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# Delivery of inhibitor of growth 4 (ING4) gene significantly inhibits proliferation and invasion and promotes apoptosis of human osteosarcoma cells

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Growing evidence has suggested that inhibitor of growth 4 (ING4), a novel member of ING family proteins, plays a critical role in the development and progression of different tumors via multiple pathways. However, the function of ING4 in human osteosarcoma remains unclear. To understand its potential roles and mechanisms in inhibiting osteosarcoma, we constructed an expression vector pEGFP-ING4 and transfected the human osteosarcoma cells using this vector. We then studied the effects of over-expressed ING4 in the transfected cells on the proliferation, apoptosis and invasion of the osteosarcoma cells. The up-regulation of ING4 in the osteosarcoma cells, arising from the stable pEGFP-ING4 gene transfection, was found to significantly inhibit the cell proliferation by the cell cycle alteration with S phase reduction and G0/G1 phase arrest, induce cell apoptosis via the activation of the mitochondria pathway, and suppress cell invasion through the down-regulation of the matrix metalloproteinase 2 (MMP-2) and MMP-9 expression. In addition, increased ING4 level evoked the blockade of NF- $\kappa$ B signaling pathway and down-regulation of its target proteins. Our work suggests that ING4 can suppress osteosarcoma progression through signaling pathways such as mitochondria pathway and NF- $\kappa$ B signaling pathway and ING4 gene therapy is a promising approach to treating osteosarcoma.

Osteosarcoma is an aggressive malignant tumor of the skeleton system characterized by the formation of osteoid tissue. It is a rare (0.2% of all malignant tumors) but the most destructive primary bone tumor for children and young adults, and usually occurs predominantly in the long bones<sup>1,2</sup>. In the past several decades, the treatment of primary malignant bone tumors mainly includes the surgical resection of the tumors and high toxicity chemotherapy. Unfortunately, the survival rates of most osteosarcoma patients are poor<sup>2,3,4,5</sup>. Increasing evidences have suggested that the development of osteosarcoma is associated with the regulation of different cancer-related genes. However, the molecular pathogenesis and etiology have not been fully elucidated up to now<sup>6</sup>. Therefore, understanding the mechanisms of functional genes related to osteosarcoma progression and identification is an important goal, which will contribute to the development of molecular targets for future therapy of osteosarcoma<sup>7</sup>.

The inhibitor of growth (ING) gene family includes ING1, ING2, ING3, ING4 and ING5. Members of ING family have generated great interest due to their novel roles as tumor suppressors<sup>8,9</sup>. One of the ING family genes, ING4, has been demonstrated to play important roles in many cancer-related cellular processes including cell proliferation, apoptosis, cycling, migration, angiogenesis, DNA damage and hypoxia<sup>8</sup>. ING4 has also been proposed to bind with p53, NF- $\kappa$ B, and HIF-1 $\alpha$  and regulate their activities<sup>8,10,11,12</sup>. Numerous studies have revealed the suppressive role of ING4 in various cancers, such as glioma, breast cancer<sup>13</sup>, gastric carcinoma<sup>14</sup>, colon cancer<sup>15</sup>, lung cancer<sup>16</sup>, ovarian carcinoma<sup>17</sup>, head and neck squamous cell carcinoma<sup>18</sup>, malignant melanoma<sup>19</sup>, and hepatocellular carcinoma<sup>20</sup>. However, the expression level and functional roles of ING4 in osteosarcoma are still unknown.

Hence, we proposed, in our current study, to study the role of ING4 in human osteosarcoma *in vitro*. Specifically, we first stably transfected human osteosarcoma cells (U-2OS) using an ING4 expression vector.

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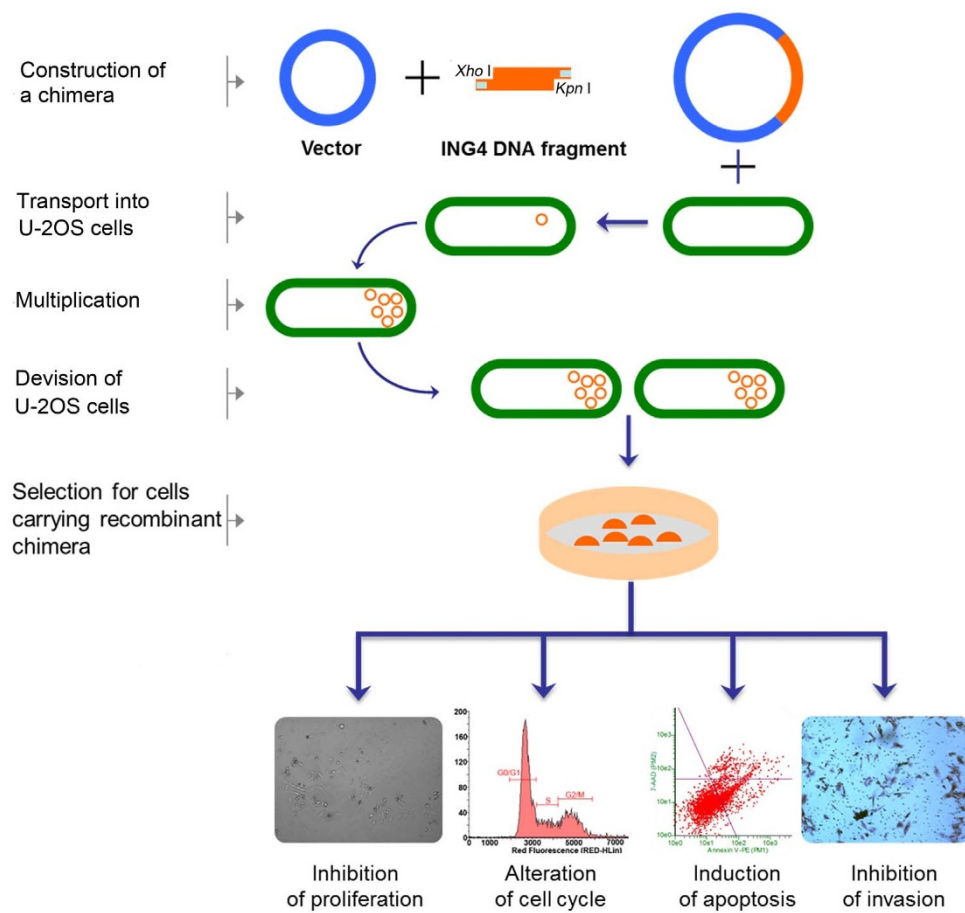
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**Figure 1** | Schematic illustration of the general idea of this study. The ING4 DNA fragments were first cloned into the pEGFP-C2 expression vector using *Xho I* and *Kpn I* restriction enzymes to produce the eukaryotic expression vector pEGFP-ING4. The vector was then transfected into human osteosarcoma cells U-2OS, and stable transfectants of pEGFP-ING4 with successful plasmid transfection were selected. Finally, the effects of overexpressed ING4 on the proliferation, cell cycle, invasion and apoptosis of U-2OS cells were evaluated.

We then determined the effects of overexpressed ING4 on the proliferation, invasion and apoptosis of U-2OS cells (Fig. 1). We also made an attempt to understand the potential mechanisms of ING4 in regulating the signaling pathway in U-2OS cells. This study will also find out whether it is possible to employ ING4 gene therapy for treating human osteosarcoma.

## Results

**ING4 overexpression in U-2OS cells by stable transfection.** We over-expressed ING4 in human osteosarcoma cell line U-2OS and stable transfectants were selected by kanamycin. U-2OS cells with positive green fluorescence were observed in stable transfectants of either pEGFP-ING4 vector or control vector (Fig. 2B, C) but were not observed in un-transfected cells (Fig. 2A). qRT-PCR and western blotting analysis were used to evaluate ING4 expression, and the results showed that both the mRNA (Fig. 2D) and protein (Fig. 2E) expression level of ING4 were significantly over-expressed in U-2OS cells with stable ING4 expression compared with un-transfected U-2OS cells ( $p < 0.01$ ) and U-2OS cells transfected with control vector ( $p < 0.01$ ).

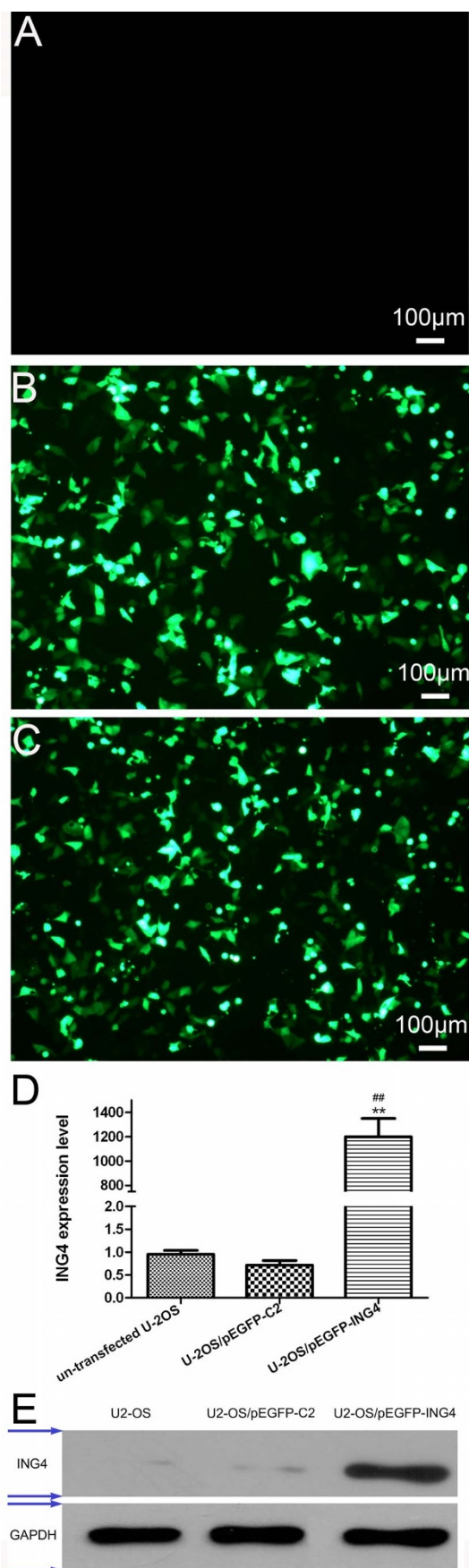
**Inhibition of osteosarcoma cell proliferation by ING4.** Cell proliferation was compared between U-2OS cells that were stably transfected by pEGFP-ING4 vector or control vector and those not transfected. As shown in Fig. 3, after U-2OS cells were cultured for 2, 3 and 4 days, the proliferation of U-2OS cells with stable ING4 overexpression exhibited a slight downward trend, and was

significantly lower than the proliferation of the control vector group and un-transfected group on day 3 and day 4 ( $p < 0.05$ ), suggesting that expression of ING4 inhibited the proliferation of U-2OS cells. The highest inhibitory rate of ING4 stably-expressed group on cell proliferation ( $82.4 \pm 3.25\%$ ) was reached on day 4. Besides, the microscope images showed that cells with stable ING4 overexpression presented shrinkage and a reduction in numbers (Fig. 3). However, there were no significant differences in the cell growth curve between the control vector group and un-transfected group. These results suggested that increased ING4 level could significantly inhibit the proliferation of osteosarcoma cells.

### Induced osteosarcoma cell cycle arrest and cell apoptosis by ING4.

To explore the potential mechanism by which ING4 suppressed cell growth, cell cycle distribution analysis by flow cytometry was performed for stably transfected and un-transfected cells. A significant increase in G0/G1 phase and reduction in S phase were observed in U-2OS cells transfected with ING4 vector, but not with the control vector group and un-transfected group (Fig. 4A,  $p < 0.05$ ). This result indicated that the overexpression of ING4 inhibited osteosarcoma cell proliferation via inducing G0/G1 phase arrest and S phase reduction.

Cell apoptosis evaluated by flow cytometry revealed that cells with stably-expressed ING4 displayed higher early apoptosis rate and the number of apoptotic cells in the ING4 transfection group was significantly higher than that in the control vector group and un-transfected group (Fig. 4B). 25.2% of U-2OS cells with stable ING4



**Figure 2 |** ING4 expression in osteosarcoma cells. Osteosarcoma cells U-2OS were transfected with pEGFP-ING4 expression vector and pEGFP-C2 control vector, respectively. Stable clones were selected with kanamycin

and checked under fluorescence microscopy (A: Un-transfected U-2OS cells; B: U-2OS cells transfected with pEGFP-C2 control vector; C: U-2OS cells transfected with pEGFP-ING4 vector). D: The mRNA expression level in U-2OS cells transfected with ING4 was increased significantly in comparison to the control vector group and un-transfected group quantified by qRT-PCR method. E: The ING4 protein expression level in U-2OS cells transfected with ING4 was increased significantly in comparison to the control vector group and un-transfected group analyzed by Western blot method. ING4 mRNA expression in the stably ING4-transfected U-2OS cells was increased significantly in comparison to the control vector group and un-transfected cells (D,  $p < 0.01$ ). Blots for different proteins were cropped and vertically stacked into one image with white area separated in between different proteins. Blue arrows indicate the horizontal cropping lines. All gels were run under the same experimental conditions. All data represent the mean  $\pm$  standard deviation of three independent experiments. \*\*: compared to the un-transfected group ( $p < 0.01$ ), #: compared to the cells transfected with control vector ( $p < 0.01$ ).

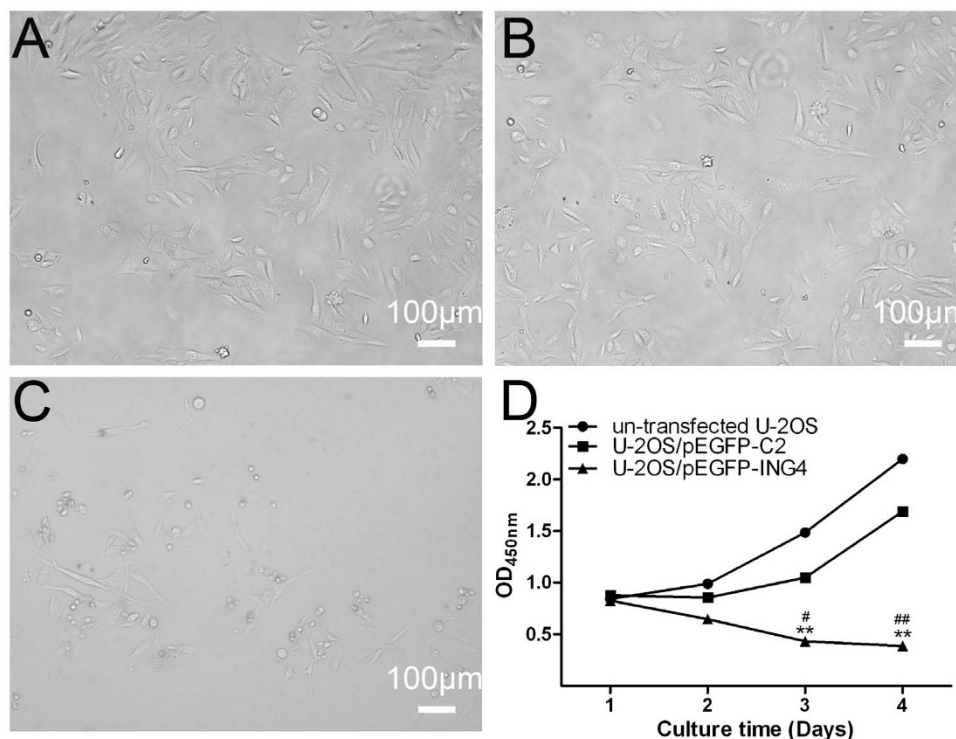
expression showed apoptosis, whereas only 3.7% of U-2OS cells transfected with control vector and 3.1% of un-transfected cells underwent apoptosis ( $p < 0.05$ ). These results indicated that ING4 could induce apoptosis of osteosarcoma cells *in vitro*.

**Suppression of osteosarcoma cells invasion by ING4.** The effects on the cell invasion of ING4 overexpression were evaluated by 24-well plate Matrigel chambers. The invasion ratio was represented as the ratio between cells invading through Matrigel membrane and those migrating through the control membrane. The crystal violet staining results and microscopic photographs (Fig. 5) showed that the number of ING4 over-expressed U-2OS cells invading through the Matrigel was reduced greatly, and the percent of invading cells decreased significantly in comparison to the control vector group and un-transfected group ( $p < 0.05$ ). However, the differences between the control vector group and un-transfected group were not significant (Fig. 5D), indicating that ING4 significantly suppressed the invasion of osteosarcoma cells *in vitro*.

**ING4 regulation of the expression of genes and proteins involved in the apoptosis, cell cycle and cell invasion.** We then proceeded to investigate the effects of ING4 on the expression of target genes and proteins involved in the apoptosis, cell cycle and cell invasion of osteosarcoma cells, as well as to study the molecular mechanism by which ING4 influenced the phenotype of osteosarcoma cells. Towards this goal, we measured the mRNA expression levels of p21, p53, Bax, Bcl-2, NF- $\kappa$ B, caspase-3, IL-8, IL-6, Cyclin D1, Cyclin E1, MMP-2 and MMP-9, which were associated with cell apoptosis, cycle and invasion, using qRT-PCR analysis. We also determined the protein expression levels of p65, Bax, p21, and IL-6 by western blotting analysis. The qRT-PCR results showed that over-expressed ING4 increased the mRNA levels of p21, Bax, caspase-3, but decreased the mRNA levels of p53, NF- $\kappa$ B, IL-6, Cyclin D1, Cyclin E1, MMP-2 and MMP-9 significantly (Fig. 6). The ratio of Bcl-2/Bax was also decreased significantly in the ING4-transfected cells due to the overexpression of ING4 in comparison to the cells transfected with the control vector ( $p < 0.05$ , Fig. 6). However, the mRNA levels of Bcl-2 and IL-8 did not change significantly. Western blotting results revealed that increased ING4 level reduced the p65 and IL-6 levels, but obviously increased the Bax and p21 levels (Fig. 7).

## Discussion

As a member of the ING family, ING4 plays a critical role in tumorigenesis of different tumors. Increasing evidence has shown that down-regulation of ING4 gene expression or deletion of ING4 gene was associated with the progression of high-grade tumors and poor prognosis of tumors, such as lung cancer<sup>21</sup>, brain tumor<sup>22,23,24</sup>, colorectal cancers<sup>25</sup>, and ovarian cancer<sup>17</sup>. In addition, recent studies have



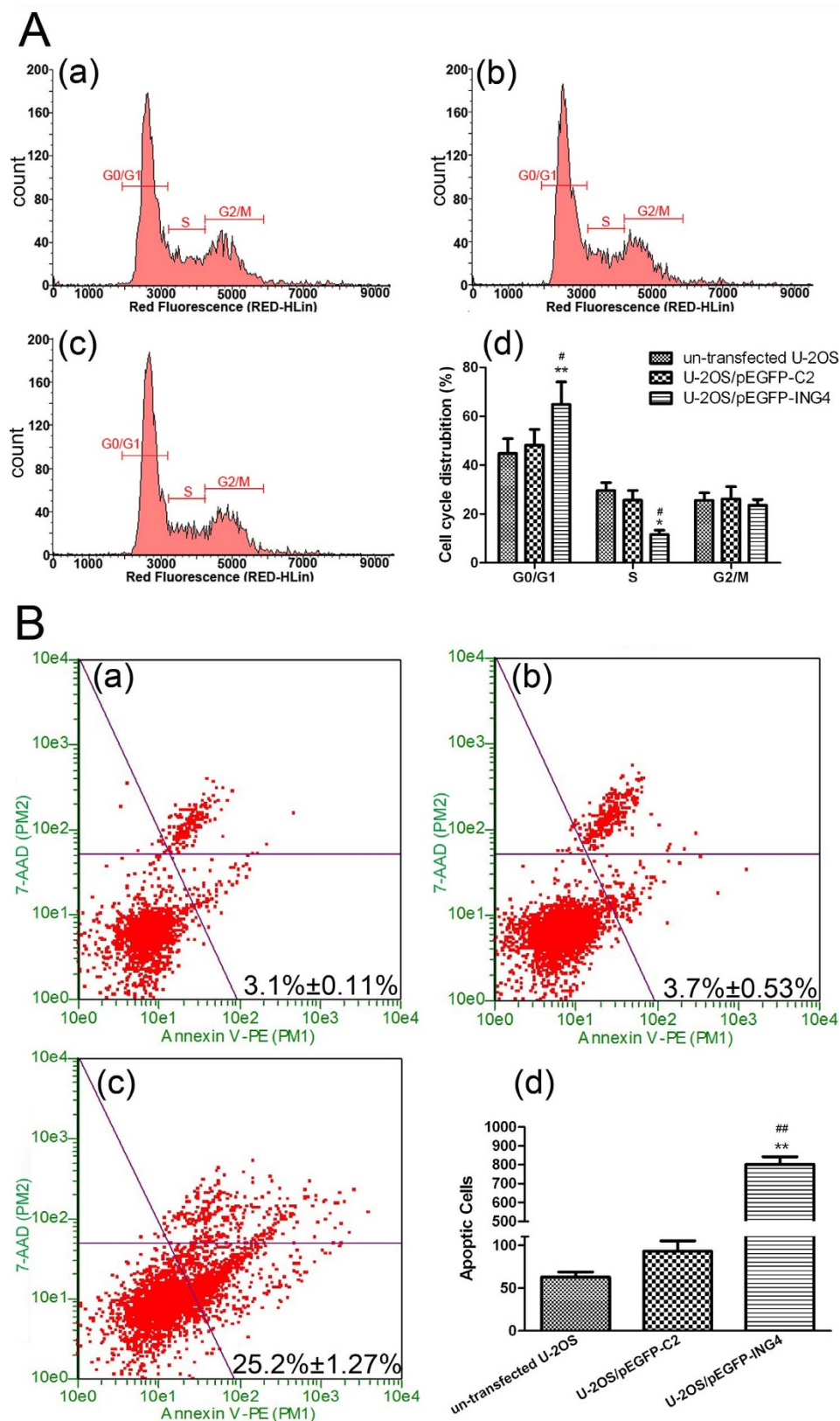
**Figure 3 | The role of ING4 in inhibiting the proliferation of osteosarcoma cells.** The number of viable cells was evaluated on day 1, 2, 3 and 4 by CCK-8 assay. Cell morphology was observed by inverted phase contrast microscope on day 3, and the microscopy image showed cells with stable ING4 presented shrinkage and a reduction in numbers (A: un-transfected U-2OS cells, B: U-2OS cells transfected with pEGFP-C2 control vector, C: U-2OS cells transfected with pEGFP-ING4 vector. Magnification:  $\times 100$ ). Cell growth curve (D) showed that the U-2OS cells with stable ING4 expression exhibited significant decrease in cell proliferation on day 2, 3 and 4 when compared with cells expressing pEGFP-C2 and un-transfected cells. D also showed that the highest inhibitory rate ( $82.4 \pm 3.25\%$ ) was reached on day 4 (D,  $p < 0.01$ ). All data represent the mean  $\pm$  standard deviation of three independent experiments. \*: compared to the un-transfected cells, #: compared to the cells transfected with control vector. One symbol,  $p < 0.05$ ; 2 symbols,  $p < 0.01$ .

also demonstrated that ING4 level is closely linked with the survival, proliferation<sup>26</sup>, apoptosis, invasion, and metastasis of tumor cells through regulating different signaling pathways<sup>19</sup>. It appears that up-regulation of ING4 may be a promising therapeutic target of many tumors. However, there have been no relevant studies about the exact roles of ING4 in human osteosarcoma as well as the relevant molecular mechanism. In the present study, we established the stable human osteosarcoma cells with sustained over-expression of ING4, and detected that stably overexpressed ING4 inhibited cell proliferation and invasion, induced cell apoptosis, and arrested cell cycle in G0/G1 phase in U2-OS cells. In addition, the exogenous ING4 influenced the mRNA and protein expression level of target genes related to apoptosis, cell cycle and cell invasion. All of the results imply that ING4 can inhibit the progression of cancer cells in osteosarcoma.

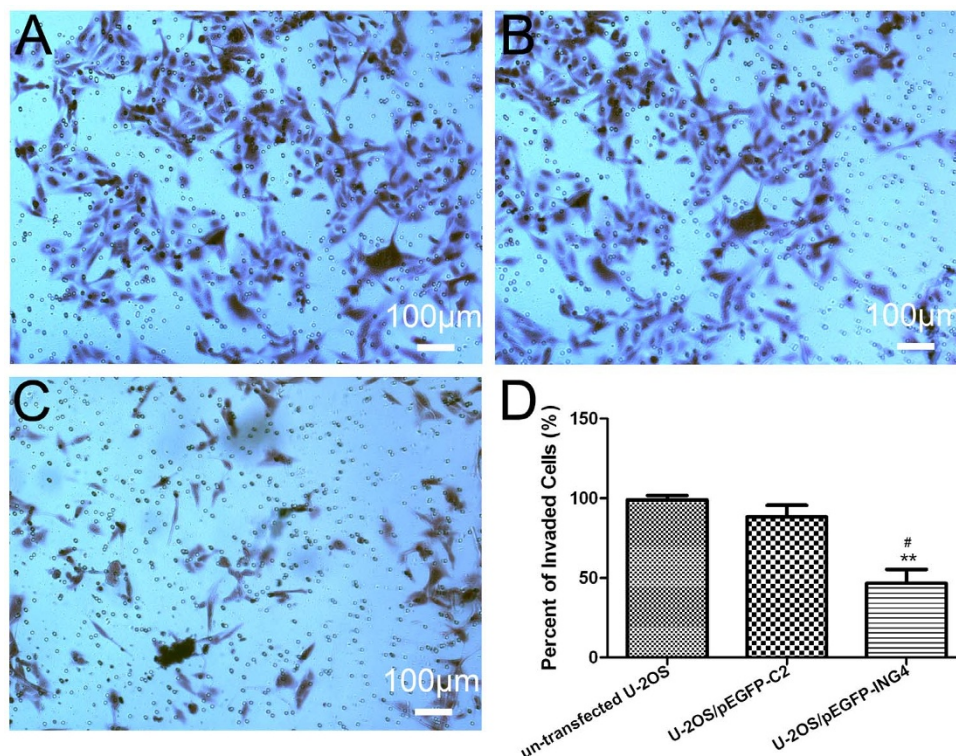
It is crucial to estimate the cell proliferation for studying the behavior of tumor. To investigate the cytotoxic effect of ING4 on human osteosarcoma cells *in vitro*, the growth of U-2OS cells was examined on the daily basis for 4 days using CCK-8 assay. The pEGFP-ING4 transfection significantly inhibited the U-2OS cell growth in a time-dependent manner compared to the control and un-transfected groups (Fig. 3), indicating that ING4 transgene expression efficiently suppressed osteosarcoma cell growth *in vitro*. To further explore the mechanism of inhibiting osteosarcoma growth *in vitro* by ING4, the flow cytometry assay was employed to measure the cell cycle parameters. We found a possible involvement of ING4 in the G0/G1 cell cycle checkpoint (Fig. 4A), further supporting that the suppression of the growth of osteosarcoma cells by ING4 possibly arose from a block in the S phase and an arrest in G0/G1 phase. It is well known that cyclins, cyclin-dependent kinases (cdks) and cdk inhibitors are crucial for cell cycle progression<sup>27</sup>. The activities of this cell cycle protein

family are negatively regulated by the cdk inhibitors through preventing cdk's phosphorylation<sup>28,29,30</sup>. As a member of the cdk inhibitors, p21 can regulate pRb phosphorylation or inhibit cyclin D1 and cyclin E activity<sup>31</sup>, which play key roles in regulating the entry of cells at the G1/S transition checkpoint<sup>32,33</sup>. Thus, we examined the effects of ING4 on several cell cycle regulatory proteins, including p21, cyclin D1 and cyclin E. The mRNA and protein expression levels of p21 were notably increased as shown in Fig. 6 and 7, respectively. However, the mRNA expression levels of cyclin D1 and cyclin E were significantly decreased (Fig. 7), suggesting that ING4 induced G0/G1 arrest in osteosarcoma cells via the up-regulation of cdk inhibitor p21. On the other hand, ING4 has also been reported to bind with other important transcription factors to modulate their activities such as p53<sup>34</sup>. Shiseki *et al* reported that ING4 induced the apoptosis of RKO colon cancer cell line in a p53-dependent manner<sup>33</sup>. We found that ING4 overexpression caused the reduction of p53 expression in osteosarcoma cells significantly (Fig. 6). Hence, we presumed that p21 up-regulation induced by ING4 in U-2OS cells was also in a p53-independent manner<sup>35</sup>.

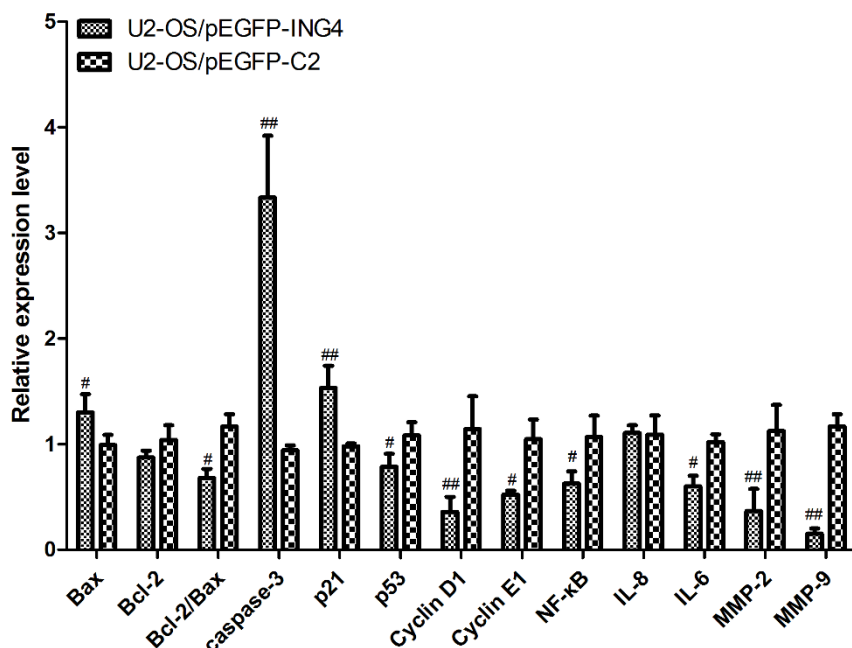
To investigate the apoptotic effect of ING4 overexpressed in U-2OS cells, cell apoptosis was examined using flow cytometry to detect labeled Annexin-V FITC/7-AAD and qRT-PCR analysis was employed to determine the expression of apoptosis-related factors. The results showed that ING4 could induce apoptosis significantly (Fig. 4B) via regulating the expression level of Bcl-2 family genes. The Bcl-2 family proteins include well-characterized regulators of apoptosis, consisting of anti-apoptotic member Bax and pro-apoptotic member Bcl-2<sup>36,37</sup>. Bax is thought to induce the opening of the mitochondrial voltage dependent anion channel. The pro-apoptosis functions of Bcl-2 family are possibly implemented through the release of Cyt-c from mitochondria and the activation of caspases 3, which in



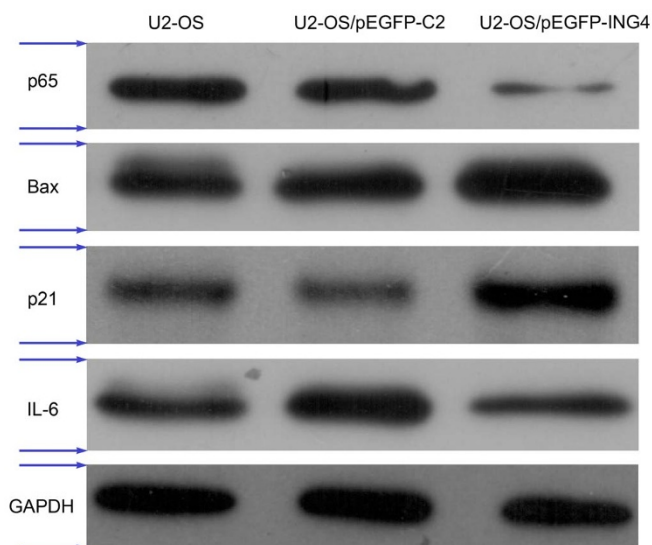
**Figure 4** | Roles of ING4 in cell cycle and apoptosis of osteosarcoma cells. (A) Cell cycle alteration induced by over-expression of ING4 in U-2OS cells according to the cell cycle analysis using PI staining. The number of cells in S phase was reduced obviously ( $p < 0.05$ ), and G0/G1 arrest was observed in the U-2OS cells transfected with ING4 (c) compared with control vector group (b) and un-transfected group (a). (B) Apoptosis induced by over-expression of ING4 in U-2OS cells, which was determined by an assay where cells were double-stained with Annexin V-PE/7-AAD. The percentage of apoptotic cells was significantly higher in pEGFP-ING4 group (c) than that in control vector group (b) and un-transfected group (a) ( $p < 0.01$ ). All data represent the mean  $\pm$  standard deviation of three independent experiments. \*\*: compared to the un-transfected group ( $p < 0.01$ ). #: compared to the cells transfected with control vector ( $p < 0.01$ ).



**Figure 5** | Effects of ING4 on the invasion of osteosarcoma cells. Invading cells were stained with crystal violet and visualized by inverted phase contrast microscope (A: un-transfected U-2OS cells, B: U-2OS cells transfected with pEGFP-C2 control vector, C: U-2OS cells transfected with pEGFP-ING4 vector, magnification:  $\times 100$ ). The invasion percent of U-2OS cells was significantly decreased in the cells transfected with ING4 when compared to those transfected with control vector or un-transfected cells (D). All data represent the mean  $\pm$  standard deviation of three independent experiments. \*\*: compared to the un-transfected cells ( $p < 0.01$ ), #: compared to the cells transfected with control vector ( $p < 0.05$ ).



**Figure 6** | Relative expression of top 12 differentially expressed genes in osteosarcoma cells transfected with pEGFP-ING4 or with a control vector pEGFP-C2 using SYBR Green qRT-PCR. In each case, the data were normalized to the expression level of  $\beta$ -actin. A comparison between the cells transfected with pEGFP-ING4 and those transfected with the control vector showed that the average mRNA expression levels of p21, Bax and caspase-3 in the stably ING4-transfected cells were markedly up-regulated, and the levels of p53, NF- $\kappa$ B, IL-6, MMP-2 and MMP-9 were significantly down-regulated for the stably ING4-transfected cells ( $p < 0.05$ ). All data represent the mean  $\pm$  standard deviation of three independent experiments. #: compared to the cells transfected with control vector (One symbol,  $p < 0.05$ ; two symbols,  $p < 0.01$ ).



**Figure 7 | The ING4-induced expression of some target proteins involved in the cell cycle and apoptosis.** Total proteins were extracted from differently treated osteosarcoma cells and analyzed by Western blotting. Over-expression of ING4 decreased the expression of IL-6 and p65, and increased the expression of Bax and p21. Expression of GAPDH was used as an internal control. Blots for different proteins were cropped and vertically stacked into one image with white area separated in between different proteins. Blue arrows indicate the horizontal cropping lines. All gels were run under the same experimental conditions.

turn initiate the mitochondrial apoptotic pathway<sup>38</sup>. Our data showed that ING4 gene overexpression significantly increased the mRNA and protein levels of Bax in U-2OS cells, and decreased the Bcl-2/Bax ratio at the mRNA level (Fig. 6). Caspase-3 was then significantly activated to induce cell apoptosis, suggesting that ING4 could induce the apoptotic progression of U-2OS cells by regulating the activation of the mitochondria pathway.

As one of the complex processes of tumor metastasis, tumor cell invasion is the most difficult problem faced by oncologists. Therefore, the inhibition of cancer invasion is one of the keys to the success of cancer treatment. It should be noted that ING4 is not an alien gene; both bone-related normal cells and cancer cells have ING4 expression. The difference lies in the fact that cancer cells show significantly reduced level of ING4 expression (Fig. S1). It is reported that ectopic expression of ING4 in different types of tumor cell lines decreased both cell spreading and cell migration<sup>39</sup>. ING4 is expressed abundantly by bone cells (Fig. S1), however, bone cancer cells have decreased level of ING4 and increasing its level will allow us to inhibit their migration and destruct them. To clarify the direct effect of ING4 expression on the invasiveness of osteosarcoma cells, a transwell assay was used and a significant reduction in the number of invasive cells was seen when the cells were transfected with pEGFP-ING4 compared with the control vector and un-transfected groups (Fig. 5). We demonstrated that ING4 had novel anti-invasive activities on U-2OS cells for the first time. As proteolytic enzymes, the matrix metalloproteinases (MMPs), especially MMP-2 and MMP-9, are important in the multiple processes of tumor cell invasion<sup>40,41</sup>. In the present study, we found that ING4 overexpression resulted in the significant down-regulation of the MMP-2 and MMP-9 expression levels ( $p < 0.01$ , Fig. 6), which may be the possible molecular mechanism of ING4-mediated anti-invasive activity in U-2OS cells. Additionally, angiogenesis is indispensable for the growth and invasion of cancer cells, as well as a prognostic marker of metastasis<sup>42</sup>. ING4 was shown to inhibit the growth of cancer cells and angiogenesis probably via an IL-6 pathway<sup>23</sup>. Our results showed that ING4 overexpressed in U-2OS cells decreased the IL-6 at both mRNA and

protein levels (Fig. 6 and Fig. 7), which was consistent with the previous study.

Results from various transfection studies have suggested that ING4 protein is implicated in the regulation of the p53 pathway<sup>43,44</sup>. However, our experiments indicated that ING4 in U-2OS cells showed p53-independent functions (Fig. 6), revealing that ING4 may participate in the cellular process of osteosarcoma through other signaling pathways. NF- $\kappa$ B regulates the expression of many genes proposed to govern tumor growth and survival, apoptosis, angiogenesis and invasion<sup>45,46</sup>, such as MMP-2 and MMP-9<sup>47,48,49</sup>, IL-6, COX-2 and CSF-3<sup>24</sup>. It has been suggested that perturbation of NF- $\kappa$ B signaling is important in tumorigenesis. Previous studies have indicated that ING4 regulated the NF- $\kappa$ B signaling pathway in a variety of tumors<sup>11</sup>. In this study, we detected the activity level of NF- $\kappa$ B in U-2OS cells transfected with exogenous ING4. Our qRT-PCR results indicated that ING4 negatively regulated NF- $\kappa$ B in U-2OS cells significantly ( $p < 0.05$ , Fig. 6). NF- $\kappa$ B is a dimeric transcription factor composed of Rel protein family members, and the activation of NF- $\kappa$ B by a number of stimuli induced the translocation of the NF- $\kappa$ B complex into the nucleus. It up-regulates the expression of genes involved in several cellular processes. To verify the role of NF- $\kappa$ B, Western blotting analysis was carried out in this present study and the results indicated that ING4 reduced the NF- $\kappa$ B signaling pathway by directly interacting with the p65 (Rel A) subunit of nuclear factor NF- $\kappa$ B (Fig. 7). It is thus concluded that ING4-mediated osteosarcoma cell proliferation and invasion inhibition may be tightly associated with the blockade of NF- $\kappa$ B signaling pathway and its downstream target proteins in the present experiment. It is interesting that previous studies have shown that the inhibition or loss of p53 expression in cancer cells improved the sensitivity of NF- $\kappa$ B signaling pathway, especially the activity of I $\kappa$ B kinase<sup>50,51</sup>. Based on our results, we can speculate that the change of NF- $\kappa$ B signaling pathway was closely related to the p53 deficiency in U-2OS cells with overexpressed ING4, and NF- $\kappa$ B activity was reduced in cells examined with lost or disrupted p53 function.

Once osteosarcoma begins, rapid bone destruction occurs and osteonecrosis area are surrounded by soft tissue mass, due to the abnormal increase of osteoclasts and rich circulation around tumors<sup>3</sup>. As a result, osteo-matrix around the osteosarcoma is not dense, suggesting that bone-matrix will not inhibit the delivery of intratumorally injected target genes to osteosarcoma. As a tumor suppressor, ING4 would be the target gene in gene therapy of osteosarcoma. Very recently, Xu *et al* reported the use of a viral vector, adeno-associated virus (AAV), to deliver ING4 gene to another osteosarcoma cell line (MG-63) and its derived tumor model<sup>52</sup>. In the present study, we further examined the anti-tumor mechanisms of ING4. First, our work studied not only the inhibition of proliferation and induction of apoptosis by foreign ING4 gene, but also the detailed mechanism by which ING4 mediates tumor invasion, both of which are indispensable for the application of gene therapy to treat osteosarcoma. Second, our work studied the influence of ING4 on the relevant osteosarcoma cell signaling pathways and the downstream target proteins of ING4, such as NF- $\kappa$ B signaling pathway and IL-6 protein, which are closely related to tumor angiogenesis. Third, our work represents the use of non-viral ING4 gene transfer for treating osteosarcoma. Specifically, our work used a commercial biocompatible non-viral transfection agent (X-tremeGENE HP DNA transfection reagent from Roche) and a plasmid vector that has the demonstrated advantages of low immunogenicity and large capacity for therapeutic DNA<sup>53</sup>. AAV as a viral vector has several disadvantages. For example, exogenous gene carried by AAV cannot be integrated into the chromosomes of the cells, and thus long-term sustained effective expression through AAV is not possible<sup>54</sup>. Moreover, AAV has potential toxicity, particularly, when physical or chemical methods are used to assist it to reach the accelerated and enhanced transduction<sup>55</sup>. In addition, AAV carries a single-stranded



DNA genome, which is transcriptionally inactive until it is converted into a double-stranded template, and AAV transduction requires high multiplicity of infection (MOI). Thus the transgene expression is usually delayed, limiting the usefulness of AAV vectors in the applications that require immediate therapeutic intervention such as in treating osteosarcoma. Our group has recently developed a novel non-viral vector that mimics the structure of viruses to achieve a high transfection efficiency and sustained gene expression by means of phage-displayed cell-targeting peptides<sup>56,57</sup>. We will use phage display to select tumor-targeting peptides and integrate these peptides into the virus-like vector<sup>56,58,59</sup> to deliver ING4 gene to osteosarcoma in the future studies.

Taken together, our results indicate that up-regulated ING4 level evokes the inhibition of proliferation and invasion as well as the induction of the apoptosis of osteosarcoma cells, which may be intensely correlated with the activation of mitochondria pathway and blockage of NF- $\kappa$ B signaling pathway. To the best of our knowledge, this is the first study highlighting the important functions and mechanisms of ING4 as a tumor suppressor molecule in human osteosarcoma. It confirms that ING4 can serve as both a potential therapeutic target and a promising prognostic marker for human osteosarcoma. It also suggests that ING4 gene therapy might be effective in treating human osteosarcoma.

In summary, we have constructed an expression vector pEGFP-ING4 and studied the effects of over-expressed ING4 on the proliferation, apoptosis and invasion of the human osteosarcoma cells. The results suggested that the up-regulation of ING4 could significantly suppress the osteosarcoma cell proliferation by a block in the S phase and an arrest in G0/G1 phase, induce cell apoptosis via the activation of the mitochondria pathway, and inhibit cell invasion through the blockade of NF- $\kappa$ B signaling pathway and down-regulation of the expression of its target proteins such as MMP-2 and MMP-9. Therefore, ING4 gene therapy is a promising approach to the treatment of human osteosarcoma.

## Methods

**Cell culture, construction of ING4 expression vector and transfection.** The human osteosarcoma cell line U-2OS was purchased from the Type Culture Collection of the Chinese Academy of Sciences. Cells were maintained in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco) in a humidified 5% CO<sub>2</sub> atmosphere at 37°C. The medium was changed every two to three days.

Total cellular RNA was extracted with Trizol reagent (Invitrogen) from human placenta. The first cDNA strand of ING4 was reverse transcribed with RNA as a template and Oligo d(T) as a primer. Then, the cDNA was amplified with specific primer pairs: 5'-GGCctcgagATGGCTGCGG GGATGTATTG-3' (sequence represented with letters in lower case: *Xho* I) and 5'-GGCggtaccCTATTTCTTCTTCGTTCTT GGGAG-3' (sequence represented with letters in lower case: *Kpn* I). The fragments were cloned into the pEGFP-C2 expression vector (Clontech) using *Xho* I and *Kpn* I restriction enzymes (Takara) to produce the eukaryotic expression vector pEGFP-ING4. The empty pEGFP-C2 was used as a control vector.

The vectors were transfected into human osteosarcoma cells U-2OS using X-tremeGENE HP DNA transfection reagent (Roche) according to the manufacturer's instructions. Since the vector contained enhanced green fluorescence protein (EGFP) gene and kanamycin resistance markers, successful plasmid transfections were observed by fluorescence microscope and stable transfectants of pEGFP-ING4 and pEGFP-C2 were selected by 1  $\mu$ g/ml kanamycin continuously. The stably transfected cells were further verified by real-time polymerase chain reaction (PCR) and western blotting analysis.

**Cell proliferation assay.** U-2OS cells were seeded in 96-well plates (1,000 cells per well) and incubated with RPMI-1640 containing 10% FBS. After incubation for 1, 2, 3 and 4 days, 10  $\mu$ l CCK-8 (cell counting kit) was added into each well, followed by continuous incubation for 4 h. The absorbance value at 450 nm was measured in a microplate reader. Each experiment was carried out in triplicate.

**Flow cytometry assay.** Cell cycle conditions were determined using propidium iodide (PI, sigma) staining by fluorescence-activated cell sorting (FACS) analysis. The cultured cells were harvested and washed in cold phosphate buffered saline (PBS), then fixed in 70% cold alcohol at 4°C overnight and washed with ice-cold PBS again. After stained with PI solution (40 mg/ml) at 37°C for 30 min in the dark, cells were analyzed on a Guava EasyCyte 5HT flow cytometer. The data were analyzed by Guava InCyte Software v2.2.2.

The apoptosis of U-2OS cells was measured by Guava Nexin Reagent (Millipore) according to the manufacturer's protocol. The cultured cells were collected and resuspended in 100  $\mu$ l medium supplemented with 1% FBS. The cells were then incubated with 100  $\mu$ l Annexin V-PE and 7-AAD labeling solution (Millipore) for 20 min at room temperature in the dark. The resultant stained cells were analyzed on Guava EasyCyte 5HT flow cytometer. The data were analyzed by Guava Nexin Software v2.2.2.

**Cell invasion assay.** Osteosarcoma cell invasion assays were evaluated by 24-well chambers inserts with 8  $\mu$ m pores coated with Matrigel (BD). Briefly,  $2 \times 10^4$  cells were seeded into the membrane of the upper Matrigel chamber in 500  $\mu$ l serum-free medium, and the medium with 5% FBS in the lower chamber served as a chemoattractant. 24 h later, cells on the opposite side of the membrane were stained with crystal violet (1 : 1000, Sigma) and photographed. Cells on the membrane were lysed by 10% acetic acid solution and absorbance value at 570 nm was measured. This assay was performed in triplicate.

**Quantitative Real-time PCR (qRT-PCR).** PrimeScript RT Master Mix and SYBR Premix Ex Taq II (Takara) were used for cDNA synthesis and SYBR Green qRT-PCR according to the manufacturer's protocols, respectively. The qRT-PCR assays were performed by Rotor gene Q (Qiagen). All reactions were carried out in triplicate and the qRT-PCR results were analyzed using the Rotor-Gene Real-Time analysis software 6.0. Then relative gene expression was calculated using the 2<sup>- $\Delta\Delta$ ct</sup> method<sup>60</sup>. The cycling protocol were set as follows: 95°C for 15 min, followed by 45 cycles with each cycle made of 95°C for 5 s and 60°C for 30 s. The primers used in the experiments were listed as follows: ING4: F, 5'-GAGGCTGATCTCAAGGAGAA-3' and R, 5'-TCC-ACAGGCATATCCAACAC-3'; p21: F, 5'-GATTAGCAGCGGAACAAGGAGT-3' and R, 5'-GGAGAAACGGGAACCAAGGACA-3'; p53: F, 5'-CAGATTGCAAGT-TCACTGCCACTA-3' and R, 5'-GATGAAGCCTGTGTAAGAACCCTCT-3'; Bax: F, 5'-TTTCTGACGGCAACTTCAACTG-3' and R, 5'-GGAGTCTCACCC-ACCACCCT-3'; Bcl-2: F, 5'-ATTGTGGCCTTCTTTGAGTTCG-3' and R, 5'-CACCTACCCAGCCTCCGTTAT-3'; NF- $\kappa$ B: F, 5'-AGCACGAATGACA-GAGCGTGT-3' and R, 5'-CATGAGCCGACCCAGCTGA-3'; Caspase-3: F, 5'-T CAGGCTGCCGTGGTACAG A-3' and R, 5'-AGCATGGCACAAAGCGACT-GGA-3'; IL-6: F, 5'-CAGACAGCCACTCCTC TTACAG-3' and R, 5'-CTCAT-CTGCACAGCTCTGGCTTG-3'; IL-8: F, 5'-ATGACTTCCAAGCTGGCGTGG-3' and R, 5'-TTATGAATTCTCAGCCCTCTTCA AAA-3'; MMP-2: F, 5'-GCTAT-GGACTTGGGAGAA-3' and R, 5'-TGGAAGCGGAATGAAAC-3'; MMP-9: F, 5'-TCCTGGAGACCTGAGAACC-3' and R, 5'-CGGCAAGTCTCCGAGT-AGTTT-3'; Cyclin D1: F, 5'-CTCGGTGTCTACTTCAAAATGT-3' and R, 5'-TCCTCGCACTTCTGTTCCT-3'; Cyclin E1: F, 5'-CGGCTCGTCCAGGAA-3' and R, 5'-TCATCTGGATCCTGCAAAAAA-3';  $\beta$ -actin: F, 5'-AAAGACCTGT-ACGCCAACAC-3' and R, 5'-GTCATACTCCTGCTT GCTGAT-3'.

**Western Blotting.** Total proteins were extracted by RIPA lysis buffer and protein concentration was determined by BCA protein assay (Peirce). For Western blot analysis, after being heated for 5 min at 95°C in a sample buffer, 20  $\mu$ g of each protein sample was separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore). Membranes were blocked in 3% bovine serum albumin for 2 h and incubated first in primary antibodies (1 : 1000) against ING4, p65, Bax, p21, IL-6 and GAPDH overnight at 4°C and then in horseradish peroxidase (HRP)-conjugated secondary antibodies (1 : 5000), followed by 3-5 washes with Tris-Buffered Saline and Tween 20 (TBST) buffer. Signals were then visualized by enhanced chemiluminescence detection reagents (Millipore) and developed with Kodak films.

**Statistical analysis.** Data were analyzed by SPSS 13.0. Statistical comparisons among groups were evaluated by One-way ANOVA and Post Hoc multiple comparison LSD. The results were presented as means  $\pm$  standard deviation (SD). A p-value less than 0.05 and 0.01 was considered statistically significant and highly significant, respectively.

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## Author contributions

Y.Zhang and C.B.M. conceived the idea. M.L., Y.Zhu, H.B.Z., L.H.L., P.H. and H.X. conducted experiments. M.L., Y.Zhu and C.B.M. analyzed data and wrote the manuscript. M.L. and Y. Zhu contributed equally to this work.

## Additional information

**Supplementary information** accompanies this paper at <http://www.nature.com/scientificreports>

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