Chemical biology research on the UCHL1–HIF axis toward development of molecular targeted anticancer drugs

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Tittle

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Abstract

Ubiquitin carboxyl-terminal hydrolase L1 (UCHL1) is a 223-amino acid protein that belongs to the UCH family of deubiquitinating enzymes.[1] UCHL1 catalyzes the release of a ubiquitin (Ub) moiety from C-terminal extended Ub, such as Ub precursors and C-terminal Ub thioesters, to generate a monomeric Ub pool via *de novo* synthesis and salvage pathways.[2] It is also reported that UCHL1 digests poly-Ub chains conjugated to β-catenin by SCF$^{β\text{-Trcp}}$ E3 ligase complex.[3] Conversely, homodimeric and membrane-associated farnesylated UCHL1 is able to catalyze the formation of Lys48-linked poly-Ub chains on α-synuclein by acting as an E3 Ub ligase.[4]

UCHL1 was originally identified as a brain-specific protein, known as protein-gene-product (PGP) 9.5, by two-dimensional polyacrylamide gel electrophoresis analysis.[5] The distribution of this enzyme in normal tissue is known to be highly limited, particularly in brain[6], and moderately expressed in the pancreas[7], testis[8], and urinary bladder.[9] Aberrant expression of UCHL1 has been reported in renal cancer[10], non-small cell lung cancer[11], gastric cancer[12], and colorectal cancer[3]. In colorectal tumor tissues, UCHL1 was shown to activate the β-catenin/TCF signaling pathway by releasing an Ub moiety conjugated to β-catenin, resulting in the promotion of tumor malignancy[13]. Previously, it was reported that UCHL1 functions as a deubiquitinating enzyme for the stabilization of HIF-1α while blockade of the UCHL1–HIF-1α pathway suppresses the formation of metastatic tumors in mouse models[14]. Since hypoxia-inducible factor (HIF) is known to have pivotal roles in tumor malignancy[15] and is considered a promising molecular target for cancer chemotherapy[16]. UCHL1 is a promising therapeutic target for tumor malignancies.

Despite UCHL1's significant promoting effects on metastasis and inducing effects on the exacerbation of malignant tumors, the development of inhibitors targeting UCHL1 is lagging behind. LDN57444 is the only specific inhibitor of UCHL1 that has been developed so far. A strategy using WP-1130 targeting the inhibition of the entire UCHL1 family of UCHs has been proposed to eliminate the abnormal UCHL1-stabilization of HIF-1α, but the protein expression of tumor protein p53 (p53) was also downregulated in this strategy, indicating that this approach is not suitable for application as an ideal HIF-1α inhibitor because of its side effects. On the other hand, LDN57444, which is a specific inhibitor of UCHL1, is also considered not applicable clinically because it induces nonspecific apoptosis due to endoplasmic reticulum stress and abnormal expression of synaptic proteins. Therefore, the development of an effective UCHL1 inhibitor with low side effects is a significant challenge in the treatment of malignant tumors.

In the development of new drugs, both *in vitro* and *in silico* approaches are widely used. Typical *in vitro* approaches are high-throughput screening, biological microarray, phenotypic screening, etc. Since it highly relies on the availability of sample libraries, *in silico* approaches, such as signature matching, virtual screening and semantic network analysis have been developed and becoming increasingly popular in drug discoveries. Compared with traditional *in vitro* approaches, *in silico* approaches are usually considered less accurate as the results are based on predictions, which means the source and quality of used data or

algorithms is crucial. Therefore, combining both two approaches has gradually become a mainstream method in drug development nowadays.

In this research, a consistent work on the demonstration of the effectiveness of UCHL1 inhibitors in HIF-1 dependent tumor malignancy and a novel UCHL1 inhibitor was successfully developed through computational drug repositioning and bioassays **(Figure 1)**. I firstly proved in spheroid models, which mimics tumor tissues in cultured cells, the feasibility of UCHL1 inhibition against HIF-1-related tumor malignancy (**Chapter 1**). Next, a highly potent UCHL1 inhibitor was discovered through *in vitro* high-throughput screening and *in silico* instructed structural modification. Finally, this novel inhibitor was found to show improved specificity and high potency in blocking the UCHL1–HIF-1 axis using cell models, leading to inhibited HIF activity and cell migration, which consequently demonstrated its efficacy (**Chapter 2**). Overall, the strategy of blocking the UCHL1–HIF axis and the identification of the novel UCHL1 inhibitor is expected to the development of effective treatments of UCHL1-related tumors.
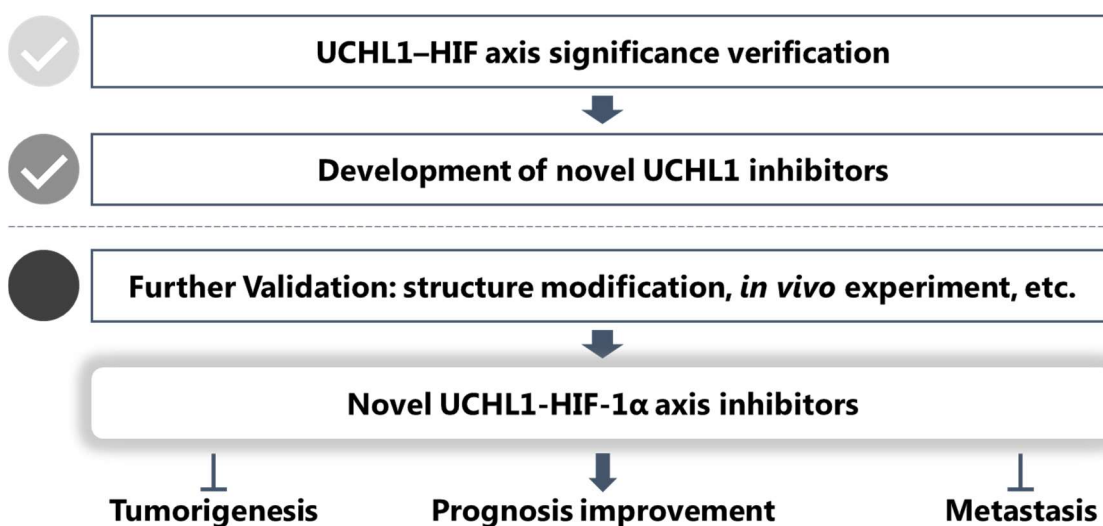

Figure 1

Chapter 1: UCHL1 promotes HIF-1-dependent tumor cell malignancy in spheroid models

In 2D monolayer culture, UCHL1 inhibition by siRNA or LDN57444, a well-known UCHL1 inhibitor, drastically lowered HIF-1α protein levels in UCHL1-expressing cells **(Figure 2A, B)**. In UCHL1 non-expressing cells, ectopic expression of UCHL1 significantly increased HIF-1α protein expression levels, which was canceled by the treatment of LDN57444 **(Figure 2C)**. When investigated with HIF-1α translocation, depletion of UCHL1 lowered HIF-1α protein expression not only in nucleus, but also in cytosol under both normoxia and hypoxia, indicating the stabilization of HIF-1α by UCHL1 is before its translocation into nucleus **(Figure 2D, E)**. Taken together, UCHL1 was demonstrated to upregulate HIF-1α protein expression, and its knockdown or pharmacological inhibition could counteract this effect.
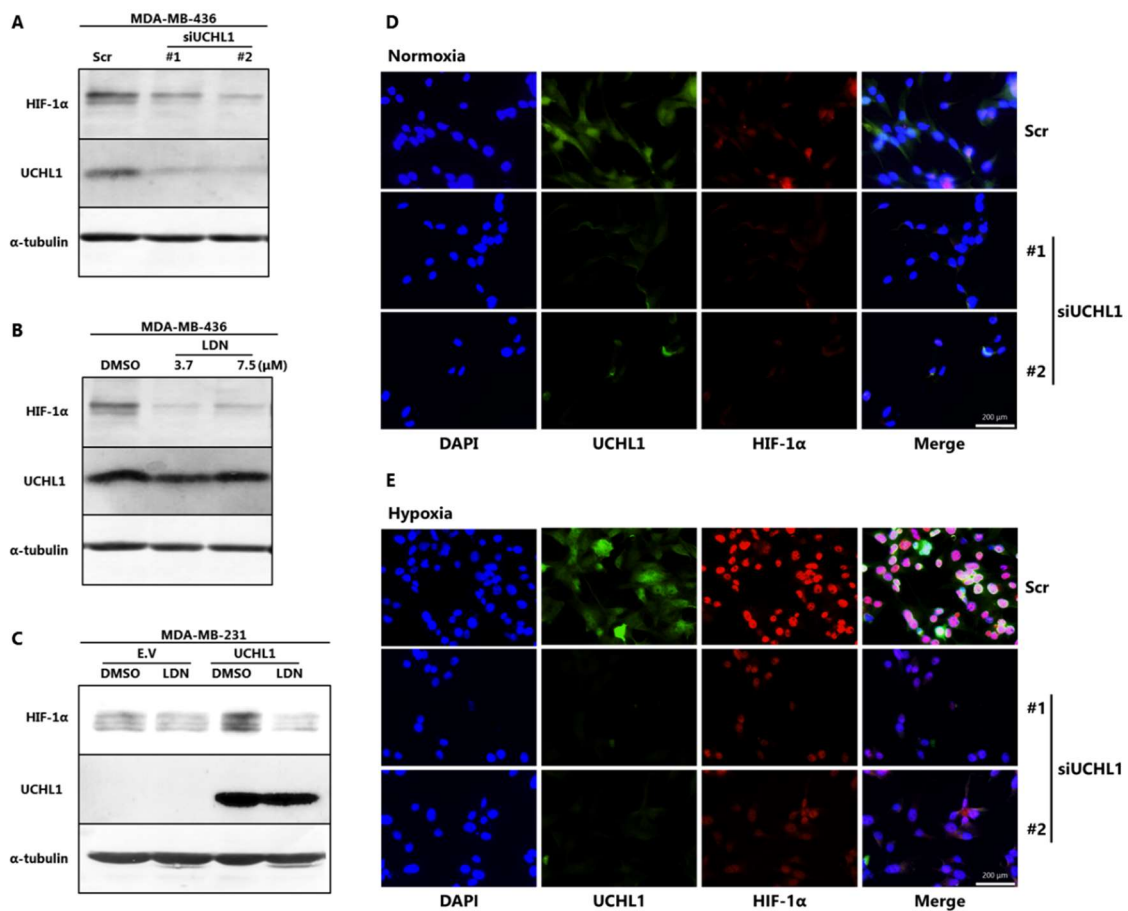

Figure 2

When further analyzed whether the UCHL1 inhibitor could affect the HIF-dependent transactivation, 5HRE-luciferase (5HRE-Luc) gene reporter assays in which HeLa cells were stably transfected with the firefly luciferase gene driven by 5HRE to measure HIF activity was conducted. The UCHL1-expressing group showed a significant increase in 5HRE-Luc activity under both normoxia and hypoxia **(Figure 3A)**. To investigate whether the UCHL1 inhibitor could affect HIF-1 activity, it was further stably transfected in HeLa/5HRE-Luc cells with UCHL1 expressing vector and treated these cells with various concentrations of LDN57444. This resulted in a significant decrease in the reporter activity in a dose-dependent manner

(IC_{50}: 3.7 µM) **(Figure 3B)**. ODD–luciferase (ODD-Luc) assays were then performed to further determine the effect of UCHL1 on HIF-1α stability. It was found that under both normoxia and hypoxia, the overexpression of UCHL1 led to a significant increase in the ODD-Luc activity while LDN57444 eliminated this effect **(Figure 3C, D)**. When confirming by immunostaining analysis, the protein expression of HIF-1α was significantly elevated in UCHL1-transfected cells while treatment with LDN57444 abrogated this increase in both nucleus and cytosol **(Figure 3E, F)**.
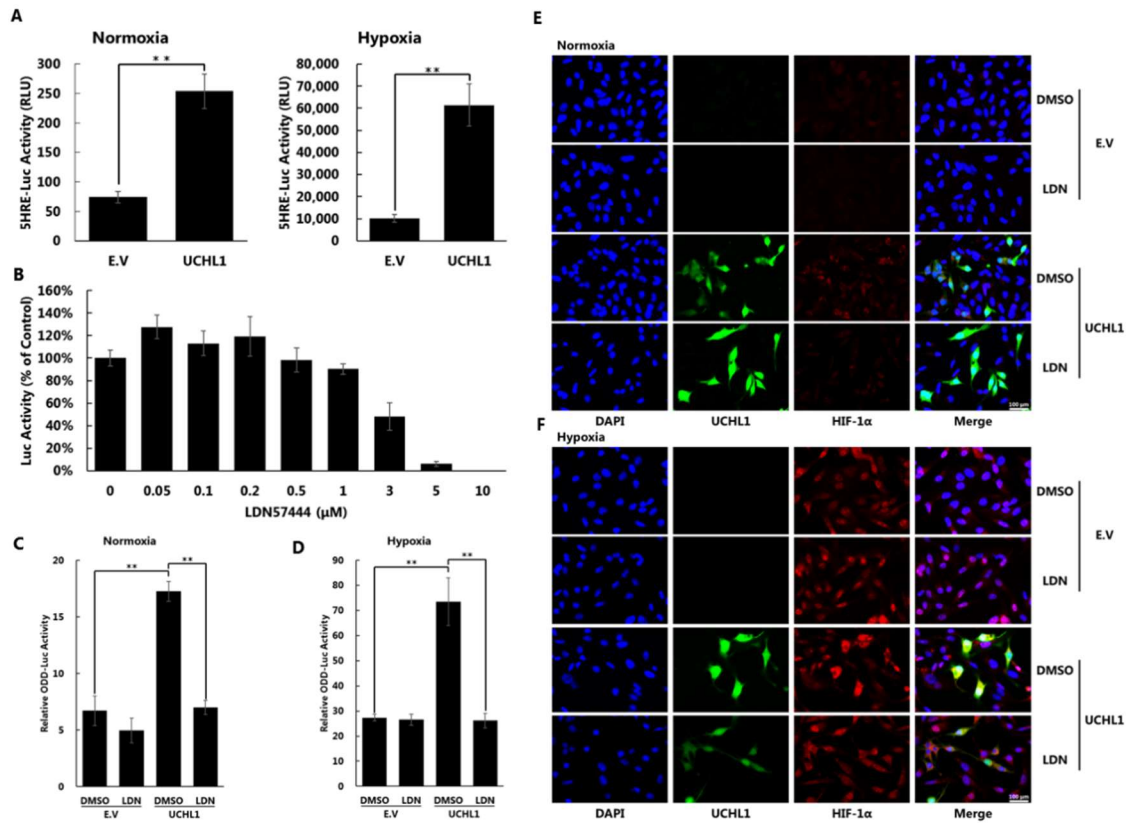
Figure 3

I then analyzed the effect of LDN57444 on the expression of two typical cancer-related HIF-1α target genes, *GLUT1* and *CA9* in both MDA-MB-231 and MDA-MB-436 cells. In UCHL1-non-expressing MDA-MB-231 cells, blockade of UCHL1 by LDN57444 had no significant influence on the expression of both genes while in UCHL1-expressing MDA-MB-436 cells, however, LDN57444 lowered the expression of both genes remarkably under and hypoxia, revealing the specificity of the UCHL1 inhibitor **(Figure 4A, B)**. To evaluate whether UCHL1 inhibitor has an anti-metastasis effect, I performed wound healing and transwell migration assays in cultured cells. Treatment with LDN57444 significantly suppressed the recovery of the scratched gap in MDA-MD-436 cells, but not in MDA-MB-231 cells **(Figure 4C, D)**. In the transwell migration assay, a similar result was obtained. LDN57444 treatment led to a striking decrease in the number of migrated MDA-MB-436 cells. Meanwhile, in MDA-MB-231 cells no such effect was confirmed **(Figure 4E, F)**. Finally, I examined whether this difference was possibly induced by a potential inhibitory effect of LDN57444 on the proliferation of MDA-MB-436 cells. I treated both MDA-MB-231 and MDA-MB-436 cells with 2.5 µM of LDN57444 and found no difference in cell number between both

cell lines after 8, 24, and 48 h **(Figure 4G)**. Overall, UCHL1 inhibition showed efficacy against UCHL1-mediated cell migration in endogenous UCHL1-expressing cells.

3D spheroid cell culture, which has been more widely applied in the preclinical studies to provide more physiologically relevant information, was performed to learn more about the link between UCHL1 and HIF1-dependent tumor malignancy. It was first compared with typical malignancy-related factors, such as equivalent diameter, spheroid area, volume, solidity, sphericity, and viable cell number when cells were

Figure 4

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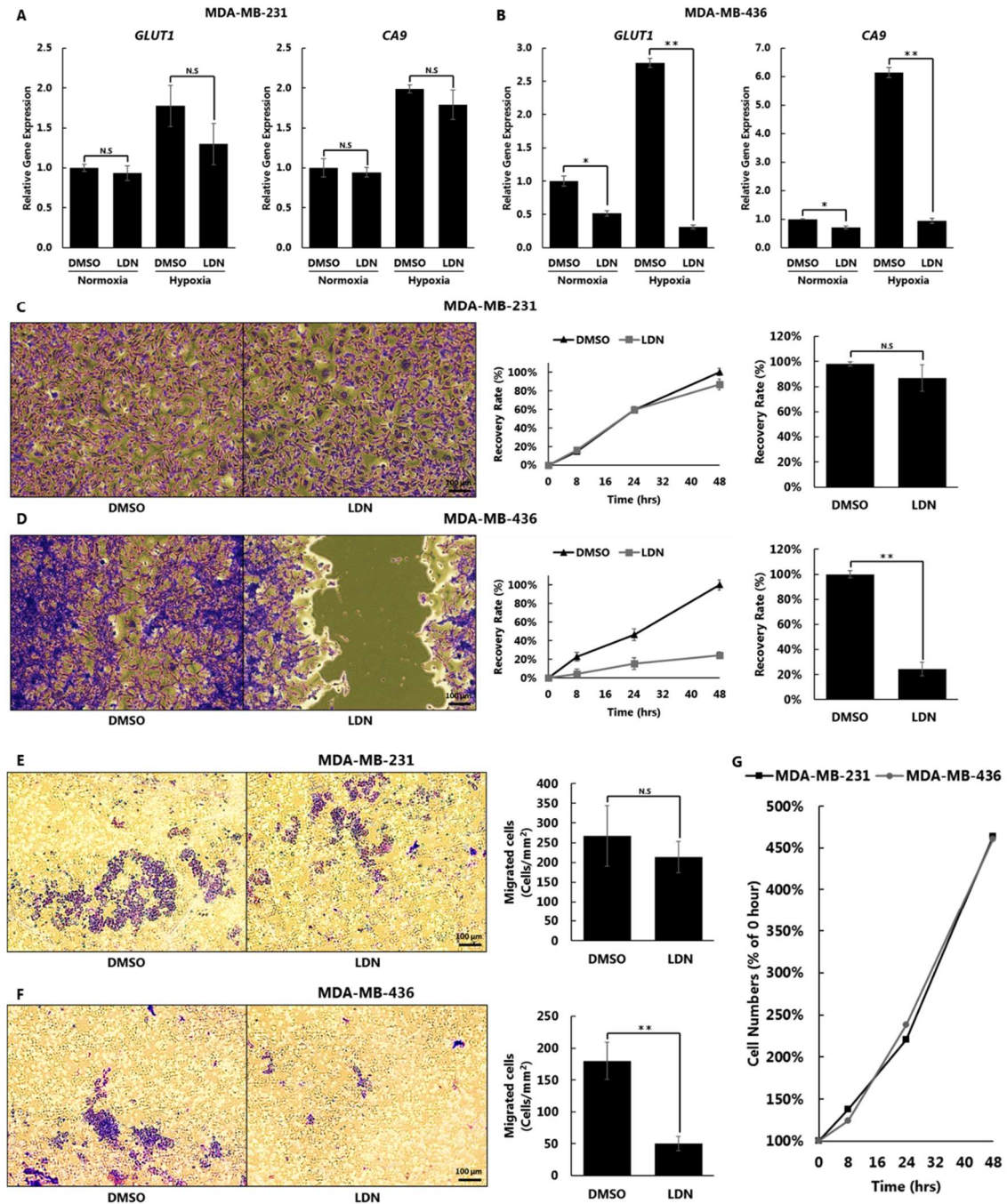
incubated in an anchorage-independent condition. In UCHL1-transfected MDA-MB-231 cells, all factors were evidently elevated **(Figure 5A, B)**. However, these factors were drastically reduced in siUCHL1-transfected MDA-MB-436 cells. In the case of UCHL1 inhibitor treatment in MDA-MB-436 cells, LDN57444 abated the diameter, area, volume, and viable cell number, but had no significant effect on solidity and sphericity **(Figure 5B)**. Transfection of UCHL1 in MDA-MB-231 cells was confirmed to rescue cells not only in the central but also in the intermediate layer of the quiescent area. Moreover, more living cells were confirmed in the proliferative area of UCHL1-transfected cells, suggesting a proliferation-promoting effect of UCHL1 under 3D culture conditions **(Figure 5C)**. When MDA-MB-436 cells were treated with LDN57444 or transfected with siRNA against *UCHL1*, global cell death was dramatically increased compared with the control group. Therefore, it was considered that the rescue of cell death and the increase in proliferation both contributed to the increase in total viable cell number **(Figure 5C)**. In 3D cell invasion assays, transfection of UCHL1 substantially promoted cell invasion under 3D culture conditions, while treatment with LDN57444 abolished this increase **(Figure 5D)**. In conclusion, UCHL1 increases malignant potential in spheroid cultured tumor cells while its deficiency or inhibition can abrogate this effect.
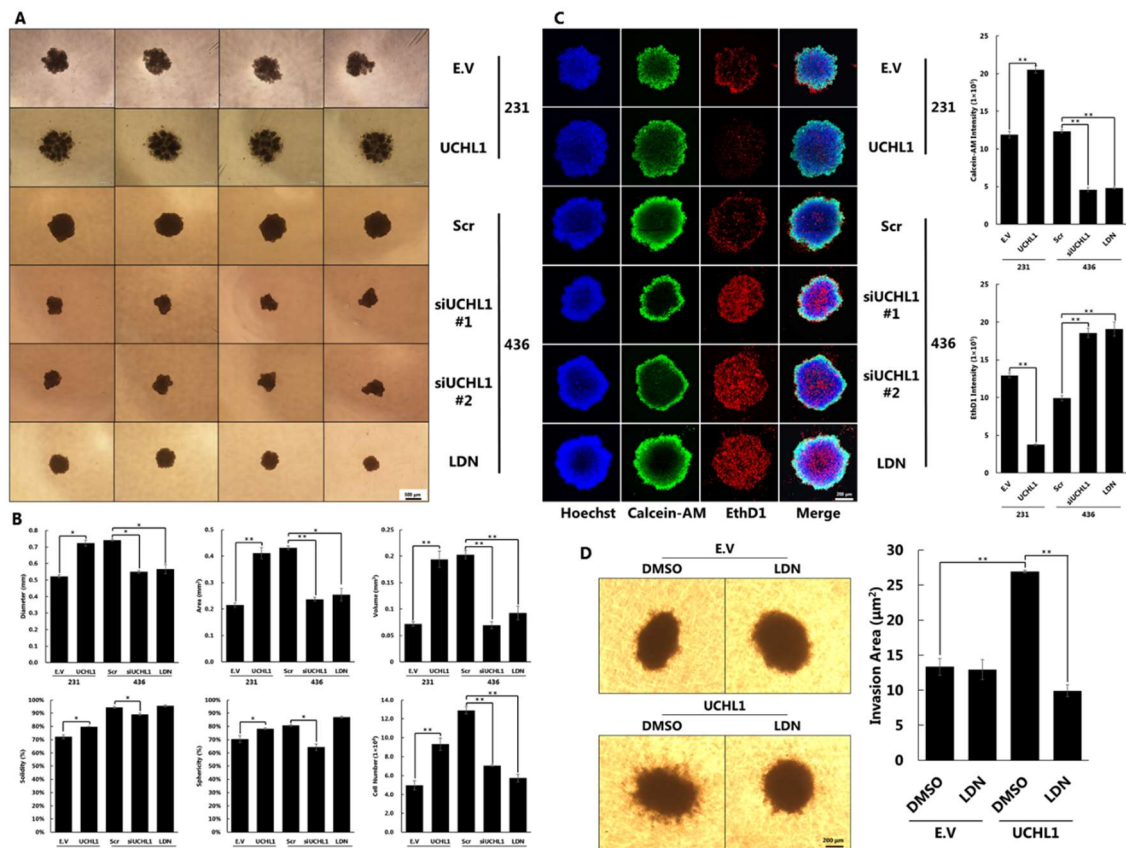

Figure 5

To examine whether the phenotypes observed previously were induced in a HIF-1α-dependent manner, a co-transfection of UCHL1 and siHIF-1α in MDA-MB-231 cells was performed **(Figure 6A)**. It was first examined whether the overexpression of UCHL1 in HIF-1α deficient cells could still affect spheroid phenotypes due to its deubiquitinating activity against other potential substrates except for HIF-1α. It was found that neither the transfection of UCHL1 nor the treatment of LDN57444 showed any effect on the invasiveness in the siHIF-1α transfected groups **(Figure 6B)**. It was further examined with other malignancy-related factors and was found that the siHIF-1α-transfected spheroids were hollowed in the central part of the sphere, despite UCHL1-transfecton, LDN57444-treatment or their combination **(Figure 6C)**. These results demonstrate the dependency on HIF-1α for UCHL1 to raise tumor cell malignancy, while the blockade of the UCHL1–HIF-1α axis abrogated this effect. The expression of two typical HIF-

Figure 6
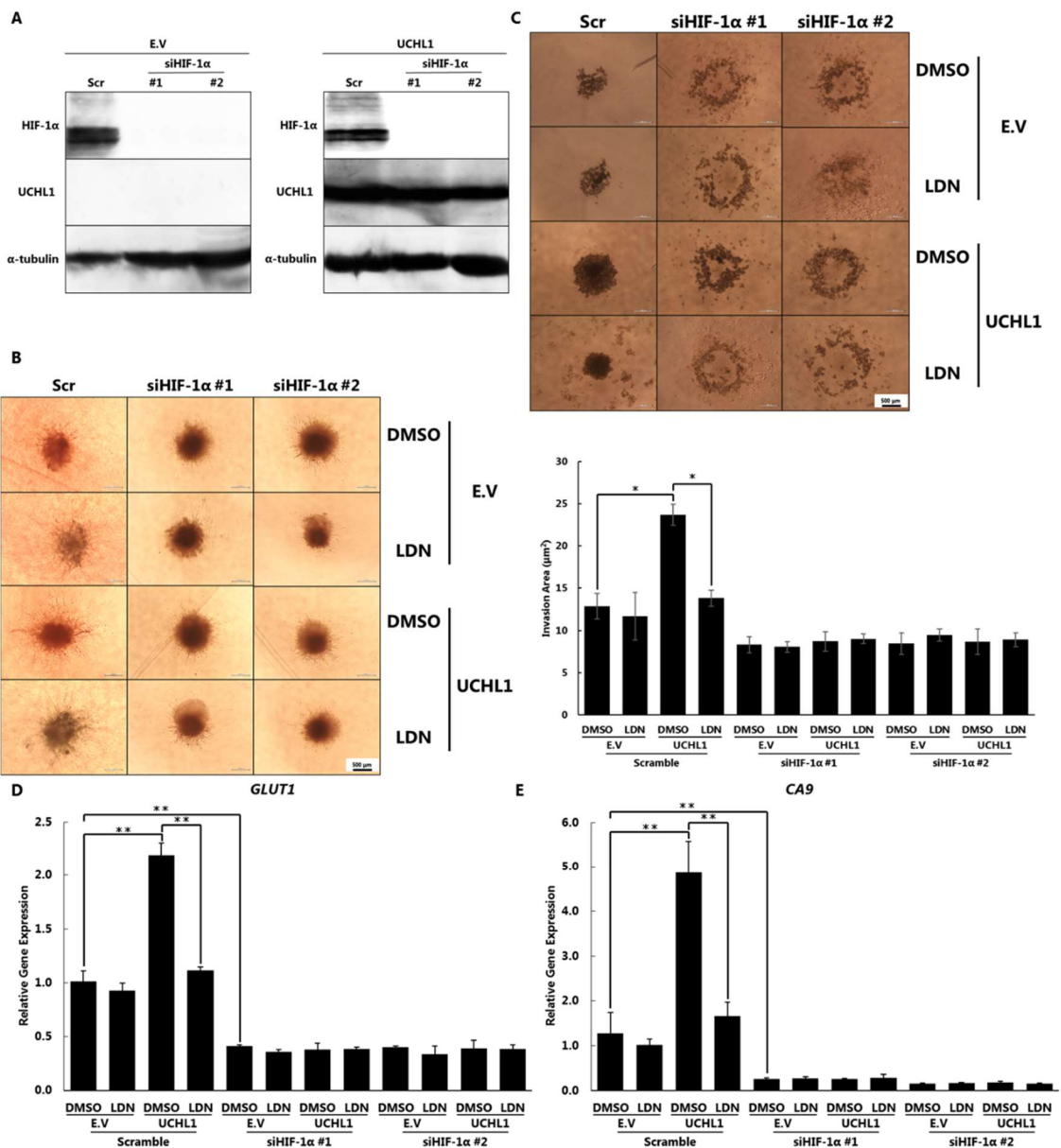
1α downstream genes, *GLUT1* and *CA9*, were also elevated by UCHL1 overexpression in MDA-MB-231 cells, which was further eliminated by LDN57444 treatment, while genes in siHIF-1α-transfected groups, UCHL1 failed to increase the expression of both **(Figure 6D, E)**. In conclusion, UCHL1 was confirmed to increase cell viability and proliferation as well as metastasis potential by stabilizing HIF-1α to increase HIF-1 activity in the spheroid 3D culture system.

Chapter 2: Development of a novel UCHL1 inhibitor by computational drug repositioning and bioassays

As known UCHL1 inhibitors have all been reported with severe side-effects such as the induction of ER stress and abnormal synapse protein expressions, the development of more effective and selective UCHL1 inhibitors remains a major challenge for the treatment of malignant tumors. Therefore, I then focused on the development of novel UCHL1 inhibitors through drug repositioning. A high-throughput screening of 4,500 known drugs and reagents was performed and seven compounds were demonstrated to be effective UCHL1 inhibitors. By further examined with their inhibition against the UCHL1–HIF axis, only two out of seven compounds were qualified to dose-dependently inhibit HIF activity as well as the UCHL1-inducued HIF-1α stabilization. In further confirmation of phenotypic migration inhibition, only one compound, Compound A, showed relatively strong selectivity towards UCHL1 harboring cells at low concentrations. Since Compound A was originally developed as a protein kinase X inhibitor that blocks protein synthesis and therefore potently inhibits cell viability, thirty Compound A analogues were designed by adding amide or bis-amide groups into the long acyl chains. Molecular docking simulations was then applied to find an analogue that has predictably the highest inhibition against UCHL1 as well as the lowest inhibition against protein kinase X. Top three ranking analogues were synthesized and only Compound A_5, was shown to exhibit a much-increased inhibitory effect against UCHL1 as well as a decreased inhibitory effect against protein kinase X.

In the analysis of the predicted interaction between UCHL1 and Compound A_5, the amino group was determined to form new hydrogen bonds with UCHL1 and helps the formation of additional interaction between UCHL1 and Compound A_5. Furthermore, ASP155 and ASP156 of UCHL1 was confirmed to be essential in the binding with Compound A_5 by newly forming charge interactions and hydrogen bonds. Therefore, UCHL1 recombinants mutated in their ASP 155 or ASP 156 or both was created to apply *in vitro* kinetics analysis and found K_m to be increased significantly in UCHL1 D155A or D156A mutants. Moreover, K_i of Compound A_5 was also much lower compared with Compound A against UCHL1 wild type (WT), D155A, D156A and D155, 156A mutants. These results revealed the significance of ASP155 and ASP156 of UCHL1 in the binding with Compound A_5. Finally, in an *in vitro* kinase assay that determines the kinase activity of protein kinase X, the inhibitory effect of Compound A_5 turned out to be almost eliminated when compared with Compound A. Taken together, Compound A_5 is a promising UCHL1 specific inhibitor.

In the determination of cytotoxicity, which is one of the strongest impacts of protein kinase X inhibition, cytotoxicity of Compound A_5 in MDA-MB-231, MDA-MB-436 cells was 4.9 and 12.6 times lower respectively, indicating a lower cytotoxicity. Then it was tested whether Compound A_5 could specifically

inhibit UCHL1–HIF axis to prevent metastasis in cellular models, Compound A_5 showed lower IC_{50} of HIF activity inhibition in UCHL1 overexpressing cells than in UCHL1 non-expressing cells. In wound healing assays, inhibitory effects on cell migration was confirmed in only MDA-MB-436 cells at a concentration of 1 or 2.5 μM of Compound A_5. One micro mol/mL of Compound A led to a decrease of HIF-1α accumulation in only UCHL1 expressing cells and eliminated the UCHL1-overexpression led HIF-1α accumulation. Finally, Compound A_5 showed no inhibitory effect against the phosphorylation of protein kinase X target protein while Compound A abundantly lowered the phosphorylation. These results above demonstrated the higher specificity and potency of our novel UCHL1 inhibitor.

Conclusion

In conclusion, this thesis represents a systematic work on the demonstration of the effectiveness of UCHL1 inhibitors in HIF-1 dependent tumor malignancy as well as the development of novel UCHL1 inhibitors through computational drug repositioning and bioassays. Compound A_5 is an ideal lead compound for the development of more potent and specific analogues. At the same time, the combination of in vitro HTS assay and computational with bioassays renders a more efficient developing process for UCHL1–HIF-1 axis. I believe that my results in this thesis will contribute to the discovery of effective UCHL1–HIF-1 axis inhibitors in future therapies of metastasis in UCHL1-related tumors, such as breast and pulmonary cancers.

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