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PRINCIPAL INVESTIGATOR: Cinzia Casella

CONTRACTING ORGANIZATION: Brown University, Providence RI
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14. ABSTRACT Which is the molecular mechanism that leads to DNA amplification and oncogenes activation in breast cancer cells? This project aims to understand the role of estrogen in inducing re-replication, thus leading to DNA amplifications. I worked on the establishment of cancer cell line models carrying an engineered replication origin to be tested for undergoing DNA amplification after estrogen treatment. Subsequent to some promising preliminary data obtained with MCF7 cells suggesting that estrogen might be able to elicit DNA amplification at this site, the <i>in vitro</i> system used was refined to have multiple possibilities on the selection of cells with DNA amplification. A new reporter construct was built and a second recombinant cell line model established from U2OS/ER-alpha cells was used. The efficiency of the Flp/FRT recombination system for the establishment of MCF7 cells carrying the new construct was too low and did not allow successful recombinants to be obtained during this time period. Moreover, the 17-β estradiol (E2) treatment of U2OS/ER-alpha recombinant cells was toxic: upon E2 treatment of cells expressing estrogen receptor alpha irreversible cell death was apparent at 24 hours. Because of the limited time of E2 exposure allowed by this system, a new experimental approach of molecular visualization of DNA re-replication has been explored.					
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1. Introduction

DNA amplification is a hallmark of human cancers that can provide proliferative advantages to malignant cells, for example through the activation of oncogenes. Moreover, DNA amplification is often correlated with disease prognosis and progression. However, mechanisms that trigger DNA amplification are not yet fully understood. This research project aims to investigate the involvement of replication origins in DNA amplification. Specifically, I am interested in exploring whether the steroid hormone estrogen, when bound adjacent to a replication origin, is able to induce re-replication leading to DNA amplification in breast cancer cells. Indeed, previous work strongly suggests that the steroid hormone ecdysone regulates site-specific DNA amplification in the fly *Sciara coprophila* through re-firing of a replication origin (Foulk *et al.*, 2006; Liew *et al.*, 2013). Moreover, recent work has shown that re-replication of deregulated origins can very efficiently generate genomic alterations (Green *et al.*, 2010; Kiang *et al.*, 2010; Finn and Li, 2013; Richardson and Li, 2014). Clarification of the molecular mechanisms underlying DNA amplification is an important step to efficiently counteract breast cancer cell proliferation and metastasis.

2. Keywords

Breast cancer, DNA amplification, estrogen receptor alpha (ER α), DNA replication, recombinant cell lines.

3. Accomplishments

The project aimed to investigate the involvement of estrogen in DNA amplification in breast cancer: the binding of the activated estrogen receptor to sites proximal to DNA replication origins could play an important role in triggering aberrant DNA replication. In particular, this mechanism could explain head-to-tail repeats in amplified DNA. To explore this mechanism, goals of the project and accomplishments achieved under these goals are described below.

Establishment of recombinant cell lines carrying a model genomic site (c-Myc replication origin next to a estrogen receptor-alpha binding site) to assess for estrogen-induced DNA amplification (Task One)

During the first two years, I successfully established recombinant MCF7/FRT cell lines carrying the core sequence of the well characterized c-Myc replication origin flanked or not by an estrogen receptor-alpha (ER α) binding element. These cell lines were established from an MCF7 Flp-In acceptor clone kindly provided by Dr. Yasuhiro Arakawa (Jikei University School of Medicine, Tokyo). These first recombinant cell lines were named MCF7/c-Myc 6xERE and MCF7/c-Myc, respectively. In the two cell lines the constructs integrated at the same genomic position through Flp/FRT homologous recombination system. The replicator activity of the engineered c-Myc replication origin was assessed by qPCR of nascent strands.

Additional MCF7 Flp-In acceptor cell lines were also established by selection and expansion of clones stably transfected with pFRT/lacZeo plasmid (Invitrogen). All acceptor cell lines have been assessed by Southern blot to select those having a single FRT genomic site. IAcceptor cells with a single FRT site are suitable for the subsequent genomic integration of a single copy of the construct containing the c-Myc replication origin. Moreover, the location of the genomic integration of the construct was mapped via either inverse PCR or Linear Amplification-Mediated PCR (LAM PCR; Schmidt *et al.*, 2007) (Figure 1).

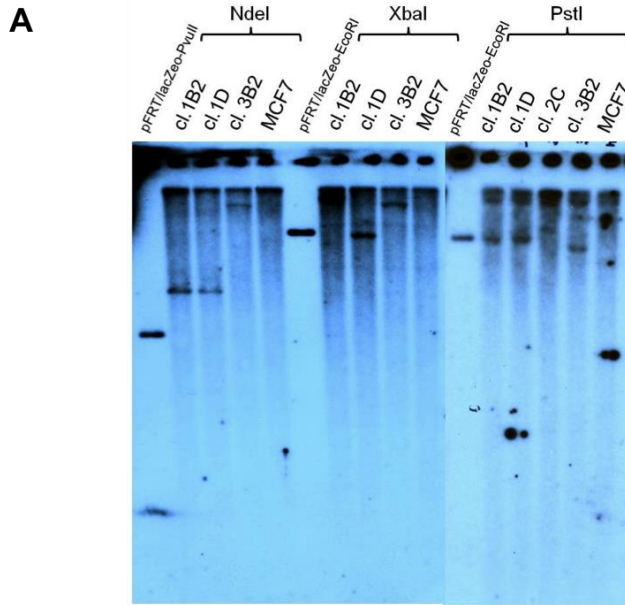


Figure 1. Acceptor MCF7 Flp-In cell lines. **A)** Southern Blots for Detection of Integrated pFRT/lacZeo. 10 µg of genomic DNA of MCF7 Flp-In clones was digested with three different restriction enzymes (NdeI, XbaI, PstI). pFRT/lacZeo plasmid sequence was detected with a ~2 kb radioactive probe. As determined by qPCR, each of the clones analyzed has a single copy of the FRT carrying plasmid. Digested pFRT/lacZeo plasmid (10 µg) and MCF7 genomic DNA were used as positive and negative controls, respectively. **B)** Genomic Insertion Site of Three MCF7 Flp-In Clones. Inverse PCR and LAM-PCR were used to identify the pFRT/lacZeo genomic integration site.

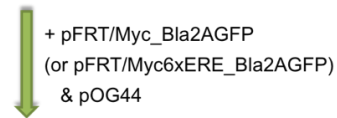
B

Flp-In clone	PCR-derived flanking genomic sequence				distance from FRT	graphical view
	chr	reference sequence	start	end		
MCF7/1D	10p14	NC_000010.10	9038847	9038705	5799 bp	
MCF7/2C	3q26.1	NC_000003.11	160936128	160935500	783 bp	
MCF7/3B2	10p14	NC_000010.10	9345977	9346339	409 bp	

After a first series of experiments performed under the goal stated in Task Two (see below), a considerable amount of work was addressed to the improvement and refinement of the vector containing the DNA replication origin to be used in monitoring ER α -dependent DNA amplification. The final construct featured a blasticidin-TagGFP2 marker encoded in a bicistronic mRNA (Figures 2 and 3). The two proteins provide the possibility for two independent approaches for the identification of cells that undergo DNA amplification at the ectopic locus: drug selection and identification via fluorescent activated cell sorting (FACS). Specifically, at the increase of the copy number of the DNA encoding the two markers because of the re-firing of the nearby ectopic c-Myc replication origin, cells will be resistant to higher concentration of blasticidin and will

encode higher level of the fluorescent protein compared to non-amplified cells. Site-specific DNA amplification is expected to occur at low frequency, so the drug selection will provide a tool to specifically expand the population of interest, while TagGFP2 will allow time course monitoring of the whole population as well as confirmation for DNA amplification in high-dose blasticidin selected cells. Upon completion of the new constructs, the expression of blasticidin and TagGFP2 was assessed in transiently transfected cells.

MCF7 Flp-In Acceptor cell line :



Recombinant cell line:

MCF7/c-Myc



MCF7/c-Myc 6xERE

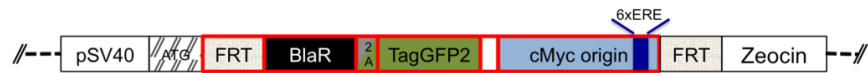


Figure 2. Schematic Representation of the Latest Version of the Construct to be Used in MCF7 Flp-In cells to Assess DNA Amplification at the Ectopic Replication Origin. The construct containing the 2.4 kb c-Myc origin is outline as red boxed. The blasticidin-2A-TagGFP2 ORF will be transcribed from the SV40 promoter located upstream of the acceptor FRT sequence integrated into the genome of the Flp-In host MCF7 cells. Moreover, the ATG start codon is present only upstream of the acceptor FRT sequence, and will be available to the blasticidin-2A-TagGFP2 ORF only when a successful homologous recombination occur at the genomic FRT site.



Figure 3. Schematic Representation of the Construct for the PiggyBAC System. In the recent years the PiggyBac system has been proved to be an efficient tool for gene delivery in mammalian cells. The pFRT.PGK-Bla2AGFP_Myc6xERE construct has been implemented with the PiggyBac-specific inverted terminal repeats (5' ITR and 3' ITR) to use the PiggyBac transposition system to generate the recombinant U2OS/ER-alpha_PGK-BlaGFP_Myc6xERE. Upon co-transfection of the vector and the PiggyBac transposase expression plasmid, the engineered c-Myc replication origin and the selection markers will be randomly integrated at chromosomal AATT sites.

During the last period of the funded award, three different MCF7 Flp-In cell lines established and characterized in Year One (namely, MCF7/2C, MCF7/1D, and MCF7/3B2) were transfected with the new constructs: pFRT.PGK-BlaGFP_Myc6xERE and the control construct pFRT.PGK-BlaGFP_Myc, the latter not containing the ER α binding site. Despite the fact that I successfully used the FRT/Flp system in the past for the site-specific integration of a DNA construct via homologous recombination in MCF7 cells, the same transfection conditions failed to produce recombinant cell lines. Transfections were repeated multiple times, and cell number scaled up to circumvent a possible lower recombination efficiency compare to what was experienced before. Of

the three different acceptor MCF7 Flp-In cell lines, each transfected with either one of the two constructs, I recovered only one recombinant clone for the control construct. The presence of the FRT site in the acceptor cells as well in the donor constructs was confirmed as well as absence of major rearrangements of the plasmid encoding for the Flippase enzyme as assessed via restriction digestion.

The new construct that allows either blasticidin selection or GFP-mediated identification of cells that undergo DNA amplification at the ectopic Myc-6xERE genomic site was however successfully integrated in U2OS/ER α cells. These cells, kindly provided by Dr. Dale Leitman (Center for Obstetrics, Gynecology and Reproductive Sciences, UCSF) conditionally express ER α upon exposure to doxycycline. In Year Two the integration of the DNA construct in U2OS/ER α was achieved through the PiggyBac system: sequences recognized by the PB transposase were added to the same construct used for MCF7 cells to generate the pPB.PGK-BlaGFP_Myc6xERE construct (Figure 3), and the enzyme proved to be very efficient in mediating the genomic integration of the target DNA. Recombinant cells were analyzed by Southern blot to assess the copy number of the integrated construct (Figure 5) and expression of both markers successfully accessed. Moreover, taking advantage of recombinant cells carrying multiple copies of the construct, the possible readout of increased TagGFP expression because of DNA amplification at the ectopic c-Myc replication origin was evaluated (Figure 6).

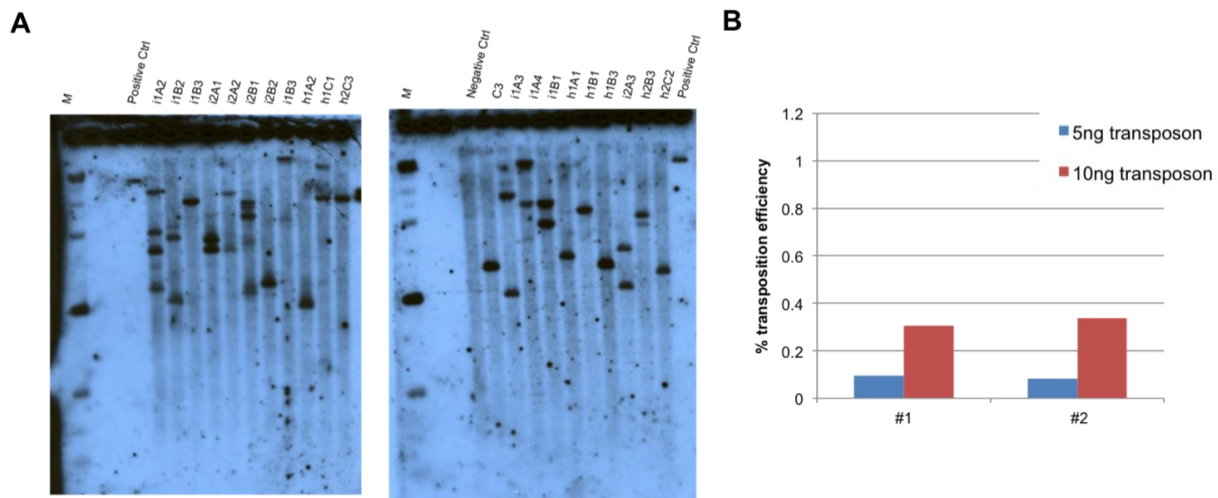


Fig 5. U2OS/ER-alpha_PGK-BlaGFP_Myc6xERE Clones. **A)** Southern blot. Upon cell transfection and blasticidin selection, 25 independent clones were isolated and Southern blot performed to identify clones with a single copy of the pPB.PGK-BlaGFP_Myc6xERE vector. Results show that the transfection condition used allowed to integrate one copy of the vector per genome in half of the cases (11 out of the 21 clones analyzed). M: 1 kb DNA ladder; Positive Ctrl: 10 ng linearized pPB.PGK-BlaGFP_Myc6xERE vector; Negative Ctrl: HindIII digested genomic DNA from non transfected U2OS/ER-alpha cells. **B)** Quantification of transposition efficiency. After 24 hr from transfection cells were diluted, re-plated and selected with 4 μ g/mL blasticidin for crystal violet staining and colony count.

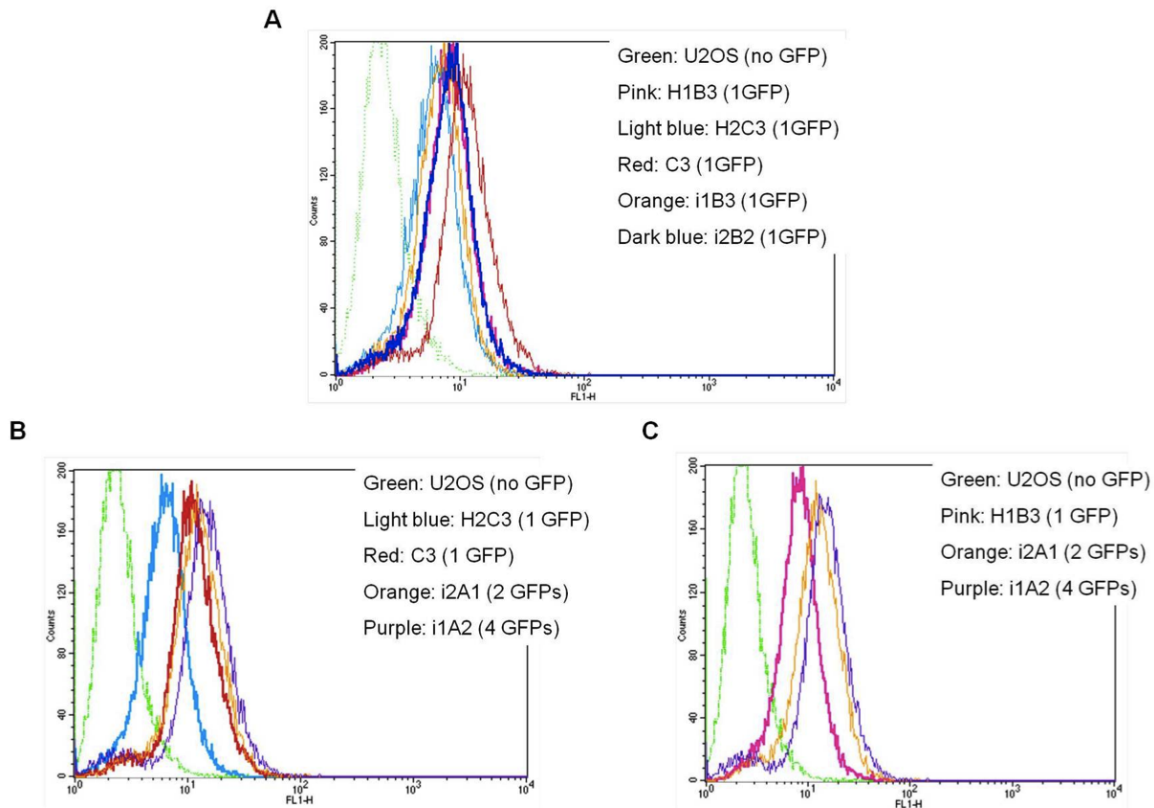


Figure 6. FACS Analysis of Basal GFP Expression in U2OS/ER-alpha_PGK-BlaGFP_Myc6xERE Clones . A) Basal expression of GFP was evaluated by FACS for 5 independent U2OS/ER-alpha_PGK-BlaGFP_Myc6xERE clones that carry a single copy of the vector. Parental U2OS/ER-alpha have been used as negative control. The 5 clones identify 3 different level of GFP expression: low (H2C3 and i1B3), medium (H1B3 and i2B2), and high (C3). B) and C) Comparative approximated analysis of clones that contain 1 copy (H2C3, H1B3 and C3), 2 copies (i2A1), and 4 copies (i1A2) of the integrated vector. GFP signal profiles show that at the increase of GFP copy number increase the intensity of the total fluorescent signal.

In order to perform a drug selection of cells that undergo DNA amplification at the ectopic c-Myc replication origin, blasticidin resistance of single integrant clones was assessed by drug titration. The blasticidin concentration effective in killing cells with a single copy of the construct shifted from 4 $\mu\text{g}/\text{mL}$ to 1 mg/mL .

After having assessed an increase in GFP signal at the increase of the construct copy number (Figure 6), the next step was to assess the sensitivity of DNA amplification detection at the ectopic c-Myc origin by detection of GFP expression levels. For this purpose, recombinant cells with 4 integrated copies of the pPB.PGK-BlaGFP_Myc6xERE construct (U2OS/i1A2) were mixed with cells having a single copy of it (U2OS/H1B3) at different ratios (Figure 7). FACS analysis of TagGFP2 expression for the different samples suggests that a 4-fold amplification might be detected when the cells represent at least a quarter of the analyzed population. Note however that this is

only an approximate evaluation, since marker expression level is influenced by the surrounding genomic sequence at the integration site.

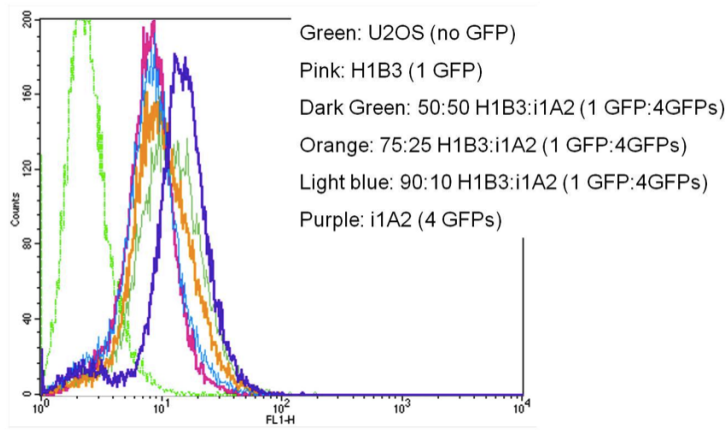


Figure 7. Evaluation of the Approximate Sensitivity of the GFP Selection Method. U2OS/ER-alpha_PGK-BlaGFP_Myc6xERE clones containing either 1 (clone H1B3) or 4 GFP (clone i1A2) coding genes have been mixed at different ratios. FACS analysis shows that with a 4-fold DNA amplification, the total fluorescent signal profile differs from the 1 GFP copy if the amplified cells constitute at least 25% of the population analyzed (dark green and orange lines). If amplified cells constitute only 10% of the entire population, a 4-fold DNA amplification will not be evident through FACS analysis. However, since GFP expression is dependent also on the surrounding genomic sequence at the integration site, the comparison between different clones provides only indicative data.

Test the ability of estrogen to induce DNA amplification at the engineered ectopic c-Myc replication origin (Task Two)

The refinement to the experimental design was aimed to allow a reliable repetition of the preliminary results obtained with a first experiment conducted in Year One with recombinant MCF7 cells, where subpopulation of cells with an average 14-fold

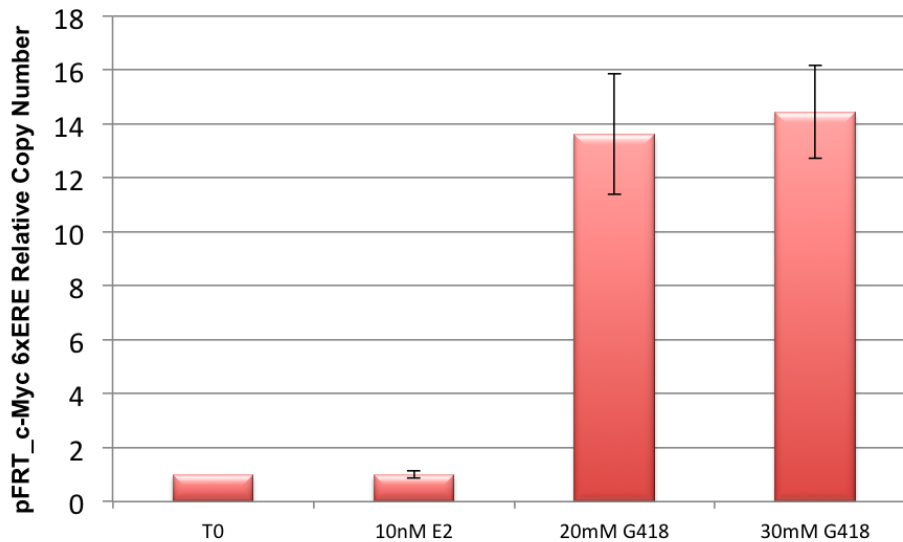


Figure 8. Evaluation of DNA Amplification at the Ectopic c-Myc Origin After E2 treatment. MCF7/ FRT c-Myc 6xERE were treated for 1 month with 10 nM E2 and then treated with 20 mM G418 and 30 mM G418. Genomic DNA was isolated from cells collected at time zero (T0), after the steroid hormone treatment (10 nM E2), and from cells survived to the high dose G418 selection (20 mM G418 and 30 mM G418). The vector specific sequence was normalized to TBP (TATA Binding Protein).

amplification at the ectopic site was identified after 1 month exposure to 10 nM 17- β estradiol (E2) (Figure 8). However, no E2-dependent DNA amplification at the engineered c-Myc6xERE replication origin was detected in subsequent replications of the experiment.

The ER α -dependent DNA amplification at the ectopic c-Myc replication origin was then tested in recombinant U2OS/ER α cells. Three clones of recombinant U2OS/ER α cells having a single integrated copy of the pPB.PGK-BlaGFP_Myc6xERE construct (namely: U2OS/i2B3, U2OS/H2C3, and U2OS/C3) were used. Cells were plated and incubated overnight in standard media (phenol red-free DMEM/F-12, 5% charcoal/dextran treated FBS, 1% penicillin/streptomycin, 500 μ g/mL Zeocin, 50 μ g/mL Hygrimycin B, 4 μ g/mL blasticidin) and after a PBS wash, cells were incubated with media containing 10 nM 17- β estradiol (E2) with or without the additional addition of 1 μ g/mL doxycyclin for the induction of ER α expression. Unexpectedly, the activation of ER α resulted in cell death that was visible already after 24 hours treatment (Figure 9). Moreover, cell death could not be reversed by changing the media, culturing cells in standard media with no addition of either doxycycline or E2. This posed a serious problem to the planned experiment. Aiming to seek for conditions compatible with E2 cell treatment, different doxycycline concentrations and times of exposure were tested (Figure 9).

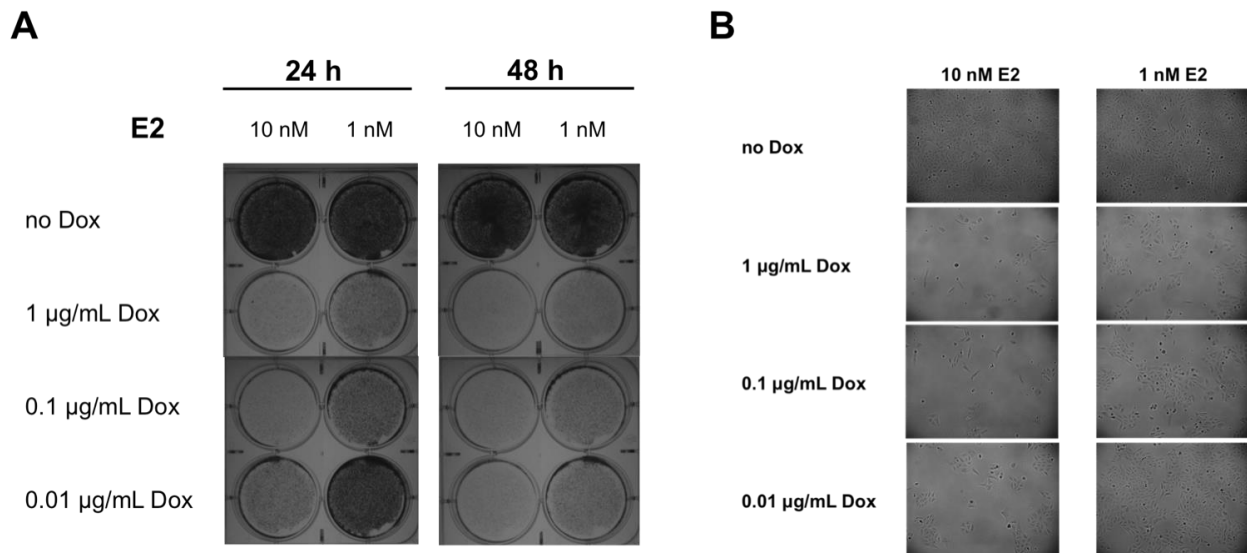


Figure 9. ER α Expression Triggers Cell Death in Recombinant U2OS/ER α Cells. Recombinant U2OS/ER α cells were treated with E2 in presence or absence of doxycycline to induce the expression of ER α . Evident cell death is apparent already at 24 hours after doxycycline induction, as demonstrated by remarkable reduction of cell density. A) crystal violet staining. B) microscope bright fields. The reduction in cell number progressively increases at the increase of time of doxycycline induction (A, 48 h induction).

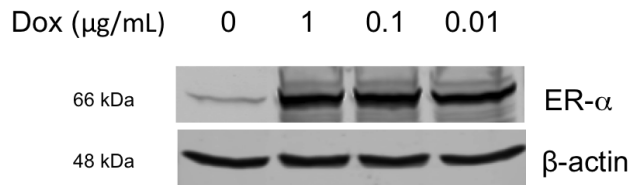
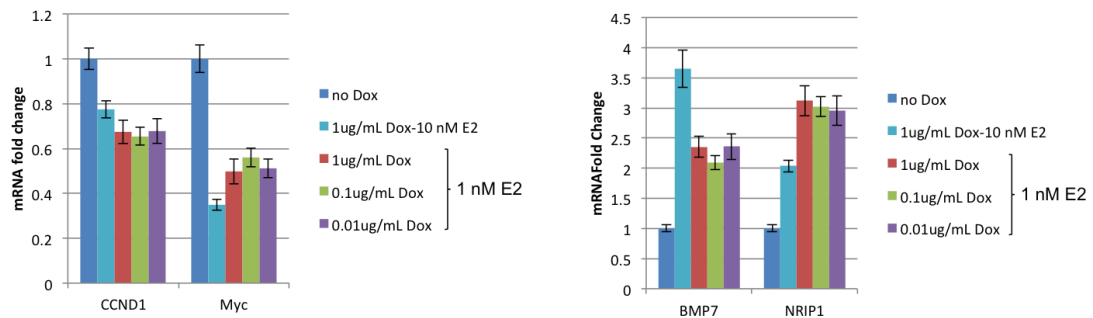
A**B**

Figure 10. ER α is Activated at Lower Doxycycline and E2 Concentration. The activation of ER α was assessed for the conditions found to have the lowest impact on cell viability, namely 24 hours treatment with 10 ng/mL doxycycline and 1 nM E2. **A)** Western blot confirmed ER α expression for all doxycycline concentration tested. **B)** qPCR evaluation of level of expression of selected ER α target genes. Data show that ER α is activated even when cells are treated with 1 nM E2.

24 hours treatment with 1 nM E2 proved to be the condition that least affected cell viability. Western blot and qPCR supported ER α activation in these experimental conditions (Figure 10).

Greatly limited by the unexpected toxicity determined by ER α expression, FACS analysis was performed on U2OS/H2C3 cells treated for 24 hours with 1 nM E2. No significant change in TagGFP expression level was detected (Figure 11).

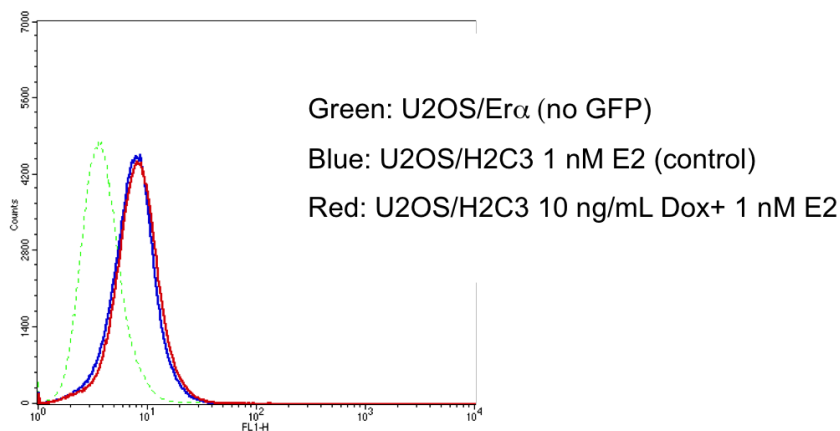


Figure 11. TagGFPs expression Do Not Change Compared to Control Cells After 24 hours E2 Treatment. U2OS/H2C3 cells, which carry a single copy of the c-Myc 6xERE construct, were plated and incubated over night in standard media and then treated for 24 hours with 1 nM E2 in presence or absence of 10 ng/mL doxycycline. Cells were trypsinized and TagGFP2 expression level analyzed by FACS. No apparent change in expression level is detected when ER α expression is induced.

Expand the assessment of estrogen-dependent DNA amplification to a genome-wide scale (Task Three)

An alternative approach to identify re-replication in the recombinant U2OS cells after E2 treatment can be to directly look at single DNA molecules by DNA combing (Bianco *et al.*, 2012). This methodology comprises the sequential pulse labeling of active proliferating cells with two nucleotide analogs, the iododeoxyuridine (IdU) and chlorodeoxyuridine (CldU). IdU and CldU are therefore incorporated in newly replicated DNA. After labeling, DNA is isolated and molecules stretched on microscope slide coverslips. IdU and CldU are then visualized by staining with fluorescent labeled antibodies. Depending on the distance of the labeled replicating DNA from the origin, different staining patterns can be visualized (Figure 12). Moreover, a specific labeling pattern will be associated with re-replicated DNA (Dorn *et al.*, 2009). Indeed, re-replication will be readily identifiable because of overlapping labeling of the same DNA molecule stretch with both IdU and CldU (Figure 13).

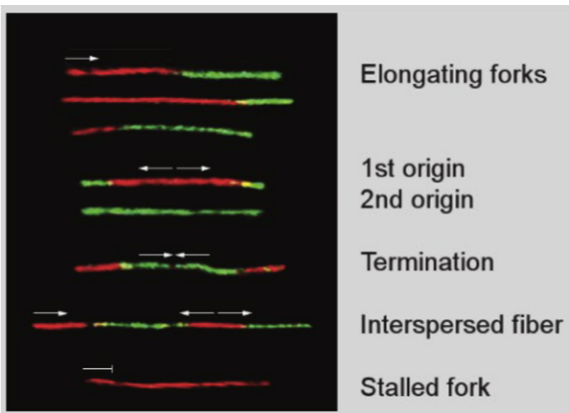


Figure 12. DNA combing Technique. DNA combing of newly synthesized molecules by sequentially pulse labeling proliferating cells with IdU (red) and CldU (green) allows the visualization of replication structures, such as actively replicating forks, the firing of new origins, two converging forks (termination), sites of closely spaced origins, and stalled forks (image from: Schwab and Niedzwiedz J Vis Exp. 2011 (56):e3255).

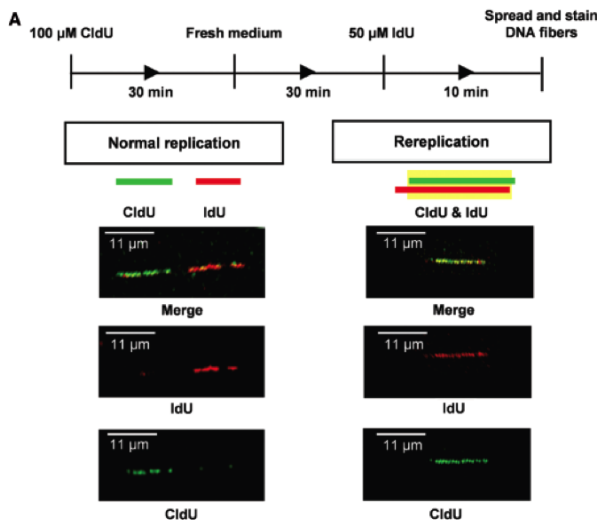


Figure 13. DNA re-replication visualized through DNA combing. When replication origin fires twice within the time window when cells are sequentially pulse labeled with nucleotide analogs, re-replication will be highlighted by the presence of overlapping signal from IdU and CldU (image from Dorn ES *et al.* Nucleic Acids Res. 2009 37(1):60-9).

In the attempt to use DNA combing to identify genome wide the presence of DNA re-replication as result of ER α activation, about 10⁷ recombinant U2OS/H2C3 cells were plated and incubated over night in standard media, and subsequently treated for 24 hours with 1 nM E2 in presence or absence of 10 ng/mL doxycycline. Cells were then incubated for 30 min with fresh pre-warmed media containing 100 μ M CldU. Media was changed to fresh pre-warmed media (no label) and the cells were left in the incubator for 30 min. This step ensures that all CldU up-taken by the cells is incorporated and there is none left during the second pulse labeling of replicating DNA. Cells were then incubated for 10 min with pre-warmed fresh media containing 100 μ M IdU. To prepare and store the samples for downstream DNA combing, cells were embedded in agarose plugs using the CHEF Genomic DNA Plug Kit (Biorad), following the manufacturer's instructions. Briefly, after the labeling, cells were washed twice with PBS, trypsinized, and counted. 10⁷ cells for each condition (i.e. with or without induction of ER α expression) were pelleted and resuspended in 0.63 mL of Cell Resuspension Buffer and the cell suspension equilibrated to 50°C. Subsequently, 0.37 mL of 2% CleanCut agarose solution was added to the cell suspension by gentle mixing to obtain a final concentration of 0.75% agarose. The suspension was transferred to plug molds (100 μ L/plug) and incubated for 15 min at 4°C. Solidified agarose plugs were collected into a 50 mL tube containing the Proteinase K Reaction Buffer and incubated over night at 50°C. Plugs treated with proteinase K were washed 3 times with 1X Wash Buffer, each time by incubating 1 hour with gentle agitation. Samples have been processed at Nick Rhind's laboratory (Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA), which has good expertise in DNA combing. Moreover, an undergraduate student of my lab, Beverly Naigles, was involved in samples processing for replicating DNA visualization, since she recently visited Dr. Aaron Bensimon's laboratory at Genomic Vision (Bagneux, France). Dr. Aaron Bensimon is a recognized authority on all aspects of DNA combing technology and his contribution to the field has been widely published.

Preliminary staining experiments were unfortunately not conclusive and highlighted the need to further optimize DNA labeling and staining. Noteworthy, DNA molecules prepared from recombinant U2OS cells were found to be shorter than expected (30-50 kb rather than the desired 120 kb). This finding may be indicative of estrogen-mediated DNA breaks, as found in other cell systems (Williamson and Lees-Miller, 2011; Savage *et al.* 2014) and which might also provide a reasonable explanation for cell death upon induction of ER α expression.

Test if estrogen receptor-alpha directly interacts with the replication machinery and which domains of the receptor are involved in such interaction (Tasks Four and Five).

A plasmid expressing a FLAG-Orc2 protein has been kindly provided by Dr. Melvin DePamphilis (NIH, Bethesda, MD). To investigate the molecular interaction between the replication machinery and the ER-alpha, I could take advantage of the U2OS/ER-alpha cells that were already in use. A FLAG-tagged ORC2, in conjunction with an induced expression of the ER-alpha are likely to be optimal conditions for a co-IP experiment. A stock of the plasmid has been prepared and the expression of the Flag-Orc2 protein has been assessed by Western blot in transfected MCF7 cells.

However, since Task Two represented the essential starting point of the project and has been proved technically more challenging than anticipated and required more efforts than foreseen, thus delaying the accomplishment of stated goals, the interaction between the replication machinery and ER α was not further investigated.

The study of estrogen-dependent DNA amplification in breast cancer as a result of a crosstalk between the estrogen receptor and an origin of DNA replication is tightly connected to the identification of DNA replication origins genome-wide. Knowing where replication origins are located in the genome will be of great importance to draw a link between DNA amplification, estrogen receptor binding sites, and re-replication. Joining the efforts of members of the laboratory of my mentor Susan Gerbi, we wrote a review article on the methods that have been used recently to map origin of DNA replication as well as factors that may influence their specification. The review article is in press at F1000Prime Reports.

Moreover, a paper was submitted on the genome-wide mapping of origin of DNA replication in MCF7 cells by nascent strand sequencing (NS-seq). NS-seq strongly relies on the ability of the enzyme Lambda exonuclease to efficiently digest parental DNA while leaving RNA-primer protected nascent strands intact. Genomics and biochemical approaches were adopted to determine if Lambda exonuclease has biases in digesting parental DNA. The careful experimental design and the use of proper controls unveiled enzyme biases towards G-quadruplex structures (G4). In fact, Lambda exonuclease does not efficiently digest DNA containing G4s and GC-rich sequences. Interestingly, a subset of the mapped replication origin resulted associated with G4, and a periodic spacing of G4 motifs and nucleosomes around these origins was observed, suggesting that G4s may position nucleosomes at these sites. I am a co-author on this paper that is in press at Genome Research.

Methods.

Establishment of Flp-In Cell Lines. Cells were transfected with pFRT/lacZeo plasmid (Invitrogen) linearized with XmnI restriction endonuclease (New England Biolabs) using FugeneHD Transfection Reagent (Promega) following the manufacturer's instruction. Briefly, a 3:1 FugeneHD (μ L): plasmid DNA (μ L) transfection mixture was incubated 10 min at room temperature and then added to log-growth phase cells about 80% confluent plated 24 h earlier. Transfection efficiency was assessed by detection of YFP expression in cells transfected at the same time with the pbabeYFP control plasmid. At 48 h after transfection, cells were selected with 200 μ g/mL Zeocin. Drug resistant clones were isolated and expanded.

Southern Blot. Southern blots were performed as described in Sambrook *et al.*, Molecular Cloning (1989). 10 μ g genomic DNA isolated from U2OS/ER-alpha pPB.PGK-BlaGFP_Myc6xERE cells was digested overnight at 37°C with 100 units HindIII (New England Biolabs) and loaded on a 1% agarose gel. The gel was sequentially soaked with gentle agitation in 0.25 HCl for 20 min, dH₂O for 2 min, and

twice in transfer buffer (0.4 M NaOH, 1 M NaCl) for 20 min). DNA was then transferred onto a positively charged nylon membrane (Hybond XL, Amersham) by overnight capillary alkaline transfer using the transfer buffer. The membrane was subsequently washed twice in 0.5 M Tris-HCl pH 7.2, 1M NaCl for 20 min, air dried, and baked at 80°C for 2 h. The membrane was then pre-hybridized in Hybridization Buffer (0.5 M Na₂HPO₄ pH 7.2, 7% SDS, 1 mM EDTA) at 60°C for 4 h. The 1052 bp ³²P labeled probe (a PCR amplified fragment of the blasticidin-2A-TagGFP ORF) was synthesized using the NEBlot kit (New England Biolabs) following the manufacturer's instruction. Free ³²P was removed from the reaction by gel filtration (Illustra ProbeQuant G-50 Micro Columns, GE Healthcare). The membrane was hybridized overnight at 60°C with Hybridization buffer supplemented with the radioactive probe (6x10⁵ cpm/mL). The membrane was washed at room temperature 5 min with 2X SSC, 0.25% SDS, twice with 2X SSC, 0.1% SDS for 20 min, twice with 1X SSC, 0.1% SDS for 20 min. Finally, the membrane was exposed to X-Ray film at -80°C with intensifying screen for 2-14 days.

Cloning. *E. coli* strain Stbl3 (Invitrogen) were used for all the cloning steps and cells were transformed by electroporation (Micropulser, BioRad). Oligonucleotides used for cloning and PCR amplifications were purchased from either Integrated DNA Technologies or Invitrogen. TagGFP2 expression plasmid was acquired from Evrogen (Moscow, Russia). Restriction enzymes, T4 DNA Ligase, DNA polymerase I-Klenow fragment, Shrimp Alkaline Phosphatase, and T4 Polynucleotide Kinase were purchased from New England Biolabs. Cloning is performed following standard protocols (www.neb.com). At each cloning step, constructs were assessed by restriction digestion and further verified by sequencing (Genewiz, Cambridge, MA). Small scale plasmid DNA is prepared using QIAprep Spin Miniprep Kit (Qiagen) following the manufacturer's instructions. High scale plasmid DNA preparations needed for human cell line transfections is performed with PureLink HiPure Plasmid Filter Purification Kit (Invitrogen) following the manufacturer's instructions.

Establishment of Recombinant MCF7/PGK-BlaGFP_Myc and MCF7/PGK-BlaGFP_Myc6xERE Cells. 3x10⁵ MCF7 Flp-In cells were co-transfected with either pFRT.PGK-BlaGFP_Myc or pFRT.PGK-BlaGFP_Myc6xERE construct and the Flippase recombinase expression plasmid pOG44 at a ratio of 1:9. FugeneHD transfection reagent was used at 3:1 FugeneHD (μL): DNA (μg) ratio according to the manufacturer's instructions. Drug selection was started after 48 hours from transfection and selective media was changed every 3-4 days.

Establishment of U2OS/ER-alpha_PGK-BlaGFP_Myc6xERE Clones. For each transfection, 2x10⁵ cells were co-transfected with pPB.PGK-BlaGFP_Myc6xERE and pCMVhyPBBase, which is the expression plasmid for the hyperactive version of the PiggyBac transposase (Yusa *et al.*, 2011). Transfection was performed with FugeneHD DNA transfection reagent (Promega) using 3.5:1 FugeneHD (μL): DNA (μg). After 48 hours from transfection, cells were trypsinized and 1/60 plated in a new dish in complete media additioned with 4 μg/mL blasticidin. Once resistant colonies were visible, single colonies were isolated and expanded. Duplicated dishes plating 1/12 of transfected cells

were stained with Crystal Violet after drug selection to determine transposition efficiency

Nascent Strand Isolation and Origin Activity Assessment. Total genomic DNA was isolated from $\sim 10^7$ exponentially growing cells using DNAzol (Invitrogen) following the manufacturer's instructions. Single-stranded DNA (ssDNA) was enriched by affinity chromatography through a benzoylated naphthoylated DEAE (BND)-cellulose column (Sigma). The resin was equilibrated with NET buffer (1 M NaCl, 1 mM EDTA, 10 mM Tris-HCl pH 8.0) and, after loading the genomic DNA, ssDNA was eluted with NET buffer supplemented with 1.8% caffeine. The enriched sample was 5'-end phosphorylated with T4 polynucleotide kinase. Subsequently, the contaminating ssDNA derived from broken DNA was degraded by overnight digestion with λ -exonuclease enzyme (New England Biolabs): only newly synthesized DNA molecules are protected from degradation by the presence of an RNA primer at their 5'-end. Contaminating Okazaki fragments (<500 bp) were subsequently removed by size fractionation on gels (1-2 kb). The assessment of origin activity at a given site was determined by quantitative PCR (qPCR). The abundance of sequences at the assayed replication origins relative to the DNA abundance at a site without replication origin activity was normalized to total genomic DNA.

RT-qPCR. Total RNA was isolated using RNeasy Kit (Qiagen) following manufacturer's instructions. cDNA was obtained from 1 μ g RNA using SuperScript III (Invitrogen) following manufacturer's instructions. After cDNA quantification (picogreen, Invitrogen), 5 ng was used to perform real time PCR. Each sample was tested in triplicate. GAPDH was used for sample normalization.

Quantitative PCR. 10 ng genomic DNA was assessed for each reaction using SYBR Green PCR Master Mix (Applied Biosystems). The amplification efficiency of each primer set was measured by testing serial dilutions of a reference sample. In each assay, samples were assessed in triplicate (expression level) or quadruplicate (DNA copy number). Assays were performed with an Applied Biosystems 7300 Real Time PCR machine.

Western Blot. Cells were washed once with ice-cold PBS and collected with a rubber spatula. Cells were then lysed in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS). After cell homogenization, insoluble material was removed by 10 min centrifugation at 13000 xg at 4°C. Total protein was determined with BCA Assay (Pierce) and 30 μ g sample in 1X Laemmli buffer and 5% β -mercaptoethanol were boiled for 10 min at 95°C and loaded onto an 8% polyacrylamide gel. After semi-dry transfer, nitrocellulose filter was blocked with TBS, 5% nonfat dry milk and probed with primary antibody diluted in TBS, 3% nonfat dry milk. Detection of the fluorescent-labeled secondary antibody was performed with Li-Cor ODYSSEY CLx scanner.

FACS Analysis on Live Cells. Cells were trypsinized, washed once with ice-cold PBS and resuspended in ice-cold PBS at $\sim 1 \times 10^6$ cells/mL. GFP fluorescence signal (ex 488 nm, em 530 nm) was measured with BD FACSCalibur Flow Cytometer performing the acquisition of at least 20×10^6 events and analysis performed with BD-CellQuest software.

What opportunities for training and professional development has the project provided? During the funded project period I participated and interacted with professors, postdocs, and Ph.D. students at meetings and seminars held at Brown University as well as in the highly renowned venue of the Cold Spring Harbor DNA replication meeting. Overall, there were numerous opportunities that provided me with great training by expanding my scientific background and setting up the platform for constructive scientific discussions.

Besides the aforementioned meeting “Eukaryotic DNA Replication and Genome Maintenance” I participated in the following:

- classes of the Bio 105/205 Course “Biology of the Eukaryotic Cell” (Profs. Susan Gerbi and Ken Miller) (audit);
- weekly laboratory group seminars in the Gerbi lab, where our own research data as well as published works were presented and discussed. In particular, a four month period was mostly dedicated to the critical discussion of papers on DNA amplification, which offered a complete and critical overview of the status of the art in the field;
- laboratory seminars held in the Brodsky lab, contributing to the discussion of research data as well as in the critical reading and presentation of cutting edge works in cancer research;
- I attended some of the Molecular and Cell Biology and Biochemistry Data Club Seminars, which have been held monthly and provided an updated overview of the research pursued in the department;
- I attended many of the Molecular and Cell Biology and Biochemistry Program Seminars, as well as some seminars of interest hold by the Pathobiology Graduate Program and the Center for Computational Molecular Biology (CCMB), including the CCMB symposium “Evolution of Cancer”.

How were the results disseminated to communities of interest? Co-author on two papers about DNA replication origins:

Urban JM, Foulk MS, **Casella C** and Gerbi SA (2015). The hunt for origins of DNA replication in multicellular eukaryotes. *F1000*, in press (doi: 10.12703/P7-30).

Foulk MS, Urban JM, **Casella C** and Gerbi SA (2015). Characterizing and controlling intrinsic biases of Lambda exonuclease in nascent strand sequencing reveals phasing between nucleosomes and G-quadruplex motifs around a subset of human replication origins. *Genome Research*, in press (doi: 10.1101/gr.183848.114).

What do you plan to do during the next reporting period to accomplish the goals? Nothing to Report (Final report).

4. Impact

What was the impact on the development of the principal discipline(s) of the project? The project aimed to test the effect of estrogen exposure on the regulation of DNA replication. DNA replication is a tightly controlled process that take place once and only once per cell cycle, ensuring that after cell division each of the two daughter cells have an identical copy of the entire genome. Subversion of the mechanisms that regulate DNA replication has been shown to be an efficient mechanism to elicit DNA amplification. DNA amplification is a hallmark of cancer and the identification of the factors and mechanisms involved in this process could provide useful tool to counteract cancer cell proliferation and metastatic spread. On the other hand, estrogen is a well known risk factor in breast cancer and previous data obtained in a model organism suggest that a steroid hormone can modulate the activity of a replication origin resulting in site-specific DNA amplification.

The technical challenges encountered during the project hampered the achievement of definitive answers on the role of estrogen in breast cancer DNA amplification. Nonetheless, in one experiment a 14-fold DNA amplification detected in cells treated with estrogen at a specific genomic site containing a binding site for estrogen receptor next to a replication origin suggests that the cross-talk of estrogen and replication origins deserves further investigation. Moreover, recent work showed that altered DNA replication is an efficient mechanism for DNA amplification. The reporter construct that carry a motif for the estrogen receptor binding next to the well characterized replication origin was refined during this project and is valuable resource to be used in *in vitro* experiments.

What was the impact on other disciplines? Nothing to Report.

What was the impact on technology transfer? Nothing to Report.

What was the impact on society beyond science and technology? Nothing to Report.

5. Changes/Problems

Accordingly to the approved request for travel authorization and early termination of the DoD-BCRP award, from 01.01.2014 until the termination of the awarded fellowship I worked on the writing of scientific papers related to the funded project.

6. Products

Journal Publications:

Urban JM, Foulk MS, **Casella C** and Gerbi SA (2015). The hunt for origins of DNA replication in multicellular eukaryotes. *F1000*, in press (doi: 10.12703/P7-30).

Foulk MS, Urban JM, **Casella C** and Gerbi SA (2015). Characterizing and controlling intrinsic biases of Lambda exonuclease in nascent strand sequencing reveals phasing between nucleosomes and G-quadruplex motifs around a subset of human replication origins. *Genome Research*, in press (doi: 10.1101/gr.183848.114).

Other Products:

Development of cell lines: Breast cancer MCF7 Flp-In cell lines developed by stable integration of pFRT/lacZeo plasmid; U2OS/ERa recombinant clones carrying an optimize construct for DNA amplification selection

7. Participants & Other Collaborating Organizations

Name:	▪ <i>Cinzia Casella</i>
Project Role:	▪ <i>PI</i>
Researcher Identifier (e.g. ORCID ID):	▪
Nearest person month worked:	▪ 32
Contribution to Project:	▪ Experiment design and execution
Funding Support:	▪

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? Nothing to Report

Other organizations were involved as partners?

Organization Name: Nick Rhind's laboratory, Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School

Location of Organization: Worcester, MA, USA

Partner's contribution to the project: collaboration (DNA combing)

8. Special Reporting Requirements

None

9. Appendices

References

Bianco JN1, Poli J, Saksouk J, Bacal J, Silva MJ, Yoshida K, Lin YL, Tourrière H, Lengronne A, Pasero P. Analysis of DNA replication profiles in budding yeast and mammalian cells using DNA combing. *Methods*. 2012; 57(2):149-57.

Dorn ES1, Chastain PD 2nd, Hall JR, Cook JG. Analysis of re-replication from deregulated origin licensing by DNA fiber spreading. *Nucleic Acids Res*. 2009; 37(1):60-9.

Finn KJ, Li JJ. Single-stranded annealing induced by re-initiation of replication origins provides a novel and efficient mechanism for generating copy number expansion via non-allelic homologous recombination. *PLoS Genet*. 2013;9(1):e1003192.

Foulk MS, Liang C, Wu N, Blitzblau HG, Smith H, Alam D, Batra M, Gerbi SA. Ecdysone induces transcription and amplification in *Sciara coprophila* DNA puff II/9A. *Dev Biol*. 2006; 299(1):151-63.

Green BM, Finn KJ, Li JJ. Loss of DNA replication control is a potent inducer of gene amplification. *Science*. 2010; 329(5994):943-6.

Kiang L, Heichinger C, Watt S, Bähler J, Nurse P. Specific replication origins promote DNA amplification in fission yeast. *J Cell Sci*. 2010; 123(Pt 18):3047-51.

Liew GM, Foulk MS, Gerbi SA. The ecdysone receptor (ScEcR-A) binds DNA puffs at the start of DNA amplification in *Sciara coprophila*. *Chromosome Res*. 2013; 21(4):345-60.

Schmidt M, Schwarzwaelder K, Bartholomae C, Zaoui K, Ball C, Pilz I, Braun S, Glimm H, von Kalle C. High-resolution insertion-site analysis by linear amplification-mediated PCR (LAM-PCR). *Nat Methods*. 2007; 4(12):1051-7.

Richardson CD, Li JJ. Regulatory mechanisms that prevent re-initiation of DNA replication can be locally modulated at origins by nearby sequence elements. *PLoS Genet*. 2014; 10(6):e1004358.

Savage KI, Matchett KB, Barros EM, Cooper KM, Irwin GW, Gorski JJ, Orr KS, Vohhodina J, Kavanagh JN, Madden AF, Powell A, Manti L, McDade SS, Park BH, Prise KM, McIntosh SA, Salto-Tellez M, Richard DJ, Elliott CT, Harkin DP. BRCA1 deficiency exacerbates estrogen-induced DNA damage and genomic instability. *Cancer Res*. 2014; 74(10):2773-84.

Williamson LM, Lees-Miller SP. Estrogen receptor α -mediated transcription induces cell cycle-dependent DNA double-strand breaks. *Carcinogenesis*. 2011; 32(3):279-85.