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N-methyl-*N*-nitro-*N*-nitrosoguanidine-mediated ING4 downregulation contributed to the angiogenesis of transformed human gastric epithelial cells

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ABSTRACT

Aims: Angiogenesis is associated with the progression and mortality of gastric cancer. Epidemiological evidences indicate that long-term *N*-nitroso compounds (NOCs) exposure predominantly contributes to the mortality of gastric cancer. Therefore, further reduced mortality of gastric cancer demands to explore the exact mechanisms of NOCs induced angiogenesis. As a tumor suppressor gene, inhibitor of growth protein 4 (ING4) plays an important role in pathological angiogenesis. In this study, we will investigate ING4 expression level in human gastric epithelial cells after the long-term low dose exposure of *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) and the pathological impact of MNNG-reduced ING4 on angiogenesis of transformed cells.

Main methods: The soft agar colony formation assay, Western blotting, immunofluorescence and wound healing assay were used to evaluate the characteristics of transformed cells. HUVEC growth and tube formation assays were performed to test the angiogenic abilities. EMSA, luciferase reporter gene assay, real-time PCR and Western blotting were used to explore the exact mechanism.

Key findings: By establishing transformed human gastric epithelial cells *via* chronic low dose treatment, a gradually ING4 downregulation was observed in the later-stage of MNNG-induced cell transformation. Moreover, we demonstrated that MNNG exposure-reduced ING4 expression significantly resulted into aggravating angiogenesis through increasing the phosphorylation level of NF-κB p65 and subsequently DAN binding activity and regulating the expressions of NF-κB p65 downstream pro-angiogenic genes, MMP-2 and MMP-9.

Significance: Our findings provided a significant mechanistic insight into angiogenesis of MNNG-transformed human gastric epithelial cell and supported the concept that ING4 may be a relevant therapeutic target for gastric cancer.

1. Introduction

Gastric cancer is the fourth most common malignancies and the second leading causes of cancer-related deaths worldwide [1]. The high mortality rate is related to the tendency for early invasion and metastasis [2]. Tumor angiogenesis has been widely believed to be the key step for the invasion and metastatic spread of gastric cancer by facilitating malignant cells to escape from the primary site and establish distant metastasis elsewhere, and inhibition of tumor angiogenesis has showed the clinical benefit in advanced gastric cancer [3]. Therefore, it is important to unravel the mechanisms of angiogenesis for prognosis judgement and future interventions of gastric cancer.

As potential carcinogen, NOCs, such as *N*-methyl-*N*-nitrosourea, MNNG, and the their precursors, such as nitrates, nitrites, amines, are ubiquitous in the environment, and humans can be exposed to them through the ways of dietary, occupation, and tobacco smoking [4]. The epidemiological data have suggested that long-term NOCs exposure predominantly contributes to the incidence and mortality of gastric cancer [5–7]. And NOCs can induce the malignant transformation of normal gastric epithelial cell *in vitro* and the formation of rat gastric cancer *in vivo* [8,9]. MNNG induced gastric cancer tissues showed striking angiogenic response [10]. However, little is known about the molecular events critical to NOCs-induced gastric cancer angiogenesis. Therefore, further reduced mortality of gastric cancer demands to explore the exact mechanism of NOCs induced angiogenesis and inform the management strategies to slow/prevent progression of gastric cancer.

Mounting evidence indicates that genetic aberrant alterations in specific proto-oncogenes and tumor suppressor genes contribute to the development and progression of gastric cancer [11]. ING4 has been identified as an important tumor suppressor gene, which is significantly down-regulated in gastric cancer and associated with prognosis of gastric cancer patients [12,13]. In addition, ING4 is involved in cell cycle arrest, apoptosis, DNA repair, chromatin modification, and

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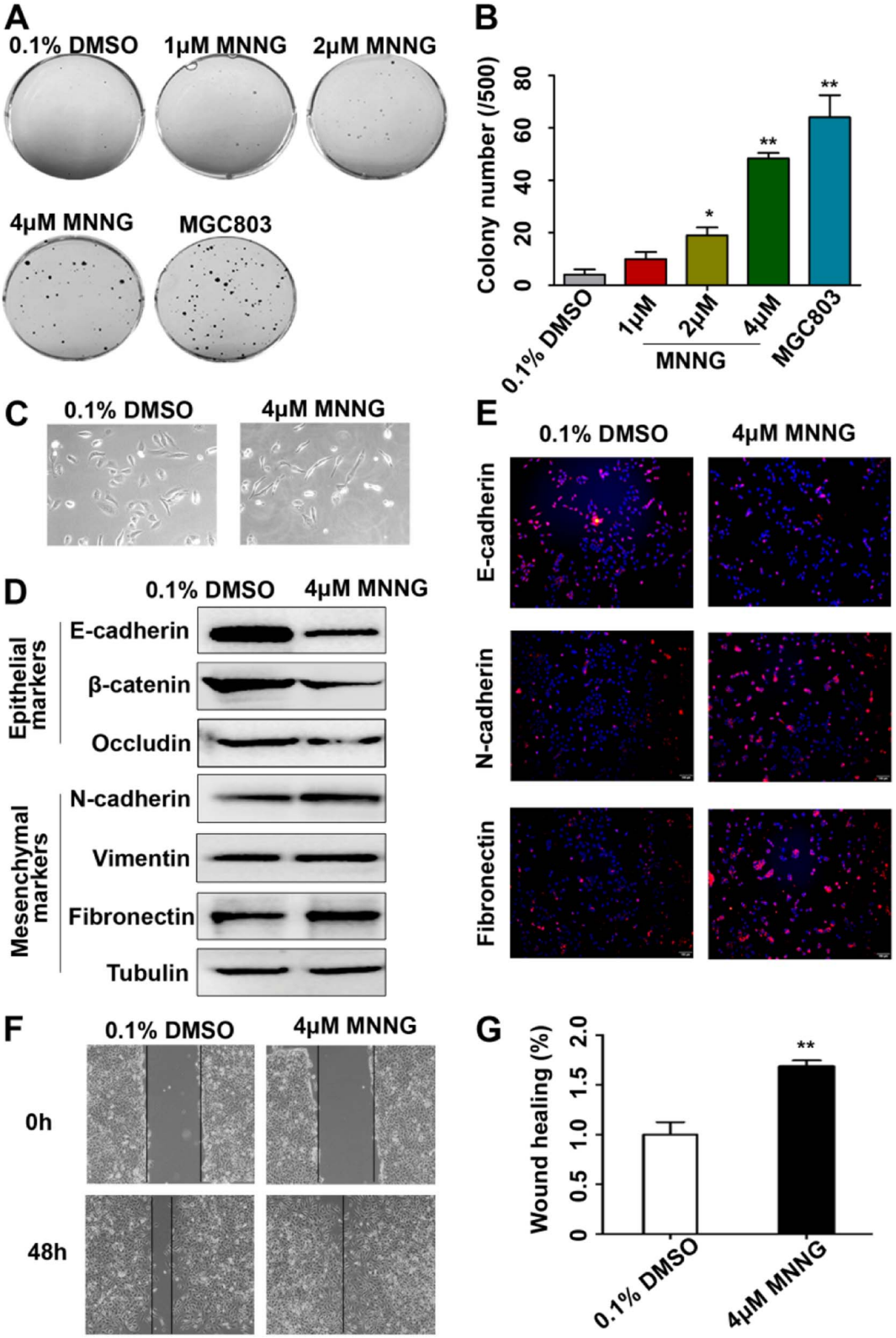

Fig. 1. Long-term, low levels of MNNG exposure induced malignant phenotypes of GES-1 cells. (A–B) The soft agar colony formation in GES-1 cells exposed to MNNG (1, 2, 4 μM) and 0.1% DMSO for 60 passages, and the number of cell colonies was counted (n = 3/group). (C) Morphology changes in GES-1 cells after exposure to 4 μM MNNG and 0.1% DMSO for 60 passages. (D) Western blotting analyzed expressions of the epithelial markers E-cadherin, β-catenin, and occludin, and the mesenchymal markers fibronectin, vimentin, and N-cadherin in GES-1 cells exposed to 4 μM MNNG and 0.1% DMSO at passage 60. (E) Immunofluorescence staining for the epithelial and mesenchymal markers. (F) Cell wound healing assays in GES-1 cells exposed to 4 μM MNNG and 0.1% DMSO at passage 60. The width mean of cell healing was measured (n = 3/group). Data were presented as means ± standard deviations. Data were presented as means ± standard deviations, $^{*}P < 0.05$, $^{**}P < 0.001$ (Student's *t*-test).