The Wistar Institute of Anatomy and Biology

Annual Progress Report: 2010 Formula Grant

Reporting Period

January 1, 2011 – June 30, 2011

Formula Grant Overview

The Wistar Institute of Anatomy and Biology received $1,582,665 in formula funds for the grant award period January 1, 2011 through June 30, 2012. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Isoform Specific p73 Regulatory Networks in Neurogenesis - This systems biology project will unravel the isoform specific regulatory networks of *TP73* (tumor protein p73) gene involving complex cross-talk with other p53 family members and other transcription factors which control the expression of p73 target genes in normal neuronal development and in perturbed cellular conditions. This problem will be addressed by integrative computational modeling and NextGen sequencing-based experimental approaches using *in vitro* neuronal differentiation of P19 cells (embryonal carcinoma) treated with retinoic acid. This work will not only pave a new way to think about how to approach the problem of gene regulatory networks, but will also lead the research effort to understand the molecular mechanisms of cancer and other developmental disorders.

Anticipated Duration of Project

1/1/2011 - 6/30/2012

Project Overview

In recent years, the notion of "one gene –> one protein –> one functional pathway" in mammalian cells has been shown to be overly simplistic. In fact, numerous genes displaying complex transcriptional regulation, involving cross-talk of multiple transcription factors (TFs) regulating multiple processes, have been known for some time. Recent evidence suggests that more than 50% of the human genes, including many developmentally regulated TFs, produce two or more protein isoforms. The p73 gene, tumor protein p53 family member, is one such gene that produces either full-length (TAp73) or N-terminally shorter (DNp73) isoforms. As integral components of complex gene regulatory networks, involving other p53 family members and different TFs, the different p73 isoforms interfere with major tumor suppressor and oncogenic pathways that either prevent or facilitate the generation of genetically and epigenetically altered cells, making isoform-specific gene regulation by p73 fundamentally important in normal development, tumor initiation and progression. However, the exact mechanism by which they

contribute to the human disease etiology remains an open question. It is apparent that, to answer this question, it is necessary to understand the role of all isoforms in the interaction network, which members of p53 protein family create**.** *Therefore, the actual challenges are in the deciphering of the molecular mechanisms by which p73 function in opposing roles, and unravel the isoform-specific regulatory networks involving complex cross-talk with other p53 family members and other TFs during normal development and disease conditions.* This challenging problem will be addressed by a systems biology approach, involving innovative computational modeling approaches and use of NextGen sequencing technologies, in *in vitro* neuronal differentiation of P19 cells (embryonal carcinoma) treated with retinoic acid. We will (1) conduct ChIP-seq (to derive direct target genes of TAp73 & DNp73) and mRNA-seq (to find the overall transcriptional outcome) experiments in differentiated cells at days 0, 4, and 7 after treating the P19 cells with retinoic acid, in a) wild-type P19 cells, b) TAp73-knock down P19 cells, and c) DNp73-knock down P19 neuronal cells; and (2) develop novel computational methods to model the combinatorial interaction of p73 isoforms (TAp73 and DNp73) with p53 family members and other TFs and decipher isoform regulatory networks of each isoform in controlling the expression of target genes during neuronal differentiation under normal and perturbed cellular conditions.

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Expected Research Outcomes and Benefits

The successful completion of this project will answer the following questions – What are the direct target genes of TAp73, when DNp73 is present and when DNp73 is lost? What are the direct target genes of DNp73, when TAp73 is present and when TAp73 is lost? What are the TF partners that co-operate with these specific isoforms in regulating different sets of target genes leading to opposing biological activities (e.g., pro-apoptosis versus anti-apoptotic) forming the core of the transcriptional regulatory network in neuronal development? The answers to these critical questions will lead to the understanding of the isoform-specific TF networks of p53 family members. This is clearly central if we are to develop rational and mechanistic therapies for complex genetic diseases that arise from the accumulated contributions of many protein–protein interactions and environmental factors. While TFs are difficult drug targets in general, knowledge of de-regulation of TF isoforms linked to their upstream triggers, such as signaling cascades, will guide both development of targeted therapeutics and personalization of treatment to an individual's particular malignant phenotype.

Summary of Research Completed

In vitro neuronal differentiation of P19 cells (embryonal carcinoma) treated with retinoic acid.

We performed *in vitro* neuronal differentiation of P19 cells treated with retinoic acid as summarized in Figure 1A. As a proof of principle, to follow the neuronal differentiation, we measured the expression of ES cell marker (Oct3/4), neuronal precursor cell marker (Nestin), and neuronal marker (Map2) during the course of nine days differentiation (Figure 1B). As expected, we observed a drop in Oct3/4 expression and an increase in Nestin expression from days 0 to 4 and a highly induced expression of Map2 by days 6 to 9. When we looked at the expression of the p73 isoforms, we found opposing behavior (Figure 1B). As cells are subjected to the differentiation program, there is a marked decrease in DNp73 expression accompanied by an increased expression of TAp73 from day 0 to 4 followed by a decline by day 9.

Strikingly, the protein expression of TAp73 increases during differentiation from day 0 to 9, although mRNA expression tends to decrease between days 4 to 9; and for DNp73, despite the rapid loss of its mRNA, no change is observed in its protein levels until day 9 during neuronal differentiation (Figure 2A). These results indicate that p73 gene expression is also regulated through translation and protein stability. Furthermore, in agreement with previous studies, we found that TAp73 is present in both nucleus and cytoplasm, while DNp73 is a nuclear protein.

ChIP-seq & RNA-seq results suggest isoform-specific gene regulation by TAp73 and DNp73.

Recent studies using neural stem cells from wild type (WT) and p73-/- mice indicate that both TAp73 and DNp73 are expressed in neural stem cells and that they are involved in both embryonic and adult neurogenesis, a process involving differentiation of neural stem cells to mature postmitotic neurons. <u>Having established that p73 isoforms are differentially expressed during differentiation of neurons, we hypothesized that TAp73 and DNp73 regulate distinct genes by interacting with different co-regulatory proteins/complexes during neurogenesis</u>. To identify the target genes of each isoform, we decided to conduct ChIP-seq experiments using commercially available isoform-specific antibodies (Imgenex Inc.). We first tested the specificity of the antibodies by Western blotting experiments on over-expressed flag-tagged TAp73 or DNp73 protein in 293T cells and found that the antibodies do not cross-react (Figure 2B). Additionally, we performed immunoprecipitation followed by Western blotting experiments, which confirmed that the antibodies are effective for immunoprecipitating the p73 protein isoforms (Figure 2C).

Next, we performed ChIP-seq experiments with the tested isoform-specific antibodies on cross-linked chromatin from undifferentiated P19 cells (day 0). Following sequencing and bioinformatics data analysis, we identified approximately 151,844 and 122,900 significant ChIP-seq peaks (p-value ≤ 0.0001) for TAp73 and DNp73, respectively; and it was found that TAp73 and DNp73 were recruited to the 5' proximal region (-5Kb to + 1Kb around transcription start site (TSS)) of 21,316 and 19,153 gene promoters, respectively (Figure 3).

We found that, for some genes like *Timeless*, the two isoforms are regulating the expression of distinct promoter-driven mRNA transcripts of the same gene (Figure 4A), while on the other hand TAp73 and DNp73 recruitment is mutually exclusive on promoters of *Spp1* and *NeuroD2* (Figure 4A). We also observed co-localization of both isoforms on some promoters, such as the downstream promoter of *RTN4* (Figure 4A). It has been suggested that DNp73 can heterodimerize with TAp73 and function as a dominant negative protein, and we speculate that this contributes to the presence of both isoforms on specific gene promoters.

Further analysis on TAp73 and DNp73 binding on -1Kb to +1Kb region of TSS has revealed that the recruitment of both isoforms is mostly mutually exclusive with 7759 and 6750 promoters being TAp73 and DNp73 specific, respectively, while both isoforms co-exist on 1600 promoters, thus identifying distinct pathways that are regulated by the p73 isoforms (Figure 4B). Our results show that, while TAp73 regulates members of mTOR, axonal guidance, synaptic long term potentiation, type II diabetes mellitus, eIF4 and p70S6K signaling pathways, DNp73 regulates FAK, Reelin, Rac, Integrin, and PAK signaling. <u>The identification of p73 isoform-specific target promoters provides a strong basis for our hypothesis that each isoform interacts with different co-regulatory complex to regulate target gene expression</u>.

To identify the transcriptional outcome/consequences of p73 isoform recruitment, we have performed mRNA-seq on undifferentiated P19 (day 0) and specified P19 cells (day 4). We observed that 16,557 and 17,462 genes are expressed in day 0 and retinoic acid treated day 4 P19 cells through 21,481 and 22,655 promoters that transcribe 31,938 and 33,272 transcripts, respectively. We have observed that some promoters targeted by TAp73 are highly expressed, while others targeted by DNp73 are repressed (shown in Figure 4A). We also found that the expression of 11,038 and 7,832 transcripts are up and down regulated by day 4 of neurodifferentiation.

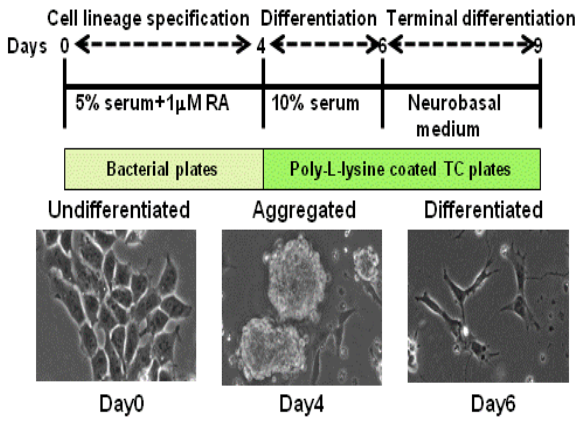


Figure 1A: In vitro differentiation of P19 cells to neurons.

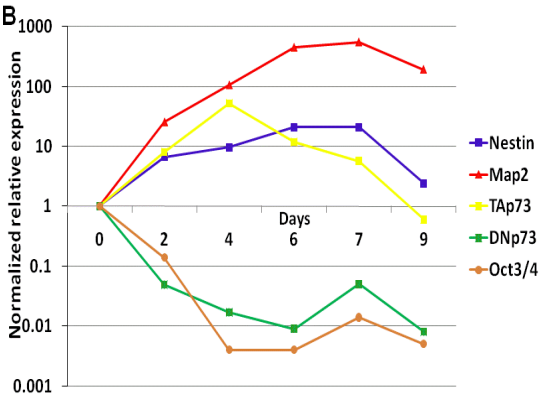
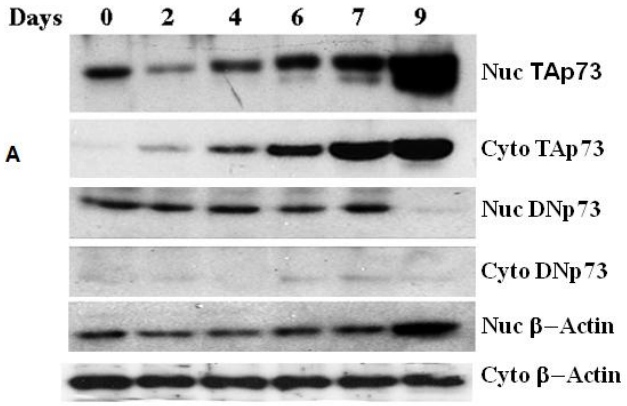
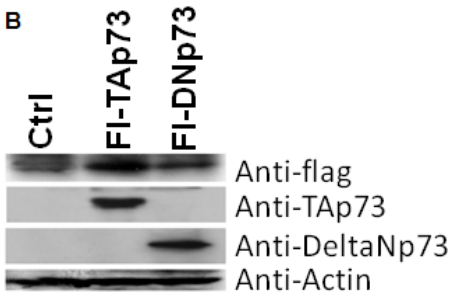


Figure 1B: mRNA expression of ES, Neuronal precursor and Neuron cell markers, along with TAp73 and DDNp73 during P19 differentiation





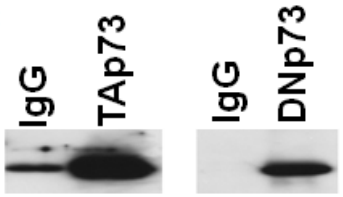


Figure 2A: TAp73 and DNp73 protein expression in nuclear and cytosolic fraction of P19 cells at different stages of differentiation.

Figure 2B: 293T cells were transfected with plasmids expression flag tagged either TAp73 or DNp73 for 48 hours before cell extracts were prepared and analyzed by Western blotting with indicated antibodies.

Figure 2C: P19 nuclear extracts were subjected to immunoprecipitation with either TAp73 or DNp73 antibodies before detection by Western blotting.

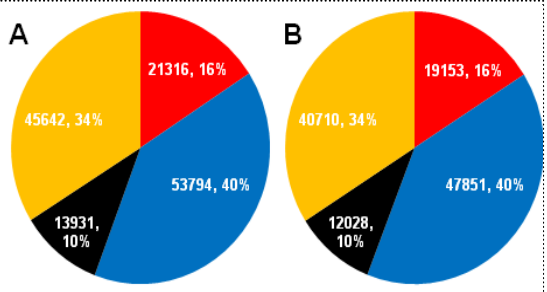


Figure 3: The distribution of genome wide TAp73 (A) and DNp73 (B) binding in TSS proximal region (-5Kb to +1Kb in red), TSS distal (-5Kb to -25Kb in black), intragenic (blue) and intergenic (yellow).

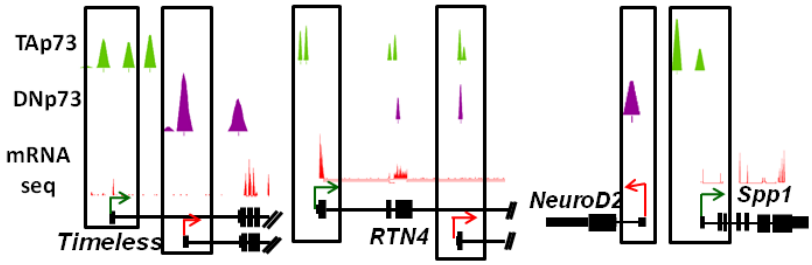


Figure 4A: Genomic binding profile of TAp73 (green) and DNp73 (purple),and mRNA-seq expression profile (red) on the promoters of *Timeless*, *RTN4*, *NeuroD2* and *Spp1* in undifferentiated day 0, P19 cells.

Figure 4B: Venn diagram shows the unique and overlapping target promoters of TAp73 and DNp73.

Research Project 2: Project Title and Purpose

SECTM1 is a Novel Mediator of Melanoma Tumorigenesis and Progression - Interaction of melanoma cells and melanoma-associated macrophages (MAMs) plays a critical role in melanoma development. However, the molecular mechanism by which this interaction contributes to melanoma progression is poorly defined. We have identified a novel factor, SECTM1, in the tumor microenvironment that can recruit and interact with MAMs. We have evidence that melanoma cells frequently express SECTM1, causing them to be more invasive and to migrate to distant sites more readily in experimental systems. In this project, we will try to determine the manner in which overexpression of SECTM1 causes melanoma invasive phenotype and the significance of SECTM1 on recruitment of macrophage associated with melanoma. This study will provide insight into the possibility that SECTM1 may be a potential new therapeutic target for melanoma therapy.

Anticipated Duration of Project

1/1/2011 - 6/30/2012

Project Overview

Progression from the earliest stages of transformation to malignant metastatic melanoma requires interaction with the tumor microenvironment. Melanoma-associated macrophages (MAMs) constitute a major component of the melanoma microenvironment, and promote melanoma progression while exerting immune suppressive functions. They also increase melanoma tumor cell migration, invasion, metastasis and angiogenesis by secreting cytokines, chemokines, angiogenesis factors and proteases. However, the specific biological network and molecular mechanisms of this interaction are poorly defined. We will present evidence that a novel molecule, SECTM1, produces a tumor-prone microenvironment for melanoma development.

The SECTM1 gene encodes a 27kD type I integral membrane protein that is cleaved to produce a 20kD secreted form (sSECTM1). SECTM1 is expressed normally in a number of cell types including neutrophils, dendritic cells, activated monocytes, CD4 T cells and many epithelial cell types. SECTM1 is also highly expressed in breast, ovarian, and prostate cancers, and some myeloid leukemia cells. To further characterize the expression pattern of SECTM1, we examined a variety of malignant tissues and demonstrated that SECTM1 is highly expressed in most melanoma cell lines and melanoma tissue samples, but not melanocytes. We found that overexpression of SECTM1 significantly increases melanoma cell migration and invasion. Expression of SECTM1 also increases tumor growth and metastasis in mice. The mechanism by which SECTM1 increases melanoma invasive phenotype is still unclear. It has recently been shown that SECTM1 activates the NF-κB pathway, known to play a critical role in melanoma progression and metastasis, suggesting that activation of the NF-κB pathway might contribute to melanoma invasive phenotype induced by SECTM1. In addition, we observed that most melanoma lesions with high levels of SECTM1 expression exhibit inflammatory cell infiltration, including MAMs; and SECTM1 increases migration of MAMs, suggesting that SECTM1 may be a significant mediator of MAMs and melanoma cell interaction.

Based on these observations, we propose the following aims to understand the role of SECTM1 in melanoma tumorigenicity and progression: (1) To fully characterize the interaction of SECTM1 on the cells of the melanoma microenvironment, with a special focus on MAMs. This will include a determination of the specific mechanism for interaction of SECTM1 and MAMs; and (2) To determine if recruitment and activation of MAMs by SECTM1 specifically contributes to melanoma cell growth and metastasis.

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Expected Research Outcomes and Benefits

Melanoma is an aggressive tumor with no effective therapies. It is well established that the interaction of melanoma with its microenvironment plays critical roles in melanoma progression. Melanoma-associated macrophages are reported to be an essential component of the melanoma microenvironment. Clinically, infiltration of macrophages correlates to the poor prognosis in melanoma patients. Moreover, it has been shown that targeting macrophages increases efficacy of chemotherapy or biotherapy, suggesting that a combination of targeting melanoma and macrophages holds promise as a new strategy for melanoma therapy.

We have evidence that SECTM1 affects tumor growth and migration and thus might be a novel target for melanoma therapy, based on the following observations. First, SECTM1 increases melanoma cell and macrophage migration and invasion. Second, although interferon alpha is one of the few FDA approved agents for metastatic melanoma, it is effective in only a small number of patients and it also induces SECTM1. SECTM1 is also induced by bortezomib, a proteasome inhibitor, which is in Phase II clinical trials for metastatic melanoma. Thus it is essential to determine if the induction of SECTM1 by these therapies limits their effectiveness. This provides a rationale to combine blockade of SECTM1 with interferon alpha and bortezomib for melanoma therapy. This project will determine how SECTM1 affects melanoma progression and functions of MAMs and will attempt to determine if inhibition of SECTM1 can be used as a target for melanoma therapy.

Summary of Research Completed

In previous studies we isolated a gene, SECTM1, that encodes a transmembrane molecule expressed on many cell types, which upon cleavage of the extracellular domain produces a 20 kDa soluble protein that is the ligand for a receptor that is expressed on T lymphocytes and NK cells. Furthermore, we have shown that this soluble protein acts as a T cell co-stimulatory molecule that activates T cells. The retained part of SECTM1 following cleavage activates the NFkappaB signaling pathway and produces a myriad of biological effects on the SECTM1 expressing cell. Our most recent studies suggest that overexpression of SECTM1 has the ability to induce a metastatic phenotype to certain melanoma cells. This is more intriguing because SECTM1 gene is induced by interferons, also used in the treatment of melanoma, but with weak results.

More recently, we have been studying the effect of SECTM1 in the tumor microenvironment and have found other cells, monocytes, that previously were not known to express the receptor for SECTM1. Our results also suggest that SECTM1 can help monocytes mature to macrophage, one of the most important tumor promoting cells in the tumor microenvironment. The studies reported here are an attempt to understand the significance of the interaction of SECTM1 in the tumor microenvironment on both tumor cells and melanoma-associated macrophages (MAMs).

Specific Aim 1. To determine the significance of SECTM1 on MAMs in the melanoma microenvironment.

a. Determine the mechanism for the interaction of SECTM1 with MAMs.

Rationale: The expression of SECTM1 in melanoma is highest in melanomas that have high macrophage numbers. The exposure of monocytes to conditioned media enriched for SECTM1 significantly increased the expression of macrophage markers. This suggested that MAMs may express the SECTM1 receptor, CD7. In our preliminary studies, we were not able to demonstrate the expression of CD7 on monocytes by flow cytometry, but CD7 mRNA could be detected by PCR.

Original Overall Design of Studies: We planned to examine the expression of CD7 on MAMs and monocytes using flow cytometry analysis with multiple antibodies to CD7. Numerous reports demonstrate different sensitivities of the available monoclonal antibodies to CD7. If we confirmed that CD7 is expressed on MAMs, we would further stain for CD7 and CD68, a macrophage marker, in melanoma lesions from different stages of melanoma patients to determine the number of CD7 expressing macrophage.

Results:

CD7 is expressed on melanoma-associated macrophages (MAMs) and dependent on M-CSF. Monocytes were cultured in the presence of melanoma tumor conditional medium (CM) with or without M-CSF for seven days. Expression of CD7 and macrophage marker CD68 was determined by flow cytometry (Figure 1). As hypothesized, CD7 is expressed on M-CSF (10 ng/ml) differentiated macrophages (Figure 1, left panel), and MAMs (Figure 1, middle panel). The induction of CD7 expression in MAMs was decreased in cells that were incubated in conditioned media containing anti-M-CSF (10 μg/ml). Our data demonstrated that CD7 is induced in tumor-associated macrophages by conditioned media, and expression of CD7 is dependent on M-CSF. It is possible that SECTM1, which is also a component of conditioned media, might help differentiate monocytes to macrophages.

SECTM1 promotes inflammatory cell infiltration in melanoma lesions by increasing monocyte differentiation to MAMs. By tissue staining for SECTM1, we observed a considerable increase in SECTM1 expression at sites where melanoma tumors had been infiltrated by immune cells. 64.5% of the 172 melanoma samples with inflammatory infiltration showed significant SECTM1 signals (Figure 2A). Supporting this, clinically normal dermal nevi with little or no inflammatory cell infiltration revealed particularly low SECTM1 expression (92% negative - data not shown), while compound nevi which have high levels of inflammatory cell infiltration express SECTM1 (38% - data not shown). This expression pattern, together with our finding that LPS and interferon-γ induce expression of SECTM1 in monocytes, suggests that SECTM1 secreted by macrophages or tumor cells may have either direct or indirect effects on melanoma progression. Therefore, we tested the ability of SECTM1 to induce the differentiation of monocytes to macrophages. Monocytes from healthy donors were cultured for seven days with melanoma cell conditioned medium from cells that have no expression of SECTM1 or have enhanced, forced expression of SECTM1. Conditioned media containing enhanced amounts of SECTM1

significantly increased the expression of macrophage markers, including CD68 and Anti-human Mature Macrophage Marker (Mφ marker) as determined by flow cytometry analysis (Figure 2B). Furthermore, SECTM1 also increased expression of SECTM1 in macrophages (Figure 2C), indicating a positive feedback on SECTM1 expression.

b. Determine the effect of SECTM1 on MAMs.

Rationale: Since SECTM1 is highly expressed in melanoma lesions by histochemistry and SECTM1 increases differentiation of monocytes to macrophages, we propose that SECTM1 may have a profound effect on MAMs in the tumor microenvironment by both recruiting and differentiating monocytes to macrophage. In particular, it would be very important if SECTM1 could recruit MAMs to melanoma lesions and further promote melanoma progression and metastasis. Therefore, we sought to determine if SECTM1 increases MAMs migration.

Initial Experimental Design and Methods: We first tested whether SECTM1 increases MAMs migration using a transwell assay. We used melanoma-conditioned medium from the melanoma cell line WM3899 and the same cell line with forced overexpression of SECTM1 (SECTM1/WM3899) as chemoattractant. We then seeded MAMs into the upper chamber of the transwell. After six hours, the migrated MAMs were counted. In order to verify whether SECTM1-induced MAMs migration is dependent on CD7, we first added blocking antibody to the MAMs to test whether blockade of receptors of SECTM1 inhibits SECTM1-induced macrophage migration. In studies being planned, we will perform knockdown expression of CD7 on MAMs using siRNA, then conduct macrophage migration assays.

Results:

SECTM1 increases migration of tumor-associated macrophages. WM3899 control and SECTM1/WM3899 cells were cultured in the presence of serum-free medium for three days, producing conditioned media. Conditioned medium was harvested and added in the lower chamber of the transwell. MAMs were added in the upper chamber. After 24 hours incubation, the migrated cells were counted with microscopy. As shown in Figure 3, more MAMs migrated to SECTM1 conditioned media than those of control medium. Furthermore, this effect can be blocked by anti-CD7 antibody (Figure 3).

c**.** *Determine the role of SECTM1 in promoting interactions between MAMs and melanoma cells using a 3D skin reconstruct model.*

Rationale**:** Soluble SECTM1 (sSECTM1) from SECTM1/WM3899 culture medium enhances differentiation of monocytes. Furthermore, stimulation of monocytes by sSECTM1 results in a significant increase in the expression of SECTM1 on monocytes, creating a positive feedback effect and thus amplifying the effect of SECTM1 on macrophages, as well as potentially producing an effect on melanoma cells. Restating this, SECTM1 production by either tumor or macrophages increases SECTM1 production by the other cell and itself. Current models studying melanoma and MAMs in 2D culture systems may not fully reflect the physiological conditions of the melanoma microenvironment. The 3D skin reconstruct model, developed in the laboratory of our close collaborator Dr. Meenhard Herlyn, The Wistar Institute, consists of epidermal

keratinocytes, melanoma cells and fibroblasts. Importantly, the skin reconstruct model can be implanted into (NOD/SCID/IL2rγnull, NSG) mice, providing a useful model for further analysis of melanoma biology *in vivo*. Our preliminary experiments demonstrated that human macrophages can survive in 3D culture conditions, making it possible to include macrophages in 3D skin reconstructs, thus facilitating the study of the interaction of MAMs with melanoma cells.

Design and Methods**:** We will use this model to study the role of SECTM1 on melanoma growth, migration and apoptosis, as well as monocyte differentiation. Specifically, we will compare 3D tumors composed of malignant cells with either low or high (overexpression) of SECTM1 by staining for Ki67 (growth), caspase-3 staining (apoptosis), and by quantitation of migration of melanoma cells into the dermis. MAMs will be added to the cells constituting the 3D tumors; and we will determine whether SECTM1 increases monocyte differentiation by immunohistochemistry staining for macrophage marker CD68, late stage macrophage marker, CD163, and SECTM1. Finally, we will use an anti-human SECTM1 monoclonal antibody to investigate whether blockade of sSECTM1 impacts these patterns of cellular functions.

Results:

We are still developing this model. We tried this assay once using 10% fetal bovine serum RPMI1640 medium (R10 medium). We found that, after seven days of incubation, no macrophages were detected in the 3D skin reconstruct system. We interpret this to indicate that all the cells died during incubation. We will modify this assay by mixing tumor conditioned medium with R10 medium, and test the role of SECTM1 in promoting interactions between MAMs and melanoma cells. Since this is a new model system, it is possible that macrophage viability is not possible to reach our endpoint.

Specific Aim 2. To determine the effect of SECTM1 induced macrophage in the tumor microenvironment.

a. *We will determine the biological effect of SECTM1 induced macrophages on melanoma progression and invasion.*

Rationale: Many laboratories have shown that melanoma-associated macrophages promote melanoma progression and invasion by secretion of chemokines or cytokines, including IL-6, IL-8, TNF-α, CCL-2, SDF-1 and EGF. Considering that SECTM1 differentiates monocytes to MAMs, it is thus also possible that SECTM1 indirectly increases melanoma cell migration and invasion via increasing the expression of cytokines and chemokines.

Design: Monocytes will be cultured in melanoma culture medium with or without SECTM1 for three and seven days. Cultured medium will be harvested. The expression of IL-6, IL-8, CCL-2, SDF-1 and EGF will be determined by enzyme-linked immunosorbent assay. If we determine which cytokine increases after SECTM1 stimulation, we will test whether blockade of this cytokine will affect melanoma cell migration and invasion assay using a transwell assay. In addition, we will also test whether melanoma conditioned medium with SECTM1 in the presence of anti-human SECTM1 antibody can inhibit this cytokine expression. If none of these cytokines and chemokines is changed with SECTM1 stimulation, we will use either a cytokine array panel

to detect the secretion of multiple cytokines and chemokines or we may extract RNA from macrophages stimulated with or without SECTM1 and use microarray to further analyze the expression of factors which may increase melanoma migration and invasion. We will verify cytokine array and microarray data by ELISA and real-time PCR, respectively. We will further conduct experiments to analyze this factor on melanoma cell migration and invasion.

Results: These studies are very preliminary. We tested conditioned-medium from one healthy donor. Results show that SECTM1 stimulated macrophages secrete more cytokines and chemokines compared to the control medium stimulated macrophages. We will test more samples to verify this result.

b. Determine whether expression of SECTM1 in melanoma can recruit MAMs into tumors in vivo using a B16 mouse melanoma model, and if it leads to melanoma progression.

Rationale: Since most melanoma lesions with high levels of SECTM1 expression exhibit inflammatory cell infiltration, including macrophages, we will perform tests to determine whether we can mimic this effect in a mouse system. We will use a B16 mouse melanoma model, which has been extensively used for studying the interaction of melanoma and its microenvironment, including macrophages.

Design: We will transfect mouse SECTM1 and control plasmid into B16 mouse melanoma cells, and enrich the SECTM1 positive B16 cell (SECTM1/B16) by flow cytometry cell sorting. One million SECTM1/B16 and control B16 melanoma cells will be subcutaneously inoculated on the back of the C57BL/6 mice for the tumor growth model. Tumor growth will be measured for two to three weeks. Tumor tissues will be harvested for immunohistochemistry analysis of macrophage infiltration. We will inject SECTM1/B16 and control B16 cells via tail vein for the lung metastasis model. We will euthanize mice by CO_2 asphyxiation on days 16 to 18 after tumor inoculation. Lungs will be harvested and photographed after three weeks. Lungs will be fixed in formalin and embedded in paraffin. Standard H&E staining will be conducted at the Wistar Histology shared facility. Immunohistochemistry analysis of macrophage infiltration to the tumor tissue and metastasis of lung will be conducted. The percent of lung metastasis areas will be analyzed using Imagine Pro software.

Results**:** We are still designing this experiment and preparing reagents.

Summary.

Our studies further support the hypotheses that some melanoma tumors overexpress the transmembrane protein SECTM1, and that production of the soluble form (sSECTM1) can induce monocyte migration and differentiation to macrophage. These macrophages also are induced to express SECTM1. The induced macrophage can then release inflammatory cytokines that have the potential to promote transition to EMT and metastasis.

Ongoing studies are designed to further test this hypothesis, and we will continue to follow the original experimental plan since all of our data remain consistent with the original hypothesis of the proposed research project.

Figure 1

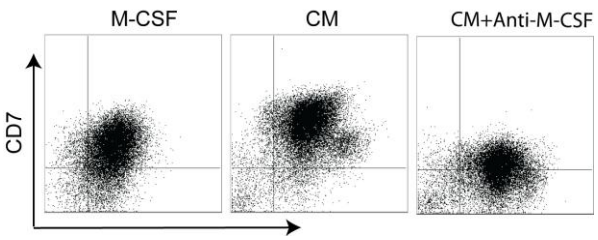


Figure 2

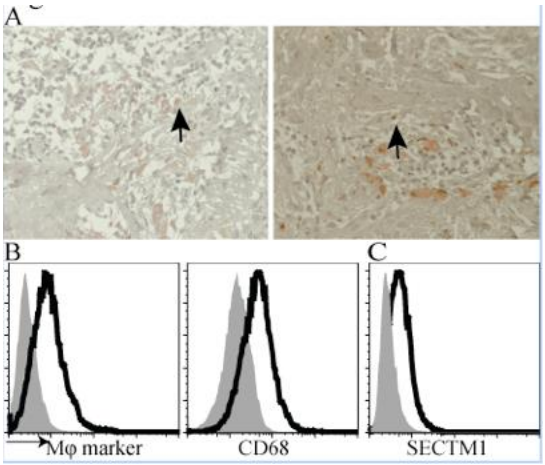
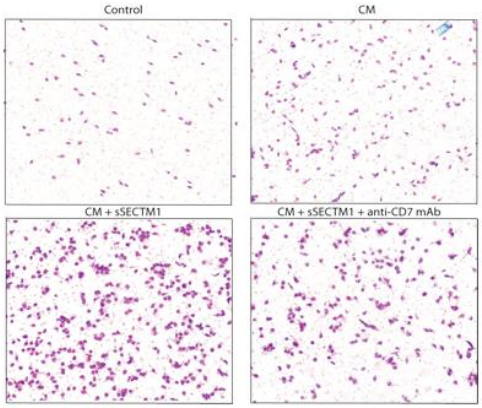


Figure 3



Research Project 3: Project Title and Purpose

Regulation of EBV Infection and Latency by Editing of Viral MicroRNAs - Primary transcripts of certain microRNA (miRNA) genes (pri-miRNAs) are subject to one type of RNA editing that converts adenosine residues into inosine. Editing of pri-miRNAs controls synthesis and function of miRNAs. Epstein-Barr virus (EBV) infects more than 90% of the world's population. EBV causes a variety of human cancers such as Burkitt's lymphoma, Hodgkin's disease, and nasopharyngeal carcinoma. EBV genome encodes multiple miRNA genes of its own. Primary transcripts of ebv-miR-BART6 (pri-miR-BART6) are edited in latently EBV-infected cells. The significance of pri-miR-BART6 RNA editing in viral latency and lytic replication will be investigated. Information obtained through this research project is essential for the future development of a new intervention for prevention and treatment of human diseases caused by EBV.

Anticipated Duration of Project

1/1/2011 - 6/30/2012

Project Overview

The broad, long-term goal of this project is to increase understanding of the mechanism that regulates Epstein-Barr virus (EBV) infection by editing of miRNAs. One type of RNA editing

involves the conversion of adenosine residues into inosine (A-to-I editing) through the action of ADAR (adenosine deaminase acting on RNA). Primary transcripts of certain miRNA genes (pri-miRNAs) are subject to this A-to-I RNA editing. Editing of pri-miRNAs modulates expression and function of miRNAs or expression of edited mature miRNAs and silencing of a set of genes different from those targeted by the unedited miRNAs. We found that primary transcripts of EBV genome-encoded miR-BART6 (pri-miR-BART6) are edited by ADAR1 in latently EBV-infected cells. Editing of a mutation-containing pri-miR-BART6 found in Daudi Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC) C666-1 cell lines suppressed processing of miR-BART6 RNAs. Mutation and A-to-I editing appear to be adaptive mechanisms that antagonize miR-BART6 activities. Most importantly, miR-BART6-5p RNAs are predicted to silence Dicer through multiple target sites located in the 3'UTR of Dicer mRNA. In this project, functions of miR-BART6 RNAs and the role played by A-to-I RNA editing in EBV infection and latency will be investigated. Two specific aims are sought:

Specific Aim 1. Determine the significance of pri-miR-BART6 RNA editing in viral latency and lytic replication. Our preliminary studies of *in vitro* miRNA processing analysis demonstrated that A-to-I editing of pri-miR-BART6 inhibits processing of pri-miR-BART6 and loading of miR-BART6-5p onto miRISC. We will investigate the *in vivo* significance of pri-miR-BART6 RNA editing in EBV-positive BL and NPC cells in different stages of latency by over expressing or knocking down ADAR1. Changes in latency state and activation of lytic replication will be examined.

Specific Aim 2. Determine the in vivo function of miR-BART6 RNAs in viral latency and lytic replication. Our preliminary results indicate that miR-BART6-5p RNAs are likely to target human Dicer mRNAs. We will determine the significance of Dicer overexpression or silencing in viral latency and lytic infection. We will examine whether Dicer knockdown would affect the state of latency and/or affect lytic replication of EBV. Furthermore, we will examine whether lytic replication is also initiated by manipulating miR-BART6-5p and Dicer levels.

Principal Investigator

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

This project is highly relevant to human diseases caused by EBV, and focuses on a previously unexplored paradigm: control of EBV infection and latency through miR-BART6 viral miRNA and its A-to-I RNA editing. However, this research area has never been investigated before. This project, based on a previously unexplored paradigm, will give a challenging thrust to EBV research. The outcome of the experiments planned in this project will have a large impact on the

understanding of EBV infection and latency. Information obtained through this research may lead to a new miRNA-based intervention for the prevention or therapeutic treatment of EBV-mediated diseases such as Burkitt's lymphoma, Hodgkin's disease, and nasopharyngeal carcinoma.

Summary of Research Completed

Overview

During the current reporting period, we completed our first milestone: Specific Aim 1, determine the significance of pri-miR-BART6 Ribonucleic Acid (RNA) editing in viral latency and lytic replication. Our preliminary studies of *in vitro* miRNA processing analysis demonstrated that A-to-I editing of pri-miR-BART6 inhibits processing of pri-miR-BART6 and loading of miR-BART6-5p onto miRISC. Furthermore, we have shown that miR-BART6-5p RNAs target Dicer. Therefore, we investigated the *in vivo* significance of pri-miR-BART6 RNA editing and functions of miR-BART6 RNAs in EBV-positive BL and NPC cells in different stages of latency. We found that miR-BART6-5p RNAs suppress the expression of Zta and Rta required for activation of lytic replication as well as EBNA2 oncogene required for transition to type III latency. Our findings revealed the important roles played by miR-BART6-5p RNAs in viral replication and latency. Finally, A-to-I editing of pri-miR-BART6 appears to be an adaptive mechanism to antagonize miR-BART6 activities.

Results

Modulation of the Epstein Barr Virus (EBV) latency state by miR-BART6-5p RNAs

We investigated the possibility that Dicer silencing by miR-BART6-5p RNAs may control the EBV infection state. We first examined the relative expression levels of miR-BART6-5p strand RNAs and Dicer among GM607, Daudi, and C666-1 cells by qRT-PCR. Much higher levels of miR-BART6-5p were detected in C666-1 cells, which have much less editing than GM607 and Daudi cells (Fig. 1A). The low levels of miR-BART6-5p in GM607 and Daudi cells are consistent with the high editing rate of pri-miR-BART6 RNAs in these cells, which affects their processing, miRISC formation, and consequently the levels of mature miR-BART6-5p RNA. As expected, Dicer levels were lowest in C666-1 cells, in inverse relation to the miR-BART6-5p levels (Fig. 1B). Accordingly, we decided to explore the significance of Dicer repression by miR-BART6-5p RNAs in C666-1 cells.

We first attempted to antagonize the miR-BART6-5p RNAs expressed in C666-1 cells by transfection of a miR-BART6-5p antagomir. As expected, the miR-BART6-5p antagomir substantially decreased the miR-BART6-5p level (~20-fold) with a concomitant increase in the Dicer levels (~2-fold), indicating that miR-BART6-5p RNAs constantly suppress and maintain Dicer at the reduced levels in C666-1 cells (Fig. 2A). We then examined the relative expression levels of several genes known to be important for either lytic infection or the state of latency – EBNA1, EBNA2, LMP1, Zta, and Rta. EBNA1 is detected in type I, II, and III latency, whereas EBNA2 and LMP1 are usually detected in type III latency. EBNA2 is essential for the transformation of B lymphocytes and plays a central role in type III latency by up-regulating

promoters of all latent EBV genes. Deficiency of the EBNA2 expression is known in type I and II latency. A weak expression of LMP1 in type II latency and its deficiency in type I latency have been reported. By contrast, Zta and Rta are essential for the initiation of the lytic EBV infection cycle. By antagonizing miR-BART6-5p, Zta and Rta increased by 2- to 3-fold, indicating that miR-BART6-5p keeps these gene products under control. Furthermore, we noticed substantial upregulation of EBNA2 oncogene expression (~5-fold) and LMP1 (~2-fold) by suppression of miR-BART6-5p RNAs, whereas no effects on EBNA1 were observed (Fig. 2A).

Transition of viral promoter usage regulated by miR-BART6-5p RNAs

The activities of the three viral promoters Cp, Wp, and Qp were also monitored (Fig. 2B). Transcription from Cp and Wp is characteristic of type III latency, whereas the Qp promoter is used in EBV-infected cells undergoing type I or II latency. We used qRT-PCR primers specific for RNAs initiating at Wp, Cp, or Qp. Significant upregulation of type III latency-specific Cp and Wp promoter activities (5.4- and 11-fold, respectively) were detected in C666-1 cells transfected with miR-BART6-5p antagomir. On the other hand, Qp promoter activities associated with type I and type II latency were completely abolished; not detectable in comparison to control (Fig. 2B). Finally, we examined the effects of miR-BART6-5p RNAs on viral replication and latency in Burkitt's lymphoma Mutu I cells, which are in type I latency, in order to access the function of miR-BART6-5p in B lymphoma cells (non-NPC cell lines). We transfected Mutu I cells with miR-BART6-5p antagomir. As we observed in C666-1 cells, the antagomir effectively reduced miR-BART6-5p levels (~10-fold) and increased Dicer levels (1.9-fold). Furthermore, significant upregulation of EBNA2, LMP1, Zta, and Rta was detected (Fig. 3A). Downregulation of Qp activities and upregulation of Cp and Wp activities were also observed in Mutu I cells transfected with the miR-BART6-5p antagomir (Fig. 3B). Together, these results suggest that miR-BART6-5p RNAs maintain not only the type II latency of C666-1 cells, but also the type I latency of Mutu I cells, by suppressing lytic replication and also inhibiting transition of these cell lines to type III latency, a more immunoresponse-prone state of the viral infection cycle.

Together, we have demonstrated that suppression of miR-BART6-5p by antagomir results in activation of EBNA2, LMP1, Zta, and Rta genes, critical for transition to type III latency or lytic replication in Mutu I and C666-1 cells, which usually remain in the less immune reactive type I and type II latency, respectively. In addition, the type III latency-specific Cp and Wp promoter activities were dramatically activated by miR-BART6-5p antagomir, whereas the type I and type II latency-specific Qp promoter activities were suppressed by the antagomir. We currently have no explanation as to how these promoter activities are up- or down-regulated. Involvement of B-cell specific factors that activate the Wp promoter has been reported. On the other hand, many factors including E2F1, Rb, and LSD1 histone demethylase have been suggested to control the Cp promoter activities. Reduction of Dicer and consequent suppression of specific miRNAs that control these factors may be one possible mechanism to effect different viral promoter activities. In conclusion, our results suggest the important roles played by EBV miR-BART6 RNAs in the regulation of viral replication and latency. Naturally occurring pri-miR-BART6 mutation and editing may be an adaptive selection to counteract the miR-BART6 function.

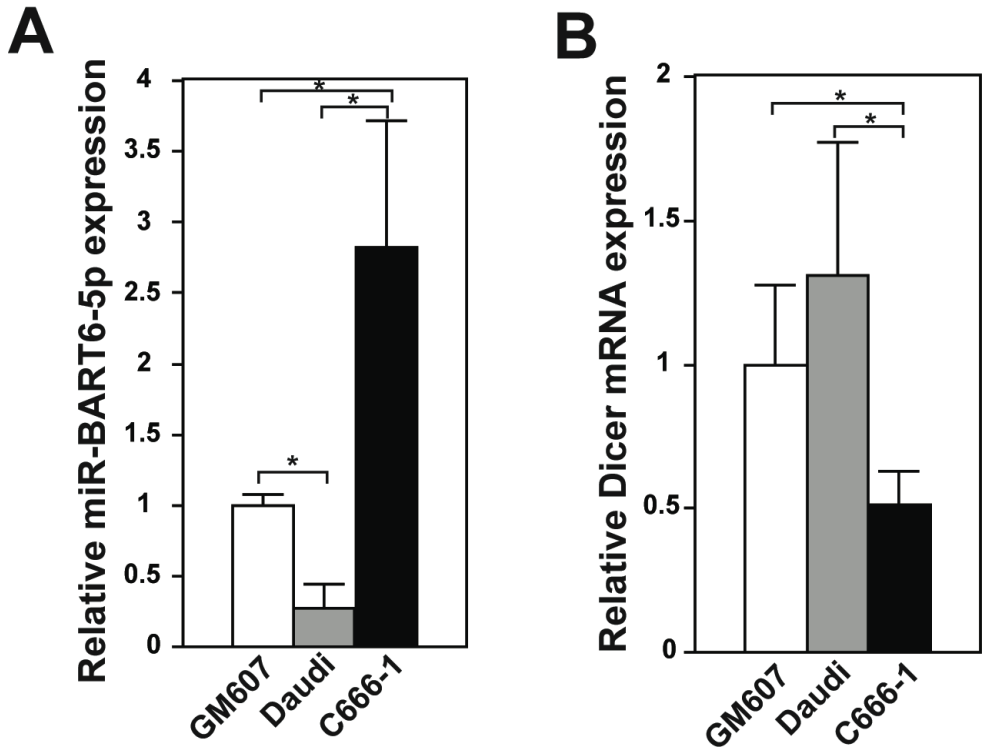

Fig. 1. Relative expression levels of miR-BART6-5p and Dicer in different cell lines. (**A**) miR-BART6-5p RNA levels were examined by qRT-PCR and normalized to β-actin mRNA level. Three independent assays were done. Mann-Whitney *U*-test, *p<0.05. Error bars, SEM (n=3). (**B**) Dicer mRNA levels were monitored by qRT-PCR and normalized to β-actin mRNA levels. Three independent assays were performed. Mann-Whitney *U*-test, *p<0.05. Error bars, SEM (n=3).

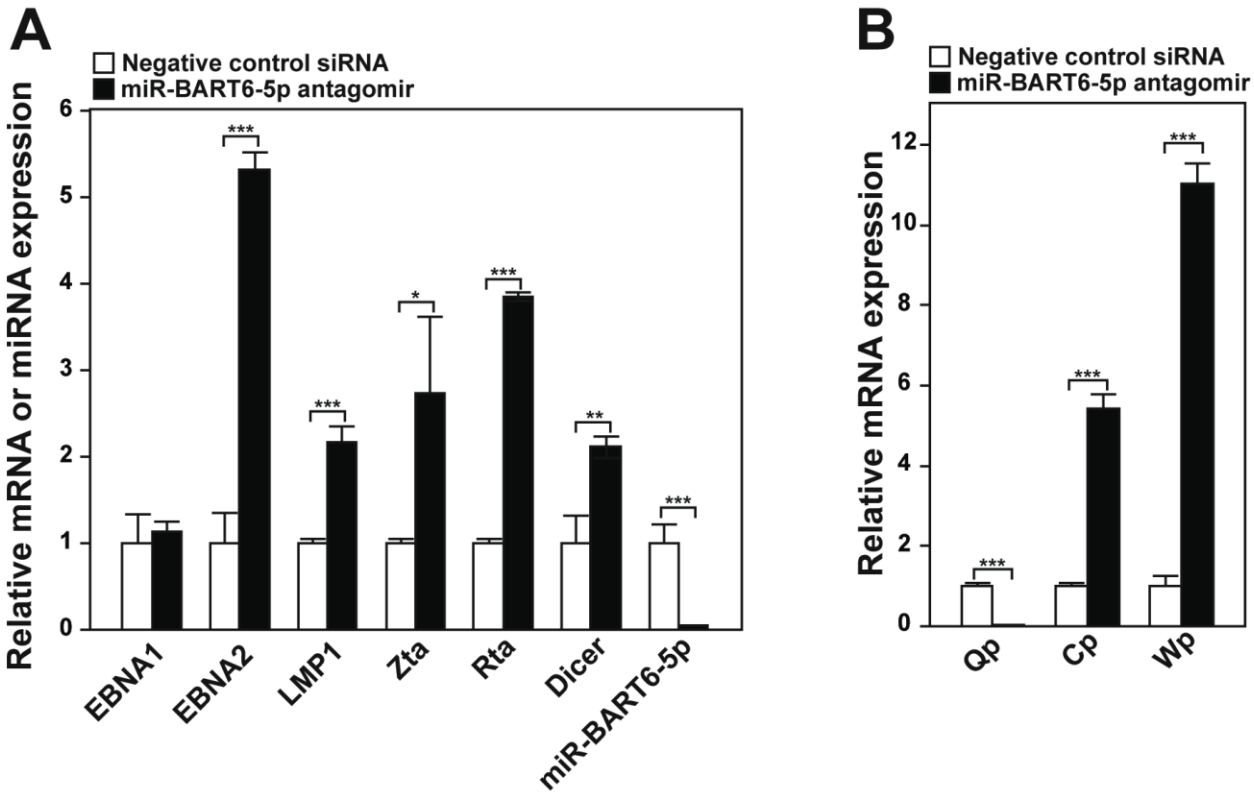

Fig. 2. Control of viral genes critical for the state of latency and lytic viral replication. **(A)** Upregulation of EBV genes critical for latency and viral replication by the miR-BART6-5p antagomir. Expression of select viral genes including miR-BART6-5p in C666-1 cells transfected with the miR-BART6-5p antagomir or control (sequence unrelated Qiagen AllStars Negative Control siRNA) was examined by qRT-PCR. Three independent assays were done. Mann-Whitney *U*-test, *p<0.01, **p<0.005, ***p<0.001. Error bars, SEM (n=3). (**B**) Changes induced by the miR-BART6-5p antagomir in the viral promoters Qp, specific for the type I latency, and Cp and Wp, specific for the type III latency. Transcripts initiated from Qp, Cp, and Wp were determined by qRT-PCR and compared to β-actin transcripts. Three independent assays were done. Mann-Whitney *U*-test, **p<0.005, ***p<0.001. Error bars, SEM (n=3).

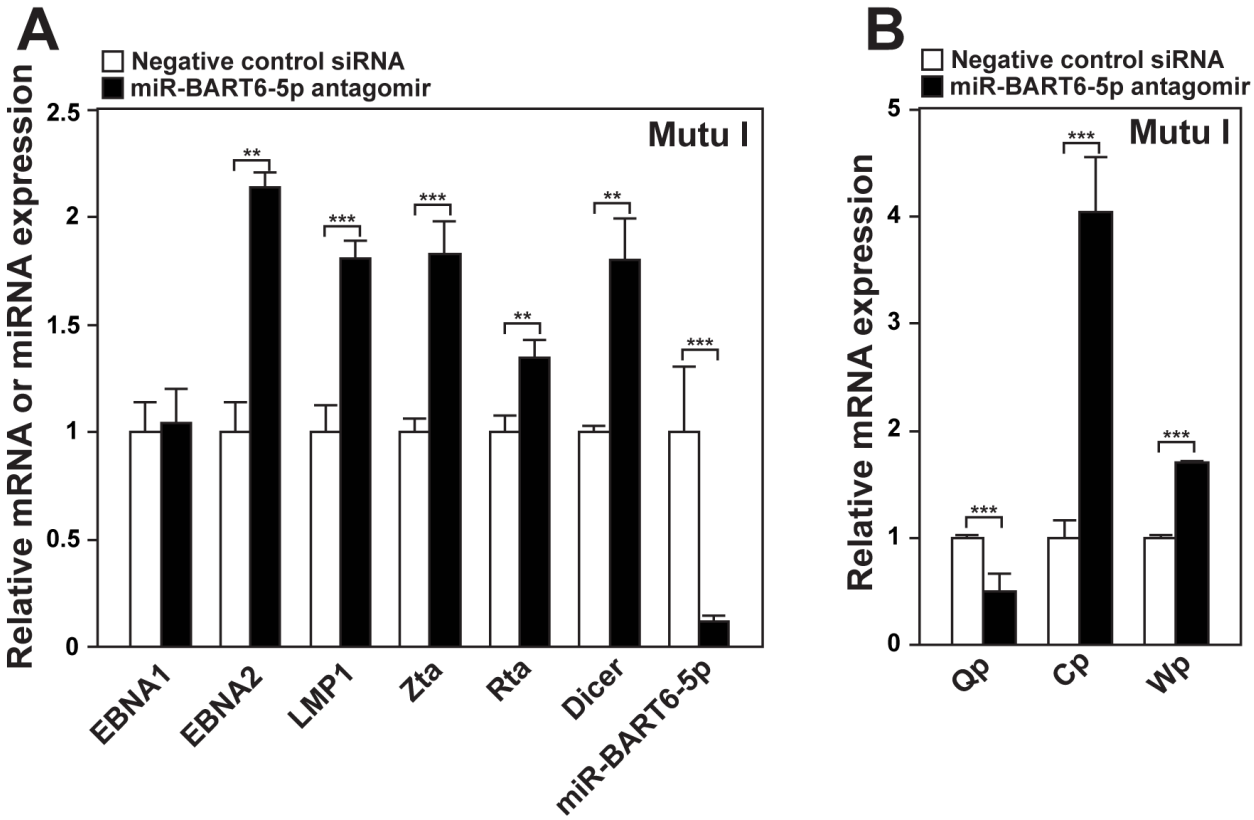

Fig. 3. The effects of miR-BART6-5p in Mutu I B lymphoma cells.
(**A**) Upregulation of EBV genes critical for latency III and lytic replication in Mutu I cells transfected with the miR-BART6-5p antagomir or control siRNA. Dicer and miR-BART6-5p levels were also monitored. Three independent qRT-PCR assays were performed. Mann-Whitney *U*-test, **p<0.005, ***p<0.001. Error bars, SEM (n=3). (**B**) Changes in viral promoter activities were induced in Mutu I cells by the miR-BART6-5p antagomir. Three independent qRT-PCR assays were done. Mann-Whitney *U*-test, ***p<0.001. Error bars, SEM (n=3).

<u>Research Infrastructure Project 4: Project Title and Purpose</u>

Laboratory Renovation Research Infrastructure - The Wistar Institute is planning essential laboratory renovations to enable development of a new programmatic initiative in "Integrated Cancer Therapeutics." This initiative is consistent with the Institute's long-term strategic plan and will provide a better understanding of the tumor microenvironment, resulting in the development of novel, more effective anticancer therapies. In support of this initiative, we plan to renovate a 1,800 square foot laboratory unit located on the first floor of the 1894 main research building of The Wistar Institute. The planned renovations, with their resultant creation of a new, state-of-the-art laboratory unit, are essential to the successful recruitment of a new leader to strengthen our integrated cancer therapeutics initiative.

Anticipated Duration of Project

1/1/2011 - 6/30/2012

Project Overview

The objective of the planned infrastructure project is to renovate laboratory space at The Wistar Institute to create a more efficient and functional layout conducive to modern research. Renovation involves removal of old, outdated equipment; demolition of existing interior walls; removing existing lab benches, sinks and plumbing; constructing and finishing new interior walls; and installing additional electrical power capacity, energy efficient lighting, data drops, distilled and potable water supply lines, drain lines, ventilation, built-in tables/shelving for equipment and new and salvaged lab benches.

Principal Investigator

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

The outcome of the planned project is to enhance the capacity of Wistar Institute researchers to conduct research involving gene expression and regulation; molecular and cellular oncogenesis; immunology; the tumor microenvironment; and diverse roles for RNA in gene regulation by providing modern physical space and related services (electric, plumbing, ventilation, internet connectivity) necessary to accommodate modern research.

Summary of Research Completed

Renovation of a 1,800 square foot laboratory unit on the first floor of the 1894 main research building of The Wistar Institute to create a new and efficient state-of-the-art laboratory capable of supporting modern biomedical research is currently in the planning phase. Plans are in progress for removal of old, outdated equipment; demolition of existing interior walls; removing existing lab benches, sinks and plumbing; constructing and finishing new interior walls; and installing additional electrical power capacity, energy efficient lighting, data drops, distilled and potable water supply lines, drain lines, ventilation, built-in tables/shelving for equipment, and new and salvaged lab benches.