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PRINCIPAL INVESTIGATOR: Maria Spies

CONTRACTING ORGANIZATION: The University of Iowa

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

In contrast to its important function in DNA repair, overexpression of human MSH2/6 mismatch recognition protein is driving the progression of basal like breast cancer and is associated with poor prognosis. This project will establish the molecular mechanisms of this MSH2/6 activity. During the first funding period we have established an experimental workflow to test the hypothesis that MSH2/6 protein targets methyltransferase DNMT1 to specific promoters and activates its enzymatic activity thus connecting the MSH2/6 overexpression with altered epigenetic regulation. Our preliminary data suggest that this hypothesis is correct. During the next funding period we will complete the biochemical and single-molecule studies testing this hypothesis.

15. SUBJECT TERMS

NONE LISTED

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1. INTRODUCTION:

Current treatment options for basal like breast cancer (BLBC) rely on cytotoxic dose-dense chemotherapy and surgery. The work under this award builds on our preliminary data that suggested that MSH2/6 may be a viable molecular target for BLBC therapy. Our goal is to define the role of MSH2/6 at different stages of BLBC pathogenesis and to establish MSH2/6 as a molecular target for BLBC therapy. As a partnering PI with expertise in biochemical mechanisms of DNA mismatch repair (MMR), Dr. Spies' lab is responsible for the biochemical and single-molecule experiments carried under this award.

2. KEYWORDS:

Basal like breast cancer, BLBC; DNA mismatch repair, MMR; Human MutS homolog MSH2/6, MutSα; human methyltransferase DNMT1; total internal reflection fluorescence microscopy, TIRFM; single-molecule pull-down, SiMPull

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: Determine the role of MSH2/6 in BLBC pathogenesis and immunotherapy

The Spies' lab involvement in completion of this specific aim will start in year three of the project

Specific Aim 2: Determine the mechanism how MSH2/6 contributes to epi/genetics and immune modulation. Below are the tasks relevant to this reporting period

- Determine DNMT1 binding efficiency to CD274 and PVR promoters.
- Characterize the MSH2/6 and DNMT1 interaction *in vitro*;
- DNMT1 and MSH2/6 dimers will be purified and the interaction between DNMT1 and MSH2/6 will be determined by single-molecule pull down (SiMPull).
- Determine the effect of MSH2-MSH6 on the DNMT1 enzymatic activity

What was accomplished under these goals?

The 2020 was a challenging year for the lab, as for the rest of the scientific community. The access to the labs was restricted in the beginning of this funding period due to the Covid-19 pandemic. We used the time during lab closure to write a comprehensive review describing our current understanding of the molecular events at stalled and damage DNA replication forks:

Kondratick, C.M., Washington, M.T., and Spies, M, *Making choices: DNA replication fork recovery mechanisms*. Seminars in Cell & Developmental Biology. 2020 [in print; https://doi.org/10.1016/j.semcdb.2020.10.001],

a comprehensive review on how single-molecule and biophysical approaches (including our work) illuminate mechanistic understanding of the Replication Protein A:

Caldwell, C.C., and Spies, M‡, *Dynamic Elements of Replication Protein A at the Crossroads of DNA Replication, Recombination, and Repair*. Critical Reviews in Biochemistry & Molecular Biology. 2020 Oct;55(5):482-507. [PubMed PMID: 32856505; PubMed Central PMCID: PMC7821911],

and a book chapter summarizing current state of DNA repair inhibitors research for cancer therapy.

Chheda, P., Spies, M, and Spies, M.A., Small-Molecule Effectors of DNA Repair Proteins: Applications for Development of Cancer Therapeutics and Research. Burger's Medicinal Chemistry, Drug Discovery and Development, 8th edition. 2020 (in print)

These three reviews set a stage for understanding the important events in DNA metabolism that may be affected by overexpression of MSH2/MSH6.

Biochemical analysis of the effect of the purified MSH2/MSH6 on the enzymatic activity of human DNMT1 and binding specificity of MSH2-MSH6:

DNMT1 recognizes hemi-methylated DNA and converts it to fully methylated sequence, the process important for the faithful of replication of inherited epigenetic patterns. During the first year of funding we have started exploring the hypothesis that MSH2-MSH6 can activate methyltransferase DNMT1 thereby enabling a crosstalk between overexpression of MSH2-MSH6 and epigenetic regulation. We have expressed and purified three DNMT1 constructs, a nearly full length protein containing self-inhibition domain, an "activated" truncated form of DNMT1 lacking self-inhibition domain, and the inhibitory domain by itself. We have developed a (Föster resonance energy transfer (FRET)-based methylation assay to test the activation of MSH2/6 by DNMT1 (**see progress report from year 1**). During the second year, we have scaled up the purification of the DNMT1 constructs and can now routinely purify large quantities of these proteins for biochemical and biophysical studies.

To increase the production of MSH2-MSH6 heterodimer and to facilitate massively parallel TIRFM-based analyses of binding specificity (a single-molecule methodology we have developed to complement the ChIP experiments), we have reengineered the expression construct for MSH2-MSH6. In this new expression system, open reading frames for MSH2 and MSH6 are cloned into pFastBac vectors, generated bachmids and baculoviruses for infection of SF9 cells. MSH6 open reading frame contains 10xHis tag and a cleavable GFP at the N-terminus. **Figure 1** shows optimization of the infection conditions (**a** and **b**), and purification of MSH2-MSH6-6xHis/GFP

Figure 1d shows that the GFP-tagged MSH2-MSH6 has similar DNA binding properties to the protein with removed GFP tag.

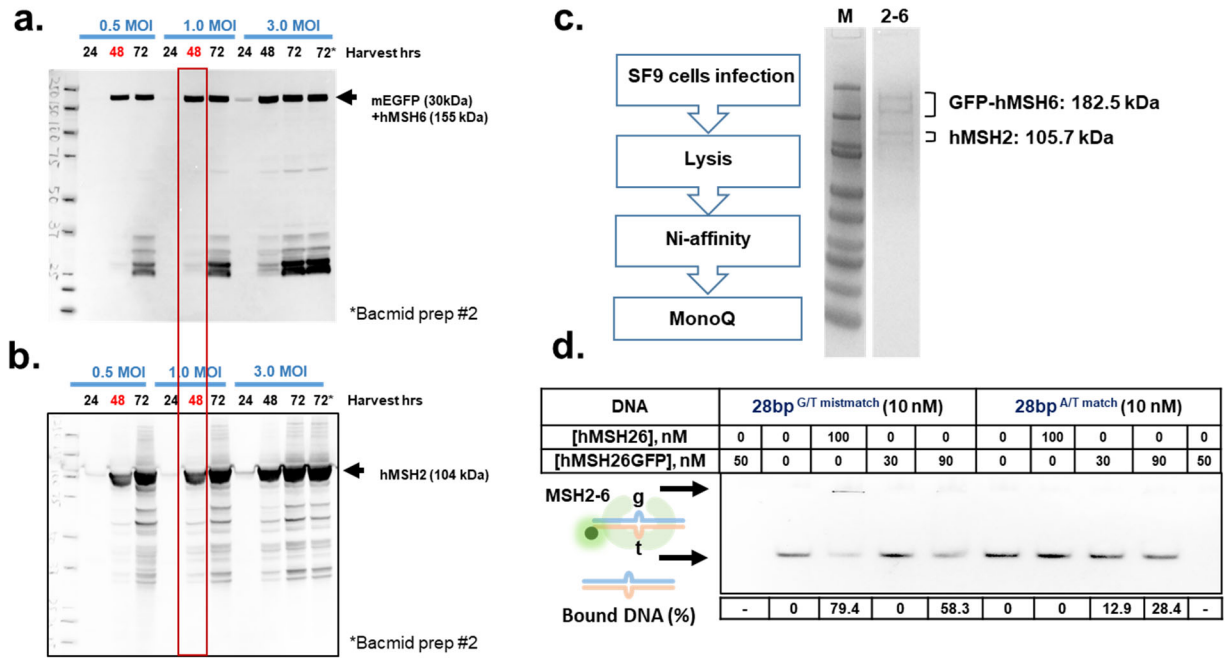

***Figure 1. New expression construct for the MSH2-MSH6. a&b**. Optimization of the infection conditions with new baculovirus. **a.** Western blot using anti-GFP antibodies, **b.** Western blot using anti-MSH2 antibodies. Conditions selected for the large scale infection are marked with the red rectangle. **c.** Purification scheme for MSH2-MSH6 and a CBB stained gel showing purified protein. **d.** Mobility shift assay showing that purified MSH2-MSH6-GFP specifically binds to mismatch-containing DNA. Note that these experiments were carried out with Cy3-labled DNA and there is a partial interference between the signals from shifted DNA and GFP. For the subsequent experiments (shown below) we have reengineered the substrate, so it is now labeled with Cy5 dye.*

We will use the new purified MSH2-MSH6 protein to complete the analysis of DNMT1 activation. Our preliminary data from year 1 were suggestive of the stimulatory effect of the mismatch bound MSH2/6 on the DNMT1 activity. We now have all necessary components of the assay, sufficient amount of proteins and the controls to complete this study during the first half of the next funding period.

Two single-molecule types of MSH2/6-DNMT1 interaction analyses were planned, SiMPull (single-molecule pull-down) and the massively parallel analysis of binding specificity (a single-molecule methodology we developed to complement the ChIP). The major time consuming step in both analysis is establishing the experimental conditions and reagents. We have completed this task for both types of analyses. Currently, we have the SiMPull fully working on the model system with purified and affinity tagged proteins. The most difficult task to overcome was establishing conditions for antibody labeling (with biotin and fluorophores) and avoiding non-specific adsorption on the TIRFM surface. Commercial antibodies used in SiMPull experiments need to be specific and available in BSA-free form. We now have a collection of antibodies (α-MSH6 ab214454, α-MSH2 ab228334, α-DNMT1 ab207601, α-BRCA1 ab215988, α-FANCJ ab250254), which we can efficiently label with biotin (for surface immobilization), Cy3 and Cy5 (for identification of the constituencies in the complexes. All experimental conditions and data analysis protocols for the

SiMPull work have been established and tested on an unrelated system (Hefel, A., Honda, M., Cronin N., Harrel, K., Patel, P., Spies, M., Smolikove, S.‡, *RPA complexes in Caenorhabditis elegans meiosis; unique roles in replication, meiotic recombination and apoptosis. Nucleic Acids Res 2021 Feb 26;49(4):2005-2026. doi: 10.1093/nar/gkaa1293*; [PMID: 33476370 PMCID: PMC7913698])

Next, two cell lines MDA-MB-231 and 4T1 will be used to visualize and quantify MSH2/6-DNMT1 interaction, as well as partitioning of MSH2/6 between complexes containing DNMT1 and genome surveillance BASC complex (visualized by anti-BRCA1 antibodies). We will also determine whether overexpression of MSH2-MSH6 sequesters BRCA1 from its other important interactions, such as complexation with FANCJ.

To distinguish whether the biochemical activity of MSH2-MSH6 (DNA binding), or the presence of the protein itself (i.e. its protein-protein interactions) promote BLBC pathogenesis, we plan to carry out the SiMPull experiments using cells grown in the presence of small-molecule inhibitor(s) of the MSH2-MSH6 interaction. Zhang lab has carried out an *in silico* screen of the NCI chemotherapeutic agents repository and identified 40 compounds that may interact with MSH2-MSH6's ATP binding site. We have screened these compounds for their ability to interfere with MSH2-MSH6 binding to a mismatch-containing dsDNA (**Figure 2**). At 10 µM, several of these compounds partially inhibited the interaction (**Figure 2b**), while two compounds (NSC77680 and NSC321596), inhibited the interaction completely. Upon further characterization, NSC321596, which is not a nucleotide analog and seems non-toxic towards NCI60 cells lines, will be invaluable for the SiMPull and ChiP-Seq experiments.

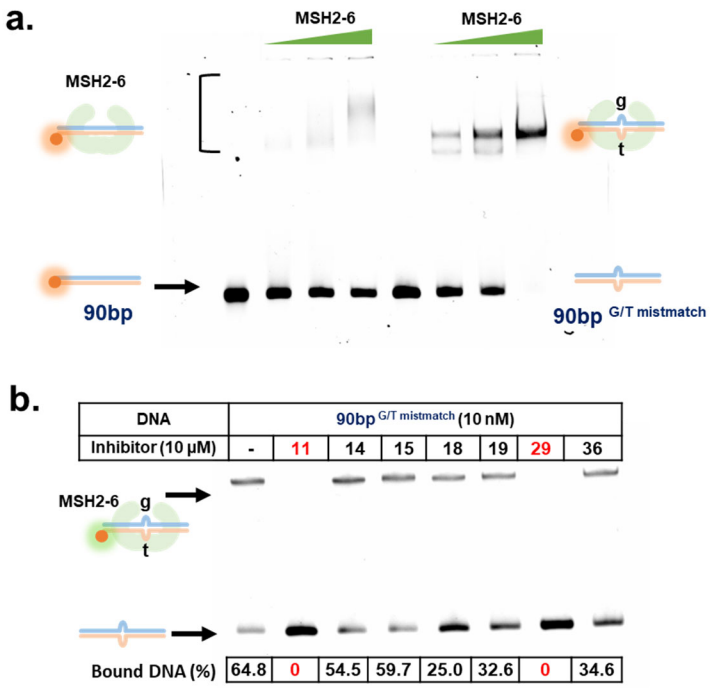

Figure 2. Confirming specificity of the purified MSH2-MSH6 interaction with mismatch-containing 90 bp substrate and inhibition of this interaction with small molecules. a. *Mobility shift assay showing that purified MSH2-MSH6 specifically binds to mismatch-containing DNA designed for SELEX experiments. Ten nM 90 bp long dsDNA with and without mismatch was incubated with the increasing concentrations of purified MSH2-MSH6 heterodimer.* ***b****. Rescreening of the most promising compounds that inhibit MSH2-MSH6-mismatched DNA interaction. Compounds 11 and 29* (NSC77680 and NSC321596, respectively) consistently and completely inhibit the interaction at 10 µM. Fraction of shifted (bound) DNA was quantifies using ImageJ.

In the second type single-molecule analysis we are combining Next Generation Sequencing with imaging by Total Internal Reflection Fluorescence Microscopy (TIRFM). This analysis will identify sequences, which MSH2-MSH6 can bind in the absence of mismatches (**Figure 3**). We have the assay working on our custom built TIRFM system, which can visualize and co-localize the data in

three colors (488 nm – GFP, 532 nm – Cy3, 640 nm – Cy5). We have also optimized the conditions for mapping the surface positions using fiduciary markers (**see Figure 3**).

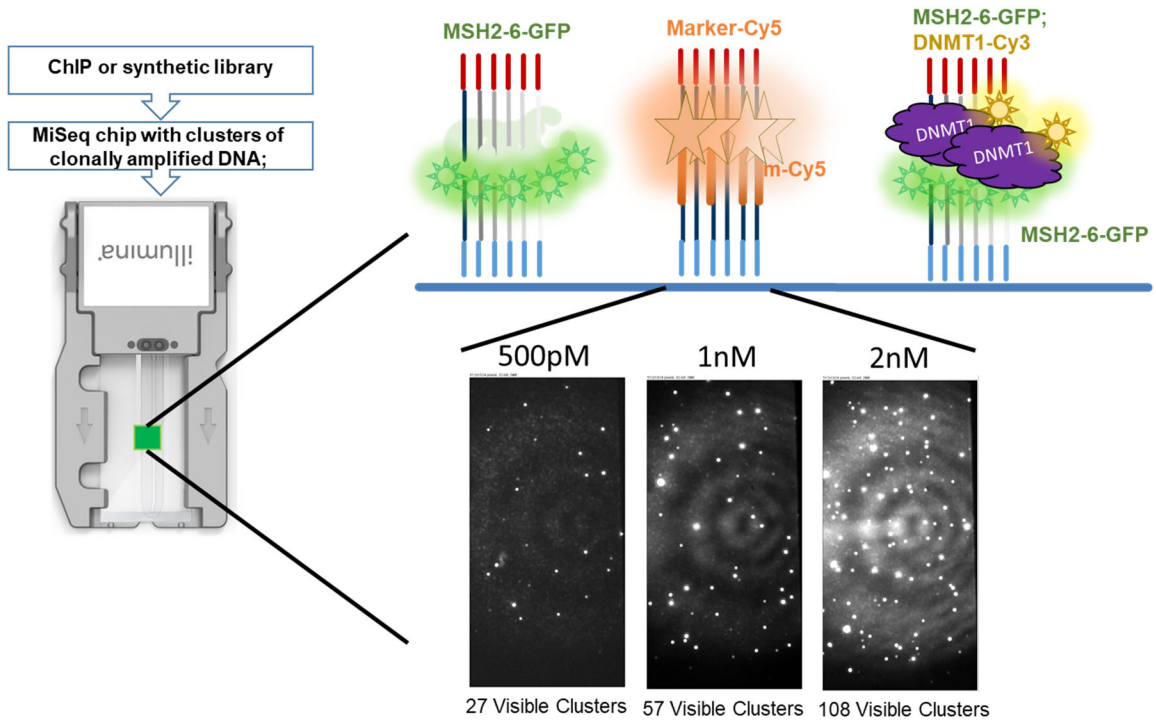

Figure 3. Developing massively parallel platform for analysis of the MSH2/6-DNA-DNMT1 interaction. *The experimental system we are developing will test the hypothesis that MSH2/6 binds not only to mismatched DNA, but also to highly flexible regions found in some promotors. Two libraries will be used: 1) We are building a library of promotor sequences from genes that are the most up or downregulated in MSH2/6 overexpressing BLBC cells. This library will be amplified and subjected to sequencing using Illimina MiSeq platform. At the end of the sequencing, we will have a MiSeq chip with clusters of clonally amplified DNA. We have adapted our total internal reflection fluorescence microscopy (TIRFM) system to image the surface of the MiSeq flow cells. Because we are spiking the library with fiduciary markers, we can correlate the position of clusters from the sequencing file with their positions observed in TIRFM. This will allow us to correlate for each cluster the binding signal for GFP-tagged MSH2/6 and Cy3-labeled DNMT1 with the DNA sequence and thereby determine binding specificity for MSH2/6 and/or DNMT1. 2) The second set of experiments will involve ChIP of the MSH2-MSH6 and using the resulting library for the analysis of MSH2-MSH6 affinity for these sequences and its ability to recruit DNMT1.*

In parallel with the single-molecule visualization of the MSH2-MSH6/DNA interaction we have initiated a SELEX-based selection of the sequences that MSH2-MSH6 can stably bind in the absence of mismatch. We are currently in the process of carrying out the first round of selection.

What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

We will complete the studies of DNMT1 activation by MSH2/6, MSH2/6-DNMT1 interaction and will establish the specificity of MSH2/6-promoter interactions. Experimental strategies will include biochemical, FRET-based analyses of the DNMT1 enzymatic activation by the MSH2/6 bound to a mismatch and/or a specific sequence, as well as single-molecule analyses of the DNMT1-MSH2/6 interactions and MSH2/6 sequence specificity. We will also complete SELEX and SiMPull studies.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

When completed, our work will characterize a previously unknown, pathological function of the MSH2/6 protein, which is primarily thought of as a guardian of genome integrity through its function in DNA mismatch repair.

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:

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

The 2020 was an extremely challenging year for the lab, as for the rest of the scientific community. The access to the lab was restricted in the beginning of this funding period due to the Covid-19 pandemic. Some of the studies we planned to finish in 2020 are still on going, but we are now on track to catch up with the proposed timeline.

Changes that had a significant impact on expenditures

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

N/A

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Chheda, P., Spies, M, and Spies, M.A., Small-Molecule Effectors of DNA Repair Proteins: Applications for Development of Cancer Therapeutics and Research. Burger's Medicinal Chemistry, Drug Discovery and Development, 8th edition. 2020 (in print)

Journal publications.

Kondratick, C.M., Washington, M.T., and Spies, M, *Making choices: DNA replication fork recovery mechanisms*. Seminars in Cell & Developmental Biology. 2020 [in print; https://doi.org/10.1016/j.semcdb.2020.10.001],

Caldwell, C.C., and Spies, M‡, *Dynamic Elements of Replication Protein A at the Crossroads of DNA Replication, Recombination, and Repair*. Critical Reviews in Biochemistry & Molecular Biology. 2020 Oct;55(5):482-507. [PubMed PMID: 32856505; PubMed Central PMCID: PMC7821911],

Books or other non-periodical, one-time publications.

Nothing to report

Other publications, conference papers and presentations.

Nothing to report

- **Website(s) or other Internet site(s)**

Nothing to report

- **Technologies or techniques**

Nothing to report

- **Inventions, patent applications, and/or licenses**

Nothing to report

- **Other Products**

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Maria Spies
Project Role:	Principal Investigator
Research Identifier (e.g. ORCID ID):	0000-0002-7375-8037
Nearest person month worked:	1
Contribution to Project:	Dr. Spies has coordinated work outlined in Specific Aim 2, Major Task 4
Funding Support:	No change.
Name:	Masayoshi Honda
Project Role:	Assistant Research Scientist
Research Identifier (e.g. ORCID ID):	0000-0002-9817-3908
Nearest person month worked:	8
Contribution to Project:	Dr. Honda has performed work outlined in Specific Aim 2, Major Task 4
Funding Support:	No change.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

No

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: This report reflects the work and progress of Maria Spies, PI.

QUAD CHARTS: N/A

9. APPENDICES: None