Protein Cell 2012, 3(10): 762–768
DOI 10.1007/s13238-012-2058-x

Protein & Cell

COMMUNICATION

Suppression of GSK3β by ERK mediates lipopolysaccharide induced cell migration in macrophage through β-catenin signaling

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Received June 10, 2012 Accepted August 27, 2012

ABSTRACT

We investigate the role of β-catenin signaling in the response of macrophage to lipopolysaccharide (LPS) using RAW264.7 cells. LPS rapidly stimulated cytosolic β-catenin accumulation. β-catenin-mediated transcription was showed to be required for LPS induced gene expression and cell migration. Mechanically, ERK activation-primed GSK3β inactivation by Akt was demonstrated to mediate the LPS induced β-catenin accumulation. Overall, our findings suggest that suppression of GSK3β by ERK stimulates β-catenin signaling therefore contributes to LPS induced cell migration in macrophage activation.

KEYWORDS LPS, ERK, GSK3β, β-catenin, cell migration

INTRODUCTION

Bacterial lipopolysaccharide (LPS) triggers a series of signaling events in innate immune responses. LPS binds to LBP and membrane-bounded CD14 and associates with Toll-like receptor 4 (TLR4) (Poltorak et al., 1998; Means et al., 1999; Lien et al., 2000). CD14 mediated LPS induced activation of MEK/ERK signaling (Guha and Mackman, 2001). As the central recognition protein in the LPS receptor complex, TLR4 causes recruitment of MyD88, IRAK, and TRAF6 to the receptor where TRAF6 is catalyzed synthesis of K63-linked polyubiquitin chains and subsequently activates NF-κB (Lomaga et al., 1999; Carpenter and O'Neill, 2009).

In contact with bacterial infection, immune cells such as macrophage play essential role to initiate defensive responses, including releasing a large number of inflammation mediators and activating a number of signaling cascades. Wnt pathway is one of the signaling cascades in macrophages. As a family of secreted proteins, Wnt regulate cell proliferation, migration and differentiation (Logan and Nusse, 2004; Moon et al., 2004; Reya and Clevers, 2005; MacDonald et al., 2009; Zhang and Ma, 2010). One of the major components of the canonical Wnt-induced signaling is β-catenin, which is rapidly degraded by the proteasome system due to phosphorylation by the Adenomatous polyposis coli (APC)-Axin-glycogen synthase kinase (GSK) 3β complex (Liu et al., 2002; Nelson and Nusse, 2004). Blocking GSK3β kinase activity, such as phosphorylation by AKT (Cross et al., 1995), leads to the cytosolic accumulation of β-catenin that freely enters into the nucleus and triggers target gene activation by displacing transcriptional repressors from DNA-bound LEF/TCF (Behrens et al., 1996; Brunner et al., 1997). The present work describes the role of ERK-mediated activation of canonical Wnt/β-catenin signaling and its role in LPS induced macrophage activation.

RESULTS

LPS stimulation induces β-catenin accumulation and promotes cell migration in macrophage

We investigated Wnt/β-catenin signaling in LPS-treated cells by western blotting and q-PCR analysis. Exposure of RAW264.7 cells to LPS markedly increased total and cytosolic β-catenin in a time course dependent manner (Fig. 1A). The β-catenin induction pattern is negative correlated with GSK3β kinase activity and positively correlated with AKT and

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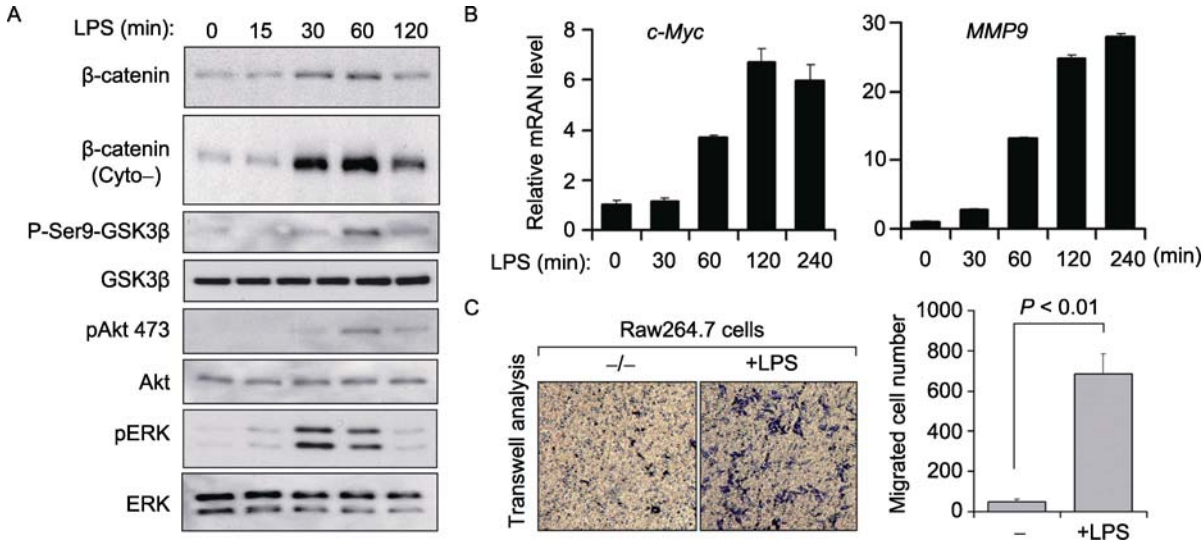

Figure 1. LPS stimulation induces β-catenin accumulation and promotes cell migration in macrophage. (A) Immunoblot analysis in RAW264.7 cells treated with LPS (100 ng/mL) as indicated. (B) qPCR analysis of *c-Myc* and *MMP9* expressions in LPS stimulated RAW264.7 cells as indicated. (C) LPS (100 ng/mL) promotes RAW264.7 cell migration using transwell migration essay. Migrated cell number was quantified in the right panel.

ERK activations by LPS (Fig. 1A). Q-PCR analysis showed that LPS induced expression of β-catenin direct target genes *c-Myc* and *MMP9* (Fig. 1B). Consistent with the *MMP9* induction, functionally as measured in transwell migration assay, LPS sharply potentiated macrophage cell motility (Fig. 1C), the characteristic that enables infiltration from blood vessels and cell migration for antigens, contributing strategically to the defending inflammation process.

Blocking β-catenin mediated transcription impairs LPS induced *MMP9* expression and reduces cell migration

To test the role of β-catenin-mediated transcription in LPS induced gene expression and macrophage migration. Dominant negative LEF-1(dnLEF-1) was introduced into RAW cells to specifically block β-catenin mediated transcription. As shown in Fig. 2A, dnLEF-1 inhibited both the basal and LPS induced *c-Myc* and *MMP9* gene expressions. Consistently in the measurement of cell motility, dnLEF-1 significantly reduced LPS induced macrophage migration (Fig. 2B). Similar effects were also observed by using specific siRNA against β-catenin (Fig. 2). These results indicate the requirement of β-catenin mediated transcription for LPS induced macrophage activation. Consistently, treatment of GSKβ kinase inhibitor SB216763 or LiCl increased β-catenin accumulation and elevated macrophage cell migration (Fig. 2C and data not shown).

Activation of both ERK and AKT are required in LPS-induced β-catenin accumulation.

Immunoblotting of Phospho-ERK (p-ERK) and phosphor-AKT 473 (p-AKT 473) has been shown to be correlated with LPS triggered β-catenin accumulation, suggesting that activation of ERK and AKT pathways may be involved in this process. To test this hypothesis, ERK and AKT kinase inhibitors were employed to uncover the mechanism underlying. As shown in Fig. 3A, treatment of RAW cells with either ERK inhibitor or Akt inhibitor, (PD95059 and Ly294002 respectively) can partially block β-catenin accumulation by LPS. Combined treatment of both inhibitors almost completely blocked LPS stimulated β-catenin accumulation, an observation similar with infection of constitutive active GSK3β mutant (S9A) (Fig. 3B). Thus both ERK and AKT pathways are involved in β-catenin accumulation by LPS.

Activated ERK associates with and primes GSK3β inactivation by AKT in response to LPS

Combined together, AKT activation (as viewed by pAkt 473) mediated GSK3β inactivation (as viewed by pSer9 GSK3β) is likely to be responsible for LPS induced β-catenin accumulation. Thus we investigate the role of ERK. Since ectopic tagged ERK and GSK3β could interact with each other in HEK293T cells (data not shown), we hypothesized that endogenous ERK may regulate GSK3β activity via association. We first performed co-immunoprecipitation assays to test whether ERK can interact with GSK3β in macrophage cells. The endogenous ERK in RAW cells could indeed be co-precipitated with endogenous GSK3β (Fig. 4A). Notably, LPS induced p-ERK associated with GSK3β and increased the level of GSK3β phosphorylation in Ser9 (Fig. 4A). Reversely, endogenous GSK3β was also found in p-ERK im-

munoprecipitation in 30 minutes treatment point of LPS (Fig. 4B). These results suggested that LPS induced activation of ERK which may subsequently facilitate GSK3β inactivation by AKT. To consolidate this, we examined in ERK2 overxpressed RAW cells the GSK3β activity and its downstream β-catenin expression. As shown in Fig. 4C, GSK3β is inactivated and β-catenin is indeed accumulated in cytosolic fraction of ERK overexpressed macrophage cells. Taken together, ERK associates with GSK3β and primes GSK3β inactivation in LPS treated macrophages.

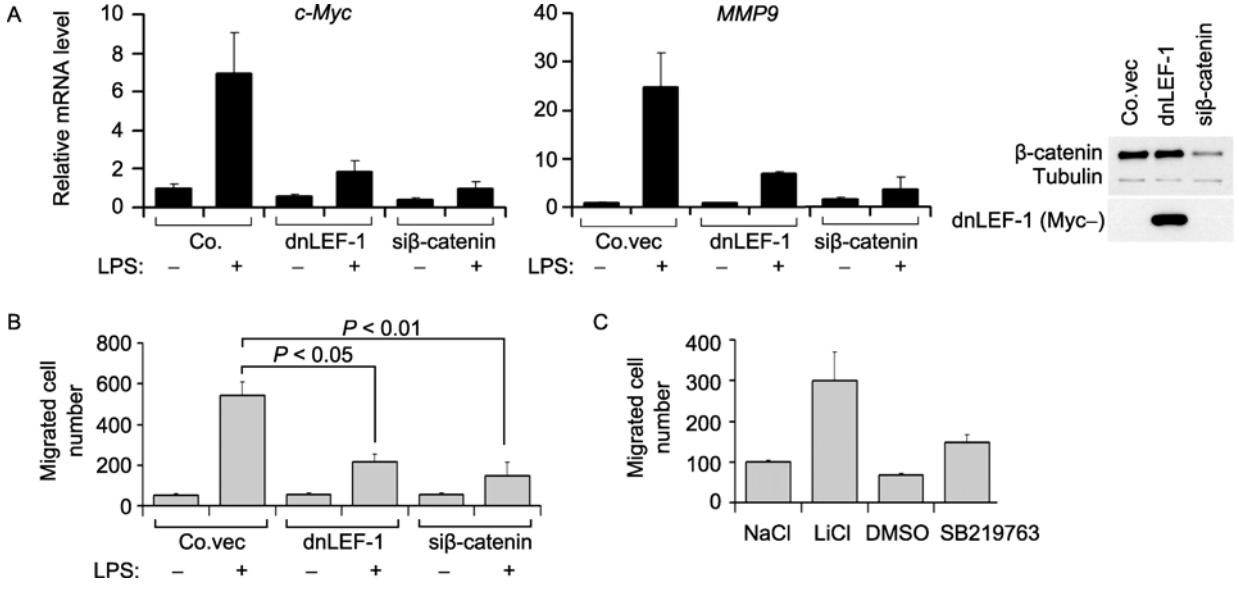

Figure 2. Ectopic expression of dominant negative LEF-1 impairs LPS induced *c-Myc* and *MMP9* expression and reduces cell migration. (A and B) Control (Co.vector and Co.siRNA) and dnLEF-1 or siβ-catenin transfected RAW264.7 cells were treated with or without LPS (100 ng/mL) for 4 h as indicated, cells were harvested for qPCR analysis (A) or employed for transwell migration essay (B). Expression of Myc tagged dominant negative LEF-1 (dnLEF-1) and β-catenin knocking down effects was shown in Fig. 2A, right panel. (C) Transwell analysis of RAW264.7 cell migration in the presence or absence of GSK3β kinase inhibitor SB216763 (10 μmol/L) or LiCl (10 mmol/L). Migrated cell number was quantified from 5 random fields.

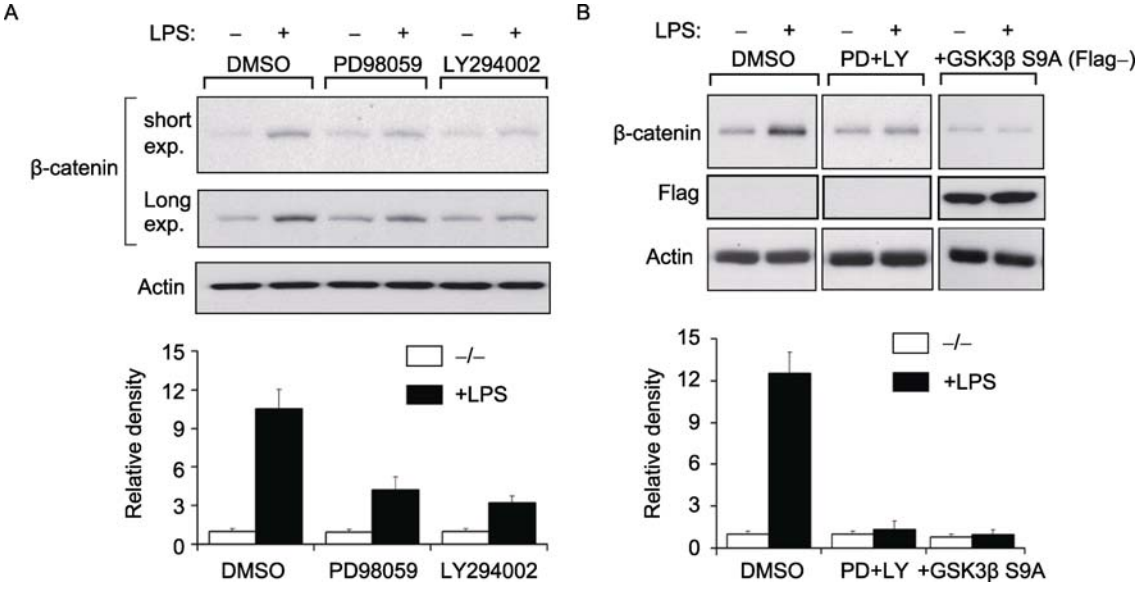

Figure 3. Activation of Erk and Akt are involved in LPS-induced β-catenin accumulation. (A) Control DMSO, PD98059 (10 μmol/L) or LY294002 (10 μmol/L) pretreated RAW264.7 cells were stimulated with or without LPS for 1 hour. Cells were harvest for immunoblottings. Statistically analysis was shown below (n = 3). (B) RAW264.7 cells were treated with control DMSO, combination of PD98059 (10 μmol/L) and LY294002 (10 μmol/L), or infected with Flag-GSK3β S9A lenti-virus. Cells were stimulated with or without LPS for 1 h and harvested for immunoblot analysis. Statistically analysis was shown below (n = 3).

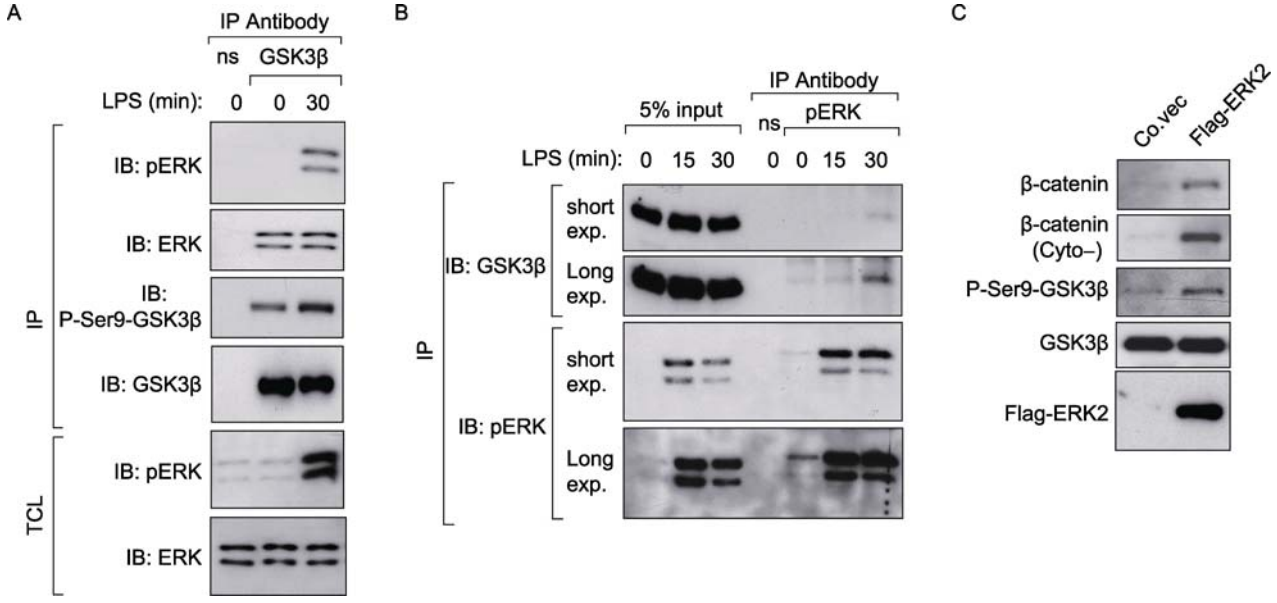

Figure 4. Erk associates with and primes GSK3β inactivation by Akt. (A) In the presence or absence of LPS (100 ng/mL) treatment, RAW264.7 cells were harvested for anti-GSK3β or control anti-none specific (*ns*) immunoprecipitation (*IP*) and immunoblottings (*IB*) as indicated. (B) Control and LPS stimulated RAW264.7 cells were harvested for anti-phosphor-ERK immunoprecipitation and immunoblottings as indicated. (C) Immunoblot analysis of Flag-ERK2 overexpressed RAW264.7 cells compared with control.

ERK activated β-catenin pathway is required for LPS induced *MMP9* expression and cell migration in macrophage

To prove the possible role of ERK-β-catenin cascades in LPS induced macrophage cell characteristics such as downstream gene activation and cell migratory capacity. We treated RAW cells with ERK inhibitor PD98059 and examined LPS induced *MMP9* expression and cell migration. Q-PCR analysis showed that PD98059 treatment significantly suppressed LPS induced *MMP9* expression and blocked RAW cell migration (Fig. 5A and 5B). These effects were clearly rescued by ectopic expression of constitutive active β-catenin mutant (SY) (Fig 5A–C). Taken together, these results showed that ERK activated β-catenin pathway is required for LPS induced *MMP9* expression and cell migration in macrophage.

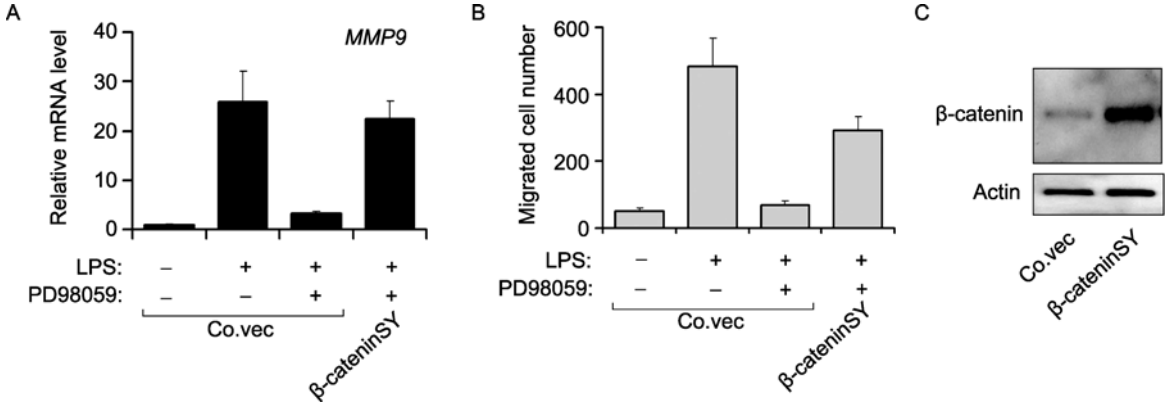

Figure 5. LPS induced *MMP9* expression and cell migration is inhibited by ERK kinase inhibitor and rescued by ectopic β-catenin SY expression. (A and B) Control vector (Co.vec) and β-catenin SY infected RAW264.7 cells were treated with LPS or PD98059 (10 μmol/L) as indicated, cells were harvested for qPCR analysis (A) or cell migration essay (B). (C) β-catenin expression was examined by immunoblotting.

DISCUSSION

As a signaling pathway with function in many aspects including cell proliferation, migration and differentiation, the Wnt/β-catenin pathway is tightly controlled by regulators at multiple steps, the amount of (cytosolic) β-catenin being the most decisive response parameter. In this study, we demonstrated that LPS rapidly induced β-catenin accumulation in macrophage. We confirmed the activation of this pathway by examining its downstream direct target genes, such as *c-Myc* and *MMP9*. As one of the MMPs, MMP9 is released by macrophage to degrade Collagen, Elastin, Fibronectin and other extracellular matrix components, which is important for migration. To elucidate the requirement of β-catenin in LPS induced effect, we specifically blocked β-catenin mediated transcription by expressing dnLEF-1 or siβ-catenin in macrophages. This indeed impaired LPS induced *MMP9* expressin and macrophage motility.

We next examined the mechanism underlying. Dickkopf-1 conditioned medium failed to block LPS induced β-catenin accumulation and LRP6 phosphorylation (Ser1490) was not observed in the presence of LPS in macrophage cells (data not shown), suggesting Wnt receptor activation is not responsible for LPS induced β-catenin activation. However, correlated to LPS triggered β-catenin accumulation, we observed inactivation of GSK3β and activation of both AKT and ERK. Moreover, both AKT and ERK inhibitors were showed to block β-catenin accumulation by LPS. Endogenous ERK was found to associate with GSK3β and facilitate its inactivation by AKT, as detected in pSer9 GSK3β level. Additionally, degradation resistant β-catenin active form rescued ERK inhibitor mitigated *MMP9* gene induction and cell migration by LPS. This further confirmed the finding that ERK potentiated β-catenin signaling cascades in LPS induced macrophage cells. Our finding supports a previous observation in hepatocellular carcinoma that ERK is activated to activate β-catenin signaling (Ding et al., 2005).

Release of MMP molecules and initiation of macrophage migration are important for host defense and direction of the adaptive immune system. When uncontrolled, they can cause significant damage on the microenvironment. By eliciting massive inflammatory mediators, pro-apoptotic factors, and matrix degrading proteases, the activated macrophage is capable of dismantling tissues to the point of inflicting serious injury. Tissue destruction by chronic inflammation has been associated with the development of tumors and other pathologies (Gordon, 2003). Therefore, the turning off of LPS induces β-catenin signaling is even more interesting than the "turning on", which is left to be studied in the future.

In conclusion, the results of the present study suggested that, in LPS stimulated macrophages, the β-catenin and its downstream signals play strategic roles in inducing cell mobility. β-catenin signaling contributes the early phage *MMP9* gene induction and cell migration. Mechanistically, rapid ERK activation primed GSK3β inactivation by AKT is involved in efficient silencing β-catenin destruction complex by LPS.

MATERIALS AND METHODS

Reagent and plasmids

β-catenin antibody (Cat. #610153) was purchased from BD Biosciences; ERK(#4695), pERK(#9106), GSK3β(#9832), pSer9 GSK3β(#9323), Akt(#4685), pAkt 473(#4058) and Actin(#4970) were purchased from Cell Signaling Technology, Inc (USA); LPS, LY294002, PD98059 were purchased from Sigma, USA. β-catenin SY, dnLEF-1 and GSK3β S9A and control constructs were previously described (Zhang et al., 2006, 2012a, 2012b, 2012c; Zhou et al., 2012a, 2012b).

Cell culture

RAW264.7 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and incubated at 37°C in a humidified atmosphere of 5% CO_2.

Transwell migration assay

As previously described (Zhang et al.,2012d,2012e), transwell assay were performed in 24-well PET inserts (Falcon 8.0 μm pore size) for migration assays. 50,000 cells were plated in transwell inserts (at least 3 replicas for each sample) and left treated with or without LPS (100 ng/mL) for 4 h. Cells in the upper part of the transwells were removed with a cotton swab; migrated cells were fixed in PFA 4% and stained with crystal Violet 0.5%. Filters were photographed and the total number of cells counted. Every experiment was repeated at least three times independently.

Real-time reverse transcription (RT)-PCR

As previously described (Zhang et al., 2012b; Zhou et al., 2011a, 2011b), total RNAs were prepared using NucleoSpin® RNA II kit. Expression level of target genes was normalized with GAPDH. Primers used for qRT-PCR are listed as follows: mGAPDH forward, 5'-AACTTTGGCATTGTGGAAGG-3'; mGAPDH reverse, 5'-ACACATTGGGGGTAGGAACA-3'; mMMP9 forward, 5'-CAAGTGGGACCATCATAACATCA-3'; mMMP9 reverse, 5'-TCTCGCGGCAAGT CTTCAG-3'; mc-Myc forward, 5'-GTCTTTCCCTACCCGCTCAAC-3'; mc-Myc reverse, 5'-GTGGAATCGGACGAGGTACAG-3'

Immunoprecipitation and immunoblotting

Immunoprecipitation was performed as previously described (Zhang et al., 2004; Nie et al., 2010; Zhang et al., 2012b; Zhou et al., 2012b); Immunoblotting was performed with specific primary antibody as indicated and secondary anti-mouse or anti-rabbit antibodies that were conjugated to horseradish peroxidase (Amersham Biosciences). Proteins were visualized by chemiluminescence.

Lentiviral transduction

Lentiviruses were produced by transfecting pLV-bc-CMV (for cDNA

expression) plasmids together with helper plasmids pCMV-VSVG, pMDLg-RRE (gag/pol), and pRSV-REV into HEK293T cells. Cell supernatants were harvested 48 h after transfection and were either used to infect cells or stored at –80°C

Preparation of cytosolic and nuclear fractions

Cytosolic fractions were prepared/isolated using the ProteoExtract kit (Calbiochem) according to the manufacturers' standard procedures.

Statistical analysis

Values are expressed as the mean ± SE derived from 3 samples and the figures show the results obtained from three independent experiments. Statistical analysis was performed using analysis by Student's *t*-test and the level of significance was put at $P < 0.01$ or $P < 0.05$ as indicated.

ACKNOWLEDGEMENTS

We are grateful to Dr. Xiao-fan Wang of Duke University for GSK3β S9A construct. This work was supported by the Netherlands Organization for Scientific Research (NWO 918.66.066), Netherlands Initiative for Regenerative Medicine and Centre for Biomedical Genetics; CSTC2012 jjjq1000; and National Nature Science Foundation of China Grants 30900748 and 31171388 (to H. H.).

ABBREVIATIONS

APC, Adenomatous polyposis coli; dnLEF-1; Dominant negative LEF-1; GSK, glycogen synthase kinase; LPS, lipopolysaccharide; p-AKT, phosphor-AKT; p-ERK, Phospho-ERK; TLR4, Toll-like receptor 4

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