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Effect of Reishi polysaccharides on human stem/progenitor cells

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ABSTRACT

The polysaccharide fraction of *Ganoderma lucidum* (F3) was found to benefit our health in many ways by influencing the activity of tissue stem/progenitor cells. In this study, F3 was found to promote the adipose tissue MSCs' aggregation and chondrosphere formation, with the increase of CAM (N-CAM, I-CAM) expressions and autokine (BMP-2, IL-11, and aggrecan) secretions, in an in vitro chondrogenesis assay. In a stem cell expansion culture, it possesses the thrombopoietin (TPO) and GM-CSF like functions to enhance the survival/renewal abilities of primitive hematopoietic stem/progenitor cells (HSCs). F3 was found to promote the dendrite growth of blood mononuclear cells (MNCs) and the expression of cell adhesion molecules in the formation of immature dendritic cells (DC). On the other hand, F3 exhibited inhibitory effects on blood endothelial progenitor (EPC) colony formation, with concomitant reduction of cell surface endoglin (CD105) and vascular endothelial growth factor receptor-3 (VEGFR-3) marker expressions, in the presence of angiogenic factors. A further cytokine array analysis revealed that F3 indeed inhibited the angiogenin synthesis and enhanced IL-1, MCP-1, MIP-1, RANTES, and GRO productions in the blood EPC derivation culture. Collectively, we have demonstrated that the polysaccharide fraction of *G. lucidum* F3 exhibits cytokine and chemokine like functions which are beneficial to human tissue stem/progenitor cells by modulating their CAM expressions and biological activities. These findings provide us a better the observation that F3 glycopolysaccharides indeed possesses anti-angiogenic and immune-modulating functions and promotes hematopoietic stem/progenitor cell homing for better human tissue protection, reducing disease progression and health.

1. Introduction

Somatic tissue stem/progenitor cells are preserved for replenishing the damaged and senescent tissue cells and for maintaining the homeostasis of healthy tissues due to their regenerative potential. Molecules that affect tissue stem/progenitor cells may thus have implications in regenerative medicine, aging, and cancer therapy. Glycoconjugates on cell surfaces also play an important role in stem cell homing, mobilizing, and transforming property.[1–7,12] Glycoconjugate compositions on cell surface adhesion molecules (CAMs) have been shown to play a key role in modulating embryonic development, aging, and cancer progression. The content of N-CAM polysialic acid (PolySia) changes dramatically during embryonic development and human breast cancer progression.[8] Changes in the glycosylation pattern of N-CAM may affect high-order structures and influence the cell–cell and cell–matrix interactive activities of neural cells.[8,9] The N-CAM glycopolysaccharide deficiency or dis-regulation has been found in various cancer metastasis (Table 1).

Reishi F3, a water soluble glycoconjugate fraction of *Ganoderma lucidum*, has been shown to contain polysaccharides that responsible to stimulating cell growth and a variety of cytokine expression, especially IL-1, Il-2, and INF-gamma for immuno-modulating, anti-tumorous activities of spleen cells.[10,11]

The polysaccharide fraction from *G. lucidum* has also been reported to enhance human myeloid and lymphoid cell activity[17] and promote immune cell function and anti-tumor activity.[13–17] It could alter cell immunophenotypic expression and enhance $CD56^+$ NK-cell cytotoxicity in cord blood.[17]

In this study we have studied the effect of this polysaccharide fraction (F3) on human mesenchymal and hematopoietic tissue stem/progenitor cells in an attempt to gain a better insight into its role in human tissue health, disease progression, and aging.

2. Materials and methods

2.1. F3 preparation

The polysaccharide fraction F3 was prepared by the methods previously described.[10,11,17] Briefly, the crude Reishi extract

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Table 1
Types of N-CAM associated with tumor metastasis

Tumor Type	N-CAM	V-CAM	I-CAM	Reference
Breast Cancer 4	DisReg. [CD56, C16]			29
Oral Cancer (SACC)	Dec	Inc	Inc	30
Lymph angiogenesis	Dec [induces Inc VEGFs]			31
Ewing tumor	DisReg			32
Prostate cancer	Unknown [Inc in neuron]			33
Neuroblastoma	Dec			34
Ovarian carcinoma	Lost			35
Adenoid cystic carcinoma cells	Dec			36
Melanoma	Dec			37
Rectal cancer	Dec		Inc	38
Lung cancer [small cell]	Inc			39
Bile duct Cancer.	Positive			40
Colorectal tumor	Dec		Inc	41
Glioma [BT4C & BT4Cn]	Dec			27

(100 g) was dissolved in 3 l of double distilled water, stirred at 4 °C for 24 h, and centrifuged for 1 h to remove the insoluble. The resulting solution was concentrated at 35 °C to give a small volume and lyophilized to generate 70 g of dark-brown powder, 2.5 g of which were dissolved in a small volume of Tris buffer (pH 7.0, 0.1 N), and purified by gel filtration chromatography using a Sephacryl S-500 column (95 × 2.6 cm) with 0.1 N Tris buffer (pH 7.0) as the eluent. The flow rate was set at 0.6 ml/min, and 7.5 ml per tube was collected. After the chromatography, each fraction was subjected to phenol–sulfuric acid test to detect sugar components. Five fractions were collected (fractions 1–5) and combined, dialyzed to remove excessive salt and lyophilized to give 450 mg of F3.[10] This polysaccharide fraction is mainly composed of beta 1,3-glucans and alpha 1,4-mannans as backbones with fucosyl glycans as side chains.[10,11,17]

2.2. Isolation of blood mononuclear cells (MNCs)

MNCs of adult peripheral and umbilical cord blood were obtained from Taipei Blood Donation Center and Taipei Municipal Wan-Fang Medical Center, following the institutional IRB guidelines. MNCs were isolated from the buffy-coat layer by density

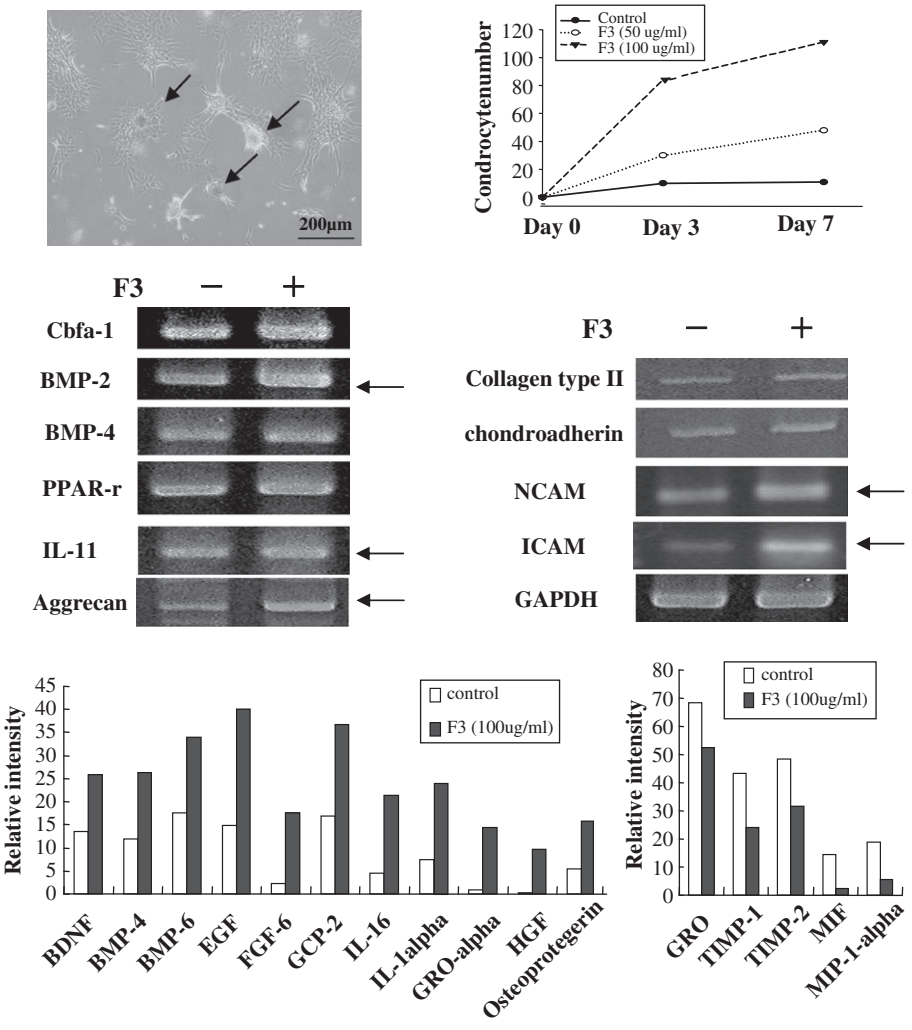
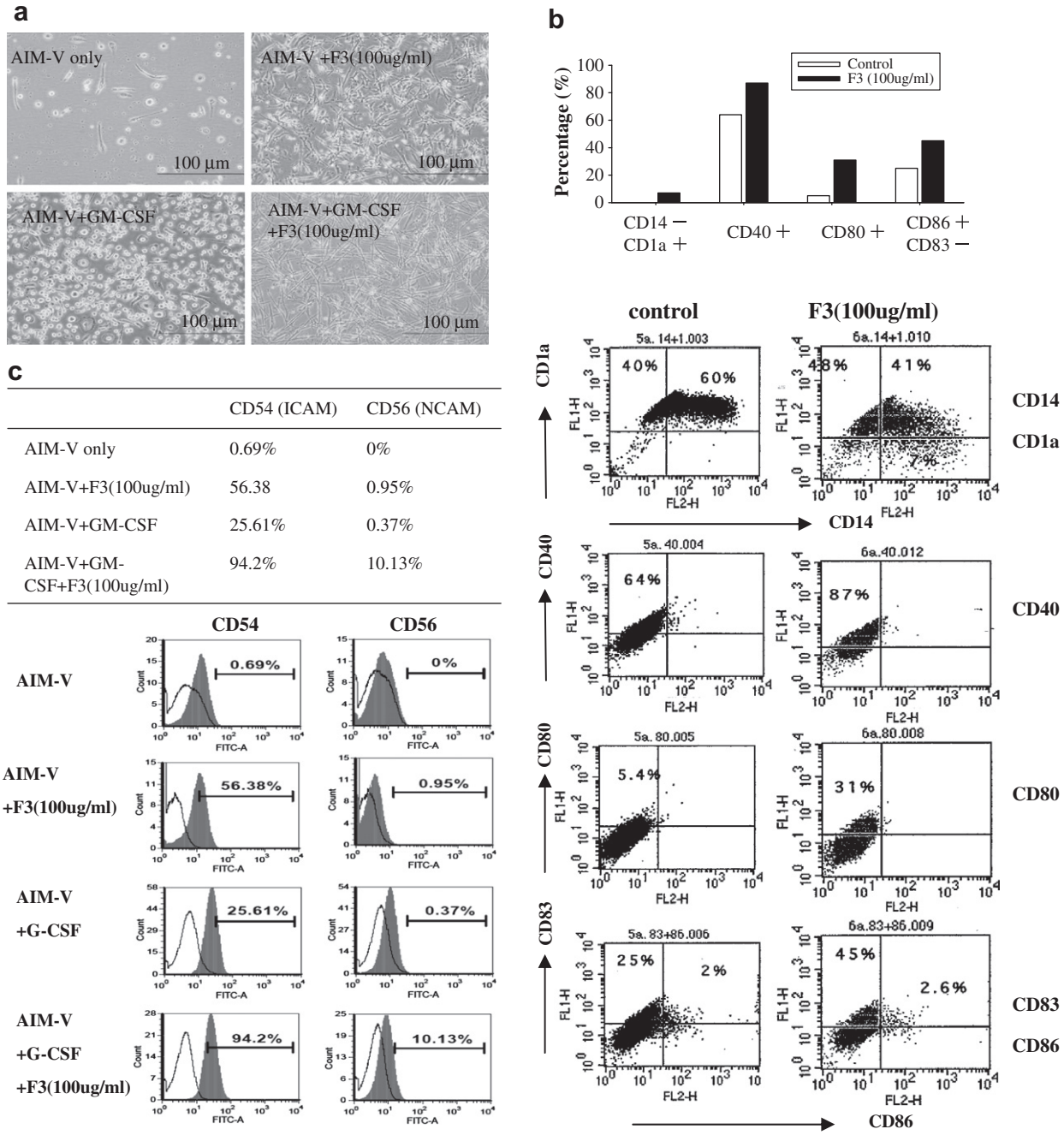


Figure 1. F3 influences the condrosphere formation, CAM gene expression, and protein synthesis of Ad-MSCs. F3 accelerates mesenchymal chondrosphere formation in the course of in vitro chondrogenesis. It accelerates cell aggregation, and forms the chondrospheres, as shown in marked arrows (upper panel). The middle panel shows the RT-PCR results on the influence of chondrosphere formation with increased gene expressions of BMP-2, IL-11, aggrecan, N-CAM, and I-CAM by F3 stimulation. The lower panel shows the protein array data indicating that F3 enhances the synthesis of proliferating growth factors and inhibiting several chemokine secretions of ad-MSCs, in the chondrogenesis culture.

gradient centrifugation, following the standard procedure. Briefly, each blood unit was diluted 1:1 (v/v) with phosphate-buffered saline in 2 mM EDTA (PBS/EDTA), and carefully loaded onto the Ficoll-Hypaque solution (GE Healthcare). After density gradient centrifugation at 2000 rpm for 40 min at room temperature, MNCs were removed from the interface and washed two to three times with PBS/EDTA.

2.3. Adipose MSC isolation

The isolation of Ad-MSCs from freshly prepared individual fat tissues were proceeded by the standard protocol reported previously[18] with some modifications. Briefly, the tissues were washed three to four times with PBS and suspended in an equal volume of PBS (37 °C) (supplemented with 1% bovine serum and 0.1% collagenase type 1 (Sigma)) for 20 min in a shaker incubator. After centrifugation at 400*g* for 10 min, the pellets were treated with RBC lysis buffer (0.2% Tris base, 0.75% NH_4Cl, pH 7.4) for 10 min at room temperature. Suspended cells were passed through a 100 μm cell sieve (Becton Dickinson). The collected cells were seeded at the density of 1 × 10E5 in each non-coating 10-cm culture plate (Falcon) containing 6–7 ml of DMEM (Gibco) low-glucose basal medium supplemented with 10% FBS (Hyclone) to grow the cells to 75% confluent, before harvesting the Ad-MSCs.



	CD54 (ICAM)	CD56 (NCAM)
AIM-V only	0.69%	0%
AIM-V+F3(100ug/ml)	56.38	0.95%
AIM-V+GM-CSF	25.61%	0.37%
AIM-V+GM-CSF+F3(100ug/ml)	94.2%	10.13%

Figure 2. F3 affects blood MNCs on dendritic cell (DC) formation in culture. (a) F3 significantly increased the attachment of MNCs, and induced morphology changes in the culture, regardless the presence of GM-CSF. (b) The immature surface markers (CD1a, CD40, CD80) increased by F3 treatment. (c) Over expression of adhesion molecules, N-CAM (>20 times) and I-CAM (>3 times).

All protocols were reviewed and approved by the IRB committee of Taipei Medical University. Liposuction aspirates from subcutaneous adipose tissue site were obtained from donors with a signed consent.

2.4. MSC chondrogenic differentiation

To initiate the chondrogenic differentiation, Ad-MSCs were seeded with a higher cell density of $2 \times 10^5/10$ μl in a DMEM low-glucose medium (Gibco) supplemented with 1% FBS (Hyclone), 6.25 μg/ml insulin (Sigma), 10 ng/ml TGF-β (R&D) and 50 nM ascorbate-2-phosphate (Sigma). After 3 days and 7 days, chondrospheres were observed and examined by microscopy and detected by Alician blue stain.

2.5. Monocytic dendritic cell derivation

2×10^7 MNCs were plated in 25 cm^2 flasks in 4 ml RPMI or AIM-V (Gibco) basal medium. After 1 h of incubation at 37 °C in a humidified atmosphere containing 5% carbon dioxide, non-adherent cells were removed, and the remaining adherent cells were further cultured in RPMI or AIM-V (Gibco) medium supplemented with human cytokine IL-4 (50 ng/ml) and GM-CSF (50 ng/ml) with/without F3 for 7 days.

2.6. Circulation endothelial progenitor cell (cEPCs) derivation

The derivation of cEPCs from freshly prepared individual MNCs were preceded by the standard protocol reported previously.[19] Briefly, 2×10^7 adult PB-MNCs were plated on a 6-well culture plate (pre-coating with 0.1% gelatin) in 1.5 ml of EBM-2 (Clone Tech) medium, supplemented with VEGF-1, FGF-2, EGF, IGF-1, and ascorbic acid. After 1 h incubation at 37 °C in 5%CO_2 and humidified atmosphere, non-adherent cells were removed. The remaining adherent cells were further cultured in a condition medium of endothelium cells. PB-MNCs derived cEPCs were grown into colony like units, consisting of multiple thin, flat cells emerging from a cluster of round cells by day 4 and harvested on the 7th day as described by Hill et al.[20].

2.7. Endothelium tube forming assay

Endothelium cell tube formation assay was performed with Matrigel basement membrane matrix (BD Biosciences) according to the manufacture's instruction. Briefly, AD-MSCs derived endothelial cells were mixed with EGM-2 (Cambrex) media in the presence of 2 mM VEGF. Capillary tube-like formation was checked every 2 h by phase contrast microscope.

2.8. Ex vivo expansion of hematopoietic stem/progenitor cells

$CD34^+$ cells were enriched from cord blood MNCs by magnetic activated cell sorting (MACs) with direct CD34 progenitor cell isolation kit (Miltenyi Biotech). The purity of the cell population was analyzed by flow cytometry. Briefly, Isolated $CD34^+$ cells were cultured at a density of 2×10^4 cells/ml for expansion in IMDM (Gibco) containing 20% FBS (Hyclone) and a cytokine (R&D) cocktail of 20 ng/ml rhTPO, 50 ng/ml rhSCF, 10 ng/ml rhIL-3, 10 ng/ml rhIL-6, and 50 ng/ml Flt-3 ligand. $CD34^+$ HSCs with or without addition of F3 were cultured in a water-saturated atmosphere with 5% CO_2 for 7 days.

2.9. Flow cytometric analysis

Cells were analyzed according to the instructions of Calibur flow cytometry manufacture (Becton Dickinson). Briefly, an aliquot of 1×10^5 cells harvested from culture dish were labeled with fluorescent isothiocyanate- (FITC) or phycoerythrin-conjugated (PE) monoclonal antibodies in 100 μl phosphate buffer for 15 min at room temperature. Cell surface markers (CD1a, CD40, CD80, CD83, CD86) and hematopoietic lineage markers (CD34, CD38, CD133, CXCR4) were analyzed and quantified.

2.10. Reverse-transcription polymerase chain reaction (RT-PCR) analysis

Total cellular RNA was isolated using RNeasy® Total RNA isolation kit (Qiagen) and cDNA was synthesized using the SuperScript™ First-strand Synthesis System (Life Technologies). Specific genes were amplified by PCR reaction using Fast-Run Taq Master Kit (Protech Technology). The primer sequences used for gene amplification were shown in the list below.

Gene name	Sequence	Annealing temperature (°C)	Product size (bp)
Aggrecan	(s)GCCTTGAGCAGTTCACCTTC (a)CTCTTCTACGGGGACAGCAG	57	372
BMP-2	(s)ACTTTTGGACACCAGGTTGG (a)AGCCACAATCCAGTCATTCC	60	379
BMP-4	(s)GAATGCTGATGGTCGTTTTATTA (a)GACGGCACTCTTGCTAGGCT	60	217
CBFA-1	(s)TGCCTGCCTGGGATCTGTAA (a)GGACGAGGCAAGAGTTTCAC	60	305
Chondro-adherin	(s)ACCTGGACCACAACAAGGTC (a)CACCTTCTCCAGGTTGGTGT	57	388
Collagen type II	(s)GAACATCACCTACCACTGCAAG (a)GCAGAGTCCTAGAGTGACTGAG	57	387
GAPDH	(s)AATCCCATCACCATCTTCCA (a)TGTGGTCATGAGTCCTTCCA	57	318
I-CAM	(s)ACCCAGTGAGGCCTTATTCC (a)TGATCACTGCAGGAAACTGG	60	221
IL-11	(s)ATGAACTGTGTTTGCCGCCTG (a)GAGCTGTAGAGCTCCCAGTGC	61	228
N-CAM	(s)CTCGGCCTTTGTGTTTCCAG (a)TGGCAGGAGATGCCAAAGAT	60	341
PPARγ	(s)TTCAGAAATGCCTTGCAGTG (a)CACCTCTTTGCTCTGCTCCT	60	332
VCAM	(s)ATGAGGGGACCACATCTACG (a)ATCTCCAGCCTGTCAAATGG	60	232

2.11. Human cytokine array analysis

Human cytokine antibody arrays (C series 1000) were used to analyze cytokines according to the manufacturer's instructions (RayBiotech, Atlanta, GA). Briefly, the cell cultured condition medium was used to hybridize with the cytokine antibody array membrane, followed by addition of biotin-antibody cocktail. HRP-conjugated streptavidin was then used for detection of signals (enhanced chemiluminescence, ECL). The signal intensity of tested sample spots was determined by one-dimensional image densitometry analysis software (Kodak Scientific Imaging) and Raybio antibody array analysis tool (RayBiotech). The relative intensities of signals were normalized against the positive controls on each array membrane. Culture medium containing FBS was also used as a control experiment to provide basal signals and exclude possible cross-reactions in the cytokine array studies.

2.12. Real-time PCR array

The cultured cellular RNA was isolated using RNeasy® Total RNA isolation kit (Qiagen) and cDNA was synthesized using the

SuperScript™ First-strand Synthesis System (Life Technologies). The cDNA was then added to the RT^2 SYBR Green qPCR Master Mix (SABiosciences). Real-time PCR was then performed on each sample using the RT^2 Profiler™ PCR Array kit for analysis of human angiogenesis and human tumor metastasis (SABiosciences). All real-time PCR reactions were done by ABI 7300 system, with the following cycling conditions: an initial denaturation at 95 °C 15 min, and 40 cycles of 95 °C 15 s, 60 °C 1 min. To analyze the real-time PCR array data, an MS-Excel sheet with macros was followed by the manufacturer's instruction at website (http://www.sabiosciences.com/pcrarraydataanalysis.php).

3. Results

3.1. F3 influences the chondrogenic differentiation potential and CAM expressions of adipose mesenchymal stem/stromal cells (Ad-MSCs)

When Ad-MSCs were subjected to chondrogenesis in the presence of F3, it was found that F3 (i) accelerates and enhances the chondrosphere formation, (ii) enhances the transcriptional expression of N-CAM, I-CAM, IL-11 (VLA-4), BMP-2, and aggrecan, and the translational expression of cell surface protein makers N-CAM (CD56) and I-CAM (CD54), and (iii) induces the secretion of proliferative chemokines, for example, GRO-a, IL-16, and GCP-2 examined by a cytokine array analysis (Fig. 1). The enhanced and accelerated chondrosphere formation by F3 can be caused by the increased intercellular interactions involving I-CAM, aggrecan, and BMP. These results implied that F3 is likely targeting to the initiation of MSC skeletal condensation.

3.2. F3 effects human monocytic dendritic cells (DCs) derivation

The effectiveness of DC derivation in cancer immune therapy heavily relies on the ex vivo transformation of blood MNCs into active DCs in culture. Adjuvants and co-stimulatory factors are the important sources of MNCs for the presentation of tumor antigen(s) and activation of the immune system. By using the RPMI and AIM-V culture systems, we tested the influence of F3 on the blood monocytic DC transformation. Our results showed that F3 significantly increased the adhesive property of blood mononuclear cells (MNCs) in the culture dish, and induced changes in cell morphology in the AIM-V culture medium, regardless the presence of DC activating cytokines. Figure 2a exemplified that F3 significantly induced the dendrite growth of the adherent blood MNCs in AIM-V culture, regardless the presence of GM-CSF. Further examining the influence of F3 on the CAM expression of the adherent MNCs, we found that F3 preferentially enhances the expression of adhesion molecules [e.g., CD54 (I-CAM) and CD56 (N-CAM)]. Figure 2c indicates the enhancement in cell adhesive molecular glycosylation of monocytes by F3, under the co-stimulations of GM-CSF and IL-4. The increased cell adhesion with enhanced CAM secretion and morphologic changes of the cultured cells were related to the increased expression of immature DC markers (CD14-CD1a+, CD40+, CD80+, and CD83-CD86+), as demonstrated by FACS analysis (Fig. 2b). The DC activating cytokine, GM-CSF, has also been known as an antimicrobial agent that may enhance the innate immune defense. In a cytokine array analysis of the DC culture medium, we found that F3 caused a significant decrease in the secretion of angiogenin, which has been known as an innate immune suppressor, and increase in the secretion of the innate immune defensive chemokines such as MIP-1, MCP-1, and GRO (Fig. 3). The data from this study indicate that, under the AIM-V culture condition, F3 exhibits GM-CSF like activity and effective adjuvant function to produce the relatively immature DC subset for better antigen presentation.

3.3. F3 effects human monocytic endothelial angiogenesis and lymph angiogenesis

Normal wound healing vasculogenesis is regulated by well balanced angiogenic and angiostatic factors. In this study, both EPC colony formation assay in liquid culture and matrix gel endothelium cell (EC) tube formation analysis have been used to probe the influence of F3 on monocytic angiogenesis. Our study showed that F3 exhibited little influence on MSC derived EC tube formation. However, it significantly diminished the potential of adult peripheral blood MNCs (PB-MNCs) derived circulatory EPC colony formation (Fig. 4a). Interestingly, in the presence of F3, the cultured cells showed a decrease in the expression of both angiogenic Flt-4 (VEGFR-3) and endoglin (CD105) molecules (Fig. 4b), in the presence of angiogenic growth factors, VEGF-1 and IGF. Both Flt-4 (VEGFR-3) and CD105 are involved in the initiation of neo-vascular angiogenesis and lymph-angiogenesis.[21,22] A real-time PCR

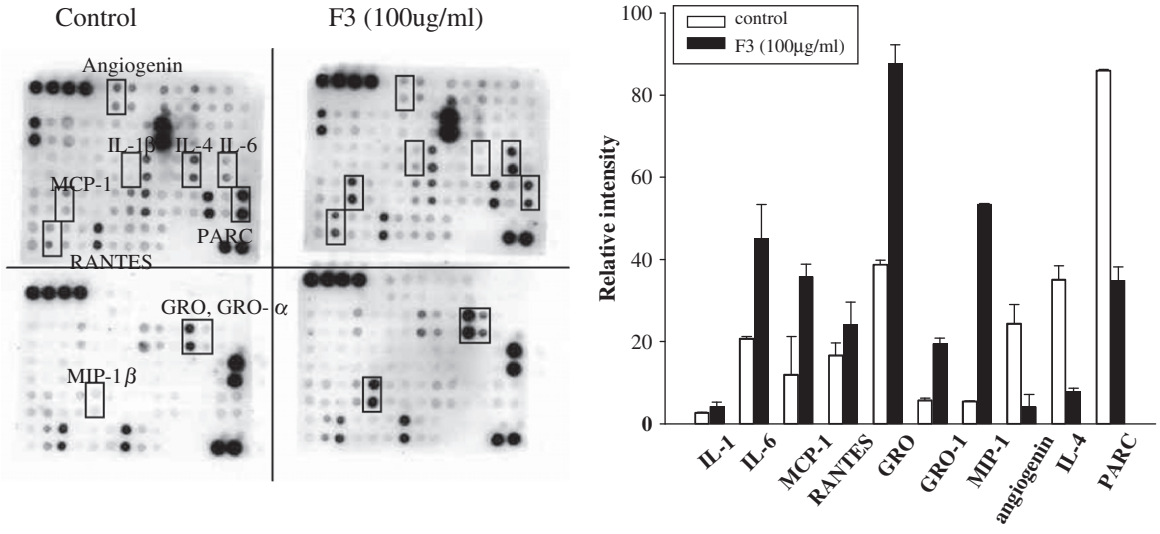


Figure 3. F3 influences human blood MNC cytokine secretion. Under co-stimulation of GM-CSF and IL-4 to MNCs, F3 influences the DCs induction in culture significantly by the reducing secretion of angiogenin and PARC, and the increasing immune chemokines synthesis of IL-6, GRO, MIP-1, MCP-1, and RANTES, show in a cytokine array analysis.

array analysis of the cultured cells further verified the down regulation of angiogenic factors (e.g., FLt-1, KDR, VEGFR-3, and CD105) as shown in Table 2. In a cytokine array analysis we further demonstrated that F3 indeed inhibited the angiogenin synthesis and enhanced the immune defensive IL-1, MCP-1, MIP-1, RANTES, and GRO chemokine productions in the blood EPC derivation culture (Fig. 4c).

3.4. F3 enhances the primitive HSCs populations at undifferentiated state in expansion culture

Despite numerous studies on the effect of cytokines on HSCs in vitro, the exact signals that govern cell renewal of undifferentiated HSCs are still not fully understood. Multiple lines of evidence indicate that thrombopoietin (TPO) contributes to the

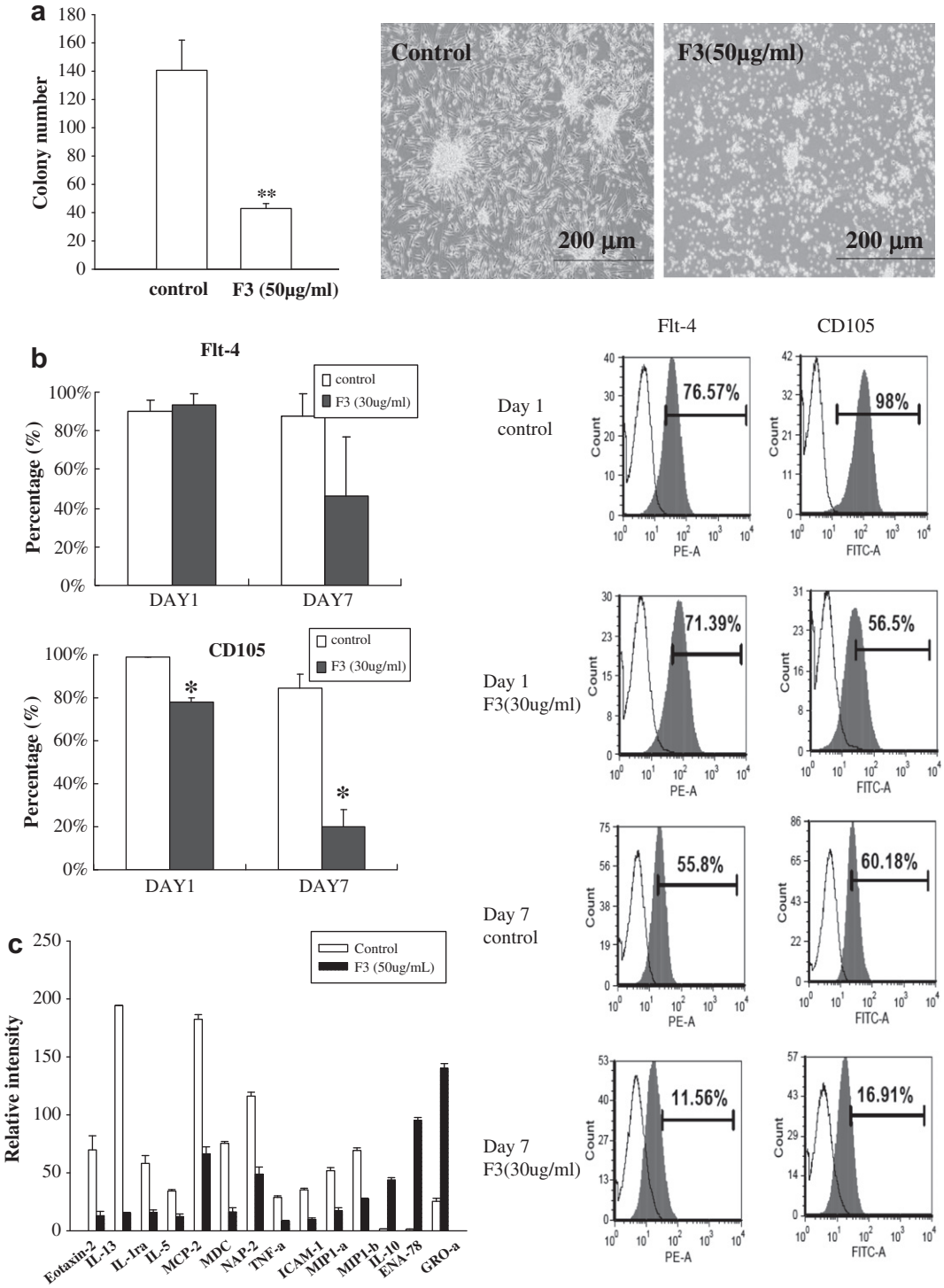


Figure 4. F3 inhibits the EPC colony formation by altering the protein secretion and reducing the angiogenic cell surface marker expression. F3 reduces the blood EPCs colony formation in culture and showing (a). F3 inhibits the expression of Flt-4 (VEGFR-3) and endoglin (CD105) expressions, analyzed by FACS (b). F3 was shown to up regulate IL-10, ENA-78, and GRO-a and down regulate Eotaxin-2, IL-13, IL-1ra, MCP-2, MDC, NAP-2, TNF-a, and I-CAM-1 secretions, analyzed by the cytokine array analysis (c).

transplantation of HSCs by supporting their cell survival and proliferation in vitro and enhances the engraftment of transplanted cells into marrow, in vivo. Accordingly, we have examined the influence of F3 on human umbilical cord blood hematopoietic stem/progenitor cells regarding their survival and proliferation in a five-cytokine cocktail liquid medium. The result showed that in the presence of F3, the primitive CD38− and CD133+ HSC subpopulation of CD34+ HSCs in the culture was kept from differentiation and thus useful for the purpose of ex vivo expansion (Fig. 5a). In a BrdU assay, it was shown that the reduction of S phase population with increase in the G0/G1 population is consistent with the increased quiescent primitive HSC cell marker expressions (data not shown). It has been showed that TPO is associated with the increase in surviving before entering the G1 phase of CD34+ HSCs in order to maintain their survival in the undifferentiated state.[23,24] In a cytokine replacement culture study, we further demonstrated that F3 indeed exhibits the TPO like function (Fig. 5b), maintains the (CD38−, CD133+, and CXCR4+) primitive subpopulations (Fig. 5c). Consistently, F3 was shown to promote the proliferation of the long-term repopulating (side-population) CD34+ HSCs in culture (Supplementary data Fig. B).

4. Discussion

G. lucidum (GI) and their extract products have been widely used for many years for health promotion. Previous studies on F3 have shown its role in promoting immune cell function and antitumor activity.[10,11,13–17] In addition, Reishi F3 has also been found to influence the CD34+ hematopoietic stem cell populations of human umbilical cord blood (CB) in culture.[17]

In stem cell transplant therapy, the successful use of neonatal cord blood and G-CSF mobilized adult peripheral blood CD34+ HS/PCs has been hampered by the loss of CXCR4 homing receptor in mobilized adult CD34+ HS/PCs, and the low engraft rate in CB transplantation due to the insufficient CD34+ HS/PCs amount and the low CD56+ natural killer (NK) cells.[25] Various attempts have been made to increase the transplant engraft efficiency by developing G-CSF substitutes and ex vivo expansion of stem cells and NK cells in culture, but little success have been shown to date.

4.1. Reishi F3 benefits ex vivo manipulations of blood CD34+ HS/PCs for transplantation therapy

In this study we have shown that F3 exhibits TPO cytokine like function to retain and possible expand the long-term engrafting primitive HSCs population in culture (Fig. 5 & Supplementary data Fig. B). The result is supportive to the earlier observation that F3 involved in cord blood CD34+ HS/PCs expansion in culture, as reported by Chien.[17] Thus, F3 may potentially benefit to improve the low cell number of umbilical cord blood (CB) and the low engrafting rate of G-CSF mobilized adult peripheral blood (PB) for clinical transplantation application.

4.2. F3 sustains the immunity of NK and immature dendritic cells (DCs) formation

F3 enhanced the expression of blood mononucleated cells adhesion molecules and promoted dendrite growth with increased $CD56^{+}$ NK cells (Fig. 2c), and immature DC cell activities (Fig. 2a and b), indicating that F3 may be used in enhancing the innate immunity and antigen presenting activity of DCs and NK. It also caused a significant decrease in the secretion of innate immune suppressor, such as angiogenin, and increase in the secretion of the innate immune defensive chemokines such as MIP-1, MCP-1, and GRO during the DC derivation culture as an immune booster.

Table 2
Fold of down-regulation or up-regulation caused by F3

Symbol	Description	Fold down-regulation
CXCL9	Chemokine (C-X-C motif) ligand 9	−365.58
IFNB1	Interferon, beta 1, fibroblast	−324.02
CXCL10	Chemokine (C-X-C motif) ligand 10	−261.55
LEP	Leptin	−39.75
IFNA1	Interferon, alpha 1	−27.55
MDK	Midkine (neurite growth-promoting factor 2)	−21.99
TNF	Tumor necrosis factor (TNF superfamily, member 2)	−19.62
FLT1	Fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)	−18.50
FGF1	Fibroblast growth factor 1 (acidic)	−16.00
ANGPT1	Angiopoietin 1	−15.85
FGFR3	Fibroblast growth factor receptor-3 (achondroplasia, thanatophoric dwarfism)	−14.68
TEK	TEK tyrosine kinase, endothelial (venous malformations, multiple cutaneous, and mucosal)	−12.93
PLXDC1	Plexin domain containing 1	−11.59
KDR	Kinase insert domain receptor (a type III receptor tyrosine kinase)	−9.13
TIMP3	TIMP metallopeptidase inhibitor 3 (Sorsby fundus dystrophy, pseudoinflammatory)	−9.02
EPHB4	EPH receptor B4	−7.89
EFNA3	Ephrin-A3	−7.00
ANGPTL4	Angiopoietin-like 4	−6.22
IL6	Interleukin 6 (interferon, beta 2)	−6.40
PLG	Plasminogen	−6.01
EFNA1	Ephrin-A1	−5.76
HAND2	Heart and neural crest derivatives expressed 2	−5.75
Symbol	**Description**	**Fold up-regulation**
PECAM1	Platelet/endothelial cell adhesion molecule (CD31 antigen)	155.90
THBS1	Thrombospondin 1	85.70
CXCL5	Chemokine (C-X-C motif) ligand 5	59.43
HIF1A	Hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	7.76
CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	6.82
PROK2	Prokineticin 2	5.26
EDG1	Endothelial differentiation, sphingolipid G-protein-coupled receptor, 1	5.21
TGFA	Transforming growth factor, alpha	5.09

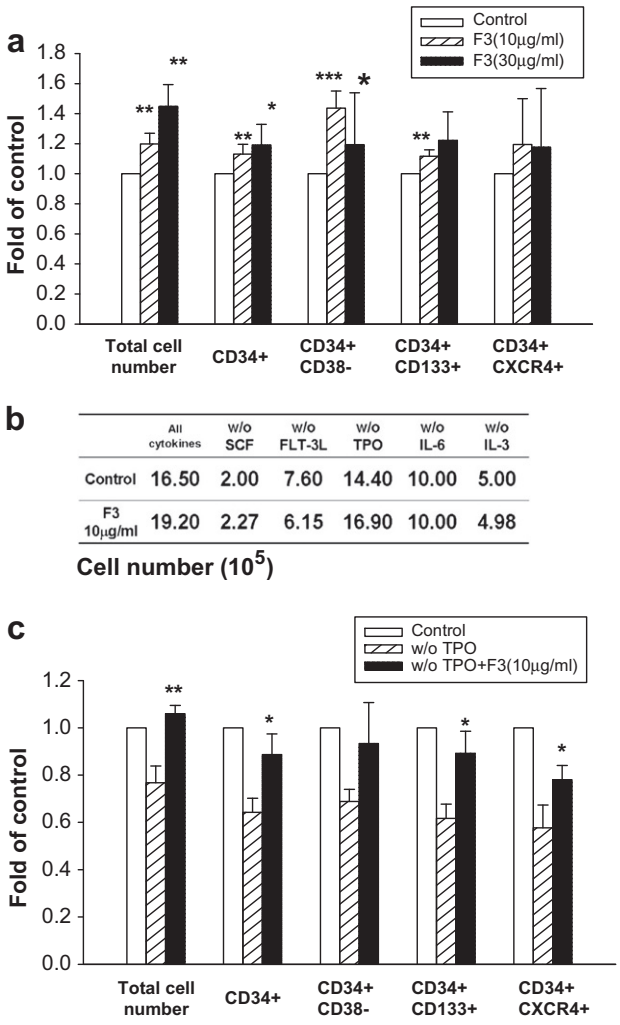


Figure 5. F3 exhibits the TPO cytokine like function to enrich cell growth and sustain the primitive stem cell populations of CD34+ HS/PCs, in culture. (a) F3 maintains the primitive HSC subpopulations (CD38−, CXCR4+, CD133+) in a cytokine cocktail culture with an average increase of 10–15%. (b) F3 exhibits the TPO like function to retain cell survival in the relatively undifferentiated state, in a cytokine replacement experiment. The cytokine cocktail for the cell culture consists of SCF, Flt-3L, TPO, IL-6, and IL-3 cytokines. (c) F3 was found to be equivalent to TPO function in maintaining CD38−, AC133+, and CXCR4+ primitive HSC subpopulations survival and undifferentiated, in a TPO replacement assay. (*, P <0.05; **, P <0.01; ***, P <0.001).

These data suggest that the potential usefulness of the Reishi polysaccharide fraction, F3, in the ex vivo manipulation of blood MNCs for cancer immunotherapeutic supplement in vivo.

4.3. F3 exhibits inhibitory effect on angiogenesis and lymph-angiogenesis

The angiogenin expression is initially in the early pregnant uterine decidual or endothelial progenitor cells (EPCs), not in the late gestational endothelial cells (ECs) vasculogenesis,[26] and the increased CAM expression in tissue cells has been shown to limit the tumor cell metastasis.[27] Table 2 showed that F3 diminished the monocytic endothelial progenitor colony formation in presences of angiogenic factor which is directly reflected to the down regulations of the transcription of tyrosine kinases (FLT1, TEK), kinase receptor (KDR), angiogenic and fibroblast growth factors and receptors (angiopoietin-1, FGF-1, and FGFR-3) for the inhibition of angiogenesis. The reduction of EPC colony formation, thus the angiogenesis by F3 can be further elucidated by the decreased EPC expression of angiogenin, Flt-1, KDR, endoglin (CD105), and VEGFR-3 (Flt-4) shown in the cytokine array protein analysis (Fig. 4). Therefore, it is predictable that the inhibitory effect of F3 on tumor growth and metastasis which may potentially benefit for the cancer metastasis prevention.

Current study has shown that F3 increases CAM expression in both hematopoietic and mesenchymal stem/progenitor cell and inhibits the EPC angiogenic and lymph-angiogenic expressions. Collectively, we have found F3 glycopolysaccharide exhibits cytokine and chemokine like functions which are cellular niche dependent and largely involved in the enrichment of cell–cell interactions, and/or cell–matrix interactions to benefit human tissue stem/progenitor cells by modulating their CAM expressions and biological activities.

Chemical analyzes of F3 conducted by our group have shown a number of linkage domains, for example, β-1,3-D-glucan, β-(1,3)-β-(1,6)-D-glucan, α-1,4-D-mannan, β-1,3-D-glucosyl-β-1,4-D-glucuronan, β-1,4-D-glucuronan and less percentage of other components have been shown in our previous reports[10,11,28] (Supplementary data Fig. A). How Reishi F3 glycopolysaccharides with these linkage domains modulate the glycosylations of human tissue progenitor surface is unclear. We are speculating that the F3 glycoconjugate linkage domains may interact with the CAM associated chemokine and cytokine receptors of human tissue progenitor cells to influence their CAM expression in culture. However, to probe the structural and functional mechanism of F3, further study on separated glycans with well defined structures in vitro and in vivo are needed, and work is in progress to address this question.

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The authors declare no conflict of interest.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.10.016.

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