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Nitric oxide promotes epidermal stem cell proliferation via FOXG1-c-Myc signalling

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ABSTRACT

Objective: Epidermal stem cells (ESCs) play a critical role in wound repair, but the mechanism underlying ESC proliferation is unclear. Here, we explored the effects of nitric oxide (NO) on ESC proliferation and the possible underlying mechanism.

Methods: The effect of NO (two NO donors, SNAP and spermine NONOate, were used) on cell proliferation was detected using cell proliferation and DNA synthesis assays. Thereafter, expression of FOXG1 and *c*-Myc induced by NO was determined by immunoblot analysis. pAdEasy-FOXG1 adenovirus and *c*-Myc siRNA plasmids were infected or transfected, respectively, into human ESCs to detect the effect of FOXG1 and *c*-Myc on NO-induced cell proliferation. Additionally, NO-induced ESC proliferation in vivo was detected by BrdU incorporation and a superficial second-degree mouse burn model. Moreover, the relationships among NO, FOXG1 and *c*-Myc were detected by western blotting, real-time PCR and dual luciferase assay.

Results: NO exerted a biphasic effect on ESC proliferation, and 100 μM SNAP and 10 μM spermine NONOate were the optimal concentrations to promote cell proliferation. Additionally, NO-promoted human ESC proliferation was mediated by FOXG1 and *c*-Myc in vitro and vivo. Furthermore, NO regulated FOXG1 expression through cGMP signalling, and NO-induced transcription of *c*-Myc was regulated by FOXG1-mediated *c*-Myc promoter activity.

Conclusion: This study showed that the biphasic effect of NO on ESC proliferation as well as NO induced ESC proliferation were regulated by the cGMP/FOXG1/c-Myc signalling pathway, suggesting that NO may serve as a new disparate target for wound healing.

1. Introduction

The skin is the largest organ of the body and primary protective barrier against the environment. The epidermis, consisting of keratinocytes with variable degrees of differentiation, is constantly self-renewing and is maintained by epidermal stem cells (ESCs) [1], skin-specific adult stem cells with a strong proliferative capacity. Following induction, ESCs differentiate into various epidermal lineages to promote self-renewal and regeneration of the epidermis, as well as wound healing [2]. Wound healing is a complex process that is mediated by various factors that are responsible for the regeneration and reorganization of damaged tissue into its normal architecture, and ESC differentiation, migration and proliferation are the basis of this process. We previously conducted work on ESC differentiation [3] and migration [4,5]; however, little is known regarding ESC proliferation and the mechanisms that stimulate ESC proliferation during wound repair.

Over the past two decades, it has become well known that NO is a critical molecule for wound healing [6,7] and the underlying cellular homeostasis [8,9]. NO regulation during wound healing depends on the modulation of NO by the different cell types involved in this complex reconstruction process [10,11]. Among its many effects, NO plays a critical role in wound healing by promoting cell migration and

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proliferation. The mechanism of NO on wound healing has been studied by several authors, but its exact signalling has not been completely elucidated [12,13]. The pathways directly downstream of NO signalling primarily consist of interactions between NO and heme-containing proteins, the most important of which is guanylate cyclase [14,15]. NO-cGMP signal transduction has been shown to regulate the migration of lung cancer cells, endothelial cells, and neural progenitor cells [16,17]. Our previous study showed that NO enhances human keratinocyte cell (HaCaT) and ESC migration [4,5]. However, little is known about NO-induced ESC proliferation and its underlying mechanisms during wound repair.

C-Myc is a major transcription factor that is located on chromosome 8q24 and accelerates cell proliferation via cyclin D and cyclin-dependent kinases-4/6 in the G1 phase [18]. C-Myc is expressed in many types of proliferating cells, especially neoplasms, as well as during wound healing [19] and has critical functions in cell proliferation, differentiation, and apoptosis. Many investigators have reported that *c*-Myc is required for the maintenance of ESCs in their niche, controls differentiation along the epidermal lineage, and regulates ESCs proliferation in wound healing [20,21]. Resent reported that NO induced *c*-Myc expression in wound healing [22], but its mechanism was unclear.

The Forkhead box G1 (FOXG1) gene is located on chromosome 14q12 and encodes a winged-helix transcriptional repressor that is important for early development of the ventral telencephalon dorsoventral patterning by integrating several signalling centres [23]. Additionally, FOXG1 controls production of specific neuronal subtypes and regulates the balance between neural progenitor cell proliferation and differentiation in the telencephalon [24]. Goubau [25] reported that FOXG1 is expressed in platelets and skin tissue in patients. Additionally, Nagao [26] and Liu [27] reported that FOXG1 and *c*-Myc have important functions in coordinating developmental control of self-renewal and cell fate choices in NSCs. Furthermore, bioinformatics analysis predicted several potential FOXG1 binding sites in the 5′ regulatory region of the *c*-Myc gene.

Our previous study found that NO can enhance migration of ESCs via the cGMP-Rho GTPase signalling pathway in wound healing [5]. In this study, we aimed to illustrate the role and mechanism of NO in the proliferation of ESCs in wound healing. We employed cultured ESCs that were prepared from human foreskin and primarily focused on the effect of NO on ESC proliferation. Additionally, we demonstrated that NO promotes ESC proliferation through cGMP/FOXG1/c-Myc signalling and that FOXG1 may induce the transcriptional activity of the *c*-Myc gene promoter, suggesting that NO may serve as a new disparate target for wound healing.

2. Materials and methods

2.1. Materials

TRIzol reagent, Lipofectamine 2000, cell culture medium (Dulbecco's modified Eagle's medium, DMEM) and keratinocyte serum-free medium (K-SFM) were purchased from Invitrogen (Carlsbad, CA, USA). The siRNA pools for *c*-Myc and control siRNA were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The dual luciferase assay systems were from Promega (Madison, WI, USA).

2.2. Isolation and culture of primary huESCs

Primary huESCs were isolated from human foreskins using a modified method of rapid adhesion to collagen IV as previously described [5]. These protocols were approved by the Ethics Committee of Southwest Hospital, Chongqing, China. All experiments were performed in accordance with ethical guidelines and regulations. Foreskin tissue was obtained from the redundant prepuce of patients who were 12–20 years old and provided informed consent. Briefly, after disinfection and rinsing three times with PBS, foreskin tissues were cut into approximately 0.5-cm^2 pieces and were digested with 0.25% Dispase II (Roche, Basel, Switzerland) overnight at 4 °C. Next, the epidermis was separated carefully and digested with 0.25% trypsin for 10 min at 37 °C. The cells were collected by filtration centrifugation and were washed with PBS. Finally, isolated cells were suspended in keratinocyte serum-free medium (K-SFM, Invitrogen, California, USA) supplemented with epidermal growth factor (0.1–0.2 ng/ml), bovine pituitary extract (20–30 mg/ml), $CaCl_2$ (0.05 mM) and 100 IU/ml of streptomycin and penicillin. Next, 5 ml of isolated suspended cells were seeded at a concentration of 2×10^5/ml in 25-mm dishes that had been coated overnight with collagen IV (100 μg/ml; Sigma, Saint Louis, USA). Cells were incubated for 10 min at 37 °C and 100% humidity in an atmosphere of 5% CO_2 in air. Non-adherent cells were then immediately rinsed off. Adherent cells were further cultured with fresh medium at 37 °C and 100% humidity in an atmosphere of 5% CO_2 in air. The medium was changed every other day. When the cells of passages 1 and 2 grew to 60–70% confluence, they were digested with TrypLE Select (Invitrogen, Carlsbad, CA, USA) for 5–10 min. The cells at the second passage were used for identification as well as for other experiments.

2.3. ^{3}H-thymidine incorporation assay and CCK-8 test

The cells were pulsed during the last 10 h with 1 mCi of [^{3}H]-thymidine before collection and were assessed for radioactivity using a scintillation counter Tri-Carb 2100 TR (Perkin Elmer Life Science, Boston, MA, USA). In all instances where radioisotopes were used, background radioactivity was subtracted before quantifying radioactivity. For the CCK-8 (Dojindo, Kumamoto, Japan) test, the cells were mixed with 10 ml of CCK-8 solution per well and were incubated for an additional 2 h at 37 °C. The amount of formazan dye generated by cellular dehydrogenase activity was measured for absorbance at 450 nm by a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The optical density values of each well represented the survival/proliferation of ESCs.

2.4. RNA isolation and real-time PCR

For quantitative PCR analysis, total RNA was extracted using an RNeasy Mini Kit (QIAGEN), and cDNA was synthesized using a First Strand cDNA Synthesis Kit (TOYOBO). Real-Time PCR analysis of human cDNA was performed using the 7500 Real-Time PCR System (Applied Biosystems) and SYBR Green. The expression values were normalized to GAPDH expression. The GAPDH primer sequences were as follows: forward, 5′-TGCACCACCAACTGCTTAGC-3′; and reverse, 5′-GGCATGGACTGTGGTCATGAG-3′. The FOXG1 primer sequences were as follows: forward, 5′-TTCAGCTACAACGCGCTCAT-3′; and reverse, 5′-ACAGATTGTGGCGGATGGAG-3′. The *c*-Myc primer sequences were as follows: forward, 5′-ACCACCAGCAGCGACTCT-3′; and reverse, 5′-GCTGTGAGGAGGTTTGCTGT-3′.

2.5. Western blotting

After ESCs were lysed, the protein concentrations were measured using a Bradford protein assay kit (Biyuntian, Beijing, China), and then, western blot analysis was performed as described previously [28]. The primary antibodies anti-*c*-Myc (1:500), anti-FOXG1(1:500) and anti-GAPDH (1:1000) were purchased from Abcam (Cambridge, MA, USA).

2.6. siRNA transient transfection

Human ESCs were transfected with 50 nm of siRNA using Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Control siRNA or siRNAs specifically targeting *c*-Myc were purchased from Qiagen (Qiagen, Hamburg, Germany). After incubation in K-SFM for 24 h, ESCs were then harvested for assays, including western blotting, the ^{3}H-TdR incorporation