2017 Research Fellowship Application : Entry # 10977

Project Information

<table>
<thead>
<tr>
<th>Project Title</th>
<th>Management of cholangiocarcinoma using extracellular vesicles loaded with microRNAs associated with melatonin production and circadian rhythm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount Requested</td>
<td>$50,000</td>
</tr>
</tbody>
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Applicant Information

<table>
<thead>
<tr>
<th>Applicant Name</th>
<th>Keisaku Sato</th>
</tr>
</thead>
<tbody>
<tr>
<td>eRA Commons Username</td>
<td>KEISAKUSATO</td>
</tr>
<tr>
<td>Degree (Select all that apply)</td>
<td>PhD</td>
</tr>
<tr>
<td>Academic Level (Select all that apply)</td>
<td>Post-Doc Fellow</td>
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Institution Information

<table>
<thead>
<tr>
<th>Institution Name</th>
<th>Texas A&amp;M Health Science Center</th>
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<tbody>
<tr>
<td>Institution City</td>
<td>College Station</td>
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<tr>
<td>Institution State</td>
<td>Texas</td>
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<tr>
<td>Institution Country</td>
<td>United States</td>
</tr>
<tr>
<td>Email</td>
<td><a href="mailto:srs-awards@tamu.edu">srs-awards@tamu.edu</a></td>
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Mentor Information
Cancer of the bile duct mucosa, cholangiocarcinoma (CCA), results from the malignant transformation of cholangiocytes, which line intrahepatic and extrahepatic bile ducts of the liver. CCA is clinically silent and mostly diagnosed as an advanced disease resulting in limited therapeutic options. While advances have been made in our understanding of the multiple endocrine and neuroendocrine factors that modulate biliary growth during the pathogenesis of chronic liver diseases and the ultimate development of CCA, unfortunately, viable therapies for the management of CCA remain elusive. Accumulating evidence suggests that disruption of circadian rhythms may be a risk factor for tumor initiation and may accelerate cancer progression. Studies have shown that melatonin inhibits cancer growth and resynchronizes dysregulated circadian rhythm circuitry, which may involve the translational control of the circadian clock genes by specific microRNAs. Other studies have also shown that extracellular vesicles with loaded microRNAs can decrease the size of CCA and improve survival of treated rats. The objective of this application is to determine the molecular mechanisms of bile duct carcinogenesis that are modulated by dysregulation of the synthesis of the circadian hormone, melatonin, and circadian rhythm gene expression, and also to seek novel therapeutic methods for CCA by transferring microRNAs into malignant cells using extracellular vesicles.

Our long-term research goal is to develop an understanding of the endogenous neuroendocrine factors regulating the carcinogenesis of the biliary mucosa, which will provide a foundation for the discovery of new pharmaceutical interventions for the prevention and treatment of CCA. Our recent studies have shown that downregulation of biliary arylalkylamine N-acetyltransferase (AANAT, the key enzyme in melatonin synthesis) expression followed by attenuated melatonin secretion is associated with CCA growth. We have also found that melatonin inhibits CCA tumor growth and AANAT overexpression induces tumor cell apoptosis. Melatonin synthesis and associated circadian rhythm gene expression could be a therapeutic target for CCA. Recent studies have shown that extracellular vesicles, which carry microRNAs can inhibit proliferation and invasion of hepatocellular carcinoma cells. Another study has demonstrated that extracellular vesicles can also inhibit CCA growth in rats. Extracellular vesicles that contain microRNAs associated with melatonin synthesis and circadian rhythm may be a candidate tool for novel therapies of CCA. The information gained from the successful completion of this application is expected to provide important insights into the mechanisms regulating biliary mucosa injury and CCA growth, and also therapeutic targets that can be pursued for novel treatments for biliary mucosa malignancy.
Cholangiocarcinoma is bile duct cancer. Bile ducts are tubes to move a fluid called bile, which helps digestion of fats in food. Cholangiocarcinoma is often fatal because this cancer can go further stages without showing any noticeable signs or symptoms. Although there are many risk factors reported to date, detailed mechanisms as well as treatments of this bile duct cancer are still unknown. Some studies suggest that a disrupted circadian rhythm, or body clock may be a risk of cancer. Various hormones are secreted in the human body according to this biological clock, and the irregular rhythm causes unbalanced hormones and this may lead to cancer or cancer growth. Melatonin is a hormone, which helps sleep and wake cycles. Melatonin production is controlled by the body clock, and melatonin supplements are used to treat jet lag and help sleep patterns who work night shifts. Melatonin production and the sleep-wake cycle may be important for cancer. We have recently found that melatonin is related to cholangiocarcinoma. Our findings to date show that melatonin decreases cancer growth and protects the liver from damage in animal models. It is important to study detailed mechanisms of bile duct cancer and effects of melatonin in cancer growth to develop a new therapy to cure this silent malignant cancer, and the body clock and its regulation could be the key for this innovation.

Specific Aims (2500 Max Characters)

This application proposes two projects: (1) aberrant expression of microRNAs that modulate AANAT expression leading to melatonin production and core circadian gene expression promoting the growth of CCA, and (2) management of CCA growth by extracellular vesicles carrying microRNAs that regulate AANAT and circadian gene expression. Our proposed studies are innovative from the standpoint that we propose that the downregulation of hepatic AANAT and subsequent biliary melatonin synthesis and the dysregulation of circadian gene expression play a critical role in the pathogenesis of CCA (Figure 1). Our preliminary data demonstrate that: (i) the upstream pathway for biliary melatonin synthesis is downregulated; and (ii) there is a dysregulation of circadian gene expression (i.e., PER1, BMAL1 and CLOCK) in CCA cell lines and human CCA biopsy samples. We present preliminary data demonstrating that the downregulation of AANAT and circadian gene expression is resulted from the alterations of the key microRNAs miR-141/miR-200a, miR-25 and miR-34a (Figure 2). Sequence analyses revealed that the 3'-UTR of CLOCK, AANAT and PER1 mRNA contain putative sites that are partially complementary to miR-141/miR-200a, miR-25 and miR-34a and evolutionarily conserved among human, mouse and rat. To experimentally validate these targets, we will measure their levels upon altering specific miRNAs. Immunoblots will reveal that blockage of endogenous microRNAs in CCA cells, which highly express miR-141/miR-200a, miR-25 and miR-34a, result in an induction of CLOCK, AANAT and PER1, respectively.

Extracellular vesicles carrying various microRNAs can be a therapeutic tool for cancer management. A latest study has shown that extracellular vesicles containing miR-195, which is downregulated in CCA cells decrease the size of cancers in rats [1]. Extracellular vesicles carrying candidate microRNAs identified in our current studies may also be useful to control CCA. Melatonin stimulation or transfection of normal cholangiocytes will produce therapeutic extracellular vesicles carrying microRNAs that inhibit cancer cell proliferation. Profiling of microRNAs for extracellular vesicles will identify other novel candidate microRNAs associated with melatonin production, circadian gene or inhibition of CCA growth. These projects will provide detailed information for association between circadian gene expression and cancer growth as well as novel treatments using extracellular vesicles.

Background and Significance (3000 Max Characters)
Cancer of the biliary mucosa, cholangiocarcinoma (CCA), results from the malignant transformation of cholangiocytes, which line intrahepatic and extrahepatic bile ducts of the liver [2]. CCA is clinically silent and mostly diagnosed as an advanced disease resulting in limited therapeutic options [3]. Recent evidence indicates that the proliferating biliary mucosa serves as a neuroendocrine compartment during the pathogenesis of liver diseases, and as such, secrete and respond to hormones, neurotransmitters and neuropeptides contributing to the autocrine and paracrine pathways that positively and/or negatively modulate biliary carcinogenesis [4].

Accumulating evidence suggests that disruption of circadian rhythms may be a risk factor for tumor initiation and may accelerate cancer progression [5, 6]. Recent studies have demonstrated the role of circadian rhythm and the key circadian hormone, melatonin, in the pathogenesis of CCA [7-9]. Aberrant expression of circadian genes has been found in various cancer types [10]. These studies have highlighted that melatonin as well as circadian genes may function as tumor suppressors due to their roles in regulating cell proliferation and apoptosis in biliary mucosa. In fact, our recent studies have shown that melatonin inhibits cancer growth and resynchronizes dysregulated circadian rhythm circuitry [11-13]. Translational control of the circadian clock genes by specific microRNAs has been shown in different types of organs including mice liver [14-16].

Extracellular vesicles are membrane-bound vesicles secreted from a wide variety of cells, and these vesicles may play an important role in liver pathology including carcinogenesis as we reviewed recently [17]. Our preliminary data using successfully isolated extracellular vesicles from cholangiocytes demonstrate alteration of physiological events in recipient cholangiocytes during bacterial infection in vitro (Figure 3). Extracellular vesicles have a great potential to regulate cell activity and control disease conditions by transferring their contents including microRNAs into recipient cells. Recent studies have shown that extracellular vesicles inhibit hepatoma growth by transferring microRNAs into cancer cells [18-20]. Our early study has shown that melatonin treatment elevates microRNA expression in extracellular vesicles secreted from intrahepatic biliary epithelial cells [21]. These findings suggest that extracellular vesicles can be a novel therapeutic tool for CCA treatment by transferring microRNAs that regulate melatonin production as well as circadian clock gene expression in cancer cells. Our contribution here is significant because it is the first step to provide the background knowledge for the development of unique therapeutic interventions (targeting melatonin using extracellular vesicles) for preventing malignant transformation of biliary mucosa after chronic cholestatic liver diseases and ameliorating the progression of established CCA.

**Project Timeline (2500 Max Characters)**

Month 1-2: Evaluation of candidate microRNAs in melatonin production or circadian gene expression in CCA cells
Silencing for candidate microRNAs identified in our current studies (miR-141/miR-200a, miR-25 and miR-34a) will be performed in CCA cells. Immunoblotting will show that reduction of these microRNAs enhances AANAT or circadian gene expression leading to attenuated proliferation of cancer cells. CCA cell transfection followed by immunoblotting and cell proliferation assay will be performed.

Month 3-6: Luciferase assay for candidate microRNAs
To determine whether CLOCK, AANAT and PER1 are bona fide targets of miR-141/miR-200a, miR-25 and miR-34a-mediated siRNA silencing, the miR-141/miR-200a, miR-25 and miR-34a binding sites in the 3'-UTR of these three genes will be cloned into a luciferase reporter. We will prove that forced expression of miR-141/miR-200a, miR-25 and miR-34a result in decreased luciferase activity when the wild-type sequences are present. Results will demonstrate that miR-141/miR-200a, miR-25 and miR-34a directly target CLOCK, AANAT and PER1, respectively.

Month 7-8: Isolation of extracellular vesicles
Normal cholangiocytes will be stimulated with melatonin and/or transfected with candidate microRNA inhibitors. Extracellular vesicles will be isolated by ultracentrifugation and analyzed by electron microscopy, nanoparticle tracking analysis and immunoblotting to confirm successful vesicle isolation. Extracellular vesicles will also be isolated from CCA cells.

Month 9-12: Evaluation of effects of extracellular vesicles in cancer cells
Normal cholangiocytes and CCA cells will be incubated with isolated extracellular vesicles. Cell viability, proliferation and migration will be analyzed. CCA-derived vesicles may induce cell proliferation or malignancy in normal cholangiocytes. Normal cholangiocytes-derived vesicles, on the other hand, may inhibit CCA proliferation, especially vesicles carrying microRNAs associated with induction of AANAT and circadian genes. Results from these experiments will present the first step for the development of novel therapeutic techniques using extracellular vesicles. Screening of microRNA panels for extracellular vesicles will also identify more candidate microRNAs that have therapeutic effects against CCA.
Literature Cited


Supporting Files - Please upload any supporting files such as charts or graphs in JPG or PDF format.

- Figure3.jpg
- Figure2.jpg
- Figure1.jpg

Budget
2017 Research Fellowship Application : Entry # 10977

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**Impact Applicant Name**

Keisaku Sato

**Impact App Title**

Postdoctoral Research Associate

**Impact App Institution**

Texas A&M Health Science Center

**Impact App Personnel 1 Name**

Gianfranco Alpini

**Impact App Per 1 Effort**

50

**Impact App Personnel 2 Name**

Keisaku Sato

**Impact App Per 2 Effort**

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**Impact Per 2 Base Salary**

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**Impact Per 2 Salary Requested**

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**Impact App Personnel Total**

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**Impact Equipment List**

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**Impact Total Requested**

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