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Hypoxia suppresses the production of matrix metalloproteinases and migration of human monocyte-derived dendritic cells

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Key Words: hypoxia, dendritic cell, matrix metalloproteinase, migration

Abbreviations: 5-AZ (5-azacytidine), TSA (Trichostatin A), MMP (matrix metalloproteinase), DCs (dendritic cells), hmDCs (human monocyte-derived dendritic cells)

## SUMMARY

As most solid tumors are hypoxic, dendritic cells (DCs) in solid tumors are also exposed to hypoxia. While many adaptation responses of tumor cells to hypoxia are known, it is yet to be determined how hypoxia affects the functions of DCs. In order to explore the effects of hypoxia on the functions of DCs, we compared the expression of surface markers, cytokines, chemokine receptors and matrix metalloproteinases (MMPs) of human monocyte-derived DCs (hmDCs) differentiated under hypoxia to those differentiated under normoxia. Both groups of hmDCs expressed similar levels of surface markers and cytokines. However, expression of MMP-9 and membrane type-1-MMP (MT1-MMP), as well as migrating activity, was significantly suppressed in hmDCs differentiated under hypoxia compared with their normoxia counterparts. We also demonstrated that Trichostatin A (TSA) restored the production of MMP-9 in hmDCs, under hypoxia. Collectively, our findings show that a hypoxic microenvironment suppresses the production of MMPs in hmDCs, most probably through the deacetylation

of promoter regions of MMPs, thus suppressing the migrating activity of hmDCs. Our results suggest that the hypoxic microenvironment in solid tumor tissues may suppress the function of DCs.

## INTRODUCTION

Aggressive tumors often have insufficient blood supply, partly because tumor cells grow faster than endothelial cells, and partly because a newly formed vascular supply is disorganized [1-4]. When cells are not supplied with sufficient blood, they are exposed to hypoxia and hypoglycemia. Recently, over-expression of the hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) protein, which is stabilized and also activated under hypoxia, has been reported in human common cancers *in vivo* [5], suggesting that tumor tissues are generally hypoxic. A number of reports have recently demonstrated that hypoxic conditions induce a variety of adaptation responses in tumor cells [6-9]. We have also demonstrated that the expression of HIF-1 $\alpha$ , as well as HIF-1-induced glycolysis is essential for *in vivo* tumorigenicity of pancreatic cancer cells [10].

However, tumor tissues are composed of both normal as well as tumor cells. In tumor tissues, various types of normal cells, such as endothelial cells and leukocytes are perceived. It is now widely recognized that macrophages

represent a prominent component of the leukocyte population in tumor tissues. These cells, often called tumor-associated macrophages (TAMs), are thought to be derived from peripheral blood monocytes, and recruited into the tumor tissues [11]. Importantly, there is accumulating evidence showing that hypoxia modulates the expression of various genes in the accumulated TAMs, in tumor tissues [11-13].

Precursors of dendritic cells (DCs) are also localized in peripheral tissues; they undergo maturation upon antigen uptake and processing [14, 15]. Mature DCs migrate from the peripheral tissues to draining lymph nodes, where they present antigens to antigen-specific T cells [14, 15]. Thus, migrating capacity of DCs is crucial for their professional function as antigen-presenting cells (APC) [16-18]. A recent report asserted that DCs generated *in vitro*, and injected into tumor tissues, did not migrate to regional lymph nodes [19]. This suggests that DCs lose their migrating activity in tumor tissues. In principle, on their way from peripheral tissues to regional lymph

nodes, DCs have to cross basement membranes and move through connective tissue. Previous reports demonstrated that MMP-9 and MMP-2 are necessary for the migration of DCs [20-22], whereas it is yet to be determined how the expression of MMP-9 and MMP-2 in DCs is regulated under hypoxic conditions.

In this study, we compare the expression of MMPs and migrating activities of human monocyte-derived DCs (hmDCs) differentiated under normoxia (hmDCs-N) to those differentiated under hypoxia (hmDCs-H).

## RESULTS

### *Expression of DC-associated markers and cytokines:*

We first examined the expression of DC-associated markers (Fig. 1a). CD14 was not expressed. CD83 was expressed on both the cells stimulated by LPS under normoxia (m-hmDCs-N), and those stimulated under hypoxia (m-hmDCs-H). CD80, CD86, HLA-DR, HLA-ABC and CD1a were also expressed on both cell counterparts (Fig. 1a). Both of them showed more than 90 % viability (Fig. 1b), and expressed similar levels of IL-10 and IL-12 (Fig. 1c, 1d and 1e). Collectively, these results suggest that hypoxia does not affect differentiation or maturation of hmDCs.

### *Expression of MMP-9 in hmDCs:*

MMP-2 was not expressed in hmDCs. The immature hmDCs (im-hmDCs) and m-hmDCs differentiated under normoxia expressed MMP-9 mRNA at 2-5 fold higher levels than those differentiated under hypoxia (Fig. 2a). The hmDCs-N also produced MMP-9 (Fig. 2b), and secreted MMP-9 at higher levels than hmDCs-H (Fig. 2c). However, the active form of

MMP-9 was detected only in the supernatants of hmDCs differentiated under normoxia (Fig. 2c). Gelatin zymography showed that hmDCs-N secreted pro-MMP-9 at higher levels than hmDCs-H, and that only hmDCs-N secreted the active form of MMP-9 (Fig. 2d). The difference in MMP-9 expression between hmDCs-H and hmDCs-N appeared in the early phase of differentiation (Fig. 2e). We then examined MMP-9 mRNA expression in the hmDCs which had differentiated under normoxia and then been incubated under hypoxia for 3 days, to investigate whether hmDCs inoculated into peripheral blood in an adoptive immunotherapy could maintain their functions after migrating to hypoxic tumor tissues. As a result, these m-hmDCs expressed half as much MMP-9 mRNA, as those that were incubated continuously under normoxia did (Fig. 2f).

*Expression of MT1-MMP in hmDCs:*

Since we did not detect MMP-2 in our hmDCs, we examined the expression of MT1-MMP, which has been reported to activate MMP-2 produced by other cells, and to play an

important role in the migration of cells that do not express MMP-2 [23, 24]. The im-hmDCs and m-hmDCs differentiated under normoxia expressed MT1-MMP mRNA about five-fold higher than those differentiated under hypoxia (Fig. 3a). We then examined MT1-MMP mRNA expression in hmDCs-N, which had been incubated for a further 3 days under hypoxia, to investigate whether DCs inoculated in an adoptive immunotherapy might also have decreased production of MT1-MMP after migrating into tumor tissues. As a result, these m-hmDCs expressed half as much MT1-MMP mRNA, as those that were incubated continuously under normoxia did (Fig. 3b).

*Expression of tissue inhibitors of MMPs in hmDCs:*

As specific tissue inhibitors of MMPs (TIMPs) are thought to regulate proteolytic activities of MMPs [25], we then examined the expression of three TIMPs in the hmDCs. TIMP-1 was expressed at higher levels in hmDCs-H than in hmDCs-N (Fig. 4). TIMP-2 and TIMP-3 were expressed at similar levels in both hypoxic and normoxic groups. As

TIMP-1 is a specific inhibitor of MMP-9, these results further indicate that hmDCs-H have lower proteolytic activities than hmDCs-N and suggest that the decreased expression of MT1-MMP and MMP-9, in our experiments, is a specific effect of hypoxia.

*Migrating activities of hmDCs:*

As MMP-9 is necessary for the migration and infiltration of DCs through the basement membrane, we then compared the migrating activities of hmDCs-N to hmDCs-H. Both im-hmDCs-N and m-hmDCs-N had significantly higher migrating activities than their counterparts, differentiated under hypoxia (Fig. 5a). As the migrating activities of the hmDCs differentiated under normoxia were suppressed by recombinant TIMP-1 protein, we attributed this result to their higher secretion of MMP-9, when compared to that of their counterparts, under hypoxia (Fig. 5b). We next examined the expression of chemokine receptors in hmDCs differentiated under hypoxia and under normoxia, respectively, because the chemokine/chemokine

receptor interaction is also essential for the migration of DCs [26]. The im-hmDCs differentiated under normoxia expressed CCR1 and CCR6 at 4-times higher levels than those differentiated under hypoxia (Fig. 5c). However, m-hmDCs-H expressed CCR7 at a slightly higher level than m-hmDCs-N. CCR7 is reported to play important roles in the homing of mature DCs to regional lymph nodes [26]. This result strongly suggests that MMP-9 might be one of the essential proteins needed for the homing of DCs from peripheral tissues to regional lymph nodes, since not withstanding the higher expression of CCR7 in m-hmDCs-H, the migration potential of these cells is significantly lower than their normoxic counterparts.

*Restoration of expression of MMP-9:*

Incubation of the m-hmDCs-H, for a further 72 h under normoxia, did not restore the production of MMP-9 or MT1-MMP (data not shown). Furthermore, neither interferon- $\alpha$ , nor interferon- $\gamma$  or interleukin-12 showed any effect on the production of MMP-9 or MT1-MMP in the hmDCs (data not

shown). Hence, we hypothesized that either methylation or deacetylation of the MMP-9 promoter region might be implicated in its suppression, caused by hypoxia. Five-azacytidine (5-AZ), a demethylating agent, enhanced the production of MMP-9 in both m-hmDCs groups, notwithstanding their culture condition (Fig. 6a). We did not detect any differences in the methylation sites of the promoter region of MMP-9 (Fig. 6b). In contrast, Trichostatin A (TSA), a specific inhibitor of histone deacetylase, restored the production of MMP-9, under hypoxic conditions (Fig. 6c). These results suggest that deacetylation, but not methylation, of the MMP-9 promoter region might be implicated in the suppression of MMP-9, caused by hypoxia.

## DISCUSSION

Most solid tumors develop regions of low oxygen tension because of an imbalance in oxygen supply and consumption. As a tumor expands, vigorous growth of cancer cells creates a hypoxic microenvironment, which, if not alleviated, may restrict tumor growth or even cause cell death [27]. Angiogenesis and increased glycolysis, two universal characteristics of solid tumors, represent an adaptation response to the hypoxic microenvironment [28]. DCs, which undergo differentiation and maturation in tumor tissues, are also in the hypoxic microenvironment.

DCs are the most efficient type of migrating APC, playing an essential role in the initiation of immune responses. To fulfill their effector function, DCs migrate through endothelial barriers and tissues [14, 15].

Although recent reports have shown that some of the mechanisms underlying DC migration are controlled by MMPs and TIMPs [25], little is known about how hypoxia regulates MMP expression and migratory capacity of DCs.

This is the first study showing the hypoxia-induced-suppression of MMP-9 and MT1-MMP production, and migrating activity of hmDCs. Here we clearly demonstrate that hmDCs-H express significantly lower levels of MMP-9 and MT1-MMP, as well as higher levels of TIMP-1, when compared to hmDCs-N. In accordance with these results, we demonstrate that hmDCs cultured under hypoxia have less capability for migration than those cultured under normoxia. Furthermore, hmDCs differentiated and matured under normoxia, and then cultured under hypoxia for another 3 days, also show a decreased expression of MMP-9 and MT1-MMP, suggesting that the hypoxic microenvironment in tumor tissues can potentially suppress the migrating activities of inoculated DCs in an adoptive immunotherapy.

Interestingly, in accordance with recent data showing that CXCR4 is induced by hypoxia in several cell types, such as monocytes, endothelial cells, TAMs and also cancer cells [29], levels of CCR7 were upregulated in m-hmDCs-H in our study. These findings suggest that hypoxia stimulates chemotactic potential to constitutively secreted chemokines,

through the enhanced expression of chemokine receptors.

However, in view of the fact that *in vitro* invasion of our DCs, under hypoxia, was still lower than that under normoxia we postulate that MMP-9 might play a pivotal role in DC migration *in vivo*, and restoration of MMP-9 production might be critical for the migration of DCs from tumours to regional lymph nodes.

From our observation, that TSA, an inhibitor of histone deacetylase, restores the production of MMP-9 and MT1-MMP in hmDCs, we postulate that deacetylation of promoter regions of MMP-9 and MT1-MMP might be the cause of suppression of MMP-9 and MT1-MMP by hypoxia. To date, two reports have described the association of histone deacetylase with MMP production. Trichostatin A was reported to stimulate MMP-9 promoter activity in HT1080 fibrosarcoma cells [30]. Also, Pender et al., confirmed that a histone deacetylase inhibitor, butyrate, up-regulated the production of MMP-7 [31].

In our results, the hypoxic microenvironment suppresses the expression of MMP-9 and MT1-MMP in hmDCs.

However, several issues remain yet to be resolved, namely, whether the suppression of MMP production caused by hypoxia is also observed in other DC subtypes, such as CD11c<sup>+</sup> Langerhans cells and interstitial DCs, derived from CD34<sup>+</sup> myeloid progenitor cells. Most importantly, it is to be determined whether this phenomenon is also observed in other species, like mouse and rat, so as to conduct proper *in vivo* studies. A number of reports have already demonstrated that hypoxia enhances the expression of MMP-9 in various types of cells (32, 33). On the other hand, several other reports state that this hypoxia-mediated increase of MMP-9 is not consistently observed [34]. Saed *et al.* reported that hypoxia decreases the expression of MMP-9, and that TGF- $\beta$ 1 increases this expression under normoxia, but decreases it under hypoxia [35]. In our experiments, adherent cells, without any stimulation, express only a low level of MMP-9 mRNA, with a gradual increase of MMP-9 mRNA expression during the culture of cells in the presence of GM-CSF and IL-4. This suggests that a hypoxic condition may suppress MMP-9 mRNA expression

induced by GM-CSF and IL-4 by disturbing the balance of the deacetylation: acetylation reaction. Initially, we had hypothesized that the expression of MMP-9 would be suppressed by hypoxia only in hematopoietic cells. However, surprisingly, the expression of MMP-9 in U-937 leukemia cells, derived from hematopoietic cells, was stimulated by hypoxia (data not shown). Our results, as well as the current literature, clearly emphasize the need for further elucidation of the relationship between hypoxia and MMPs. We believe that there exists the possibility that this hypoxia-induced decrease of MMP production may be specific to hmDCs only, or DCs in general, and is thus of empirical importance to DC-based tumor immunotherapy. Moreover, the examination of MMP production in other immune cells, such as T cells and B cells, under hypoxia as compared to normoxia, might reveal challenging results regarding the overall immune system scenario.

Collectively, our findings indicate that a hypoxic microenvironment suppresses migrating activities of hmDCs,

by stifling their production of MMP-9 and MT1-MMP, strongly implying that production of MMPs is reduced in hmDCs within tumor tissues, thus hindering the stimulation of an adequate immune response.

## MATERIALS AND METHODS

### *Reagents:*

Interleukin-4 (IL-4), granulocyte/macrophage-colony stimulating factor (GM-CSF) and regulated upon activation, normal T expressed and secreted (RANTES) were purchased from R&D Systems (Minneapolis, MN). Human interferons IFN- $\alpha$  and IFN- $\gamma$  were purchased from JCR Pharmaceuticals Co. Ltd. (Tokyo, Japan) and PBL Biomedical Laboratories (Piscataway, NJ), respectively.

Lipopolysaccharide (LPS), Ficoll-Paque, Human  $\alpha$ IFN, Trichostatin A (TSA), and gelatin were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS) was purchased from HyClone (HYCLONE, Utah). FITC-conjugated mAbs against human CD83, CD80, CD14, HLA-ABC, HLA-DR, un-conjugated mAbs against human CD1a and CD86, and isotype control antibodies were purchased from Immunotech (Marseille, France). FITC-conjugated anti-mouse IgG+IgM (H+L) was purchased from Jackson ImmnoResearch Laboratories, Inc. (West Grove, PA). Trizol reagent, QuantiTect<sup>TM</sup> SYBR<sup>®</sup> Green PCR Master Mix, ABI PRISM<sup>®</sup> 7900HT sequence Detection

System and TaqMan® Reverse Transcription Reagents were purchased from Life Technologies (Frederick, MD), QIAGEN (Germany) and Applied Biosystems (Foster City, CA), respectively. Anti-human MMP-9 and TIMP-1 antibodies were purchased from Daiichi Pure Chemical Co. Ltd. (Tokyo, Japan). Mouse anti-human  $\beta$  actin antibody, sheep anti-mouse Ig antibodies, Western blotting detection reagent and BD BioCoat Matrigel Invasion Chamber were purchased from Chemicon International, Inc. (Temecula, CA), Amersham LIFE SCIENCE, Inc. (Arlington Heights, IL), and BD Biosciences (Mississauga, Ont., Canada), respectively.

*Generation of human monocyte-derived dendritic cells:*

Human monocyte-derived DCs were prepared as previously reported [36, 37]. Briefly, adherent cells were cultured in the presence of 1000 U/ml of GM-CSF and IL-4 for 6 days under hypoxia or normoxia. According to the previous definition of tumor hypoxia (median  $pO_2 < 10$  mmHg, approximately 1.25%) [38], the cells in the hypoxic group were incubated at 1 %  $O_2$  in a hypoxic chamber gassed with

94 % N<sub>2</sub> and 5 % CO<sub>2</sub> (Wakenyaku Co. Ltd., Tokyo). On day 6, the cells were separated into 2 groups, respectively. Cells of group 1 were cultured in the same condition for one day and used as immature DCs (im-hmDCs). Cells of group 2 were treated with LPS (1 µg/ml) for one day and then used as mature DCs (m-hmDCs). In some experiments, 5-azacitidine (5-Az), or Trichostatin A (TSA), was added to the monocyte cultures 3 days after incubation, during DC differentiation, at 5 µM or 12.5 µM.

*Phenotypic analysis by FACS:*

The cells in the respective groups were incubated with FITC-conjugated specific mAbs against CD83, CD14, CD80, HLA-ABC and HLA-DR. They were then incubated with unconjugated mouse Abs against CD86 and CD1a and FITC-conjugated Anti-Mouse IgG+IgM (H+L). After incubation, the cells were analyzed using a FACScan (Beckton Dickinson).

*Real-Time PCR:*

Each cDNA (10 ng) was amplified in triplicate with the use of the SYBR-Green PCR assay kit and then detected on an ABI PRISM® 7900HT Sequence Detection System. The  $\beta$ -actin RNA was used to standardize the total amount of cDNA. The primers used were as follows:

MMP-9: CCTTTTGAGGGCGACCTCCAAG and CTGGATGACGATGTCTGCGT;

MT1-MMP: CCGATGTGGTGTTCAGACA and TGGCCTCGTATGTGGCATACT;

TIMP1: ACAGACGGCCTTCTGCAATTC and GGTGTAGACGAACCGGATGTCA;

TIMP2: GTTCAAAGGGCCTGAGAAGGA and CCAGGGCACGATGAAGTCA;

TIMP3: GCCTTCTGCAACTCCGACAT and TCTCGGAAGCTTCCGTATGG;

CCR1: CGAAAGCCTACGAGAGTGGAA and CGGACAGCTTTGGATTTCTTCT;

CCR6: TCGCCATTGTACAGGCGACTA and CGCTGCCTTGGGTGTTGTAT;

CCR7: GGTGGCTCTCCTTGTCATTTTC and GGGAGGAACCAGGCTTTAAAGT;

$\beta$ -actin: GTCCTCCTGAGCGCAAGT and TCGTCATACTCCTGCTTGCTGAT.

Relative mRNA levels were determined by comparing the PCR cycle thresholds between the cDNA of the gene of interest and that of  $\beta$ -actin.

*PCR amplification of bisulfide-treated DNA and sequencing:*

Six fragments of the human MMP-9 promoter region containing CpG dinucleotides were amplified according to the previously described method (Chicoine et al, 2002). The PCR products were cloned into the pCR4-TOPO vector (Invitrogen) and then sequenced.

*Western blot analysis:*

Cell-free supernatants were collected from DCs cultured for 24 hours in serum-free RPMI1640 medium. A supernatant of the human fibrosarcoma cell line HT1080 was used as a positive control. After SDS-PAGE, proteins were blotted onto nitrocellulose membranes (Bio-Rad, Hercules, CA) and stained with anti-MMP-9 antibody. Each cell lysate was also analyzed by Western blot analysis, in order to detect intracellular MMP-9 protein. The membranes were developed with the use of an ECL detection kit (Amersham, Tokyo, Japan).

*Gelatin zymography:*

Enzymatic activity was analyzed by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate (SDS) and gelatine as described previously [39].

*Matrigel invasion assay:*

The Matrigel invasion assay was performed in Matrigel-coated Transwell chambers (BD Bioscience) according to the manufacturer's instructions. Recombinant human RANTES and 6Ckine were added in the culture media, in the lower chambers, at a concentration of 100 ng/ml. After 6-8 hours incubation, the number of hmDCs, which migrated to the lower surface of the membrane, was counted.

*Statistical analysis:*

Statistical differences among the groups were determined by a two-tailed *Student's t* test as for independent samples. Mean values were considered significantly different when *p* values were less than 0.05.

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## FIGURE LEGENDS

Fig.1. Generation of im-hmDCs and m-hmDCs.

a. Surface marker expression on im-hmDCs and m-hmDCs differentiated under normoxia (blue line) and hypoxia (pink line). The green lines represent staining with a control isotype-matched antibody.

b. Viability of the im-hmDCs and m-hmDCs differentiated under normoxia and hypoxia.

c. IL-10 mRNA expression in hmDCs.

d. IL-12 p40 mRNA expression in hmDCs.

e. IL-12 p35 mRNA expression in hmDCs.

Fig.2. Expression of MMP-9 in hmDCs.

a. MMP-9 mRNA expression in im-hmDCs and m-hmDCs differentiated under hypoxia and normoxia. Mononuclear cells were obtained from three different donors.

b. MMP-9 protein in cell lysates derived from im-hmDCs and m-hmDCs generated under normoxia and hypoxia.

- c. Secreted MMP-9 protein in conditioned media obtained from im-hmDCs and m-hmDCs generated under normoxia and hypoxia.
- d. Gelatin zymography showing MMP-9 activity in conditioned media obtained from im-hmDCs and m-hmDCs generated under normoxia and hypoxia.
- e. Sequentially examined expression of MMP-9 during the differentiation of human monocytes into DCs.
- f. mRNA expression of MMP-9 in m-hmDCs-N, cultured for a further 3 days under normoxia (indicated as N (normoxia) ➡ N (normoxia)) and m-hmDCs-N, cultured for a further 3 days under hypoxia (indicated as N (normoxia) ➡ H (hypoxia)).

Fig.3. Expression of MT1-MMP

- a. Expression of MT1-MMP mRNA in hmDCs differentiated under normoxia and hypoxia.
- b. mRNA expressions of MT1-MMP in m-hmDCs-N, cultured for a further 3 days under normoxia (indicated as N (normoxia) ➡ N (normoxia)) and m-hmDCs-N cultured for a further 3 days under hypoxia (indicated as N (normoxia) ➡ H (hypoxia)). A

representative result of three different experiments is shown.

#### Fig.4. Expression of TIMPs

Relative copy numbers of TIMP-1, TIMP-2 and TIMP-3 are shown. TIMP-1 expression was enhanced by hypoxia, whereas expression of TIMP-2 and TIMP-3 was not affected.

Fig.5. Migrating activities of hmDCs differentiated under normoxia and hypoxia.

a. Migrating activities through Matrigel-coated transwell membranes of im-hmDCs (chemoattractant - RANTES) and m-hmDCs (chemoattractant - 6Ckine).

b. Migrating activities of hmDCs differentiated under normoxia were specifically inhibited by recombinant TIMP-1 (100 ng/ml).

c. Expressions of CCR1, CCR6 (im-hmDCs) and CCR7 (m-hmDCs) in DCs differentiated under hypoxia and under normoxia. A representative result of three different experiments is shown.

Fig.6. Restoration of MMP-9 expression.

a. 5-azacytidine did not restore the expression of MMP-9 in m-hmDCs, suppressed by hypoxia, however it enhanced the expression of MMP-9 both under normoxia and under hypoxia.

b. Representative possible methylated sites in the sequence of the MMP-9 promoter region of m-hmDCs differentiated under hypoxia and under normoxia. There is no difference between the promoter region of m-hmDCs differentiated under hypoxia and under normoxia.

c. TSA restored the expression of MMP-9 in m-hmDCs. A representative result of three experiments is shown.

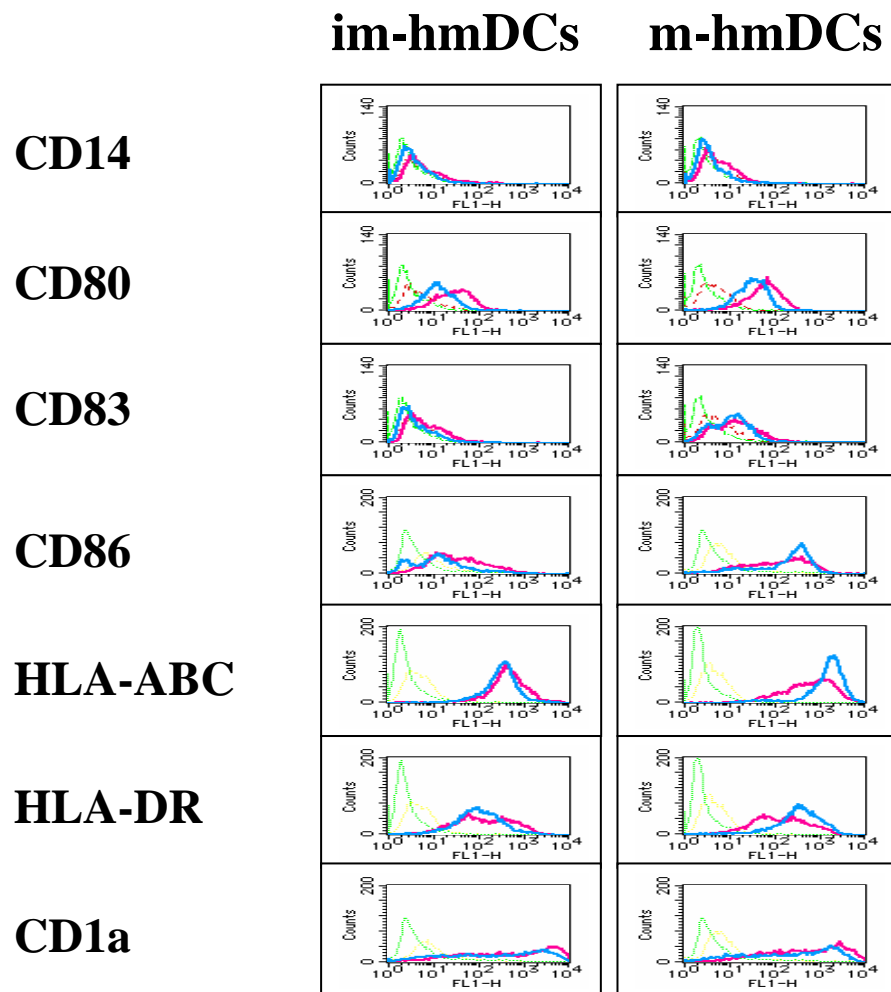


Fig. 1a

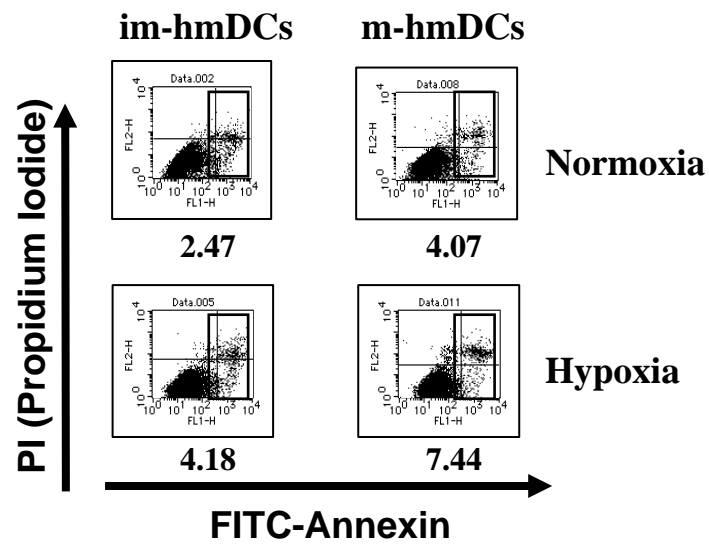
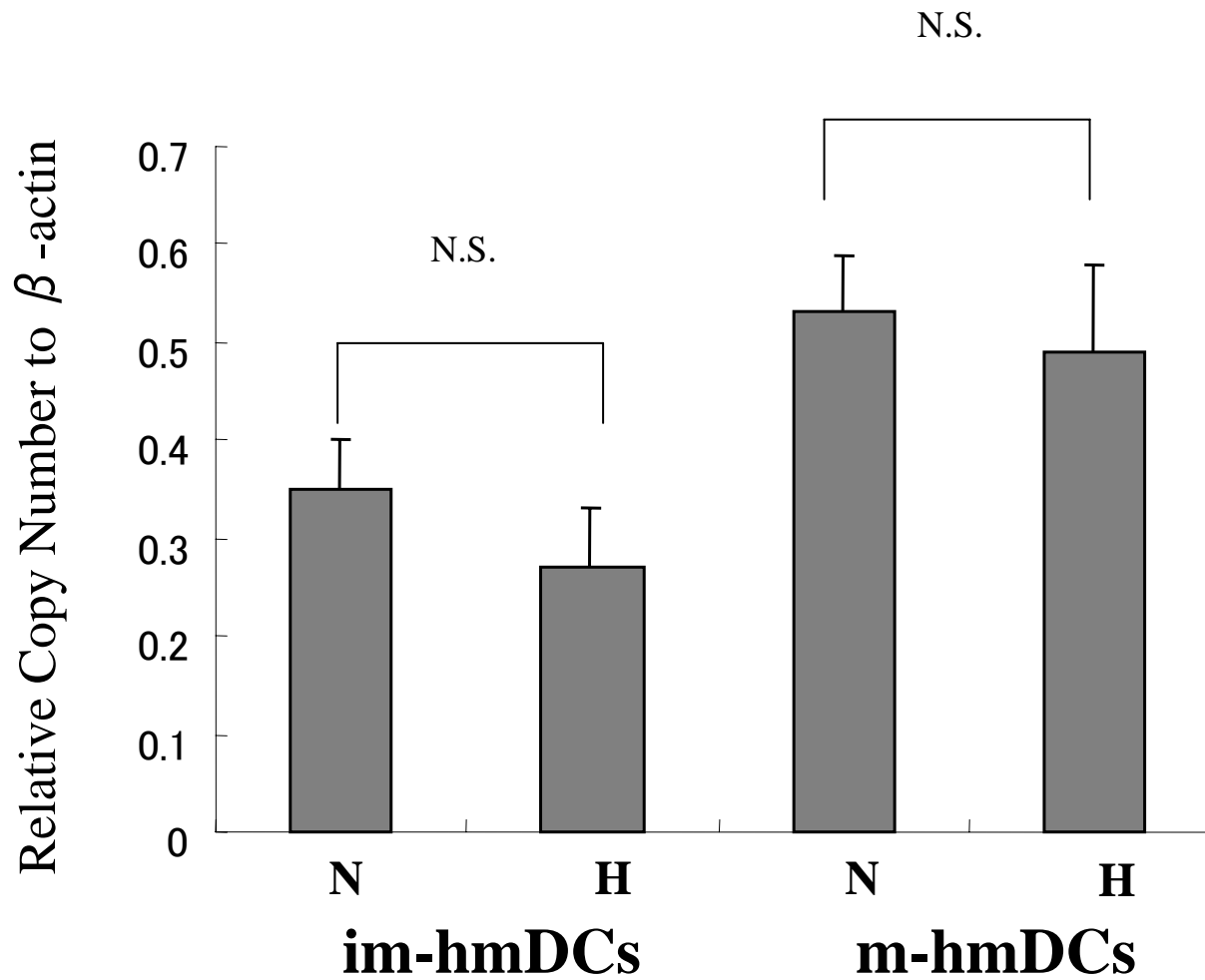


Fig. 1b



IL-10 mRNA

Fig. 1c

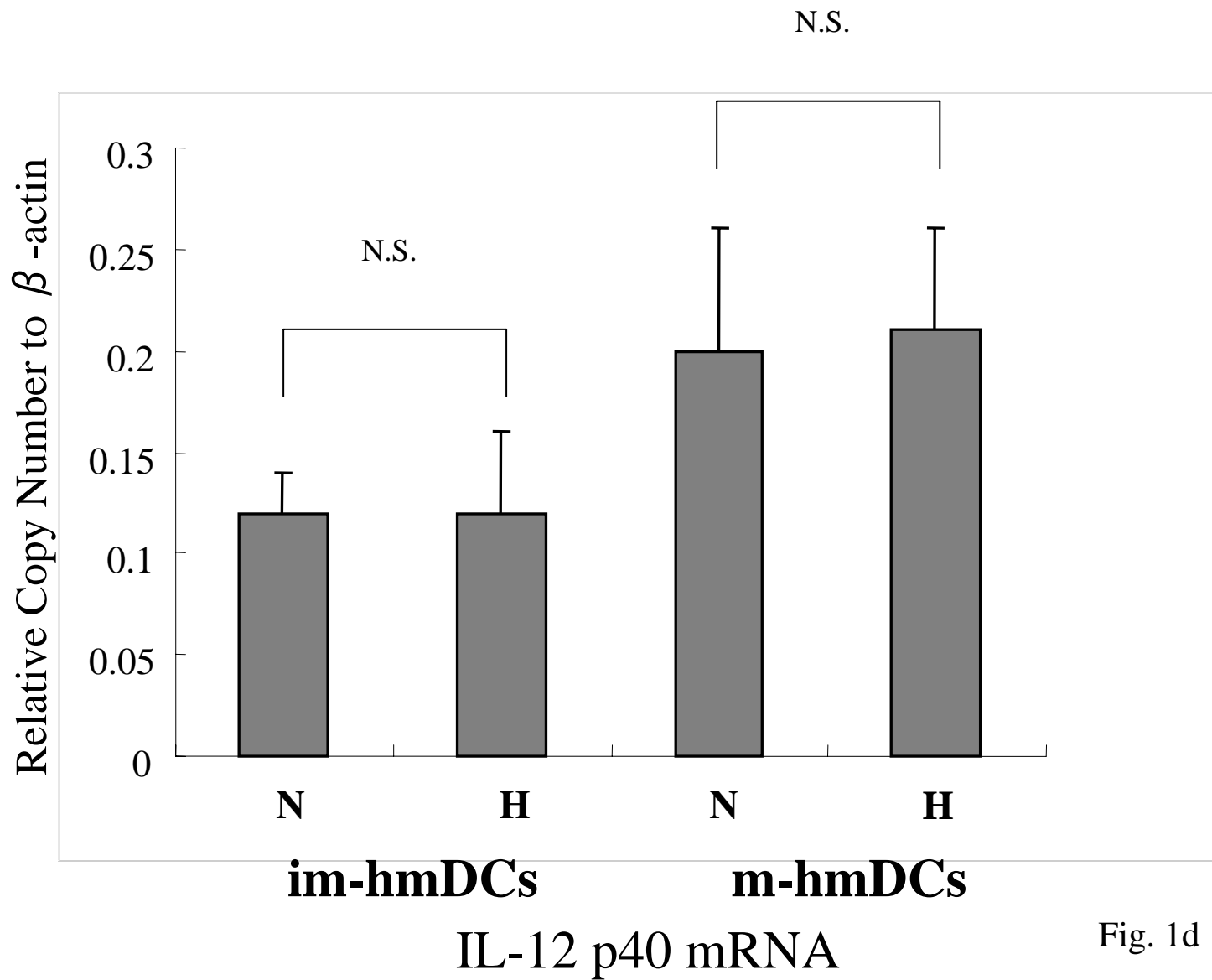


Fig. 1d

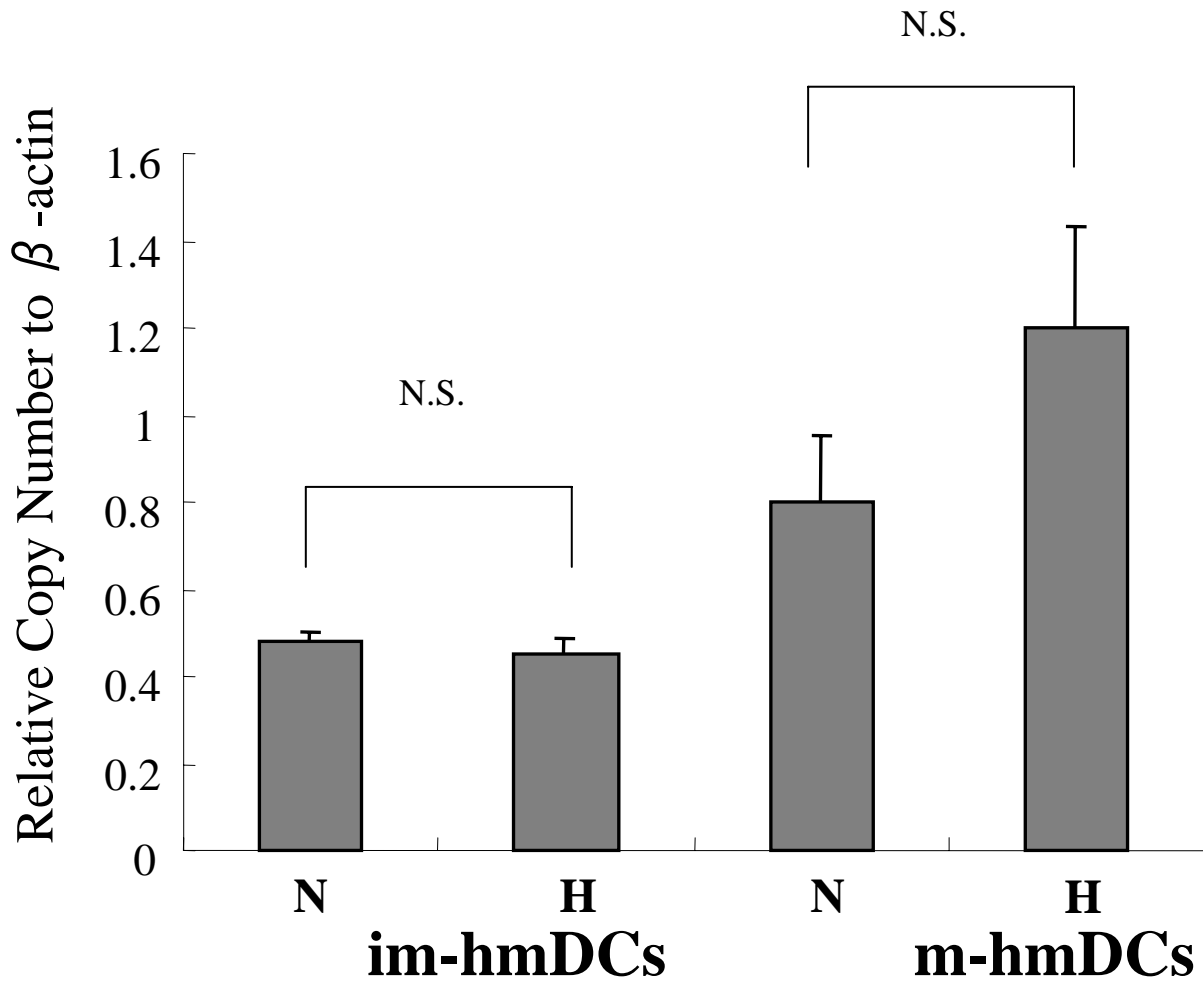


Fig. 1e

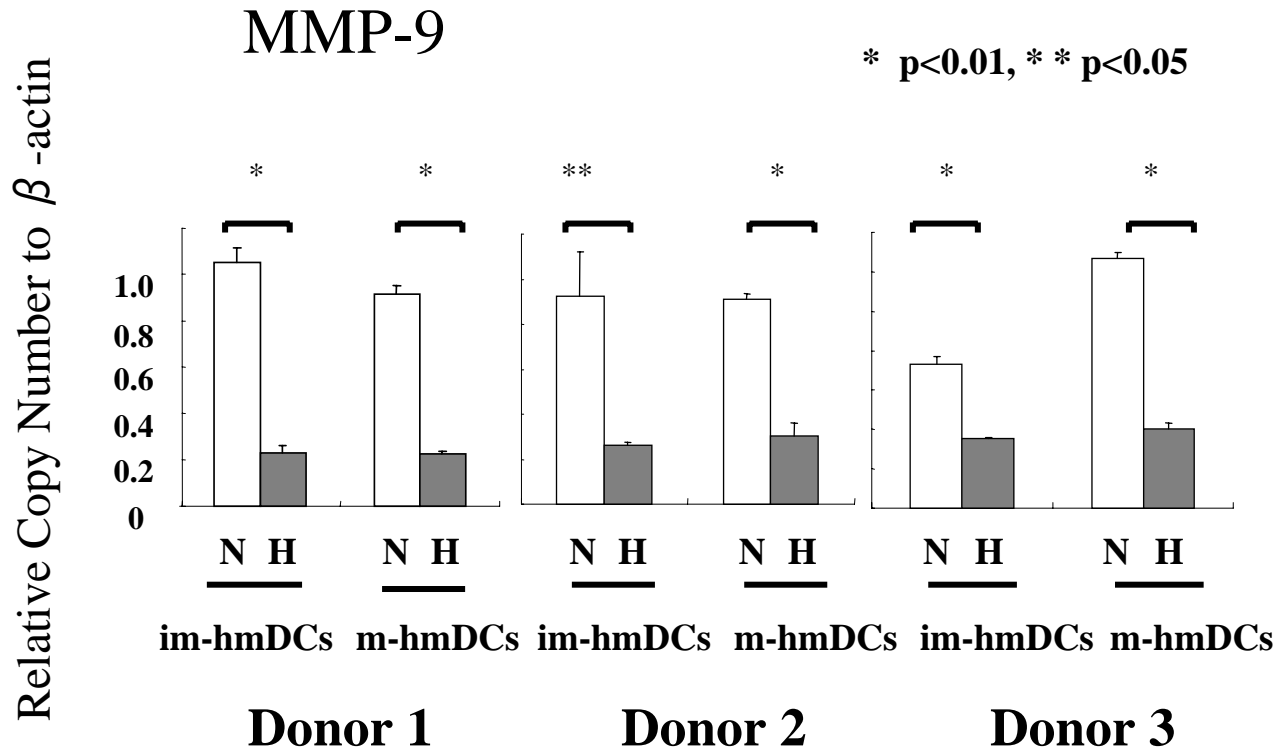


Fig. 2a

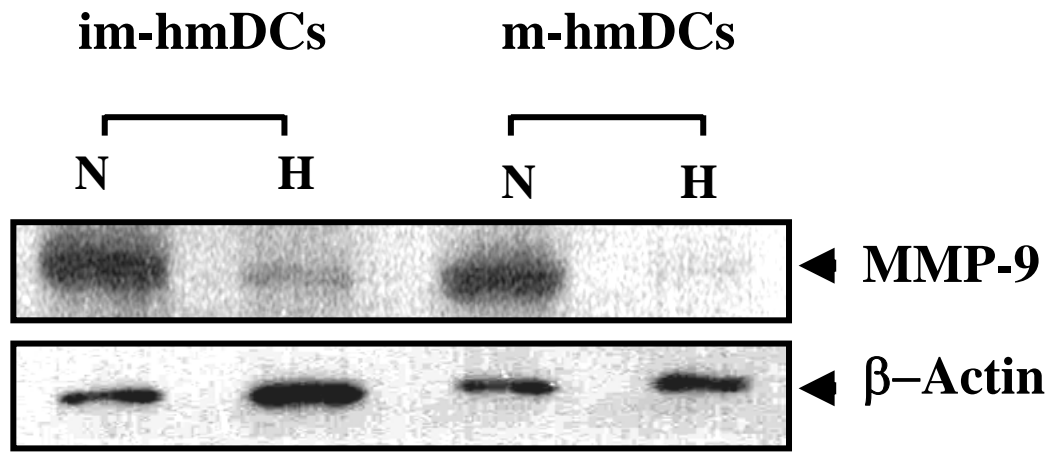


Fig. 2b

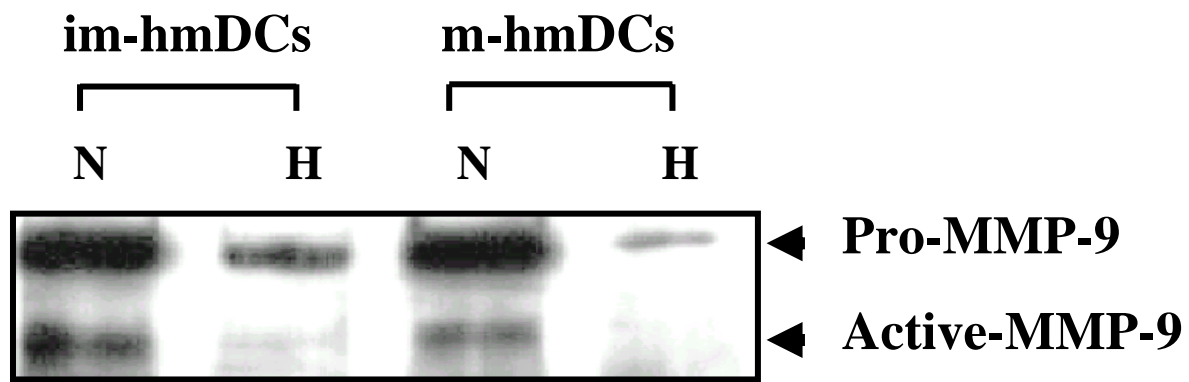


Fig. 2c

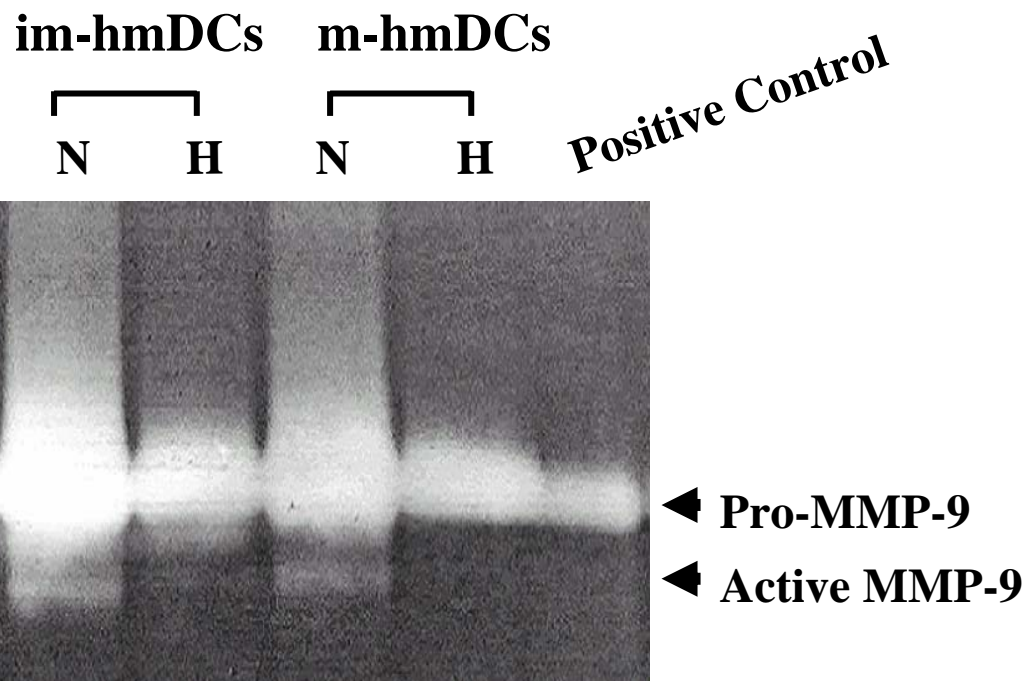


Fig. 2d

# MMP-9

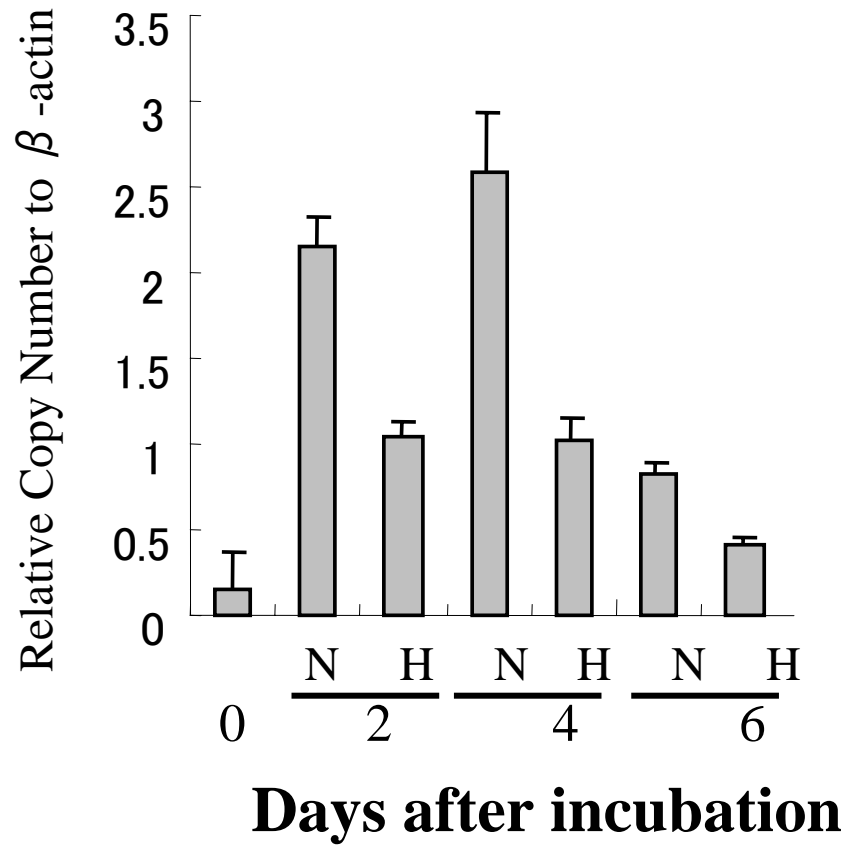


Fig. 2e

# MMP-9

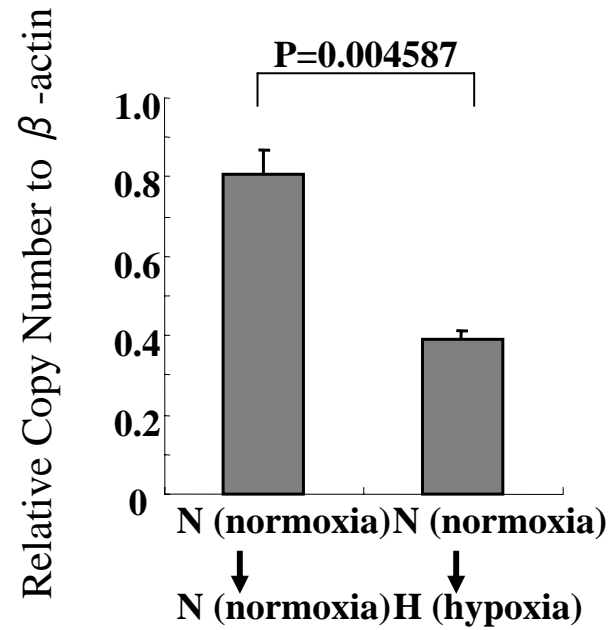


Fig. 2f

# MT1-MMP

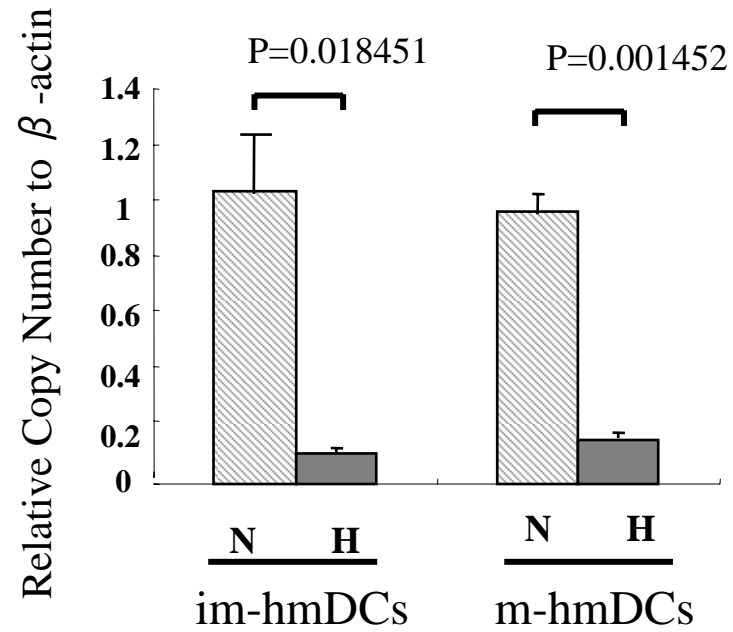


Fig. 3a

# MT1-MMP

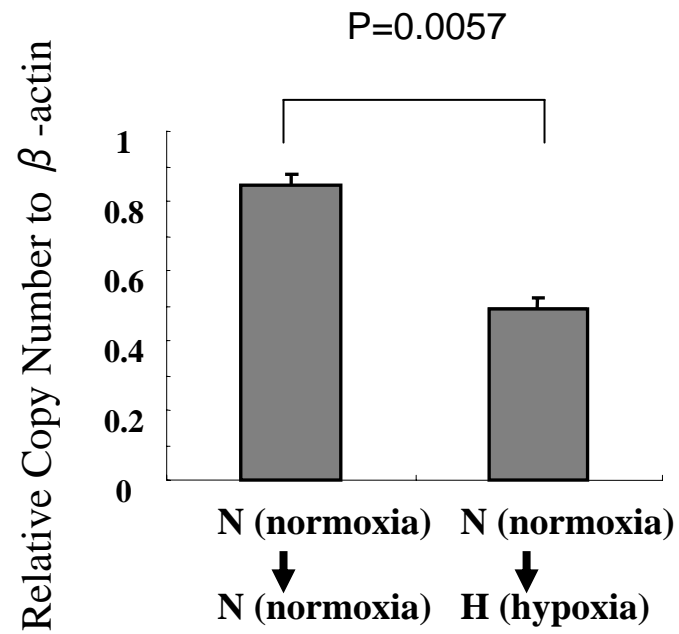


Fig. 3b

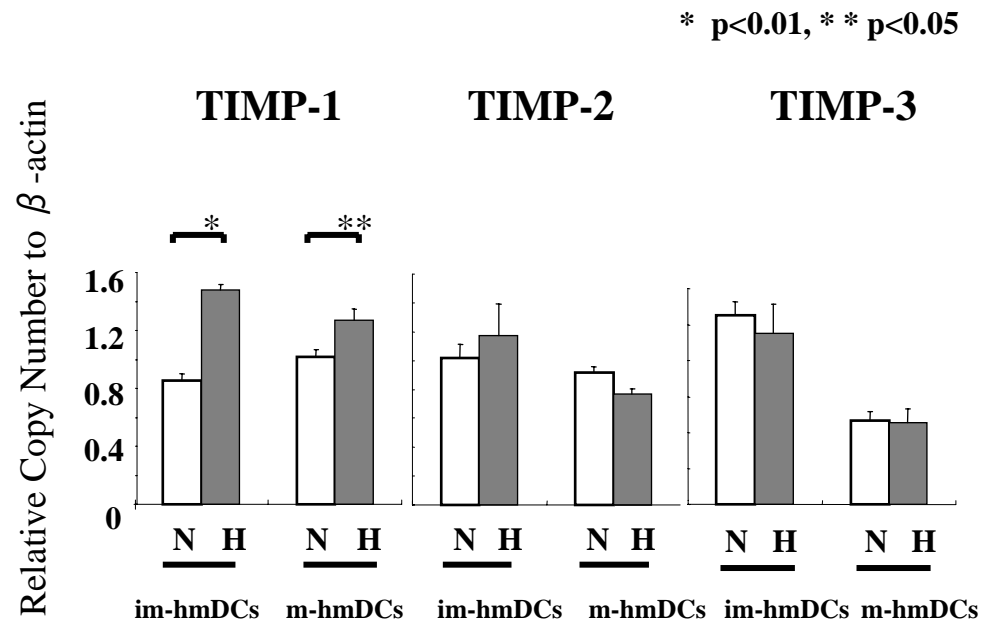


Fig. 4

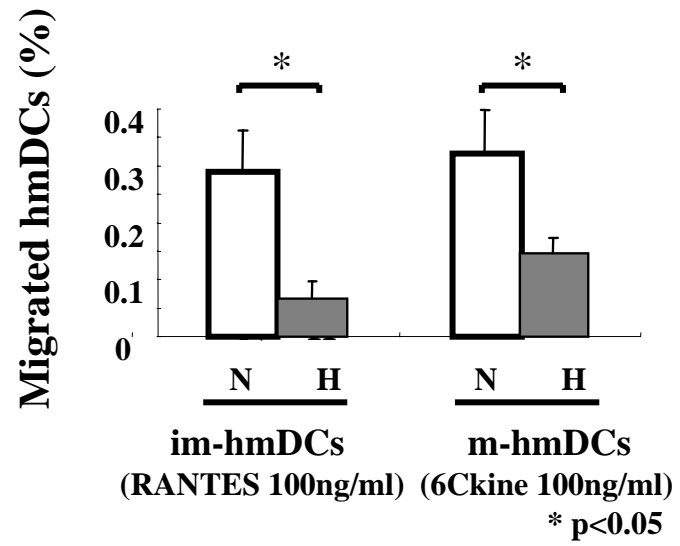
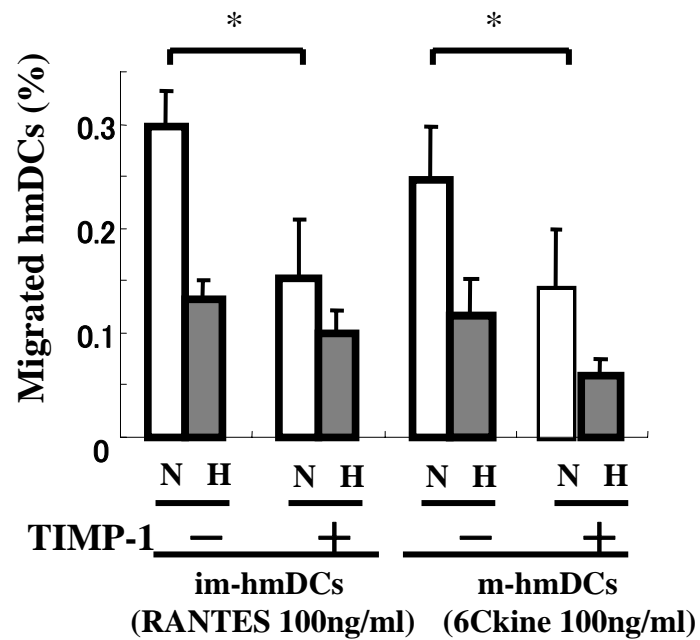


Fig. 5a



\* p<0.05

Fig. 5b

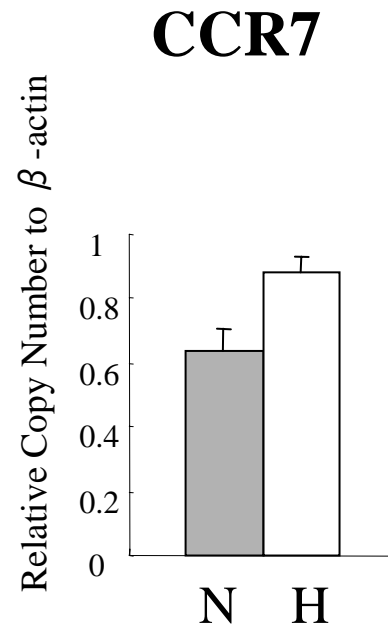
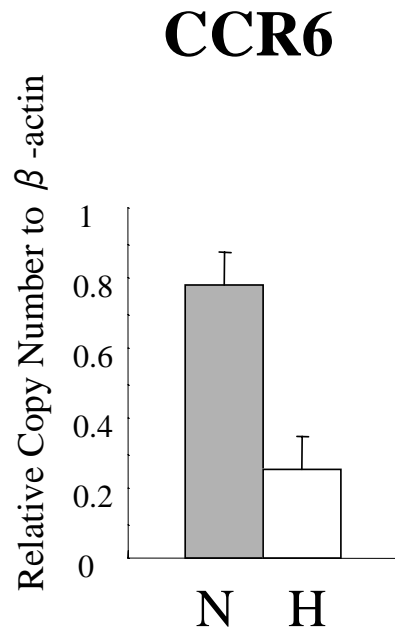
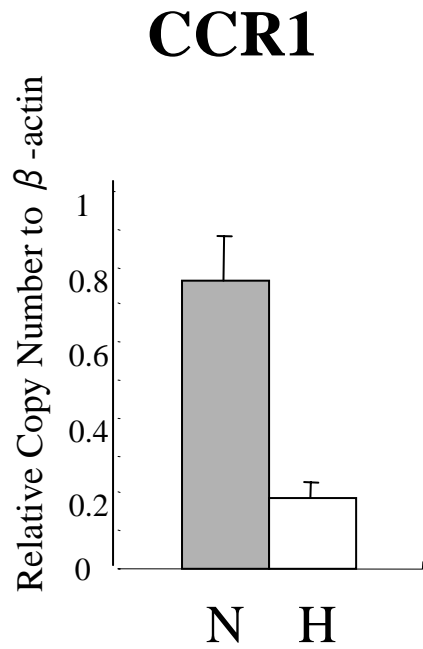


Fig. 5c

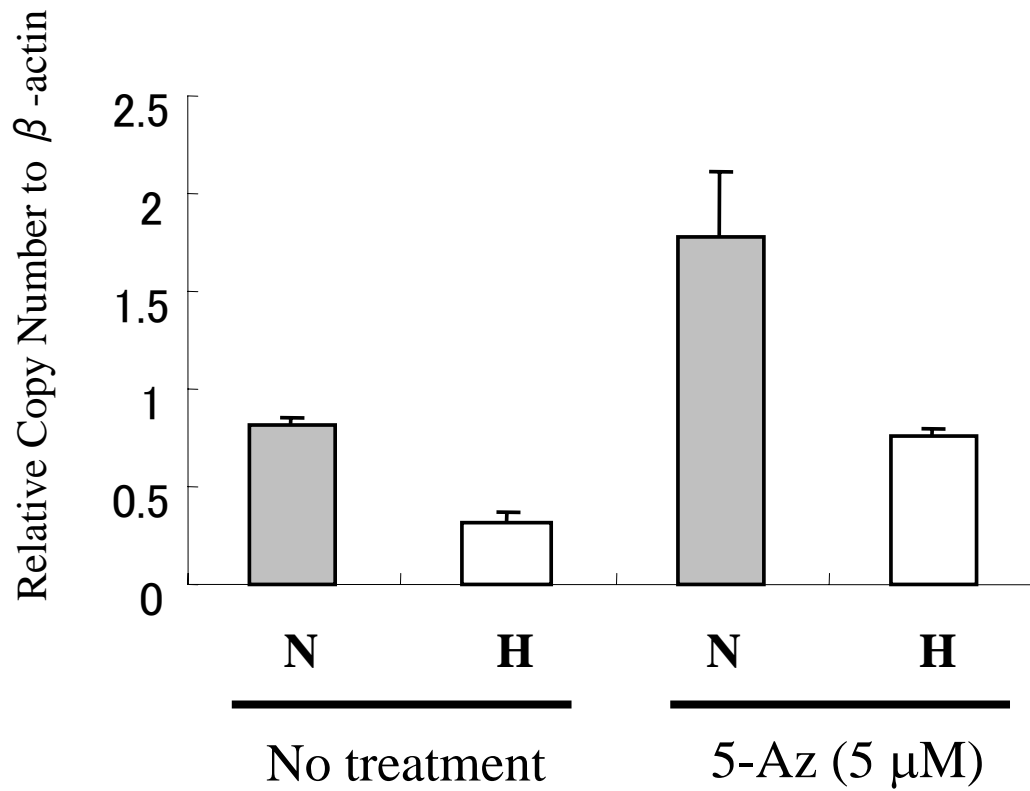


Fig. 6a



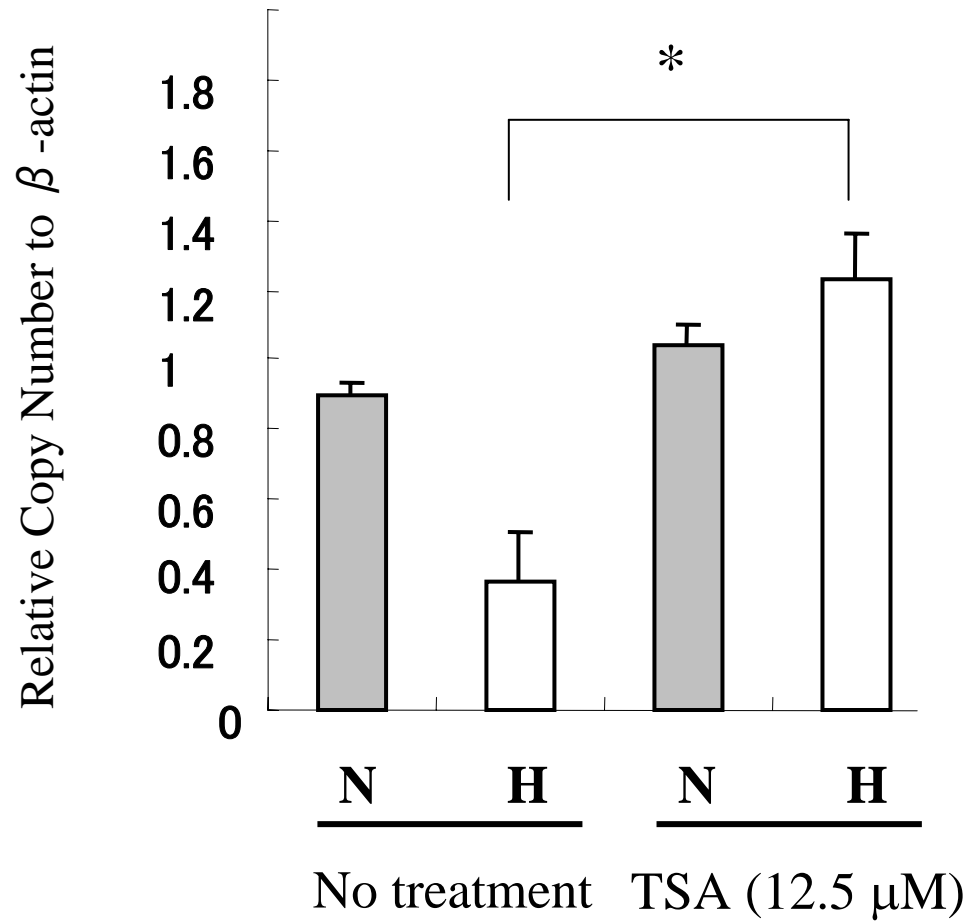


Fig. 6c