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General Summary

Polyamines (putrescine, spermidine, and spermine) are ubiquitous biogenic amines that bind to nucleic acids and are essential for proliferation. Cellular polyamine contents are maintained by a feedback mechanism involving the key regulatory proteins antizymes (AZs). The AZs are expressed by translational frameshifting that is induced by polyamines and negatively regulate cellular polyamines. Three AZ isoforms (AZ1-3) are present in mammals. The AZs are further regulated by proteins termed antizyme inhibitors (Azins). Cancer cells generally contain elevated levels of polyamines. Our goal is to clarify the mechanism and biological significance of the elaborate regulatory system and to develop polyamine-related research or diagnostic tools.

Research Activities

Role of AZ2 in c-MYC degradation

We have previously found in cultured cells that AZ2 accelerates c-MYC degradation by the proteasome in an ubiquitin-independent manner. When investigating the roles of AZ2-mediated c-MYC degradation, we noticed that hypoxia and undernutrition have been reported to cause downregulation of c-MYC. Knockdown of AZ2 under these conditions inhibited downregulation of c-MYC and increased the frameshift efficiency of AZ2. Thus, our results suggest a novel pathway of hypoxia-induced c-MYC degradation mediated by polyamines and AZ2 in an ubiquitin-independent manner. c-MYC has been found in both the nucleolus and the nucleus. Proteasome inhibition leads to c-MYC accumulation within the nucleolus. Interestingly, AZ2 also localized to the nucleolus in the presence of proteasome inhibitor. Furthermore, when c-MYC and AZ2 were coexpressed in the cells, their nucleolar localizations were identical. These results suggest that AZ2 regulates c-MYC in the nucleolus.

Fluorescent visualization of cancer cells by monitoring of cellular polyamines

We are developing a method to visualize cancer cells by combining the polyamine-dependent frameshift mechanism of AZ, an endogenous cellular polyamine sensor, and fluorescent protein techniques. Last year a reporter, constructed from the entire protein-coding region of human AZ1 messenger (m) RNA and the enhanced green fluorescent protein (EGFP) gene that is inserted immediately downstream of the pseudoknot structure, showed increases in both EGFP fluorescence and the frameshift product in response to the addition of polyamines to the culture medium. This year, we further improved this construct by inserting the ECFP gene immediately in front of AZ1 mRNA as a nonframeshifting control and by inserting the Keima-Red gene immediately downstream

of the pseudoknot structure as a frameshifting confirmation. However, the construct did not respond to the addition of polyamines to the culture medium. Reconsideration of fluorescent reporter genes and their insertion positions is needed to increase the polyamine response.

Characterization of hematopoietic stem cells in the livers of fetal AZ1 knockout mice
We have previously shown that knockout of the AZ1 gene increases tissue polyamine levels, decreases the number of multipotent hematopoietic progenitor cells (MPPs) in fetal liver, and results in severe anemia and embryonic death. Because asymmetric division of hematopoietic stem cells (HSCs) generates MPPs, we used a long-term competitive bone-marrow repopulating assay to evaluate the hematopoietic ability of HSCs in the livers of *AZ1*$^{-/-}$ fetal mice. Consequently, we found that the number of repopulating units/HSC was lower in the livers of *AZ1*$^{-/-}$ fetal mice than in those of wild-type mice. This result possibly explains the decrease in MPPs in the livers of *AZ1*$^{-/-}$ fetal mice.

Multiple forms of mouse Azin1 mRNA are differentially regulated by polyamines
Azin1, a positive regulator of cellular polyamines, is induced by various proliferative stimuli and is repressed by polyamines. We have found multiple forms of *Azin1* transcript formed by alternative splicing and initiation of transcription from putative alternative start sites. This year, we first showed that a novel splice variant, *Azin1*-X, with a premature termination codon encoding a C-terminal truncated form of protein, is subject to nonsense-mediated mRNA decay. Next, 2-difluoromethylornithine, an inhibitor of polyamine synthesis, increased both transcription from the canonical transcription start site and the ratio of the full-length mRNA to Azin1-X mRNA; in contrast, polyamines showed the opposite effects. Thus, polyamines regulate 2 novel steps of *Azin1* expression, namely transcription and a particular splicing pattern, both of which may affect the level of mRNA encoding the full-length active Azin1 protein.

Polyamine-related research with stable isotopes
We have identified ATP citrate lyase (ACLY) as a candidate AZ2-interacting protein. We found that nuclear localization of ACLY was increased in the presence of AZ2, although localization of ACLY is predominantly cytoplasmic when expressed alone. We are establishing assay systems for ACLY activity and histone acetylation which employ a mass spectrometer with a stable isotope-labeled citrate (^{13}C) to reveal the effect of AZ2 on ACLY activity. We are also attempting to determine polyamine flux by means of liquid chromatography/tandem mass spectrometry using stable isotope-labeled ornithine as a metabolic substrate of polyamines to clarify the effect of an Azin1 variant on polyamine levels in the cell. To eliminate the effect of impurities in samples, we are also establishing a liquid chromatography/tandem mass spectrometry detection method using stable isotope-labeled polyamines as internal standards for absolute quantification.

Isolation and analyses of polyamine-binding RNA aptamers
RNA aptamers have the potential applications for both clinical and research. In

particular, aptamers are useful for exploring RNA-binding sequences and structures for target molecules. We are revealing general polyamine-binding RNA sequences and structures by analyzing polyamine-binding sites on isolated RNA aptamers. A part of a previously isolated antispermine aptamer consisting of a stem-loop with bulge-out structure has affinity for spermine. We found that a bulge region with 4 neighboring base pairs possessed a lower affinity for spermine. Moreover, the other stem region also possessed a weak binding activity. Interestingly, the existence of both regions is important for the strong binding activity. Some base replacements in one region abolished the binding activity of the other region. This finding indicates that these 2 regions work together to achieve the full binding activity.

Analysis of the molecular mechanism of carcinogenesis in ovarian clear cell carcinoma
Amplification of chromosome 17q21-24 has frequently been observed in ovarian clear cell carcinoma (CCC). However, the driver gene of the region has not been identified. Aberrant expression of microRNAs has been shown to be involved in oncogenesis. MicroRNA-21 (miR-21), encoded on 17q21-24, is a frequently overexpressed miRNA in many types of cancer. Analysis of clinical samples revealed overexpression of miR-21 and repression of phosphatase and tensin homologue (PTEN) in cases of CCC with amplification of 17q21-q24. We profiled *miR-21*, *PTEN* mRNA, and PTEN protein expression in 5 CCC cell lines. We selected RMG-II for function analysis because it was the CCC cell line that had the most prominent miR-21 overexpression and lowest PTEN protein expression. Knockdown of miR-21 in RMG-II cells increased *PTEN* mRNA and protein expression and cell viability but did not change invasive ability. We identified *PTEN* as a target gene for miR-21 using a dual luciferase reporter assay. Our study revealed that miR-21 is a possible driver gene other than *PPM1D* for 17q21-24 amplification in CCC.