

## 研究简报

## RhoB分子靶蛋白MYH9的鉴定

唐蓓<sup>1\*</sup> 唐彦<sup>2</sup><sup>1</sup>重庆师范大学生命科学学院, 重庆 401331; <sup>2</sup>云南省中医学院, 昆明 650200

**摘要** 鉴定能与活性RhoB分子结合的靶蛋白。制备GST融合的活性RhoB蛋白(GST-RhoB), 与LPS刺激的DC2.4细胞裂解物混合后实施pull-down实验, 沉降复合物通过SDS-PAGE分析、金染色后, 对与GST-RhoB结合的蛋白条带进行基质辅助激光解吸电离飞行时间质谱(MALDI-TOF-MS)分析。然后制备小鼠的树突状细胞, LPS刺激12 h后, 进行荧光标记的抗体染色。激光共聚焦显微镜下观察RhoB与MYH9的细胞内定位。通过MALDI-TOF质谱分析, 鉴定到一个新的可以与RhoB的活性形式结合的马达分子MYH9。激光共聚焦显微镜的结果表明, RhoB在LPS刺激前后均与MYH9在树突状细胞内共定位。该研究首次发现MYH9可与活性RhoB结合, 可能是RhoB下游的一个靶蛋白。

**关键词** RhoB; 靶蛋白; 鉴定; MYH9

## Identification of Target Protein MYH9 with RhoB

Tang Bei<sup>1\*</sup>, Tang Yan<sup>2</sup><sup>1</sup>College of Life Sciences, Chongqing Normal University, Chongqing 401331, China;<sup>2</sup>Yunnan University of Traditional Chinese Medicine, Kunming 650200, China)

**Abstract** To identify the target molecules associated with active RhoB, GST-fusion protein of the active form of RhoB (GST-RhoB) was prepared and immune-precipitated with LPS-treated DC2.4 cell lysates. The precipitated samples were subjected to SDS-PAGE and then was stained with Colloidal Gold Total Protein Stain. Molecular mass analysis of the protein which was associated with GST-RhoB in the lysates was performed by Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS). With dendritic cells (DCs) which were prepared from mice incubating with or without LPS for 12 hours, the subcellular colocalization of RhoB and its interacting partner were detected by confocal microscopy after labeling with antibody. The results indicated that motor molecule MYH9 was identified as a novel associating protein of active RhoB by MALDI-TOF mass spectrometry. Confocal microscopy analysis revealed that MYH9 colocalized with RhoB in DCs regardless of LPS stimulation. These results suggest that MYH9 is associated with RhoB and it may be a specific target of RhoB.

**Key words** RhoB; target molecules; identification; MYH9

收稿日期: 2012-10-23 接受日期: 2012-12-20

重庆市教委科学技术研究项目(批准号: KJ120603)资助的课题

\*通讯作者。Tel: 023-65910315, E-mail: xiaobt26@yahoo.com.cn

Received: October 23, 2012 Accepted: December 20, 2012

This work was supported by Chongqing Education Department Science and Technology Project (Grant No.KJ120603)

\*Corresponding author. Tel: +86-23-65910315, E-mail: xiaobt26@yahoo.com.cn

网络出版时间: 2013-03-04 14:56 URL: http://www.cnki.net/kcms/detail/31.2035.Q.20130304.1456.001.html

Rho家族蛋白是Ras超家族中最早被克隆出来的蛋白。它们是一组分子量大约为20~25 kDa的三磷酸鸟苷(guanosine triphosphate, GTP)结合蛋白,具有GTP酶活性,因此被称为Rho GTP酶。Ras家族成员B(ras homolog gene family, member B, RhoB)为Rho家族中的成员,通过作用于靶蛋白而产生多种生物学效应,在调节细胞骨架重组、囊泡转运、细胞周期、细胞运动和凋亡等过程中发挥了重要作用<sup>[1-4]</sup>。

RhoB有GTP结合的活性形式和GDP结合的非活性形式两种。鸟苷酸交换因子(guanine nucleotide-exchange factors, GEF)可使Rho蛋白由GDP形式转变为GTP形式。其中鸟苷酸交换因子H1(guanine nucleotide-exchange factors H1, GEFH1)可促进RhoB的活化<sup>[5-6]</sup>,活化的RhoB再通过募集靶蛋白发挥其相应的功能。但RhoB活化后,通过哪些靶蛋白发挥作用尚不清楚。我们在研究中发现,活化的RhoB可与非肌性马达分子重链9(nonmuscle myosin heavy chain 9, MYH9)结合,提示MYH9可能在RhoB介导的囊泡转运中发挥作用。

实验材料: C57BL/6小鼠、DC2.4细胞、pGEX-CA-RhoB(表达RhoB的活性蛋白)和pGEX-6p-3质粒来自大阪大学免疫发生学研究室。RhoB活性分析试剂盒(Upstate公司);金染色液(Colloidal Gold Total Protein Stain)购自BIO-RAD公司;兔抗鼠MYH9多克隆抗体(BAB公司);鼠抗RhoB(c-5)抗体(Santa Cruz公司);BD cytofix/cytoperm™ plus kit(BD公司);Glutathione-Sepharose 4 Fast Flow(GE Healthcare公司)。

细胞培养: DC2.4树突状细胞系的培养采用添加了10%小牛血清的RPMI-1640培养液,每两天换一次液。C57BL/6小鼠骨髓来源树突状细胞(dendritic cells, DCs)的分离和培养参考文献[7],具体过程如下:无菌条件下取小鼠后肢股骨、胫骨和肱骨,用RPMI-1640培养液冲洗骨髓腔制备骨髓悬液,红细胞裂解液去除红细胞后,用RPMI-1640全培养液调细胞密度为 $2 \times 10^6/\text{mL}$ ,加入GM-CSF(1 000 U/mL),混匀,10 mL/皿分装于100 mm培养皿中,于37 °C、5% CO<sub>2</sub>条件下培养。每两天换一次液,去除非贴壁细胞,并补充等剂量的GM-CSF,到第6天时未成熟DCs的纯度可达80%以上。在第6天,加入终浓度为100 ng/mL的脂多糖LPS进行刺激。

## GST pull-down实验

将GST和GST-CA-RhoB融合蛋白表达质粒转化到DH5 $\alpha$ 中,挑取单个转化子接种到3 mL LB培养基中,37 °C培养过夜,然后转到300 mL培养液中37 °C大量培养至菌液D值达到0.20~0.25后,加入IPTG至终浓度为0.1 mmol/L,继续振荡培养6 h。离心收集菌体,用NP-40缓冲液重悬,加PMSF至终浓度为0.1 mmol/L,超声破菌后离心收集上清液。加入适量Glutathione-Sepharose 4 Fast Flow珠子,4 °C旋转孵育1 h,收集Glutathione-Sepharose 4 Fast Flow,充分漂洗后,即得到结合GST和GST-CA-RhoB融合蛋白的Glutathione-Sepharose 4 Fast Flow。收集LPS刺激12 h的DC2.4细胞,用细胞裂解液(NP-40缓冲液,蛋白酶抑制剂)裂解,收取上清,分别加入等量结合于Glutathione-Sepharose 4 Fast Flow的GST、GST-CA-RhoB融合蛋白于4 °C旋转孵育1.5 h,离心收集沉淀, NP-40缓冲液充分漂洗,沉降复合物中加入适量的蛋白质电泳上样缓冲液,进行SDS-PAGE分析。

## MALDI-TOF质谱分析RhoB的结合蛋白

将DC2.4细胞裂解物与GST融合的RhoB活性蛋

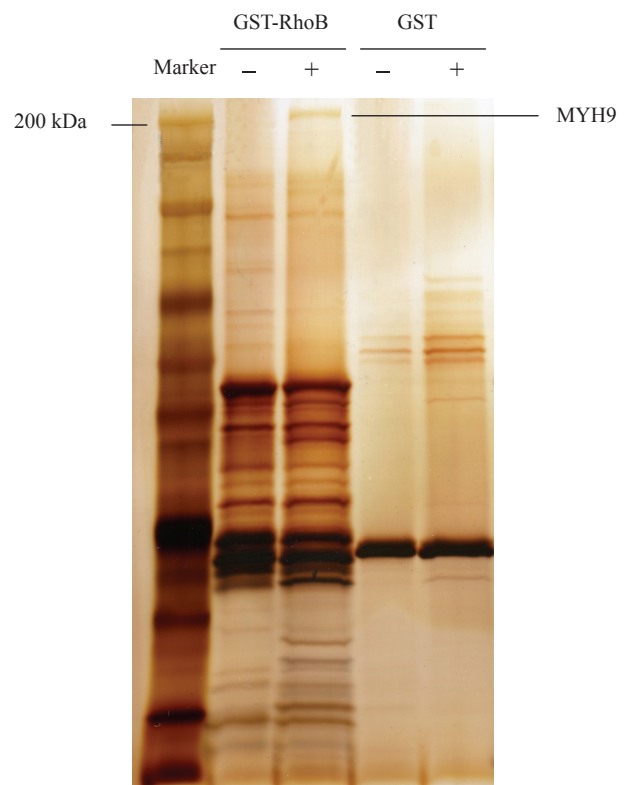


图1 GST pull-down实验结果  
Fig.1 The result of GST pull-down

表1 MALDI-TOF质谱分析结果

Table 1 The results of MALDI-TOF mass spectrometry analysis

头 Head	尾 Tail	观察值 Observed value	理论值 Theoretical value	△值 Delta value	相应序列 Corresponding sequence
1 632	1 638	859.483	859.464	+0.019	[K]NREEAIK[Q]
1 836	1 842	916.541	916.533	+0.008	[K]QVRRTEK[K]
1 042	1 048	930.528	930.501	+0.027	[K]QRQELEK[T]
738	745	962.544	962.517	+0.027	[K]QACVLMIK[A]
396	403	964.520	964.521	-0.001	[K]VGRDYVQK[A]
1 493	1 500	972.553	972.548	+0.005	[K]AELERLNLK[Q]
229	237	1 024.495	1 024.481	+0.014	[K]NDNSSRFQK[F]
1 165	1 173	1 101.631	1 101.627	+0.004	[K]REQEVSILK[K]
802	810	1 119.580	1 119.594	-0.014	[K]RQQQLTAMK[V]
546	555	1 122.599	1 122.591	+0.008	[K]VVQEQGTHPK[F]
408	419	1 305.671	1 305.669	+0.002	[K]EQADFAIEALAK[A]
641	651	1 315.685	1 315.683	+0.002	[K]GMFRTVQGQLYK[E]
1 556	1 566	1 330.729	1 330.752	-0.023	[K]LRLEVNLAQMK[A]
1 393	1 404	1 416.710	1 416.733	-0.023	[K]DLEGLSQRLEEK[V]
1 776	1 788	1 627.810	1 627.826	-0.016	[K]NENARQQLERQNK[E]
1 261	1 274	1 636.822	1 636.829	-0.007	[K]FSEGERVRETELADK[V]
746	760	1 706.890	1 706.908	-0.018	[K]ALELDSNLYRIGQSK[V]
1 220	1 234	1 729.852	1 729.872	-0.020	[K]QTLENERGELANEVK[A]

注: 鉴定为非肌性马达分子重链9蛋白(编号gi|17978023|emb|CAC85955.1)

Note: identification to gi|17978023|emb|CAC85955.1|nonmuscle heavy chain myosin II-A(MYH9)[*Mus musculus*]

## MYH9蛋白序列(匹配肽段用粗体显示)

## MYH9 protein sequence(matched peptides shown in bold)

1	MAQQAADKYL	YVDKNFINNP	LAQADWAACK	LVWVPSKNG	FEPASLKEEV	GEEAIVELVE	NGKKVKVNKD	DIQKMNPPKF
81	SKVEDMAELT	CLNEASVLHN	LKERYYSGLI	YTYSGLFCVV	INPYKNLPIY	SEEIVEMYKG	KKRHEMPPHI	YAITDTAYRS
161	MMQDREDQSI	LCTGESGAGK	TENTKKVIQY	LAHVASSHKS	KKDQGELERQ	LLQANPILEA	FGNAKTVKND	<b>NSSRFQK</b> FIR
241	INFDVNGYIV	GANIETYLLE	KSRAIRQAKE	ERTFHIFYYL	LSGAGEHLKT	DLLLEPYNKY	RFLSNHVTI	PGQDQKDMFQ
321	ETMEAMRIMG	IPEDQMGLL	RVISGLQLG	NIAFKKERNT	DQASMPDNTA	AQKVSHLLGI	NVTDFTRGIL	TPRIK <b>VGRDY</b>
401	<b>VQKAQTKEQA</b>	<b>DFAIALAKA</b>	TYERMFRWL	LRINKALDKT	KRQGASFIGI	LDIAGFEIFD	LNSFEQLCIN	YTNEKLQQLF
481	NHTMFILEQE	EYQREGIEWN	FIDFGLDLQP	CIDLIEKPAG	PPGILALLDE	ECWFPKATDK	SFVEK <b>VVQEQ</b>	<b>GTHPK</b> FQKPK
561	QLKDKADFCI	IHYAGKVQDYK	ADEWLMKNMD	PLNDNIATLL	HQSSDKFVSE	LWKDVDRIG	LDQVAGMSET	ALPGAFKTRK
641	<b>GMFRTVQGLY</b>	<b>KEQLAKLMAT</b>	LRNTNPNFVR	CIIPNHEKKA	GKLDPHLVLD	QLRCNGVLEG	IRICRQGFNP	RVVFQEFRQR
721	YEILTPNSIP	KGFMQDG <b>QAC</b>	<b>VLMIKALELD</b>	<b>SNLYRIGQSK</b>	VFFRAGVLAH	LEEERDLKIT	DVIIGFQACC	RGYLARKAFA
801	<b>KRQQQLTAMK</b>	VLQRNCAAYL	RLRNWQWWRL	FTKVKPLLNS	IRHEDELLAK	EAELTKVREK	HLAAENRLTE	METMQSQLMA
881	EKLQLEQLQ	AETELCAEAE	ELRARLTAKK	QELEEICHDL	EARVEEEER	CQYLQAEKKK	MQQNIQELEE	QLEEEESARQ
961	KLQLEKVTTE	AKLKKLEEDQ	IIMEDQNCKL	AKEKLLLEDR	VAEFTTNLME	EEKSKSLAK	LKNKHEAMIT	DLEERLRREE
1041	<b>KQRQELEKTR</b>	RKLEGDSTDL	SDQIAELQAQ	IAELKMQLAK	KEEELQAALA	RVEEEAAQKN	MALKKIRELE	TQISELQEDL
1121	ESERASRNKA	EKQKRDGEE	LEALKTELED	TLDSTAAQQE	LR <b>SKREQEVS</b>	<b>ILKKTLEDEA</b>	KTHEAQIQEM	RQKHSQAVEE
1201	LADQLEQTKR	VKATLEKAK <b>Q</b>	<b>TLENERGELA</b>	<b>NEVK</b> ALLQGK	GDSEHKRKKV	EAQLQELQVK	<b>FSEGERVTE</b>	<b>LADK</b> VTKLQV
1281	ELDSVTGLLS	QSDSKSSKLT	KDFSALESQ	QDTQELLQEE	NRQKLSLSTK	LKQMEDEKNS	FREQLEEEEEE	AKRNLEKQIA
1361	TLHAQVDTMK	KKMEDGVGCL	ETAEEAKRRL	<b>QKDLEGLSQR</b>	<b>LEEK</b> VAAAYDK	LEKTKTRLQQ	ELDDLVDLD	HQRQSVSNLE
1441	KKQKQKFDQLL	AEEKTISAKY	AEERDRAEAE	AREKETKALS	LARALEEAME	<b>QKAELERLNLK</b>	QFRTEMEDLM	SSKDDVQKSV
1521	HELEKSKRAL	EQQVEEMKQ	LEEELEDELQA	TEDAK <b>LRLEV</b>	<b>NLQAMKAQFE</b>	RDLQGRDEQS	EEKKQLVRQ	VREMAELED
1601	ERKQRSMAMA	ARKKLEMDLK	DLEAHIDTAN	<b>KNREEAIKQL</b>	RKLQAQMKDC	MRELDDTRAS	REEILAQAKE	NEKKLSMEA
1681	EMIQLEELA	AAERAKRQAQ	QERDELADEI	ANSSGKGALA	LEEKRRLEAR	IALLEEELEE	EQGNTELIND	RLKKANLQID
1761	QINTDLNLER	SHAQKNENAR	<b>QQLERQNKEL</b>	KAKLQEMESA	VKSKYKASIA	ALEAKIAQLE	EQLDNETKER	QAASK <b>QVRRT</b>
1841	<b>EKKLKDVLQ</b>	VEDERRNAEQ	FKDQADKAST	RLKQLKRQLE	EAEEEAQRAN	ASRRKLQREL	EDATETADAM	NREVSSLKKN
1921	LRRGDLPFVV	TRRIVRKGTG	DCSDEEVDGK	ADGADAKAAE				

白进行蛋白沉降实验,取10  $\mu$ L沉降复合物进行12% SDS-PAGE,并将蛋白质转至PVDF膜,用TTBS缓冲液洗膜后金染色(Colloidal Gold Total Protein Stain),风干后,对比样品的差异条带进行基质辅助激光解吸电离飞行时间质谱(Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry, MALDI-TOF-MS)分析,根据其肽质量指纹图谱进行数据库查询。

沉淀产物与单纯GST融合的RhoB活性蛋白比较,并以GST沉降的DC2.4细胞裂解物为对照,经SDS-PAGE实验金染色后,在200 kDa处发现一条差异条带(图1)。对差异条带进行了MALDI-TOF质谱分析,分析结果见表1,根据肽质量指纹图谱进行数据库查询后,鉴定出差异常条带为MYH9蛋白,这说明活化的RhoB可与MYH9结合。

### 激光共聚焦显微镜观察RhoB与MYH9的细胞内定位

培养小鼠骨髓来源的DC,在第6天用LPS刺激12 h,细胞经PBS清洗后用4%多聚甲醛室温下固定20 min, PBS清洗3次,然后用1 $\times$ perm/wash缓冲液室温作用20 min, PBS清洗后,一抗用1:250兔抗鼠MYH9多克隆抗体、1:25鼠抗RhoB单克隆抗体室温下孵育1 h, PBS清洗3次后,二抗用Alexa Fluor-594标记的羊抗兔IgG和Alexa Fluor-488标记的羊抗鼠

IgG室温下孵育30 min, PBS洗涤后,用Fluorescent mounting封片,共聚焦显微镜63 $\times$ /1.4油浸物镜下观察,并摄取图像。用波长488 nm的激发光观察FITC标记的绿色荧光, 594 nm的激发光观察Alexa Fluor标记的红色荧光。

细胞经固定,用荧光标记的抗RhoB、MYH9的抗体染色后,在激光共聚焦显微镜下RhoB显示为绿色荧光, MYH9显示为红色荧光,结果如图2所示。如图中箭头所示,无论LPS刺激前还是刺激后,MYH9都与RhoB共定位。

近年来,分子生物学技术的快速发展为寻找目标分子作用的靶蛋白提供了重要手段,其中以生物质谱、生物信息学和蛋白质复合物亲和纯化等为主要研究方法的亲和蛋白质组学是有效而常用的研究手段。本研究利用GST沉降技术,以带GST标签的RhoB活性蛋白为“诱饵蛋白”,捕获了与之相互作用的蛋白质,经MALDI-TOF质谱分析和数据库查询,鉴定出该蛋白质为马达分子MYH9。

RhoB作为小G蛋白,发挥其生物学功能是通过从GDP结合的非活性形式转化为GTP结合的活性形式,然后再与靶蛋白结合而实现的。RhoB参与了很多生物学过程,如细胞骨架重组、囊泡转运、细胞运动等,通过pull-down实验,我们发现,MYH9可以与RhoB的活性形式结合。接着我们用LPS刺激DCs,以激活RhoB所在的信号转导通路LPS-TLR4...

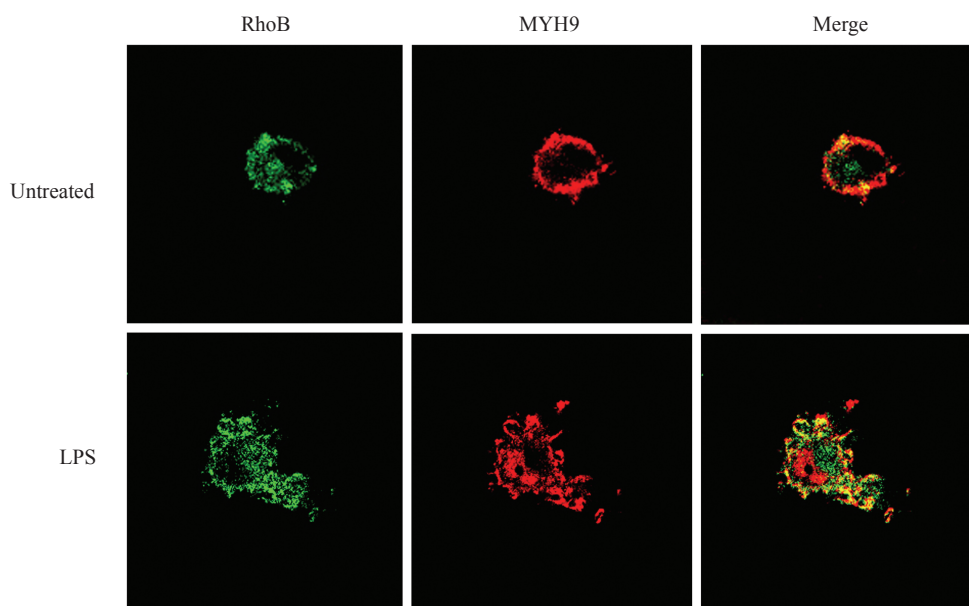


图2 MYH9与RhoB在DC中共定位

Fig.2 MYH9 co-localized with RhoB in DC



RhoB(信号传导途径由LPS传给TLR4, 再通过中间的一些蛋白传递给RhoB), 从而使RhoB转化为活性形式。通过激光共聚焦显微镜观察, 发现在LPS刺激前后RhoB都与MYH9在DC细胞中共定位。在LPS刺激后RhoB转化为活性形式因此与MYH9共定位, 但为何在LPS刺激前的RhoB也可与MYH9共定位呢? 通过查阅文献, 发现在LPS刺激前, DC细胞中就存在着GTP结合的活性RhoB, 在LPS刺激后, 活性RhoB的量增加, 并在刺激3 h时达到最大<sup>[6]</sup>。激光共聚焦显微镜的实验结果进一步表明RhoB与MYH9有关, 提示MYH9可能是RhoB募集的一种靶蛋白。

MYH9编码220 kDa的传统肌球蛋白重链, 既是构成细胞骨架、肌球蛋白和质膜等的成分, 也是一种马达分子, 在细胞运动、细胞连接、细胞骨架重组、肌动蛋白介导的移动以及蛋白转运中发挥着重要作用<sup>[8-10]</sup>。该基因若发生突变, 还可引起称为MYH9综合征的遗传性疾病<sup>[11]</sup>。由于肌球蛋白作为马达分子驱动由肌动蛋白介导的胞内转运, MYH9可与RhoB的活性形式结合, 提示其可能作为RhoB下游的一种马达分子, 驱动由RhoB介导的囊泡转运。RhoB分子参与很多蛋白质的胞内转运, MYH9可能是RhoB下游的靶蛋白这一结果可为研究这些蛋白的转运过程及信号转导途径等提供研究思路。

### 参考文献 (References)

- 1 Huang M, Prendergast GC. RhoB in cancer suppression. *Histol Histopathol* 2006; 21(2): 213-8.
- 2 Huang M, Satchell L, Duhadaway JB, Prendergast GC, Laury-Kleintop LD. RhoB links PDGF signaling to cell migration by coordinating activation and localization of Cdc42 and Rac. *J Cell Biochem* 2011; 112(6): 1572-84.
- 3 Barberan S, McNair K, Iqbal K, Smith NC, Prendergast GC, Stone TW, *et al.* Altered apoptotic responses in neurons lacking RhoB GTPase. *Eur J Neurosci* 2011; 34(11): 1737-46.
- 4 Huang M, Duhadaway JB, Prendergast GC, Laury-Kleintop LD. RhoB regulates PDGFR-beta trafficking and signaling in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 2007; 27(12): 2597-605.
- 5 Ren Y, Li R, Zheng Y, Busch H. Cloning and characterization of GEF-H1, a microtubule-associated guanine nucleotide exchange factor for Rac and Rho GTPases. *J Biol Chem* 1998; 273(52): 34954-60.
- 6 Kamon H, Kawabe T, Kitamura H, Lee J, Kamimura D, Kaisho T, *et al.* TRIF-GEFH1-RhoB pathway is involved in MHCII expression on dendritic cells that is critical for CD4 T-cell activation. *EMBO J* 2006; 25(17): 4108-19.
- 7 Park SJ, Nakagawa T, Kitamura H, Atsumi T, Kamon H, Sawa S, *et al.* IL-6 regulates *in vivo* dendritic cell differentiation through STAT3 activation. *J Immunol* 2004; 173(6): 3844.
- 8 Jacobelli J, Chmura SA, Buxton DB, Davis MM, Krummel MF. A single class II myosin modulates T cell motility and stopping, but not synapse formation. *Nat Immunol* 2004; 5(5): 531-8.
- 9 Takeda K, Kishi H, Ma X, Yu ZX, Adelstein RS. Ablation and mutation of nonmuscle myosin heavy chain II-B results in a defect in cardiac myocyte cytokinesis. *Circ Res* 2003; 93(4): 330-7.
- 10 Conti MA, Even-Ram S, Liu C, Yamada KM, Adelstein RS. Defects in cell adhesion and the visceral endoderm following ablation of nonmuscle myosin heavy chain II-A in mice. *J Biol Chem* 2004; 279(40): 41263-6.
- 11 Zhang Y, Conti MA, Malide D, Dong F, Wang A, Shmist YA, *et al.* Mouse models of MYH9-related disease: Mutations in nonmuscle myosin II-A. *Blood* 2012; 119(1): 238-50.