The DLC-1 tumor suppressor is involved in regulating immunomodulation of human mesenchymal stem cells through interacting with the Notch1 protein

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Abstract

Immunomodulatory activities of human mesenchymal stem cells (hMSCs) have been widely accepted as the most critical function of the cells for exerting its therapeutic effects. The activities include the inhibition by hMSCs on pro-inflammatory CD4 + -T lymphocytes, and the release of immunomodulatory molecules, like IDO1. However, the detailed mechanisms responsible for regulating the immunomodulation of hMSCs still remain largely unknown. Previously, the Notch1 protein has been demonstrated to be able to promote the immunomodulation of hMSCs through inhibiting CD4 + -Th1 lymphocyte proliferation and enhancing IDO1 expression. The present study further revealed that it was the Notch1-Hey1 axis, rather than the Notch1-Hes1 axis, that was likely responsible for mediating the immunomodulation of the Notch1 signaling. Meanwhile, following a previously proposed hypothesis to identify proteasome-regulated protein(s) for limiting the activity of the Notch1 signaling in hMSCS, the DLC-1 tumor suppressor was identified to be such a candidate protein, which was subjected to protein degradation mediated by the DDB1 and FBXW5 E3 ligases . It was further shown that the DLC-1 signaling composing of DLC-1, Rock1 and FBXW5 proteins was involved in inhibiting the immunomodulation of hMSCs. More importantly, the immunomodulation was achieved through an interaction between the DLC-1-FBXW5-Rock1 signaling and the Notch1-Hey1 signaling . In fact, the present study a novel function of DLC-1 tumor suppressor as well as proposed a new mutual exclusion mechanism likely responsible for fine-tuning the immunomodulation of hMSCs.

Background

Human mesenchymal stem cells (hMSCs) is a group of fibroblast-like multipotent cells existing in almost all tissues with a limited self-renewal and differentiation potential to multiple cell lineages of endoderm, mesoderm and ectoderm [1]. The hMSCs of various tissue origins also exhibit unique immunomodulatory activities, which make hMSCs the most popular cell type used in stem cell-based therapies [2, 3]. As the most important quality attributes of biological effectiveness, the immunomodulation of hMSCs should be thoroughly investigated to achieve the best clinical effects.

The immunomodulation of hMSCs are manifested in part by their abilities to modulate almost all types of immune cells, such as T and B lymphocytes, natural killer cells [4], macrophages [5], and neutrophils [6]. In modulating $CD4^+$ T lymphocytes, hMSCs can inhibit proliferation and activity of pro-inflammatory lymphocytes, such as Th1 (Type 1 T helper) and Th17 (Type 17 T helper) subpopulations and promote polarization of regulatory T lymphocytes (Tregs) through both cell-cell interaction and/or secretion of immunomodulatory molecules [7]. Among the key molecules, the IDO1 (Indoleamine 2,3-dioxygenase 1) protein still represents a major focus of the studies of immunomodulation of hMSCs [8].

IDO1, or indoleamine 2,3 deoxygenase 1, is a rate-limiting enzyme for metabolizing tryptophan into kynurenine [9]. Its expression is induced by pro-inflammatory molecules, such as IFN-γ (interferon-gamma), TNF-α (Tumor Necrosis Factor alpha) or IL-1α (interleukin-1 alpha) [10]. The importance of IDO1 in the immunomodulation of hMSCs has been established partially through employing either IDO1

silencing or small molecule IDO1 inhibitor, i.e. 1-methyl-L-tryptophan (1-L-MT). The IDO1 inhibition can cause significant reduction of various immunomodulatory activities of hMSCs, such as the reduction in inhibiting Th1 lymphocyte proliferation or promoting Treg polarization [8, 11]. However, even though the IDO1 activities have been characterized in substantial details, the molecular mechanisms, particularly the cell signaling pathways for regulating the activities, still remain largely unknown.

Among various cell signaling pathways, the Notch1 signaling has been previously revealed for promoting the immunomodulation of hMSCs [8]. The Notch1 signaling is activated sequentially through binding of Notch1 proteins to their ligands on surface of adjacent cells, then two successive proteolytic cleavages mediated by TNF-α converting enzyme (TACE) and γ-secretase/presenilin complex [8]. Different types of γ-secretase inhibitors have been used as experimental tools to unveil novel functions of Notch signaling [12]. Among the most commonly used inhibitors is a small peptide inhibitor GSI-I (Gamma-secretase inhibitor I), which shares both structural and functional similarities with Botezomib, a proteasome inhibitor used frequently in cancer research [13]. The cleavage by γ-secretase results in release of Notch intracellular domain of (NICD) from plasma membrane and its translocation into the nucleus, where it binds CSL (CBF1/RBP-Jk; Su(H)/Suppressor of Hairless; Lag-1) protein complex and turns the complex from transcriptional repressor into transcriptional activator with consequent activation of downstream effector proteins [14].

Among the downstream effectors of Notch1, Hes1 (hairy and enhancer of split-1) and Hey1 (Hairy/enhancer-of-split related with YRPW motif protein 1) have been more intensely studied [15]. However, these two effectors may represent different aspects of Notch signaling. For example, over-expression of Hey1, but not Hes1, induced over 80-fold decrease in Col2a1 (Collagen Type II Alpha 1 Chain) transcription in a three-dimension differentiation induction model, suggesting that the Notch signaling played an inhibitory role on chondrogenic differentiation, in which the Notch-Hey-1 axis, rather than the Notch-Hes1 axis, was most likely involved [16]. In addition, Hes1 and Hey1 might be differently involved in tissue development-whereas Hes1 was involved in the development of brain, skin and adipose tissues, Hey1 was associated with the development of heart and vasculature [17]. All these findings thus suggested that different Notch signaling axis may mediate different functional aspects of hMSCs.

The DLC-1 (deleted in liver cancer 1) protein has been established as a tumor suppressor with abilities to inhibit growth, migration, invasion and metastasis of a large variety of common cancers [18–20]. While the DLC-1 gene expresses in almost all normal tissues, it is frequently absent or dramatically down-regulated in tumor tissues mainly due to genomic deletion and/or aberrant methylation of the promoter region of the gene [21]. In addition, the DLC-1 protein was subjected to cytoplasmic sequestration and proteasome-mediated degradation [22, 23], which was directed by the CUL4A–DDB1–FBXW5 E3 ubiquitin ligase complex [24].

The DLC-1 protein is a multi-domain protein comprising of SAM (Sterile alpha motif), RhoGAP (Rho GTPase-activating protein) and START (StAR-related lipid-transfer) domains in its N-terminus, middle and C-terminus, respectively. In addition, it possesses a bipartite nuclear localizing sequence (NLS)

responsible for DLC-1 protein nuclear translocation and a serine-rich region likely for regulating the NLS (nuclear localization signal) activity [23]. Among the major functional domains, the RhoGAP domain is conserved for catalyzing hydrolysis of GTP (Guanosine-5'-triphosphate) into GDP (guanosine diphosphate) and subsequent inactivation of small Rho subfamily proteins, such as RhoA/B/C (Ras homologous A/B/C) and Cdc42 (Cell division control protein 42 homolog). A prominent downstream effector of small Rho proteins is the Rock1/2 (Rho-associated protein kinase 1/2), which transduce various activities of the Rho proteins in different cells [25].

In this study, we discovered a mutual exclusion crosstalk between the DLC-1 signaling and the Notch signaling in hUC-MSCs (human umbilical-cord-derived mesenchymal stem cells) from searching the candidate proteins subjective to proteasome-mediated protein degradation. The crosstalk detailed that the DLC-1 signaling represented by the FBXW5, DLC-1 and Rock1 proteins was inhibitory to the immunomodulation of hUC-MSCs and able to interact with the Notch1 signaling represented by the Notch1-Hey1 axis in a mutual exclusion manner, thus providing a fine-tuning mechanism in the regulation of the immunomodulation of hMSCs.

Methods

1. Materials:

Cells: hUC-MSCs was gifted anonymously from TuoHua Biotech company (Siping, China), where the cells were isolated and purified from Wharton's Jelly of a discarded umbilical cord. The expression of the featured surface markers of hMSCs, the differentiation potentials to osteocytes, chondrocytes and adipocytes, and microbiological safety were tested for hUC-MSCs in our laboratory; PBMCs (Peripheral blood mononuclear cells) were freshly isolated using a conventional Ficoll method [26] from whole blood of healthy donors provided anonymously from local Red Cross. All data analysis associated with the use of hUC-MSCs and PBMCs in this study was conducted anonymously. Antibodies: the antibodies against IDO1, NICD1, DDB-1 and phosphor-STAT1(Y701) were from Cell Signaling (Danvers, MA); the antibodies against DLC-1, from BD Bioscience (Franklin Lakes, NJ); the antibodies against ubiquitin, Mcl-1, Hes1, Hey1, Rock1 and Rock2, from Santa Cruz Biotechnology (Dallas, TX); the antibodies against Cullin 4A and FBXW5, from Abcam (Cambridge, MA); the antibody against β-actin, from Sigma (Milwaukee, WI); HRP (Horseradish peroxidase)-conjugated anti-mouse or anti-rabbit secondary antibodies, from GE Healthcare (Piscataway, NJ). All antibodies conjugated with different fluorescent dyes were from BD Bioscience. Constructs: pIDO1-Luc, pcDNA3.1-DLC-1, -DLC-1-Δ622, -DLC-1-662, -DLC-1-R718E containing a point mutation in RhoGAP domain, and -DLC-1-RhoGAP, which is the RhoGAP domain only mutant, were constructed in previous studies [8, 23]. Chemicals: GSI-I (Z-Leu-Leu-Nle-al), a γ-secretase inhibitor, was purchased from Sigma Aldrich (St. Louis, MI); Bortezomib, a proteasome inhibitor, was from ChemieTek (Indianapolis, IN); Y27632, a pan-Rock inhibitor, from EMD Millipore (Darmstadt, Germany).

2. Detection of hMSCs surface markers

The hUC-MSCs surface markers, i.e. CD105, CD90 and CD73, were detected via a flow cytometry assay using BD Stemflow hMSC Analysis Kit following the previously described procedures [8, 27].

3. Osteogenic differentiation assay

The osteogenic differentiation of hUC-MSCs was examined by detecting the expression of RGC32, an effective biomarker of osteogenesis, via a real-time PCR (Polymerase chain reaction) [8].

4. PCR

Conventional semi-quantitative RT-PCR was employed to detect mRNA expression of Hes-1 and Hey-1 in hUC-MSCs following various treatments. The expression of GAPDH was used as an internal control for the semi-quantitation. The sets of primer sequences were: AGCACAGACCCAAGTGTGCTG and GAAGGTGACACTGCGTTGGG, for HES-1 ; ACGAGAATGGAAACTTGAGTTCGGC and CCCAAACTCCGATAGTCCATAGCAAG, for HEY-1 ; ACCACAGTCCATGCCATCAC and TCCACCACCCTGTTGCTGT, for GAPDH.

Real-time PCR with SYBR Green quantification were set up with 1/20 of each cDNA (complementary DNA) preparation in Applied Biosystems® 7500 Real-Time PCR Systems (Thermo Fisher scientific, Waltham, MA). Quantitative analysis was conducted by normalizing the expression level of the testing gene to that of GAPDH (Glyceraldehyde 3-phosphate dehydrogenase). The primer sequences were: GAAGTTCTGGGTCCTTTCATC and GCATGGATCGTCTGTTCTAATA, for RGC32 (response gene to complement 32 protein); AGGCTGGAGAGGCGGCTAAG and TGGAAGGTGACACTGCGTTGG, for HES1; GGATCACCTGAAAATGCTGCATAC and CCGAAATCCCAAACTCCGATAG, for HEY1; GCCGGACACCATGATCCTAAC and GAGCCTCAATGGCATCTCTGT, for DLC-1; GTGTGAACCATGAGAAGTATGA and TAGAGGCAGGGATGATGTT, for GAPDH.

5. Th1 lymphocyte proliferation assay

The effect of hUC-MSCs on inhibiting proliferation of Th1 lymphocytes from PHA-induced PBMCs was detected by following the previously reported procedures [8].

6. Western blotting

The procedures of conventional Western blotting were followed to monitor changes in expression of relevant proteins in hUC-MSCs following various treatments [8].

7. Immunoprecipitation (IP) for detecting the expression of poly-ubiquitinated proteins

Cell lysates extracted using RIPA buffer from 1.5×10^6 cells treated with GSI-I or Bortezomib were incubated with 1 ∝g antibody of the targeted protein for IP at 4^oC overnight, then incubated with protein A/G agarose at 4^oC for 1 h. After washing three times at room temperature, the agarose-bound cell lysates were then analyzed by Western blotting using ubiquitin antibody.

8. Transient cDNA or siRNA transfection

The procedures reported previously for siRNA transfection were followed for silencing the expression of Notch1, DLC-1, Hes-1 or Hey-1 in hUC-MSCs and the silencing effect for each target was determined by Western blotting or RT-PCR [8]. The siRNA sequences were CACCAGUUUGAAUGGUCAAtt for Notch1; AGAACAGCACCUCUGGGAUtt for DLC-1, CGAGGUGACCCGCUUCCUGtt (1#) [28] and AGACGAAGAGCAAGAAUAAtt (2#) [29] for Hes1, and GUGCGGACGAGAAUGGAAAtt (1#) and GACCGGAUCAAUAACAGUUtt (2#) for Hey1 [30]. The siRNA for Rock1 (sc-29473) and Rock2 (sc-29474) were purchased from Santa Cruz Biotechnology (Dallas, TX). The randomly scrambled siRNA was used as negative control.

9. Construction of Notch1 and NICD expression vector

The pcDNA3.1-Notch1 containing full-length Notch1 cDNA was kindly provided by Dr. Jon C. Aster [31]. The cDNA of NICD1 was amplified by RT-PCR from total RNA of hUC-MSCs and constructed into the pcDNA3.1 expression vector. The primer sequences used for amplifying NICD were: 5'-GCTCTAGAGTGCTGCTGTCCCGCAAGCG-3' and 5'-CCCAAGCTTTTCAACTTCCCTTCTCCAACATCATTTC-3', in which Xbal and HindIII restriction sites were designed for subcloning.

10. Luciferase assay for detecting IDO1 promoter activity

The pIDO1-Luc vector was constructed and characterized in a previous study [8]. The procedures reported previously were followed for detecting IDO1 promoter activity in response to IFN-γ in hUC-MSCs [8].

11. Data analysis

The data collected from the tests for cell viability, surface markers, luciferase-based promoter activity, and Th1 lymphocyte proliferation were expressed as means ± SEM of at least three separate experiments. Comparison between group means was assessed using one-way analysis of variance with Newman–Keuls posttest (GraphPad Prism 4.0 Software, Inc., San Diego, CA, USA). The difference with $P < 0.05$ was considered significant.

Results

The treatment with GSI-I elevated DLC-1 protein level in hUC-MSCs through proteasome inhibition

Given the dual inhibitory activities of GSI-I, we speculated from the previous study that the effect of GSI-I on immunomodulation of hUC-MSCs was likely the consequence of interaction between Notch1 and other protein(s) that were subjected to proteasome-mediated protein degradation [8]. To identify the candidate proteins interacting with Notch1, we examined expression of different proteins likely targeted by the proteasome-mediated degradation in hUC-MSC cells. Among the candidate proteins examined were Mcl-1 and DLC-1, both of which were subjective to the proteasome-mediated protein degradation [24, 32]. Through Western blotting and immunoprecipitation, we found that the GSI-I treatment significantly elevated protein level for Mcl-1 and DLC-1 with the increase also in polyubiquitinated form of both proteins (Fig. 1A & B), suggesting that both proteins were subjected to the proteasome-targeted degradation in hUC-MSCs.

The DLC-1 protein was involved in regulating the expression of surface markers of hUC-MSCs

Given that the Notch1 protein is involved in regulating the expression of the featured surface markers of hUC-MSCs, like CD73, CD90 and CD105, and osteogenic differentiation, which was measured in part by the transcription of RGC32, a surrogate marker of the osteogenesis of hUC-MSCs [8]. To determine the possible involvement of DLC-1 in regulating surface markers and osteogenic differentiation of hUC-MSCs, we transfected a hUC-MSCs cell line with siDLC-1 in the presence of 2.5-5 ∝M GSI-I, then tested the expression of both the surface markers and RGC32. After confirming that the effect of DLC-1 silencing from siDLC-1 transfection (Fig. 2A), we observed that, whereas the GSI-I treatment reduced the expression of CD73, CD90 and CD105 with the most significant reduction seeing in CD105, and RGC32 expression, the siDLC-1 transfection moderately reversed the GSI-I-induced reduction of all three surface markers but showed no effect on RGC32 expression (Fig. <link rid="fig2">2</link>B-1 & <link rid="fig2">2</link>B-2), thus suggesting that the GSI-I-induced DLC-1 elevation contributed to the GSI-I-induced reduction in surface markers, but not in osteogenic differentiation.

DLC-1 inhibited the immunomodulation of hUC-MSCs

Given that the immunomodulation of hMSCs can be represented by its inhibition of Th1 lymphocyte proliferation and IFN-γ-induced IDO1 expression [8], we next transfected siDLC-1 or DLC-1 cDNA into hUC-MSCs and then examined the effect of the transfection on both Th1 lymphocyte proliferation and IDO1 expression. After confirming the effect of each transfection on DLC-1 expression (Fig. 3A & B), it was observed through flow cytometry assay that, whereas the siDLC-1 transfection further enhanced the ability of hUC-MSCs to reduce Th1 proliferation as well as significantly reversed the GSI-I-induced inhibition of Th1 lymphocyte proliferation, the DLC-1 cDNA transfection caused a significant reduction of

the inhibitory effect of hUC-MSC on Th1 proliferation (Fig. 3A & B). Meanwhile, through the IDO1 promoter assay, in which hUC-MSCs were co-transfected with pIDO1-Luc plus siDLC-1, or pIDO1-Luc plus DLC-1 cDNA for 24 h, as followed by the treatment with 10 ng/ml IFN-γ for another 24 h before measuring the luciferase activity from each transfection. It was observed that, comparing with each negative control, DLC-1 overexpression significantly reduced the IFN-γ-induced IDO1 promoter activity, whereas the DLC-1 silencing increased the promoter activity (Fig. 3C & D), all thus suggesting for the first time that DLC-1 played an inhibitory role in the immunomodulation of hUC-MSCs.

The activity of DLC-1 on the immunomodulation of hUC-MSCs appeared to be RhoGAP domain-dependent and RhoGAP domain-independent

To determine which functional domain(s) were responsible for DLC-1's activity of inhibiting the immunomodulation, we then tested the effect of different DLC-1 mutants, i.e. the mutant with the deletion of N-terminus (DLC-1-Δ662), C-terminus (DLC-1-622) or RhoGAP domain point mutation (DLC-1-R718E), or a RhoGAP domain only mutant, on the INF-γ-induced IDO1 promoter activity in hUC-MSCs. The schemes of all DLC-1 mutants were shown in Fig. 4A. It was observed in the co-transfection of DLC-1 or its mutant cDNAs with pIDO1-Luc that wild type DLC-1 (wtDLC-1) inhibited over 50% of, the DLC-1-622 mutant showed no effect, and all other mutant showed even an increase in, the IDO1 promoter activity (Fig. 4B). In addition, the wtDLC-1 and the DLC-1-622 and DLC-1-Δ662 mutants reduced, but the DLC-1-R718E mutant increased, the IDO1 protein expression (Fig. 4C), all thus suggesting that the inhibitory effect of DLC-1 on IDO1 might be both RhoGAP domain-dependent and RhoGAP domain-independent.

The effect of DLC-1 in hUC-MSCs was achieved through inhibiting the Notch signaling

After associating the DLC-1 protein with the immunomodulation, we then attempted to link it with the Notch1 protein in regulating the immunomodulation of hUC-MSCs. To test the possible association, we first constructed the NICD1 expression vector following the literature [31] and validated its expression after transfecting the construct into hUC-MSCs (Fig. 5A). Next, we tried to determine the relationship between DLC-1 and Notch1 in regulating IDO1 promoter activity after co-transfecting DLC-1 cDNA with either siNotch1 or NICD1 cDNA. It was found that, whereas the transfection with either siNotch1 or DLC-1 cDNA alone caused a similar reduction of the promoter activity, and siNotch1 plus DLC-1 cDNA further reduced the activity (Fig. 5B). Meanwhile, it was found that, whereas the NICD1 transfection alone caused a significant increase in the promoter activity, the effect was partially reversed by DLC-1 cDNA transfection (Fig. 5B), thus suggesting that the inhibitory effect of DLC-1 on IDO1 was achieved at least in part through inhibiting the Notch1 function.

A mutual exclusive relationship existed between DLC-1 and Notch1 in hUC-MSCs

To further reveal the relationship between DLC-1 and Notch1, we next examined the changes of Notch1 cleavage/activation in hUC-MSCs following the transfection with siDLC-1 [8]. Interestingly, it was found that the siDLC-1 transfection caused a significant increase in both basal NICD1 and the GSI-I-reduced NICD1 (Fig. 6A). Meanwhile, the transfection with DLC-1 cDNA alone resulted in a significant reduction of NICD1 (Fig. 6B). Furthermore, it was observed that, whereas the DLC-1 cDNA transfection almost completely abolished the effect of inducing IDO1 from the NICD1 cDNA transfeciton, the NICD1 cDNA transfection also inhibited the effect of the DLC-1 cDNA on IDO1 expression (Fig. 6C), all thus together strongly supporting the existence of a mutual exclusive relationship between DLC-1 and Notch1, which determined the eventual expression level of the IDO1.

Regarding the likely effect of DLC-1 on NICD1 nuclear translocation, we revealed that, whereas the siDLC-1 transfection clearly increased NICD1 protein level in both the cytoplasm and the nucleus, the DLC-1 cDNA transfection caused a significant reduction of NICD1 in both compartments with a more reduction seen in the nucleus than the cytoplasm (Fig. 6D), thus indeed suggesting that DLC-1 reduced NICD1 protein nuclear translocation.

The Notch-Hey1 axis, but not the Notch-Hes1 axis, was involved in promoting the immunomodulation of hUC-MSCs

Both Hey1 and Hes1 have been well established as two prominent downstream effectors of the Notch signaling, but may behave differently in mediating different functions of the Notch1 signaling [16, 33]. Therefore, it was of great interest to determine a possible distinction between these two effectors in mediating the immunomodulation of hUC-MSCs. By employing gene silencing with either each specific siRNA and then validating via by RT-PCR, (Fig. 7A), we found that the Hes1 silencing caused a slight increase, but the Hey1 silencing resulted in a dramatic decrease, in the IFN-γ-induced IDO1 promoter activity (Fig. 7B). Meanwhile, it was also observed that it was the Hey1 silencing, but not Hes1 silencing, that was able to significantly reduce the inhibition of Th1 proliferation by hUC-MSCs (Fig. 7C), thus indicating that it was the Notch1-Hey1 axis, but not the Notch1-Hes1 axis, that was involved in the immunomodulation of hMSCs.

Since it was reported that the Notch inhibitor DAPT exerted its inhibition more specifically on Hes1 than Hey1 [33], we then utilized the test of DAPT in comparison with GSI-I as a tool to distinguish between Hes1 and Hey1. It was observed through RT-PCR that, while the treatment with 10 ∝M GSI-I equally reduced both Hes1 and Hey1 expression, the treatment with 10 ∝M DAPT only reduced Hes1 expression, thus confrming that DAPT was a Hes1-specific inhibitor (Fig. 7D). It was next observed that, whereas the GSI-I treatment reduced both IDO1 protein expression in hUC-MSCs and the inhibition of Th1 lymphocyte proliferation by hUC-MSCs, the DAPT treatment unexpectedly increased the IFN-γ-induced IDO1 protein

expression and showed no effect on Th1 lymphocyte proliferation (Fig. 7E & F), thus further supporting that it was the Notch1-Hey1 axis, but not Notch1-Hes1 axis, that promoted the immunomodulation of hUC-MSCs.

The Hey1 protein served as a key molecule in mediating the mutual exclusion relationship between DLC-1 and Notch1

After charactering the differences between Hey1 and Hes1, we then attempted to determine whether the Hey1 protein was involved in the mutual exclusion relationship between DLC-1 and Notch1. We then tested the gene expression for Hey1 and Hes1 using RT-PCR after transfection with either siDLC-1 or DLC-1 cDNA. It was found that the transfection with DLC-1 cDNA caused a slight reduction of Hes1, but a significant reduction of Hey1. In contrast, the siDLC-1 transfection resulted in a remarkable reduction of Hes1, but a significant elevation of Hey1 (Figure s1A, B & C). On the other hand, it was observed via Western blotting that, whereas the siHes1 transfection induced an apparent increase in the IFN-γ-induced IDO1 expression but a clear decrease in DLC-1 expression, the siHey1 transfection induced a remarkable decrease in the IDO1 expression, but a clear increase in DLC-1 expression (Figure s1B). Therefore, all these findings together strongly demonstrated that it was Hey1, but not Hes1, that served as the key signaling molecule involved in the mutual exclusion relationship between DLC-1 and Notch1.

The Notch1-Hey1 axis regulates DLC-1 protein stability through modulating the expression of FBXW5 E3 ligase

Given that the DLC-1 protein could serve as a degradation target of the CUL4A-DDB1-FBXW5 E3 complex in tumor cells [24], it was of great interest to also determine the involvement of these E3-ligase proteins in regulating DLC-1 protein stability and in the relationship between DLC-1 and Notch1 in hUC-MSCs. For this purpose, we silenced the expression of each E3-ligase protein in hUC-MSCs, then determined the effect of each silencing on DLC-1 protein expression, as well as on the immunomodulation of hUC-MSCs. Interestingly, after confirming the silencing effect of each E3 ligase by Western blotting (Figure s2A), we observed that the silencing of either DDB1 or FBXW5, but not CUL4A, significantly elevated DLC-1 protein level with a more significance seen in FBXW5 silencing. In addition, the silencing of either FBXW5 or DDB1 was accompanied by a significant reduction in IDO1 and p-STAT1 with a more significance seen again in FBXW5 silencing (Figure s2A). More interestingly, it was also observed that, comparing with DDB1 and CUL4A, the silencing of FBXW5 caused a significant reduction in the inhibition of Th1 lymphocyte proliferation (Figure s2B). These findings thus supported that FBXW5 was the major E3 ligase for regulating DLC-1 protein stability and subsequent immunomodulation of hUC-MSCs.

To further pursue the possibility that the E3 ligase(s) for DLC-1 and Notch1-Hey1 signaling could be mutually regulated, we first tested the expression of NICD1 and Hey1 following the silencing of each E3 ligase. We then found that, whereas the FBXW5 silencing led to a significant reduction in both NICD1 and

Hey1, the silencing for DDB1 or CUL4A showed almost no such effect (Figure s2A). Next, we examined the effect of silencing of Hes1 or Hey1 on the expression of all E3 ligases. It was then observed that, whereas the Hes1 silencing showed no effect on all E3 ligase proteins, the Hey1 silencing however caused a significant reduction only in FBXW5 protein, thus concluding that the Notch1-Hey1 signaling and FBXW5 could be mutually inhibitory for regulating DLC-1 protein stability and subsequent immunomodulation of hMSCs (Figure s2C).

The inhibition of Rock1, but not Rock2, inhibited the immunomodulation of hUC-MSCs

Given that the Rock1/2 proteins serve as the key signaling proteins downstream of the DLC-1 signaling, we next attempted to determine whether they played a role in regulating the immunomodulation of hUC-MSCs. We then employed Y27632, a Rock1/2 small molecule inhibitor, to treat hUC-MSCs and then tested the effect of the treatment on the IFN-γ-induced IDO1 expression and promoter activity in hUC-MSCs. Unexpectedly, we found that the Y27632 treatment resulted in a significant dose-dependent increase in the IDO1 protein expression and promoter activity (Figure s3A & s3B). To understand the seemingly contradictory effect of Y27632 from that of DLC-1 on IDO1, we then tested via Western blotting the protein expression of Rock1, Rock2 together with DLC-1, NICD1, Hes1 and Hey1. Interestingly, it was found that the Y27632 treatment resulted in a dose-dependent reduction of both Rock1 and Rock2 with a much more significant reduction in Rock2. In addition, it also caused a dose-dependent decrease in DLC-1, but an increase in both NICD1 and Hey1 (Figure s3A). Moreover, consistent to the increase in IDO1, the hUC-MSCs pretreated with Y27632 exhibited a significantly enhanced inhibition of Th1 lymphocyte proliferation (Figure s3C), all thus suggesting that the treatment of Y27632 in fact mimicked the activity of the Notch1 signaling in enhancing the immunomodulation of hMSCs. Considering that Y27632 is an inhibitor of both Rock1 and Rock2, we next transfected either siRock1 or siRock2 before examining the expression of the relevant proteins, thus attempting to distinguish the effect between Rock1 and Rock2 in the immunomodulation of hMSCs. The results showed that the transfection with siRock2, but not siRock1, achieved the same effect as Y27632 on the expression of IDO1 and DLC-1, whereas the siRock1 transfection resulted in a clear reduction of IDO1 and unexpectedly an increase in Rock2 (Figure s3D), suggesting that the effect of Y27632 observed above on the immunomodulation of hMSCs was attributable to the inhibition of Rock2, but not Rock1, and Rock1 and Rock2 appeared exerting differently in the regulation of the immunomodulation with Rock1 seemingly being pro-immunomodulatory and Rock2 anti-immunomodulatory. It can be further suggested that Rock1 likely represented the downstream inhibitory target of the DLC-1 signaling in inhibiting the immunomodulation of hUC-MSCs, while Rock2 might serve as a negative feedback regulator of the DLC-1 signaling in this perspective.

Discussion

The unique immunomodulatory activities confer hMSCs a great versatility of managing various inflammatory/immune situations to treat a large variety of uncontrolled inflammatory and abnormal

immune diseases. It is reasonable to understand that the fine-tuned regulatory mechanisms must be always available for ensuring hMSCs to effectively sense the inflammatory environment of various degrees for precisely modulating the corresponding inflammations utilizing different regulatory capacities. Therefore, the endeavor to understand the fine-tuned mechanisms endowing the versatility is extremely important to fully appreciate the immunomodulation of hMSCs. With a set of novel evidence, the present study demonstrates that a crosstalk between two important cell signaling represents a means of fine-tuning the immunomodulation of hMSCs.

The two signaling revealed in this study are the Notch1-Hey1 signaling and the FBXW5-DLC-1-Rock1 signaling, which regulate the immunomodulation of hMSCs in opposite directions. Whereas the Notch1-Hey1 signaling promotes, the FBXW5-DLC-1-Rock1 signaling inhibits, the immunomodulation of hMSCs. More importantly, the activities of these two signaling are mutually exclusive, thus making the fine-tuning of the immunomodulation of hMSCs available.

The mutual exclusion mechanism presented in this study is built up on a hypothesis proposed previously that some protein(s) subjective to proteasome-mediated protein degradation may antagonize the Notch1 signaling in regulating the immunomodulation of hUC-MSCs [8]. Although the search for DLC-1 represents the authors' preferential focus due to long time interest in it, the result from the search indeed supports that DLC-1 is the right candidate according to the hypothesis. From thoroughly analyzing the key members of each signaling and their involvement in the relationship between DLC-1 and Notch1, a sophisticated mutual exclusion mechanism between the two signaling then emerges.

On the side of the DLC-1 signaling, the DLC-1 tumor suppressor is identified for the first time inhibiting the immunomodulation of hUC-MSCs. This novel activity of DLC-1 is supported by the evidence that the change in DLC-1 expression is directly associated with the IFN-γ-induced IDO1 expression and the inhibition of Th1 lymphocyte proliferation by hUC-MSCs (Fig. 3). In this regard, the activity of DLC-1 appeared to be both RhoGAP domain-dependent and RhoGAP domain-independent, although the structural integrity of the protein is needed (Fig. 4). Nevertheless, this novel finding may be of great importance as MSCs exist abundantly in tumor microenvironment and contribute to the immunosuppression of tumor progression, especially the metastatic progression [34]. If DLC-1 can help restrict the immunosuppression MSCs, it then exerts its tumor suppressor functions in both seeds (tumor cells) and soil (mesenchymal cells) in tumor microenvironment.

When functioning in hMSCs for dealing with non-tumor diseases, the regulation of DLC-1 by proteasome-mediated protein degradation, as revealed in this study, may provide a flexible mechanism allowing DLC-1 to exert its activity in hMSCs on the basis of necessity. However, the genomic deletion and/or aberrant methylation occurring frequently for DLC-1 in different human cancers represent the more rigid mechanism(s) necessary for tumor cells to achieve a complete knock-down of tumor suppressor functions of DLC-1.

Concerning the E3 ligases responsible for DLC-1 protein degradation, DDB1 and FBXW5 are the key E3 ligases in hMSCs. However, in a set of lung cancer cells, the CULT4, DDB1 and FBXW5 E3 ligases formed

in a complex are involved in DLC-1 protein degradation [24]. In a different study, DDB1 and FBXW7, which is in the same family with FBXW5, is sufficient to form an E3 ligase complex without CUL4A for degrading MYC proteins [35], supporting that two E3 ligases, i.e. DDB1 and FBXW5, are sufficient for mediating DLC-1 degradation in hMSCs. The discrepancy between lung cancer cells and hUC-MSCs in composition of E3 ligase proteins for degrading DLC-1 may represent different regulation stringencies needed in different types of cells. The regulation for DLC-1 protein stability may be more stringent in parenchymal cells with three E3 ligases than that in mesenchymal cells using two E3 ligases, thus providing more flexibility for DLC-1 to regulate the immunomodulation of hMSCS.

On the side of the Notch1 signaling, we first advance our understanding about the involvement of Notch1 in the immunomodulation of hMSCs by manifesting that the Notch1 signaling diverges at the level of Hey1 and Hes1 regarding the immunomodulation of hMSCs. The new evidence, provided from the experiments employing the inhibitory tools of either gene silencing or γ-secretase inhibitors GSI-I and DAPT, suggest that the Notch1-Hey1 axis, rather than the Notch1-Hes1 axis, is likely involved in the immunomodulation of hMSCs (Fig. 7 and s1). As the Notch signaling possesses a large variety of functions, the distinguished roles between Hey1 and Hes1 downstream of the Notch1 signaling are highly rational because different downstream effectors of any functionally diverse cell signaling may represent different functional perspective of the signaling. More importantly, the distinction between Hey1 and Hes1 provides a sophisticated multi-level basis on the side of the Notch signaling for establishing the mutual exclusion relationship between Notch1 and DLC-1.

The multi-level mutual exclusion relationship between the two signaling is identified by employing a general approach, in which the function of each key molecule of one signaling is characterized for its association with the function of the opposite signaling in terms of the regulation of the immunomodulation of hMSCs. Through the characterization, each of the DLC-1, FBXW5 and Rock1 proteins along the DLC-1 signaling shows the ability to inhibit the expression of NICD1 and Hey1 and the associated immunomodulation of hMSCS (Fig. 7). Similarly, either Notch1 or Hey1 demonstrates their activity of reducing the expression of DLC-1, but inducing the expression of FBXW5 (Figure s2). Interestingly, although both FBXW5 and DDB1 are directly involved in regulating DLC-1 protein stability, it is FBXW5, but not DDB1, that is directly involved in the mutual exclusion relationship, thus leading to a possibility that, within the E3 ligases responsible for degrading DLC-1, the FBXW5 protein exerts more likely as a sensor for detecting the degradation signal transduced from the Notch1 signaling.

The characterization also leads to the conclusion that the mutual exclusion relationship between the DLC-1 and Notch1 signaling is formed by the intra-signaling and inter-signaling transduction. The intra-signaling transduction along the DLC-1 signaling is in the order of FBXW5-DLC-1-Rock1. The inter-signaling transduction concerns mutual crosstalk between the two signaling. It can be understood that the crosstalk can be initiated at multiple levels of either signaling and then guided through the intra-signaling and the inter-signaling to the opposite signaling with the consequence of the mutual inhibition/exclusion of the two signaling regarding the regulation of the immunomodulation of hMSCs. For example, the inter-signaling transduction can be transduced to the level of Hey1 through the Notch1

signaling and then transduced to the FBWX5 protein of the DLC-1 signaling for achieving the enhanced immunomodulation by inhibiting the DLC-1 signaling. Through both the signaling transduction, the multi-level mutual exclusion mechanism can then provide a sophisticated automation system to fine-tune the immunomodulation of hMSCs, which can then be under a constantly dynamic control to sense and meet different needs of immunomodulation in various inflammatory environments.

With the framework the crosstalk between the two signaling being proposed, a set of preliminary data provided in this study also suggests the existence of negative feedback control mechanism within each signaling for limiting the activity of that signaling. Within the DLC-1 signaling, the Rock2 protein may serve as a negative feedback molecule for limiting the activity of the DLC-1 signaling as the Rock2 inhibition induced by siRock2 or Y27632, which exhibits a more preferential inhibition on Rock2 than Rock1 in our experiments, inhibits DLC-1 protein expression while elevating IDO1 expression (Figure s3). Similarly, within the Notch1 signaling, the Hes1 protein may serve as a negative feedback molecule for inhibiting the Notch1 signaling as the Hes1 silencing can elevate NICD1 and IDO1, but decreases DLC-1, showing an apparently opposite effect to the Hey1 silencing. The negative feedback control branched from the downstream effector of each signaling represents a common and effective control mechanism seen in different cell signaling pathways [36] and provides an additional level of sophistication to the mutual exclusion crosstalk. Nonetheless, further investigation for the importance of the negative feedback mechanisms is warranted.

While more mechanistic studies are needed for fully understanding the mutual exclusion mechanism, one previous observation about the association of Caveolin-1 with the activity of γ-secretase may help interpret the signaling transduction from DLC-1 to Notch1. The DLC-1 protein can bind the Caveolin-1 protein in a START domain-dependent manner [37, 38]. The binding may lead to a negative regulation of γ-secretase activity as the Caveolin-1 protein is involved in the attenuation of γ-secretase-mediated proteolysis of Notch1 [39]. Nevertheless, further investigations are warranted to address all relevant questions before fully understanding the mutual exclusion crosstalk between these two signaling.

Conclusions

In summary, the present study significantly advances our understanding about the regulation of the immunomodulation of hMSCs. It demonstrates for the first time that the DLC-1 tumor suppressor can function as an inhibitor of the immunomodulation of hMSCs, thus further emphasizing the importance of the DLC-1 tumor suppressor in regulating both tumor ‘seed’ and tumor ‘soil’, both of which are critical for tumor development and progression according to the ‘seed-soil’ theory [40]. On top of the findings about DLC-1, the present study in fact discovers a sophisticated multi-level mutual exclusion mechanism, which well fits the need of fine-tuning the immunomodulation of hMSCs. It is believed that all the findings will contribute to the development of new biomarkers and technologies for precisely and effectively evaluating and controlling the immunomodulatory activities of hMSCs.

Abbreviations

1-L-MT:1-methyl-L-tryptophan; Cdc42:Cell division control protein 42 homolog; cDNA:complementary DNA; Col2a1:Collagen Type II Alpha 1 Chain; CSL:CBF1/RBP-Jk; Su(H)/Suppressor of Hairless; Lag-1; CUL4A:Cullin-4A; DDB1:Damage Specific DNA Binding Protein 1; DLC-1:deleted in liver cancer 1; FBXW5:F-box/WD repeat-containing protein 5; GDP:guanosine diphosphate; GTP:Guanosine-5'-triphosphate; Hes1:hairy and enhancer of split-1; Hey:Hairy/enhancer-of-split related with YRPW motif protein 1; hMSC:human mesenchymal stem cells; HRP:Horseradish peroxidase; hUC-MSCs:human umbilical-cord-derived mesenchymal stem cells; IDO1:Indoleamine 2,3-dioxygenase 1; IL-1a:interleukin-1 alpha; IFN-γ:interferon-gamma; IP:Immunoprecipitation; Mcl-1:Myeloid cell leukemia-1; NICD:Notch intracellular domain; NLS:nuclear localization signal; PBMCs:Peripheral blood mononuclear cells; PCR:Polymerase chain reaction; RGC32:response gene to complement 32 protein; Rho:Ras homologous; RhoGAP:Rho GTPase-activating protein; Rock1/2:Rho-associated protein kinase 1/2; RT-PCR:Real-time Polymerase chain reaction; SAM:Sterile alpha motif; siRNA:Small interfering RNA; START:StAR-related lipid-transfer; STAT1:Signal transducer and activator of transcription 1; TACE:TNF-α converting enzyme; Th1:Type 1 T helper; Th17:Type 17 T helper; TNFα:Tumor Necrosis Factor alpha; Treg:regulatory T lymphocytes.

Declarations

Acknowledgement

Not Relevant

Authors’ contributions

Bao-Zhu Yuan and Tao Na designed the experiments. Kehua Zhang performed the osteogenic differentiation and Th1 lymphocyte proliferation assay and analyzed the corresponding results. Tao Na performed the other experiments and analyzed the results. Bao-Zhu Yuan and Tao Na wrote the paper.

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Availability of data and materials

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request

Ethics approval and consent to participate

None of the cell lines used in the present study required ethics approval for their use.

Consent for publication

Not relevant.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

The treatment with GSI-I elevates DLC-1 protein level in hUC-MSCs via proteasome inhibition. At 24 h after GSI-I treatment，Western blotting shows a dose-dependent increase in protein expression for DLC-1(A-1) or Mc-1(A-2)，while the immunoprecipitation from the cell lysates using the antibodies against either DLC-1 (B-1) or Mcl-1 (B-2) shows an increase in the polyubiquinated form of both proteins.

Figure 2

DLC-1 moderately regulates CD105 expression in hUC-MSCs. The siDLC-1 transfection effectively reduces DLC-1 protein expression seen in Western blotting (A), moderately reverses the GSI-1-induced reduction of CD105 tested in a flow cytometry assay (B), but shows no effect on the expression of RGC32 gene as examined by RT-PCR (C).

Figure 3

DLC-1 inhibits the immunomodulation of hUC-MSCs. A. The DLC-1-silencing in hUC-MSCs, confirmed by Western blotting after siDLC-1 transfection (A-1), enhances the inhibitory effect of hUC-MSCs with or without GSI-1 on Th1 lymphocyte proliferation, as tested via a flow cytometry in Th1 lymphocyte proliferation assay (A-2). The original spectrogram of a representative Th1 lymphocyte analysis (A-3). Th1 lymphocytes are CD8-/IFN-γ+ cells as circled in the spectrogram. All sepctrograms are arranged alphabetical order corresponding to the groups in A-2. B. The DLC-1 overexpression in hUC-MSCs, confirmed by Western blotting after DLC-1 cDNA transfection (B-1), blunts the inhibitory effect of hUC-MSCs on the Th1 proliferation, as tested via a flow cytometry in the Th1 lymphocyte proliferation assay

(B-2). PIB represents the treatment of PBMCs used in the Th1 proliferation assay with PMA, Ionomycin and Brefeidin. The original spectrogram of a representative Th1 lymphocyte analysis (B-3) . Th1 lymphocytes are CD8-/IFN-γ+ cells as circled in the spectrogram. All sepctrograms are arranged alphabetical order corresponding to the groups in B-2. C. The co-transfection of siDLC-1 with pIDO1-Luc in hUC-MSCs shows that DLC-1 silencing enhances the IFN-γ-induced IDO-1 promoter activity. D. The co-transfection of DLC-1 cDNA with pIDO1-Luc in hUC-MSCs indicates that DLC-1 over-expression inhibits the IDO1 promoter activity.

Figure 4

The inhibition by DLC-1 on the immunomodulation of hUC-MSCs is RhoGAP domain dependent and independent. A. The schemes of all DLC-1 mutants used in this study are presented. B. In the co-transfection of pIDO-1-Luc with the cDNA of wild type DLC-1 (wtDLC-1) or its mutants in hUC-MSCs, wtDLC-1 shows an approximately fifty percent reduction of the IFN-γ-induced IDO1 promoter activity, while all mutants exhibit either no effect (DLC-1-662) or even enhanced effect (DLC-1-662, -Δ662, -R714E and DLC-1/RhoGAP), on the promoter activity. C. In the transfection with cDNA of wtDLC-1 or its mutants in hUC-MSCs, wtDLC-1, DLC-1-662, or DLC-Δ662 cause a significant reduction in IDO1 expression, while DLC-1/RhoGAP shows no effect on, and DLC-1/RhoGAP results in, a slight increase in the IDO1 expression.

Figure 5

DLC-1 inhibits the immunomodulation of hUC-MSCs through inhibiting Notch1. A. In the IDO1 promoter assay from the co-trasnsfection of DLC-1 cDNA with siNotch1, the Notch1 silencing is confirmed by Western blotting (A-1). While DLC-1 alone or siNotch1 alone reduces, the co-transfecton further inhibits, the IDO1 promoter activity (A-2). B. In the IDO1 promoter assay from the co-trasnsfection of DLC-1 cDNA with NICD1 cDNA, the NICD1 over-expression is confirmed by Western blotting (B-1). DLC-1 inhibits the NICD1-elevated IDO-1 promoter activity (B-2). The IDO1 promoter activity is measured from the transfection with pIDO1-Luc followed by the treatment with IFN-γ in hUC-MSCs.

Figure 6

A mutual exclusion relationship exists between DLC-1 and Notch1 in hUC-MSCs. Western blotting shows that the siDLC-1 transfection reduces both basal and GSI-I-induced NICD1 (A); the transfection with DLC-1 cDNA causes a significant reduction of basal NICD1 level (B); in the co-transfection studies, whereas the transfection with NICD1 cDNA alone shows an over-expression of NICD1 and increases, and the transfection with DLC-1 cDNA alone reduces, the IDO1 expression, the co-transfection of both cDNAs inhibits NICD1 over-expression from the NICD1 gene expression and the IDO1 expression (C); in analyzing

the partition in cellular compartments of DLC-1 and NICD1 proteins, the siDLC-1 transfection clearly increases the NICD1 level in both the cytoplasm and the nucleus, whereas the DLC-1 cDNA transfection reduces it also in both cellular compartments with a more significant reduction seen in the nucleus than the cytoplasm (D). In all the experiments, the siControl and pcDNA3.1 are used as control or for keeping a balance of the transfection material in siRNA transfection and plasmid DNA transfection, respectively.

Figure 7

DLC-1 inhibits the activity of the Notch-Hey1 axis, but not the Notch-Hes1 axis, in the immunomodulation of hUC-MSCs. A. RT-PCR validates that the transfection with siHes1 (1#) and siHey1 (1#) can effectively inhibits the gene expression of Hes1 and Hey1, respectively. B. The IDO1 promoter activity assay indicates that, comparing with siControl, the siHes1 transfection causes a slight increase, but the siHey1 transfection results in a dramatic decrease, in the IDO1 promoter activity. C. The Th1 lymphocyte proliferation assay shows that the transfection with siHey1, but not siHes1, is able to significantly reduce the inhibition of Th1 proliferation by hUC-MSCs. D. RT-PCR shows that the treatment with GSI-I can equally reduce the gene expression of both Hes1 and Hey1, whereas DAPT can only reduce that of Hes1. E. Western blotting shows that DAPT causes a slight increase in, but GSI-I results in, the IDO1 expression. F. The Th1 lymphocyte proliferation assay indicates that GSI-I reduces, but DARP exhibits no effect on, the inhibition of Th1 lymphocyte proliferation by hUC-MSCs.

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