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ORIGINAL ARTICLE

Heme-binding-mediated negative regulation of the tryptophan metabolic enzyme indoleamine 2,3-dioxygenase 1 (IDO1) by IDO2

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Indoleamine 2,3-dioxygenases (IDOs) are tryptophan-catabolizing enzymes with immunomodulatory functions. However, the biological role of IDO2 and its relationship with IDO1 are unknown. To assess the relationship between IDO2 and IDO1, we investigated the effects of co-expression of human (h) IDO2 on hIDO1 activity. Cells co-expressing hIDO1 and hIDO2 showed reduced tryptophan metabolic activity compared with those expressing hIDO1 only. In a proteomic analysis, hIDO1-expressing cells exhibited enhanced expression of proteins related to the cell cycle and amino acid metabolism, and decreased expression of proteins related to cell survival. However, cells co-expressing hIDO1 and hIDO2 showed enhanced expression of negative regulators of cell apoptosis compared with those expressing hIDO1 only. Co-expression of hIDO1 and hIDO2 rescued the cell death induced by tryptophan-depletion through hIDO1 activity. Cells expressing only hIDO2 exhibited no marked differences in proteome profiles or cell growth compared with mock-transfectants. Cellular tryptophan metabolic activity and cell death were restored by co-expressing the hIDO2 mutant substituting the histidine 360 residue for alanine. These results demonstrate that hIDO2 plays a novel role as a negative regulator of hIDO1 by competing for heme-binding with hIDO1, and provide information useful for development of therapeutic strategies to control cancer and immunological disorders that target IDO molecules.
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INTRODUCTION

Indoleamine 2,3-dioxygenase 1 (IDO1) and its paralog IDO2[1,2] are involved in tryptophan catabolism. IDO molecules have immunosuppressive functions, as IDO1 is required for maternal tolerance to fetal tissue and L, D-1-methyl tryptophan (1MT)-triggered rejection of the fetal tissue.[3] IDO1 has been implicated in various diseases, including cancers, chronic infection and autoimmunity.[4] Genetic ablation of *Ido1* leads to upregulation of *Ido2* in the epididymis, suggesting a possible functional overlap between these two molecules.[5] In this study, we evaluated the function interaction between IDO1 and IDO2, focusing on the impact of IDO2 co-expression on IDO1 catalytic activity.

IDO1 is the major rate-limiting enzyme that catalyzes conversion of tryptophan to kynurenine, an initial step in tryptophan degradation by the kynurenine pathway.[6,7] Human (h) IDO1 shares 58% sequence homology with mouse IDO1.[8] IDO1 is widely expressed in various tissues, including lung, small intestine, placenta, spleen, central nervous system and epididymis,[5,9,10] and its expression is increased markedly in various cancers. IDO1 plays a role in immune tolerance,[11,12] promoting tumorigenesis.[13–15] IDO1-mediated tryptophan depletion is considered to be one of the immune suppressive mechanisms and a target pathway for development of anticancer drugs.

IDO2 was identified by *in silico* mapping of the National Center for Biotechnology Information human genome sequence using the IDO1 sequence as a probe.[2] It was mapped to chromosome 8p12, immediately downstream of the *IDO1* gene. The hIDO2 protein shows 43% amino acid homology with hIDO1.[16] IDO2 is detectable in placenta, brain, liver, kidney and the epididymis of mice[5] and some human gastric,

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colon and pancreatic cancer cell tumors.[17,18] However, the functional activity of IDO2 is obscure. IDO2 is considered to be biologically inactive or active only under specific (albeit uncharacterized) conditions.[19] Kynurenine is not detectable in HEK293 cells transfected with hIDO2,[20,21] but is present at low levels in a cell line transfected with an inducible construct.[2] IDO1 and IDO2 seem to be regulated differently because the *levo* (L)-1MT isomer eliminates IDO1 activity,[2,18,19] whereas the *dextro* (D)-1MT stereoisomer exclusively blocks IDO2 activity.[2] Because IDO2 has a very-low level of catalytic activity, it may have a distinct biological role. IDO2, but not IDO1, is a critical mediator of arthritis development and autoantibody production,[22] suggesting separate *in vivo* functions for IDO1 and IDO2. However, the IDO2 blocker D-1MT has been evaluated in clinical trials as an anticancer treatment, suggesting that IDO2 may play a cooperative role with IDO1 in immune regulation. Both IDO1 and IDO2 are expressed in normal mouse epididymis, but IDO2 is highly upregulated in the epididymis of IDO1-deficient mice.[5] High IDO2 expression is insufficient to compensate for kynurenine production. Upregulation/alteration of IDO2 splice variants was detected in peritoneal macrophages of IDO1-deficient mice, but no such changes in IDO1 expression or activity were observed in IDO2-deficient mice.[23] Therefore, we presume that IDO2 plays a particular biological role and that interplay and/or interactive-regulation between IDO1 and IDO2 is possible.

In this study, we investigated the influence of hIDO2 co-expression on hIDO1 catalytic activity to assess the interplay between these two molecules, using a hIDO1 and hIDO2-overexpressing cell line. The results reveal a novel regulatory effect of hIDO2 on hIDO1 catalytic activity and the possible underlying mechanism.

MATERIALS AND METHODS

Plasmid DNA

hIDO1 and hIDO2 (named hIDO1-2) recombinant DNA, the hIDO1-coding region tagged with Flag, or hIDO2 tagged with hemagglutinin (HA) were subcloned into bicistronic vectors including the internal ribosomal entry site or the porcine teschovirus-1 (P2A) peptide to construct hIDO1 and hIDO2.[24] The enhanced green fluorescent protein (eGFP) or mCherry was included as a reporter gene. The hIDO2 (H; H360A) mutant, with alanine replacing histidine at position 360, was constructed by polymerase chain reaction-based site-directed mutagenesis using the forward primer 5′–CTGCGGAGCTATGCCATCACCATG–3′ and the reverse primer, 5′–CATGGTGATGGCATAGCTCCGAG–3′.

Cell culture and construction of hIDO-expressing HEK293 stable cell lines

Human embryonic kidney (HEK293) cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% bovine calf serum, penicillin, streptomycin and gentamycin. HEK293 cells were transfected with 2 μg of the hIDO1, hIDO2, hIDO1-2 or hIDO1-2 (H) hIDO DNA constructs using the calcium phosphate or polyethylenimine (PEI) (Sigma-Aldrich, St Louis, MO, USA) transfection method to construct hIDO1-, hIDO2- and hIDO1-hIDO2-expressing HEK293 stable cell lines. Transfected HEK293 cells were selected with 20 μg ml^{-1} blasticidin (Invitrogen, Carlsbad, CA, USA) including 50 μM L-tryptophan (L-Trp) (Sigma-Aldrich) for 2–4 weeks, and single-cell originated hIDO-expressing stable cell lines were isolated by flow cytometry analysis and western blotting.

Kynurenine assay

The catalytic activity of the hIDOs was measured by means of a colorimetric kynurenine assay.[25] Briefly, stable hIDO1-expressing HEK293 cells were transfected with hIDO2 wild-type and hIDO2 mutant DNAs using PEI. The next day, the medium was exchanged for fresh medium containing 100 μM L-Trp, and the culture supernatants were harvested after additional 24 h incubation. The hIDO-expressing HEK293 stable cells were seeded onto a 96-well plate at 2×10^5 cells per well in the absence or presence of 100 μM L-Trp, and the culture supernatants were harvested after 24 h incubation. A 160 μl aliquot of culture supernatant was transferred to a 96-well round-bottom plate (SPL Life Sciences, Pocheon, Korea); 10 μl of 30% trichloroacetic acid (Sigma-Aldrich) were added to each well and plates were incubated for 30 min at 50 °C to hydrolyze N-formylkynurenine to kynurenine. The samples were then centrifuged at 3000 *g* for 10 min. Then, 100 μl aliquots of the supernatant were harvested and mixed with 100 μl of freshly prepared Ehrlich's reagent (2% 4-demethylaminobenzaldehyde in glacial acetic acid; Tokyo Chemical Industry, Tokyo, Japan). Absorbance at 492 nm was measured using a microplate reader (BioTek, Winooski, VT, USA) with the Gen5 software (BioTek) after 10 min incubation.

Proteomic analysis

The hIDO1-, hIDO2- and hIDO1-2-expressing HEK293 stable cell lines were subjected to proteomic analysis. Proteins were quantitated and identified using isobaric tags for relative and absolute quantitation [26] and mass spectrometry. A total of 2557 proteins were analyzed using the International Protein Index Human Database with the proteomic tools. Normalized intensities for those proteins were determined using a hierarchical clustering algorithm. Analysis of variance was performed to identify differentially expressed proteins with a threshold false discovery rate. Significantly differentially expressed proteins were classified based on their biological functions. Enrichment of each biological category was examined statistically using Fisher's exact test.

Cell proliferation assay

The stable hIDO-expressing HEK293 cell lines were seeded in 96-well plates at a density of 4×10^4 per well. Cell proliferation rates were then measured with the colorimetric WST-1 assay (ITSBio, Seoul, Korea) over 3 days in the absence or presence of 25 μM L-Trp. Absorbance at 450 nm was measured using a microplate reader with the Gen5 software.

Flow cytometry analysis

The stable hIDO1-expressing HEK293 cell lines transfected with hIDO2 and hIDO2 (H) mutant DNAs were fixed in 4% paraformaldehyde for 10 min at 48 h posttransfection. Fixed cells were washed with Triton buffer (0.5% Triton X-100, 0.1% BSA in PBS) and stained with anti-HA epitope (Applied Biological Materials, Richmond, BC, Canada) primary antibodies, and an allophycocyanin-conjugated rat anti-mouse secondary IgG antibody (BD Biosciences, Franklin Lakes, NJ, USA). The stained cells were washed and analyzed by FACSCalibur (BD Bioscience) running the FlowJo software (Tree Star, Ashland, OR, USA).

Western blotting assay

The stable hIDO-expressing HEK293 cell lines were harvested and lysed with RIPA buffer (1 ml/1×10^7 cells; 150 mM NaCl, 50 mM Tris-HCl [pH 7.4], 1% NP-40, 0.1% sodium deoxycholate, 1 mM EDTA and protease inhibitors). Cell lysates were loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and stained with mouse Flag epitope (Sigma-Aldrich) and anti-HA epitope (Applied Biological Materials) primary antibodies and a horseradish peroxidase-conjugated goat anti-mouse secondary IgG antibody (KOMA Biotechnology, Seoul, Korea). A monoclonal anti-β-actin (Sigma-Aldrich) antibody was used as the loading control. An enhanced chemiluminescence reagent (Intron, Seoul, Korea) was used to visualize the protein signals.

Statistical procedures

The statistical analysis was performed using the two-tailed *t*-test with the GraphPad Prism ver. 5 software (GraphPad Software, San Diego, CA, USA). A value of $P<0.05$ was taken to indicate statistical significance.

RESULTS

Overexpression of hIDO2 leads to decreased hIDO1 activity at the single-cell level

We measured kynurenine production by hIDO1 and hIDO2 (hIDO1-2)-overexpressing HEK293 cell lines in comparison with those produced by HEK293 cells expressing either hIDO1 or hIDO2 to investigate the influence of hIDO2 on hIDO1 functional activity. Transfection with a plasmid in which recombinant DNAs encoding Flag-tagged hIDO1 and HA-tagged hIDO2 were linked with the 2A peptide was used to establish hIDO1 and hIDO2-co-expressing HEK293 cells (Figure 1a). HEK293 cells transfected to co-express Flag-tagged hIDO1 and HA-tagged hIDO2, and control cells transfected to express either Flag-tagged hIDO1 or HA-tagged hIDO2, were cultured with 20 μg ml^{-1} blasticidin for 2–4 weeks. More than five single-cell originating cell lines expressing hIDO1 and hIDO2 at similar levels were selected from each group of transfectants, and their expression levels were confirmed by Western blotting using anti-Flag and HA antibodies (Figure 1b).

The stable cell lines were subjected to kynurenine assay after an overnight incubation. Cells expressing hIDO1 only had high tryptophan catalytic activity and produced 64.54 ± 2.19 μM kynurenine, whereas those expressing only hIDO2 showed significantly lower tryptophan metabolic activity, as evidenced by their production of $\leqslant 2$ μM kynurenine. These results agree with those of previous studies.[20,21] Interestingly, kynurenine production by HEK293 cells co-expressing hIDO1 and hIDO2 was significantly lower (mean, 56.40 ± 2.75 μM) than that by cells expressing only hIDO1, despite the similar hIDO1 expression of these two cell lines. This difference in L-Trp catalytic activity between the two cell lines was confirmed upon addition of 100 μM L-Trp to the culture medium. L-Trp supplementation increased kynurenine production by both co-expressing cells and those expressing only hIDO1 (87.57 ± 4.01 and 111.67 ± 4.43 μM, respectively) (Figure 1c). This difference remained when the IDO1 inhibitor L-1MT was added to the medium; however, this treatment decreased kynurenine production by both cell types in a dose-dependent manner. Therefore, co-expression of hIDO2 reduced the catalytic activity of hIDO1.

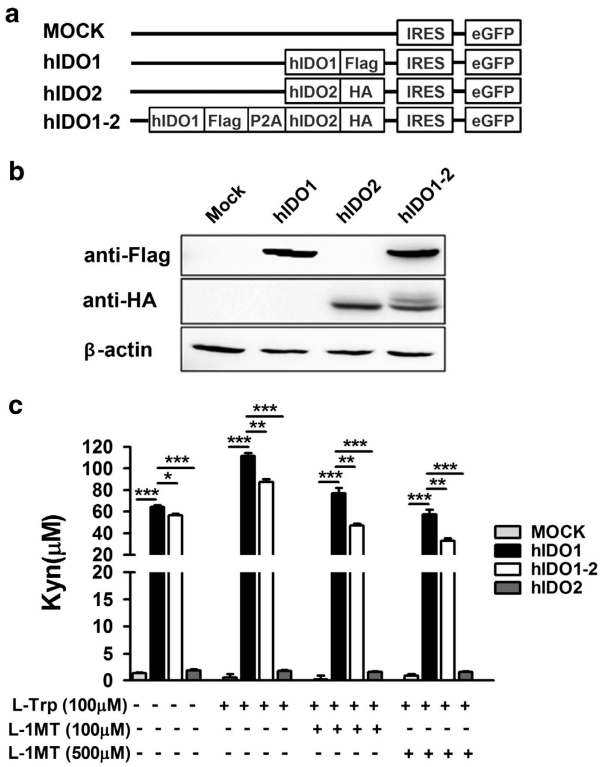

Figure 1 Inhibition of human indoleamine 2,3-dioxygenase 1 (hIDO1) enzymatic activity by co-expressing hIDO2 at the single-cell level. (**a**) Diagram of DNA constructs for expression of hIDO1, hIDO2, or both hIDO1 and hIDO2 (hIDO1-2) tagged with Flag, hemagglutinin epitopes, and fused with the eGFP gene. A self-cleaving 2A peptide (26 amino acids) between hIDO1-Flag and hIDO2-hemagglutinin was used for efficient expression of multiple genes. (**b**) Western blotting was performed using extracts of HEK293 cells expressing hIDO1-Flag, hIDO2-HA, or both proteins. HEK293 cells were transfected with plasmid DNA as shown in **a**, and subsequently selected with 2 μg ml^{-1} blasticidin for 2–4 weeks to generate stable cell lines. hIDO1 or hIDO2 protein levels were determined by staining with anti-Flag and -hemagglutinin monoclonal antibodies, respectively, using the LAS 4000 Mini. β-actin was used as the internal control. (**c**) Kynurenine production was measured with a quantitative colorimetric assay using Ehrlich's reagent. hIDO-expressing stable cell lines were incubated for 24 h with complete medium including 100 μM L-tryptophan (L-Trp) in the absence or presence of 100 or 500 μM of the IDO inhibitor L-1MT. $*P<0.05$, $**P<0.01$, $***P<0.001$. Data shown (**b** and **c**) represent at least three independent experiments.

Differential protein expression profiles of the hIDO1-, hIDO2- and hIDO1-2-expressing stable cell lines and implications for cell survival

We compared the protein profiles of cells expressing either hIDO1 or hIDO2 with those co-expressing hIDO1 and hIDO2 (hIDO1-2) to evaluate the effect of hIDO2 co-expression on

the catalytic activity of hIDO1 (Figure 2). Among the 2557 proteins analyzed, 159 were identified by analysis of variance as differentially expressed among the groups with fold-changes >1.5. A heat map analysis showed differential protein expression among the stable hIDO1-, hIDO2- and hIDO1-2-expressing HEK293 cell lines (Figure 2a). The top six biological classifications were identified by Fisher's exact test (Figure 2b). Unexpectedly, hIDO2 overexpression did not result in marked changes in protein profile compared with mock-transfected control cells. However, hIDO1 overexpression enhanced the expression of proteins related to the cell cycle and amino acid metabolism compared with mock-transfected cells, suggesting enhanced metabolic activity in hIDO1-expressing cells. Additionally, the expression of cell survival/negative regulation of cell death-related proteins also showed considerable changes. Among the 159 proteins, 69 were expressed at higher levels by cells co-expressing hIDO1 and hIDO2 compared with cells expressing only hIDO1 (Table 1); 46 proteins were expressed at lower levels (Table 2). Seven of the 67 proteins whose expression was enhanced in cells co-expressing hIDO1 and

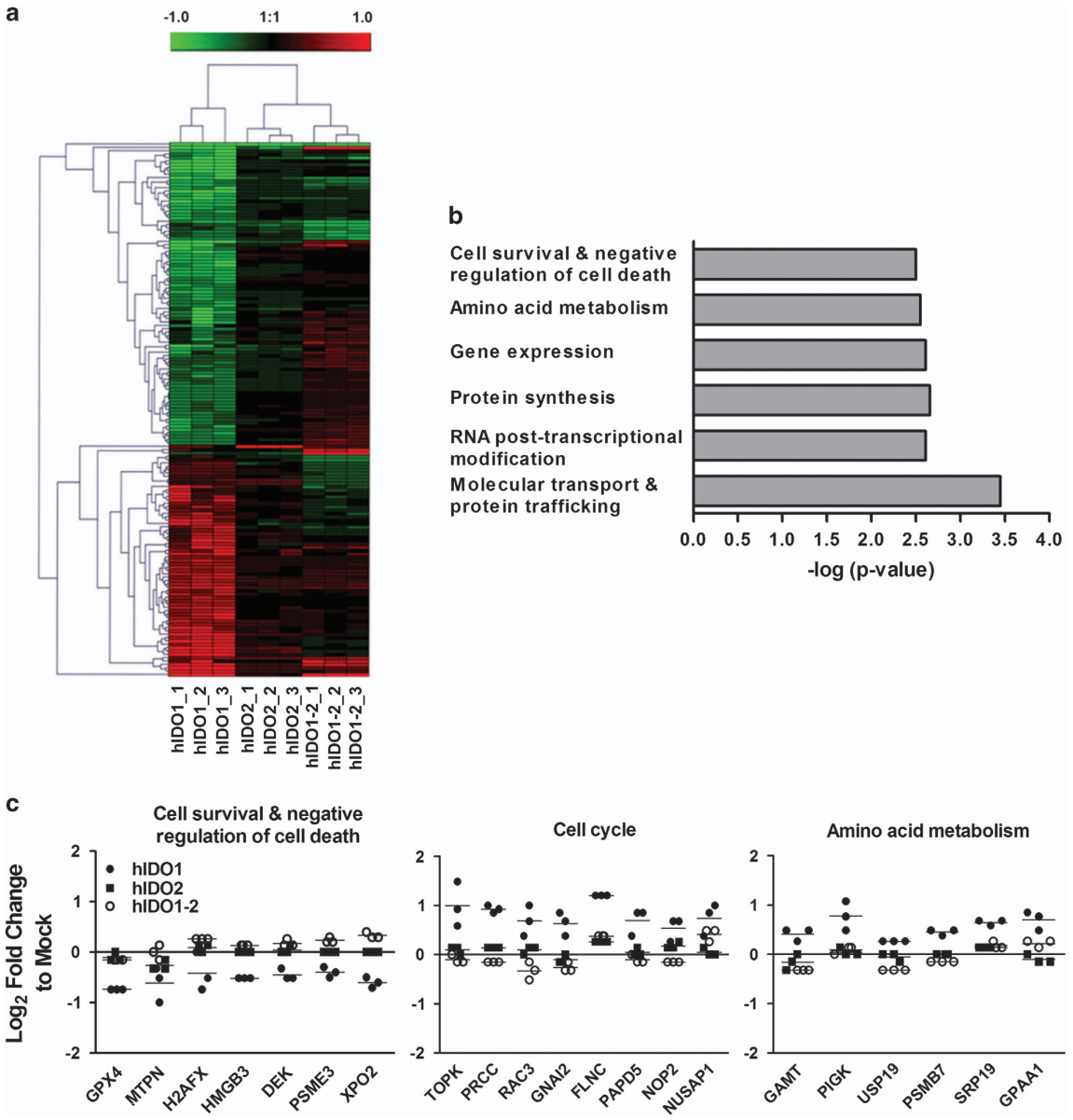

Figure 2 Evaluation of the protein profiles of human indoleamine 2,3-dioxygenase 1 (hIDO1)-, hIDO2-, or hIDO1-2-overexpressing HEK293 stable cells using a proteomic approach. (**a**) Hierarchical clustering analysis of 159 differentially expressed proteins identified by analysis of variance at a false discovery rate <0.05. Scale bar at the top indicates fold change compared to mock control on a log2 scale. (**b**) Functional classifications of the 159 proteins. Significant biological classes were identified by Fisher's exact test. The top six significant categories with *P*-values are shown. (**c**) Fold changes in expression of proteins related to cell survival/negative regulation of cell death, amino acid metabolism, and cell cycle compared with the mock control are plotted on a log2 scale.

Table 1 The 69 proteins detected at higher levels (>1.5-fold) in cells co-expressing human indoleamine 2,3-dioxygenase 1 (hIDO1) and hIDO2 than in cells expressing only hIDO1

Accession no.	*Symbol*	*Protein names*	*Fold changes hIDO1-2 vs hIDO1*
Q71U36	TUBA1A	Tubulin alpha-1A chain	8.316
P84077	ARF1	ADP-ribosylation factor 1	3.249
Q96P16	RPRD1A	Isoform 1 of regulation of nuclear pre-mRNA domain-containing protein 1A	2.644
Q15758	ATB(0)	Neutral amino acid transporter B(0)	2.598
P08107	HSP71	Heat shock 70-kDa protein 1A/1B	2.321
P49207	RPL34	60S ribosomal protein L34	2.224
Q13561	DCTN2	Isoform 2 of dynactin subunit 2	2.014
P30046	DOPD	D-dopachrome decarboxylase	2.008
O60333	KIF1B	Isoform 3 of kinesin-like protein KIF1B	1.988
O75503	CLN5	ceroid-lipofuscinosis neuronal protein 5	1.919
P61970	NUTF2	Nuclear transport factor 2	1.908
Q14202	ZMYM3	Isoform 1 of zinc finger MYM-type protein 3	1.904
P00918	CA2	Carbonic anhydrase 2	1.904
P05387	RPLP2	60S acidic ribosomal protein P2	1.904
P49643	PRIM2	Isoform 1 of DNA primase large subunit	1.888
O75832	PSD10	26S proteasome non-ATPase regulatory subunit 10	1.866
P55060	XPO2	Isoform 1 of Exportin-2	1.853
P62495	ERF1	Eukaryotic peptide chain release factor subunit 1	1.833
Q9BWJ5	SF3B5	Splicing factor 3B subunit 5	1.800
P39687	ANP32A	Acidic leucine-rich nuclear phosphoprotein 32 family member A	1.798
P41567	eIF1	Eukaryotic translation initiation factor 1	1.794
P15954	COX7C	Cytochrome c oxidase subunit 7C, mitochondrial	1.788
O01805	ACBP1	Isoform 1 of Acyl-CoA-binding protein	1.788
A5D904	RPS9	40S ribosomal protein S9	1.771
A6NKZ8	YI016	Putative tubulin beta chain-like protein ENSP00000290377	1.771
Q96AG4	LRRC59	Leucine-rich repeat-containing protein 59	1.761
O60762	DPM1	Dolichol-phosphate mannosyltransferase	1.752
O95248	MTMR5	Isoform 1 of myotubularin-related protein 5	1.726
Q9Y224	C14orf166	UPF0568 protein C14orf166	1.726
P62851	RPS25	40S ribosomal protein S25	1.720
Q9NTK5	OLA1	Isoform 1 of Obg-like ATPase 1	1.714
H0Y9X3	PDCD6	Programmed cell death protein 6	1.699
Q9BTT0	ANP32E	Acidic leucine-rich nuclear phosphoprotein 32 family member E	1.699
Q9UBX3	DIC	Isoform 1 of mitochondrial dicarboxylate carrier	1.667
Q6PD74	AAGAB	Alpha- and gamma-adaptin-binding protein p34	1.667
Q7Z524	HUMEEP	HUMEEP	1.667
Q9UL15	BAG5	Isoform 1 of BAG family molecular chaperone regulator 5	1.654
P61289	PSME3	Isoform 1 of Proteasome activator complex subunit 3	1.640
Q15008	PSMD6	26S proteasome non-ATPase regulatory subunit 6	1.640
Q7L5N1	CSN6	COP9 signalosome complex subunit 6	1.603
P16104	H2AFX	Histone H2A.x	1.602
O15145	ARPC3	Actin-related protein 2/3 complex subunit 3	1.594
Q99471	PFDN5	Prefoldin subunit 5	1.594
O43808	PMP34	Peroxisomal membrane protein PMP34	1.587
Q96IL1	DIAPH1	Isoform 1 of protein diaphanous homolog 1	1.583
Q00688	FKBP3	Peptidyl-prolyl cis-trans isomerase FKBP3	1.582
Q86XI2	CNDG2	Isoform 2 of condensin-2 complex subunit G2	1.578
P99999	CYC	Cytochrome c	1.571
P62805	HIST4H4	Histone H4	1.571
P62913	RPL11	Isoform 1 of 60S ribosomal protein L11	1.571
O15347	HMGB3	High mobility group protein B3	1.571
A5D7K0	BLVRA	Biliverdin reductase A	1.565

Table 1 (Continued)

Accession no.	*Symbol*	*Protein names*	*Fold changes hIDO1-2 vs hIDO1*
Q12125	GET4	Isoform 2 of Golgi to ER traffic protein 4 homolog	1.552
P35659	DEK	Protein DEK	1.547
Q96CD2	PPCDC	Isoform 1 of Phosphopantothenoylcysteine decarboxylase	1.547
P31949	S100-A11	Protein S100-A11	1.541
Q96IX5	USMG5	Up-regulated during skeletal muscle growth protein 5	1.539
P62330	ARF6	ADP-ribosylation factor 6	1.529
P58546	MTPN	Myotrophin	1.523
Q53H12	AGK	Isoform 1 of acylglycerol kinase, mitochondrial	1.523
Q13084	MRPL28	39S ribosomal protein L28, mitochondrial	1.511
Q9C005	DPY30	Protein dpy-30 homolog	1.508
P62277	RPS13	40S ribosomal protein S13	1.504
Q99986	VRK1	Serine/threonine-protein kinase VRK1	1.504
B4DJ38	B4DJ38	cDNA FLJ56092, highly similar to pentatricopeptide repeat protein 1	1.504
Q9Y5S9	RBM8A	Isoform 1 of RNA-binding protein 8A	1.503
Q92973	TNPO1	Isoform 1 of transportin-1	1.503
Q5BJH1	PSAP	Isoform Sap-mu-0 of proactivator polypeptide	1.503
P36969	GPX4	phospholipid hydroperoxide glutathione peroxidase	1.500

hIDO2 (GPX4,[27] MTPN,[28] H2AFX,[29] HMGB3,[30] DEK,[31] PSME3[32] and XPO2[33]) were related to cell morphogenesis, proliferation or negative regulation of cell death/apoptosis (Figure 2c). The expression levels of these proteins were not enhanced markedly in cells expressing only IDO2. IDO1 levels were slightly (1.5×) higher in the co-expressing cells than in those expressing hIDO1 only. Therefore, the proteins whose expression was enhanced in co-expressing cells would likely be those suppressed by hIDO1 over-expression, and suppression of which was reversed in the presence of hIDO2, regardless of its function.

Co-expression of hIDO2 alleviate cell growth arrest and death

The proteomic analysis indicated that the expression levels of negative regulators of cell death were enhanced in cells co-expressing hIDO1 and hIDO2. Therefore, we postulated that high catalytic activity of hIDO1 would increase susceptibility to cell death (likely because of rapid depletion of tryptophan in the culture medium). Moreover, the reduced hIDO1 activity because of hIDO2 co-expression would rescue the cells from death. To test this, we compared the growth and death of cells co-expressing hIDO1 and hIDO2 and cells expressing hIDO1 only. Cell proliferation and morphological changes were examined under a microscope (Figure 3a); proliferation was also assessed using the quantitative colorimetric WST assay (Figure 3b). Microscopic observations and the WST assay showed that the proliferation and growth of cells over-expressing only hIDO1 were limited, whereas cells over-expressing hIDO2 and mock-transfected cells continued to proliferate. Interestingly, cells that over-expressed hIDO1 together with hIDO2 showed two-fold greater proliferation and enhanced survival compared to cells overexpressing only hIDO1. Supplementation of the culture medium with L-Trp increased the difference in survival between the two cell lines (Figure 3c). These results indicate that hIDO1 overexpression rapidly induced cell death, which was alleviated by hIDO2 co-expression because of a reduction in the catalytic activity of hIDO1.

hIDO1 functional activity is modulated via the hIDO2-heme-binding site

Based on our results, we hypothesized that the negative regulation of hIDO1 activity by hIDO2 co-expression may be because of competition rather than any specific hIDO2 functional activity. hIDO2 has two immunoreceptor tyrosine-based inhibition (ITIM) motifs, which play roles in immune cell signal transduction;[34,35] and a heme-binding site, which is essential for hIDO1 catalytic activity.[34,36,37] These two sites correspond to regions in the IDO2 sequence, in which mutations or alternative splicing occur.[2] Therefore, we postulated that hIDO2 suppresses hIDO1 catalytic activity through heme-binding site-based competition with hIDO1. To test this, we generated a hIDO2 mutant, in which the histidine residue at position 360 was substituted for alanine (H360A) to abrogate hIDO2 heme-binding activity. Then, we examined the effects of co-expression of this hIDO2 mutant with hIDO1 (Figure 4a). A DNA construct harboring the HA-tagged hIDO2 mutant was transfected into stable hIDO1-expressing cell lines at various doses, and kynurenine production by cells expressing H360A was compared with that of control cells transfected with wild-type hIDO2 and mock-transfected control cells. Flow cytometry analysis confirmed dose-dependent expression of the wide type or mutant form of hIDO2 after transfection; however, the levels

Table 2 Forty-six proteins detected at lower levels (>1.5-fold) in cells co-expressing human indoleamine 2,3-dioxygenase 1 (hIDO1) and hIDO2 than in cells expressing hIDO1 only

Accession no.	*Symbol*	*Protein names*	*Fold changes hIDO1-2 vs hIDO1*
Q15427	SF3B4	Splicing factor 3B subunit 4	2.61
P21333	FLNA	Isoform 2 of Filamin-A	2.538
P05204	HMG-17	Non-histone chromosomal protein HMG-17	2.263
Q96KB5	TOPK	Lymphokine-activated killer T-cell-originated protein kinase	2.144
Q92733	PRCC	Proline-rich protein PRCC	2.109
P60763	RAC3	Ras-related C3 botulinum toxin substrate 3	2.021
Q9H2H9	SLC38A1	Sodium-coupled neutral amino acid transporter 1	2.003
Q8ND24	RNF214	RING finger protein 214	1.945
P67936	TPM4	Isoform 1 of tropomyosin alpha-4 chain	1.921
P04897	GNAI2	Isoform 2 of Guanine nucleotide-binding protein G(i) subunit alpha-2	1.866
P49069	CAMLG	Calcium signal-modulating cyclophilin ligand	1.775
Q14315	FLNC	Isoform 1 of Filamin-C	1.769
Q9H7N4	SFR19	Splicing factor, arginine/serine-rich 19	1.753
Q8NDF8	PAPD5	PAP-associated domain-containing protein 5 isoform b	1.732
P05114	HMG-14	Non-histone chromosomal protein HMG-14	1.727
O43157	PLXNB1	Isoform 2 of Plexin-B1	1.685
Q14353	GAMT	Guanidinoacetate N-methyltransferase	1.662
O43542	XRCC3	DNA repair protein XRCC3	1.655
A4D105	RPA3	Replication protein A 14 kDa subunit	1.65
Q8NEY8	PPHLN1	Isoform 1 of Periphilin-1	1.65
Q9Y679	AUP1	Isoform short of ancient ubiquitous protein 1	1.644
P37802	TAGLN2	Transgelin-2	1.625
Q9BZX2	UCK2	Isoform 1 of Uridine-cytidine kinase 2	1.625
P46087	NOP2	Putative ribosomal RNA methyltransferase 1	1.615
Q6VN20	RANBP10	Ran-binding protein 10	1.615
Q9Y2R4	DDX52	Probable ATP-dependent RNA helicase DDX52	1.614
Q9H061	TMEM126A	Transmembrane protein 126A	1.61
Q9NZT2	OGFR	Isoform 1 of opioid growth factor receptor	1.609
Q9BYB4	GNB1L	Isoform 1 of guanine nucleotide-binding protein subunit beta-like protein 1	1.609
Q92643	PIGK	GPI-anchor transamidase	1.604
Q9UPT8	ZC3H4	Zinc finger CCCH domain-containing protein 4	1.6
Q9UBV2	SE1L1	Isoform 1 of protein sel-1 homolog 1	1.594
Q9UPN9	TRIM33	Isoform alpha of E3 ubiquitin-protein ligase TRIM33	1.589
Q9H6R0	DHX33	Highly similar to putative ATP-dependent RNA helicase DHX33	1.583
O95453	PARN	Poly(A)-specific ribonuclease PARN	1.572
P11021	GRP78	78 kDa glucose-regulated protein	1.571
Q9NXE4	SMPD4	Isoform 4 of sphingomyelin phosphodiesterase 4	1.542
Q9NRL3	STRN4	Striatin-4	1.534
Q9NYV4	CDK12	Isoform 1 of cyclin-dependent kinase 12	1.53
Q13610	PWP1	Periodic tryptophan protein 1 homolog	1.521
Q99436	PSMB7	Proteasome subunit beta type-7	1.518
P24390	KDELR1	ER lumen protein retaining receptor 1	1.515
Q6P9B9	INTS5	Integrator complex subunit 5	1.515
P49821	NDUFV1	Isoform 2 of Nadh dehydrogenase (ubiquinone) flavoprotein 3	1.513
Q9ULV3	CIZ1	Isoform 2 of cip1-interacting zinc finger protein	1.5
O94966	USP19	Isoform 1 of ubiquitin carboxyl-terminal hydrolase 19	1.5

were consistent among the hIDO2 forms transfected with an identical dose (Figure 4b). The hIDO2 did not exert suppressive effects when H360A (hIDO2 heme-binding mutant) was co-expressed with hIDO1, as kynurenine production increased significantly compared with that in hIDO2-transfected control cells in a DNA-dose-dependent manner (Figure 4c). These results suggest that negative regulation by hIDO2 is based on competition with hIDO1 for heme binding.

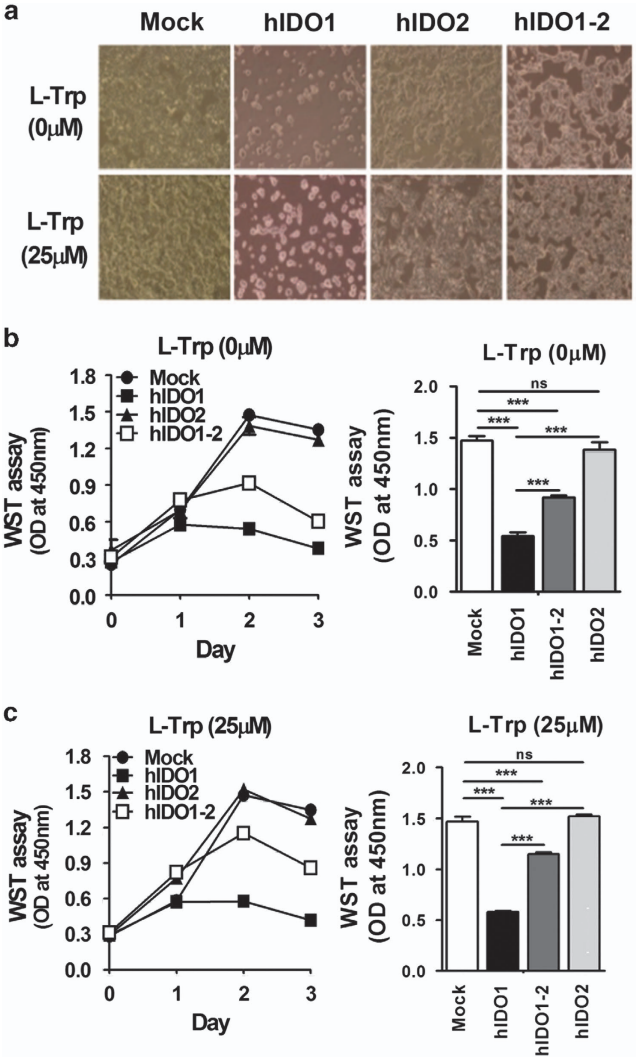

Figure 3 Alleviation of cell growth arrest and death by co-expression of hIDO2. (**a**) Morphological changes in hIDO1-, hIDO2- or hIDO1-2-expressing HEK293 stable cells in the absence or presence of 25 μM L-Trp were detected on day 3 during the cell proliferation assay (×100 magnification). Cell proliferation was measured by WST colorimetric assay during the 3-day culture period in the (**b**) absence or (**c**) presence of 25 μM L-Trp. Absorbance at 450 nm was measured using a microplate reader. Cell proliferation values on day 2 were subjected to statistical analysis. All experiments were performed independently three times. ns, not significant, $***P<0.001$.

Effect of the IDO inhibitor L-1MT on hIDO1 functional activity upon co-expression with hIDO2 or hIDO2 (H) genes

Next, we generated stable cell lines from hIDO1-expressing cells that stably co-expressed the hIDO2 wild-type or the H360A mutant. These cells were subjected to kynurenine assay following growth in medium supplemented with L-Trp. Cell lines expressing hIDO2 at similar levels were selected after flow cytometric analysis of levels of HA-tagged, wild-type and mutant hIDO2. Co-expression of the H360A mutant did not suppress hIDO1 catalytic activity to the same degree as did co-expression of wild-type IDO2 (Figure 5a). Although L-1MT co-treatment significantly reduced hIDO1 activity, such trends were still maintained (Figure 5a). Therefore, the suppressive effects of hIDO2 were focused on hIDO1 catalytic activity.

Next, we investigated whether the H360A mutant could restore the reduction in growth rate caused by hIDO1-mediated tryptophan depletion. WST proliferation assays demonstrated that co-expression of the H360A mutant did not negatively affect hIDO1-mediated cell death compared with co-expression of wild-type hIDO2 during the 3-day culture period. This resulted in early arrest of the growth of cells co-expressing the H360A mutant and hIDO1, as well as cells expressing hIDO1 only (Figure 5b). These results confirm that hIDO2 negatively regulates hIDO1 catalytic activity via its heme-binding domain.

DISCUSSION

Our findings demonstrated that hIDO2 functioned as a negative regulator of hIDO1 catalytic activity, an effect mediated by its heme-binding activity.

In contrast to the established biological role of IDO1 in tryptophan catabolism and as an immune modulator implicated in tumors and inflammatory diseases, the biological relevance of IDO2 is unknown. Here, we demonstrated that hIDO2 suppressed the catalytic activity of hIDO1. Kynurenine assays revealed that IDO2 had negligible catalytic activity, in agreement with previous reports that IDO2 is inactive under physiological conditions and that its catalytic activity corresponds to only 3–5% of that of IDO1.[16,38] Although IDO2 is a candidate target of D-1MT, which has been evaluated in clinical trials for treatment of cancers,[39] the physical evidence supporting this notion is reliant on inhibition of the already very-low catalytic activity of IDO2. We did not detect inhibition by D-1MT of hIDO1 catalytic activity (data not shown), consistent with a previous report that IDO1 is not a D-1MT target.[19] We could not determine whether hIDO2 catalytic activity is the D-1MT target because hIDO2 catalytic activity was negligible. These results suggest that IDO2 does not play a role as an active catalytic enzyme, and suggest that the anticancer effect of D-1MT may be dependent on negative regulation of IDO1 catalytic activity by IDO2, as IDO2 is a target of D-1MT.

Co-expression of IDO1 and IDO2 has been detected in tumor cells, dendritic cells and the epididymis.[5,9] The IDO2 protein is highly upregulated in the epididymis of IDO1-deficient mice, suggesting that it contributes to immune modulation or maintenance of IDO1 tryptophan metabolism[5]. IDO2 upregulation in IDO1-deficient mice suggests a functional interaction between these two molecules. Here, we provide evidence that IDO2 co-expression suppresses IDO1 catalytic activity, and that this effect is dependent on the presence of an intact heme-binding site. This finding indicates that the IDO2 heme-binding site is the active site, suggesting the presence of competition for heme-binding upon co-expression of IDO1 and IDO2. Therefore, although IDO2 is inactive during tryptophan metabolism, a mutation in the heme-binding site or a change in

its expression level could regulate IDO1 catalytic activity, even if the IDO1 expression level is unaffected.

In addition to their catalytic activity, IDO1 and IDO2 have signaling activities because of their two putative ITIM-motifs.[34,35] The ITIM-motif is responsible for transforming growth factor (TGF-β)-mediated signaling, and the TGF-β-IDO axis activates the noncanonical nuclear factor-κB pathway through SHP.[34] Signaling activity regulates IDO expression. In this study, mutation of the active ITIM-motif at position 266[34] resulted in maintenance of the heme-binding-mediated negative regulation by hIDO2 of hIDO1 catalytic activity (data not shown). Therefore, the hIDO2-ITIM-motif may be irrelevant to the catalytic function and suppression of hIDO1 catalytic activity by hIDO2.

Our data demonstrate that hIDO2 plays a role as a negative modulator of hIDO1 activity, rather than as a catalytic enzyme. This is further supported by the independent and separately grouped protein profiles of hIDO2- and hIDO1-expressing cells. Although we did not perform a functional assessment of these molecules in immunological tolerance in this study, IDO1-dependent generation of T-regulatory cells has been reported to be defective in IDO2-deficient mice,[23] supporting our suggestion of a functional interaction between IDO1 and IDO2. However, the fact that IDO2 deficiency reduces inflammatory cytokine production after immune stimulation suggests a non-redundant function.[23] In addition, IDO1- and IDO2-deficient mice showed different susceptibilities to antibody-mediated autoimmunity.[22] Therefore, IDO2 might play a separate role. The proteomes of cells expressing only hIDO2 showed few changes compared with those of mock-transfectants, suggesting that hIDO2 rarely functions under nonstimulatory normal conditions (Figure 2a). Therefore, it is possible that hIDO2 expression and function are induced by environmental changes such as inflammation or increased needs for tryptophan catabolism. One possible scenario is that hIDO2, when over-expressed in the presence of hIDO1, reduces hIDO1 catalytic activity through competition for heme-binding, but may play independent roles related to immune modulation through its ITIM-motif under activating conditions.

In summary, our results suggest a novel role for hIDO2 as a negative regulator of hIDO1 catalytic activity, an effect associated with the heme-binding site. These results provide information useful for both understanding the role of IDO2

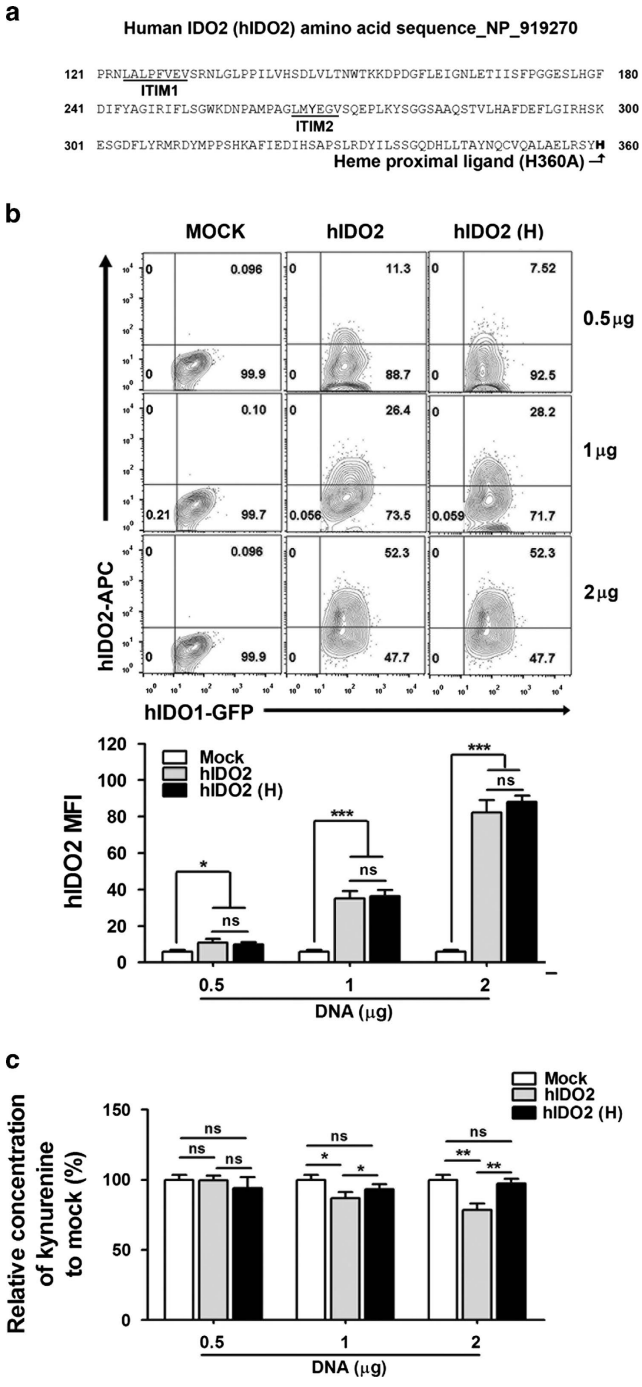

Figure 4 Human indoleamine 2,3-dioxygenase 2 (hIDO2) modulates hIDO1 functional activity through the heme-binding histidine residue 360 in a dose-dependent manner. (**a**) The hIDO2 amino acid sequence obtained from the National Center for Biotechnology Information (GenBank accession number: NP_919270) annotated with the putative immunoreceptor tyrosine-based inhibition ITIM1 and ITIM2 motifs, and heme proximal ligand. The hIDO2 (H) mutant was constructed by substituting histidine 360 for alanine (H360A). Mutated residues are shown in bold. (**b**) Flow cytometric analysis of protein expression after transient transfection with hIDO2 or hIDO2 (H) constructs into the green florescent protein-expressing hIDO1 stable cell line. Plasmid DNAs (0.5, 1, and 2 μg) were subjected to transfection using polyethyleneimine. hIDO2 and hIDO2 (H) protein levels were compared after gating green florescent protein-positive hIDO1-expressing cells stained with an anti-hemagglutinin primary antibody and allophycocyanin-conjugated secondary antibodies 48 h after transfection. The mean hIDO2 fluorescence intensity values of all transfected cells were plotted. (**c**) Kynurenine production was measured using Ehrlich's reagent and calculated compared to the mock-transfectant at 48 h posttransfection. Empty vector was used as the mock control. ns, not significant, $*P<0.05$, $**P<0.01$, $***P<0.001$.

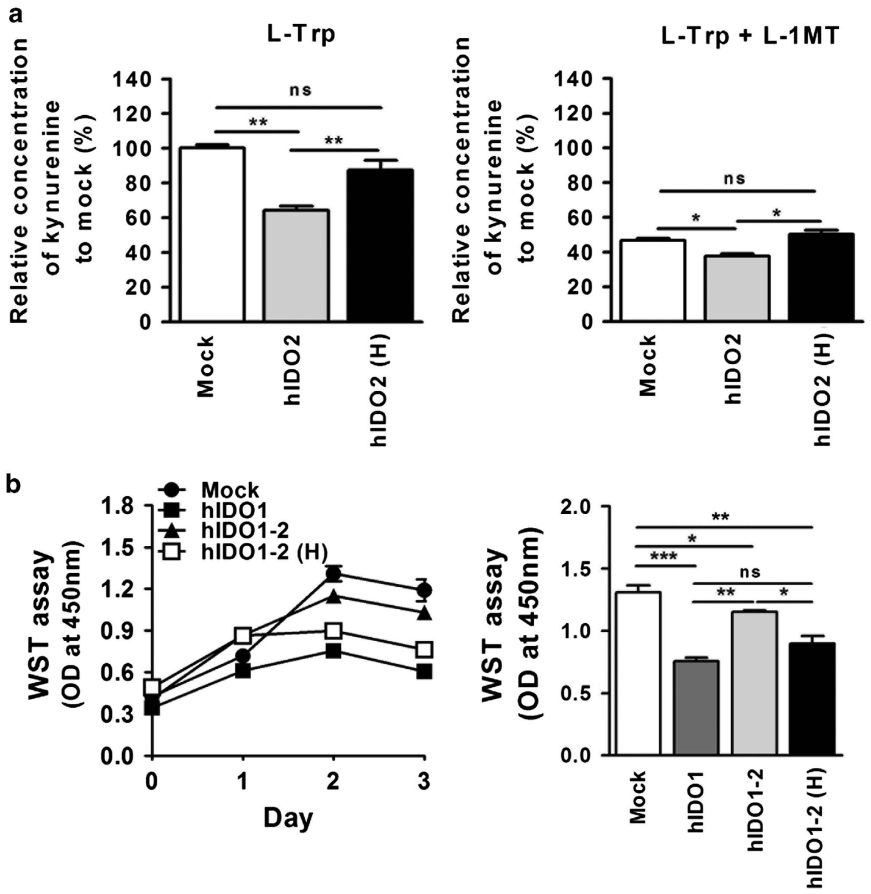

Figure 5 Effects of the indoleamine 2,3-dioxygenase 1 (IDO1) inhibitor L-1MT on human (h) IDO1 activity in cells co-expressing hIDO2 (H) mutants. (**a**) Kynurenine production was measured in culture supernatants of stable hIDO1-expressing cells transfected with 2 μg hIDO2 or hIDO2 (H) mutant DNAs. Transfected cells were incubated for 24 h with complete medium including 100 μM L-Trp in the absence or presence of 100 μM L-1MT. (**b**) Proliferation of stable hIDO1-, hIDO1-2- and hIDO1-2 (H)-expressing HEK293 cells was measured by WST assay during the 3-day culture period. Cell proliferation values on day 2 were subjected to statistical analysis. Absorbance at 450 nm was measured using a microplate reader with the Gen5 software. ns, not significant, $*P<0.05$, $**P<0.01$, $***P<0.001$.

and the development of IDO1-targeted therapeutic strategies for cancers and immunological diseases.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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