This is provided as an example proposal.
It is important that you follow the current guidelines.
The mentor letter is included.

PURF COVER PAGE

TITLE OF PROJECT: Characterization of intracellular ATP's role in the early steps of cancer metastasis

NAME OF APPLICANT: Maria Evers
CAMPUS/LOCAL ADDRESS: 197 West State St. Apt. B, Athens OH 45701
E-MAIL ADDRESS: me391615@ohio.edu
DEPARTMENT: Edison Biotechnology Institute

BUDGET: Total Request $1,500
(May not exceed $1,500)

CLASS RANK: (circle one) Freshman Sophomore (Junior) Senior
GPA: 3.824
EXPECTED DATE OF GRADUATION: May 2020 *

* Note: Students must be enrolled and maintain undergraduate student status during the proposed project period.

FACULTY MENTOR INFORMATION:
NAME: Dr. Xiaozhuo Chen
E-MAIL ADDRESS: chenx@ohio.edu
CAMPUS ADDRESS: 109 Konneker Research Labs
DEPARTMENT: Biomedical Sciences & Edison Biotechnology
DEPARTMENT ADMIN/EMAIL: Rachel Beha / beha@ohio.edu

We the undersigned have read the PURF Guidelines and understand the responsibilities we undertake should funding be granted.
We certify that the application has been conceived, written and completed by the student.

Student signature: Maria D. Evers Date: 9/20/18
Faculty signature: Xiao Z. Chen Date: 9/20/18
Faculty Advisor's Dept. Chair signature: Tom Kosol Date: 10/1/18

IRB AND IACUC APPROVAL:
To ensure that the University is in compliance with all federal regulations, complete the checklist below. *Note: your proposal can be approved prior to IRB or IACUC approval (put "pending" or "to be submitted" instead of approval number), but funding will be withheld until notification of approval or exemption.*

Yes	*No*	*Office of Research Compliance*	*Policy #*
	X	Human Subjects in Research (including surveys, interviews, educational interventions): Institutional Review Board (IRB) Approval #: Expiration Date:	19.052
	X	Animal Species: Institutional Animal Care & Use Committee (IACUC) Approval #: Expiration Date:	19.049

☑ **Optional:**
If selected for funding, I give permission to the Research Division to use my proposal as an example during training and workshop exercises. (Sign below)
Signature: Maria D. Evers Date: 9/20/18

2. Abstract

Metastasis is the leading cause of death in cancer patients, yet is one of the least understood processes in cancer. ATP has recently been found to be a main contributor to the progression of cancer and cancer metastasis, which is spread of primary cancer to distant organs. In this proposed project, I will further investigate ATP's intracellular role in cancer metastasis utilizing a genetically engineered human non-small cell lung cancer (NSCLC) cell line. Completion of this project is likely to lead to better understanding of metastasis which could help find new ways to slow down this process.

3. Project Narrative

Project Description: More than 90% of all cancer related deaths involve a late stage cancer cell spread called metastasis [1]. Metastasis is the development of secondary malignant growths at a distance from a primary site of cancer. Delaying or preventing metastasis is the key to substantially prolonging cancer patients' survival, and potentially transforming cancer from a terminal illness into a chronic disease. Dr. Xiaozhuo Chen's lab, where I have been working, has been one of the few to pioneer the study of a very powerful, multifunctional molecule utilized by cancer: extracellular ATP (eATP). ATP is unique among millions of different biological molecules in that it exhibits functions as an energy molecule, a signaling molecule, an extracellular messenger, and a genetic material. ATP can function as either an energy or signaling molecule by transferring a phosphate group to enzymes or kinases, activating them to perform enzymatic / signaling reactions. Intratumoral eATP concentrations are 1,000 to 10,000 times higher than in normal tissues of the same origin [2], and is essential to the survival and progression of cancer. We have recently shown that eATP is capable of independently initiating a process called epithelial to mesenchymal transition (EMT) [3] via purinergic receptor signaling, primarily P2X7 [4]. Normal purinergic receptor signaling is for cell movement during embryonic development. However, tumors have hijacked the purinergic receptor signaling pathway, activated by their eATP rich environment, to induce the spread of cancer cells to surrounding normal tissues and eventually other distant organs by EMT. EMT is an essential process for the initiation and early steps of cancer metastasis. During the EMT process, epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties [5]. It should be noted that, like eATP, transforming growth factor beta (TGF-β) is a cytokine that is also known to induce

EMT via the P2X7 signaling pathway. TGF-β has been long thought to be the key player in P2X7 mediated EMT. However, my preliminary research has supported our hypothesis that eATP, as a signaling molecule, is capable of independently inducing greater cancer cell migration and invasion than TGF-β (figure 1). Concentrations used in this study for TGF-β and eATP were consistent with literature values [2, 6]. The results support our speculation

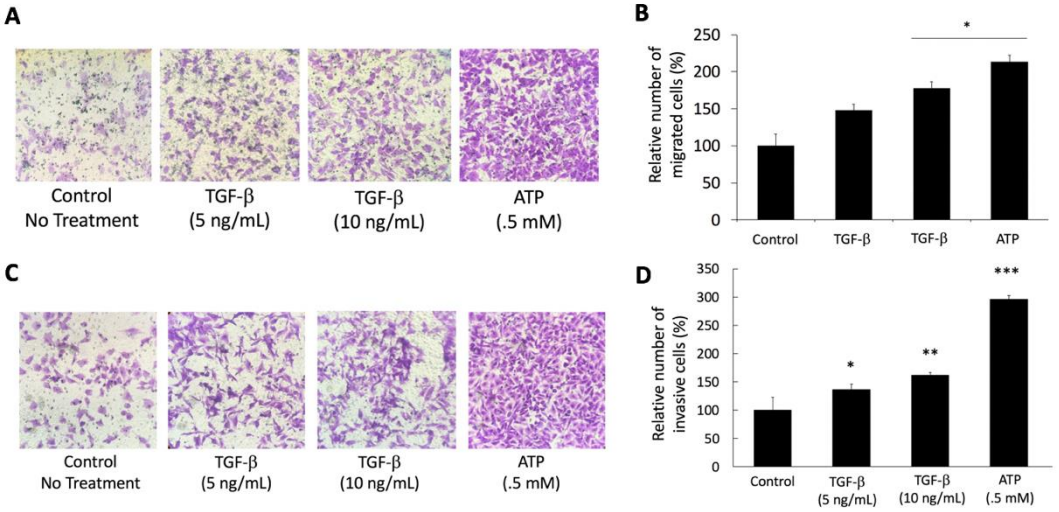

Figure 1: Effect of ATP and TGF-β on the migration and invasion of A549 cells using Transwell assay. Cells were treated with various concentrations of ATP or TGF-β in a migration assay (16h) or an invasion assay (20h). Then cells were stained with crystal violet and observed under the microscope with x200 magnification. **(A)** Representative images of migrated cells. **(B)** Quantification of migrated cells relative to control. **(C)** Representative images of invaded cells. **(D)** Quantification of invaded cells relative to control. *P < 0.05, **P < 0.01, ***P< 0.001

that eATP may play a much larger role in inducing EMT and cancer metastasis than TGF-β. Additionally, further studies have shown that reduction of eATP significantly diminishes cancer cell invasion in a dose dependent manner (figure 2), without inhibition of TGF-β signaling, further suggesting that eATP is the more powerful player in this mechanism. In addition to initiating P2X7 signaling extracellularly, ATP also intracellularly contributes to

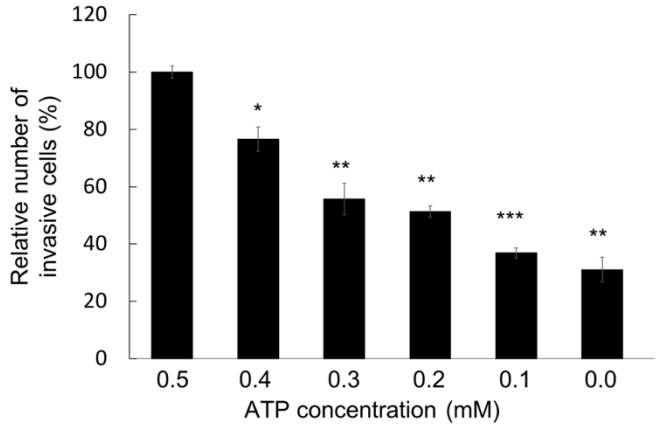

Figure 2: Reduction of the extracellular ATP concentration (below the threshold of P2X7 activation) significantly decreases the amount of invasive cancer cells. *P < 0.05, **P < 0.01, ***P< 0.001

induction of EMT by phosphorylating proteins in the P2X7 signaling pathway, which we have yet to investigate. Phosphorylation of proteins is the process by which an ATP molecule transfers one of its phosphate groups to a protein, rendering that protein "active." This active protein will then cause a chain reaction that results in EMT. As recently cited by Nature Reviews Cancer, our lab has found that cancer cells are able to uptake eATP via a process called macropinocytosis for many uses [3,4,7]. We hypothesize that the increased intracellular ATP concentration as a result of macropinocytosis contributes to the intracellular function of ATP in P2X7 mediated EMT (figure 3).

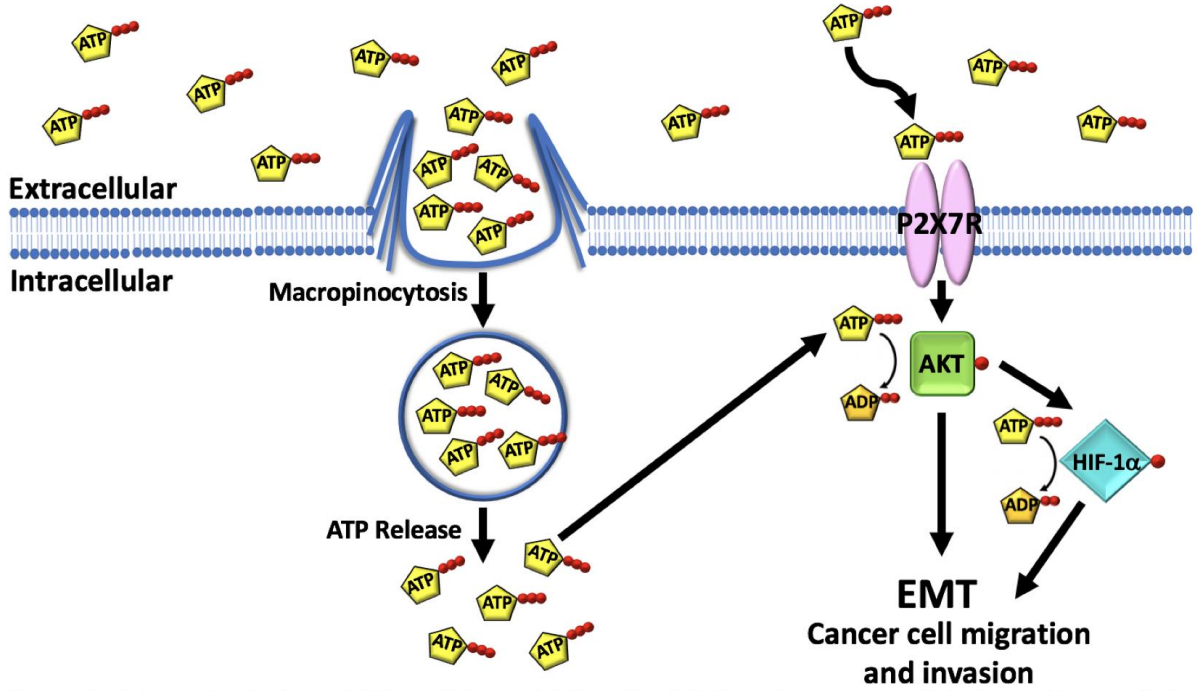

Figure 3: A hypothetical model for wildtype A549 cells. ATP functions extracellularly and intracellularly in the P2X7 signaling pathway. Extracellular ATP activates the P2X7R and initiates a downstream signaling cascade. Extracellular ATP is taken up by cancer cells via macropinocytosis, and is then used to further phosphorylate and activate proteins in the P2X7 signaling pathway, leading to EMT and cancer cell migration and invasion.

To investigate this, our lab has recently generated a *SNX5* knockout cell line (A549snx5ko) utilizing CRISPR-Cas9, a genetic engineering method. The *SNX5* gene is a necessary gene involved in macropinocytosis in human NSCLC cells. The resultant KO cell line, A549snx5ko, exhibits significantly reduced macropinocytosis and a slower growth rate.

Methods: I hypothesize that A549snx5ko cells show reduced ATP internalization and therefore reduced EMT induction compared to the wildtype A549 lung cancer cells. I will complete all of the proposed studies, with the technical assistance of my thesis advisor, Dr. Chen.

Specific Aim 1: Determine and compare intracellular ATP levels in A549snx5ko cells and control A549 cells. ATP assays to measure the intracellular ATP concentration of ATP treated A549snx5ko and wildtype A549 cell lines will be completed. Extracellular ATP concentrations of 0, 0.1, 0.2, 0.3, 0.4, 0.5, 1 mM ATP will be used, which are consistent with the reported in vivo concentrations. The completion of this aim will confirm and quantify the reduction of macropinocytosis in A549snx5ko cells versus the wildtype A549 cells.

Specific Aim 2: Compare the consequences of intracellular ATP levels in EMT induction and protein phosphorylation in the P2X7 signaling pathway. The effect of decreased intracellular ATP levels on cancer cell migration and invasion will be determined utilizing transwell assays. In the transwell migration assay, cancer cells migrate through a porous membrane and adhere to the other side of the membrane. In the transwell invasion assay, cancer cells will first invade through a gelatinous protein layer, and then migrate through a porous membrane and adhere to the other side, imitating the real cancer invasion process. Intracellular ATP contributes to intracellular signaling in the EMT pathway, and therefore increases EMT-induced cancer migration and invasion. Reduction of intracellular ATP in A549snx5ko cells will decrease EMT, and also cancer migration and invasion. Next, the effect on phosphorylation, or activation of signaling proteins, in the P2X7 signaling pathway will be determined with Western Blots, a method for qualitatively measuring the presence of specific proteins. Two crucial proteins in the P2X7 signaling pathway, AKT and HIF1-α, have been chosen to measure. In all these assays, wildtype A549 cells will be used as a control for baselines of migration, invasion

and protein phosphorylation. The completion of this aim will quantify the differences between the knocked out cancer cell line and the wildtype cell line, thus determining the contributions of macropinocytosis and intracellular ATP in these processes.

Data analysis: All experimental samples are in triplicate, and all experiments will be repeated at least once. Student's t-test will be performed to evaluate the differences between any two treated groups. $P<0.05$ is considered significant. Data analysis software ANOVA will be utilized for any statistical analysis.

Timeline: Upon funding in November, I will order the products required for the project. I will immediately begin working on the project. In November, I will complete the ATP assays. In January and February, I will complete the transwell migration and invasion assays, and protein phosphorylation study. Results will be presented at the 2019 Ohio University Student Exposition and the 2019 annual American Association of Cancer Research (AACR) meeting. In total, I expect to spend approximately 130-160 hours on this project.

Student's Role: For my honors thesis, I have chosen and designed a mechanism study of extracellular ATP-induced epithelial mesenchymal transition (EMT). This proposed study will comprise approximately one third of my thesis study. I will independently perform all of the studies discussed in this proposal, with the technical assistance of my thesis advisor, Dr. Chen.

Significance: Cancer is the second leading cause of death in the United States. Metastasis is responsible for 90% of solid tumor-related deaths, and is one of the least understood processes in cancer. Additionally, cancer metabolism and the study of ATP in cancer is a relatively new field in which not much is known. The completion of this project will contribute new knowledge to the scientific community and result in a better understanding of ATP's role in cancer metastasis.

4. Bibliography

1. Seyfried, T. N., & Huysentruyt, L. C. (2013). On the Origin of Cancer Metastasis. *Critical Reviews in Oncogenesis*, *18*(1-2), 43–73.

2. Pellegatti P, Raffaghello L, Bianchi G, Piccardi F, Pistoia V, Di Virgilio F (2008) Increased Level of Extracellular ATP at Tumor Sites: *In Vivo* Imaging with Plasma Membrane Luciferase. PLoS ONE 3(7): e2599.

3. Virgilio, F.D., Sarti, A.C., Falzoni, S., Marchi, E.D., & Adinolfi, E. (2018). Extracellular ATP and P2 purinergic signalling in the tumour microenvironment. *Nature Reviews Cancer*, 1-18.

4. Wang X, Li Y, Qian Y, Cao Y, Shriwas P, Zhang H, Chen X. Extracellular ATP, as an energy and phosphorylating molecule, induces different types of drug resistances in cancer cells through ATP internalization and intracellular ATP level increase. Oncotarget. 2017 September 23;8:87860-87877. doi: 10.18632/oncotarget.21231.

5. Lamouille, S., Xu, J., & Derynck, R. (2014). Molecular mechanisms of epithelial–mesenchymal transition. *Nature Reviews. Molecular Cell Biology*, *15*(3), 178–196.

6. Kim JH, Jang YS, Eom KS, Hwang YI, Kang HR, Jang SH, Kim CH, Park YB, Lee MG, Hyun IG, Jung KS, Kim DG. Transtorming Growth Factor β1 Induces Epithelial-to-Mesenchymal Transition of A549 Cells. J Korean Med Sci. 2007 Oct;22(5):898-904.

7. Qian, Y. et al. Extracellular ATP is internalized by macropinocytosis and induces intracellular ATP increase and drug resistance in cancer cells. Cancer Lett. 351, 242–251 (2014). 75.

5. Biographical Information

Since beginning my studies at Ohio University, I have taken many biological sciences and chemistry courses, including upper level classes, and have achieved a GPA of 3.824. As a student in the Honors Tutorial College, I have participated in 3 tutorials with Dr. Chen, my thesis advisor. During these tutorials, I learned the basics of cancer including cancer metabolism, genetics, and the hallmarks of cancer. I also learned about our labs research, and how to critically read / present scientific work. I began attending weekly lab meetings in January 2017 and began benchtop research and presenting research at group meetings in May 2017. During this time, I learned how to successfully perform assays and experiments commonly used in cancer research, and produce data of publishable quality. During my sophomore year, I received $800 in HTC Dean's funding for my preliminary research, which I presented at the Student Exposition in 2018 Additionally, I have been working on a graduate student's research, and am a coauthor on a manuscript in preparation for submission, which will be submitted near the end of 2018. I have also designed a research project for my honors thesis, which I have recently started working on.

6. Budget

Item	Justification	Source	Cost
ATP Assay	Specific Aim 1	https://www.caymanchem.com/product/700410	$189
Transwell Plates	Specific Aim 2	https://ecatalog.corning.com/life-sciences/b2c/US/en/Permeable-Supports/HTS/Transwell%C2%AE-Permeable-Supports%2C-Polycarbonate-%28PC%29-Membrane/p/3422?clear=true	$230
Phospho-AKT Antibody	Specific Aim 2	https://www.cellsignal.com/products/primary-antibodies/phospho-akt-ser473-antibody/9271	$297
Phospho-HIF1A Antibody	Specific Aim 2	https://www.cellsignal.com/products/primary-antibodies/hif-1a-antibody/3716	$255
Cell Culture Supplies	Maintain and growth of cell lines	Includes: cell culture media, PBS, FBS, Penicillin, trypsin, formaldehyde, cell culture plates, centrifuge tubes, serological pipets, micropipette tips	$250
Western blot supplies	Specific Aim 2	Gels, secondary antibody, nitrocellulose membranes	$179
Shipping Costs			$100
		Total:	$1500

Cost of poster printing for the Student Expo will be covered by HTC bios. Cell culture supplies and western blot supplies are a shared lab expenses and cost listed reflects my contribution to the purchase of these supplies.

OHIO UNIVERSITY

College of Arts and Sciences

Program in Molecular and Cellular Biology
Porter Hall 414
Athens OH 45701-2979

September 20, 2018

Dear PURF Award Review Committee,

I am writing this letter to strongly support my undergraduate student Ms. Maria Evers for her application for the Provost's undergraduate research fund. I am writing this supporting letter as her academic advisor for her undergraduate thesis.

Maria first began her time in my lab as a freshman. She began by taking multiple tutorials with me in order to learn the basics of cancer biology and cancer research, and attended weekly lab meetings. Once she had a stronger foundation and understanding of our research, she began working on her own first small cancer research project, which she excelled at. Once Maria showed her deeper understanding of cancer biology and lab skills in performing experiments, I offered her a paid position in my lab, as she has become an integral and contributing member of our research team. She is now working with a graduate student in my lab on cancer research for an upcoming publication, as well as independent cancer research for her thesis.

The project proposed by Maria is an integral part of her undergraduate thesis. For her thesis she has chosen a mechanism study of extracellular ATP-induced epithelial mesenchymal transition (EMT), which is an early and essential step for metastasis of cancer. Metastasis is involved in up to 90% of cancer related death. This application deals with characterization of the roles of intracellular ATP in the early steps of cancer metastasis, which consists of about one third of her proposed thesis study. This project will help Maria to learn how cancer and metastasis develop, preparing her for her desired future graduate study and her career in cancer and biomedical research.

Maria has worked in my lab for almost 2 years. Maria is an excellent student from the Honors Tutorial College with a cumulative GPA of 3.824. She is also a quick learner with a sharp mind and dexterous hands in the lab. She has shown passion for research and has proven her ability to generate high quality publishable results. With this funding, she will have the opportunity to further grow as a young researcher as she learns new lab skills, moving her one step closer to the completion of her thesis and publication of her research. As her thesis advisor, I will supervise Maria on a weekly basis, discussing experimental designs and results, trouble shooting with her for potential experimental / technical problems, and advising her on data analysis. I will also guide and assist her writing of posters, manuscripts, and her thesis. Furthermore, I will accompany her to the regional and national conferences for her research presentations.

Working at the Edison Biotechnology Institute, I have had the privilege of working with several undergraduate students as mentors over the years. This is a valuable opportunity that will prepare Maria to become an independent and productive cancer researcher in the future.

Sincerely,

Xiaozhuo Chen, Ph.D.
Associate Professor of Biomedical Sciences
EBI and Department of Biomedical Sciences, HCOM, Ohio University