論文の内容の要旨

Analysis of gene expression dynamics in circadian clock synchronization and Akt signaling pathway by optical perturbation systems

（概日時計同調機構と Akt シグナル伝達経路における
光摂動系を用いた遺伝子発現動態解析）

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Transmission of genetic information to the expression of a protein is a fundamental process in living cells. Transcriptional regulation including gene expression plays an important role in the process. For example, dynamic changes in the gene expression are observed upon cellular fate decision, adaptation to the environment, and in response to stimulation. Because of its fundamental role in the cellular system, gene expression dynamics are tightly controlled in both space and time. Additionally, transcription is regulated by numerous molecular species including transcripts, proteins, and metabolites. Thus, in order to understand the causative relationships in transcriptional regulation, a framework that is able to analyze these characters is necessary.

Conventionally, a perturbation system to disrupt the cellular system are widely used to analyze the molecular mechanism underlying transcriptional regulation. However, conventional perturbation systems lack either temporal resolution or target specificity of the stimulation. Optical perturbation system, represented by the emerging biological technique called optogenetics, is a promising approach to overcome these issues. Therefore, I aimed to demonstrate that temporally precise and target specific characteristics of the optical perturbation system contribute to the understanding of the causative relationships on transcriptional regulation.

In this thesis, I describe two topics for the analysis of transcription dynamics using optical perturbation system. In the first topic, I focused on the temporal precision of optical perturbation system and selected the circadian clock system as a target of analysis. The circadian clock is a cell-autonomous oscillatory system with the periodic temporal pattern of gene expression. In circadian clock synchronization, a circadian-time dependent process is necessary to reset the

oscillation of circadian timing to a certain phase. To understand the time-dependent process involved in the synchronization, I utilized UV exposure as an optical perturbation which can simulate the circadian clock system in precise timing. Using UV exposure, I describe circadian-time dependent temporal activation pattern of molecules involved in circadian clock synchronization upon UV exposure.

In the second topic, I focused on the target specificity of optogenetics to identify the effect of single protein function on transcriptional regulation. For this purpose, an optogenetic tool to activate the specific protein, Akt kinase, is utilized. In addition, to understand the causative regulation on transcription, the integrative multi-omics analysis was conducted. The integrative multi-omics analysis is an approach to integrate collective information from multiple omics measurement to reveal the network of cellular signaling across different molecular species. By combining these two methods, I describe the signaling network across multiple molecular species induced by activation of Akt. In this network, collective information about the outputs of Akt specific activation is included, leading to the identification of the Akt responsible signaling pathway.

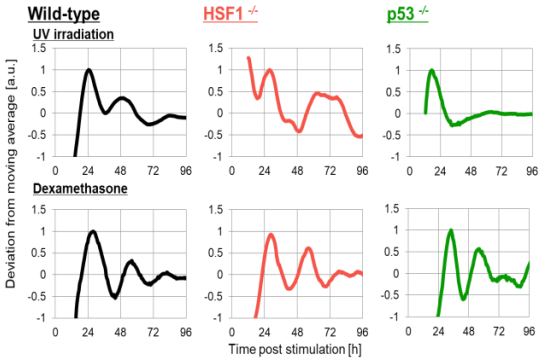

Figure 1. Per2-Luc expression after UV irradiation. Wild-type, HSF1$^{-/-}$ and p53$^{-/-}$ MEFs cells harboring Per2-Luc reporter were irradiated with UV or treated with Dexamethasone for synchronization. Temporal luminescence profiles are shown.

Circadian clock and stress-responsive pathways are reported to have a strong relationship, where synchronization of the circadian clock by stress stimulation engages activation of stress response pathways, and dysfunction of circadian clock alters the function of stress-responsive pathways. I selected UV exposure as a perturbation to induce cellular stress and observed its effect on circadian clock synchronization. Firstly, by using Per2-Luc reporter to monitor the transcriptional regulation on clock gene *Per2*, dose-dependent induction of Per2-Luc after UV irradiation was observed. Upregulation of Per2-Luc was abolished in HSF1 knock-out cell-line, indicating that heat-shock responsive transcription factor HSF1 is necessary for the induction of *Per2* upon UV exposure. In contrast, Per2-Luc measurement after UV irradiation in p53$^{-/-}$ cells demonstrated long-lasting induction of Per2-Luc expression, indicating DNA damage responsive transcription factor p53 is required for down-regulation of Per2-Luc after UV irradiation (**Figure 1**). These results suggest stress-responsive transcription factors HSF1 and p53 are both required

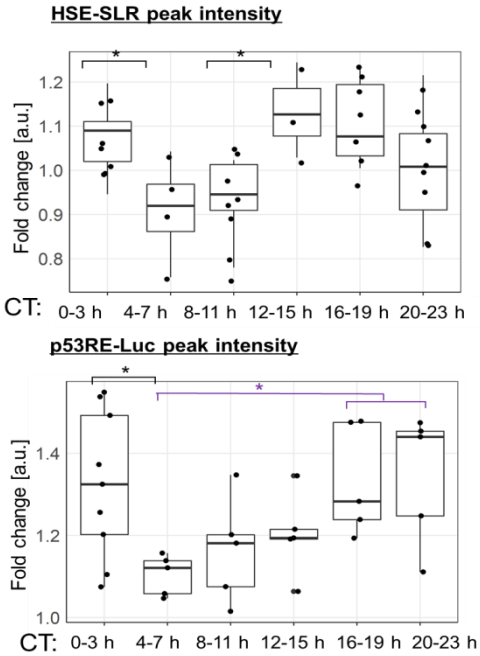

Figure 2. Circadian time-dependent activation of HSF1 and p53 after UV irradiation. Cells harboring HSF1 or p53 reporter (HSE-SLR or p53RE-Luc) were treated with Dexamethasone for pre-synchronization. UV was irradiated at various circadian times and fold change of each reporter were quantified. N = 4, *: p<0.05.

for the synchronization.

I also discovered a sequential relationship between HSF1 and p53 upon UV irradiation. Reporter gene assays revealed that p53 activity was altered in HSF1 knock-out cells, while HSF1 activity was not affected by the p53 deficiency. For the molecular basis of HSF1 and p53 relationships, I hypothesized a protein-protein interaction exists for transcriptional regulation of p53 by HSF1. Split luciferase complementation assay of HSF1－p53 interaction showed interaction increase after UV irradiation, suggesting that HSF1－p53 interactions modulate p53 transcriptional activity.

Finally, owing to temporally precise delivery of UV exposure, I analyzed circadian time-dependent activation of HSF1 and p53 by the reporter gene assays. Circadian time-dependent phase shift was observed in Per2-Luc, which implies UV-induced synchronization is time-dependent. HSE and p53RE were also time-dependently activated, suggesting that time-dependent synchronization is associated with time-dependent responses of stress-responsive transcription factors, HSF1 and p53 (**Figure 2**). Taken together, it was revealed that circadian time-dependent synchronization was induced by time-dependent activation of stress-responsive transcription factors, HSF1 and p53. This result demonstrates that the optical perturbation system is beneficial for the analysis of temporal regulation of gene expression dynamics.

Serine-threonine kinase Akt is a hub signaling protein in the insulin signaling with activation pattern highly correlated to that of insulin. Akt is regarded as a regulator of insulin-mediated metabolic processes. However, the extent to which Akt is responsible for controlling the metabolic pathways remains elusive. By utilizing optogenetics as an optical perturbation tool that can specifically control Akt activity, I aimed to construct a signaling network responsive to Akt activation from multiple omics measurement by globally identifying signaling molecules affected by the optical perturbation.

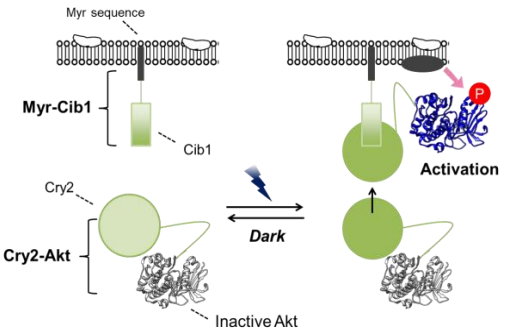

Figure 3. Design of optical tools for the analysis of cellular signaling. Schematics of the PA-Akt system. Cry2-Akt upon illumination translocates to the plasma membrane and is subsequently phosphorylated by upstream kinases.

Optogenetics is a biological method that utilizes light to control protein function. To manipulate the activity of Akt, Photoactivatable Akt (PA-Akt) system consisting of Cry2 fused Akt (Cry2-Akt) and plasma membrane-anchored Cib1 (Myr-Cib1) was used to trigger subcellular translocation of Cry2-Akt upon light stimulation thereby activating Akt (**Figure 3**). Using PA-Akt system, selective activation of Akt is achieved. In order to analyze the effect of Akt perturbation across multiple molecular species, quantification of the phosphorylation level of Akt substrates, transcripts, and metabolites was performed. These results were then integrated to identify responsible signaling pathways. The results of Akt specific activation were compared with that of insulin stimulation to identify signaling pathways that Akt is responsible to regulate.

I first evaluated the functionality of PA-Akt system in insulin target cells. For this aim, temporal dynamics of subcellular localization changes of Cry2-Akt upon light illumination were analyzed using a confocal microscope. Light-induced localization changes to the plasma membrane and subsequent diffusion to the cytosol in the dark condition was observed, demonstrating reversible

activation of PA-Akt system. Next, photo-induced activation of Cry2-Akt activity was tested by quantifying the phosphorylation level of Cry2-Akt after the illumination. Since temporal activation pattern of signaling molecules affects activity pattern of downstream molecules, light illumination pattern for PA-Akt system was adjusted to induce phosphorylation of Cry2-Akt analogous to that of insulin stimulation. The phosphorylation level of Cry2-Akt increased along with illuminated time, as observed with insulin stimulation. These results indicate that PA-Akt system allows selective activation of Akt.

I then investigated the phosphorylation pattern of Akt substrates in 3T3-L1 adipocyte and C2C12 myocyte cells. After Akt specific activation, temporal phosphorylation pattern of Akt substrates and a kinase from Akt independent pathway, Erk, was measured (**Figure 4**). The result suggests that selective activation of Akt is not sufficient to induce phosphorylation of some of Akt substrates. Transcriptional regulation by Akt specific activation was analyzed by the transcriptome analysis. Of measured 13,561 transcripts, 444 transcripts were changed by specific Akt activation. Among them, 100 transcripts were also changed by insulin stimulation. This result suggests that transcriptional regulation by Akt-specific activation is limited to certain genes. From the collected mass spectrometry data, an abundance of 128 metabolites was analyzed. A number of changed metabolites was different between two stimulations; 13 metabolites were common, and 15 in total were stimulation-dependent. Commonly changed and insulin specifically changed metabolites included molecules from the glycolysis pathway. Finally, the integrative multi-omics analysis identified that among the glycolysis pathway, regulation induced by Akt-specific activation was limited to lower glycolysis, while regulation of upper glycolysis requires non-Akt pathway for the activation. Taken together, a combination of specific molecule activation by optogenetics and integrative multi-omics analysis succeeded in the identification of Akt responsible metabolic pathways, demonstrating molecule specific activation by the optogenetics contributes to the analysis of the regulation mechanism of a specific molecule.

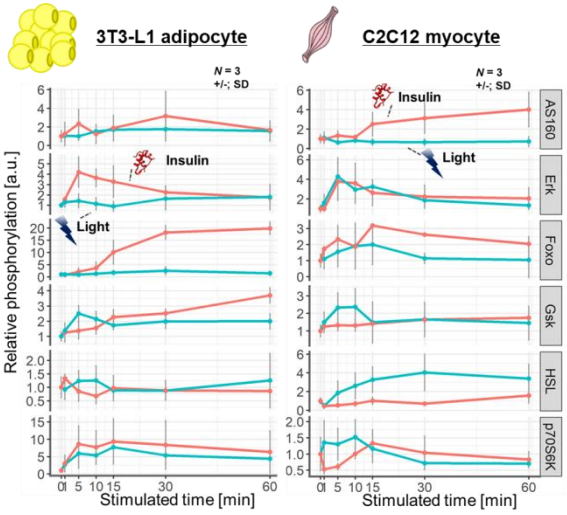

Figure 4. Quantification and comparison of phosphorylation of Akt substrates induced by Akt specific activation and insulin stimulation.
Temporal phosphorylation level changes of Akt substrates, AS160, FoxO, Gsk, HSL, p70S6K and non-Akt substrate, Erk after each stimulation was quantified. n = 3, +/-: SD.

In this dissertation, I described two examples of transcriptional regulation analysis using optical perturbation systems. First, I demonstrated that precise perturbation of the circadian clock system contributes to the identification of time-dependent processes. Second, owing to the molecule specific activation by the optogenetic tool, I succeeded in identifying the molecular mechanism, including the regulation of transcripts, driving the Akt-dependent metabolic pathways. These analyses represent that the character of an optical perturbation system is beneficial for the analysis of gene expression dynamics, which would pave the way for further understanding of biological phenomena.