Am J Transl Res 2017;9(1):167-174
www.ajtr.org /ISSN:1943-8141/AJTR0036028

Original Article

GSK3β activity is essential for senescence-associated heterochromatin foci (SAHF) formation induced by HMGA2 in WI38 cells

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Received July 17, 2016; Accepted December 2, 2016; Epub January 15, 2017; Published January 30, 2017

Abstract: Cellular senescence is an irreversible form of cell cycle arrest, which is often characterized by domains of facultative heterochromatin substructures also known as senescence-associated heterochromatin foci (SAHF). SAHF assembly is likely mediated through the downregulation of the Wnt pathway, which inhibits Glycogen Synthase Kinase 3 Beta (GSK3β) in cells undergoing replicative senescence. Alternatively, High Mobility Group AT-Hook 2 (HMGA2) can also induce SAHF formation in primary cells, through a process in which the involved cell signaling pathway is unknown. Accordingly, it is important to determine whether GSK3β and the Wnt pathway are necessary during HMGA2-induced SAHF formation. In this study, we developed a senescence model for SAHF assembly in WI38 cell through ectopic expression of HMGA2. In this model, typical senescent features were identified, including elevated SA-β-galactosidase staining and the downregulation of the Wnt pathway. We also showed that the GSK3β inhibitor LiCl can partly disable SAHF formation through the HMGA2 overexpression in WI38 cells. However, the disabled SAHF formation resulting from the inactivity of GSK3β in our senescence model cannot be restored through ectopic overexpression of Catenin Beta 1 (*CTNNB1*), a downstream transcription factor of the Wnt pathway. This indicates that the GSK3β activity alone, and not those of downstream target genes, is crucial for the HMGA2-induced SAHF formation following the downregulation of the Wnt pathway.

Keywords: HMGA2, SAHF, GSK3β, Wnt2

Introduction

Normal cells undergo growth arrest after a certain number of divisions. Cellular senescence is essentially an irreversible proliferation arrest of primary cells [1] due to an exhaustion of replicative potential [2]. During such an arrest, senescent cells do have metabolic activity, but they are morphologically and physiologically significantly different from normal cells. One notable difference is that they tend to have a more enhanced staining for β-galactosidase (SA-β-gal) in replicative senescence triggered by telomere shortening [3, 4].

Senescence-associated heterochromatin foci (SAHF) is a known downstream consequence of cellular senescence caused by high-intensity RAS signaling and telomere dysfunction [5]. During SAHF formation, there are many specific histone modifications, such as H3Lys9 dimethylation (H3K9me2) and trimethylation (H3K9me3). Moreover, SAHF are typically characterized by a colocalization of defined marker proteins, such as HP1β, HIRA, etc. [6, 7]. Particularly noteworthy is the Retinoblastoma-associated protein (Rb) or, to be more specific, the hypophosphorylated Rb [8], a marker protein essential for the formation of SAHF [9, 10].

Different from replicative senescence, some studies found that even in younger cells, SAHF formation might be directly induced by the downregulation of the Wnt signaling pathway, a key regulator of stem cells mainly by promoting their proliferation and division. On the other

hand, the abnormal activation of Wnt signaling has frequently been associated with cancer development and progression [11]. Under normal conditions, the protein level of Catenin Beta 1 (CTNNB1, also known as β-catenin) in the cytoplasm is tightly regulated by phosphorylation-dependent and ubiquitin-mediated degradation. Under pathological conditions, extracellular Wnt ligands bind to their cognate transmembrane receptors, resulting in the inhibition of β-catenin phosphorylation by Glycogen Synthase Kinase 3 Beta (GSK3β, also known as GSK3β) and the prevention of β-catenin ubiquitination and degradation. This leads to the cytoplasmic accumulation of β-catenin, which later translocates to the nucleus. The stabilized β-catenin works together with transcription factors like the T cell factor (TCF) and lymphoid enhancer factor (LEF) family, etc., leading to the activation of Wnt/β-catenin responsive genes, such as C-Myc (*MYC*) and Cyclin D1 (*CCND1*). In addition, research by Ye et al. showed that the repression of *WNT2* was a trigger for the formation of SAHF and the onset of cell senescence in primary human cells, most likely through GSK3β-mediated phosphorylation of HIRA [12]. Once the SAHF are formed, the process tends to become self-sustaining and perpetuates the senescent cell cycle arrest [13].

This self-sustaining mechanism of SAHF formation may contribute to antitumor response. Even in a promitogenic environment, once cells become senescent, the genes that drive cell proliferation and division will be repressed [14, 15]. This is crucial for us to understand issues like how to suppress tumor development and progression.

Noteworthy, the activation of some oncogenes like RAS can also trigger cell senescence in a large number of normal cells [16-18]. Oncogene-induced senescence (OIS) is a robust and sustained anti-proliferative response to oncogenic stress which can effectively inhibit tumor progression [19]. Accordingly, senescence is actually a cellular response that, like apoptosis and necrosis, limits cell proliferation [20]. The High Mobility Group AT-Hook 2 (HMGA2) protein, which has been known as a kind of tumorigenic protein, also contributes to maintaining a stable state of SAHF [21]. The HMGA2 protein, through the AT-hook domains, can bind to the minor groove of DNA in the AT-rich regions [22]. This protein is also an essential structural component of the SAHF and is abundant in senescent cell chromatin. It has been reported that HMGA2 is only expressed in embryos during early development or in patients with metastatic malignant tumors [23-26]. In these tumors, the Wnt signaling pathway is more likely to be activated [27]. Surprisingly, the overexpression of the HMGA2 protein is sufficient to induce SAHF formation and other senescence-related phenotypes in primary IMR90 cells. Thus, we consider it necessary to determine whether the onset of HMGA2-induced senescence is accompanied by the downregulation of Wnt signaling.

In this study, we focused on investigating whether HMGA2-induced senescence is related to downregulated Wnt signaling and found that this phenomenon can be prevented by LiCl, a specific inhibitor of GSK3β [28]. However, it is independent from the modification of β-catenin. Consequently, like in other models, senescence of primary human cells induced by HMGA2 is also dependent on the activity of GSK3β.

Materials and methods

Cell culture and reagents

The WI38 and HEK293T cell lines were purchased from the ATCC (Manassas, VA, USA) and from the Institute of Cell Biology (Shanghai, China), respectively. WI38 cells were maintained in minimum essential medium (MEM, Gibco, Carlsbad, CA, USA) and HEK293T cells in Dulbecco's MEM (DMEM; Gibco), supplemented with 10% FBS, 100 mg/mL penicillin and 100 mg/mL streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C.

Vector construction and viral infections

The pWPXLD lentiviral vector was used. The HMGA2 sequence was amplified by RT-PCR with total RNA from senescent WI38 cells. The PCR product was inserted into the Pme I and BamH I sites of the pWPXLD vector and fused with the EGFP gene.

SA-β-gal staining and DAPI staining

SA-β-gal staining and DAPI staining of the cells were performed as previously described (10).

GSK3β activity assay

The GSK3β activity assay was performed as previously described [28]. Briefly, the intensity

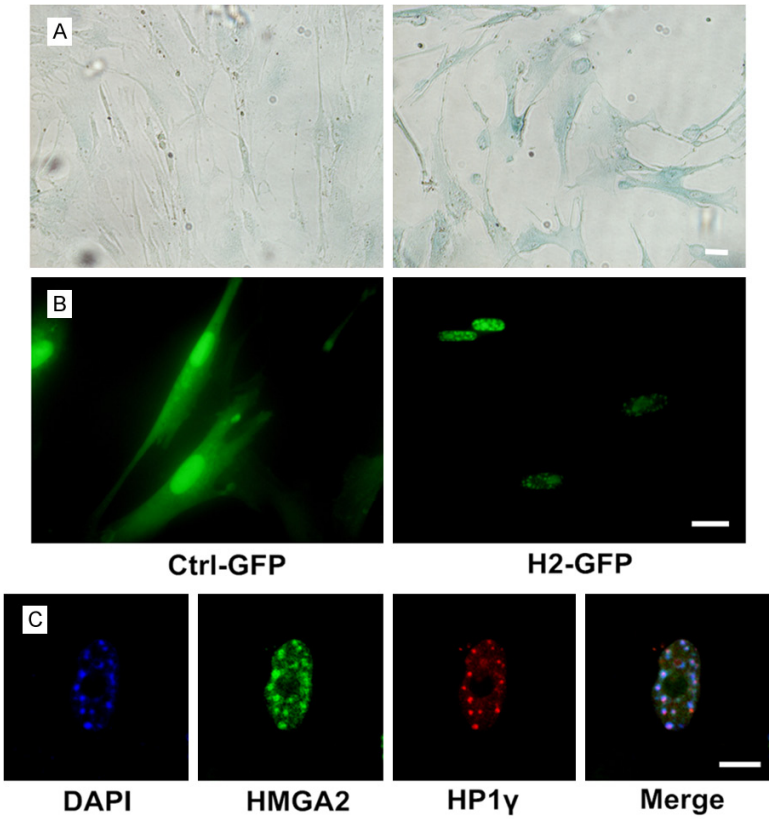

Figure 1. HMGA2 induced the senescence phenotype and formation of SAHF in WI38 cell line. A. β-gal staining shown the increased SA-β-galactosidase activity in WI38 cells 5 days after HMGA2-GFP overexpression indicating that HMGA2 induced senescence in these cells. Scale bar: 20 μm. B. Fluorescent images of WI38 cells displaying SAHF-like foci 5 days after HMGA2-GFP overexpression. Scale bar: 20 μm. C. Confocal immunofluorescent images of co-localization of HMGA2 in chromatin foci with the SAHF markers HP1γ in WI38 cells 5 d after over-expression of HMGA2-GFP, which indicates that the foci induced by HMGA2 are indeed senescence-associated heterochromatin foci (SAHF). Scale bar: 10 μm.

of the immunoreactive bands of GSK3β and phospho-Ser-9-GSK3β was assessed and correlated to the relative amount of each immunoreactivity protein using the ImageJ software (NIH, Bethesda, MA, USA). The activity of GSK3β protein was calculated from the unphosphorylated fraction of GSK3β, using the following formula: Unphosphorylated GSK-3β = (Total GSK3β-phospho-Ser-9-GSK3β)/Total GSK3β ×100%.

Real-time RT-PCR

Total cellular RNA was extracted using Trizol reagent according to the manufacturer's protocol (TaKaRa, Tokyo, Japan). Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using the ImProm-II TM Reverse Transcription System (Promega, Fitchburg, WI, USA). To assess possible gene amplification, semi-quantitative RT-PCR was performed using r-taq. The sequence-specific primers in the quantitative RT-PCR were as follow: *WNT2* F: GTTACCCAGACATCATGCGTT, R: GGGTGGTACAGTTCCAGCG; *ACTB* F: TCGTGCGTGACATTAAGGAG, R: ATGCCAGGGTACATGGTGGT.

Western blot analysis

Western blot analysis was performed as described previously (18). The primary antibodies used were the following: anti-phospho-Ser-9-GSK-3β (1:1000; CST Inc., Danvers, MA, USA), anti-GSK3β (1:1000; CST Inc.), anti-β-catenin (1:1000; CST Inc.), anti-Cyclin D1 (1:1000; Santa Cruz Inc., Santa Cruz, CA, USA), HMGA2 (1:5000; Epitomics) and anti-b-actin (1:10,000; Sungene, Gatersleben, Germany).

Immunofluorescence

WI38 cells were grown on coverslips in six-well plates, washed three times with PBS, and then fixed in 4% formaldehyde solution for 10 min before being permeabilized with 0.2% Triton X-100 in PBS for 10 min. Coverslips were blocked with 5% BSA in PBS for 1 h at room temperature and then were incubated with the respective primary antibodies for 1 h. The following primary antibodies were used: anti-H3K9me3 (1:200, Upstate) and anti-HP1γ (1:200, Chemicon). The plates were then washed with TBST and incubated for 1 h with the TRITC-conjugated secondary antibodies at 1:400 dilutions. Cells were further washed in TBST, and DNA was visualized using DAPI (1 mg/mL). Confocal images were captured under a confocal laser-scanning microscope.

Reporter essay

The luciferase assays in this study were performed as previously described [30]. The cells

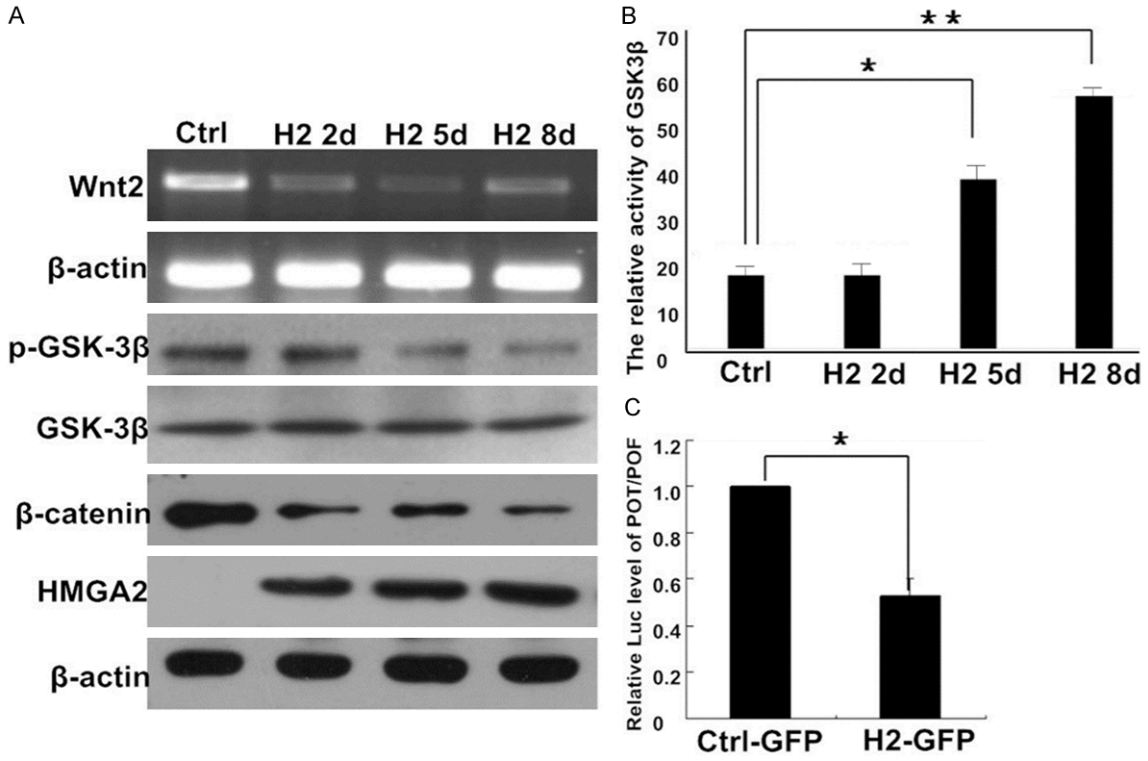

Figure 2. Canonical Wnt Signaling is Down-regulated in HMGA2 Induced SAHF Formation. (A) RT-PCR analysis showing the down-regulation of Wnt2 & Western-blot analysis showing the downregulation of β-catenin and the phosphorylated GSK3β, but not the unphosphorylated GSK3β, in WI38 cells after HMGA2 (H2) overexpression at the indicated time points in days (d). β-actin, a housekeeping gene, was used for normalization here. These results show that HMGA2 downregulated the Wnt pathway in WI38 cell line. (B) The increased GSK3β activity was measured at the indicated time points in days (d) after HMGA2 (H2) overexpression and the GSK3β activity analyzed by the overall quantitation of the GSK3β and phosphorylated GSK3β in (A), the details are described in the Methods section. Statistical analysis was performed using the Student's t-test and the statistical significance is indicated by "*" and "**" which denote a *P* value of < 0.05 and < 0.01, respectively. (C) Reporter assay showing the downregulation of the activity of downstream transcription factor of the Wnt pathway indicated by POT/POF. Statistical analysis was performed using the Student's t-test and the statistical significance is indicated by "*" which denotes a *P* value of < 0.05.

were transfected with 1 μg TOP Flash or FOP Flash plasmid (Upstate Biotechnology, Lake Placid, NY, USA), 1 μg pSV β-galactosidase vector (Promega, Fitchburg, WI, USA) and/or empty vector using Lipofectamine (Roche, Penzberg, Germany). Luciferase and beta-galactosidase activities were measured 48 h after transfection according to standard methods and were used to evaluate transfection efficiency (Promega).

Results

HMGA2 can induce the senescence phenotype in WI38 cells

In order to determine whether the HMGA2 protein can induce the senescence phenotype in primary WI38 cells, the HMGA2 protein fused with GFP was expressed in WI38 cells using a lentiviral delivery system driven by a strong EF1a promoter. The result demonstrated that the ectopic overexpression of HMGA2 resulted in both, elevated SA-β-galactosidase staining (**Figure 1A**) and SAHF-like chromatin foci in the nuclei (**Figure 1B**). These results indicated that HMGA2 can induce the senescence phenotype and the formation of SAHF in WI38 cells.

Downregulation of the Wnt pathway during the HMGA2-induced SAHF formation

To test whether the onset of cell senescence involves Wnt signaling, we overexpressed HMGA2 in WI38 primary cells. The results revealed that overexpression of HMGA2 led to

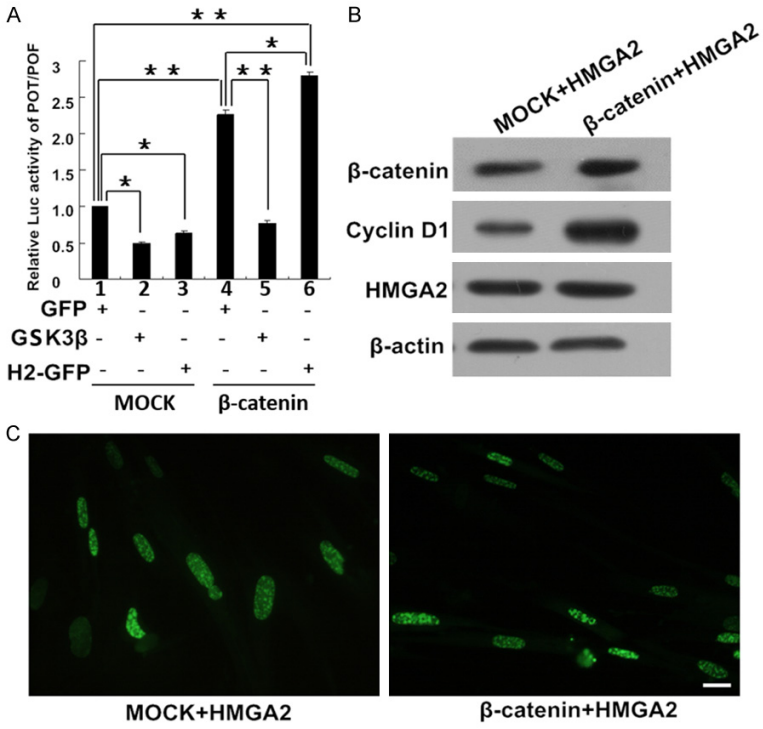

Figure 3. HMGA2-induced SAHF formation does not involve β-catenin regulated downstream factors in WI38 cells. A. POT/POF reporter assay in WI38 cells transfected with either GFP, GSK3β or HMGA2 (1, 2, 3) and co-transfected with each of GFP, GSK-3β, HMGA2 and β-catenin (4, 5, 6). Overexpression of β-catenin increased the activity of a downstream gene promoter (compare 3 with 6), a process independent from HMGA2 (compare 1 with 4), whereas GSK3β inhibited such a process (compare 4 with 5). Statistical analysis was performed using the Student's t-test and the statistical significance is indicated by "*" and "**", which denote *P* value of < 0.05 and < 0.01, respectively. B. Western-blotting showing upregulation of Cyclin D1, a downstream target gene of the Wnt pathway, induced by ectopic overexpression of β-catenin in WI38 cells. Overexpression of β-catenin increased the expression level of the Wnt downstream genes. C. The Fluorescent images of WI38 cells displaying the SAHF formation 5 days (d) after transfection with Mock+HMGA2-GFP or β-catenin+HMGA2-GFP (Scale bar: 20 μm), indicating that in WI38 cells the HMGA2-induced SAHF formation is independent from β-catenin-regulated downstream factors.

dramatically decreased mRNA levels of WNT2 in WI38 primary cell line, as shown in **Figure 2A**. The fraction of phosphorylated GSK3β (inactive) protein was decreased, while the overall level of GSK3β remained unchanged. The data also showed an enhanced GSK3β activity in the HMGA2-overexpressing WI38 cells (**Figure 2B**), which was evaluated by a Quantitation software based on the results of Western blotting analysis (**Figure 2A**). The Western blot analysis also revealed the degradation of β-catenin and decreased expression of Cyclin D1, a downstream cell circle regulatory protein (**Figure 2A**). The down-regulated activity of the downstream response elements of WNT was also detected by the Dual-Luciferase Reporter Assay System (**Figure 2C**).

β-catenin and down-stream target genes are not involved in HMGA2-induced SAHF formation

To test the effects of β-catenin on the promoter activity of Wnt target genes, we analyzed the activity of β-catenin-responsive promoter genes fused with a luciferase reporter gene. The HEK293T cells were transiently transfected with a plasmid expressing either MOCK+HMGA2 or β-catenin+HMGA and the effect of the genes expression was analyzed. The results demonstrated that overexpression of β-catenin increased the activity of a downstream gene promoter (compare 3 with 6), through a process that did not involve HMGA2 (compare 1 with 4). On the other hand, GSK3β inhibited such a process (compare 4 with 5). To further confirm the potential involvement of β-catenin and down-stream target gene in the HMGA2-induced SAHF formation, we examined the effects of co-expressing β-catenin and HMGA2 on SAHF formation in WI38 cells. We found that β-catenin increased the expression of CCND1, a target gene of the Wnt-pathway (**Figure 3B**). However, the SAHF formation induced by HMGA2 is independent from β-catenin and its down-stream target genes (**Figure 3C**).

GSK-3β is essential for SAHF formation induced by HMGA2 in WI38 cells

With respect to the finding that β-catenin is not involved in the HMGA2-induced SAHF formation in WI38 cells, we presume that the GSK3β may be an important enzyme in this process. Previous studies using embryological models suggested that LiCl mimicked Wnt/β-catenin

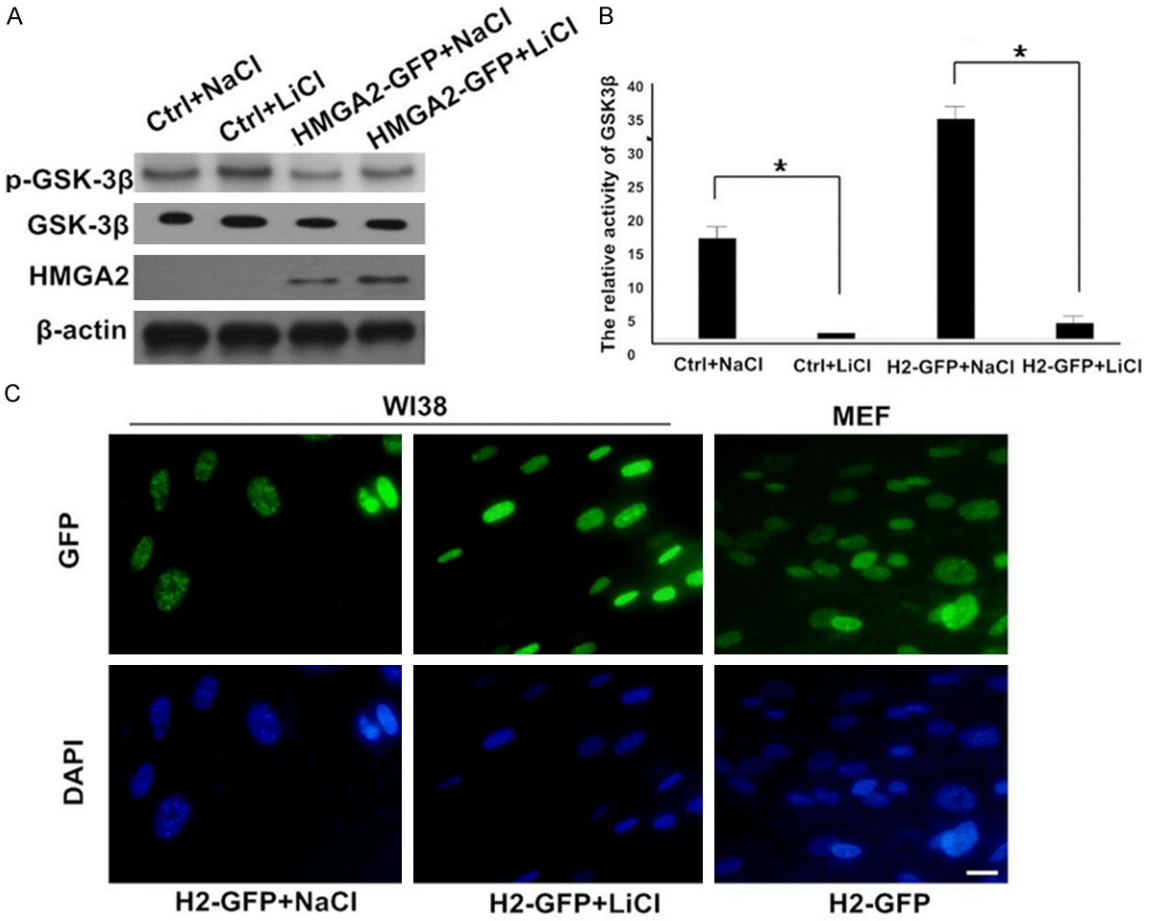

Figure 4. The activity of GSK3β is required for the SAHF formation induced by HMGA2 in WI38 cells. (A) Western-blotting showing changes in phosphorylated GSK3β and unphosphorylated GSK3β after adding a specific inhibitor (LiCl) or mock inhibitor (NaCl) in WI38 cells with or without HMGA2-GFP. (B) Activity of GSK3β analyzed by the quantitation of (A). LiCl effectively inhibited the GSK3β activity regardless of whether HMGA2 existed or not. Statistical analysis was performed using the Student's t-test and the statistical significance is defined by "*" which denotes a *P* value of < 0.05. (C) Fluorescent images showing changes in SAHF formation in WI38 cells expressing HMGA2 incubated with NaCl or LiCl, as well as the absence of SAHF formation in MEF cells expressing only HMGA2-GFP. Scale bar: 20 μm. GSK3β is essential for SAHF formation induced by HMGA2 in WI38 cells.

activation by inhibiting GSK3β activity through phosphorylation at Ser-9 [8, 9]. This effect was further confirmed in our model after we evaluated the effect of LiCl on GSK3β in WI38 cells expressing GFP and HMGA2-GFP using a lentivirus system. After 24 h serum deprivation, cells were incubated with LiCl (5 mM) for 0-24 h. Inhibition of GSK3β (i.e. by GSK3β Ser-9 phosphorylation) was assessed by immunoblot analysis using a phospho-Ser-9-specific antibody. In MOCK (NaCl) experiments incubated with HMGA2-GFP cell lines, the level of inactive phosphorylated GSK3β was low, whereas exposure to LiCl dose-dependently increased the inactive, phosphorylated form of GSK3β (**Figure 4A**). The relative activity of GSK3β was evaluated by the quantitation software based on the results presented in **Figure 4A**. The results revealed that treatment with LiCl abrogated the SAHF formation induced by HMGA2-GFP in WI38 cells (**Figure 4C**). A similar phenomenon has also been observed in MEF cells with high levels of HMGA2-GFP expression, possibly a result of low GSK3β activity in these cells [29]. Overall, these data proved that GSK3β plays a crucial role in the HMGA2-induced SAHF formation in WI38 cells.

Discussion

This study showed that a major signal for SAHF formation was the down-regulation of Wnt sig-

naling induced by HMGA2 in senescent cells. However, this process was independent from β-catenin-related downstream transcription factors of the Wnt signaling pathway. In **Figure 3**, Although the protein activity and transcript level of β-catenin both decreased after HMGA2 expression (**Figure 3**), the ectopic expression of β-catenin did not prevent the SAHF formation induced by HMGA2. These results revealed that the expression of Wnt target genes had no effects on the HMGA2-induced SAHF formation. However, we found that LiCl, an inhibitor of GSK3β, can prevent the SAHF formation induced by HMGA2. Accordingly, the activity of GSK3β might hold the key to understand the HMGA2-induced SAHF formation in WI38 cells (**Figure 3**). We hypothesize that it might be dependent upon phosphorylation of HIRA, a protein involved in the formation of SAHF, as this phosphorylation process have been found to occur in the model of SAHF formation induced by the oncogene Ras in IMR90 cells [9]. We also found that HMGA2 was unable to induce SAHF formation in MEF cells, due to the inactivity of GSK3β in this cell line. However, the precise mechanism involving the downstream targets of GSK3β has yet to be studied.

Compared with previous studies, we confirmed that Rb is an essential factor in Ras- and HMGA2-induced SAHF formation [10]. Meanwhile, the involvement of GSK3β and the Wnt pathway was similarly found in the SAHF formation induced by Ras or HMGA2 expression. Consequently, there is no difference between the mechanisms of SAHF formation induced by Ras and HMGA2 in primary WI38 cells. We also posited that HMGA2-induced senescence might be similar to the Ras-induced senescence, since both Ras and HMGA2 are regarded as oncogenes in many carcinomas. HMGA2-induced senescence can also be considered as an immediate premature senescence expedited by an aberrant oncogene to protect these cells from transformation. Our results to some extent explain how HMGA2 acts as an oncogene or tumor suppressor, depending on different cellular contexts, though the precise underlying mechanisms remain open to further discussion.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (31300624, 81470684), Clinical Special Fund of Jiangsu Province (b12014032), Postdoctoral Science Foundation of China (2013M530473, 2015M571818), Six Major Categories Talent (2014-WSN-043, 2011-WS-074), Innovation and Entrepreneurship Training Program for College Students in Jiangsu Province (2015-10313003Z, 201510313003, KYLX14-1455, 201610313002Z), Colleges and universities Foundation in Jiangsu Province (16621632, 16KJB320016). We would also like to thank Tiantian Sun and Xiaohui Yang for helping proofread this paper.

Disclosure of conflict of interest

None.

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