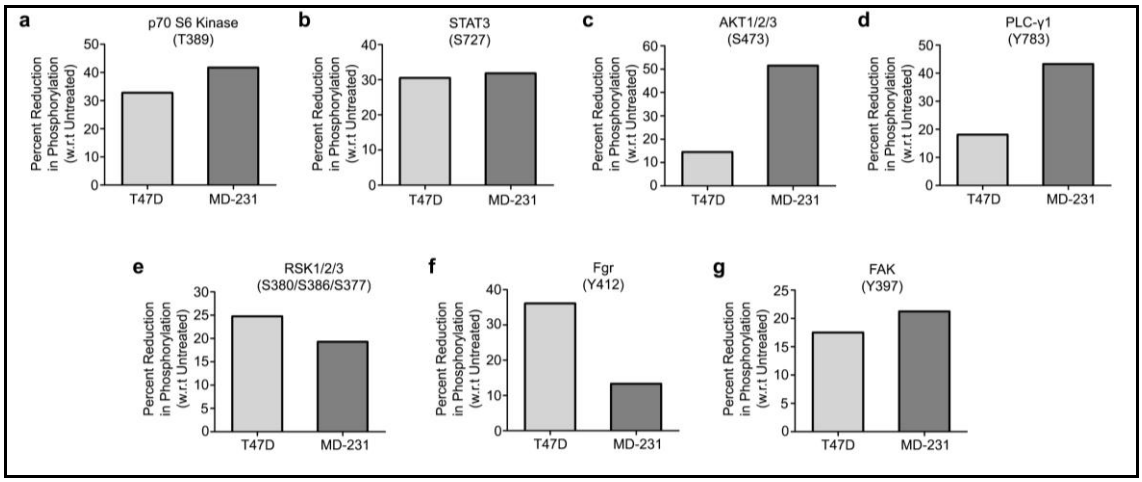
The activation of kinases like EGFR and ERK1/2 has been reported to play an important role in the de-regulation of cellular processes that are associated with the metastatic capacity of breast cancer cells [146]. Here, we set out to assess the effect of progesterone on the activation of kinases in breast cancer cells using a human phospho-kinase array platform. To verify the effect of progesterone independent of the progesterone receptor (PR) status of the cells, we selected both PR-positive (T47D) and PR-negative (MDA-MB-231) breast cancer-derived cells for our study (III-Table 1). Untreated cells were used as negative controls.

Sr. No.	Cell Line	Literature reported Receptor Status			Validation of Receptor Status by RT-PCR		
		PR	ER	HER2	PR	ER	HER2
1.	BT474	+	+	+	+	+	+
2.	T47D	+	+	-	+	+	-
3.	MCF7	+	+	-	+	+	-
4.	ZR-75-1	-	+	-	-	+	-
5.	MDA-MB-231	-	-	-	-	-	-
6.	BT-549	-	-	-	-	-	-

III-Table- 1: Selection of breast cell lines and validation of PR/ER/Her2 hormone receptor status

A panel of breast cancer cell lines with varying receptor statuses, as reported in literature, was selected for studying the effects of progesterone. The validation status of PR/ER/Her2 transcript expression in the cell lines is indicated as "+" (positive) or "-" (negative).

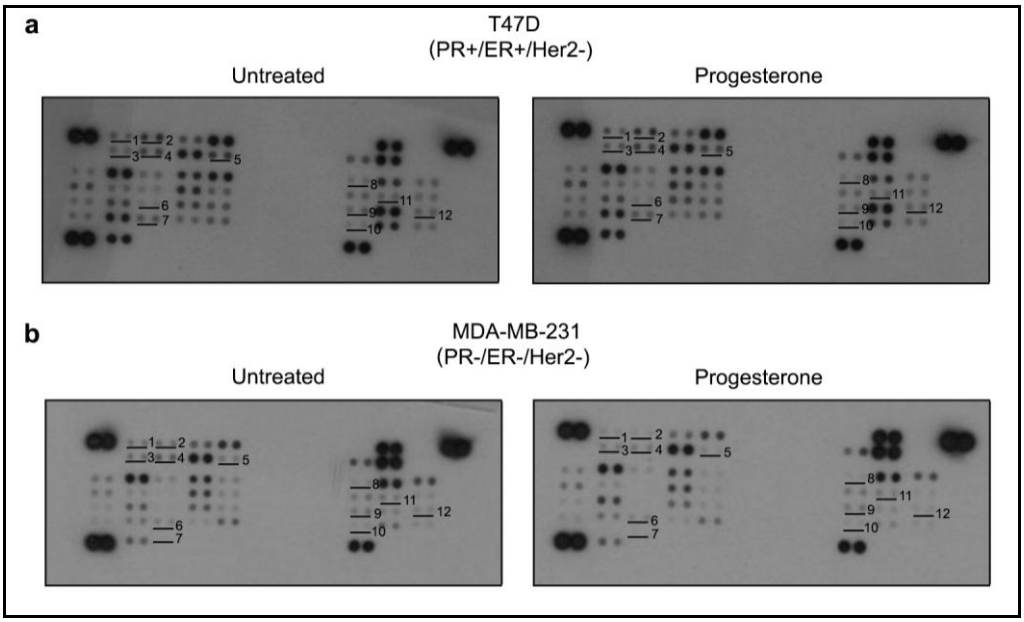
As reported before, we observed a breast cancer cell-specific phosphorylation of p53 (S392/S46/S15) and AMPK (T183), which were subsequently used as internal positive controls [147, 148]. Based on differential phosphorylation analyses of the T47D and MDA-MB-231 cells, 7 out of 43 kinases tested were found to be de-phosphorylated in the progesterone treated cells (III-Figure-1a-g and III-Figure-2). Of these, p70 S6 kinase and STAT3 showed the highest decrease in phosphorylation (30%) while FAK, AKT and RSK1/2/3 showed a 20% decrease in both the cell lines in response to progesterone treatment.



III-Figure- 1: Kinase phosphorylation is modulated by progesterone in breast cancer cells

The percentage of reduction in phosphorylation in response to progesterone was calculated relative to that in untreated cells and is plotted for each of the differentially phosphorylated kinases (panels a-g). In the bar plot the light grey bar indicates phosphorylation reduction in T47D cells and the dark grey bar indicates phosphorylation reduction in MDA-MB-231 cells.

In addition, we observed a reduction in phosphorylation of the ERK1/2 (T202/Y204, T185/Y187), EGFR (Y1068), MSK1/2 (S376/S360), p38α (T180/Y182) and p27 (T198) kinases upon treatment with progesterone (III-Figure-2), as reported earlier [91], and validated the results by Western blot analysis (III-Figure-3a). Consistent with earlier reports [91], we also observed a significant up-regulation of a dual specificity phosphatase, DUSP1, upon treatment with progesterone in breast cancer cells that could possibly mediate the effect observed (III-Figure-3b).

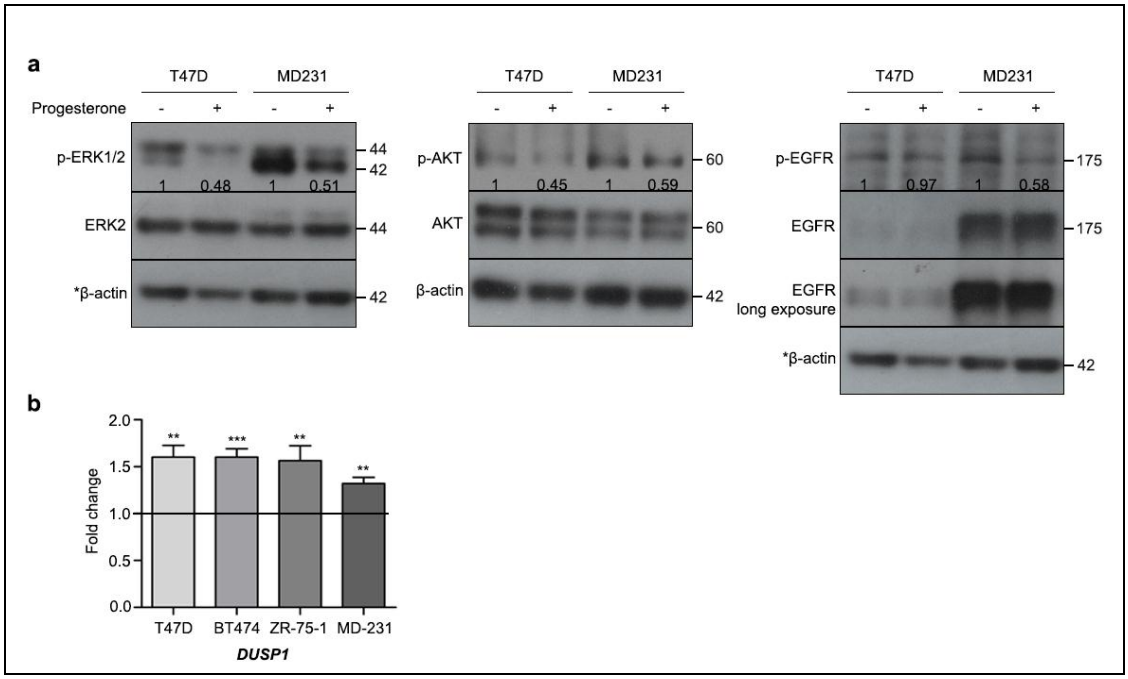


III-Figure- 2: Differentially phosphorylated kinases in response to progesterone treatment of breast cancer cells

The figure represents X-ray films exposed to nitrocellulose membrane for 5min with untreated and progesterone-treated lysates from **a)** T47D and **b)** MDA-MB-231 cells. Black lines and numbers on the right of the spots indicate the location of differentially phosphorylated kinases in untreated and progesterone-treated sets for both cells. The annotation of the spots is as follows:
1- p38α; **2**- ERK1/2; **3**- EGFR; **4**- MSK1/2; **5**- AKT1/2/3; **6**- Fgr; **7**- FAK; **8**- p70S6K; **9**- p27; **10**- STAT3; **11**- RSK1/2/3; **12**- PLC-γ1.

Taken together, our results indicate that progesterone can reduce the phosphorylation of 12 out of 43 kinases tested in a PR-independent manner, which could affect cellular

signaling pathways downstream to these kinases with a concomitant increase in the expression of a dual-specificity phosphatase, *DUSP1*, that could mediate the de-phosphorylation of these kinases [91].



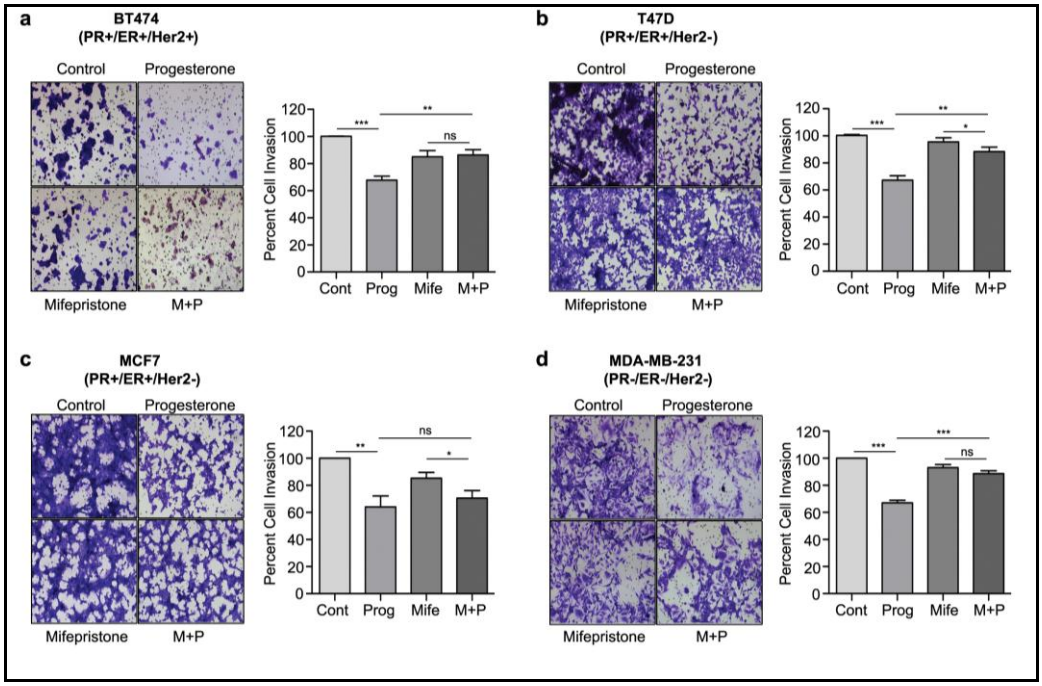
III-Figure- 3: Progesterone suppresses phosphorylation of kinases involved in cell migration and invasion in breast cancer cells by up-regulating DUSP1 (modified from the manuscript)

a) Western blot analysis of p-EGFR (Y1086), p-AKT (S473) and p-ERK1/2 (T202/Y204) was performed in T47D and MDA-MB-231 (MD231) cells treated with progesterone. Total protein for each phospho-kinase was also probed. Numbers on blot indicate ratio of intensity of phosphorylation for each kinase with respect to its total protein levels. β-actin was used as internal loading control. "*****" mark on β-actin indicates same β-actin blot used for p-ERK1/2 and p-EGFR panels. "-" on the western blot panel indicates control, while "+" indicates progesterone treatment. **b)** Real-time PCR analysis of *DUSP1* was performed in breast cancer cells treated with progesterone. Graph has been plotted as fold-change for *DUSP1* with respect to *GAPDH* for control and progesterone-treated cells; horizontal black line indicates expression of *DUSP1* in control cells. Figure is representative of three independent experiments performed in triplicates. *P*-value was calculated using student's unpaired t-test. ** indicates *P*-value <0.001; *** indicates *P*-value <0.0001.

Based on the known involvement of EGFR, AKT and ERK1/2 in the invasion and migration of breast cancer cells and the finding that their inhibition may block this phenotype [142, 143, 149] or *vice versa*, i.e., metastases inhibitors may inhibit the phosphorylation of FAK in PR-negative MDA-MB-231 cells [150] or lung cancer cells [99], we set out to analyze the *in vitro* effects of progesterone on breast cancer cells. Using a Matrigel chamber assay in conjunction with cells with varying PR/ER/HER2 statuses (III-Table-1) we found that progesterone could decrease the invasion capacity of different breast cancer-derived cells (BT474, T47D, MCF7 and MDA-MB-231), irrespective of their hormone receptor status (III-Figure-4a-d). This result suggests that targeted activation of kinases by progesterone may bring about phenotypic changes in breast cancer-derived cells independent of their PR-status, potentially decreasing their metastatic capacity similar to combinatorial EGFR and AKT inhibition, which is known to affect the invasion of breast and other cancer cells mediated by matrix metalloproteinases [144, 151]. Importantly, we found that PR blocking by mifepristone had no significant effect on the invasive capacities of the cells, again suggesting that progesterone may induce suppression of invasion in breast cancer cells in a predominantly PR-independent manner (III-Figure-4a-d).

Next we performed scratch wound healing assays to assess whether breast cancer cell migration is affected by progesterone. Similar to the effect of progesterone on breast cancer cell invasion, we observed a significant decrease in cellular migration in response to progesterone over the period of 20h in a PR independent manner (III-Figure-5). The non-essential role of PR that we observed in the inhibition of migration of breast cancer-derived cells in response to progesterone, specifically in the PR-negative MDA-MB-231, ZR-75-1 and BT-549 cells, may be mediated by interaction

of progesterone with the glucocorticoid receptor (GR) or the membrane progesterone receptor (mPR), as has been reported before [77, 98, 99].

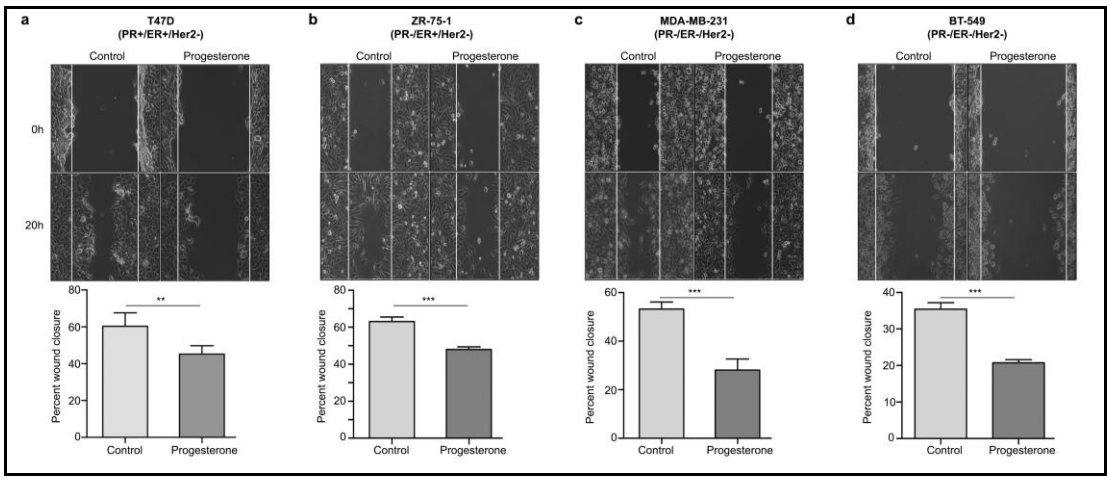


III-Figure- 4: Progesterone inhibits breast cancer cell invasion

Invasion assays were performed with breast cancer-derived cell lines **a**) BT474, **b**) T47D, **c**) MCF7 and d) MDA-MB-231 treated with progesterone, mifepristone or a combination of mifepristone and progesterone (M+P). The bar plot represents the percentage of cell invasion for each panel. The figure is representative of three independent experiments performed in triplicates. *P*-values were calculated using student's unpaired t-test. ** $p < 0.001$; *** $p < 0001$; ns not significant.

Of note, it has also been reported that treatment with glucocorticoids may similarly decrease the migration of PR-negative MDA-MB-231 cells [152], which suggests that redundant pathways may underlie the progesterone response in a PR-independent manner [83]. Consistent with these observations, we found that blocking PR by mifepristone prior to exposing the cells to progesterone did not rescue the effect of progesterone, suggesting that the progesterone-mediated suppression of migration in breast cancer cells is predominantly mediated in a PR-independent manner (III-

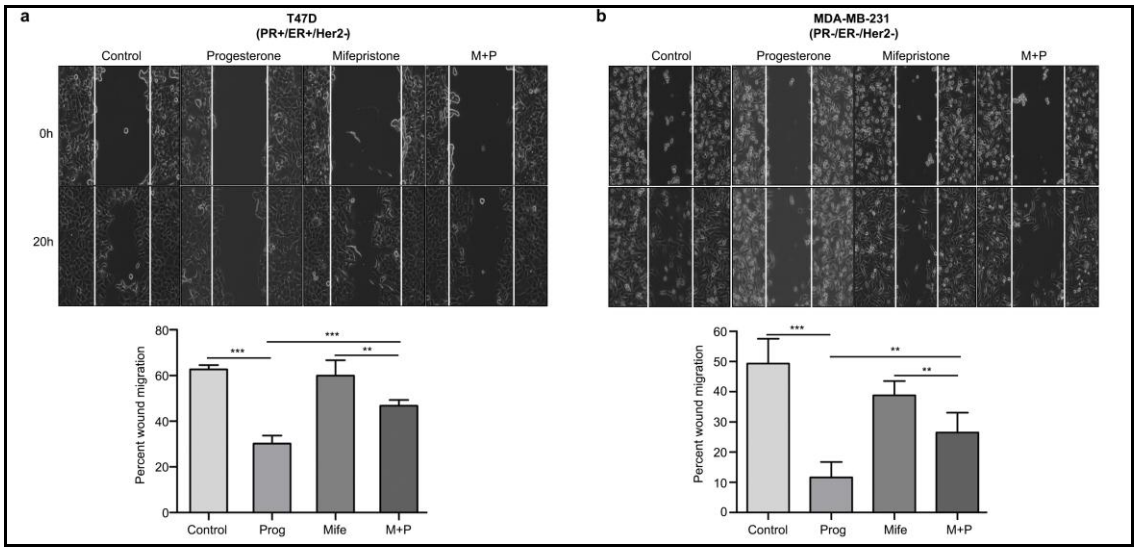
Figure-6a and 6b). This result corroborates a clinical study in which it was found that progesterone may reduce the recurrence of node-positive breast cancer in patients independent of their PR status [128].



III-Figure- 5: Migration of breast cancer cells decreases in response to progesterone treatment

Scratch wounds were made in breast cancer-derived cell lines **a**) T47D, **b**) ZR-75-1, **c**) MDA-MB-231 and **d**) BT-549, with differing receptor statuses. Subsequently, the cells were treated with alcohol (control) and progesterone for 20hrs and followed in time for migration. The bar plots indicate the percentages of cellular migration, with direct comparisons between control and progesterone treated cells. The figures are representative of three independent experiments performed in triplicates. *P*-values were calculated using student's unpaired t-test. ** $p < 0.001$; *** $p < 0.0001$.

A recent *in vitro* study, however, suggested that the PR status may play an essential role as no significant effect was observed in PR-negative MDA-MB-231 cells in response to R5020, which is a synthetic progestin [153]. But, it has also been shown that the downstream effects of progesterone and progestin may be variable [103] and this notion, together with possible variations that may occur during cell line passage, could account for the phenotypic differences observed in MDA-MB-231 cells. Moreover, we found that the progesterone-mediated suppression of migration and invasion also occurred in other PR-negative breast cancer-derived cells, i.e., ZR-75-1 and BT-549, which has not been reported before.



III-Figure- 6: Mifepristone antagonizes the effect of progesterone on cell migration

a) T47D and **b)** MDA-MB-231 cells were treated with alcohol (control), progesterone, mifepristone and mifepristone+progesterone for 20hrs and followed for time-lapse cellular migration assay. Bar plots indicate percentage cellular migration of the cells, with a comparison between control and progesterone; mifepristone and mifepristone+progesterone; and progesterone and mifepristone+progesterone treated cells. Figures are representative of three independent experiments performed in triplicates. *P*-value was calculated using student's unpaired t-test. ** indicates *P*-value<0.001; *** indicates *P*-value<0.0001.

In summary, we present a first lead to model a randomized in vitro study to systematically elucidate the role of kinases that may underlie the clinical outcome of pre-operative progesterone intervention in breast cancer patients.

IV. *miR-129-2* MEDIATES DOWN-REGULATION OF PROGESTERONE RECEPTOR IN RESPONSE TO PROGESTERONE IN BREAST CANCER CELLS

[An excerpt; as published in *Cancer Biology and Therapy* (2017); 18(10):801-805]

Abstract

Objective

Hormonal therapy is an important component of first line of treatment for breast cancer. Response to hormonal therapy is influenced by the progesterone receptor (PR)-status of breast cancer patients. However as an early effect, exposure to progesterone decreases expression of PR in breast cancer cells. An understanding of the mechanism underlying down-regulation of PR could help improve response to hormonal therapy.

Methods

We performed small RNA sequencing of breast cancer cells for identification of microRNAs targeting *PR* in response to progesterone treatment. Biochemical approaches were used to validate the findings in breast cancer cells.

Results

Analysis of small RNA sequencing of four breast cancer cell lines treated with progesterone revealed an up-regulation of *miR-129-2* independent of the PR status of the cells. We show that *miR-129-2* targets 3'UTR of PR to down-regulate its expression. Furthermore, inhibition of *miR-129-2* expression rescues the down-regulation of PR in breast cancer cells. Also, the expression levels of *miR-129-2* was observed to be elevated in patients with low expression of PR in the TCGA cohort (n=359).

Conclusion

miR-129-2 mediates down-regulation of PR in breast cancer cells in response to progesterone, while anti-*miR-129-2* could potentiate PR expression levels among patients with inadequate PR levels. Thus, modulation of activity of *miR-129-2* could stabilize PR expression and potentially improve response to hormonal therapy under adjuvant or neo-adjuvant settings.

4.1 Introduction

Breast cancer is the most prevalent cancer among women worldwide. Despite all advances in early diagnosis and treatment, nearly 30% node-negative and 70% node-positive patients relapse with metastatic disease [21]. Treatment of breast cancer patients is influenced by the presence of estrogen receptor (ER) and progesterone receptor (PR). ER/PR positive patients tend to respond better to hormonal therapy and have a lower risk of relapse compared to ER-positive, PR-negative patients [59]. The down-regulation of *PR* expression in breast cancer cells is caused either by methylation at *PR* promoter [154], or in response to progesterone by post translational modification of the PR protein by CUEDC2 and MAPK [63, 64]. Growing evidence also suggest microRNAs to respond to steroid hormones and suppress the activity of respective hormone receptor [65]. For instance, miR-18a, miR-19b and miR-20b (paralogous pri-microRNAs) down-regulate the expression of ER in response to estrogen in breast cancer [61]. Comparatively, similar regulation of microRNA expression in response to progesterone has been less explored [155]. In order to study the progesterone-regulated microRNAs targeting PR, we performed small RNA sequencing of breast cancer cell lines treated with progesterone. The differentially expressed microRNAs were used to identify microRNAs that target 3'UTR of PR. Our

analysis reveals *miR-129-2* targets PR and is up-regulated in response to progesterone. The association of *miR-129-2* and PR was functionally validated by luciferase assay. Also western blot analysis suggests that inhibition of *miR-129-2* stabilizes PR in breast cancer cells even in presence of progesterone. Moreover, patients with high *miR-129-2* levels had significantly lower expression of PR as compared to patients with no *miR-129-2* expression in The Cancer Genome Atlas cohort.

4.2 Materials and Methods

4.2.1 Breast Cell lines

T47D, BT474, MDA-MB-231 and MCF7 breast cancer cell lines were cultured, grown and confirmed the ER/PR/Her2 receptor status as explained in Chapter-III section 3.2.1. Additionally, the human embryonic kidney 293FT cells were obtained from Invitrogen and cultured in DMEM medium supplemented with 10%FBS. All the cell lines were authenticated using STR profiling and made mycoplasma-free as described in section 3.2.1.

4.2.2 Progesterone treatment, RNA isolation and protein sample preparation

Progesterone treatment, RNA isolation and protein sample preparation were performed as explained in sections 3.2.2, 3.2.3, 3.2.5 and 3.2.6 of Chapter-III. For western blotting, primary antibodies used were PR-AB (sc-810, 1:300 dilution) and β-actin (sc-1616-R, 1:4000 dilution). Secondary antibody used was goat anti-mouse (sc-2005, 1:3000 dilution) and goat anti-rabbit (sc-2004, 1:3000 dilution).

4.2.3 Small RNA sequencing analysis

Small RNA sequencing was performed on single lane of Illumina HiSeq 1000 with eight multiplex libraries from the four breast cancer cell lines. The reads obtained from deep sequencing of small RNAs were subjected to Illumina adaptor trimming using FastX tool kit and were size filtered to select for candidate miRNA's (14 to 24 bases) from a pool of small RNA sequences using in-house perl script. The size separated reads were then mapped onto human miRNA reads obtained from miRBase (version 21) using Bowtie2 (version 2.1.0) [156] with 0 mismatches in the first 8 bases. MicroRNAs were quantified followed by normalisation by read per million using in-house script. Deregulated miRNAs with >= 3fold change were retained for further analysis. For searching microRNAs targeting *PR* 3'UTR, differentially expressed microRNAs in response to progesterone were compared to microRNAs predicted to target *PR* using 6 algorithms (TargetScan, miRanda, miRWalk, miRMap, RNA22 and RNAhybrid).

4.2.4 Quantitative Real-time PCR

Transcript levels of candidate microRNA's were analyzed by quantitative real time PCR. 1µg total RNA was used for cDNA synthesis using Mir-X miRNA First-Strand Synthesis Kit (Clontech Takara). For analyzing transcript levels of de-regulated genes, cDNA was synthesized using High capacity cDNA reverse transcription kit (Applied Biosystems). cDNA from each cell line with the two treatment conditions were then subjected to quantitative real-time PCR analysis using Roche Light-Cycler-II 480 instrument using the Mir-X miRNA qRT-PCR SYBR Kit (2X) Master Mix (Clontech Takara) for microRNAs and Roche real-time master mix (Roche) for genes.

Expression change of candidate microRNAs and genes de-regulated by progesterone was calculated by the $2^{-\Delta\Delta C}{}_{T}$ method. *U6* small RNA (primers provided by Clontech Takara) was used as an internal control for microRNAs and *GAPDH* was used for genes. Primer sequences for each microRNA and gene used for validation purpose are given in IV-Table-1.

IV-Table- 1: Primer sequences used for real-time PCR validation of genes and microRNAs

Sr. No.	OAD Number	Primer Sequence	Gene/ microRNA Name
1	OAD 121	5' AGCCCACAATACAGCTTCGAG	*PR-A/B*
	OAD 122	3' TTTCGACCTCCAAGGACCAT	
2	OAD 123	5' CCTGAAGTTTCGGCCATACCT	*PR-B*
	OAD 124	3' AGCAGTCCGCTGTCCTTTTCT	
3	OAD 328	5' AATCCCATCACCATCTTCCA	*GAPDH*
	OAD 329	3' TGGACTCCACGACGTACTCA	
4	OAD 551	5' AAGCCCTTACCCCAAAAAGCA	*miR-129-2*

4.2.5 Cloning of microRNA/*PR* 3′UTR and Luciferase assay

A 400bp sequence of *miR-129-2* containing the seed sequence was PCR amplified using genomic DNA isolated from T47D. Amplicons were cloned in a T/A cloning vector (Fermentas, USA) followed by sub-cloning in BamHI and HindIII sites of pCDNA 3.1 (-) expression vector (Invitrogen). *PR*-3′UTR of 1000bp was PCR amplified using T47D cDNA. Amplicons were cloned in a T/A cloning vector followed by sub-cloning between XbaI sites in pGL3-promoter vector (Luciferase

Expressing vector, Promega). For the Luciferase assay, 293FT cells (50,000 cells/well) were transfected using lipofectamine 2000 reagent (Life Technologies) with the combination of these constructs along with Renilla luciferase vector (for normalizing transfection efficiency) and 5nM miR-inhibitors (SIGMA, HSTUD0162) in separate wells. 48 hours post-transfection cells were lyzed and luciferase assay was performed to measure luminescence (Berthold Luminometer, Germany). Experiment was performed in triplicates and differences between group showing *p*-values <0.05 (calculated using an unpaired student's t-test) were considered significant.

4.2.6 Transfection of microRNA inhibitor in breast cancer cells:

T47D cells were grown up to 60% confluence and transfected with 25nM negative control microRNA inhibitor (against *miR-29a*) and *miR-129-2* inhibitor (SIGMA, HSTUD0162). Post-transfection, cells were incubated for 48h and then treated with progesterone for 6h. Cell lysate was prepared and western blot analysis was performed.

4.3 Results:

4.3.1 Identification of progesterone responsive microRNAs targeting PR expression in breast cancer cells

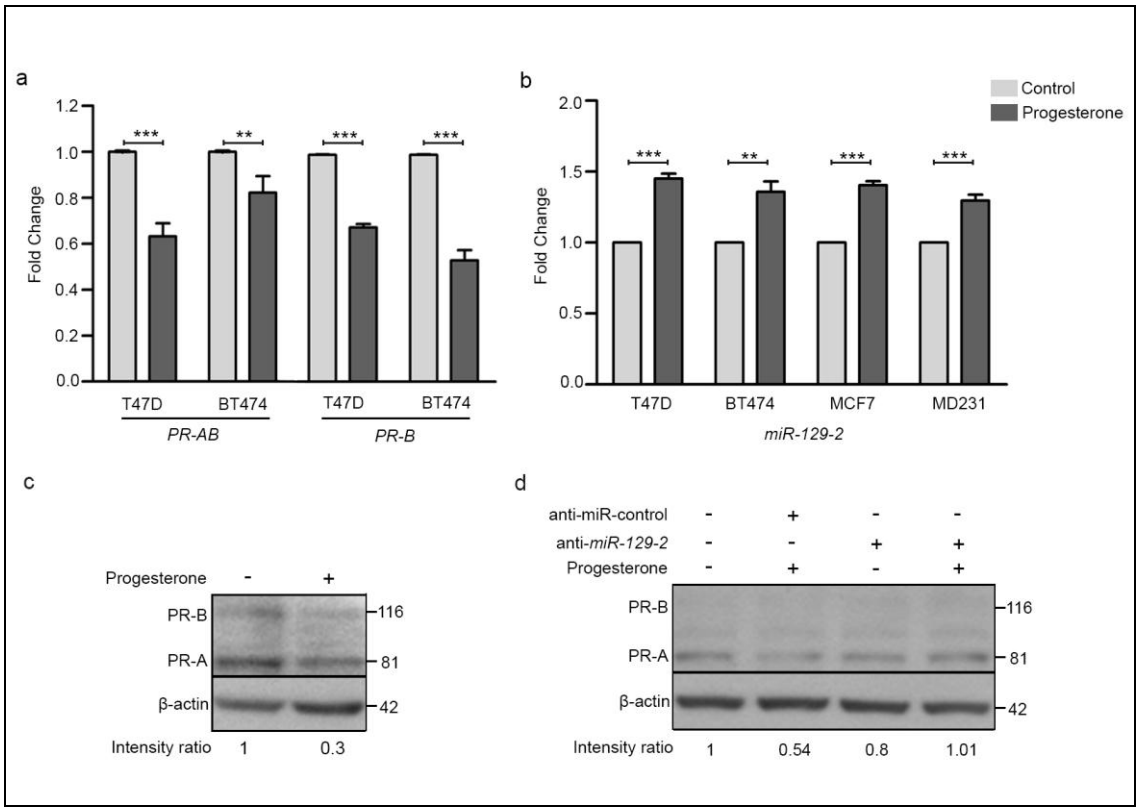
Consistent with earlier reports, we observed down-regulation of *PR* transcripts when T47D and BT474 cells were treated with 10nM progesterone for 6h (IV-Figure-1a). Similarly, progesterone reduced the expression level of PR protein in T47D cells (IV-Figure-1c). To understand the role of microRNA's involved in regulation of *PR* expression, we performed small RNA sequencing of three PR-positive T47D, BT474, MCF7 and one PR-negative MDA-MB-231 cell line for identifying microRNAs

which could down-regulate PR expression in response to progesterone treatment for 6h. On an average we obtained 22 million reads per sample per cell line. The sequence reads were mapped to human microRNA sequences obtained from miRBase (version 21) to identify median 800 mature microRNA sequences. These microRNA reads were used for identification of differentially expressed microRNAs. We used a fold-change cut-off of 3-fold difference and observed that progesterone had an effect in both directions by up-regulating and down-regulating the microRNAs and all the four cells had different number of de-regulated microRNAs (IV-Table-2). Of these, 98 microRNAs were up-regulated in T47D, 96 in BT474, 189 in MCF7 and 106 in MDA-MB-231 cells in response to progesterone. Intriguingly, expression of *miR-513a-5p* shown to be differentially up-regulated in response to synthetic progestin (medroxy progesterone acetate, MPA) by microarray-based analysis in T47D cells was not observed in any of the four breast cancer cells in this study [155], possibly due to variable downstream effects elicited by synthetic progestin (MPA) and progesterone [103] or distinct platform specific threshold involved in these studies. The up-regulated microRNAs found across the four breast cancer cells were further used to search microRNAs targeting 3'UTR of *PR* gene. Of the 6 different algorithms used, we found three microRNAs (*miR-3908, miR-129-2-3p and miR-3140-3p*) that were predicted to target 3' UTR of *PR* and showed an increased expression relative to levels in control. When expression of these microRNAs was checked in the TCGA breast cancer cohort (n=359), only *miR-129-2* was found to be expressed. Next, the up-regulation of *miR-129-2* in response to progesterone could be validated by real-time PCR in our panel of cells (IV-Figure-1b). While the progestin-regulated *miR-513a-5p* could be validated only at 100nM progestin (MPA) as reported by Cochrane

et al. [155], we could validate consistent up-regulation of *miR-129-2* in response to 10nM progesterone that was used for small RNA sequencing analysis, inclusive of PR-negative breast cancer cells (IV-Figure-1b). Thus we observed that progesterone mediated up-regulation of *miR-129-2* was independent of the PR expression of cells.

IV-Table- 2: MicroRNAs de-regulated by progesterone in breast cancer cells can target 3'UTR of PR
Statistics of small RNA sequencing reads generated by NGS and number of microRNAs identified in cells in response to progesterone. De-regulated microRNAs with fold-change cut-off of 3-fold were considered as differentially expressed in response to progesterone.

	Total Number of reads	**Reads aligned to microRNA**	**Total microRNAs Identified**	**Total Number of Unique microRNAs**	**Total Number of De-regulated microRNAs**	**MicroRNAs Up-regulated (>3fold)**	**MicroRNAs Down-regulated (<3fold)**
BT474 Untreated	16268290	2194148	929	1002	132	96	36
BT474 Treated	15319206	518237	643				
T47D Untreated	21478910	1959325	1032	1082	222	98	124
T47D Treated	20723322	300141	579				
MCF7 Untreated	22424923	80184	465	649	327	189	138
MCF7 Treated	15346700	164835	544				
MDA-MB-231 Untreated	37102512	8211	263	365	201	106	95
MDA-MB-231 Treated	31362605	6793	282				

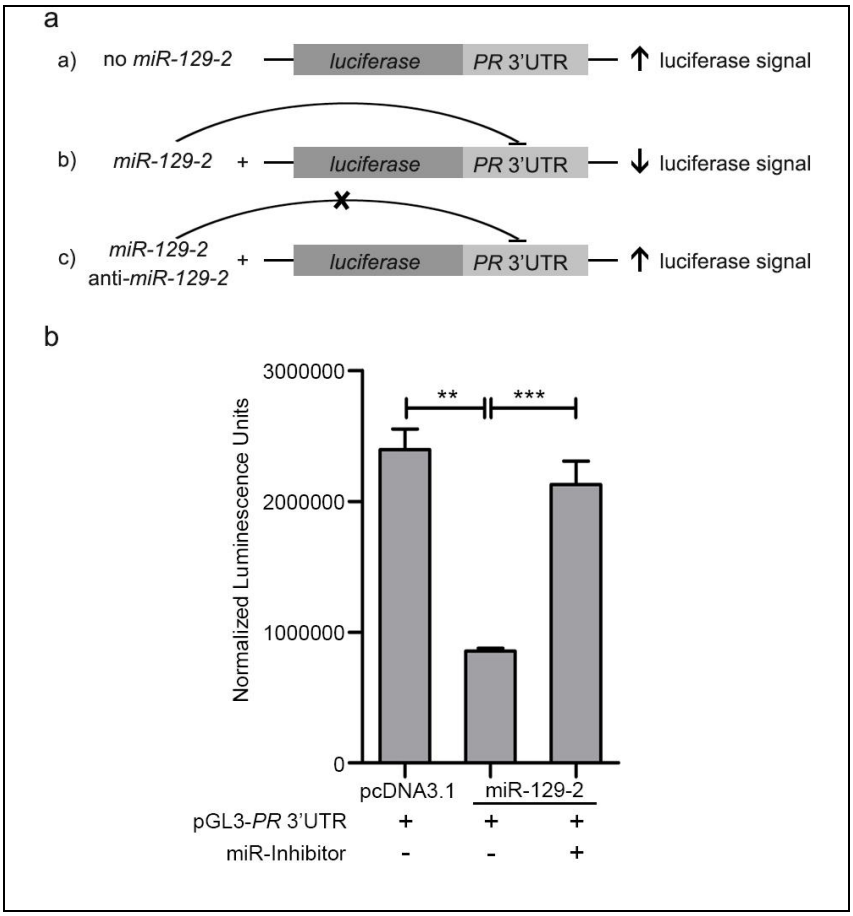


IV-Figure- 1: Progesterone receptor is down-regulated in breast cancer cell lines in response to progesterone (modified from the manuscript)

a) Transcript levels of *PR* were measured using real-time PCR in T47D and BT474 cells treated with 10nM progesterone for 6h. Graph has been plotted as fold change expression of *PR* normalized to *GAPDH* in progesterone-treated versus control. Analysis is representative of three independent experiments and *P*-value was calculated using student's unpaired t-test. **b)** Transcript levels of *miR-129-2* under similar progesterone treatment conditions were measured by real-time PCR and plotted as fold change in progesterone versus control of T47D, BT474, MCF7 and MDA-MB-231 cells obtained after normalization to expression of *U6* small RNA. Transcript levels in both control and progesterone-treated cells have been shown. Analysis is representative of three independent experiments and *P*-value was calculated using student's unpaired t-test. ** indicates *P*-value <0.001; *** indicates *P*-value <0.0001. **c)** Western blot analysis of PR (PR-A and PR-B) in response to progesterone treatment in T47D cells. β-actin was used internal protein loading control. Numbers on blot indicate ratio of intensity of PR with respect to β-actin for each lane. Western blot analysis is representative of two independent experiments. **d)** Western blot analysis of PR (PR-A and PR-B) in T47D cells treated with either anti-miR-control or anti-*miR-129-2*. As indicated in the panel, cells were either treated with progesterone or untreated. β-actin was used as internal protein loading control. Numbers on blot indicate ratio of intensity of PR with respect to β-actin for each lane. Western blot analysis is representative of two independent experiments.

4.3.2 Functional validation of *miR-129-2* based regulation of progesterone receptor

In our attempt to functionally characterize the association of *miR-129-2* with *PR*, a ~1000 bp 3'UTR of *PR* (containing seed sequence for *miR-129-2*) was cloned downstream to *luciferase* gene in a pGL3-promoter vector. The sequence for premature *miR-129-2* (~400bp) was cloned in pcDNA3.1 vector. Three set of transfections were performed— pGL3-*PR* 3'UTR; pGL3-*PR* 3'UTR with pcDNA-3.1-*miR-129-2*; and combination of pGL3-*PR* 3'UTR, pcDNA3.1-*miR-129-2* and anti-*miR-129-2* (IV-Figure-2a). The expression of *firefly luciferase* gene was checked using luciferase reporter assay system (Promega) and normalized to renilla expression, which was used as an internal control, in each of these sets. Our analysis suggests that upon over-expression of *miR-129-2*, the luciferase signal was significantly reduced as compared to signal in vector only cells. Addition of microRNA inhibitor against *miR-129-2* (anti-*miR-129-2*), a double stranded RNA sequence which is complimentary to and specifically targets *miR-129-2*, reversed the repression and showed an increase in luciferase signal (IV-Figure-2b).



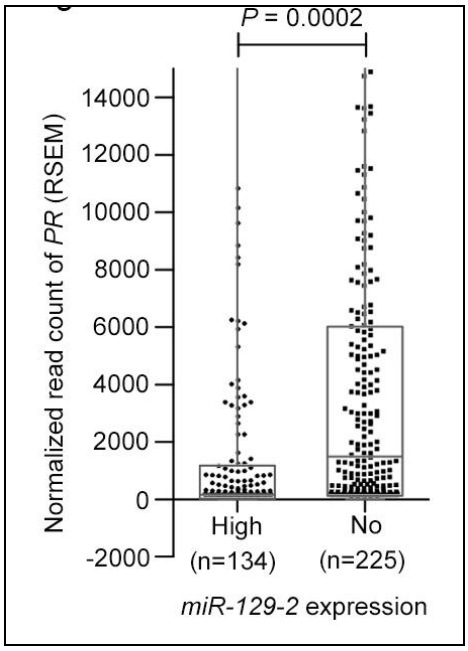
IV-Figure- 2: Validation of *miR-129-2*-based regulation of *PR*

a) pCDNA3.1-*miR-129-2* and pGL3- *PR* 3'UTR in different combinations with anti-*miR-129-2* were co-transfected in 293FT cells and luciferase signal in each condition was measured, as shown in the figure. **b)** Quantified luminescence units normalized to renilla expression was plotted for each of the sets mentioned above. Analysis is representative of three independent experiments and the *P*-value was calculated using student's t-test. ** indicates *P*-value <0.001; *** indicates *P*-value <0.0001.

Next, we inhibited *miR-129-2* in T47D cells and compared the PR expression in these cells with the expression in cells transfected with negative control (targeting *miR-29a*) inhibitor. Our western blot analysis suggests that upon exposure to progesterone, T47D cells transfected with negative control showed decrease in PR expression, while PR showed stable expression in cells transfected with *miR-129-2* inhibitor even in the presence of progesterone treatment (IV-Figure-1d). Thus our results provide basis for

direct interaction of *miR-129-2* with *PR*, where in addition to previous findings, we demonstrate that over-expression of *miR-129-2* mimics the effect of progesterone treatment to down-regulate PR and that inhibition of *miR-129-2* abrogates its interaction with *PR* in breast cancer cells. Taken together, these studies emphasize the plurality in microRNA-mediated feedback regulation of PR.

Next, we analyzed the TCGA breast cancer cohort (n=359) for studying expression of PR in breast cancer patients with high *miR-129-2* expression and in absence of *miR-129-2* expression. When expression of PR was checked in patients with high *miR-129-2* expression (n=134) versus patients with absence of *miR-129-2* expression (n=225) we observed a significantly higher expression of PR in patients with absence of *miR-129-2* expression as compared to patients with high expression of *miR-129-2* (*P*=0.0002) as shown in IV-Figure-3. Thus a further in-depth analysis needs to be carried out to ascertain the exact role of *miR-129-2* in survival of breast cancer patients.



IV-Figure- 3: Expression of *miR-129-2* in breast cancer patients in TCGA dataset (Modified from manuscript)

Expression plot for *PR* in breast cancer patients with high *miR-129-2* expression (n=134) and with absence of *miR-129-2* expression (n=225) in the TCGA cohort. The box-plot is overlaid with dot-plot wherein each point represents patient sample. Y-axis indicates normalized read count (RSEM) values for *PR* in a total of 359 breast cancer patients where expression of *PR* and *miR-129-2* was available. *P*-value (*P*=0.0002) was calculated using student's unpaired t-test with Welch's correction. (Comment: The TCGA provides only level-3 data as openly accessible, which lacks adjacent normal sample data. Thus, to get the best possible contrast between the two groups using available (only tumor) dataset, we restricted to top quartile (high) *miR-129-2* expressing patients versus no *miR-129-2* expressing patients for PR expression, wherein we observe a significant anti-correlation in their expression)

4.4 Discussion

Small RNA sequencing analysis of progesterone treated breast cancer cell lines led to the identification of a novel *PR*-targeting microRNA *miR-129-2*. Since the increased expression of *miR-129-2* was independent of the PR-status of breast cancer cells, a possible role of other steroid hormone receptors like membrane progesterone receptor or glucocorticoid receptor as suggested in literature [77, 99] to mediate the role of progesterone in these cells remains to be systematically analyzed. Consistent with our

finding, analysis of TCGA breast cancer dataset suggests a significantly decreased expression of PR in patients with elevated expression of *miR-129-2* as compared to patients with no *miR-129-2* expression, indicating a possibility for the decreased expression of PR in patients with low PR expression. It has been observed that factors like loss of PR or menopausal status of women can alter the response to hormonal therapy [157]. Some studies have indicated that the absence of PR could underlie tumors resistance to hormonal therapy [59], or could potentially increase the risk of relapse [158]. Hence we propose that stabilization of PR expression in patients with tumors expressing low PR levels by blocking activity of such microRNAs using specific microRNA inhibitors, along with other treatment modalities, could be potentially helpful in enhancing the response of patients to hormonal therapies. In support of this notion, our *in vitro* luciferase assay and western blot results using *miR-129-2* inhibitor suggest that inhibition of *miR-129-2* can increase the expression of PR. Thus, we validate under *in vitro* settings that addition of progesterone leads to up-regulation of *miR-129-2,* which suppresses the expression of *PR* in breast cancer cells; and, the inhibition of *miR-129-2* reinstates the *PR* expression in these breast cancer cells even in presence of progesterone. Also since microRNAs are being assessed for their use in clinics [159], strategies like microRNA sponges and chemically modified antisense oligonucleotides (inhibitors) hold promise as a promising line of treatment of breast cancer that need to be exhaustively explored with larger datasets [160]. Thus, our study suggests an underlying mechanism to a possible clinical consequence in response to progesterone treatment among patients with varying PR expression levels. Also, it is suggestive of treatment with anti-*miR-129-2* among those patients expressing inadequate PR levels, under adjuvant and neo-adjuvant settings, before

considering for hormonal therapy. Whether modulation of activity of *miR-129-2* could stabilize PR expression and potentially improve response to hormonal therapy remains to be validated as an immediate follow up to this pilot study.

V. DIFFERENTIAL REGULATION OF *SGK1* BY PROGESTERONE ACTIVATES *AP-1/NDRG1* GENOMIC AXIS IN PR-POSITIVE AND NEGATIVE BREAST CANCER CELLS

Abstract

Pre-operative progesterone intervention has been shown to confer a survival benefit to breast cancer patients independent of their progesterone receptor (PR) status, raising a question about how progesterone affects the outcome of PR-negative cells. Here, we identify up-regulation of a *Serum- and glucocorticoid-regulated kinase* gene, *SGK1* and an *N-Myc Downstream Regulated Gene 1*, *NDRG1*, along with down-regulation of *miR-29a* and *miR-101-1* targeting 3'UTR region of *SGK1*, to differential extents in a *PR* dependent manner in breast cancer cells. We further demonstrate a novel dual-phase transcriptional and post-transcriptional regulation of *SGK1* in response to progesterone leading to up-regulation of a tumor metastasis suppressor gene, *NDRG1*, mediated by a set of AP-1 network genes. The *NDRG1* further inactivates a set of kinases impeding the invasion and migration of breast cancer cells. In summary, we propose a model for the mode of action of progesterone in breast cancer deciphering the molecular basis of a randomized clinical trial studying the effect of progesterone in breast cancer with a potential to improve the prognosis of breast cancer patients for receiving pre-operative progesterone treatment.

5.1 Introduction:

The increasing complexity of multicellular organisms correlates with the increasing number of microRNAs than the number of coding genes encoded by the genome [161, 162], reflecting a gradual increase in the extent and intricacy of gene regulation [163]. Hierarchically, microRNAs function downstream of transcriptional regulation of genes since microRNAs represses post transcription of mRNAs [164]. However, emerging evidence suggests that transcriptional and post-transcriptional regulation is often highly coordinated [165, 166]. Hormones, for instance, have been hypothesized to regulate expression of target genes at the transcriptional and post-transcriptional level [61, 167]. Estrogen up-regulates the expression of progesterone receptor (PR) by transcriptionally recruiting estrogen receptor (ER) at the promoter, and post-transcriptionally, by silencing expression of microRNAs targeting 3'UTR of *PR* in breast cancer cells [168]. A similar example for the *ATP1B1* gene has been reported [155]. However, systematic approaches to discern dual-regulated molecular targets of hormones in breast cancer remains poorly understood.

Understanding the molecular basis of clinical phenomena in response to therapeutic interventions has been an important point of intersection between medical and biological sciences. While clinical benefit of preoperative endocrine therapy is well documented in literature [169, 170], more recently, we described the first randomized trial with preoperative progesterone resulting in greater than 10% absolute improvement in 5-year disease-free survival among node-positive breast cancer patients [171]. Of several hypothesis-generating results from this study, the impact of progesterone on PR-negative patients particularly lends itself to a systematic characterization of molecular changes that progesterone may induce in breast cells.

Gene expression studies probing the targets of progesterone have either been performed restrictively in PR-positive breast cancer cell lines or in the presence of other hormones [58, 80, 92, 172, 173]. Although few studies suggest a beneficial effect of progesterone, progesterone-responsive genes in PR-negative cells have not been studied [58, 80, 92, 174]. In order to identify targets of progesterone independent of PR status of cells, we set to perform an integrated genomic profiling of a panel of PR-positive and PR-negative breast cancer cell lines treated with progesterone, followed by functional analysis of the components found to be significantly altered. This study details the molecular action of progesterone on breast cancer cells, mediated by the up-regulation of a genomic axis inclusive of a tumor metastasis suppressor gene in breast cancer, independent of the PR status of cells.

5.2 Materials and Methods:

5.2.1 Breast Cell lines

T47D, BT474, MDA-MB-231, ZR-75-1 and MCF7 breast cancer cell lines were cultured and the PR/ER/Her2 receptor status confirmed as explained in chapter-III section 3.2.1 and provided in V-Table-1. The immortalized normal-like breast cell line 184A1 was obtained as a gift from Dr. Slamon's Laboratory (Department of Medicine, UCLA, USA) and was cultured in DMEM:F12 medium (HiMedia) supplemented with 28.18IU Insulin, 20ng/ml EGF and 500ng/ml Hydrocortisone. All the cell lines were authenticated using STR profiling (V-Table-2) and made mycoplasma-free as described in section 3.2.1.

V-Table- 1: Selection of breast cell lines and validation of PR/ER/Her2 hormone receptor status

A panel of breast cell lines with varying receptor status, as reported in literature, was selected for studying the effect of progesterone independent of the receptor status. The PR/ER/Her2 transcript expression of all the cell lines was confirmed by reverse transcriptase-PCR and using gene expression array analysis. The RT-PCR analysis for PR/ER/Her2 for T47D, MCF7, BT474, ZR-75-1 and MDA-MB-231 is as described earlier [174] and chapter-III section 3.3.

Sr. No	Breast Cell lines	Literature reported Receptor status			Validation of Receptor status at Expression level								
					Expression array analysis			RT-PCR					
		PR	ER	HER2	PR	ER	HER2	PR	ER	HER2	GR	PGRMC1	SERBP1
1	T47D	+	+	-	+	+	-	+	+	-	+	+	+
2	MCF7	+	+	-	+	+	-	+	+	-	+	+	+
3	BT474	+	+	+	+	+	+	+	+	+	+	+	+
4	ZR-75-1	-	+	-	-	+	-	-	+	-	+	+	+
5	MDA-MB-231	-	-	-	-	-	-	-	-	-	+	+	+
6	184A1 (Immortalized cell line)	-	-	-	-	-	-	-	-	-	+	+	+

V-Table- 2: STR profiling of breast cell lines

Query for STR	10-marker STR profile as per GeneMarker HID software											Authenticity of cell line as identified
	TH01	D21S11	D5S818	D13S317	D7S820	D16S539	CSF1PO	AMEL	vWA	TPOX	% Match	
T47D	6	28,31	12	12	11	10	11,13	X	14	11	100	T47D
BT-474	7,8	28,32.2	11,13	11	9,12	9,11	10,11	X	15,16	8	100	BT-474
MCF7	6	30	11,12	11	8,9	11,12	10	X	14,15	9,12	100	MCF7
MD-231	7,9.3	30,33.2	12	13	8,9	12	12,13	X	15,18	8,9	100	MD-231
ZR-75-1	8,9.3	29	13	9	8,11	11	10,11	X	16,18	8	92%	ZR-75-1
184A1	9.3	29,30	11,13	11	9,11	11,12	10,11	X	18,19	11	100	184A1

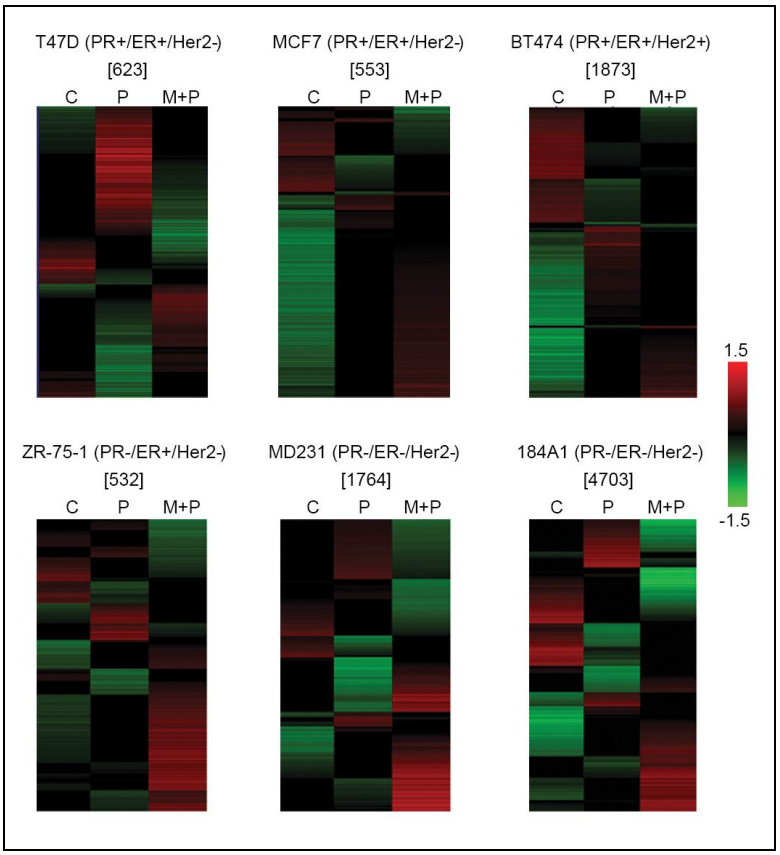
5.2.2 Progesterone treatment and RNA isolation

Breast cancer cells were treated with progesterone and RNA isolation was performed as described earlier [174, 175] and in chapter-IV section 4.2.2. Additionally, in case of Mifepristone+Progesterone (M+P) combination treatment, 100nM RU486 (Mifepristone) was added to the cells for 2h followed by 10nM Progesterone treatment for 6h in the same medium. Equal amount of alcohol was used as vehicle control. The treatment conditions for progesterone and mifepristone were standardized based on the expression change of three known candidate genes (V-Figure-3A). Various time durations and progesterone concentration combinations were tried and a consistent up-regulation of *STAT5A*, *EZF* and *F3* was obtained upon treatment with 10nM progesterone for 6hrs, consistent with [109]. Similarly, 2hrs treatment with 100nM mifepristone led to inhibition of expression of these candidate genes. Both these time and concentration combinations have been used consistently for the progesterone and mifepristone treatment of cells.

5.2.3 Gene Expression profiling

Gene expression profiling was performed using BeadChip Illumina microarray platform. Raw data (.idat files) of BeadChip Illumina platform were converted to readable format using Genome studio software (version V2011.1). Probe level data were converted into gene centric and used for further processing. Bioconductor lumi package was used for pre-processing the data which includes quality control steps, background correction, normalization, log transformation etc. and finally differential gene analysis. Robust Spline Normalisation (RSN) and Variance Stabilization

Transformation (VST) methods were used for normalization and transformation respectively. To specifically select for highly variable genes, unexpressed, non-annotated and false positive genes were excluded from the analysis. Median Absolute Deviation (MAD) was carried out to make samples comparable to each other. The differential gene expression cut-off was set as $0.5 \leq \log$ (Fold Change) ≤ -0.5 in at least one out of the three conditions (Control; Progesterone; Mifepristone+Progesterone). Heatmaps were constructed using MeV software (version 4.9.0) (V-Figure-1). All possible comparisons were taken into consideration as Progesterone versus Control (P Vs C), Mifepristone + Progesterone versus Control (M+P Vs C) and Progesterone versus Mifepristone + Progesterone (P Vs M+P). From our analysis, we identified 623, 553, 1873, 532, 1764 and 4703 differentially expressed genes in T47D, MCF7, BT474, ZR-75-1, MDA-MB-231 and 184A1 breast cell lines respectively.



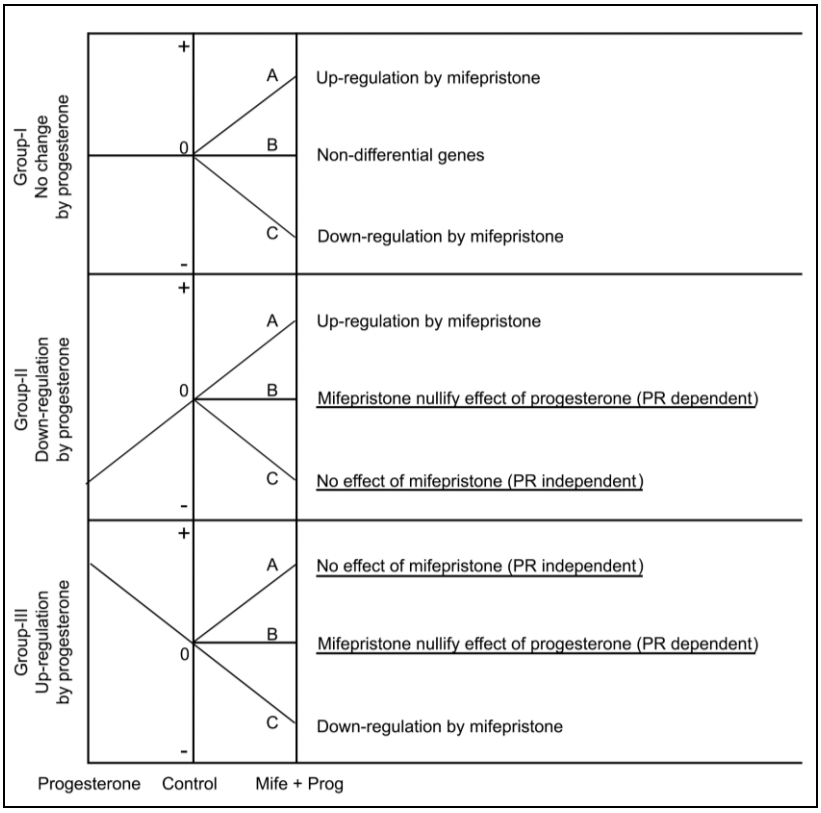
V-Figure- 1: Gene expression profile of breast cell lines with different receptor statuses

Heat map representation of gene expression profile for each breast cell line (n=6) has been shown, with three treatment conditions viz. control, progesterone or mifepristone+progesterone (M+P). In the figure, red lines indicate up-regulation; black lines indicate no change; green lines indicate down-regulation of gene in response to the treatment conditions. The differential gene expression cut-off was set as $1.5 \leq \log(\text{Fold Change}) \leq -1.5$. Numbers in square bracket indicate total number of differentially expressed genes for each cell line.

5.2.4 Integrated Analysis

A weighted gene co-expression network was constructed based on the gene expression data to identify gene modules up-regulated in response to progesterone [176] across all the breast cancer cells. *SGK1* and *NDRG1* were found to be the top up-regulated and recurrent gene in response to progesterone across multiple cells (V-Figure-2). Next, we analyzed our small RNA sequencing data (described in chapter-IV, section 4.2.3) to identify differentially expressed microRNA in response to progesterone

treatment predicted to bind to 3'UTR of *SGK1* gene using 6 different microRNA binding site prediction tools [175]. Bioinformatics prediction analysis revealed identification of *miR-29a* and *miR-101-1* to target the 3'UTR of *SGK1* that were down-regulated in response to progesterone treatment.



V-Figure- 2: Hypothetical model for all possible pattern of gene expression in microarray analysis with three treatment conditions

To study the pattern of gene expression, a hypothetical model was constructed. Comparison of expression changes in progesterone treated or the combined mifepristone+progesterone (Mife+Prog) treatment are to be compared with the central line of control. Depending on the up-regulation or down-regulation of any gene, the possible outcomes upon the Mife+Prog treatment have been shown in Groups- I, II and III. The subgroups of biological interest are highlighted with an underline.

5.2.5 Small RNA sequencing analysis

To identify the microRNA's targeting *SGK1*, we analyzed our small RNA sequencing data, described earlier [175] and used in chapter-IV. The sequencing was performed on a single lane of Illumina HiSeq 1000 platform with four breast cancer cell lines

(T47D, BT474, MCF7 and MDA-MB-231). For identifying microRNAs targeting 3'UTR of *SGK1*, differentially expressed microRNAs in response to progesterone were overlapped with microRNAs predicted to target *SGK1* using the 6 algorithms used in our earlier study [175].

5.2.6 Quantitative Real-time PCR

Transcript levels of candidate genes and microRNAs were analysed by quantitative real time PCR as previously described for genes and microRNAs [174, 175]. Briefly, cDNA from each cell line with the three conditions (control, progesterone and M+P) were then subjected to qRT-PCR analysis. *GAPDH* gene was used as an internal control and average of C_T values from each condition was used to normalize the C_T values of candidate genes in each cell line. In case of microRNAs, *U6* small RNA was used as an internal control for normalizing the expression of microRNAs. Expression change of candidate genes and microRNAs was calculated by the $2^{-\Delta\Delta C_T}$ method. Primer sequences used for real-time PCR validation of genes and microRNAs have been provided in V-Table-3.

V-Table- 3: Primer sequences used for real-time PCR validation of genes and microRNAs

Sr. No	OAD Number	Primer sequence	Gene/ microRNA name
1.	OAD 137	5’ GGCGCTTCAGGCACTACAA	*F3*
	OAD 138	3’ TTGATTGACGGGTTTGGGTTC	
2.	OAD 139	5’ GCAGAGTCCGTGACAGAGG	*STAT5A*
	OAD 140	3’ CCACAGGTAGGGACAGAGTCT	
3.	OAD 141	5’ CCCACATGAAGCGACTTCCC	*EZF*
	OAD 142	3’ CAGGTCCAGGAGATCGTTGAA	
4.	OAD 233	5’ GCAGAAGAAGTGTTCTATGCAGT	*SGK1*
	OAD 234	3’ CCGCTCCGACATAATATGCTT	
5.	OAD 457	5’ GCCTCCTTCCCCGCAGGG	*NDRG1*
	OAD 458	3’ GCCCAAACTGTTGAAGGACTCC	
6.	OAD 567	5’ GCATTGGCAGGAGGGGCAAGG	*FOS*
	OAD 568	3’ CAGCTCCCTCCTCCGGTTGCG	
7.	OAD 577	5’ CCCAAGAACGTGACAGATGAG	*JUN*
	OAD 578	3’ TGCCCCGTTGACCGGCTGC	
8.	OAD 573	5’ GGCGAGCAGCCCTACGAGC	*EGR1*
	OAD 574	3’ GTATAGGTGATGGGGGGCAGTC	
9.	OAD 571	5’ CCTGCAGTACCCCACTCTACG	*DUSP1*
	OAD 572	3’ CCCAAGGCATCCAGCATGTCC	
10.	OAD 328	5’ AATCCCATCACCATCTTCCA	*GAPDH*
	OAD 329	3’ GGACTCCACGACGTACTCA	
11.	OAD 552	5’ TAGCACCATCTGAAATCGGTTA	*miR-29a*
12.	OAD 625	5’ CAGTTATCACAGTGCTGATGCT	*miR-101-1*

5.2.7 Over-expression and knockdown studies

For over-expression of *SGK1*, two retrovirus-based constructs were used-- pBABE-puro-*SGK1* which expresses wild type *SGK1* (WT-*SGK1*); while pWZL-Neo-Myr-

Flag-*SGK1* expresses myristoylated *SGK1* (Myr-*SGK1*) (a kind gift from Dr. Shaida Andrabi, University of Kashmir). Over-expression clones for each of the constructs were selected in 1µg puromycin and 1600µg neomycin, respectively. Untransfected T47D and MD-231 cells were used as control for over-expression of *SGK1*. For the knockdown of *SGK1*, *NDRG1* and *EGR1* genes in T47D and MDA-MB-231 cells, three lentiviral shRNA constructs (PLATINUM Select Human MLP lentiviral shRNA-mir vector, Transomic technologies) each, against these genes, were used for genetic depletion. sh-non-targeting (sh-NT) was used as vector/scrambled control. Positive clones were selected using 1µg puromycin. Over-expression and knockdown experiments were performed in T47D (PR-positive) and MDA-MB-231 (PR-negative) breast cancer cells.

5.2.8 Protein sample preparation and Western blot analysis

Protein samples were prepared and western blots were developed as described earlier [174]. Briefly, cells were serum starved and treated with progesterone for 8h or left untreated. Cell lysates were prepared and equal amounts of lysate were resolved using 10% SDS-PAGE and transferred to a PVDF membrane by wet-transfer method. The immunoblots were then incubated with primary antibodies against SGK1 (Cell Signaling Technology, 1210S, Dilution 1:800); NDRG1 (Cell Signaling Technology, 9485S, Dilution 1:800); p-NDRG1 (Cell Signaling Technology, 5482S, Dilution 1:800); p-SGK1 (Abcam, ab55281, Dilution 1:500); EGR1 (Santa Cruz Biotechnology, sc-515830, Dilution 1:1000); β-actin (I-19)-R (Santa Cruz Biotechnology, sc-1616-R; Dilution 1:3000); p-EGFR (Y1068) (Cell Signaling, 3777S, Dilution 1:500), p-Akt (S473) (Cell Signaling, 4060S, Dilution 1:500); p-

ERK1/2 (T202/Y204) (Cell Signaling, 9101S, Dilution 1:1000); EGFR (1005) (Santa Cruz Biotechnology, sc-03, Dilution 1:1000); AKT (11E7) (Cell Signaling, 4685S, Dilution 1:1000); and ERK2 (c-14) (Santa Cruz Biotechnology, sc-154, Dilution 1:1000). Goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, sc-2004, Dilution 1:3000); and goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, sc-2005, Dilution 1:3000) were used as secondary antibodies.

5.2.9 Treatment with SGK1 inhibitor

Cells were grown till 70-80% confluence in a 6-well dish and then serum-starved using low glucose DMEM medium (HiMedia) for 24hrs. 1.0µM concentration of GSK650394A (SGK1-inhibitor) was added to respective wells for 4hrs, as discussed in other studies [2]. After 4hrs, medium was removed and fresh low-glucose medium was added to cells. Where indicated, cells were then treated with 10nM progesterone for 6hrs and then used for RNA or protein isolation.

5.2.10 Invasion Assay

Cell invasion assay was performed as described earlier [174]. Briefly, 35000 cells were allowed to invade matrigel in boyden chamber for 16-18h at 37ºC. Cells were observed under upright microscope, ten random fields were chosen and number of cells in each field were counted and plotted as percentage cell invasion.

5.2.11 Wound healing assay

Wound scratch migration assay and analysis was performed as described earlier [174] and section 3.2.8 of Chapter-III. Briefly, confluent cell monolayer in a 6-well plate was serum-starved for 18hrs or treated with mitomycin-C for 2hrs and subjected to a scratch manually with a sterile small pipette tip. Cell culture media was replaced with low-glucose phenol-red free DMEM containing 10% charcoal-stripped FBS. Cell migration was monitored for 20hrs and distance traversed by cells was quantified using ImageJ.

5.2.12 Dual-luciferase assay with microRNAs/*SGK1* 3'UTR

Cloning of microRNA sequences and *SGK1* 3'UTR was performed as described earlier [175]. Briefly, a 400bp sequence of *miR-29a* and *miR-101-1* containing the seed sequence was PCR amplified using genomic DNA isolated from T47D. Amplicons were cloned in a T/A cloning vector (Fermentas, USA) followed by sub-cloning in BamHI and HindIII sites of pCDNA 3.1 (-) expression vector (Invitrogen). *SGK1*-3′UTR of 1000bp was PCR amplified using T47D cDNA. Amplicons were cloned in a T/A cloning vector followed by sub-cloning between XbaI sites in a pGL3-promoter vector (Luciferase Expressing vector, Promega). For the dual-luciferase assay, 293FT cells (50,000 cells/well) were transfected using lipofectamine 2000 reagent (Life Technologies) with combination of these constructs along with a Renilla luciferase vector (for normalizing transfection efficiency) in separate wells. 5nM miR-inhibitors (anti-miR's) (SIGMA) were also transfected in combination to expression vectors for specifically inhibiting the activity of both the microRNAs. 48 hours post-transfection, cells were lyzed and luciferase assay was performed to

measure Firefly luciferase activity post normalization to Renilla luciferase values (Berthold Luminometer, Germany). Experiment was performed in triplicates and differences between group showing *P*-values <0.05 (calculated using an unpaired student's t-test) were considered significant.

5.2.13 Transfection of microRNA inhibitor in breast cancer cells

T47D cells were grown up to 60% confluence and transfected with 25nM negative control miR-inhibitor (anti-*miR-129-2*); anti-*miR-29a*; and anti-*miR-101-1* (SIGMA). Post-transfection, cells were incubated for 48h and then treated with progesterone for 6h as described above. Cell lysate was prepared and western blot analysis was performed to study expression of SGK1.

5.2.14 Statistical analysis

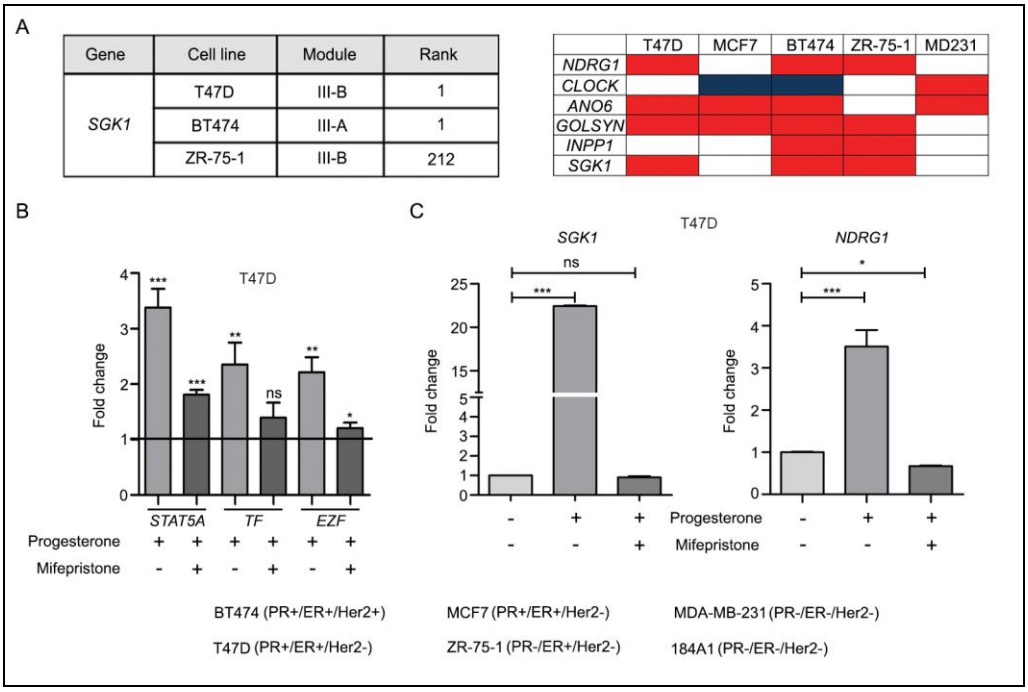
The statistical analysis was performed using GraphPad Prism software (GraphPad, version 5). Student's unpaired t-test was used to determine the statistical significance.

5.3 Results:

5.3.1 Gene expression analyses reveal a novel dual-phase regulation of *SGK1* by progesterone in breast cancer cells

An integrated analysis of micro array based mRNA expression profile and deep sequencing of non-coding small RNA of breast cancer cells (as described in 5.2.4 and 5.2.5) led us to identify up-regulation of a *Serum- and Glucocorticoid-regulated Kinase* gene, *SGK1* and a *N-Myc Downstream Regulated Gene 1*, *NDRG1*, along with

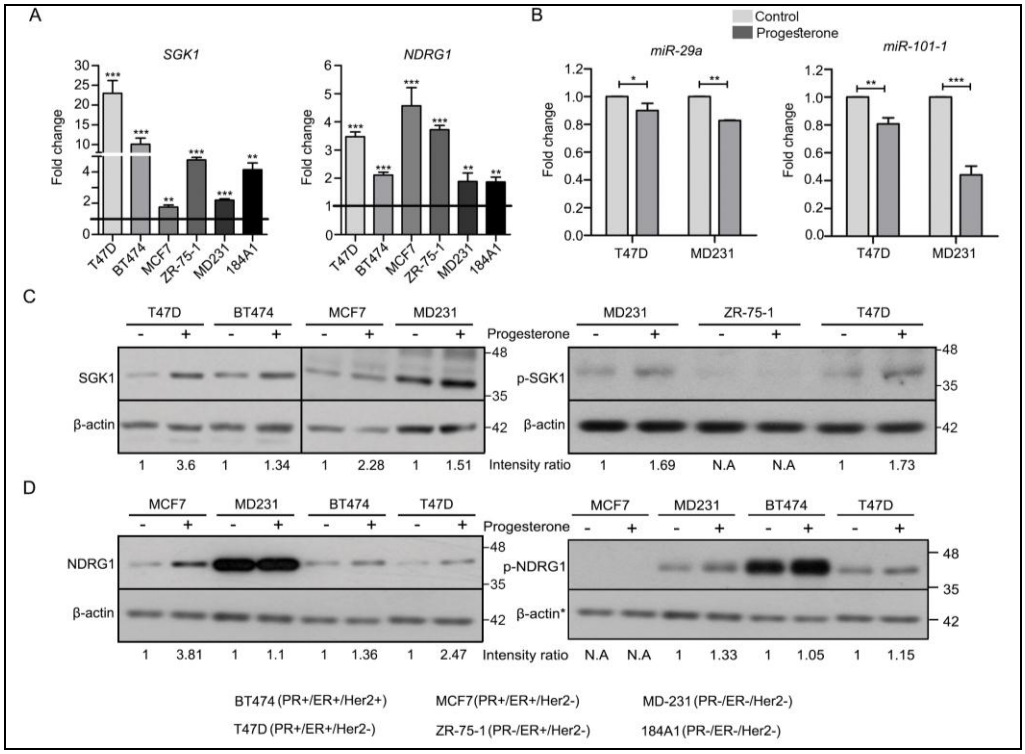
down-regulation of *miR-29a* and *miR-101-1*, predicted to bind the 3'UTR region of *SGK1*, independent of the hormonal receptor status of the cells (V-Figure-3; V-Table-4 and V-Table-5).



V-Figure- 3: Identification and validation of candidate genes and standardization of progesterone treatment in breast cancer cells

A) Representation of expression array results; left panel indicates the WGCNA results with *SGK1* being the top up-regulated gene in cells indicated, in response to progesterone. Figure panel on the right indicates results of the recurrent gene expression analysis. Genes de-regulated in more than three cell lines were considered to be recurrently expressed in response to progesterone. Red colour in the figure indicates up-regulation, blue colour indicates down-regulation, and no colour/blank stands for no change in gene expression in response to progesterone treatment. **B)** Standardization of progesterone treatment was based on expression changes of three known progesterone regulated genes (*STAT5A*, *EZF* and *F3*), studied using quantitative real time PCR analysis. Data has been plotted as expression change in response to progesterone or mifepristone+progesterone treatment, with respect to control (horizontal black line). Figure is representative of three independent experiments performed in triplicates in T47D cells (PR-positive). **C)** Real time PCR validation for expression of *SGK1* and *NDRG1* in T47D cells (PR-positive) in response to progesterone and mifepristone+progesterone treatment. Data has been plotted as fold change for individual gene with respect to expression in control cells and normalized with respect to expression of *GAPDH*. Figure is representative of two independent experiments performed in triplicates. *P*-value was calculated using student's unpaired t-test. * indicates $P<0.05$; ** indicates $P<0.005$; *** indicates $P<0.0005$; ns indicates not significant.

The up-regulation of *SGK1,* known to harbor multiple progesterone response element [177, 178], and *NDRG1* were observed to be relatively higher among the PR-positive cells, while *miR-29a* and *miR-101-1* were lower in PR-negative cells in response to progesterone (V-Figure-4A-D). Interestingly, SGK1 activation (induction of phospho-SGK1 in response to progesterone) was found to be comparable in breast cancer cells, regardless of their PR status (V-Figure-4C).

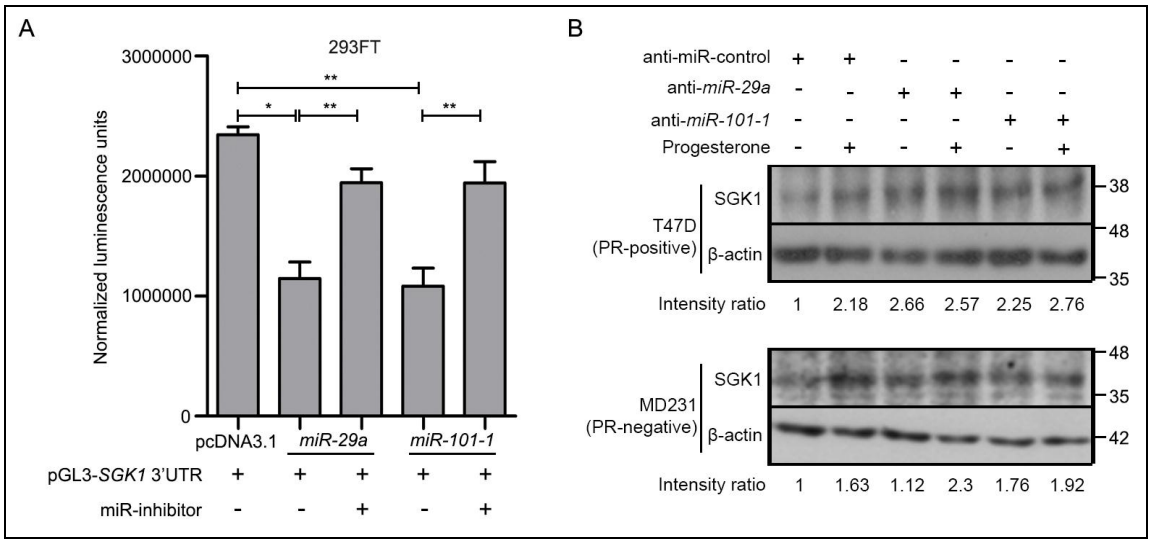


V-Figure- 4: Validation of expression of *SGK1* and *NDRG1*, and *miR-29a* and *miR-101-1* expression in breast cell lines treated with progesterone

A) Quantitative real-time PCR analysis for validation of expression of *SGK1* and *NDRG1* transcripts in breast cell lines in response to progesterone. Expression of both the genes was normalized with respect to expression of *GAPDH* in each cell line. Data has been plotted as fold change for each gene with respect to the expression in control sample of each cell line. Horizontal black line indicates gene expression in control cells. Figure is representative of three independent experiments performed in triplicates. *P*-value was calculated using student's unpaired t-test. * indicates $P<0.05$, ** indicates $P<0.005$ and *** indicates $P<0.0005$. **B)** Transcript levels of *miR-29a* and *miR-101-1* were measured using real-time PCR analysis in T47D and MDA-MB-231 cells treated with progesterone. Graph has been plotted as expression fold change of the two microRNAs normalized to expression of *U6* small RNA in progesterone-

treated versus control. Transcript levels in both control and progesterone-treated cells have been shown. Figure is representative of three independent experiments performed in triplicates. **C)** Western blot analysis for SGK1 (left panel) and p-SGK1 (right panel) in breast cancer cells treated with progesterone. "-" indicates control, while "+" indicates progesterone-treated samples. β-actin was used as an internal loading control. Numbers on blot indicate intensity ratio for SGK1 and p-SGK1, normalized to β-actin levels in respective cell lines. Both the western blot analysis are representative of three independent experiments. **D)** Western blot analysis of NDRG1 (left panel) and p-NDRG1 (right panel) in breast cancer cells treated with (indicated by "+") and without (indicated by "-") progesterone. "*" mark on β-actin indicates same β-actin was used as loading control for western blot of NDRG1 and p-NDRG1. Numbers on blot indicate intensity ratio for NDRG1 normalized with respect to β-actin levels, while p-NDRG1 levels have been normalized with respect to total NDRG1 expression. The analysis is representative of three independent experiments.

Next, we validated *SGK1* as a target of *miR-29a* and *miR-101-1* by co-expressing the microRNAs along with firefly luciferase reporter genes cloned upstream to 3'UTR region of *SGK1*. Ectopic expression of both the microRNAs decreased the firefly luciferase activity in 293FT cells expressing the 3'UTR of *SGK1*. Consistent with the findings, transfection with anti-miR's targeting *miR-29a* and *miR-101-1* not only rescued the repression of luciferase activity in 293FT cells (V-Figure-5A); but also led to sustained expression levels of SGK1 based on western blot analysis of breast cancer cells (V-Figure-5B). Taken together, the data suggests convergence of dual mode of regulation at *SGK1* in response to progesterone treatment, along with up-regulation of *NDRG1* in multiple breast cancer cell lines independent of their PR status.



V-Figure- 5: Functional validation of *miR-29a* and *miR-101-1* mediated regulation of expression of *SGK1*

A) Quantification of luminescence units normalized to renilla luciferase activity has been plotted for: pCDNA3.1-*miR-29a* or pCDNA3.1-*miR-101-1* and pGL3-*SGK1* 3'UTR in different combinations with anti-*miR-29a* or anti-*miR-101-1* in 293FT cells. Figure is representative of three independent experiments performed in 293FT cells. *P*-value was calculated using student's unpaired t-test. * indicates *P*-value<0.05; ** indicates *P*-value <0.001; *** indicates *P*-value <0.0001. **B)** Western blot analysis of SGK1 in T47D (PR-positive, top panel) and MDA-MB-231 (PR-negative, bottom panel) treated with anti-miR-control, anti-*miR-29a* or anti-*miR-101-1*. As indicated in the panel, cells were either treated with progesterone or untreated. β-actin was used as an internal protein loading control. Numbers on the blot indicate intensity ratio of expression of SGK1 with respect to the anti-miR-control lane and expression normalized with respect to individual β-actin levels. The western blot analysis is representative of three independent experiments.

V-Table- 4: Relative log-fold change of expression of genes-of-interest in response to progesterone as identified in microarray gene expression analysis and fold change from real-time PCR analysis

	Gene name	T47D	BT474	MCF7	ZR-75-1	MDA-MB-231
Microarray	*SGK1*	3.510	2.818	0.3748	0.6782	-0.0945
	NDRG1	1.818	1.14	0.0890	0.5606	0.2172
Real-time PCR	*SGK1*	22.9801	10.0952	1.7566	4.7698	2.1947
	NDRG1	3.4690	2.1108	4.5726	3.5433	1.8818

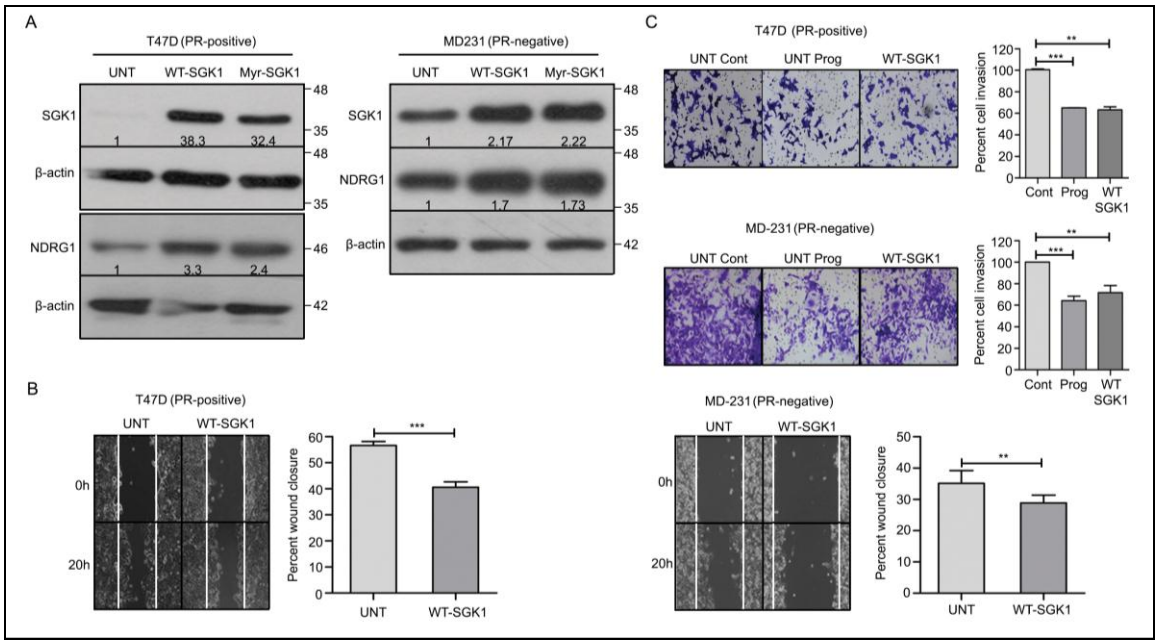
V-Table- 5: Relative log-fold change of expression of microRNAs-of-interest in response to progesterone as identified in small RNA sequencing and fold change from real-time PCR analysis

	MicroRNA name	T47D	BT474	MDA-MB-231
Small RNA sequencing	*miR-29a*	-1.605	-1.713	N.D.
	miR-101-1	-1.605	-1.713	N.D.
Real-time PCR	*miR-29a*	0.9001	1.1217	0.8274
	miR-101-1	0.8074	1.2204	0.4402

N.D: not determined in small RNA sequencing

5.3.2 *SGK1* over expression mimics progesterone treatment to up-regulate *NDRG1*

SGK1 has been shown to phosphorylate NDRG1 [179]. However, the role of SGK1 in regulating the expression of NDRG1 remained to be explored. Moreover, since phospho-NDRG1 levels did not change in response to progesterone treatment (V-Figure-4D, right panel), we checked the hypothesis whether SGK1, instead of only phosphorylating NDRG1, could regulate the expression of NDRG1 in breast cancer cells. Indeed, *SGK1* when overexpressed in PR-positive T47D and PR-negative MDA-MB-231 breast cancer cells mimics the effect of progesterone by up-regulating the expression of *NDRG1* (V-Figure-6A), which in turn lead to a significant reduction in cell migration and cell invasion (V-Figure-6B and 6C).

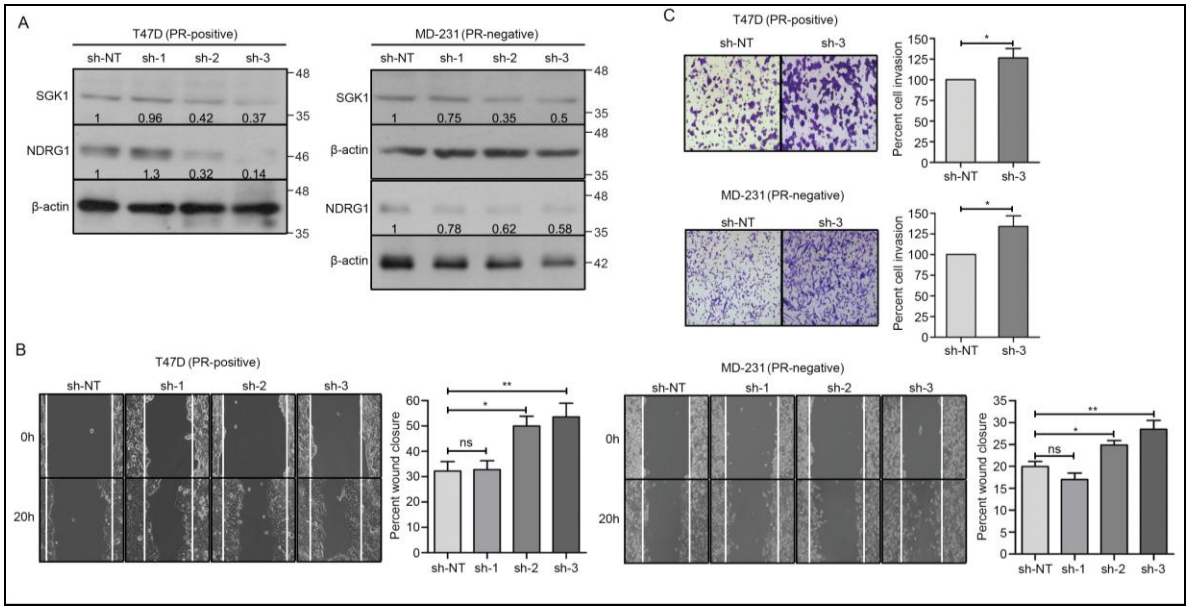


V-Figure- 6: Ectopic expression of *SGK1* mimics the effect of progesterone in breast cancer cells

A) Western blot analysis indicating expression of SGK1 and NDRG1 in T47D (PR-positive, left panel) and MDA-MB-231 cells (PR-negative, right panel) over-expressing *SGK1*. β-actin was used as an internal loading control. Numbers on blot indicate intensity ratio for SGK1 and NDRG1, normalized to respective β-actin levels. The analysis is representative of three independent experiments. **B)** Cell migration of T47D (left panel) and MDA-MB-231 (right panel) cells over-expressing *SGK1* was compared to un-transfected parent cells in a wound scratch assay. Bar plots indicate percentage cellular migration of the cells, with a direct comparison between untransfected cells and cells over-expressing *SGK1* and the analysis is representative of three independent experiments performed in triplicates. **C)** In cells over-expressing *SGK1*, cell invasion was studied in T47D (top panel) and MDA-MB-231 (bottom panel) and percent cell invasion was compared with respective parent cells. Parent cells treated with progesterone were also used to compare the level of cell invasion upon *SGK1*-over-expression. Bar plot depicts percentage cell invasion and figure is representative of three independent experiments, performed in triplicates. *P*-value was calculated using student's unpaired t-test. ** indicates $P < 0.005$; *** indicates $P < 0.0005$.

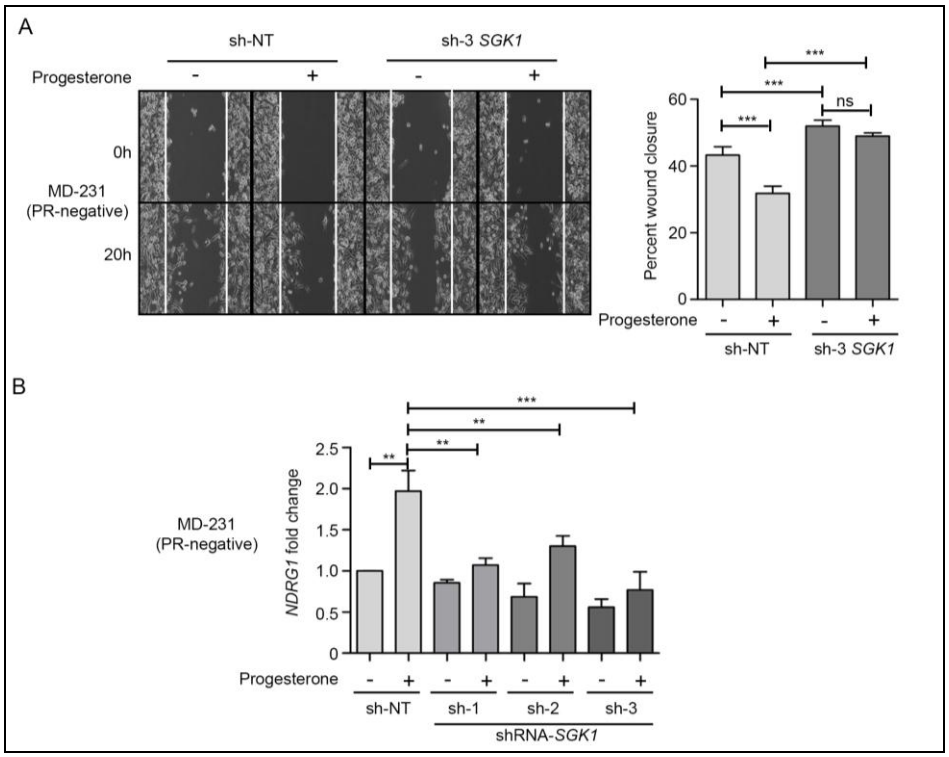
In a reciprocal approach, depletion of *SGK1* in T47D and MDA-MB-231 cells lead to decrease in expression of *NDRG1* (V-Figure-7A) with inverse effect observed on migration and invasion of the breast cancer cells (V-Figure-7B and 7C), regardless of progesterone treatment (V-Figure8A and 8B). Furthermore, consistent with the genetic perturbation, pharmacological inhibition of SGK1 with 1μM GSK650394A

similarly block the effect of progesterone on breast cancer cell migration and cell invasion, suggesting an essential role of *SGK1*/*NDRG1*-axis downstream to progesterone in breast cancer cells independent of their hormonal receptor status (V-Figure-9).



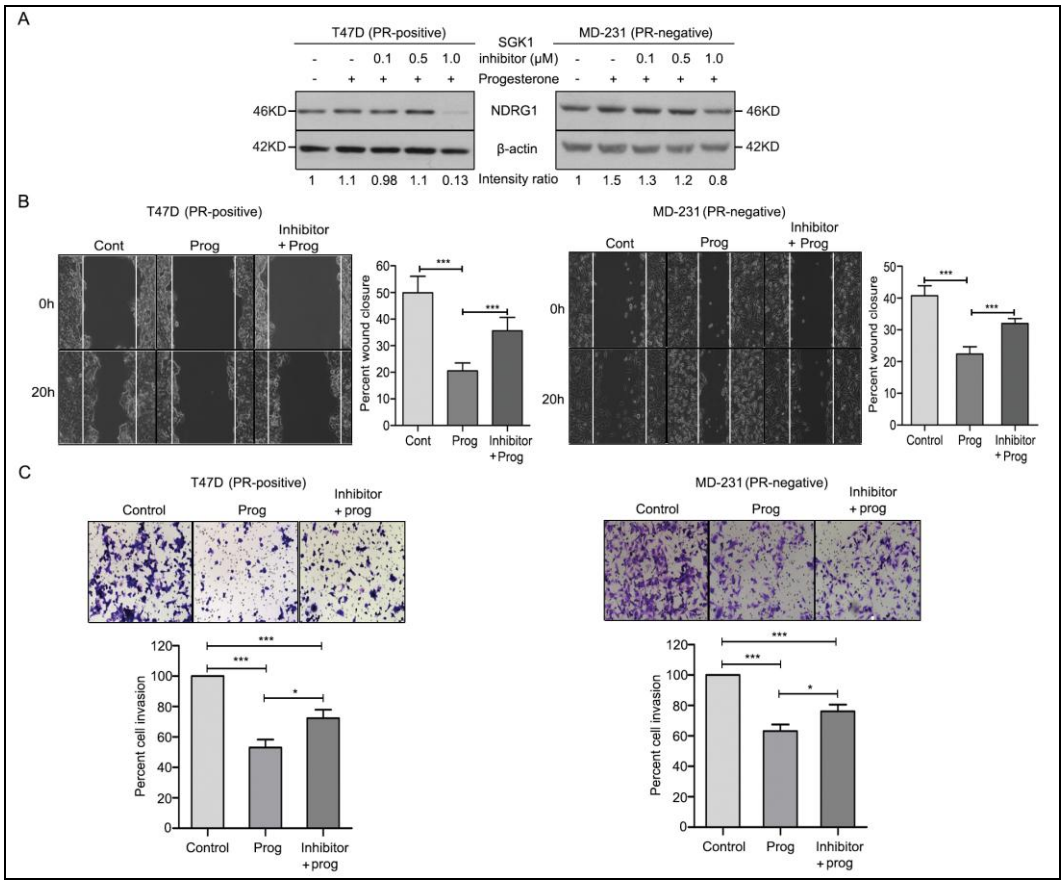
V-Figure- 7: Knockdown of *SGK1* decreases expression of *NDRG1* and increases cell migration and invasion in breast cancer cells

A) Western blot analysis depicting expression of SGK1 and NDRG1 in T47D (PR-positive, left panel) and MDA-MB-231 (PR-negative, right panel) upon depleting the expression of *SGK1*. Expression of SGK1 and NDRG1 for each knockdown clone was compared with expression in sh-Non-targeting (sh-NT) clone. β-actin was used as loading control. Numbers on blot indicate intensity ratio for expression of SGK1 and NDRG1, normalized to respective β-actin levels. The analysis is representative of three independent experiments. **B)** Cell migration was studied upon knockdown of *SGK1* in T47D (left panel) and MDA-MB-231 (right panel) cells. The distance traversed by migrating cells was calculated from the start point to the migrated point over a period of 20h. Data has been plotted as representative percentage wound closure observed in three independent experiments performed in triplicates. **C)** Invasion assay upon depletion of *SGK1* as compared to sh-NT clone of T47D (top panel) and MDA-MB-231 (bottom panel) cells respectively. Bar plot represents percentage cell invasion with respect to invasion in sh-NT clone. The analysis is representative of three independent experiments performed in triplicates. *P*-value was calculated using student's unpaired t-test. * indicates $P<0.05$; ** indicates $P<0.005$; *** indicates $P<0.0005$.



V-Figure- 8: Depletion of *SGK1* renders breast cancer cells partially responsive to progesterone

A) Cell migration assay upon depletion of *SGK1* in MDA-MB-231 cells (PR-negative), in the presence and absence of progesterone treatment. Cells were monitored by time-lapse wound healing assay for 20h. Cell migration from 0h to 20h time-point has been plotted as percentage wound closure and the comparison is with respect to sh-NT clone. The analysis is representative of three independent experiments performed in triplicates. **B)** Transcript levels of *NDRG1* have been analyzed in MDA-MB-231 cells (PR-negative) upon depletion of *SGK1*, in the presence and absence of progesterone stimulation. Data has been plotted as fold change of *NDRG1* with respect to expression in untreated sh-NT cells and individual *SGK1* knockdown clones and *NDRG1* expression normalized with respect to *GAPDH*. The analysis is representative of three independent experiments performed in triplicates. *P*-value was calculated using student's unpaired t-test. * indicates $P<0.05$; ** indicates $P<0.001$; *** indicates $P<0.0001$; ns indicates not significant.

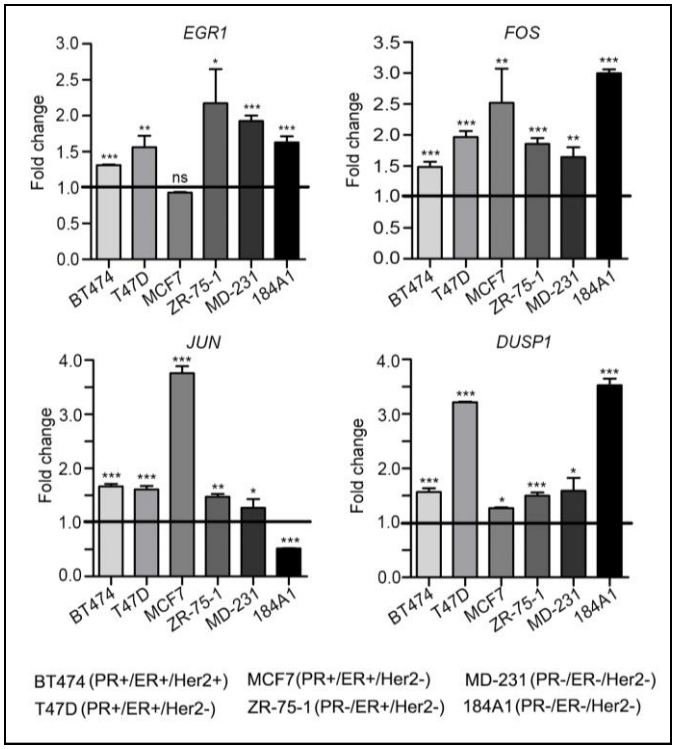


V-Figure- 9: SGK1-inhibitor phenocopies the effect of depletion of *SGK1* in breast cancer cells

A) Western blot analysis representing the expression of NDRG1 in T47D (PR-positive) and MDA-MB-231 cells (PR-negative) treated with the SGK1-inhibitor and progesterone. Expression of NDRG1 was normalized with respect to β-actin levels in respective cell lines. The numbers on the blot indicate intensity ratio of expression of NDRG1 with respect to untreated cells in each cell line. The western blot analysis is representative of three independent experiments. **B)** Cell migration assay of breast cancer cells treated with *SGK1*-inhibitor and progesterone. The motility of cells from initial to 20h time-point was plotted as percentage cell migration and the comparison was between control, progesterone-treated and SGK1-inhibitor+progesterone treated conditions. Bar plot indicates percent wound closure in each of the three treatment conditions. Analysis is representative of two independent experiments performed in triplicates. **C)** Cellular invasion assay was performed with *SGK1* inhibitor in T47D and MDA-MB-231 cells. Panels show cells with no treatment, progesterone treated, and those with both inhibitor and progesterone combination. Bar plot represents percentage cell invasion for each of the treatment conditions. Figure is representative of two independent experiments performed in triplicates. *P*-value was calculated using student's unpaired t-test. * indicates $P<0.05$; ** indicates $P<0.005$; *** indicates $P<0.0005$.

5.3.3 AP-1 transcription factors mediate up-regulation of *NDRG1*

NDRG1 is known to be regulated by AP-1 (*FOS*/*JUN*) and *EGR1* in response to stress-induced activation of kinases such *p38*, *JNK* and *ERK* [180-183]. We recently showed that progesterone modulates the effect of surgical stress in primary breast cancer patients [184]. Thus, we asked if *NDRG1* could be regulated by AP-1 network genes in response to progesterone-induced activation of *SGK1*, in a similar manner in breast cancer cells. Indeed, treatment with progesterone or over-expression of *SGK1* led to several fold over-expression of the AP-1 network genes in a panel of breast cell lines irrespective of their PR status (V-Figure-10, V-Figure-11A and V-Figure-12A).

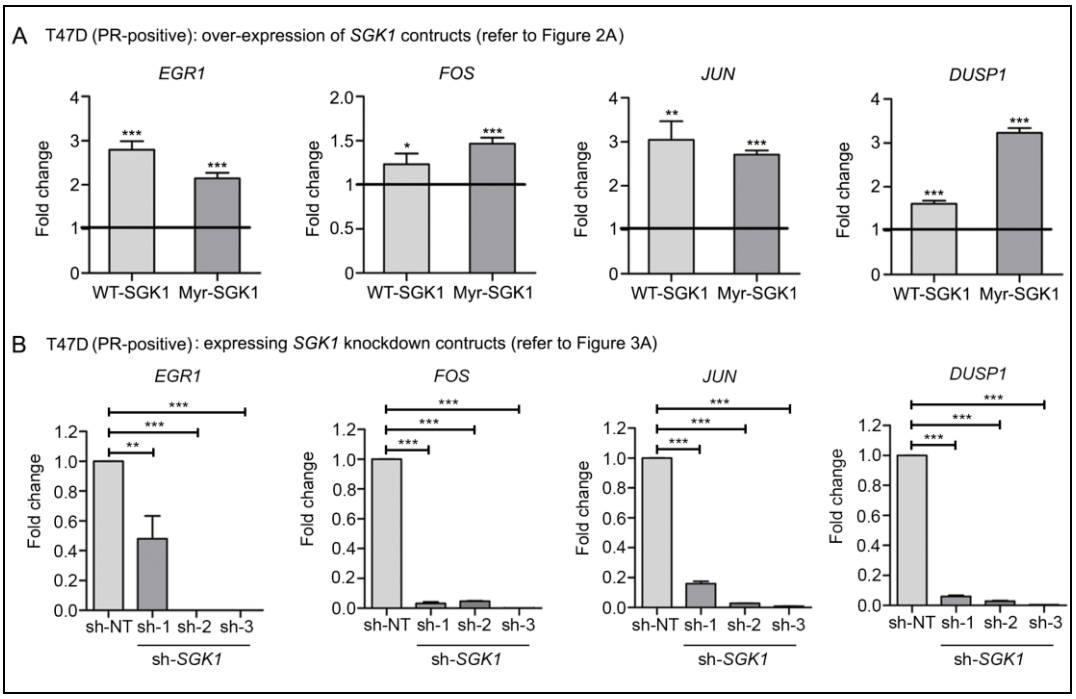


V-Figure- 10: Progesterone up-regulates expression of the AP-1 network genes in breast cell lines

In the panel of breast cell lines representing different receptor status, transcript levels of AP-1 network genes (*EGR1*, *FOS*, *JUN* and *DUSP1*) has been studied using quantitative real-time PCR analysis. Expression of individual genes in the control sample has been set as 1 and represented as a horizontal black line. Data has been plotted as expression fold change upon progesterone treatment with respect to expression in control. *GAPDH* has been used to normalize the gene expression. The

figure is representative of three independent experiments performed in triplicates. *P*-value was calculated using student's unpaired t-test. * indicates $P<0.05$; ** indicates $P<0.001$; *** indicates $P<0.0001$; ns indicates not significant.

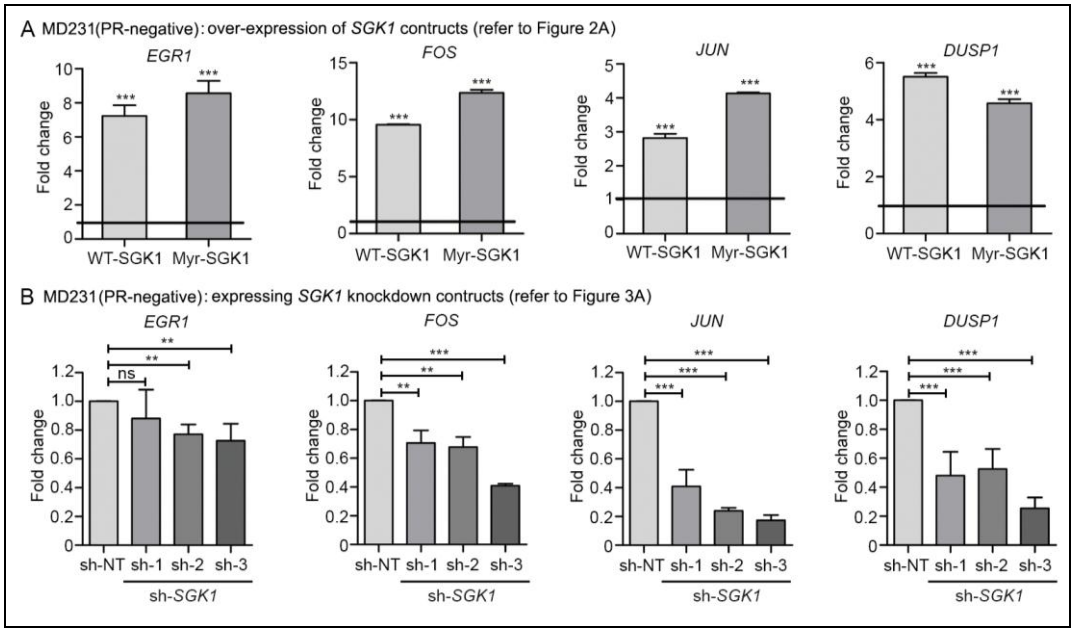
Consistent with this finding, knockdown of *SGK1* significantly reduced the expression of AP-1 network genes (V-Figure-11B and V-Figure-12B), and depleting the expression of an AP-1 network gene *EGR1* abrogated the expression of *NDRG1* (V-Figure-13A), a downstream component of the pathway, in T47D and MDA-MB-231. Taken together, these results suggest that progesterone and *SGK1* regulate the expression of *NDRG1* via the AP-1 network genes.



V-Figure- 11: *SGK1* regulates expression of the AP-1 network genes in breast cancer cells

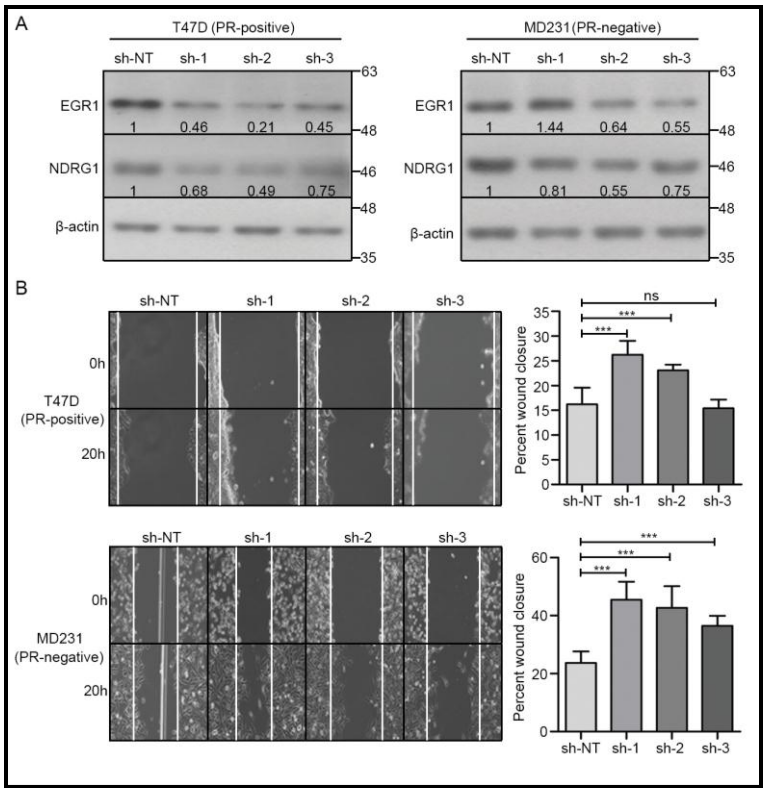
Transcript levels of AP-1 network genes (*EGR1*, *FOS*, *JUN* and *DUSP1*) were studied using quantitative real-time PCR, in T47D (PR-positive) cells **A)** over-expressing *SGK1*, and **B)** upon knockdown of *SGK1*. For expression analysis upon over-expression of *SGK1*, horizontal black line indicates transcript levels for the AP-1 network genes in untransfected cells. In case of analysis upon knockdown of *SGK1*, transcript levels of AP-1 network genes were compared against sh-NT clone. Data has been plotted as fold change for each individual gene with respect to expression in sh-

NT clone and normalized with respect to *GAPDH*. Both the real-time PCR analyses are representative of two independent experiments performed in triplicates. *P*-value was calculated using student's unpaired t-test. * indicates $P<0.05$; ** indicates $P<0.005$; *** indicates $P<0.0005$.



V-Figure- 12: *SGK1* regulates expression of the AP-1 network genes in MDA-MB-231 cells

Expression of AP-1 network genes was studied upon **A)** over-expression, and **B)** knockdown of *SGK1* in MDA-MB-231 cells (PR-negative) using quantitative real-time PCR. For expression analysis upon *SGK1* over-expression, data has been plotted as fold change for each gene with respect to expression in untransfected parent cells. The gene expression in untransfected cells has been represented using horizontal black line. For expression analysis in *SGK1* knockdown clones, data has been plotted as fold change for each individual gene with respect to sh-NT clone. Expression of all genes in both experiments has been normalized with respect to expression of *GAPDH*. Both the real-time PCR analyses are representative of two independent experiments performed in triplicates. The efficiency of over-expression and knockdown of *SGK1* can be referred in Figure 2A and 3A, respectively. *P*-value was calculated using student's unpaired t-test. * indicates $P<0.05$; ** indicates $P<0.005$; *** indicates $P<0.0005$; ns indicates not significant.



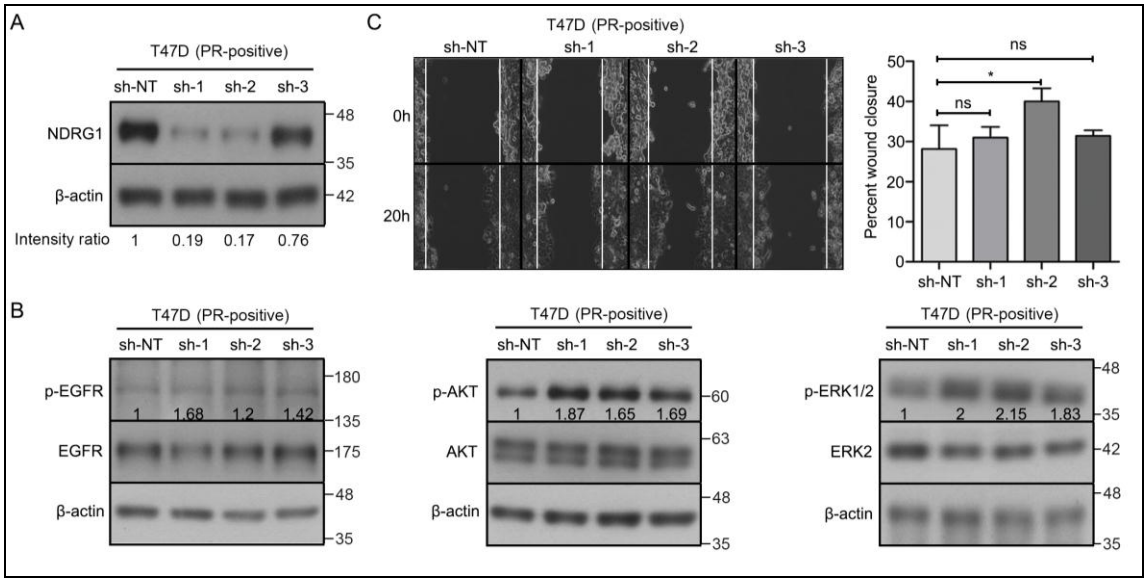
V-Figure- 13: Knockdown of *EGR1* decreases expression of NDRG1 in breast cancer cells

A) Western blot analysis of EGR1 and NDRG1 in T47D (PR-positive, left panel) and MDA-MB-231 (PR-negative, right panel) cells upon genetic depletion of *EGR1*. sh-NT was used as vector control. β-actin protein was used as loading control for western blot. Numbers on blot indicate intensity ratio for expression of EGR1 and NDRG1, normalized to respective β-actin levels. Western blot analysis is representative of three independent experiments. **B)** Cell migration analysis upon depletion of *EGR1* in T47D (top panel) and MDA-MB-231 (bottom panel) breast cancer cells. Cells were monitored by time-lapse wound healing assay for 20h. Cell migration from 0h to 20h time-point was plotted as percentage wound closure and the comparison was with respect to sh-NT clone. The analysis is representative of three independent experiments performed in triplicates. *P*-value was calculated using student's unpaired t-test. *** indicates $P<0.0001$; ns indicates not significant.

5.3.4 *SGK1/NDRG1*-axis inactivates EGFR- MAPK pathway to inhibit migration and invasion of breast cancer cells

We recently showed that progesterone decreases the activation of multiple kinases like EGFR, AKT1 and ERK1/2 in breast cancer cells, leading to suppression of cell migration [174]. To test, if *NDRG1* mediate inactivation of EGFR/AKT1/ERK1/2

kinases in response to progesterone**,** we knocked down the expression of *NDRG1* in T47D and MDA-MB-231 cells (V-Figure-14A and V-Figure-15A).

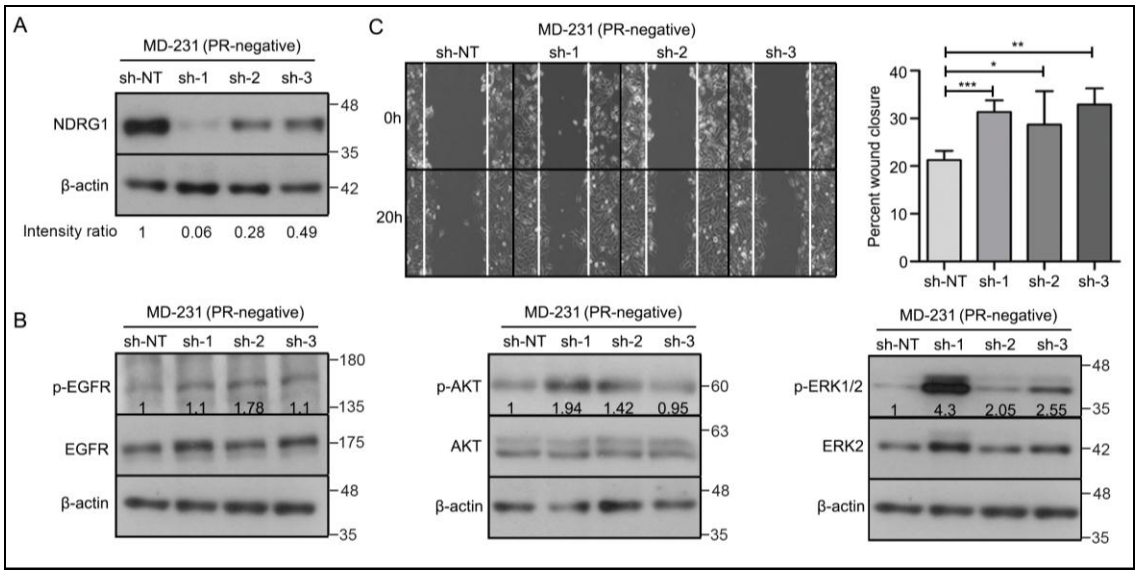


V-Figure- 14: *NDRG1* regulates the activation of multiple cellular kinases and cell migration in T47D cells

A) Western blot analysis depicting knockdown of *NDRG1* in T47D cells (PR-positive). sh-NT was used as vector control for *NDRG1* expression. β-actin protein was used as loading control for western blot. Numbers on the blot indicate intensity ratio for NDRG1 expression normalized to respective β-actin levels. Analysis is representative of three independent experiments. **B)** Western blot analysis of p-EGFR (Y1086), p-AKT (S473) and p-ERK1/2 (T202/Y204) in *NDRG1* knockdown clones of T47D cells. β-actin used as loading control for western blot. Numbers on blot indicate intensity ratio for phosphorylation levels of EGFR, AKT and ERK1/2, normalized to respective total protein levels (EGFR, AKT and ERK2). Figure is representative of three independent experiments. **C)** Cell migration analysis upon depletion of *NDRG1* in T47D breast cancer cells has been shown. Cells were monitored by time-lapse wound healing assay for 20h. Cell migration from initial to 20h time-point was plotted as percentage wound closure and the comparison was with respect to sh-NT. Figure is representative of three independent experiments performed in triplicates. *P*-value was calculated using student's unpaired t-test. * indicates $P<0.05$; ns indicates not significant.

Interestingly, 2 of 3 shRNA clones targeting *NDRG1* significantly increased the phosphorylation of EGFR (Y1068), AKT (S473) and ERK1/2 (T202/Y204) (V-Figure-14B and V-Figure-15B). Furthermore, breast cancer cells expressing constructs

targeting *EGR1* and *NDRG1* displayed an increase in breast cancer cell migration (V-Figure-14C, 15C and 13B). Taken together, our results suggest that *SGK1/NDRG1-axis* mediate regulation of activation of kinases involved in breast cancer cell migration, independent of their hormonal receptor status.



V-Figure- 15: Depletion of *NDRG1* activates multiple cellular kinases and increases migration of MDA-MB-231 cells

A) Western blot analysis depicting knockdown of *NDRG1* in MD-231 breast cancer cells (PR-negative). sh-NT was used as vector control for *NDRG1* expression. β-actin protein was used as loading control for western blot. Numbers on blot indicate intensity ratio for NDRG1, normalized to respective β-actin levels. Analysis is representative of three independent experiments. **B)** Western blot analysis of p-EGFR (Y1086), p-AKT (S473) and p-ERK1/2 (T202/Y204) in *NDRG1* knockdown clones of MD231 cells. β-actin has been used as loading control for western blot. Numbers on blot indicate intensity ratio for phosphorylation levels of EGFR, AKT and ERK1/2, normalized to respective total protein levels (EGFR, AKT and ERK2). Western blot analysis is representative of three independent experiments. **C)** Migration of cells was measured from 0-20h by using time-lapse wound healing assay. Data was plotted as percentage wound closure, with comparison between sh-NT and each shRNA clone. Figure is representative of three independent experiments performed in triplicates. *P*-value was calculated using Student's unpaired t-test. * indicates $P<0.05$; *** indicates $P<0.0001$.

5.4 Discussion:

Preoperative endocrine therapies, in contrast to neoadjuvant chemotherapy, are much simpler and economical to deliver. An understanding of the targets could thus be of immense potential utility in monitoring the response of hormones in human cancer. This study details the underlying molecular mechanism associated with benefits of preoperative progesterone treatment as observed in our randomized trial [171]. We present an intricate convergence model indicating a dual-phase regulation downstream to progesterone treatment to regulate the expression of a *Serum- and Glucocorticoid-regulated Kinase* gene 1, *SGK1*: predominantly driven as a direct transcriptional target, consistent with earlier reports [177, 178], in PR-positive breast cancer cells; and, down-regulation of *miR-29a* and *miR-101-1* targeting *SGK1* with relatively distinct effect in PR-negative breast cells in response to progesterone. In addition, as described earlier [174], glucocorticoid receptor *GR* or the membrane progesterone receptor (*PGRMC1* and *SERBP1*) likely mediates effect of progesterone in PR-negative breast cancer cells that are uniformly expressed across breast cancer cells (V-Table-2). The stringent up-regulation of *SGK1* in response to progesterone lead to an activation of a tumor metastasis suppressor gene, *NDRG1*, via a set of AP-1 network genes to inactivate AKT1, ERK1/2 and EGFR kinases, impeding the invasion and migration of breast cancer cells. As *NDRG1* is known to be regulated by AP-1 network genes in response to stress-induced activation of kinases [180-183], this model confirms and extends our recent report that progesterone modulates the effect of surgical stress by up-regulation of *SGK1* in primary breast cancer patients [184], affecting the invasive characteristics of breast cancer cells most likely by regulating their migration.

Interestingly, *SGK1* and *NDRG1* are known to be down-regulated in human cancers as compared to adjacent normal tissues and that increased expression of both these genes has been associated with better survival of cancer patients [185-189]. Even the recently described panel of 38 gene signature that predict favorable prognosis of breast cancer patients include *SGK1* [58]. Thus, enhanced expression of *SGK1* and *NDRG1* could explain better survival of breast cancer patients [171]. Our study suggests that *SGK1* up-regulates the expression of *NDRG1*, with no significant change in phosphorylation of NDRG1 in breast cancer cells. We describe that *SGK1* regulates the expression of *NDRG1* via regulation of expression of *EGR1*, a transcription factor from the AP-1 network genes, in breast cancer independent of the PR status of cells. In summary, we propose a model for the mode of action of progesterone in breast cancer deciphering the molecular basis of a randomized clinical trial studying the effect of progesterone in breast cancer (VI-Figure-2). While there have been attempts to understand the effect of progesterone as a physiological hormone [92], we provide the first mechanistic insights into the role of progesterone in breast cancer, detailing the genetic event leading to clinical observation of better survival of breast cancer patients treated with pre-operative progesterone [171]. However, whether these molecular targets of progesterone could help in stratification of breast cancer patients and aid in better prognosis, remains to be studied.

VI. DISCUSSION:

Breast cancer is the most commonly occurring cancer in females across the world with a mortality rate of about 50% [190]. Despite recent developments in diagnostic and treatment methods, patients continue to develop metastatic tumors and relapse [21]. Hormones influence physiology and development of the normal breast tissue and can also alter the outcome of breast cancer [191-193]. Early observational studies to monitor the effect of hormones on outcome of breast cancer surgery performed in different phases of menstrual cycle showed that progesterone improved the outcome of pre-menopausal patients [123, 124, 194-196]. Taking leads from these studies, a randomized clinical trial with pre-operative progesterone intervention was performed in Tata Memorial Hospital to establish an association of progesterone with breast cancer in a large cohort of operable breast cancer patients. It was observed that progesterone improved the disease-free and overall survival of node-positive breast cancer patients independent of their menopausal or PR status [128]. The survival benefits obtained using a single injection of progesterone was an encouraging outcome, however, a multicentre validation may help in the wider usability of progesterone as a therapy for treatment of breast cancer patients [140]. This was a hypothesis generating clinical outcome since in most other cases the effect of endocrine therapy was purely based on the presence of corresponding hormone receptors or the menopausal status of the patients [34, 40, 157]. However, the mechanism of action of progesterone remained to be elucidated in breast cancer to explain the outcome of this randomized clinical trial with pre-operative progesterone treatment. This led to the origin of my thesis where I have focused on identifying the

underlying effects of progesterone on breast cancer, using a functional genomics and proteomics approach performed in a panel of breast-derived cell lines. To understand the PR-independent targets of progesterone, I have selected multiple breast cancer and immortalized normal-like cell lines with different hormone receptor statuses and breast cancer subtypes. I hypothesized that progesterone could induce transient transcriptional and proteomic changes in breast cancer cells leading to phenotypic alterations that can be captured using functional genomic and phenotypic analysis.

6.1 Phenotypic and proteomic changes induced by progesterone

Breast cancer poses a unique threat with capacity to metastasize and causes more mortality compared to the primary tumor itself [46, 48, 197]. Surprisingly, as observed in the clinical trial, breast cancer patients with adjacent lymphnode-involvement showed a better survival upon pre-operative progesterone intervention. This suggests that progesterone might have possibly targeted breast cancer cells primed with metastatic property, leading to regression of tumor by controlling the dissemination of cancer cells during surgery and their establishment in distant organs [128]. While the effects of progesterone have been well studied for its context-dependent proliferative and growth-inhibitory role in breast cells, functional studies have not performed to study the role of progesterone in regulation of cell migration and invasion independent of the PR status of cells [69, 91-93, 198]. It is well established that over-expression or activation of kinases like EGFR or AKT1 provides an opportunity for breast cancer cells to invade from the site of primary tumor and escape to distant organs [146, 151]. To study whether progesterone affects the activation of kinases in breast cancer cells, I performed an unbiased proteomic analysis using a proteome profiling assay which accommodates a panel of 43 kinases

that are known to regulate migratory properties of cancer cells. I observed a significant decrease in phosphorylation of 12 of 43 kinases like EGFR, AKT1, ERK1/2, FAK and so on, in PR-positive and PR-negative breast cancer cells treated with progesterone. Concomitantly, I observed a significant reduction in cell migration and invasion in breast cancer cells independent of their PR statuses. Of note, I found that PR plays a non-essential role in mediating this effect of progesterone, since blocking the activity of PR using mifepristone did not hamper the inhibition of cell invasion and migration of breast cancer cells. Thus, consistent with studies targeting kinase activity using kinase-inhibitors [98, 142], I show that progesterone inhibits cell migration and invasion of breast cancer cells by decreasing the phosphorylation of multiple kinases. As a plausible explanation to the PR-independent mode of action, I hypothesize that progesterone might be working in synergism with other steroid hormone receptors like the GR or mPR which are known to associate with progesterone [77, 83, 99]. Although the effect of progesterone on cell invasion and migration of breast cancer cells has been studied in the context of PR isoforms or by their over-expression in MDA-MB-231 cell line with varying outcome [153, 199, 200], my study, using additional PR-negative breast cancer cells like ZR-75-1 and BT-549 that have not been studied before, suggests a consistent decrease in these cellular phenotypes independent of the PR expression.

6.2 Genomics analysis of progesterone-induced changes in microRNAs and their target genes

Endocrine therapy has helped in improving the survival of patients with hormone receptor positive breast cancer. Moreover, studies have suggested that patients treated with tamoxifen or aromatase inhibitors which target ER activity, show better response

in presence of PR and show reduced relapse as compared to patients with PR-negative tumors [53, 59, 201]. Also, PR has been observed as a prognostic marker for lower metastatic potential of breast cancer [52]. Though patients show an initial response to endocrine therapy, a significant number of patients develop resistance, primarily due to loss of PR expression over the period of progression of the disease. While over-expression of growth factor receptors like Her2 or EGFR has been attributed to loss of PR, few other pathways leading to degradation and silencing of PR have been studied in breast cancer, with some of these pathways mediated in response to progesterone [53, 62, 64, 202]. Hormones are known to decrease the expression of their receptors, for instance, estrogen represses the expression of *ER* by up-regulating microRNAs in breast cancer [61]. However, whether progesterone also suppresses expression of *PR* in a similar way is not well studied. To identify microRNAs targeting *PR* in response to progesterone, I performed small RNA sequencing of breast cancer cell lines treated with progesterone and identified *miR-129-2* as a direct target of progesterone which decreases the expression of *PR* in breast cancer. I functionally validated the physical association of *miR-129-2* with PR and observed that their expression is inversely correlated in breast cancer patients in the TCGA cohort. Interestingly, I observed that inhibition of activity of *miR-129-2* in breast cancer cells using anti-*miR-129-2* inhibitor helped in rescuing the expression of PR even in the presence of progesterone. Inhibiting the activity of such microRNAs in breast cancer patients expressing low PR levels may help in stabilizing the expression of PR and concomitantly improve the response to endocrine therapies. However, this is a preliminary observation and usage of anti-*miR-129-2* requires validation in breast cancer patients. Nevertheless, my study has identified a novel microRNA that mediates the effect of progesterone by

suppressing the expression of PR. Knowledge of such microRNAs targeting PR may provide an opportunity to reinstate the expression of PR in breast cancer.

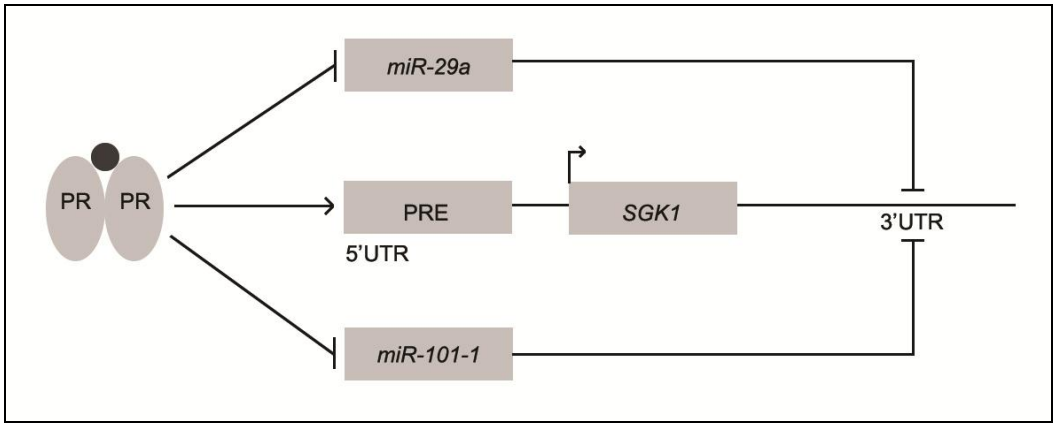
6.3 Functional genomics analysis of progesterone-treated breast cancer cell lines

As discussed in the first part of my thesis, I observed a consistent decrease in cell invasion and migration of breast cancer cells, possibly via inhibition of phosphorylation of multiple kinases in response to progesterone. While this suggests that progesterone may regulate the metastatic potential of breast cancer, the underlying molecular mechanism of action of progesterone remained to be elucidated. With this notion, I set to identify the transcriptional targets of progesterone by performing a microarray gene expression analysis of breast cancer cells. Most of the earlier studies have performed gene expression analysis in PR-positive cell lines and identified isoform-specific targets of PR rather than genes regulated by progesterone [58, 109, 203]. For my thesis work, I focused on identifying the targets of progesterone independent of the PR expression of the cells. As a strategy to identify true targets of progesterone, I depleted the culture medium of all growth factors and hormone-like compounds prior to addition of progesterone to the cells. The expression array analysis helped in the identification of *Serum and glucocorticoid-regulated kinase 1*, *SGK1* and *N-Myc downstream regulated gene 1*, *NDRG1*, as direct targets up-regulated in response to progesterone in multiple breast cancer cells. Moreover, the results suggest that progesterone increased the phosphorylation of SGK1 as well, suggesting an increased activity of SGK1 in breast cancer cells. By performing genetic perturbation experiments, I observed that *SGK1* regulates the expression of *NDRG1* in breast cancer, rather than just phosphorylating it as reported earlier. I could identify that progesterone and *SGK1* mediate the increased expression of *NDRG1* via

the AP-1 network genes *viz. EGR1* and *FOS-JUN*. As a proof of concept, I show that depletion of *EGR1* decreases the expression of *NDRG1* in breast cancer cells. Furthermore, I observed that *SGK1* mimics the activity of progesterone to suppress the cell migration and invasion of breast cancer independent of the PR status, possibly via the increased expression of *NDRG1*, a known tumor metastasis suppressor gene [189, 204]. Consistent with its known activity [205], I observed that depletion of *NDRG1* gave cells an opportunity to activate EGFR, AKT1 and ERK1/2 kinases in breast cancer cells irrespective of their PR status. Moreover, these cells showed an enhanced migratory potential. While *SGK1* has been shown to be a direct target of progesterone [206], I observed an intricate regulatory network being regulated by *SGK1*, leading to the suppression of cell invasion and migration of breast cancer cells.

In a parallel study, I observed that the expression of *SGK1* is under a dual-regulatory mode in response to progesterone. While expression of *SGK1* is known to be up-regulated by progesterone via PRE in the promoter region of *SGK1*, I show that progesterone maintains sustained expression of *SGK1* by suppressing the expression of *miR-29a* and *miR-101-1* in breast cancer. Using a biochemical approach, I observed an association of *miR-29a* and *miR-101-1* with the 3'UTR of *SGK1* and that inhibition of these microRNAs stabilizes expression of SGK1 in breast cancer. Since progesterone is able to up-regulate expression of *SGK1* from the promoter and 3'UTR of *SGK1*, we propose a dual-regulatory model of regulation of expression of *SGK1* in response to progesterone in breast cancer, irrespective of the PR status of cells (VI-Figure-1). Additional experiments like CRISPR-based knockout of *SGK1*, and over-expression of a SGK1 kinase-dead mutant or dominant-negative form of SGK1 in breast cancer cells would strengthen the interpretation made in my work This is the

first study to show that progesterone regulates expression of *SGK1* in a dual-regulatory manner in breast cancer and provides a proof-of-concept for presence of such molecular targets of progesterone.



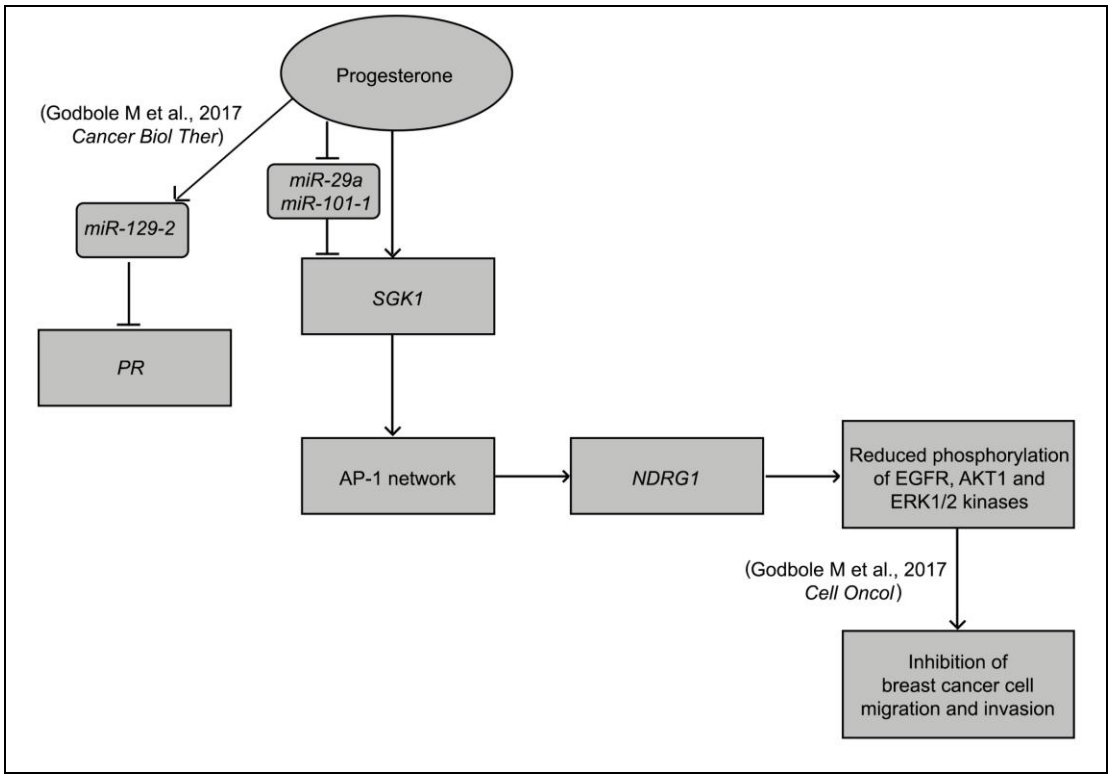
VI-Figure- 1: Proposed dual-regulatory model for regulation of expression of *SGK1*
The model describes regulation of expression of *SGK1* by progesterone at two sites. Firstly, progesterone transcriptionally activates *SGK1* via association of PR-PRE at promoter. Secondly, progesterone decreases expression of *miR-29a* and *miR-101-1* and thus stabilizes expression of *SGK1* in breast cancer.

Though we could not correlate the expression of *miR-29a* and *miR-101-1* with expression of *SGK1* in the TCGA breast cancer patient cohort, orthologous methodologies can possibly be used to check the expression of these microRNAs and *SGK1* in patient samples. Moreover, I believe that an extended analysis of our microarray gene expression and small RNA sequencing results should provide more dual-regulated targets of progesterone. Also, the expression of microRNAs validated in our study was found to be independent of the PR status of the cells. Since microRNAs are known to be housed as 'intronic' or 'intergenic' [155, 207-209], a thorough analysis needs to be performed for understanding their mode of regulation in response to progesterone. Preliminary analysis suggests that the microRNAs could either be expressed along with a gene responsive to progesterone (intronic, *miR-29a*

and *miR-101-1*) or could have their own promoter which responds to progesterone (intergenic, *miR-129-2*) in breast cancer cells, which remains to be confirmed experimentally.

6.4 Conclusion:

In overall, my thesis provides first leads to systematically model the effects of pre-operative progesterone intervention in breast cancer patients, where the up-regulation of *SGK1* leads to sustained activity of progesterone to inhibit the activation of kinases and suppress the cell migration and invasion of breast cancer cells via increased expression of *NDRG1*, regulated by *SGK1* and the AP-1 network genes, independent of the PR status of cells. While on the other hand, I observe that of the several microRNAs de-regulated in cell lines, progesterone fine-tunes the expression of *miR-129-2* and *miR-29a* and *miR-101-1* that target *PR* and *SGK1* in breast cancer cells. To explain this tightly regulated pathway, I propose a working model explaining the effect of progesterone on breast cancer (VI-Figure-2). Elevated expression of tumor metastasis suppressor genes like *NDRG1* could possibly explain the effect of pre-operative progesterone intervention of breast cancer patients independent of their PR status. Though my study focuses on the transient changes induced by progesterone in breast cancer, I am optimistic that the targets identified in the study and the beneficial role of increased expression of *SGK1* and *NDRG1* in survival of breast cancer patients reported earlier may help in better prognosis or selection of breast cancer patients for progesterone treatment [186, 210, 211].



VI-Figure- 2: Working model for progesterone-mediated regulation of coding and non-coding genes leading to suppression of cell migration and invasion in breast cancer

However, I do concur with the notion that progesterone could be regulating multiple pathways in breast cancer, functioning along with the model described in my study. Hence, I propose that the microarray gene expression and small RNA sequencing analysis performed in my study should help in identifying additional targets of progesterone and expand the model described herein. Moreover, details of how progesterone acts in PR-negative breast cancer cells, by performing experiments with PR/GR/mPR knockdown, along with in-depth study of *SGK1*-mediated regulation of *NDRG1* and downstream signaling, remains to be elucidated. And, the epigenetic modifications induced by progesterone could be studied to better understand the

regulation of the candidate genes expressed or repressed in breast cancer cells. Additionally, the mode of regulation of the microRNAs identified in this study remains to be explored. Also, non-coding RNAs like lncRNAs are emerging as targets of hormones like estrogen. These lncRNAs have been shown to mediate several functional roles in cancer cells, including endocrine resistance and metastasis of cells [212, 213]. Thus in extension to identifying the coding targets, lncRNAs and epigenetic modifications de-regulated by progesterone can also be identified by performing similar functional genomics approaches in breast cancer cells treated with progesterone.

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VIII. APPENDIX

8.1 APPENDIX: List of differentially expressed microRNAs in breast cancer cells in response to progesterone.

Numbers in bracket indicate log-fold change of gene expression in response to progesterone treatment

BT474 (PR+/ER+/Her2+)	**T47D (PR+/ER+/Her2-)**	**MCF7 (PR+/ER+/Her2-)**	**MDA-MB-231 (PR-/ER-/Her2-)**
UP-REGULATED			
miR-1226-5p (3.124)	miR-3908 (6.254)	miR-193b-5p (5.946)	miR-4485-3p (9.204)
miR-4485-3p (3.124)	miR-6764-5p (3.841)	miR-3065-5p (5.225)	miR-200c-3p (9.204)
miR-4756-3p (2.763)	miR-1273e (3.459)	miR-1268b (5.225)	miR-153-5p (9.204)
miR-518f-3p (2.423)	miR-1304-5p (2.938)	miR-199a-3p (4.97)	miR-96-5p (8.79)
miR-4451 (2.41)	miR-548t-5p (2.938)	miR-3127-5p (4.97)	miR-148b-3p (8.79)
miR-5096 (2.281)	miR-6817-5p (2.938)	miR-449c-5p (4.97)	miR-25-5p (8.79)
miR-1278 (2.281)	miR-6832-5p (2.938)	miR-6880-5p (4.659)	miR-7977 (8.207)
miR-5683 (2.281)	miR-3934-5p (2.938)	miR-449a (4.659)	miR-615-5p (8.207)
miR-6798-3p (2.281)	miR-6783-5p (2.864)	miR-95-3p (4.659)	miR-4682 (8.207)
miR-4741 (2.281)	miR-548d-3p (2.864)	miR-424-5p (4.659)	miR-924 (8.207)
DOWN-REGULATED			
miR-569 (-3.684)	*miR-3615 (-4.756)*	*miR-25-5p (-5.669)*	*miR-887-3p (-8.931)*
miR-937-3p (-3.578)	*miR-612 (-4.314)*	*miR-7161-3p (-5.264)*	*miR-365a-3p (-8.517)*
miR-196a-3p (-3.402)	*miR-558 (-3.671)*	*miR-4483 (-5.264)*	*miR-205-5p (-8.517)*
miR-744-3 (-3.052)	*miR-625-3p (-3.551)*	*miR-548t-3p (-4.697)*	*miR-149-5p (-8.517)*
miR-6886-5p (-2.97)	*miR-4664-3p (-3.551)*	*miR-570-5p (-4.697)*	*miR-148a-5p (-8.517)*
miR-26a-1-3p (-2.884)	*miR-186-3p (-3.419)*	*miR-3182 (-4.697)*	*miR-1908-5p (-8.517)*
miR-548e-5p (-2.792)	*miR-3677-3p (-3.419)*	*miR-935 (-4.697)*	*miR-532-3p (-7.934)*
miR-4783-3p (-2.792)	*miR-3659 (-3.34)*	*miR-147b (-4.697)*	*miR-4477b (-7.934)*
miR-218-5p (-2.588)	*miR-23b-5p (-3.196)*	*miR-3940-3p (-4.697)*	*miR-4661-5p (-7.934)*
miR-3613-5p (-2.588)	*miR-3619-5p (-3.196)*	*miR-942-5p (-4.697)*	*miR-4286 (-7.934)*

8.2 APPENDIX: List of differentially expressed genes upon progesterone treatment of breast-derived cell lines.

Numbers in bracket indicate log-fold change of gene expression in response to progesterone treatment

BT474 (PR+/ER+/Her2+)	**T47D (PR+/ER+/Her2-)**	**MCF7 (PR+/ER+/Her2-)**	**ZR-75-1 (PR-/ER+/Her2-)**	**MDA-MB-231 (PR-/ER-/Her2-)**	**184A1 (PR-/ER-/Her2-)**
UP-REGULATED					
GAPDHL6 (3.2186)	SGK (3.6077)	KIAA1370 (1.2780)	ARHGAP26 (1.4063)	C7ORF54 (1.9061)	GAPDHL6 (3.2186)
LOC341230 (2.8178)	SGK1 (3.5101)	ATAD2 (1.1675)	SCNN1G (1.3262)	HS.571887 (1.52969)	LOC341230 (2.8178)
LOC440311 (2.7937)	STAT5A (2.45918)	PCM1 (1.1638)	HMGCS2 (1.3247)	HS.436134 (1.52152)	LOC440311 (2.79372)
CCDC85B (2.71265)	GOLSYN (2.3103)	PHF3 (1.1625)	LOC346887 (1.2952)	HS.572444 (1.43157)	CCDC85B (2.71265)
KRT8P9 (2.5719)	ANKRD35 (2.1517)	MKLN1 (1.1501)	SUPT7L (1.2732)	HS.528873 (1.39739)	KRT8P9 (2.5719)
LOC644584 (2.5712)	RANBP3L (2.1146)	STXBP3 (1.1076)	C17ORF77 (1.2055)	HS.143018 (1.3908)	LOC644584 (2.57125)
LOC650298 (2.5155)	SLC25A18 (1.9226)	RBM25 (1.0859)	LOC100133242 (1.1250)	HS.157344 (1.26579)	LOC650298 (2.5155)
GPC1 (2.4914)	PLLP (1.91349)	FAM102B (1.0849)	ZNF689 (1.1154)	LRP5L (1.2045)	GPC1 (2.4914)
LOC391019 (2.4827)	NDRG1 (1.8181)	HS.473191 (1.0695)	ALOXE3 (1.0496)	HS.481659 (1.2037)	LOC391019 (2.4827)
LOC644936 (2.3761)	CMTM7 (1.7547)	LOC729978 (1.0543)	LOC644681 (1.0016)	HS.184721 (1.19539)	LOC644936 (2.3761)
DOWN-REGULATED					
DDX49 (-1.3715)	*LOC645762 (-1.7277)*	*PTMA (-1.1156)*	*LOC100132564 (-1.6473)*	*CEBPZ (-1.9835)*	*ADO (-3.1315)*
CLOCK (-1.3580)	*LOC643308 (-1.5930)*	*KRT18P28 (-1.007)*	*MSTO2P (-1.6147)*	*IL8 (-1.9794)*	*STX8 (-2.8209)*
SNPH (-1.230)	*PTGR2 (-1.4984)*	*LOC388344 (-0.882)*	*LOC644655 (-1.4671)*	*YEATS4 (-1.8307)*	*FLJ44124 (-2.7283)*
KRT8P9 (-1.211)	*HEY2 (-1.4359)*	*LOC100134273 (-0.8700)*	*LOC100132561 (-1.239)*	*HNRNPL (-1.7735)*	*LOC643357 (-2.5947)*
LOC728441 (-1.1526)	*LOC728572 (-1.3458)*	*LOC650276 (-0.7701)*	*HS.539123 (-1.2356)*	*TAF1B (-1.7522)*	*KIAA1826 (-2.4668)*
MAF1 (-1.1324)	*KRT18P28 (-1.3258)*	*LOC100133697 (-0.7687)*	*C3ORF41 (-1.1430)*	*TMEM126B (-1.6825)*	*KIAA1618 (-2.3665)*
LOC72905 (-1.1320)	*MYB (-1.2410)*	*LOC441550 (-0.7682)*	*HS.564701 (-1.1060)*	*IFI44 (-1.6782)*	*IFI16 (-2.2911)*
GPR68 (-1.1193)	*C6ORF141 (-1.2135)*	*LOC100129028 (-0.7682)*	*FGFBP1 (-1.0936)*	*CCDC90B (-1.6495)*	*LOC100132391 (-2.2611)*
CISH (-1.0615)	*LOC402644 (-1.1966)*	*LOC728649 (-0.7676)*	*LOC645927 (-1.0579)*	*NDUFAF2 (-1.6427)*	*ZYG11B (-2.2497)*
GPC1 (-1.0569)	*FAM65C (-1.18813)*	*LOC100131205 (-0.7562)*	*HS.545044 (-1.0390)*	*IFIT1 (-1.6381)*	*DPYSL2 (-2.2309)*

IX. REPRINTS OF PUBLICATIONS

Cell Oncol. (2017) 40:411–417
DOI 10.1007/s13402-017-0330-z

REPORT

Progesterone suppresses the invasion and migration of breast cancer cells irrespective of their progesterone receptor status - a short report

Mukul Godbole[1,2] · **Kanishka Tiwary**[1] · **Rajendra Badwe**[3] · **Sudeep Gupta**[4] · **Amit Dutt**[1,2]

Accepted: 17 May 2017 / Published online: 26 June 2017

Abstract
Purpose Pre-operative progesterone treatment of breast cancer has been shown to confer survival benefits to patients independent of their progesterone receptor (PR) status. The underlying mechanism and the question whether such an effect can also be observed in PR negative breast cancer cells remain to be resolved.
Methods We performed proteome profiling of PR-positive and PR-negative breast cancer cells in response to progesterone using a phospho-kinase array platform. Western blotting was used to validate the results. Cell-based phenotypic assays were conducted using PR-positive and PR-negative breast cancer cells to assess the effect of progesterone.
Results We found that progesterone induces dephosphorylation of 12 out of 43 kinases tested, which are mostly involved in cellular invasion and migration regulation. Consistent with this observation, we found through cell-based phenotypic assays that progesterone inhibits the invasion and migration of breast cancer cells independent of their PR status.
Conclusion Our results indicate that progesterone can inhibit breast cancer cell invasion and migration mediated by the dephosphorylation of kinases. This inhibition appears to be independent of the PR status of the breast cancer cells. In a broader context, our study may provide a basis for an association between progesterone treatment and recurrence reduction in breast cancer patients, thereby providing a lead for modelling a randomized in vitro study.

Keywords Breast cancer · Progesterone · Cell invasion · Cell migration · Phosphoproteomics · Metastasis

Electronic supplementary material The online version of this article (doi:10.1007/s13402-017-0330-z) contains supplementary material, which is available to authorized users.

✉ Rajendra Badwe
badwera@tmc.gov.in

✉ Sudeep Gupta
sudeep.gupta@actrec.gov.in

✉ Amit Dutt
adutt@actrec.gov.in

[1] Integrated Genomics Laboratory, Advanced Centre for Treatment, Research and Education In Cancer, Tata Memorial Centre, Navi Mumbai 410210, India

[2] Training School Complex, Homi Bhabha National Institute, Anushakti Nagar, Mumbai, India

[3] Department of Surgical Oncology, Tata Memorial Centre, Tata Memorial Hospital, Mumbai 400012, India

[4] Department of Medical Oncology, Tata Memorial Centre, Mumbai 400012, India

1 Introduction

Metastasis is a major cause of recurrence in breast cancer patients [1]. As a standard mode of treatment, patients with metastatic breast cancer are subjected to adjuvant hormonal therapy [2]. Also, pre-operative progesterone treatment has been shown to reduce the recurrence rate among node-positive patients, independent of their progesterone receptor (PR) status [3]. While the invasion and migration of breast cancer cells play major roles in establishing metastases [4, 5], in vitro cell-based observations on the effect of progesterone, restricted to PR-positive cells, have corroborated the clinical observations [6–8]. As yet, however, the effect of progesterone on the invasion and migration of PR-negative breast cancer cells remains to be systematically explored [9].

The metastatic nature of breast cancer cells is known to be affected by multiple molecular factors, including the activation of protein kinases [10]. The protein kinases EGFR, AKT

or FAK have, for instance, been found to activate the processes of migration and invasion of breast cancer cells [11, 12]. In addition, it has been found that these kinases may act synergistically and that abrogating their activation may decrease the invasive capacity of breast cancer cells [13]. Also, pathways downstream of these kinases may serve to restrain cell invasion and migration [10]. Although these kinases have been found to be affected by steroid hormone receptors [14], it remains to be explored whether they mediate the responses to progesterone in breast cancer cells.

To address the question whether progesterone can regulate cellular migration and invasion of breast cancer cells independent of their PR status, we selected a panel of breast cancer-derived cell lines with different PR statuses. Next, we performed a phospho-proteomic screening of kinases associated with migration and invasion using a human proteome phospho-kinase array platform, and studied their phosphorylation status after treating the respective cells with progesterone. Our cell-based phenotypic and biochemical analysis results suggest that progesterone may mitigate the invasion and migration of breast cancer cells, irrespective of their PR status.

2 Materials and methods

2.1 Breast cancer-derived cells

The BT474, T47D, MCF7, ZR-75-1, MDA-MB-231 and BT-549 breast cancer-derived cell lines were obtained as a gift from Dr. Slamon's Laboratory (Department of Medicine, UCLA, USA). The cell lines were authenticated by DNA short tandem repeat (STR) profiling using the Promega GenePrint 10 system in conjunction with the GeneMarker HID software tool and the ATCC database. The cells were tested for mycoplasma and, if necessary, made mycoplasma-free using an EZKill Mycoplasma Removal reagent (HiMedia). BT474, T47D, MCF7 and MDA-MB-231 cells were cultured in DMEM medium (Gibco), whereas ZR-75-1 and BT-549 were cultured in RPMI-1640 medium (Gibco). BT-549 cells were supplemented with 0.023 IU/ml insulin. All culture media were supplemented with 10% (*v*/v) FBS (Gibco), 2.5 mg/ml Amphotericin-B (Abbott) and 1.25 μl/ml Gentamycin (Abbott). The cells were cultured at 37 °C in a 5% CO_2 incubator. The PR/ER/Her2 receptor statuses of all the cells as reported in [15] were validated by reverse transcriptase-PCR.

2.2 Progesterone treatment

Cells were grown to 70–80% confluence and then serum starved in DMEM low glucose medium (HiMedia) for a period of 24 h. Next, the cells were treated with 10 nM 17-α hydroxy-progesterone caproate (progesterone) (MP Biomedicals) in the same medium for 6 h. In case of mifepristone + progesterone (M + P) combination treatment, 100 nM RU486 (mifepristone) was added for 2 h followed by 10 nM progesterone treatment for 6 h in the same medium. An equal volume of alcohol was used as vehicle control.

2.3 Protein sample preparation

Cells were grown to a 70–80% confluence in a 100 mm culture dish and washed thoroughly with sterile 1× PBS. Next, the cells were subjected to 24 h serum starvation (using DMEM low glucose phenol-red free medium) followed by progesterone treatment for 8 h. Alcohol was used as vehicle control. After progesterone treatment, the cells were harvested using a sterile cell scraper and cell lysates were prepared in RIPA Buffer (Sigma-Aldrich) supplemented with a protease-inhibitor cocktail solution (Sigma-Aldrich) and 0.1 M DTT. After intermittently tapping and vortexing the samples on ice, cell debris was pelleted by centrifugation at 14000 rpm after which the protein concentrations were determined using BCA reagent (MP Biomedicals). Bovine serum albumin was used as a standard and the estimations were performed in triplicate.

2.4 Phospho-kinase activation profiling

Kinase activation profiling of T47D (PR-positive) and MDA-MB-231 (PR-negative) breast cancer-derived cells was performed using a Human Phospho-kinase array kit (ARY003B; R&D Systems) according to the manufacturer's instructions. Briefly, cells were grown in T75 flasks till 70–80% confluence was reached, serum-starved for another 24 h (in serum-free phenol-red-free DMEM medium) and treated with progesterone for 8 h. Next, the cells were harvested, washed with 1× PBS and lysed, after which 400 μg protein from untreated and progesterone-treated samples was incubated overnight at 4 °C with a pre-blocked antibody array nitrocellulose membrane. Subsequently, the membranes were incubated with detection antibodies and probed using streptavidin-HRP, after which signals were developed using chemi-reagents provided with the kit. Exposures to X-ray films were taken from 10 s to 10 min (till saturation was reached). Signal densities of reference spots on both membranes were compared between each pair of membranes used for the control and progesterone-treated samples. The pixel density of each spot, in duplicate, was calculated using ImageJ Array Analyzer plugin. The average pixel density for the duplicate spots for each of the kinases was subtracted from the negative control density. The average pixel densities for control and progesterone-treated samples were plotted as percent phosphorylation for each phospho-kinase. The differential phosphorylation cut-off value was set at 20% increase or decrease in phosphorylation of kinases in response to progesterone.

2.5 Western blotting

Equal amounts of cell lysate were resolved by 10% SDS-PAGE and transferred to PVDF membranes using a wet transfer method. Primary antibodies directed against p-EGFR (Y1068) (Cell Signaling, 3777S; Dilution 1:500), p-AKT (S473) (Cell Signaling, 4060S; Dilution 1:500), p-ERK1/2 (T202/Y204) (Cell Signaling, 9101S; Dilution 1:1000), total EGFR (1005) (Santa Cruz Biotechnology, sc-03; Dilution 1:1000), total AKT (11E7) (Cell Signaling, 4685S; Dilution 1:1000), total ERK2 (c-14) (Santa Cruz Biotechnology, sc-154; Dilution 1:1000) and β-actin (I-19)-R (Santa Cruz Biotechnology, sc-1616-R; Dilution 1:3000) were diluted in 3% BSA solution prepared in 1× TBST and incubated over night at 4 °C. A goat anti-rabbit IgG-HRP secondary antibody (Santa Cruz Biotechnology, sc-2004; Dilution 1:3000) was used for the detection of primary antibody binding. ECL Western Blotting Substrate (Pierce) and Takara Chemiluminescence substrate (ClonTech Takara) were used for visualization of the protein bands on X-ray films (Fuji Films).

2.6 RNA extraction and real-time PCR

For RNA extraction from alcohol and progesterone-treated breast cancer-derived cells, the respective cells were treated with progesterone for 6 h. Next, TRIzol reagent (Invitrogen) was used to lyse the cells after which RNA was isolated according to the manufacturer's protocol. RNA concentrations were measured using NanoDrop. For assessment of the *DUSP1* transcript levels, cDNA was synthesized using a High capacity cDNA reverse transcription kit (Applied Biosystems) and subjected to quantitative real-time PCR using a Roche Light-Cycler-II 480 instrument in conjunction with a Roche real-time master mix (Roche). Expression changes were calculated using the $2^{-\Delta\Delta CT}$ method. *GAPDH* was used as internal control for normalization. The primer sequences used for *DUSP1* were Forward primer OAD-571: CCTGCAGTACCCCACTCTACG; Reverse primer OAD-572: CCCAAGGCATCCAGCATGTCC and for *GAPDH* Forward primer OAD-328: AATCCCATCACCATCTTCCA ; Reverse primer OAD-329: TGGACTCCACGACG TACTCA.

2.7 Cell invasion assay

A Matrigel invasion assay was performed using 24-well Transwell inserts (Corning) coated with 100 μg matrigel and allowed to settle for 24 h at 37 °C. Next, 35,000 cells suspended in 350 μl serum-free medium were seeded into the upper chamber and 600 μl of 10% serum-containing medium was added to the lower chamber. After this, the cells were allowed to invade for 16-18 h at 37 °C, followed by fixation of the invaded cells and staining by crystal violet. After mounting the membrane using DPX on a slide, the cells were observed under an upright microscope. Ten random fields were chosen after which the number of cells in each field was counted and plotted as percentage cell invasion.

2.8 Scratch wound healing assay

Confluent cell monolayers in 6-well plates were subjected to a scratch with a sterile pipette tip. After this, the cells were briefly rinsed using 1× PBS to remove debris and subsequently incubated with low-glucose phenol-red free DMEM medium containing 10% charcoal-stripped FBS (Gibco). The cells were treated with 10 nM progesterone or 100 nM mifepristone or a combination of both. Alcohol was used as a vehicle control. Cell migration at the wound surface was measured during a period of 20 h under an inverted microscope. Quantification was performed using the ImageJ wound healing plugin tool by measuring the distance of the wound edge of the migrating cells from the start point to the migrated point in three separate wounds in three independent experiments.

3 Results and discussion

The activation of kinases like EGFR and ERK1/2 has been reported to play an important role in the de-regulation of cellular processes that are associated with the metastatic capacity of breast cancer cells [16]. Here, we set out to assess the effect of progesterone on the activation of kinases in breast cancer cells using a human phospho-kinase array platform. To verify the effect of progesterone independent of the progesterone receptor (PR) status of the cells, we selected both PR-positive (T47D) and PR-negative (MDA-MB-231) breast cancer-derived cells for our study (Table 1). Untreated cells were used as negative controls. As reported before, we observed a breast cancer cell-specific phosphorylation of p53 (S392/S46/S15) and AMPK (T183), which were subsequently used as internal positive controls [17, 18]. Based on differential phosphorylation analyses of the T47D and MDA-MB-231 cells, 7 out of 43 kinases tested were found to be dephosphorylated in the progesterone treated cells (Fig. 1a-g and Supplementary Fig. 1). Of these, p70 S6 kinase and STAT3 showed the highest decrease in phosphorylation (30%) while FAK, AKT and RSK1/2/3 showed a 20% decrease in both the cell lines in response to progesterone treatment. In addition, we observed a reduction in phosphorylation of the ERK1/2 (T202/Y204, T185/Y187), EGFR (Y1068), MSK1/2 (S376/S360), p38α (T180/Y182) and p27 (T198) kinases upon treatment with progesterone (Supplementary Fig. 1), as reported earlier [19], and validated the results by Western blot analysis (Supplementary Fig. 2a). Consistent with earlier reports [19], we also observed a significant up-

Table 1 Selection of breast cell lines and validation of PR/ER/Her2 hormone receptor status. A panel of breast cancer cell lines with varying receptor statuses, as reported in the literature, was selected for studying the effects of progesterone. The validation status of PR/ER/Her2 transcript expression in the cell lines is indicated as "+" (positive) or "-" (negative)

Sr. No.	Cell Line	Literature reported receptor status			Validation of receptor status by RT-PCR		
		PR	ER	HER2	PR	ER	HER2
1.	BT474	+	+	+	+	+	+
2.	T47D	+	+	−	+	+	−
3.	MCF7	+	+	−	+	+	−
4.	ZR-75-1	−	+	−	−	+	−
5.	MDA-MB-231	−	−	−	−	−	−
6.	BT-549	−	−	−	−	−	−

regulation of a dual specificity phosphatase, DUSP1, upon treatment with progesterone in breast cancer cells that could possibly mediate the effect observed (Supplementary Fig. 2b). Taken together, our results indicate that progesterone can reduce the phosphorylation of 12 out of 43 kinases tested in a PR-independent manner, which could affect cellular signaling pathways downstream to these kinases with a concomitant increase in the expression of a dual-specificity phosphatase, DUSP1, that could mediate the de-phosphorylation of these kinases [19].

Based on the known involvement of EGFR, AKT and ERK1/2 in the invasion and migration of breast cancer cells and the finding that their inhibition may block this phenotype [11, 12, 20] or vice versa, i.e., metastases inhibitors may inhibit the phosphorylation of FAK in PR-negative MDA-MB-231 cells [21] or lung cancer cells [22], we set out to analyze the in vitro effects of progesterone on breast cancer cells. Using a Matrigel chamber assay in conjunction with cells with varying PR/ER/Her2 statuses (Table 1) we found that progesterone could decrease the invasion capacity of different breast cancer-derived cells (BT474, T47D, MCF7 and MDA-MB-231), irrespective of their hormone receptor status (Fig. 2a-d). This result suggests that targeted activation of kinases by progesterone may bring about phenotypic changes in breast cancer-derived cells independent of their PR-status, potentially decreasing their metastatic capacity similar to combinatorial EGFR and AKT inhibition, which is known to affect the invasion of breast and other cancer cells mediated by matrix metalloproteinases [13, 23]. Importantly, we found that PR blocking by mifepristone had no significant effect on the invasive capacities of the cells, again suggesting that progesterone may induce suppression of invasion in breast cancer cells in a predominantly PR-independent manner (Fig. 2a-d).

Next we performed scratch wound healing assays to assess whether breast cancer cell migration is affected by progesterone. Similar to the effect of progesterone on breast cancer cell invasion, we observed a significant decrease in cellular migration in response to progesterone over the period of 20 h in a PR-independent manner (Fig. 3a-d). The non-essential role of PR that we observed in the inhibition of migration of breast

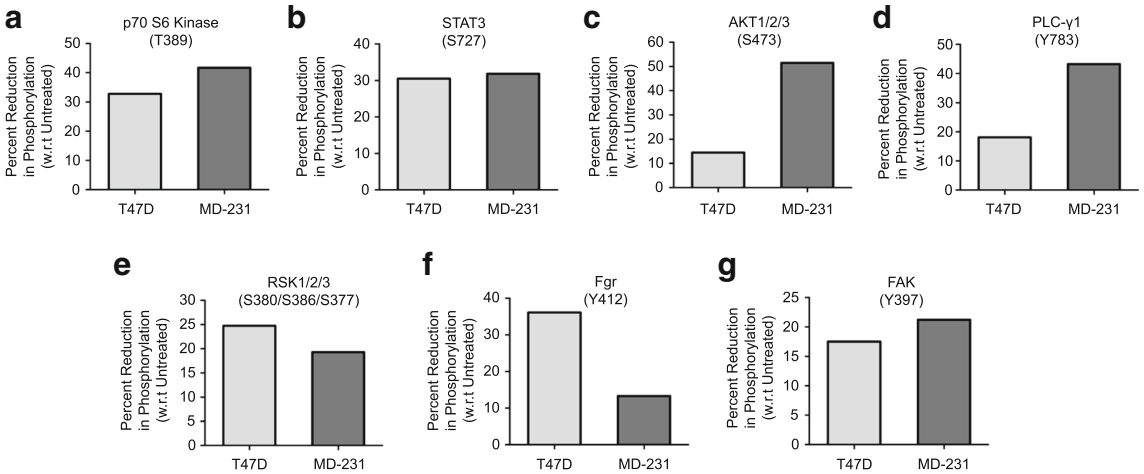

Fig. 1 Kinase phosphorylation is modulated by progesterone in breast cancer cells. The percentage of reduction in phosphorylation in response to progesterone was calculated relative to that in untreated cells and is plotted for each of the differentially phosphorylated kinases (panels **a**-**g**). In the bar plot the light grey bar indicates phosphorylation reduction in T47D cells and the dark grey bar indicates phosphorylation reduction in MDA-MB-231 cells

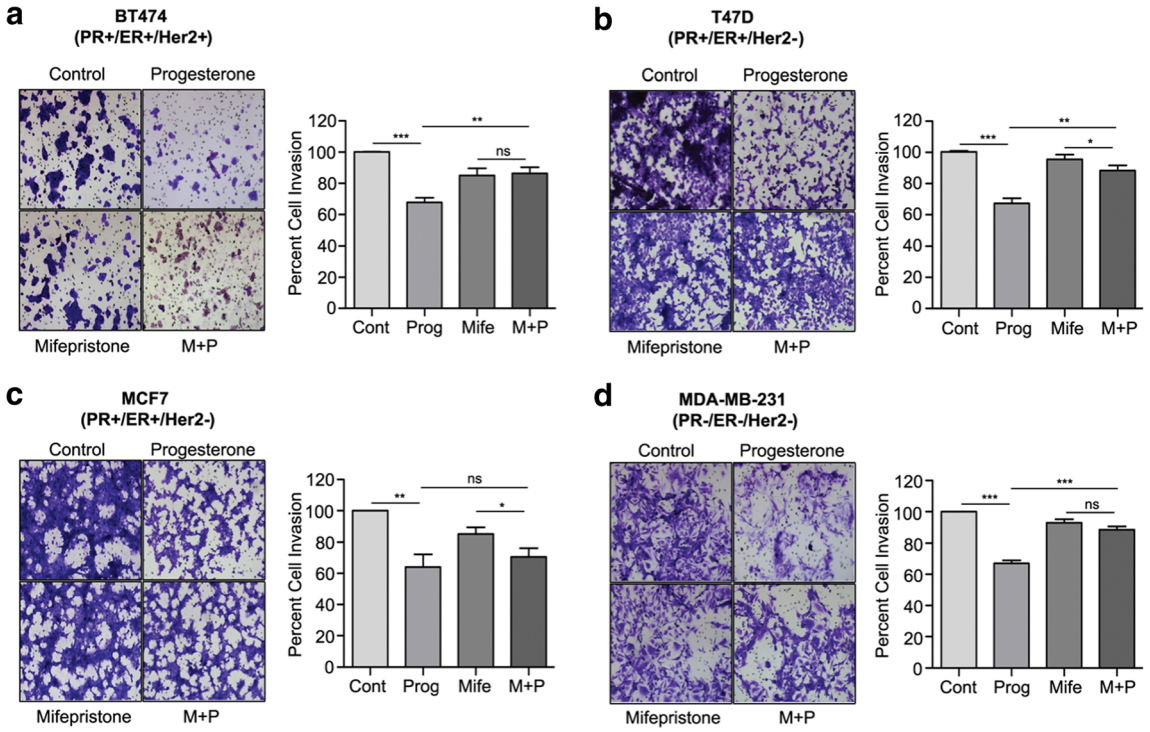

Fig. 2 Progesterone inhibits breast cancer cell invasion. Invasion assays were performed with breast cancer-derived cell lines (**a**) BT474, (**b**) T47D, (**c**) MCF7 and d) MDA-MB-231 treated with progesterone, mifepristone or a combination of mifepristone and progesterone (M + P). The bar plot represents the percentage of cell invasion for each panel. The figure is representative of three independent experiments performed in triplicates. *P*-values were calculated using student's unpaired t-test. ** $p < 0.001$; *** $p < 0001$; ns not significant

cancer-derived cells in response to progesterone, specifically in the PR-negative MDA-MB-231, ZR-75-1 and BT-549 cells, may be mediated by interaction of progesterone with the glucocorticoid receptor (GR) or the membrane progesterone receptor (mPR), as has been reported before [22, 24, 25]. Of note, it has also been reported that treatment with

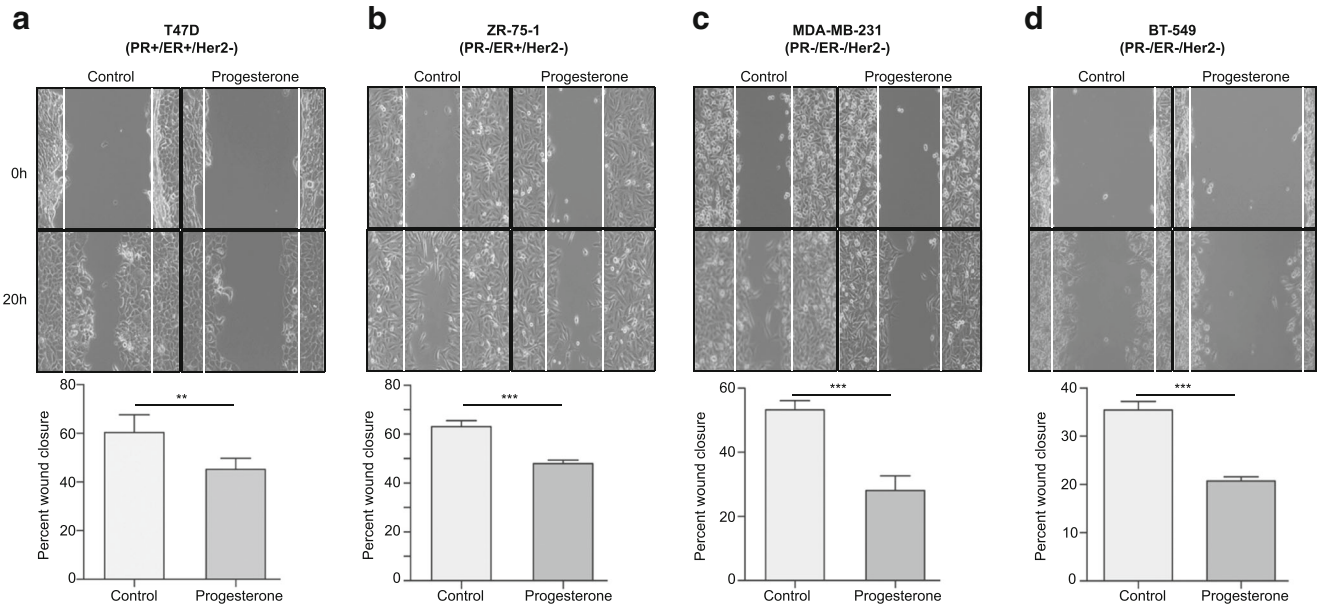

Fig. 3 Migration of breast cancer cells decreases in response to progesterone treatment. Scratch wounds were made in breast cancer-derived cell lines (**a**) T47D, (**b**) ZR-75-1, (**c**) MDA-MB-231 and (**d**) BT-549, with differing receptor statuses. Subsequently, the cells were treated with alcohol (control) and progesterone for 20 h and followed in time for migration. The bar plots indicate the percentages of cellular migration, with direct comparisons between control and progesterone treated cells. The figures are representative of three independent experiments performed in triplicates. *P*-values were calculated using student's unpaired t-test. ** $p < 0.001$; *** $p < 0.0001$

glucocorticoids may similarly decrease the migration of PR-negative MDA-MB-231 cells [26], which suggests that redundant pathways may underlie the progesterone response in a PR-independent manner [27]. Consistent with these observations, we found that blocking PR by mifepristone prior to exposing the cells to progesterone did not rescue the effect of progesterone, suggesting that the progesterone-mediated suppression of migration in breast cancer cells is predominantly mediated in a PR-independent manner (Supplementary Fig. 3a and b). This result corroborates a clinical study in which it was found that progesterone may reduce the recurrence of node-positive breast cancer in patients independent of their PR status [3]. A recent in vitro study, however, suggested that the PR status may play an essential role as no significant effect was observed in PR-negative MDA-MB-231 cells in response to R5020, which is a synthetic progestin [28]. But, it has also been shown that the downstream effects of progesterone and progestin may be variable [29] and this notion, together with possible variations that may occur during cell line passage, could account for the phenotypic differences observed in MDA-MB-231 cells. Moreover, we found that the progesterone-mediated suppression of migration and invasion also occurred in other PR-negative breast cancer-derived cells, i.e., ZR-75-1 and BT-549, which has not been reported before.

In summary, we present a first lead to model a randomized in vitro study to systematically elucidate the role of kinases that may underlie the clinical outcome of pre-operative progesterone intervention in breast cancer patients.

Acknowledgements We thank all members of the Dutt laboratory for critically reviewing the manuscript and Pawan Upadhyay for STR profiling. A.D. is supported by an Intermediate Fellowship from the Wellcome Trust/DBT India Alliance (IA/I/11/2500278), intramural grants [IRB project 2712], and by a grant from DBT (BT/MED/30/VNCI-Hr-RCA/2015). M.G is supported by a research fellowship from the Homi Bhabha National Institute (HBNI), ACTREC-TMC. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Compliance with ethical standards

Conflict of interest None declared

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Cancer Biology & Therapy

ISSN: 1538-4047 (Print) 1555-8576 (Online) Journal homepage: http://www.tandfonline.com/loi/kcbt20

miR-129-2 mediates down-regulation of progesterone receptor in response to progesterone in breast cancer cells

Mukul Godbole, Pratik Chandrani, Nilesh Gardi, Hemant Dhamne, Kuldeep Patel, Neelima Yadav, Sudeep Gupta, Rajendra Badwe & Amit Dutt

To cite this article: Mukul Godbole, Pratik Chandrani, Nilesh Gardi, Hemant Dhamne, Kuldeep Patel, Neelima Yadav, Sudeep Gupta, Rajendra Badwe & Amit Dutt (2017) miR-129-2 mediates down-regulation of progesterone receptor in response to progesterone in breast cancer cells, Cancer Biology & Therapy, 18:10, 801-805, DOI: 10.1080/15384047.2017.1373216

To link to this article: https://doi.org/10.1080/15384047.2017.1373216

CANCER BIOLOGY & THERAPY
2017, VOL. 18, NO. 10, 801–805
https://doi.org/10.1080/15384047.2017.1373216

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BRIEF REPORT

miR-129-2 mediates down-regulation of progesterone receptor in response to progesterone in breast cancer cells

Mukul Godbole[a,b], Pratik Chandrani[a,b], Nilesh Gardi[a,b], Hemant Dhamne[a], Kuldeep Patel[a], Neelima Yadav[a,b], Sudeep Gupta[c], Rajendra Badwe[d], and Amit Dutt [a,b]

[a]Integrated Genomics Laboratory, Advanced Centre for Treatment, Research and Education In Cancer, Tata Memorial Centre, Maharashtra, Navi Mumbai, India; [b]Integrated Cancer Genomics Laboratory, Homi Bhabha National Institute, Training School Complex, Anushakti Nagar, Maharashtra, Mumbai, India; [c]Department of Medical Oncology, Tata Memorial Centre, Maharashtra, Mumbai, India; [d]Department of Surgical Oncology, Tata Memorial Hospital, Tata Memorial Centre, Maharashtra, Mumbai, India

ABSTRACT

Objective: Hormonal therapy is an important component of first line of treatment for breast cancer. Response to hormonal therapy is influenced by the progesterone receptor (PR)-status of breast cancer patients. However as an early effect, exposure to progesterone decreases expression of PR in breast cancer cells. An understanding of the mechanism underlying down-regulation of PR could help improve response to hormonal therapy. Methods: We performed small RNA sequencing of breast cancer cells for identification of microRNAs targeting *PR* in response to progesterone treatment. Biochemical approaches were used to validate the findings in breast cancer cells. Results: Analysis of small RNA sequencing of four breast cancer cell lines treated with progesterone revealed an up-regulation of *miR-129-2* independent of the PR status of the cells. We show that *miR-129-2* targets 3′UTR of PR to down-regulate its expression. Furthermore, inhibition of *miR-129-2* expression rescues the down-regulation of PR in breast cancer cells. Also, the expression levels of *miR-129-2* was observed to be elevated in patients with low expression of PR in the TCGA cohort (n = 359). Conclusion: *miR-129-2* mediates down-regulation of PR in breast cancer cells in response to progesterone, while anti-*miR-129-2* could potentiate PR expression levels among patients with inadequate PR levels. Thus, modulation of activity of *miR-129-2* could stabilize PR expression and potentially improve response to hormonal therapy under adjuvant or neo-adjuvant settings.

ARTICLE HISTORY
Received 5 May 2017
Revised 27 July 2017
Accepted 24 August 2017

KEYWORDS
Breast cancer; hormonal therapy; progesterone; progesterone receptor; microRNA; cancer genomics; small RNA sequencing

Introduction

Breast cancer is the most prevalent cancer among women worldwide. Despite all advances in early diagnosis and treatment, nearly 30% node-negative and 70% node-positive patients relapse with metastatic disease.[1] Treatment of breast cancer patients is influenced by the presence of estrogen receptor (ER) and progesterone receptor (PR). ER/PR positive patients tend to respond better to hormonal therapy and have a lower risk of relapse compared to ER-positive, PR-negative patients.[2] The down-regulation of *PR* expression in breast cancer cells is caused either by methylation at *PR* promoter[3], or in response to progesterone by post translational modification of the PR protein by CUEDC2 and MAPK.[4,5] Growing evidence also suggest microRNAs to respond to steroid hormones and suppress the activity of respective hormone receptor.[6] For instance, miR-18a, miR-19b and miR-20b (paralogous pri-microRNAs) down-regulate the expression of ER in response to estrogen in breast cancer.[7] Comparatively, similar regulation of microRNA expression in response to progesterone has been less explored.[8] In order to study the progesterone-regulated microRNAs targeting PR, we performed small RNA sequencing of breast cancer cell lines treated with progesterone. The differentially expressed microRNAs were used to identify microRNAs that target 3′UTR of PR. Our analysis reveals *miR-129-2* targets PR and is up-regulated in response to progesterone. The association of *miR-129-2* and PR was functionally validated by luciferase assay. Also western blot analysis suggests that inhibition of *miR-129-2* stabilizes PR in breast cancer cells even in presence of progesterone. Moreover, patients with high *miR-129-2* levels had significantly lower expression of PR as compared to patients with no *miR-129-2* expression in The Cancer Genome Atlas cohort.

Results

Identification of progesterone responsive microRNAs targeting PR expression in breast cancer cells

Consistent with earlier reports, we observed down-regulation of *PR* transcripts when T47D and BT474 cells were treated with

CONTACT Amit Dutt adutt@actrec.gov.in Integrated Genomics Laboratory, Advanced Centre for Treatment, Research and Education In Cancer, Tata Memorial Centre, Navi Mumbai-410210, India; Rajendra Badwe badwera@tmc.gov.in Department of Medical Surgical Oncology, Tata Memorial Centre, Mumbai-400012, India; Sudeep Gupta sudeep.gupta@actrec.gov.in Department of Medical Oncology, Tata Memorial Hospital, Tata Memorial Centre, Mumbai-400012, India.
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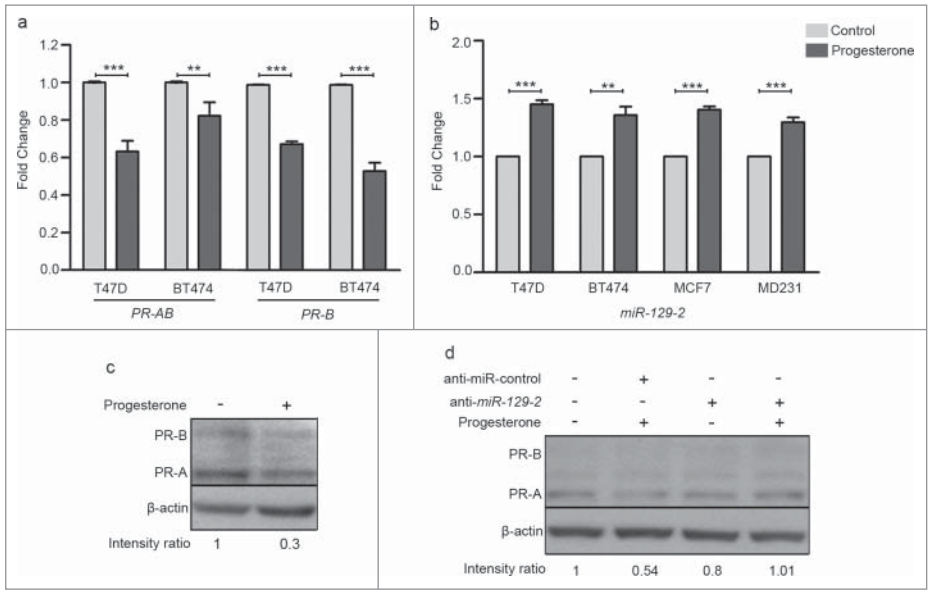

Figure 1. Progesterone receptor is down-regulated in breast cancer cell lines in response to progesterone. (a) Transcript levels of *PR* were measured using real-time PCR in T47D and BT474 cells treated with 10 nM progesterone for 6 h. Graph has been plotted as fold change expression of *PR* normalized to *GAPDH* in progesterone-treated versus control. Analysis is representative of three independent experiments and *P*-value was calculated using student's unpaired t-test. (b) Transcript levels of *miR-129-2* under similar progesterone treatment conditions were measured by real-time PCR and plotted as fold change in progesterone versus control of T47D, BT474, MCF7 and MDA-MB-231 cells obtained after normalization to expression of *U6* small RNA. Transcript levels in both control and progesterone-treated cells have been shown. Analysis is representative of three independent experiments and *P*-value was calculated using student's unpaired t-test. ** indicates *P*-value <0.001; *** indicates *P*-value <0.0001. (c) Western blot analysis of PR (PR-A and PR-B) in response to progesterone treatment in T47D cells. β-actin was used internal protein loading control. Numbers on blot indicate ratio of intensity of PR with respect to β-actin for each lane. Western blot analysis is representative of two independent experiments. (d) Western blot analysis of PR (PR-A and PR-B) in T47D cells treated with either anti-miR-control or anti-*miR-129-2*. As indicated in the panel, cells were either treated with progesterone or untreated. β-actin was used as internal protein loading control. Numbers on blot indicate ratio of intensity of PR with respect to β-actin for each lane. Western blot analysis is representative of two independent experiments.

10 nM progesterone for 6 h (Fig. 1a). Similarly, progesterone reduced the expression level of PR protein in T47D cells (Fig. 1c). To understand the role of microRNA's involved in regulation of *PR* expression, we performed small RNA sequencing of three PR-positive T47D, BT474, MCF7 and one PR-negative MDA-MB-231 cell line for identifying microRNAs which could down-regulate PR expression in response to progesterone treatment for 6 h. On an average we obtained 22 million reads per sample per cell line. The sequence reads were mapped to human microRNA sequences obtained from miRBase (version 21) to identify median 800 mature microRNA sequences. These microRNA reads were used for identification of differentially expressed microRNAs. We used a fold-change cut-off of 3-fold difference and observed that progesterone had an effect in both directions by up-regulating and down-regulating the microRNAs and all the four cells had different number of de-regulated microRNAs (Supplementary Table 1). Of these, 98 microRNAs were up-regulated in T47D, 96 in BT474, 189 in MCF7 and 106 in MDA-MB-231 cells in response to progesterone. Intriguingly, expression of *miR-513a-5p* shown to be differentially up-regulated in response to synthetic progestin (medroxy progesterone acetate, MPA) by microarray-based analysis in T47D cells was not observed in any of the four breast cancer cells in this study[8], possibly due to variable downstream effects elicited by synthetic progestin (MPA) and progesterone[9] or distinct platform specific threshold involved in these studies. The up-regulated microRNAs found across the four breast cancer cells were further used to search microRNAs targeting 3′UTR of *PR* gene. Of the 6 different algorithms used, we found three microRNAs (*miR-3908, miR-129-2-3p and miR-3140-3p*) that were predicted to target 3′ UTR of *PR* and showed an increased expression relative to levels in control. When expression of these microRNAs was checked in the TCGA breast cancer cohort (n = 359), only *miR-129-2* was found to be expressed. Next, the up-regulation of *miR-129-2* in response to progesterone could be validated by real-time PCR in our panel of cells (Fig. 1b). While the progestin-regulated *miR-513a-5p* could be validated only at 100 nM progestin (MPA) as reported by Cochrane et al.[8], we could validate consistent up-regulation of *miR-129-2* in response to 10 nM progesterone that was used for small RNA sequencing analysis, inclusive of PR-negative breast cancer cells (Fig. 1b). Thus we observed that progesterone mediated up-regulation of *miR-129-2* was independent of the PR expression of cells.

Functional validation of miR-129-2 *based regulation of progesterone receptor*

In our attempt to functionally characterize the association of *miR-129-2* with *PR*, a ~1000 bp 3′UTR of *PR* (containing seed sequence for *miR-129-2*) was cloned downstream to *luciferase* gene in a pGL3-promoter vector. The sequence for premature *miR-129-2* (~400bp) was cloned in pcDNA3.1 vector. Three set of transfections were performed— pGL3-*PR* 3′UTR; pGL3-*PR* 3′UTR with pcDNA-3.1-*miR-129-2*; and, combination of pGL3-*PR* 3′UTR, pcDNA3.1-*miR-129-2* and anti-*miR-129-2* (Fig. 2a). The expression of *firefly luciferase* gene was analyzed using firefly luciferase reporter assay system and normalized to renilla expression, which was used as internal control, in each of these sets. Our analysis suggests that upon over-expression of *miR-129-2*, the luciferase signal was significantly reduced as

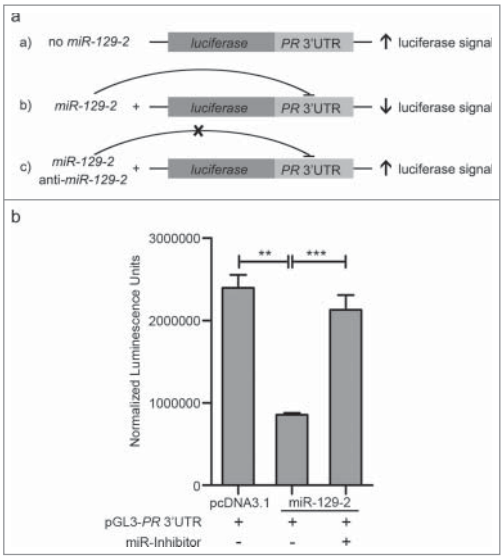

Figure 2. Validation of *miR-129-2*-based regulation of *PR*. (a) pCDNA3.1-*miR-129-2* and pGL3- *PR* 3′UTR in different combinations with anti-*miR-129-2* were co-transfected in 293FT cells and luciferase signal in each condition was measured, as shown in the figure. (b) Quantified luminescence units normalized to renilla expression was plotted for each of the sets mentioned above. Analysis is representative of three independent experiments and the *P*-value was calculated using student's t-test. ** indicates *P*-value <0.001; *** indicates *P*-value <0.0001.

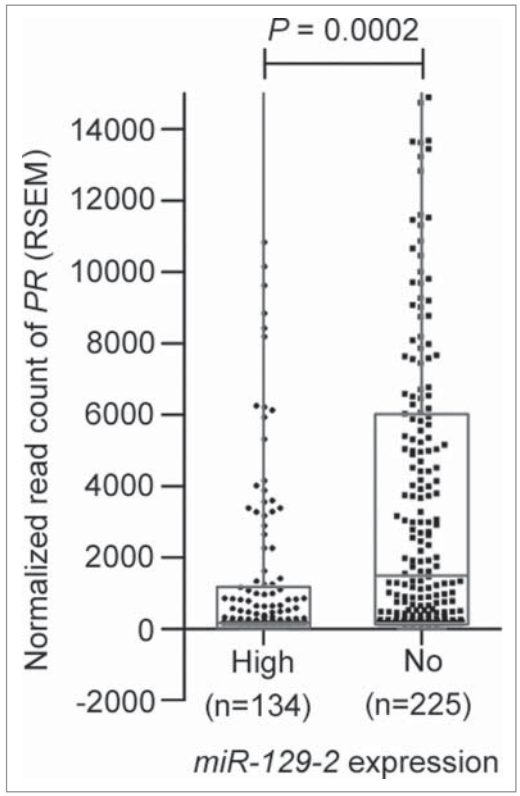

Figure 3. Expression of *miR-129-2* in breast cancer patients in TCGA dataset. Expression plot for *PR* in breast cancer patients with high *miR-129-2* expression (n = 134) and with absence of *miR-129-2* expression (n = 225) in the TCGA cohort. The box-plot is overlaid with dot-plot wherein each point represents patient sample. Y-axis indicates normalized read count (RSEM) values for *PR* in a total of 359 breast cancer patients where expression of *PR* and *miR-129-2* was available. *P*-value (*P* = 0.0002) was calculated using student's unpaired t-test with Welch's correction.

compared to signal in vector only cells. Addition of microRNA inhibitor against *miR-129-2* (anti-*miR-129-2*), a double stranded RNA sequence which is complimentary to and specifically targets *miR-129-2*, reversed the repression and showed an increase in luciferase signal (Fig. 2b). Next, we inhibited *miR-129-2* in T47D cells and compared the PR expression in these cells with the expression in cells transfected with negative control (targeting *miR-29a*) inhibitor. Our western blot analysis suggests that upon exposure to progesterone, T47D cells transfected with negative control showed decrease in PR expression, while PR showed stable expression in cells transfected with *miR-129-2* inhibitor even in the presence of progesterone treatment (Fig. 1d). Thus our results provide basis for direct interaction of *miR-129-2* with *PR*, where in addition to previous findings, we demonstrate that over-expression of *miR-129-2* mimics the effect of progesterone treatment to down-regulate PR and that inhibition of *miR-129-2* abrogates its interaction with *PR* in breast cancer cells. Taken together, these studies emphasize the plurality in microRNA-mediated feedback regulation of PR.

Next, we analyzed the TCGA breast cancer cohort (n = 359) for studying expression of PR in breast cancer patients with high *miR-129-2* expression and in absence of *miR-129-2* expression. When expression of PR was checked in patients with high *miR-129-2* expression (n = 134) versus patients with absence of *miR-129-2* expression (n = 225) we observed a significantly higher expression of PR in patients with absence of *miR-129-2* expression as compared to patients with high expression of *miR-129-2* (*P* = 0.0002) as shown in Fig. 3. Thus a further in-depth analysis needs to be carried out to ascertain the exact role of *miR-129-2* in survival of breast cancer patients.

Discussion

Small RNA sequencing analysis of progesterone treated breast cancer cell lines led to the identification of a novel *PR*-targeting microRNA *miR-129-2*. Since the increased expression of *miR-129-2* was independent of the PR-status of breast cancer cells, a possible role of other steroid hormone receptors like membrane progesterone receptor or glucocorticoid receptor as suggested in literature[10,11] to mediate the role of progesterone in these cells remains to be systematically analyzed. Consistent with our finding, analysis of TCGA breast cancer dataset suggests a significantly decreased expression of PR in patients with elevated expression of *miR-129-2* as compared to patients with no *miR-129-2* expression, indicating a possibility for the decreased expression of PR in patients with low PR expression. It has been observed that factors like loss of PR or menopausal status of women can alter the response to hormonal therapy.[12] Some studies have indicated that the absence of PR could underlie tumors resistance to hormonal therapy[2], or could potentially increase the risk of relapse[13]. Hence we propose that stabilization of PR expression in patients with tumors expressing low PR levels by blocking activity of such microRNAs using specific microRNA inhibitors, along with other treatment modalities, could be potentially helpful in enhancing the response of patients to hormonal therapies. In support of this notion, our *in vitro* luciferase assay and western blot results using *miR-129-2* inhibitor suggest that inhibition of *miR-129-2* can increase the expression of PR. Thus, we validate under *in vitro* settings that addition of progesterone leads to up-regulation of *miR-129-2*, which suppresses the expression of *PR* in breast cancer cells; and, the inhibition of *miR-129-2*

reinstates the *PR* expression in these breast cancer cells even in presence of progesterone. Also since microRNAs are being assessed for their use in clinics[14], strategies like microRNA sponges and chemically modified antisense oligonucleotides (inhibitors) hold promise as a promising line of treatment of breast cancer that need to be exhaustively explored with larger datasets.[15] Thus, our study suggests an underlying mechanism to a possible clinical consequence in response to progesterone treatment among patients with varying PR expression levels. Also, it is suggestive of treatment with anti-*miR-129-2* among those patients expressing inadequate PR levels, under adjuvant and neo-adjuvant settings, before considering for hormonal therapy. Whether modulation of activity of *miR-129-2* could stabilize PR expression and potentially improve response to hormonal therapy remains to be validated as an immediate follow up to this pilot study.

Materials and methods

Breast cell lines

T47D, BT474, MDA-MB-231 and MCF7 breast cancer cell lines were obtained from Dr. Slamon's laboratory (Department of Medicine, UCLA, USA). Human embryonic kidney 293FT cells were obtained from Invitrogen. The cell lines were authenticated by DNA Short Tandem Repeat (STR) profiling using the Promega GenePrint 10 system and the analysis was performed using the GeneMarker HID software and the ATCC database. Cells in culture were tested for mycoplasma and were made mycoplasma-free using EZKill Mycoplasma Removal reagent (HiMedia). All the cell lines were grown in DMEM medium (Gibco) supplemented with 10% (v/v) FBS (Gibco), 2.5 mg/ml Amphotericin-B (Abbott) and 1.25 μl/ml Gentamycin (Abbott) and were cultured at 37°C in a 5% CO_2 incubator. The ER/PR/Her2 receptor status of breast cancer cells was validated by RT-PCR.

Progesterone treatment, RNA isolation and protein sample preparation

Cells were grown to 70–80% confluence and then serum starved in a phenol-red free DMEM low glucose medium (HiMedia) for a period of 24h. In the same medium, cells were treated with 10 nM concentration of 17-α hydroxy-progesterone caproate (progesterone) (MP Biomedicals) for 6 h. Equal amount of alcohol was used as vehicle control. After 6 h of progesterone treatment, TRIzol reagent (Invitrogen) was used to lyze the cells. RNA was isolated according to the manufacturer's protocol. The RNA concentration was measured using NanoDrop. For western blotting, proteins were isolated from cells using RIPA buffer and separated on a 10% SDS-PAGE and transferred onto PVDF membrane for probing with primary antibody. Primary antibodies used were PR-AB (sc-810, 1:300 dilution) and β-actin (sc-1616-R, 1:4000 dilution). Secondary antibody used was goat anti-mouse (sc-2005, 1:3000 dilution) and goat anti-rabbit (sc-2004, 1:3000 dilution).

Small RNA sequencing analysis

Small RNA sequencing was performed on single lane of Illumina HiSeq 1000 with eight multiplex libraries from the four breast cancer cell lines. The reads obtained from deep sequencing of small RNAs were subjected to Illumina adaptor trimming using FastX tool kit and were size filtered to select for candidate miRNA's (14 to 24 bases) from a pool of small RNA sequences using in-house perl script. The size separated reads were then mapped onto human miRNA reads obtained from miRBase (version 21) using Bowtie2 (version 2.1.0)[16] with 0 mismatches in the first 8 bases. MicroRNAs were quantified followed by normalisation by read per million using in-house script. Deregulated miRNAs with $> = 3$ fold change were retained for further analysis. For searching microRNAs targeting *PR* 3′UTR, differentially expressed microRNAs in response to progesterone were compared to microRNAs predicted to target *PR* using 6 algorithms (TargetScan, miRanda, miRWalk, miRMap, RNA22 and RNAhybrid).

Quantitative real-time PCR

Transcript levels of candidate microRNA's were analyzed by quantitative real time PCR. 1 μg total RNA was used for cDNA synthesis using Mir-X miRNA First-Strand Synthesis Kit (Clontech Takara). For analyzing transcript levels of de-regulated genes, cDNA was synthesized using High capacity cDNA reverse transcription kit (Applied Biosystems). cDNA from each cell line with the two treatment conditions were then subjected to quantitative real-time PCR analysis using Roche Light-Cycler-II 480 instrument using the Mir-X miRNA qRT-PCR SYBR Kit (2X) Master Mix (Clontech Takara) for microRNAs and Roche real-time master mix (Roche) for genes. Expression change of candidate miRNAs and genes de-regulated by progesterone was calculated by the $2^{-\Delta\Delta C_T}$ method. *U6* small RNA (primers provided by Clontech Takara) was used as an internal control for microRNAs and *GAPDH* was used for genes. Primer sequences for each microRNA and gene used for validation purpose are given in Supplementary table 2.

Cloning of microRNA/PR 3′UTR and luciferase assay

A 400bp sequence of *miR-129-2* containing the seed sequence was PCR amplified using genomic DNA isolated from T47D. Amplicons were cloned in a T/A cloning vector (Fermentas, USA) followed by sub-cloning in BamHI and HindIII sites of pCDNA 3.1 (−) expression vector (In vitrogen). *PR*-3′UTR of 1000bp was PCR amplified using T47D cDNA. Amplicons were cloned in a T/A cloning vector followed by sub-cloning between XbaI sites in pGL3-promoter vector (Luciferase Expressing vector, Promega). For the Luciferase assay, 293FT cells (50,000 cells/well) were transfected using lipofectamine 2000 reagent (Life Technologies) with the combination of these constructs along with Renilla luciferase vector (for normalizing transfection efficiency) and 5 nM miR-inhibitors (SIGMA, HSTUD0162) in separate wells. 48 hours post-transfection cells were lyzed and luciferase assay was performed to measure luminescence (Berthold Luminometer, Germany). Experiment was performed in triplicates and differences between group showing p-values <0.05 (calculated using an unpaired student's t-test) were considered significant.

Transfection of microRNA inhibitor in breast cancer cells

T47D cells were grown up to 60% confluence and transfected with 25 nM negative control miRNA inhibitor (against *miR-29a*) and *miR-129-2* inhibitor (SIGMA, HSTUD0162). Post-transfection, cells were incubated for 48 h and then treated with progesterone for 6 h. Cell lysate was prepared and western blot analysis was performed.

Disclosure of potential conflict of interest

No potential conflict of interest was reported by the authors.

Acknowledgments

All members of the Dutt laboratory for critically reviewing the manuscript and Pawan Upadhyay for STR profiling of breast cancer cells. Small RNA sequencing were performed at Genotypic Pvt Ltd, Bangalore, India.

Authors' contributions

M.G. and A.D designed the research; M.G., P.C., N.G., H.D., K.P. and N.Y. performed the research; M.G., P.C. and A.D. analyzed the data; M.G., S.G., R.B. and A.D. wrote the paper. All the authors have read and approved the manuscript.

Funding

A.D. is supported by an Intermediate Fellowship from the Wellcome Trust/DBT India Alliance (IA/I/11/2500278), intramural grants [IRB project 2712], and by a grant from DBT (BT/MED/30/VNCI-Hr-RCA/2015). M.G., P.C., N.G. and N.Y. are supported by research fellowship from Homi Bhabha National Institute (HBNI), ACTREC-TMC. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

ORCID

Amit Dutt http://orcid.org/0000-0002-1119-4774

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