The Regulation of Polymine Homeostasis in Cancer

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Michael N. Hall, Professor at the Biozentrum of the University of Basel, Switzerland, will be Principal Investigator of the proposed project. Arkaitz Carracedo, an Ikerbasque Research Professor at CIC bioGUNE in Derio, Spain, will be co-Principal Investigator. Christoph Müller is a junior investigator in Michael Hall's laboratory at the Biozentrum in Basel. Administrative responsibility will be located at the Biozentrum.

Background

Polyamines (putrescine, spermidine, and spermine) are among the most abundant metabolites cells (up to millimolar concentrations) and are important for cell growth and survival (*1, 2*). The polycationic nature of these metabolites allows them to interact with negatively charged cellular components, e.g., DNA, RNA, proteins and phospholipids. Due to these interactions, polyamines play a role in diverse biological processes, such as maintaining chromatin architecture, regulating ion channels, maintaining membrane stability, free-radical homeostasis, transcription, and translation (*2, 3*). Thus, polyamines are essential for viability and indispensable for proliferation (*4*). Accordingly, polyamine levels are elevated in various types of cancer (*5, 6*). Furthermore, polyamine levels decline with age (*7, 8*), and polyamine supplementation can increase lifespan in multiple organisms including mice (*9*).

Polyamine homeostasis is also important. While a certain polyamine concentration is required for cellular fitness, too much polyamine is cytotoxic due to the generation of reactive oxygen species (*10*). Thus, polyamine metabolism and transport are tightly regulated to achieve a proper "Goldilocks" level of polyamines. Apart from *de novo* synthesis, polyamines can be obtained from diet or from intestinal microorganisms (*8*). Specialized transport systems mediate cellular uptake and release of polyamines. Although polyamine transport is not well understood in mammalian cells, several transporters have been reported to be involved specifically in the uptake of polyamines, in particular members of the P5B-ATPase family (*11*). While the identification of these transporters has provided valuable insight on how cells take up polyamines, the mechanism(s) regulating polyamine uptake is completely unknown. Similarly, polyamine secretion has been shown to involve vesicular transporters (*12*), but the role of polyamine release and how it is regulated has not been investigated. This project aims to identify the mechanisms by which polyamine uptake and release are regulated to maintain appropriate intracellular polyamine levels, in the context of the age- related disease cancer.

Cancer cells require an unusually high level of polyamines. For example, we have observed that tumor samples from hepatocellular carcinoma (HCC) patients and an mTOR-driven HCC mouse model display high polyamine levels despite down-regulated polyamine synthesis (*13*), indicating tumor cells strongly rely on increased polyamine uptake. We have indeed confirmed an increased uptake in cancer cells. Thus, our HCC mouse model and available HCC cell lines collectively provide a good system to study the regulation of polyamine uptake. The Carracedo (co-PI) laboratory has reported high polyamine levels in prostate cancer due to a rewiring of methionine metabolism toward polyamine synthesis (*14*). Furthermore, prostate cancer cells rapidly respond to certain stresses by exporting polyamines. The Hall and Carracedo laboratories propose to exploit the unique and combined advantages that HCC and prostate cancer provide to investigate mechanisms that mediate polyamine homeostasis. Our findings may yield novel strategies for the treatment of cancer and other aspects of aging.

Research Plan

Cancer cells depend on high polyamine levels to sustain high rates of proliferation. However, drugs targeting enzymes in polyamine synthesis, such as the ODC1 inhibitor DFMO, have been largely unsuccessful in clinical trials. This is due to the ability of cancer cells to increase polyamine uptake upon inhibition of synthesis. Upregulation of polyamine transport following polyamine depletion has been observed across many different cell types (*15, 16*). However, polyamine homeostasis also entails preventing accumulation of excessively high and thus toxic polyamine levels. Mechanisms underlying polyamine homeostasis are poorly understood but based on our unpublished observations, appear to involve transcriptional regulation (*17*). We propose to study the regulation of polyamine uptake and release using two separate approaches, a genome-wide CRISPR knockout (KO) selection and a computational approach to identify promotor motifs in known polyamine transporter genes.

Genome-Wide CRISPR KO selection/screen

We propose to perform a genome-wide CRISPR KO selection, using HCC and prostate cancer cells, to identify proteins involved in the regulation of polyamine import and release (*18, 19*). We will use the GeCKO v2 gRNA library that targets the entire genome with six gRNAs per gene (*20*). After lentiviral infection, the cells will be treated with puromycin to select for cells that integrated the Cas9/gRNA-containing plasmids. These cells will then be challenged with polyamine synthesis inhibition and selected based on proliferation (subprojects 1 and 2) or exposed to hypoxia and screened for expression of the cellular marker LAMP1 (subproject 3).

The combination of challenge and selection will result in enrichment or depletion of certain gRNAs. The frequency of every gRNA present in the selected cells will be analyzed by deep next-generation sequencing (NGS). The three proposed subprojects are described below in more detail.

Subproject 1. Positive selection to identify positive regulators of polyamine import

In a positive selection, cells with the phenotype of interest will be selected (survival benefit), in this case mitoguazone resistance. Mitoguazone (MGBG) is an inhibitor of AMD1, an important enzyme in polyamine biosynthesis. Treatment of cells with MGBG results in polyamine depletion and growth arrest. MGBG is taken up by the polyamine transport machinery as loss of the polyamine transporter ATP13A3 protects cells from MGBG toxicity (*21*).

We will challenge the pooled KO cells with MGBG at an IC_{50} for several days. Cells that have been transfected with gRNAs targeting genes positively controlling polyamine uptake will be enriched as they will have taken up less MGBG. Surviving cells will be pooled and amplicon libraries will be generated by PCR. Deep NGS will be used to rank enriched gRNAs, revealing targets directly (e.g., polyamine transporters such as ATP13A3) or indirectly (e.g., signaling or transcription factors) involved in polyamine uptake. Positive selections have the advantage of requiring fewer false positives in comparison to the negative selection described below.

Subproject 2. Selection to identify positive and negative regulators of polyamine import

In a negative selection, cells with the phenotype of interest are depleted in the presence of a biological challenge. The gRNAs remaining in the surviving cells will be compared to those present in unchallenged control cells to identify genes of interest (absent in challenged cells) and to rule out unrelated, essential genes (absent in control and challenged cells). In this approach we will combine positive and negative selection to obtain negative and positive regulators.

The pooled KO cells will be treated with the polyamine synthesis inhibitor DFMO for several days, while simultaneously supplementing the growth medium with polyamines. Over the time of the experiment, cells that retained or even increased polyamine uptake will be enriched (positively selected), while cells that have lost the ability to import polyamines will be depleted (negatively selected). Genomic DNA will be isolated and integrated gRNAs will be amplified by PCR. Bioinformatic analyses of deep NGS data will reveal enriched and depleted gRNAs in challenged vs. control cells that correspond to negative and positive regulators of polyamine uptake, respectively. We note that the first subproject described above could be modified to also identify negative regulators (e.g., negatively select for MGBG hypersensitivity), but we believe this would be technically difficult and thus unlikely to be successful.

Subproject 3. Cell sorting to identify regulators of polyamine secretion

This screen applies fluorescence-activated cell sorting to identify cells with the phenotype of interest. Our preliminary data demonstrate that pharmacological or microenvironmental perturbations elicit polyamine secretion. We can monitor the process by staining for the lysosomal protein LAMP1 that is enriched at the plasma membrane in cells

secreting polyamines.

Pooled KO cells will be subjected to hypoxic stress for 4 hours. After staining cells for LAMP1, we will sort cells using FACS for high and low levels of the marker corresponding to high and low polyamine export, respectively. The differences in gRNA frequencies of the two LAMP1 populations will be analyzed by NGS to reveal genes that negatively or positively regulate polyamine secretion.

Computational analysis of polyamine transporter promotors

We and others have found that P5B-ATPases play an important role in polyamine uptake. We have observed transcriptional upregulation of this family of transporters upon inhibition of polyamine synthesis. In addition, KO of any of these transporters is accompanied by transcriptional upregulation of other transporters of the family. To specifically investigate the transcriptional regulation of polyamine transport, we will perform computational analyses of promotor regions of known polyamine transporter genes. This approach will allow us to identify motifs of transcriptional regulation and create a short-list of possible transcription factors that will be combined with the targets of the CRISPR screens and investigated as described below.

Validation of the targets

Candidate targets identified by the methods presented above will be subjected to individual knockdown (KD) in arrayed validation experiments. The genes of interest will be knocked down using a pool of specific siRNAs and polyamine transport will be assessed using radio-labeled polyamines and targeted metabolomics in polyamine uptake and release experiments. If the target is a transcription factor, assays such as ChIP-seq analysis and electrophoretic mobility shift assay (EMSA) will be performed in the presence and absence of polyamines. If the candidate target is a signaling factor, e.g., a kinase, efforts will be initiated to place it in a signaling pathway. For example, we will investigate whether the signaling factor controls a candidate transcription factor. To identify possible regulatory mechanisms, we will perform luciferase-based promotor activity assays with varying polyamine concentrations and upon KD or KO of the identified transcription factor. We will also determine whether polyamines bind directly to candidate regulators. In addition, overexpression of the regulatory proteins will be analyzed for their ability to perturb polyamine transport. To disrupt polyamine homeostasis in cancer, we will consider combinatorial treatments to simultaneously target polyamine synthesis, import and secretion. A reduction of polyamine concentration and therefore proliferation in cancer cells could be achieved by inhibition of polyamine synthesis and import, while at the same time stimulating polyamine secretion. These approaches will be applied first in cell lines and, if successful, tested in mouse cancer models available in the Hall and Carracedo laboratories.

Experimental Milestones

Milestone 1: Generation of a cell line panel with robust polyamine uptake and sensitivity toward MGBG. Identification of cell lines that upregulate polyamine secretion upon different stresses.

Milestone 2: Generation of gRNA/Cas9 containing lentiviruses.

Milestone 3: Performance of the selections/screen(s) and data analysis. Computational analyses of promotor regions.

Milestone 4: Validation of the targets using siRNA screens and other *in vitro* and *in vivo* experiments.

Collaboration Agreement

This project will be an international collaboration between the Hall (Biozentrum, Switzerland) and Carracedo (CIC bioGUNE, Spain) laboratories. The project will involve a team of young scientists from both laboratories and will be led by Dr. Christoph Müller, himself a young investigator (postdoc), in the Hall laboratory. The CRISPR KO screen will be established in the Hall laboratory and applied to cell lines in both laboratories. The Carracedo laboratory will use computational analyses to identify possible transcription factors involved in polyamine transport. Downstream *in vitro* and *in vivo* validation will be performed in both laboratories.

Results will be published in peer-reviewed scientific journals and the Balzan Foundation will be acknowledged as a funding source.

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