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Comparison of intrabody-mediated degradation of mHTT through ubiquitin independent vs. dependent pathways

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Abstract:

Huntington's disease (HD) is a progressive brain disorder that causes various symptoms, such as uncontrolled movements, emotional problems, and cognitive deficits. In US there are more than 40,000 patients with HD disease and the global prevalence is between 1/10,000 to 1/20,000 based on the information from Huntington's Disease Society of America. HD is caused by a mutation in Huntingtin gene (HTT) coding for the mutant Huntington (mHtt) protein. The mHtt protein causes neural cell death, leading to symptoms in HD patients. Degrons are degradation signals located in some proteins that direct misfolded or aggregated proteins to Ubiquitin–Proteasome System (UPS) for degradation. Intrabody is a small recombinant antibody that targets antigens intracellularly. Anti-mHtt intrabodies were found to be able to promote mHtt degradation and degrons can enhance the intrabody-mediated mHtt degradation. By involving various degrons, intrabodies can work through either ubiquitin-dependent or ubiquitin-independent pathway to degrade mutant proteins. However, the mechanism underlying the mHtt degradation mediated by intrabody is not fully understood and the efficiency of each degradation pathway involved is unclear. To investigate which of the above pathways is more efficient, we compared the intrabody-mediated degradation pathways with CL1 vs. PEST degrons fused to the intrabody. Results showed that the ubiquitin-dependent proteasome pathway involving CL1 degron is more efficient in mHtt degradation than the ubiquitin-independent proteasome pathway involving PEST degron. These results provide the scientific foundation of developing a potential intrabody-mediated treatment for HD disease via the ubiquitin-dependent pathway.

Keywords: mutant Huntingtin, intrabody, degron, proteolysis, aggregation

1. Introduction

Huntington's disease (HD) is a progressive neurological disorder characterized by structural and functional changes in specific brain regions, leading to a range of symptoms, such as uncontrolled movements, emotional problems, and loss of thinking ability (cognitive deficits) [1-3]. It is an autosomal dominant disorder caused by a genomic expansion mutation to the *Huntingtin (HTT) gene* on chromosome 4. This gene possesses a repeating trinucleotide sequence of cytosine–adenine–guanine (CAG) coding for a polyglutamine (PolyQ) in the N-terminal region of the Huntington (Htt) protein. Normally, the PolyQ is usually present in low amounts with an average of 15 – 20 repeats [4]. However, when *HTT* is mutated and the number of repeats exceeds ~40, the mutant Huntington (mHtt) protein will misfold, aggregate and become toxic, causing neurodegeneration or neural cell death [1, 2]. The striatum (Caudate nucleus and Putamen) is the part of the brain that is mostly affected by HD.

In HD, the misfolding and aggregation of mHtt proteins form inclusions in striatal neurons, a process believed to underlie neurodegeneration and cell death. The length of the CAG repeats plays a critical role in determining the mHtt aggregation process [3]. An important intracellular pathway for reducing the levels of misfolded or aggregated proteins is the normal cellular protein cleaning process: Ubiquitin-Proteasome System (UPS), which is found to be dysfunctional and compromised in mHtt clearance in HD [3, 5]. Therefore, degrading mutant huntingtin protein (mHtt) or inhibiting mHtt aggregate formation is considered to be the therapeutic approaches. Currently, there is no cure for HD. Available treatment is only to ameliorate and pacify the symptoms [1].

Intrabody is a small recombinant antibody fragment that is engineered to be expressed and target antigens intracellularly. In recent years, intrabodies targeting the misfolded or aggregated proteins for degradation has attracted scientists' interest and has been studied as a potential treatment strategy for various diseases by multiple research groups. Intrabodies exhibit many advantages over conventional antibodies, including high specificity and affinity for target epitopes, but without the Fc domain that triggers inflammation. Therapeutic potential of intrabodies is being explored for HD by a few scientific groups. It has been shown that scFv-C4 introbody was generated and successfully decreased mHtt aggregation in HD cell model [6-9] and *Drosophila* model [10]. However, the neuroprotective effect of anti-mHtt intrabody was found to be limited or decreased in previous study using mouse model [11]. To enhance intrabody's function in mHtt degradation and facilitate its future use in clinical applications for HD treatment, Dr. Butler group in NY optimized scFv-C4 intrabody by fusing it with the PEST degron of mouse Ornithine Decarboxylase (mODC), which reduces Htt exon 1 protein fragments significantly compared to scFV-C4 alone [12].

Degron (aka degradation signal) is a specific amino acid sequence inherently located in some proteins that direct misfolded or aggregated proteins to Ubiquitin–Proteasome System (UPS) and trigger protein degradation [13]. In Butler, et al., 2012 study, it was found that anti-mHtt intrabodies (Figure 1) fused with PEST degron can bind and inactivate toxic intracellular mHtt proteins, prevent misfolding, promote degradation and block aberrant protein-protein interactions, indicating the promise of intrabody fused with degron being developed as potential agents for clinical treatment of HD.

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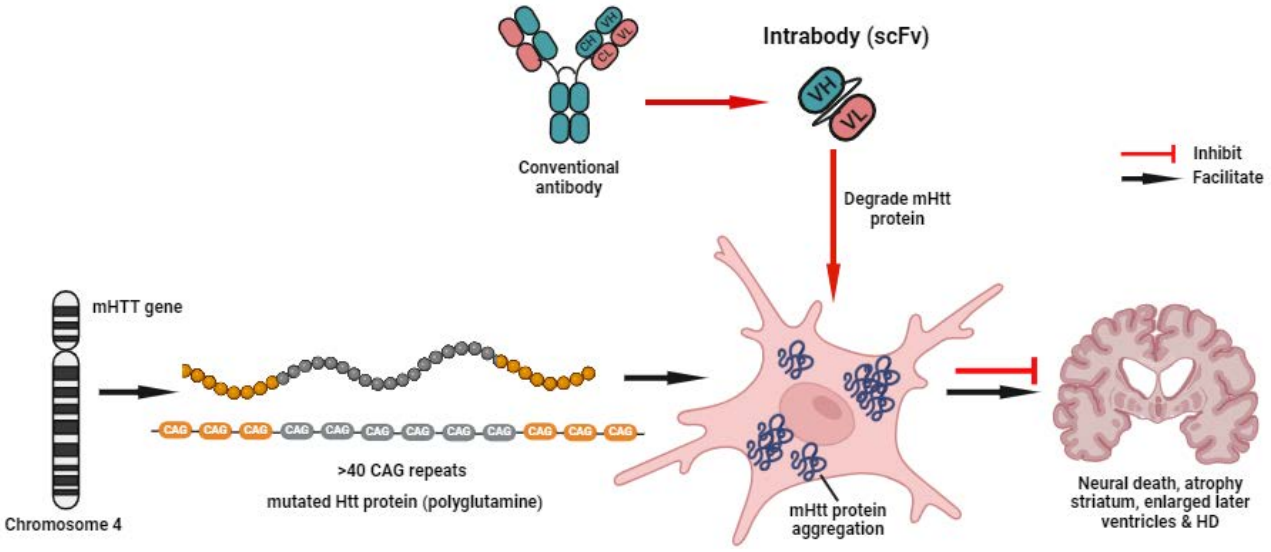

Figure 1. Schematic of development strategy for recombinant scFv intrabody from conventional antibody IgG and how it degrades mHtt protein aggregation and remedies neurodegeneration. The scFv intrabody is assembled by linking the genes that encode heavy (VH) and variable light (VL) chains of a conventional antibody together with a flexible linker. The intrabody is selectively expressed intracellularly to remedy neural death in HD brains.

In HD, the function of UPS is inhibited by aggregated mHtt proteins [5]. Intrabodies expressed intracellularly can promote UPS function through ubiquitin-dependent proteasome system or ubiquitin-independent proteasome system depending on the various degrons associated. Proteins tagged with CL1 degron were found to be degraded via ubiquitin-dependent pathway [14-15], whereas those fused with the PEST degron are degraded via ubiquitin-independent pathway [16-17]. However, it is not clear which pathway: ubiquitin-dependent proteasome system (via CL1 degron) or ubiquitin-independent proteasome system (via PEST degron) is more efficient in intrabody-mediated mHtt degradation in HD (Figure 2).

To explore a potential efficient treatment approach for HD, in this study, we compared intrabody-mediated degradation of mHtt via the ubiquitin-dependent proteasome pathway involving the CL1 degron vs. the ubiquitin-independent proteasome pathway involving the PEST degron. The finding of this study will help us better understand the molecular mechanism of mHtt degradation and provide a scientific foundation for the discovery of an effective treatment for HD.

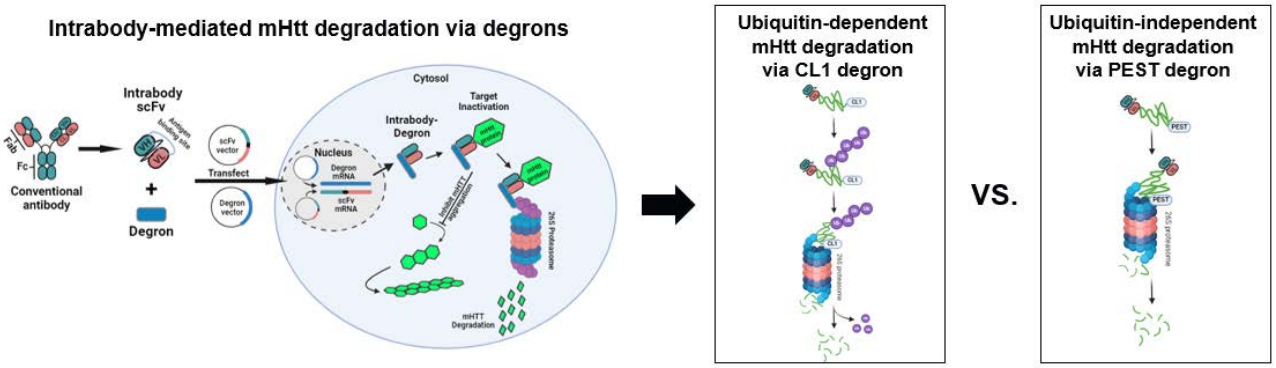

Figure 2. Schematic of intrabody-mediated protein degradation pathways: ubiquitin-dependent proteasome pathway via CL1 degron vs. ubiquitin-independent proteasome pathway via PEST degron. Left: Sequence of events starting from transfection: Plasmids carrying the intrabody and degron are transfected into cells → The cell will then make an mRNA encoding the intrabody-degron that is translated into a fusion protein at the Ribosome → The intrabody-degron prevents aggregation of target protein (mutated or misfolded) and

the target protein is degraded by the 26S proteasome. Middle: The intrabody and CL1 degron bind to the misfolded or aggregated protein with ubiquitination and direct it to the 26S proteasome for degradation. Right: The intrabody and PEST degron bind to the misfolded or aggregated protein without ubiquitination and direct it to the 26S proteasome for degradation.

2. Materials and Methods

This study is an *in vitro* study using HD cell model created from HEK293 cells. To create a successful HD cell model, plasmid carrying *mHTTex1-25Q-mRFP* or *mHTTex1-72Q-mRFP* was transfected into HEK293 cells, which were analysed at 24 hours, 48 hours and 72 hours post-transfection for mHtt expression/aggregation using live-cell fluorescence microscopy imaging. To compare the intrabody-mediated mHtt degradation via the ubiquitin-dependent vs. the ubiquitin-independent pathways, HEK293 cells were co-transfected with plasmid carrying *mHTTex1-72Q-EM48-eGFP* and plasmid containing *scFv-B8-HA-PEST-mCherry*, *scFv-C4-HA-PEST-mCherry* or *scFv-C4-HA-CL1-mCherry*, and transfected cells were analysed at 48 hours post-transfection with live-cell fluorescence microscopy imaging and western blot. See Figure 3 an example of the experimental plan diagram.

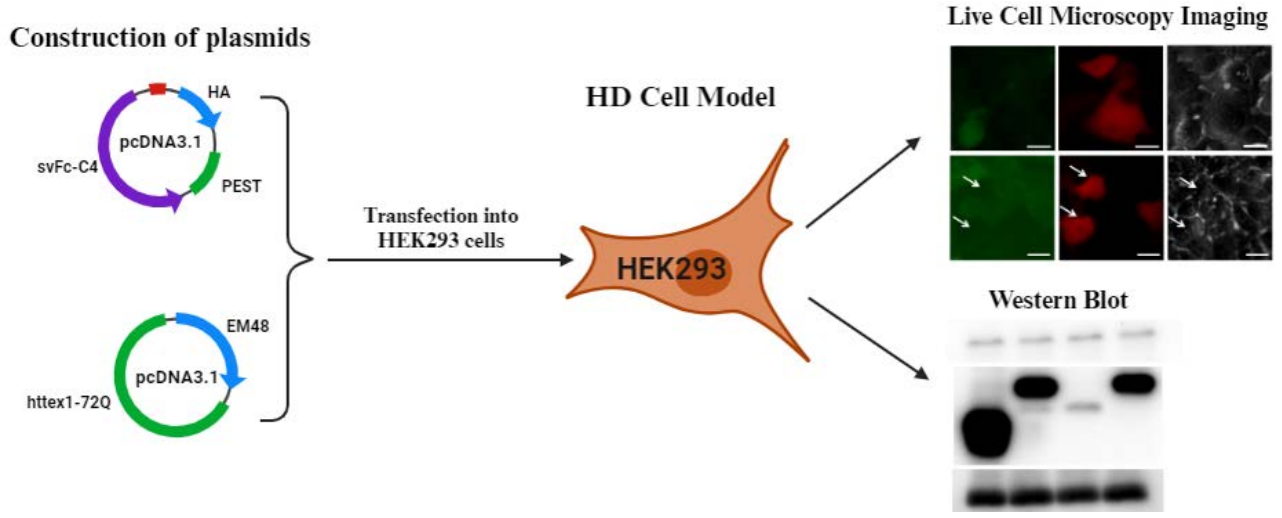

Figure 3. An example of the experimental plan diagram.

2.1 Cell Culture, Plasmids Construction and Cell Transfection

HEK293 cells were cultured following standard protocols. Cells were grown in DMEM medium supplemented with 10% heat inactivated bovine serum, 2 mM glutamine, 1 mM streptomycin and 100 international units of penicillin. The plasmids carrying *mHTTex1-25Q-mRFP*, *mHTTex1-75Q-mRFP*, *mHTTex1-72Q-EM48*, *scFv-B8-HA-PEST*, *scFv-C4-HA-PEST* or *scFv-C4-HA-CL1* in pcDNA3.1 (Invitrogen) were designed by Dr. Butler with input from me and synthesized by Vector Builder for testing. Cell transfection was performed as previously described [6]. For co-transfection, intrabody plasmids were applied at 1:1 ratio to httex1 plasmid, and cells were analyzed 48 hours post-transfection.

For the mHTTex1-25Q-mRFP and mHTTex1-72Q-mRFP, the 25Q (91 amino acids) and 72Q (138 amino acids) sequences were from the HTT exon 1 (previously reported plasmid, p416 25Q GPD and GAL 72Q+ProGFPp416, respectively, Scheme 1, [18]), and assembled with mRFP (from previously reported plasmid, pPRISM-TagRFP-gcry-mRFP, [19]).

Exon 1 sequence: MATLEKLMKAFESLKSFQQQQQQQ(n)
PPPPPPPPPPPQLPQPPPQAQPLLPQPQPPPPPPPPPPGPAVAEEPLHR

Scheme 1. HTT Exon 1 sequence.

2.2 Live-Cell Fluorescence Microscopy Imaging

For HD cell model creation, live-cell fluorescence imaging was taken on an inverted microscope Axiovert 200M at 24 hours, 48 hours and 72 hours post-transfection to visualize

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the cell population and cell morphology when most of the cells expressed the fluorescent protein at detectable levels. For the ubiquitin-dependent pathway vs. the ubiquitin-independent pathway comparison, fluorescence imaging was taken on an inverted microscope Axiovert 200M at 48 hours post-transfection. Cell plates were placed in a smaller internal chamber that was continuously perfused with humidified 5% CO_2. The imaging set up was controlled by AxioVision software and ImageJ (National Institutes of Health) was used for image processing and analysis.

2.3 Protein Extraction and Western Blot

Expression of scFv intrabody and mHtt was examined by Western blot analysis of the transfected cell extracts, by using an anti-Flag Ab (Sigma). Protein extraction and western blot were performed following a protocol described previously [12]. HA-tagged intrabody, EM48-tagged mHtt and endogenous Histone H3 were probed with monoclonal anti-HA, anti-EM48 and anti-H3 antibodies, respectively, provided by Dr. Butler group. ImageJ (National Institutes of Health) was used for image processing and analysis.

3. Results

3.1 mHtt aggregation is observed at 48- and 72-hours post-transfection

To investigate intrabody-mediated mHtt degradation pathways via various degrons, an HD cell model needs to be created. Non-neuronal HEK293 cells can exhibit some of the pathological features of HD, including mHtt aggregation and cytotoxicity, and so are used by various scientific research groups for HD disease study [20].

In this study, HEK293 cells are used to create the HD cell model. Empty vector (pcDNA3.1), mHTTex1-25Q-mRFP and mHTTex1-72Q-mRFP were transfected to HEK293 cells, respectively. Live-cell fluorescence microscopy imaging was taken to observe the mHtt aggregation at 24 hours, 48 hours and 72 hours after transfection. Empty vector transfected cell group serves as a control, which has no mHtt expression in cells at all time points. For both mHTTex1-25Q-RFP and mHTTex1-72Q-RFP transfected groups, diffuse mHtt (Figure 4) was observed at 24 hours after transfection in transfected cells. The diffuse mHtt then gets aggregated gradually over time. At 48 hours, there is still diffuse mHtt, whereas at 72 hours most of mHTT is aggregated (Figure 5), indicating the transfection is successful and mHtt is expressed intracellularly. Additionally, it shows that mHTTex1-72Q-RFP transfected cells form more dense aggregates than mHTTex1-25Q-RFP, demonstrating that mHTTex1-72Q-RFP transfected cells show more robust protein aggregation phenotype. The result also demonstrated that mHtt aggregates starting at 48 hours post-transfection and becomes significant at 72 hours. These findings (72 polyQ for a robust HD cell model creation, and 48-hour post-transfection for mHtt aggregation) were used as the basis for experimental design in the next step of the study.

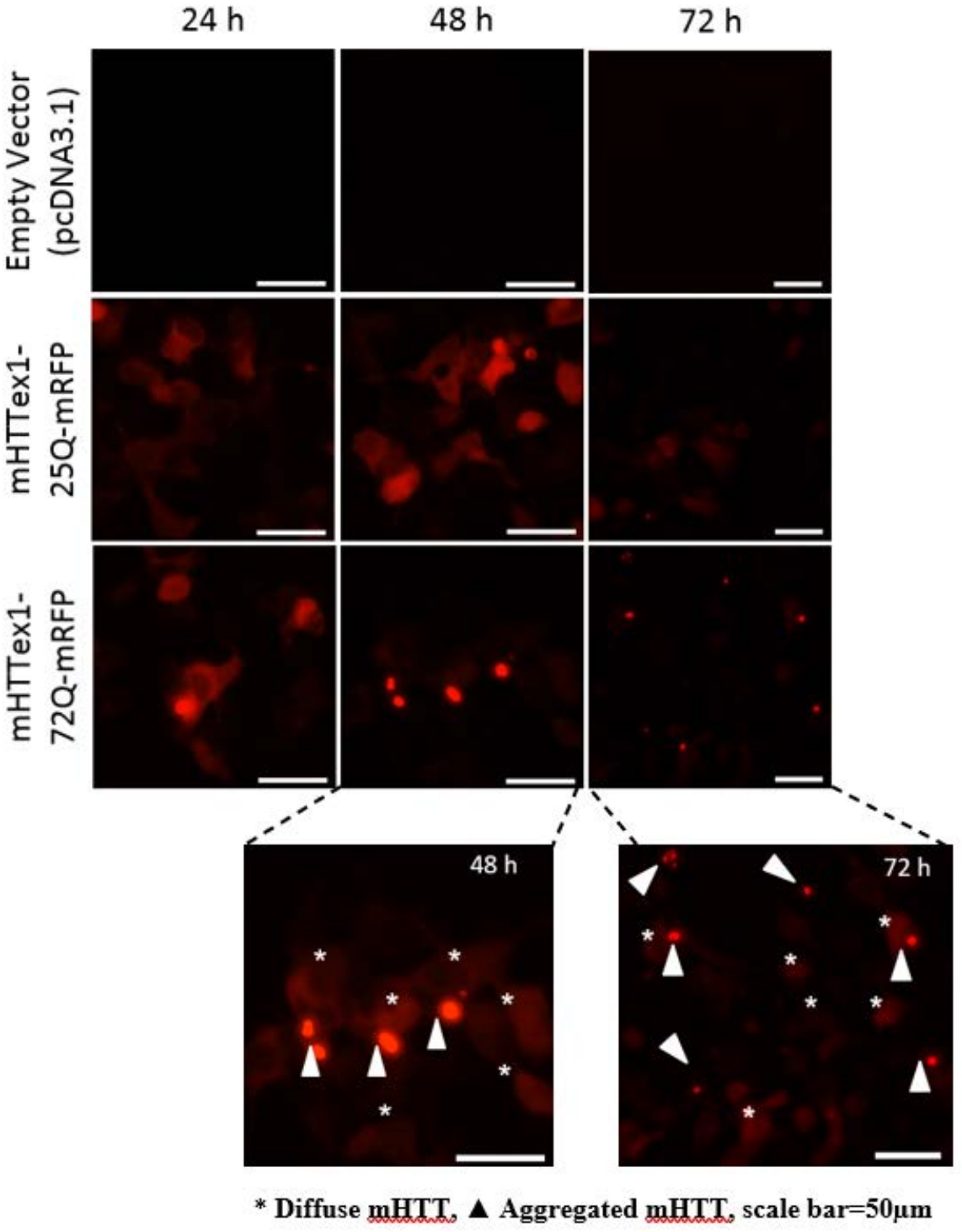

Figure 4. Live-cell fluorescence imaging of mHtt transfected HEK293 cells. Empty vector (pcDNA3.1), mHTTex1-25Q-mRFP and mHTTex1-72Q-mRFP were transfected to HEK293 cells, respectively. Diffuse mHtt (*) was observed in both mHtt transfected cell groups starting at 24 hours after transfection and mHtt aggregates starting at 48 hours with more dense aggregation observed at 72 hours (▲). Additionally, mHTTex1-72Q-mRFP transfected cells form more dense aggregates than mHTTex1-25Q-mRFP, indicating that expression of a slightly longer mHtt aa sequence in cells builds a more robust HD cell model.

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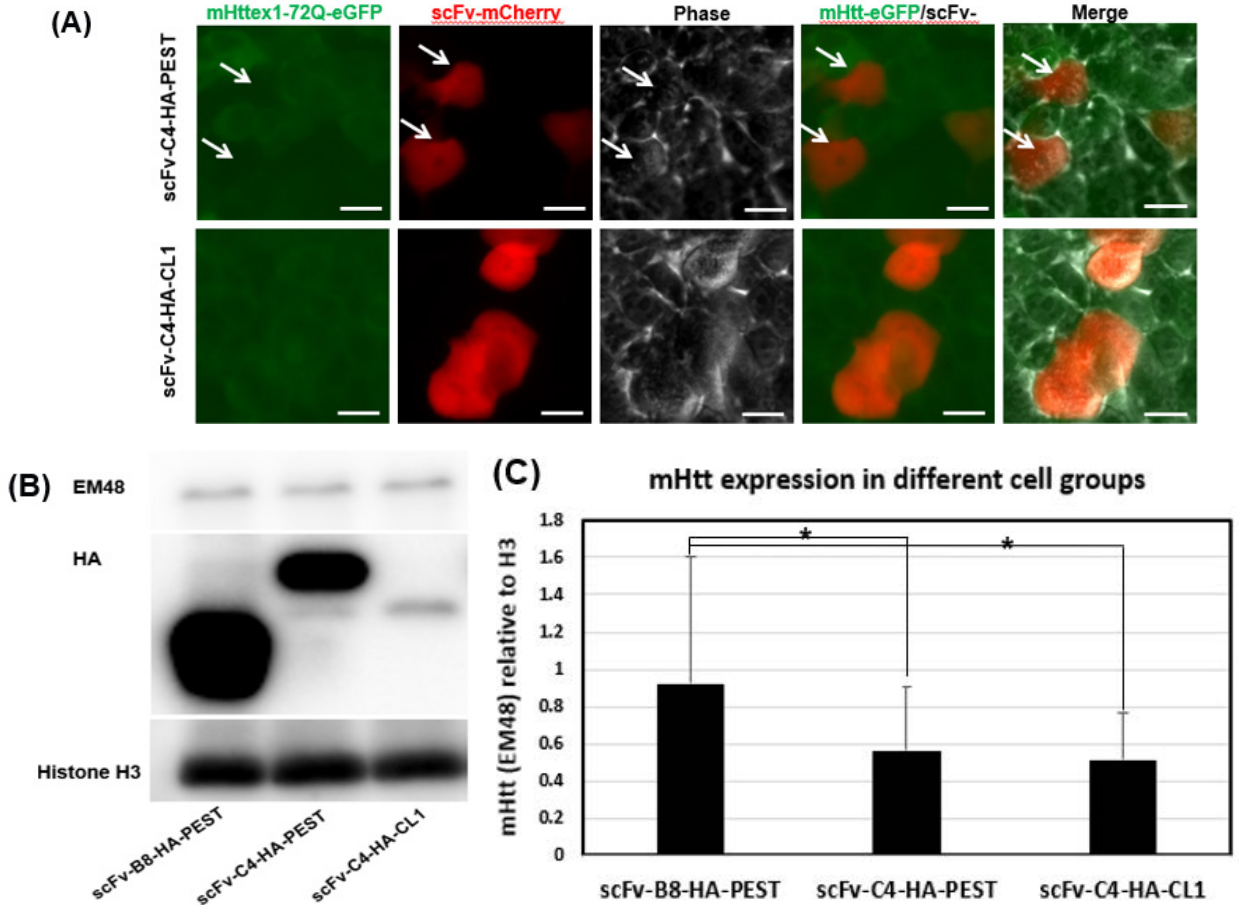
3.2 CL1 degron enhances intrabody-mediated mHtt degradation more efficiently than PEST degron

It has been shown previously that degrons enhance intrabody-mediated mHtt aggregation and degradation [12]. To further investigate whether the ubiquitin-independent pathway via PEST degron or the ubiquitin-dependent pathway via CL1 degron is more efficient in mHtt degradation, HEK293 cells were co-transfected with mHTTex1-72Q and intrabody (scFv-C4) plasmids (see Materials and Methods). scFv-B8 is an intrabody that doesn't specifically bind to mHtt, therefore cells transfected with scFv-B8 are the control group. Fluorescence imaging taken at 48 hours post-transfection demonstrated that cells co-transfected with intrabody scFv-C4-HA-PEST-mCherry or scFv-C4-HA-CL1-mCherry have diffuse mHtt instead of aggregated mHtt in the cell (green fluorescence), indicating that mHtt aggregation might be decreased by intrabodies in both groups. The overall mHtt expression in scFv-C4-HA-CL1 transfected cells is lower than scFv-C4-HA-PEST transfected cells (Fig 5A left column), suggesting that CL1 degron enhances intrabody-mediated mHtt degradation with more efficacy than PEST degron does. Western blot results also showed that these two cell groups express a significantly lower total mHtt protein level compared to the control group (* $P<0.05$), with scFv-C4-HA-CL1 transfected cells having slightly less mHtt than scFv-C4-HA-PEST transfected cells (Fig 5C). Additionally, Fig 5D showed that the intrabody (scFV-C4) expression in scFv-C4-HA-CL1 transfected cells is significantly lower than scFv-C4-HA-PEST transfected cells ($^{\#}$ $P<0.05$). More decrease of mHtt protein level in scFv-C4-HA-CL1 transfected cells observed, when relatively lower intrabody expression in these cells, indicates that CL1 degron enhances intrabody-mediated mHtt degradation more efficiently than PEST degron. In another word, the ubiquitin-dependent proteasome pathway involving CL1 degron is more efficient in intrabody-mediated mHtt degradation than the ubiquitin-independent proteasome pathway involving PEST degron.



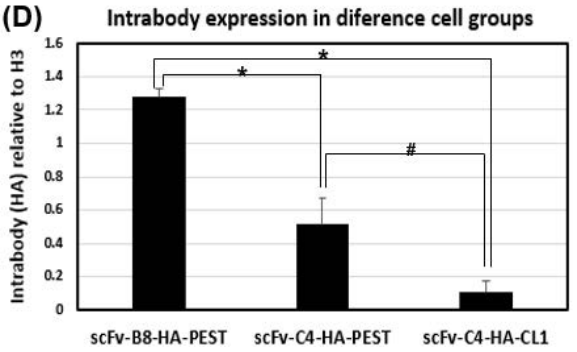


Figure 5. scFv-C4-HA-CL1 enhances eGFP-tagged mHttex1-72Q degradation more efficiently than scFv-C4-HA-PEST in co-transfected HEK293 cells. (A) Representative live-cell fluorescence imaging of scFv-C4-HA-PEST transfected HEK293 cells and scFv-C4-HA-CL1 transfected HEK293 cells. From left to right: Green fluorescence indicates mHttex1-72Q, red fluorescence indicates intrabody (scFv-C4-HA-CL1 or scFv-C4-HA-CL1 PEST). Phase image is to confirm uniform cell integrity. Merge is with all fluorescence channels open and overlapped with phase image. White arrows show an example of intrabody-mediated mHtt degradation in scFv-C4-HA-CL1 transfected cells, where green fluorescence is invisible with red fluorescence strongly displayed at the same location in the cell, indicating the mHtt degradation by intrabody. Scale bar = 20 μm. (B) Western blot probed for mHtt (EM48), scFv-C4 or scFV-B8 intrabody (HA) and endogenous Histone (H3). (C) Quantitative comparison of mHtt (EM48 relative to endogenous H3) amongst all cell groups. (D) Quantitative comparison of intrabody (HA relative to H3) amongst all cell groups.

4. Discussion

4.1 Cell lines for HD disease model

Multiple cell lines, such as human induced pluripotent stem cells (hiPSCs) or neural stem cells (NSCs), are available to create Huntington's Disease cell model. HEK293 cells are easy-to-culture, easy-to-transfect cells and can be expanded indefinitely. HEK293 cells also show neuronal lineage cells characteristics, such as the potential to propagate highly neurotropic viruses and inducible synaptogenesis [21]. It has been also demonstrated that edited HEK 293 cells containing ~100 and 150 CAG repeats at the HTT locus undergo a wide spectrum of pathological changes characteristic of Huntington's Disease [22]. These cells are popular cellular models for many studies. To studying protein degradation pathways mediated by intrabody in HD, non-neuronal HEK293 cells are used. In our study, various plasmids were co-transfected to HEK293 and successfully generated proteasome reporter systems.

4.2 mHtt aggregation decreased by ubiquitin proteasome system

From the mHttex1-72Q transfection experiment, it was demonstrated that HD cell model can be successfully created with aggregated mHtt observed in cells starting at 48 hours post-transfection. However, in the co-transfection study, it showed that aggregated mHtt was not observed at 48 hours post-transfection in cells expressing the scFv-C4-CL1 fusion protein or the scFv-C4-PEST fusion protein, indicating that degrons (CL1 and PEST) indeed enhance intrabody-mediated mHtt aggregation in general, no matter which protein degradation pathway is involved. Furthermore, the ubiquitin-dependent proteasome pathway via CL1 degron showed more efficacy in mHtt degradation than the ubiquitin-independent proteasome pathway via PEST degron, suggesting that the ubiquitin-dependent proteasome pathway triggered by CL1 degron plays an important and effective role in intrabody-mediated mHtt degradation. These results provide the scientific foundation of developing a more efficient treatment strategy for HD. The various degrons combined with intrabody facilitating degradation of mutant protein can be potentially used as a treatment strategy for neurodegenerative disease.

5. Conclusion

The inclusions formed by aggregation of mHtt proteins are the characteristics of HD. Approaches that can inhibit mHtt aggregation or facilitate mHtt degradation are the therapeutic directions. Our study investigated the intrabody-mediated mHtt degradation through degrons and compared the ubiquitin-dependent versus the ubiquitin-independent degradation pathways involving various

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degrons using HD HEK293 cell model. This *in vitro* study demonstrated that in mHttex1-72Q and intrabody (scFv-C4) co-transfected cells, the ubiquitin-dependent proteasome pathway involving CL1 degron enhances intrabody-mediated mHtt degradation more efficiently than the ubiquitin-independent proteasome pathway involving PEST degron. This study provides the scientific foundation to efficiently degrade mHtt protein using intrabodies bound with degrons and creates a potential therapeutic approach for HD. Further studies will be essential to fully explore the mHtt degradation mechanism using *in vivo* animal models, then advance to clinical applications towards a potential treatment for Huntington disease.

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