論 文 の 内 容 の 要 旨

Thesis Summary

論文題目 **Profiling of Antimicrobial Drugs Based on Yeast Single Cell Phenomics**
（酵母の単細胞フェノミクスに基づく抗菌薬のプロファイリング）

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[INTRODUCTION]

Antimicrobial drug discovery is the most challenging field (Livermore, 2011; Silver, 2011). For several decades, most of the drug discovery efforts have concentrated on target-based screens which generates protein disease model first and finds a candidate lead molecule. In contrast, less-biased phenotypic screening approaches shown promises in potentially improving success rates of drug development (Eggert, 2013; Schenone *et al.*, 2013). For comprehensive drug screening, various chemical genomic approaches have been developed to systematically search for targeted chemical libraries of compounds that potently and selectively modulate the functions of target proteins. Among others, single-cell phenomics technology, a microscopy-based chemical genomic method, has emerged as a critical set of tools for enhancing the power of analyses (Ohya *et al.*, 2015). It involves the acquisition of high-dimensional phenotypic profiles of a single cell in response to drug stimuli on a genome-wide scale from which the drug target is estimated by comparing morphological profiles of chemical perturbation with that of gene disruption. Using image-processing system CalMorph, Ohnuki *et al.* (2010) demonstrated that a drug target could be predicted from dose-dependent morphological changes induced by the drug. Later, the high-dimensional morphological data generated in a similar way has been exploited in identifying new targets of drugs as well as exploring their mode of action (Iwaki *et al.*, 2013; Okada *et al.*, 2014; Poitrowiski *et al.*, 2015). Though it is considered that our current approach is powerful, much more simpler, efficient and complementary methods are required as significant tools for drug discovery and development. Therefore, I envisaged gaining further insights into the currently available antimicrobial drugs, and propose a new method(s) for discovering molecular targets and mode of action of novel drugs.

[RESULTS and DISCUSSION]

1. Morphological profiling of the antifungal agents

1.1 Expected similarities

To confirm the MoA of the antifungal agents, a comparison was made between morphological profiles of the cells treated with the agents and those of non-essential deletion mutants with defects in their MoA-related genes. Treatment with two ergosterol biosynthesis inhibitors (FCZ and TBF) resulted in significant morphological similarities with an *erg28*, a mutant of endoplasmic reticulum membrane protein (Mo *et al.*, 2004), while AMF's target was established to be Erg2, a mutant of C-8 sterol isomerase (Rahier *et al.*, 2008) required for ergosterol biosynthesis (Fig. 1).

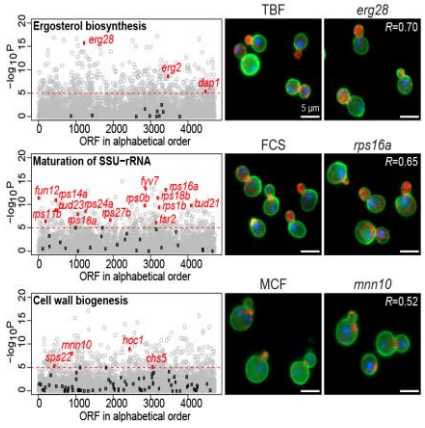

Fig.1. Morphological profiling of antifungal agents. WT cells treated with different concentration of each drugs and morphological profiles were compared with that of 4718 non-essential gene deletion mutants.

FCZ: fluconazole; **TBF**: terbinafine; **AMF**: amorolfine; **FCS**: flucytosine; **ECB**: echinocandin B; **MCF**: micafungin; **HXU**: hydroxyurea; **CMA**: concanamycin A; **BMC**: bleomycin; **MCZ**: miconazole; **NMZ**: nikkomycin Z; **LVS**: Lovastatin; **TCM**: Tunicamycin; **HYG**: hygromycin B; **BTZ**: bortezomib, **BML**: benomyl; **CAF**: Caffeine; **MoA**: mode of action

Though targets of FCZ and TBF are essential genes, our similarity search system uncovered functionally related genes in the pathway. FCS, a nucleic acid biosynthesis inhibitor, treated cells were similar to SSU-rRNA mutants (Fig. 1). Moreover, the morphology of cells treated with two cell wall biosynthesis inhibitors (ECB and MCF) shared significant similarity with that of two mutants (*hoc1* and *mnn10*), which have defects in "cell wall biogenesis" (Fig. 1). Analysis of ECB-MCF correlation revealed strong similarity between cell wall drugs. This is reasonable because even if the two compounds are distinct but of the same target. This indicates the validity of morphometric approach in finding likely targets of the drugs including previously unreported ones.

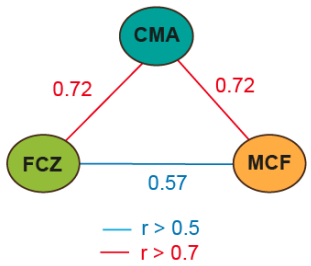

Fig.2. Correlational network. To construct the network map, the correlation coefficient of the morphological profile was determined from 102 PC scores of a pair of agent considered as estimated by the morphological profiling approach.

1.2 Unexpected similarities

Strikingly, I found some unexpected correlation between the cells treated with ergosterol-affecting agent (FCZ) and vacuolar proton-transporting V-type ATPase (V-ATPase)-affecting agent (CMA) (Fig. 2). The GO term analysis as well as phenotypic profiling revealed high morphological similarity between the ergosterol-affecting agents (FCZ and AMF) and *vma* mutants (Fig. 3). These results suggested a role of ergosterol in V-ATPase function, although there is little indirect evidence (Zhang *et al.*, 2010). Importantly, cells treated with ergosterol inhibitors showed reduction in ergosterol content as well as of vacuolar quinacrine fluorescence in wild-type yeast cells (Fig. 4), implying that the antifungal drugs had impaired vacuolar acidification. Taken together, these data suggest that ergosterol depletion is a likely mechanism of antifungal activity for disrupting V-ATPase function. Likewise, GO term analysis using mutants that were similar to ECB- and MCF-treated cells showed enrichment in several genes related to vacuolar function. I assumed that this unexpected similarity might be due to the cell wall perturbation induced by defective vacuoles. To this effect, robust morphological similarity observed between inhibitors of cell wall synthesis (ECB, and MCF) and the V-ATPase inhibitor (CMA) may support this idea (Fig. 2). Finally, I asked whether this association could be explained by cell wall defects across the cells treated with those antifungal drugs of two different classes. To comprehend the impact of drug treatments on the cell wall structure, the susceptibility of yeast cells to zymolyase was assessed (Lussier *et al.*, 1997; Ovalle *et al.*, 1998). I found that preincubation of yeast cells with CMA and cell wall-affecting drugs resulted in an increased sensitivity to zymolyase compared with the mock-treated cells (Fig. 4). Therefore, I concluded that unexpected similar phenotypes between V-ATPase- and cell wall-deficient cells are due to the functional connection between these two cellular processes.

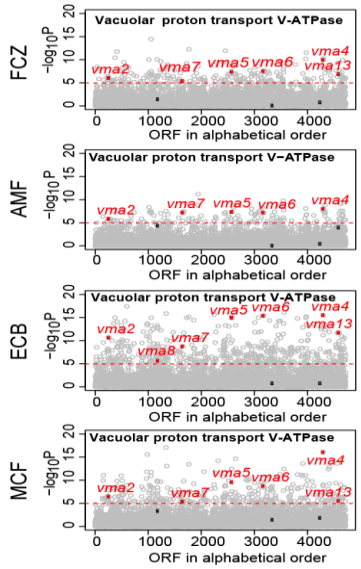

Fig.3. Morphological analysis of ergosterol and cell wall agents for V-ATPase mutants' profile.

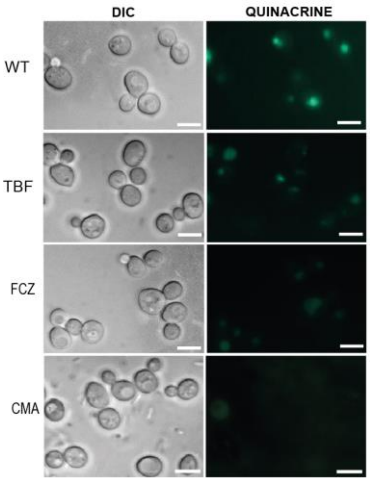

Fig.4. Ergosterol inhibitors disrupted the V-ATPase function. 5h drug-treated cells stained with quinacrine.

2. New phenotypic profiling method for identifying the targets of bioactive compounds

Though comparison between chemical-induced phenotypes and genetic perturbation is a powerful tool to understand the MoA of antifungal agents, it requires mutant information in advance. I aimed to develop a simpler method that classifies antifungal agents without any mutant information. Therein, by introducing machine learning technique, linear discriminant analysis was performed on quantified morphological data of training dataset (FCZ, TBF, AMF, FCS, ECB, MCF)

and the resulting linear discriminant (LD) scores of the phenotypic responses were depicted on 2-dimesional space to visually classify the features of each agent (Fig. 5, colored circles). When the test agents form a group with a particular training set compound, they are likely to have similar cellular targets. As proof-of-principle, I used 10 characterized compounds which the system classified the modes of action of the small molecules.

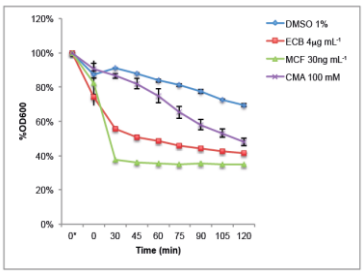

Fig. 4. Zymolyase assay. The sensitivity was determined after 4h incubation of V-ATPase and cell wall drugs to Zymolyase digestion.

Of these test compounds, the new image-profiling method suggested caffeine and nikkomycin Z, known to affect yeast cell wall, were significantly associated with a cluster of distinguished cell wall disrupting drugs, echinocandin B and micafungin (Fig. 5). Some of the nucleic acid synthesis interfering compounds such as hydroxyurea, bleomycin, and hygromycin B were also classified along with FCS, an inhibitor of fungal DNA and RNA synthesis and protein translation (Fig. 5). Miconazole and amorolfine, which interfere with fungal sterol synthetic pathway, were classified into cluster of ergosterol synthesis inhibitor (Fig. 5). Intriguingly, the systematic classifier drew new insights into antifungal agents. For example, miconazole was assigned to ergosterol biosynthesis inhibitors, but plotted near DNA affecting agents' class.

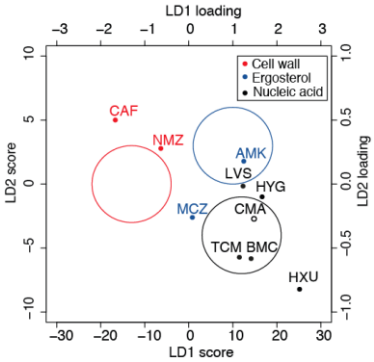

Fig. 5. Target profiling of test antifungal compounds. Classification of antifungal drugs was made on the basis of their difference in morphological profiles.

Recent mechanistic studies (Najim *et al.*, 2015) showed that miconazole affects pathways regulating DNA synthesis via interfering the role of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (MEK) signaling in estrogen receptor positive MCF-7 breast carcinoma cells. This suggests the power of the method in efficiently indicating drugs MoA from their morphologic signatures. Though I expected CMA to be classified into cell wall synthesis inhibitors, considering its MoA, it was notably ascribed to a different drug group - nucleic acid inhibitors (Fig. 5). To my surprise, a very recent research evidences demonstrated an unexpected finding that the yeast vacuole plays a positive essential role in initiation of the cell-cycle and its functional loss results in a specific arrest of cells in G1 phase (Jin & Weisman, 2015). Taken together, these data indicate that high-content system was successful in profiling phenotype by drug function.

3. Sensitive and high-throughput profiling of the responses of antimicrobial agents from single cells

Though the significance of natural products as well as image analysis to the modern drug development is still considerable, drug discovery has become frustratingly inefficient that requires new paradigms. To maximize the efficiency of high throughput screening, I sought to develop and benchmark a novel image-profiling pipeline that links bioactive natural products to their cellular targets using small amount of bioactive compounds. To this end, I developed an integrated method that combines high-content microscopy and yeast non-essential deletion mutant collection in drug-hypersensitive background. Dose-dependent morphological phenotypes of the drug-hypersensitive strain were compared with the new panel of morphological data composed of ~2,000 representative mutant strains. Using smaller amount of six well-characterized compounds (hydroxyurea, bortezomib, Methyl Methanesulfonate, echinocandin B, tunicamycin and benomyl), the scheme successfully established the target genes of these compounds and

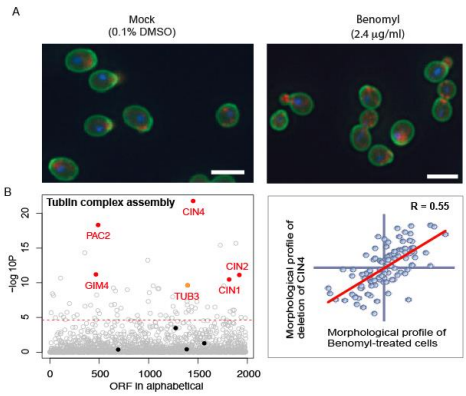

Fig. 6. Morphological profile of benomyl. (A) Morphological changes induced BML treatment. (B) The comparison of drug-induced morphological profiles with 1984 of nonessential gene deletion in triple mutant background established the target genes. R is correlation coefficients between CIN4 mutant and benomyl-treated cells.

functionally related genes in the cellular pathways (for example, benomyl; Fig. 6), demonstrating the applicability of this approach to prospective new drug discovery.

[Conclusion]

In a nutshell, high-content profiling of antifungal drugs increased our understanding of yeast cell biology and pharmacology of antifungal agents. The alternative profiling system that focuses on predicting drug target without mutant information has successfully classified well-characterized compounds and can be used to develop new compounds. A more sensitive high throughput profiling method was also established to determine the drug target and to make it possible to find a new compound with unknown mechanism. I believe that this strategy will facilitate the development of drugs for rare and/or neglected diseases as well as potentially useful novel compounds from scarce natural products to treat various diseases.

[Publication]

Abraham Abera Gebre, Hiroki Okada, Cholgwang Kim, Karen Kubo, Shinsuke Ohnuki, Yoshikazu Ohya. (2015). **Profiling of the effects of antifungal agents on yeast cells based on morphometric analysis**. *FEMS Yeast Res.* 2015, 15(5): fov040. Doi: 10.1093/femsyr/fov040.

Jeff S. Piotrowski, Sheena C. Li, Raamesh Deshpande, Scott W. Simpkins, Justin Nelson, Jacqueline M. Barber, Hamid Safizadeh, Hiroki Okada, Abraham Abera Gebre, Karen Kubo, Nikko Torres, Marissa Leblanc, Kerry Andrusiak, Reika Okamoto, Mami Yoshimura, Yoko Yashiroda, Katsuhiko Shirahige, Anastasia Baryshnikova, Grant Brown, Tamio Saito, Michael Costanzo, Yoshikazu Ohya, Hiroyuki Osada, Minoru Yoshida, Chad L. Myers, Charles Boone. **Functional Annotation of Chemical Libraries across Diverse Biological Processes**. (Submitted)