<div align="center">論文の内容の要旨</div>

論文題目　　Applications of RNA Interference in Analyzing Biological Pathway and Gene Therapy: Analysis of YY1/p73 Pathway and Application of PHD2-silencing in Angiogenesis
(RNA interference のバイオロジカルパスウェイ解析と遺伝子治療への応用： YY1/p73 パスウェイの解析と PHD2 サイレンシングを用いた血管新生応用）

氏　　　名　　呉　寿栄

Introduction

RNA interference (RNAi) was first discovered in 1998 by Fire *et al*. in *Caenorhabditis elegans*, and has been found in evolutionarily diverse organisms, such as plants, flies and protozoan. It is a naturally occurring mechanism that controls gene expression at the post-transcriptional level caused by double-stranded RNAs (dsRNAs). Soon after its discovery, RNAi was shown to be able to achieve in mammalian cells using small-interfering RNAs (siRNAs), an intermediate product in the RNAi pathway that could bypass the interferon response, which is known to be induced by long dsRNAs. In RNAi, dsRNAs were cleaved into of 21-23 nucleotides (nts) siRNAs by Dicer, a member of the RNAse III family. These siRNAs are then incorporated into RNA-Induced Silencing Complexes (RISCs), which then bind to the target mRNA having sequences complementary to the incorporated siRNA, leading to the degradation of the target mRNA, and subsequently, resulted in the selective silencing of specific proteins. This characteristic of RNAi makes it a valuable tool both in laboratory research and therapeutic field.

In term of laboratory research, the use of RNAi has already led to the characterization of novel oncogenes, including protein kinase Cε (PRKCE) in breast cancer, abnormal spindle homologue, microcephaly associated (ASPM) in glioblastoma, and mutant KRAS in non-small-cell lung cancer.

Meanwhile, in the therapeutic field, besides its high target specificity and knock down effect, the relatively short turnaround time for efficacy testing of potential therapeutic RNAi molecules, and the fact that even newly discovered pathogens are theoretically amenable to rapid targeting have caused great excitement about the potential of RNAi for treating a wide range of diseases. In fact, the first clinical applications of RNAi

have been directed at the treatment of wet, age-related macular degeneration (AMD) and respiratory syncytial virus (RSV) infection. Furthermore, therapies based on RNAi are also in preclinical development for other viral disease, neurodegenerative disorders and cancers.

In the first part of this thesis, I described the application of RNAi technology in studying genes involved in the transcriptional activity induction of p73, a structural homologue of p53 gene, and I found a novel cooperation of Yin Yang 1 (YY1) and E2F1 in inducing it transcriptional activity. In the second part, I described an example for the application of RNAi-based technology in therapeutic field, i.e., using RNAi for silencing Prolyl Hydroxylase Domain 2 (PHD2) gene, and subsequently, enhanced angiogenesis.

Yin Yang 1 induces transcriptional activity of p73 through cooperation with E2F1

Yin Yang 1 (YY1) is a multifunctional transcription factor, which can act as transcriptional activator, repressor, or initiator element binding protein. Nowadays, YY1 has already been known to have a fundamental role in normal biological processes such as embryogenesis, differentiation, DNA replication, and cellular proliferation. Additionally, YY1 has also been shown to be involved in neuronal differentiation. Recently, YY1 has been reported to possess a potential function in cancer biology. It is important to explain the biological function of YY1 via studying the putative interactions between YY1 and cell cycle regulators, death genes, and furthermore, transcription factors and cofactors in the suppression or progression of various physiological phenomena.

Transcription factor p73 is a structural homologue of tumor suppressor gene p53 and plays an important role in tumorigenesis, cellular differentiation and development. Although recently transcription factor E2F1 has been identified as a vital regulatory factor for p73 transcription, the upstream and downstream regulation of p73 pathway remains to be understood.

To understand the upstream regulation of p73 pathway, I established a p73-promoter based luciferase reporter system for performing screening using siRNA expression vectors. During the initiation process of searching genes related with E2F1, I identified the transcription factor YY1 as a novel regulator of p73 transcription. In contrast to its function in p53 pathway, in which knocking down of YY1 resulted in the enhance of p21, a p53 downstream gene, I found that co-transfecting YY1-siRNA vectors and p73 luciferase

reporter vectors led to the decrease of both p73 luciferase reporter activity and p73 message level for around 60%, indicating that YY1 might act as a positive regulator of p73 promoter. Consistently, overexpression of YY1, as well as E2F1, increased the activity of the p73 luciferase reporter vectors in a plasmid-dose dependent manner. Together, these results had shown the unsuspected role of YY1 in regulating p73 transcription level, i.e., the possibility that YY1 upregulate the p73 promoter activity.
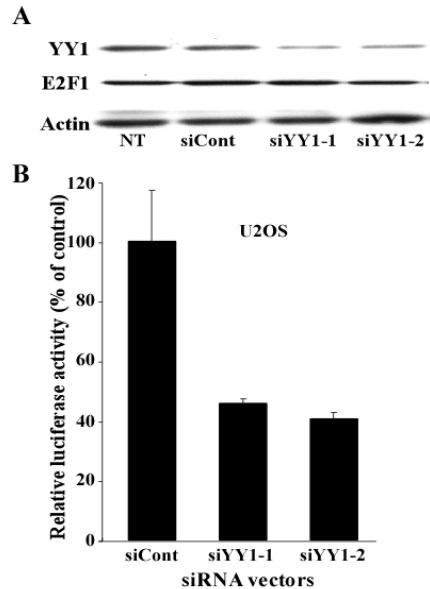

Figure 1. (A) Western blotting results demonstrated specific knockdown effect of YY1-siRNA vectors. (B) Knocking down of YY1 using YY1-siRNA vectors led to the decrease of the activity of p73 luciferase reporter.

On the other hand, it has been reported that E2F1 could recruit the p73 promoter and activate its transactivation. To further reveal the regulatory mechanism of p73 by YY1, we overexpressed YY1 and E2F1, and found out that compared to the overexpression of either YY1 or E2F1 alone, the transcriptional activity of p73 was further enhanced when both of them were overexpressed together, suggesting that YY1 could regulate p73 transcriptional activity in a synergistic fashion with E2F1. This synergistic effect of YY1 and E2F1 on p73 promoter is also implicated in physiological event, as in the cells in which YY1 or E2F1 was knocked down, the induction of the activation of p73 luciferase reporter using a doxorubicin, a DNA damaging reagent which has been known to activate p73 promoter E2F1-dependently could be suppressed to 50%. Moreover, through immunofluorescence staining and immunoprecipitation experiments, we found that YY1 and E2F1 sublocalized together mainly in the nuclei, and associated physically.

Figure 2. Overexpression of both E2F1 and YY1 together resulted in further enhancement of the activity of p73 luciferase reporter.

These data demonstrated two novel roles of YY1. First, YY1 could function as a cooperator of E2F1, a mediator of cellular proliferation. The

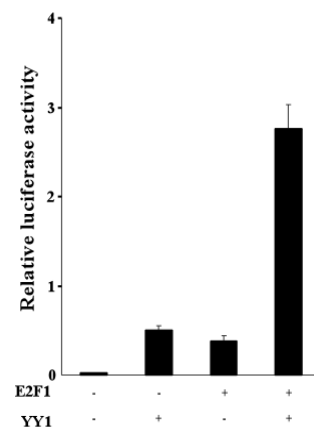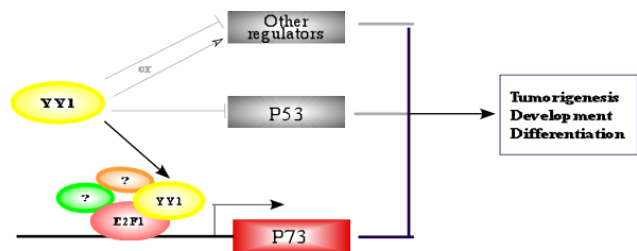Figure 3. A model of the co-regulation of YY1 and E2F1 on p73

cooperation between E2F1 and YY1 may be at least partially responsible for determining the physiological

function (i.e. oncogenic or tumor suppressive function) of E2F1 by modulating specificity and sensitivity of E2F1 on different promoter. Second, YY1 could be involved in the regulation of p73, which has been reported to be able to promote cellular growth in a synergistic manner with the proto-oncogene c-Jun. Data in this study that tumor activator gene YY1 up-regulates p73 transcription is also consistent with this result. Altogether, I demonstrated an example for using RNAi technology in analyzing biological pathway, and uncovered a novel function of YY1 on the E2F1-mediated p73 regulation, indicating its roles in tumorigenesis, development and differentiation.

Enhancement of Angiogenesis through Stabilization of Hypoxia Inducible Factor-1 by Silencing Prolyl Hydroxylase Domain 2 Gene

Therapeutic angiogenesis aims to improve neovascularization in ischemic tissues by delivery of angiogenic factors or DNA vectors encoding those proteins. Vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF2) are pro-angiogenic molecules and well investigated for therapeutic angiogenesis in previous studies. Therapeutic angiogenesis using these growth factors has been extensively investigated. However, although phase I clinical trials already had shown that the delivery of angiogenic proteins was safe, phase II trials have not demonstrated efficacy as expected, suggesting that the administration of a single angiogenic growth factor might be insufficient to induce functional vessels. Based on these disappointing results, therapeutic angiogenesis studies were altered into the combinatorial application of angiogenic growth factors. Combination of VEGF plus FGF2, VEGF plus angiopoietin-1 (Ang1), VEGF plus platelet-derived growth factor-BB (PDGF-BB), and PDGF-BB plus FGF2 have been reported to have a synergistic effect on neovascularization in experimental animal models. Collectively, these results showed that angiogenesis is a complex process, which includes the temporally and spatially orchestrated expression of multiple angiogenic factors induced by multiple cell types.

Hypoxia inducible factor-1 (HIF-1) is a heterodimeric α/β transcription factor that functions as a central regulator of oxygen homeostasis, which is degraded O_2-dependently. In oxygenated cells, HIF-1 has a very short half-life (less than 5 min at 21% O_2), while the reduction of O_2 concentration enhances HIF-1 stability by suppressing its ubiquitin-proteasome degradation. More than 60 HIF-1 target genes have been identified

so far, including VEGF and other angiogenic factors. Transgenic expression of HIF-1 in mouse skin induced the increased vascularization without excessive permeability. Furthermore, constitutively active form of HIF-1 is sufficient to induce angiogenesis in nonischemic animal tissue.

Meanwhile, in mammalian cells, three prolyl hydroxylase domain (PHD) protein isoforms, PHD1, PHD2 and PHD3 have been determined to hydroxylate two prolyl residues (P402 and/or P564) in the oxygen-dependent degradation domain (ODDD) of HIF-1. Under the normoxic condition, PHD2 is the key limiting enzyme targeting HIF-1α for hydroxylation, which subsequently led to the ubiquitination and proteosomal degradation of HIF-1α.

Figure 4. Western blotting results demonstrated that silencing of PHD2 using PHD2-siRNA vector resulted in the upregulation of HIF-1α

Here I demonstrated the possibility of a potent molecular therapy for therapeutic angiogenesis using RNAi technology, i.e., by silencing PHD2 in order to enhance the ex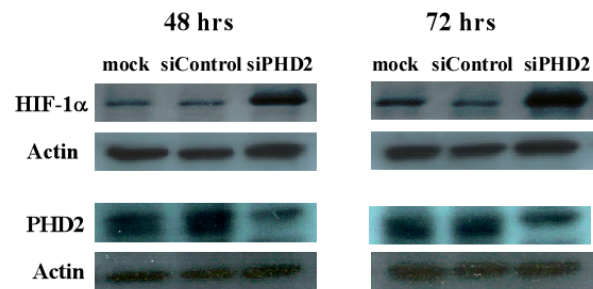pression of angiogenic growth factors and to induce neovascularization through HIF-1 activation. siRNA and siRNA expression vectors were used for silencing murine PHD2 expression, which resulted in the stabilization of HIF-1, but not HIF-2, and subsequently, in the upregulation of multiple angiogogenic growth factors, especially VEGF and FGF2, in mouse fibroblast cells. Moreover, by using PHD2-siRNA vector, the increase of VEGF secretion was observed for as long as 18 days post-transfection. Furthermore, *in vitro* treatment of human umbilical vein endothelial cells (HUVECs) with conditioned medium from PHD2-siRNA transfected NIH3T3 cells increased cells proliferation.

Finally, i*n vivo* experiment showed that implantation of Matrigel plugs mixed with NIH3T3 cells transfected with PHD2-siRNA vector is sufficient to induce angiogenesis in mice.
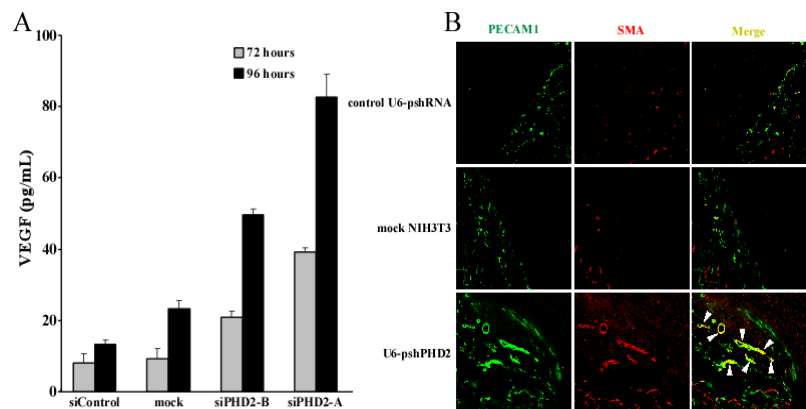

Figure 5. ELISA results showed that the silencing of PHD2 using PHD2-siRNA vector resulted in the up-regulation of VEGF protein (A), and subsequently, the increase of angiogenesis in experimental animal model was observed in the immunohistochemistry (B) against a vascular endothelium marker, platelet/endothelial cell adhesion molecule 1 (PECAM1, green), and a pericytes marker, smooth muscle-alpha actin (SMA, red).

These results indicated that PHD2 silencing induces expressions of multiple angiogenic growth factors by stabilizing HIF-1α, and that implantation of PHD2-knocked down cells is sufficient to induce angiogenesis *in vivo*. Thus, RNAi-mediated PHD2 silencing might offer a potential tool for angiogenic therapy.

YY1 Interacts with Hypoxia-Inducible Factor 1α and Inhibits Transcriptional Activity of HIF-1α

Hypoxia inducible factor 1 (HIF-1) is a heterodimeric α,β transcription factor that functions amaster regulator of oxygen homeostasis in metazoan species. HIF-1 is expressed in most tissues, but HIF-1 activity is determined by the expression and activity of itsα subunit. The half-life of the HIF-1α is determine by oxygen-dependent prolyl hydroxylation, which is required for hydroxylation of Prolyl hydroxylase domain proteins (PHD) and binding of the von Hippel-Lindau protein (VHL), the recognition component of an E3 ubiquitin ligase that targets HIF1-a for ubiquitination and degradation. Here, we demonstrate that Yin Yang 1, the protein product of a widely expressed gene, is involved in the functional activation of HIF1-α. YY1 gain-of-function promotes the inhibition of HIF-1-mediated transcription. YY1 loss-of-function caused by RNA interference increases HIF-1α protein levels, HIF-1-mediated transcription under nonhypoxic conditions. These data indicate that YY1 is an essential component of a multiprotein complex that regulates HIF-1α levels in an O_2-dependent manner, and YY1 has an important role in the hypoxia signaling.
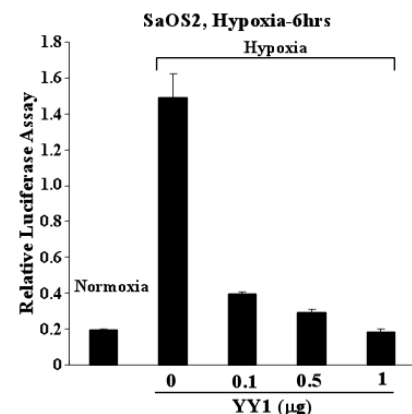

Figure 6 Overexpression of YY1 could suppress the activation of HIF-1α induced by hypoxia

Conclusions and Future Perspectives

In this thesis, firstly, I developed a method for analyzing the genes involved in the E2F1 signal introduction by using the vector-based shRNA library. During the initiation process of searching genes related with E2F1, I found the previously unsuspected role of YY1 on the E2F1-mediated p73 regulation. The fact that YY1 affects p53 family members opened up an attractive possibility that needs further investigation, that is, YY1 might function as a key integrator or modulator of various pathways in the network that includes p53 family members and regulators concerned with various biological processes. Then,

YY1 might be a good tarter for cancer therapy. Here, I also showed that the genome-wide RNAi approach is a powerful tool in gene function analysis and target identification. As for the disease therapy, this technique enable high-throughput screening, thus, the cost, time, validation and others aspects of drugs discovery could be significantly reduced. In addition, in chapter `four` of this thesis, by using gene-loss and gene-gain assay our data firstly indicate that YY1 is an essential component of a multiprotein complex that regulates HIF-1α levels in an O_2-dependent manner, and YY1 has an important role in the hypoxia signalling. The further study is required for analyzing the role of YY1 in hypoxia signalling.

In the RNAi-based angiogenesis study, we have demonstrated that implantation of cells, of which PHD2 were silenced, induced neovascularization in the *in vivo* Matrigel plug assay, providing a basis for further therapeutic application. For practical therapeutic applications, the delivery systems should be developed to deliver the siRNA expression vector and/or siRNA to the target sites and to have them function in an efficient and safe manner. In this regard, our laboratory have explored polymeric micelle-based nanocarriers for plasmid DNA and siRNA, and demonstrated their *in vitro* and *in vivo* efficacies, including efficient gene transfer to primary cells, *in vivo* transfection to a rabbit carotid artery, and transfection-mediated bone regeneration. Thus, I will further investigate the therapeutic effects of PHD2-silencing on neoangiogenesis by using polymeric micelle-based nanocarriers.

Collectively, RNAi not only has a profound effect on studies of gene regulation, it has also led to the development of a new class of therapeutic agents based on small dsRNA. I believe that the results shown in this thesis contribute to the further application of RNAi in both gene regulation and RNAi-based therapeutic fields in human disease.