

Ablation of galectin-3 induces p27^{KIP1}-dependent premature senescence without oncogenic stress

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Premature senescence induced by oncogenic stimuli or tumor suppressor activation plays opposing roles in tumorigenesis. Here, we propose that galectin-3, a β -galactoside-binding lectin, regulates premature senescence without oncogenic stress. We detected premature senescence, decreased Skp2, and increased p27^{KIP1} expression in galectin-3 knockout MEFs and galectin-3-depleted gastric cancer cells. Interestingly, galectin-3 depletion did not affect other senescence inducers such as p14^{ARF}, p16^{INK4A}, and p21^{WAF1/CIP1}, suggesting that galectin-3-regulated senescence is p27^{KIP1} dependent. We demonstrate that galectin-3 depletion decreases retinoblastoma protein (Rb) phosphorylation (Ser780, Ser807/811), cyclin D1 and CDK4 expression, and E2F1 transcriptional activation. Galectin-3 directly interacts with the cyclin D1/CDK4 complex and promotes hyperphosphorylation of Rb. It also blocks the inhibition of E2F1 transcription, thereby increasing the expression of Skp2 and reducing the stability of p27^{KIP1} to promote the proliferation of gastric cancer cells. Xenograft mice with galectin-3-depleted gastric cancer cells display tumor growth retardation that is reversed by Skp2 overexpression. Increased expression of galectin-3 is also associated with the advanced TNM (tumor, lymph node, metastasis) system, clinicopathological stage, and lymph node metastases. The probability of survival was significantly decreased in gastric cancer patients with galectin-3^{high} p27^{KIP1-low} cells. Taken together, our results show that galectin-3 may accelerate gastric tumorigenesis by inhibiting premature senescence.

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Uncontrolled cellular proliferation is a hallmark of tumorigenesis that often occurs because of the failure of intrinsic tumor suppressor mechanisms such as premature senescence.^{1,2} This process is triggered by the activation of oncogenes, tumor suppressor genes, or the DNA damage response. Aberrant oncogene activation (Ras and Myc) or excessive mitogenic signaling such as growth factor receptor signaling can induce senescence through two different pathways: the ARF/p53/p21 pathway or the p16^{INK4A}/pRb pathway. Tumor suppressor genes such as pRb, PTEN, VHL, and NF1 also induce cellular senescence by suppressing the oncogenes or directly controlling the two pathways.³ These senescence signals induce cells to withdraw from the cell cycle. Such senescent cells are usually larger, flattened out, and display increased lysosomal β -galactosidase activity.⁴

In this study, we demonstrate that galectin-3 inhibits premature senescence and is therefore involved in gastric tumorigenesis. Galectin-3, a type of chimeric 31-kDa galactoside-binding protein, is involved in cell–cell and cell–matrix interactions, pro-mRNA splicing, angiogenesis, tumorigenesis, and metastases.^{5–7} It is well established⁸ that galectin-3 shows widespread distribution among different types of cells and tissues and is found in the nucleus and cytoplasm. It is also secreted extracellularly via the nonclassical pathway and

can thus be found on the cell surface or in the extracellular space. Our research has focused on the functions of nuclear galectin-3 because its expression is associated with poor prognosis of cancer therapy and is known to regulate tumorigenesis by binding transcription factors such as AP-1, TTF-1, SP-1, and TCF-4.^{8–10} We knocked down galectin-3 using small interfering RNA (siRNA) in human gastric cancer cells and observed the induction of premature senescence and changes in the expression of cell cycle regulators such as decreased S-phase kinase-associated protein 2 (Skp2) and increased p27^{KIP1}. Low levels of p27^{KIP1} are known to affect tumor progression and indicate poor prognosis of breast, colorectal, and hematologic malignancies.^{11–13} The expression of p27^{KIP1} is regulated by the ubiquitin system that involves an SCF (Skp1-cullin-F-box protein) ubiquitin ligase complex in which Skp2 acts as a specific substrate-recognizing subunit.^{14,15} Skp2 plays an oncogenic role through the promotion of p27^{KIP1} degradation, and numerous studies have examined the prognostic significance of Skp2 in human cancers.^{16,17} Inactivation of Skp2 cannot induce cellular senescence, but potentiates oncogenic activation and inactivates tumor suppressor gene-induced cellular senescence.¹⁸ Oncogenic stress and Skp2 inactivation-driven senescence depend on Atf4, p27^{KIP1}, and p21^{WAF1/CIP1}, but not on the

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Abbreviations: Skp2, S-phase kinase-associated protein 2; Rb, retinoblastoma; TNM, tumor, lymph node, metastasis; WT, wild type; KO, knockout; MEF, mouse embryonic fibroblast; siRNA, small interfering RNA; CRD, carbohydrate-recognition-binding domain

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ARF-p53 pathway. Interestingly, downregulation of galectin-3 reduces Skp2 expression and only induces p27^{KIP1} expression. Although p27^{KIP1} has been implicated in senescence, its precise role in this process is unclear.

In this study, we prepared galectin-3 knockout (galectin-3^{-/-}) mouse embryonic fibroblasts (MEFs) and galectin-3-depleted gastric cancer cells and determined that galectin-3 is dependent on p27^{KIP1} for the regulation of premature senescence without oncogenic stress. We used mice xenografted with gastric cancer cells to examine the gene function in gastric tumorigenesis. In addition, we analyzed the clinicopathological correlation between the expression of galectin-3, Skp2, and p27^{KIP1} in gastric cancer patients.

Results

Depletion of galectin-3 induces G1 cell cycle arrest in a p27^{KIP1}-dependent manner. We screened 12 human gastric cancer cell lines to examine the levels of galectin-3. We also determined the relationship between the expression of galectin-3 and the tumorigenicity/metastatic potential of gastric cancer cell lines. We chose the AGS (high expression of galectin-3) cell line to attempt galectin-3 depletion and the SNU-638 (galectin-3 null) cell line for the study of galectin-3 upregulation (Supplementary Figure 1). Treatment of AGS cells with 10 μM galectin-3 siRNA (Gal3 siRNA) for 2 days reduced cell proliferation by 50% (Figure 1a). Interestingly,

galectin-3 depletion was accompanied by decreased Skp2 and increased p27^{KIP1} expression. Although the expression of p16^{INK4A} and p21^{WAF1/CIP1} remained unchanged, p14^{ARF} levels were found to decrease slightly (Figure 1b). Galectin-3 depletion reduced Skp2 mRNA but increased p27^{KIP1} protein expression without a change in the mRNA expression. This suggests that galectin-3 regulates the level of p27^{KIP1} protein by Skp2-mediated ubiquitin activity (Figure 1c). Moreover, the depletion of galectin-3 significantly increased the number of G₁ cell cycle-arrested cells and the sub-G₁ populations, whereas co-depletion of galectin-3 and p27^{KIP1} produced equivalent proliferation to scRNA control group cell populations (Figures 1d and e).

Increased premature senescence and decreased cell proliferation in galectin-3^{-/-} MEFs.

The growth and premature senescence of galectin-3^{-/-} and wild-type (galectin-3^{+/+}) MEFs were analyzed using SA-β-galactosidase staining. Galectin-3^{-/-} MEFs showed growth retardation when compared with galectin-3^{+/+} MEFs (Figure 2a). Moreover, 55% of the galectin-3^{-/-} MEFs displayed premature senescence, whereas only 10.8% of the wild-type (WT) MEFs went through senescence at passage 6 (Figure 2b). A significant decrease in the Skp2 mRNA and protein levels was observed in the galectin-3^{+/+} MEFs. Although the mRNA level remained unchanged, there was a detectable increase in the p27^{KIP1} protein levels in

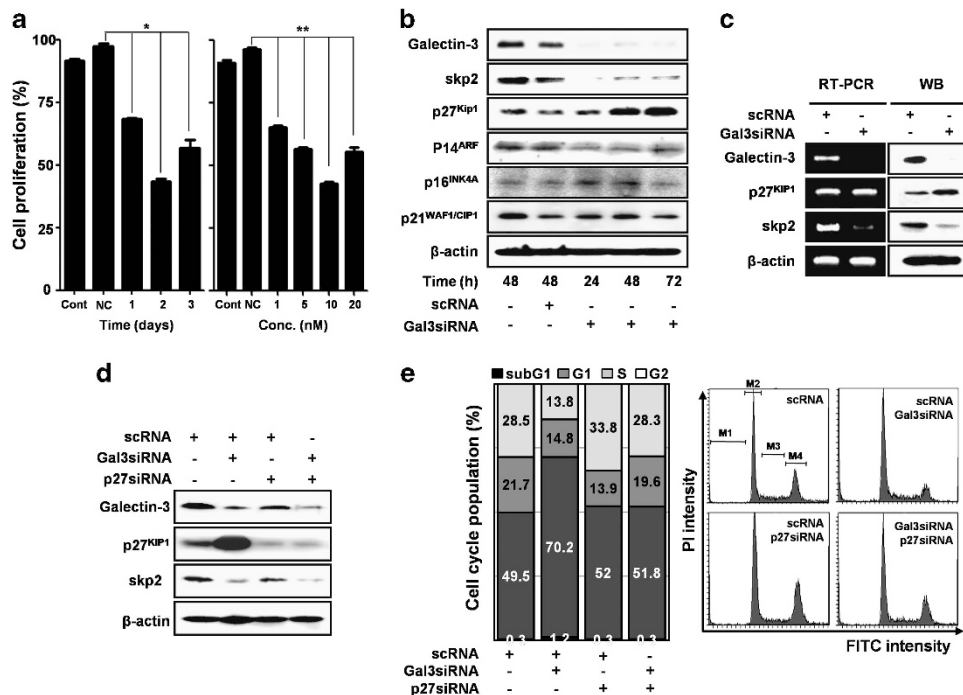


Figure 1 Inhibition of galectin-3 reduces the proliferation of gastric cancer cells in a Skp2- and p27^{KIP1}-dependent manner. (a) Cell proliferation after silencing of galectin-3 using siRNA transfection with respect to time (1–3 days) and concentration (1–20 nM) in the AGS human gastric cancer cells. The error bars indicate 95% confidence intervals; *, **P < 0.0001 using one-way ANOVA. (b) Detection of the levels of galectin-3, Skp2, p27^{KIP1}, p14^{ARF}, p16^{INK4A}, and p21^{WAF1/CIP1} after silencing of galectin-3 using siRNA transfection in the AGS human gastric cancer cells. β-Actin was used as the normalization control. (c) Detection of mRNA and protein expression of galectin-3, p27^{KIP1}, and Skp2 after transfection with galectin-3 siRNA for 48 h, as analyzed by RT-PCR and immunoblotting, respectively. β-Actin was used as the normalization control. (d and e) After transfection of AGS cells with galectin-3 siRNA, p27^{KIP1} siRNA, and co-transfection of AGS cells with both siRNAs (d) the protein expression of galectin-3, p27^{KIP1}, and Skp2 was analyzed by western blotting. β-Actin was used as the normalization control. (e) Cell cycle populations were examined by PI staining. A quantitative graph (left panel) and histogram (right panel) are generated. Marking of histogram is as follows: M₁-SubG₁, M₂-G₁, M₃-S, and M₄-G₂

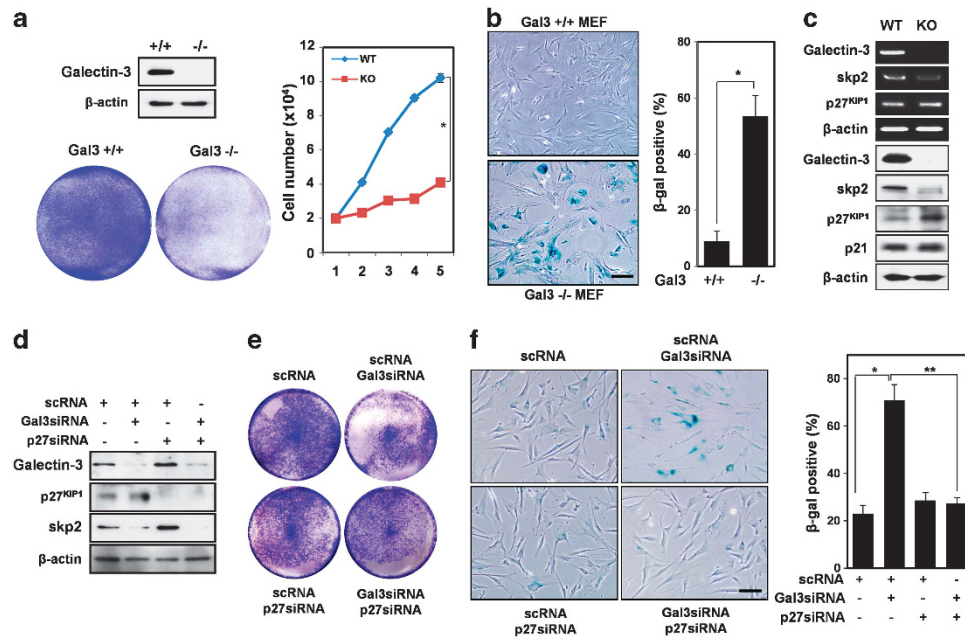


Figure 2 Decreased cell proliferation and increased premature senescence in galectin-3 knockout mouse embryonic fibroblasts (MEFs) and galectin-3-depleted human skin fibroblasts. (a) Expression levels of galectin-3 in galectin-3^{-/-} and galectin-3^{+/+} MEFs were analyzed by western blotting (upper panel). Galectin-3^{-/-} and galectin-3^{+/+} MEFs were stained with crystal violet (lower panel), and the graph shows the percentage of cells in the microscopic fields (right panel). The error bars indicate 95% confidence intervals; **P* = 0.0397 using the two-sided *t*-test. (b) Cell lysates of galectin-3^{-/-} and galectin-3^{+/+} MEFs were prepared and analyzed by RT-PCR using primers specific for p27^{KIP1}, Skp2, and galectin-3. GAPDH served as the normalization control. Protein levels were also detected using antibodies against p27^{KIP1}, p21^{WAF1/CIP1}, Skp2, and galectin-3 by western blotting. β-Actin was used as the normalization control. (c) Premature senescence was induced in galectin-3^{-/-} MEFs. Galectin-3^{-/-} and galectin-3^{+/+} MEFs were stained for β-galactosidase activity. The graph shows the percentage of β-galactosidase-positive cells (right panel). The error bars indicate 95% confidence intervals; **P* < 0.0001 using the two-sided *t*-test. The scale bar indicates 100 μm. (d) After 48 h of transfection with control siRNA, galectin-3 siRNA, p27^{KIP1} siRNA, and both siRNAs in human skin fibroblasts, the cells were stained with crystal violet. (e) Cell lysates were subjected to western blotting using the indicated antibodies. (f) Premature senescence was detected by β-galactosidase activity. The graph shows the percentage of β-galactosidase-positive cells (right panel). The error bars indicate 95% confidence intervals; **P* < 0.0001 and ***P* = 0.0003 using the two-sided *t*-test

galectin-3^{-/-} MEFs. No change in p21^{WAF1/CIP1} expression was observed in either of the cell types (Figure 2c). To further examine the effect of galectin-3 on p27^{KIP1} expression and premature senescence, we prepared human foreskin fibroblasts and silenced the expression of galectin-3 or p27^{KIP1} using specific siRNAs (Figure 2d). Depletion of galectin-3 resulted in growth retardation (Figure 2e) and premature senescence (Figure 2f) in the human foreskin fibroblasts, whereas additional depletion of p27^{KIP1} reversed the effect of galectin-3 on these cells. Thus, galectin-3 appears to inhibit premature senescence through the inhibition of the expression of p27^{KIP1} protein.

Increased premature senescence by inhibition of galectin-3 without oncogenic stress is p27^{KIP1} dependent, but p53 independent. To examine whether premature senescence induced by galectin-3 depletion is affected by p53, we employed two gastric cancer cell lines: AGS (with WT p53) and SNU-601 (p53 is nonfunctional because of the point mutation R273L). Upon galectin-3 depletion, both the cell types exhibited growth retardation and additional p27^{KIP1} ablation reversed this growth retardation (Figures 3a and c and supplementary Figures 2a and c). Similarly, premature senescence, which was accelerated by galectin-3 depletion in both the cell lines, was rescued by the additional p27^{KIP1} inhibition (Figures 3b and d and Supplementary Figures 2b and d). There was no difference in the induction of

premature senescence based on the p53 status, suggesting that galectin-3 depletion induces senescence in a p27^{KIP1}-dependent and p53-independent manner. Galectin-3 depletion alone can induce premature senescence in the gastric cancer cells. Conversely, galectin-3 overexpression can rescue this galectin-3 knockdown-mediated premature senescence (Supplementary Figure 3). Taken together, these results demonstrate that galectin-3 regulates premature senescence in the gastric cancer cells.

Overexpression of galectin-3 or Skp2 induces a decrease in p27^{KIP1} protein expression. When galectin-3 was overexpressed in the galectin-3-null SNU-638 cells, the expression of Skp2 and p27^{KIP1} was found to increase and decrease, respectively; additional Skp2 depletion enhanced the p27^{KIP1} expression (Figure 3e). Although cell proliferation was increased by galectin-3 overexpression, additional Skp2 depletion restored it to the control levels (Figure 3f). Increase in the G₁ cell cycle population induced by Skp2 depletion was also rescued by galectin-3 overexpression (Figure 3g).

We also prepared an Skp2 overexpression vector and transiently transfected it into the SNU-638 cells. Increased p27^{KIP1} expression resulting from galectin-3 depletion was significantly diminished by Skp2 overexpression (Figure 4a). Although cell proliferation increased by Skp2 overexpression, it was not significantly affected by galectin-3 depletion (Figure 4b). An increase in the G₁ cell cycle population

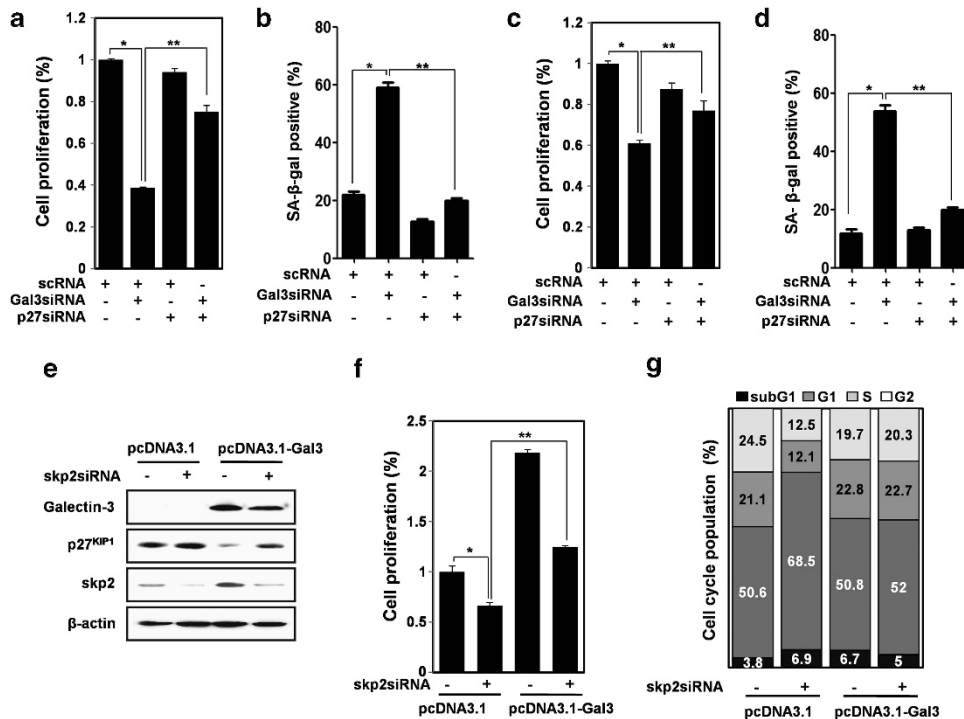


Figure 3 Increased premature senescence caused by the inhibition of galectin-3 is p27^{KIP1} dependent, but p53 independent. (a) After 48 h of transfection with control siRNA, galectin-3 siRNA, p27^{KIP1} siRNA, and both siRNAs in the (a and b) AGS cells and (c and d) SNU601 cells, (a) cell proliferation was evaluated. The error bars indicate 95% confidence intervals; * $P < 0.0001$ and ** $P = 0.0047$ using a two-sided *t*-test. (b) Premature senescence was detected by β -galactosidase activity. The graph shows the percentage of β -galactosidase-positive cells. The error bars indicate 95% confidence intervals; * $P < 0.0001$ and ** $P < 0.0001$ using the two-sided *t*-test. (c) Cell proliferation was determined and the data were presented as a histogram. The error bars indicate 95% confidence intervals; * $P < 0.0001$ and ** $P = 0.0139$ using the two-sided *t*-test. (d) Premature senescence was analyzed by determining the β -galactosidase activity. The graph shows the percentage of β -galactosidase-positive cells. The error bars indicate 95% confidence intervals; * $P = 0.0008$ and ** $P = 0.0013$ using the two-sided *t*-test. (e–g) After transfection with the galectin-3 plasmids, Skp2 siRNA was additionally transfected into the SNU-638 cells. (e) Expression of the indicated proteins was detected by western blotting. (f) Cell proliferation and (g) cell cycle population analyses were performed by Ez-Cytox assay and PI staining, respectively. The error bars indicate 95% confidence intervals; * $P = 0.0005$ and ** $P = 0.0067$ using the two-sided *t*-test

induced by galectin-3 depletion was also compensated for by Skp2 overexpression (Figure 4c). These data suggest that overexpression of both galectin-3 and Skp2 results in reduced p27^{KIP1} expression and cell cycle progression.

Depletion of Skp2 induces premature senescence through an increase in p27^{KIP1} protein levels. As the ablation of galectin-3 induces premature senescence through a decrease in Skp2, we examined Skp2-dependent p27^{KIP1} expression and premature senescence in the gastric cancer cells. When Skp2 was depleted, p27^{KIP1} expression increased (Figure 4d) and cell proliferation decreased (Figure 4e and Supplementary Figure 4a). However, this decrease was abrogated by p27^{KIP1} depletion. The increase in premature senescence (Figure 4f and Supplementary Figure 4b) and the G₁ cell cycle population (Figure 4g and Supplementary Figure 4c) caused by Skp2 depletion was restored to the control levels by p27^{KIP1} depletion. This suggests that galectin-3-regulated Skp2 is important for the regulation of p27^{KIP1} expression for inducing premature senescence and inhibiting the proliferation of gastric cancer cells.

Inhibition of galectin-3 reduces phosphorylation of retinoblastoma protein (Rb) and expression levels of cyclin D1 and CDK4. In order to determine how galectin-3 regulates Skp2 and p27^{KIP1} expression, we examined the

expression of the G₁/S phase-related genes such as Rb, E2F1, cyclin D1, and CDK4 (Figure 5). Galectin-3 depletion drastically decreased the phosphorylated forms of Rb (Ser 780, Ser 807/811) and the expression of cyclin D1 and CDK4, whereas total Rb and E2F1 levels remained unchanged (Figure 5a and Supplementary Figure 5a). Interestingly, PTEN was slightly increased and k-Ras and c-Myc were decreased by galectin-3 depletion, suggesting that tumor suppressors or oncogenic stresses are not involved in galectin-3-induced premature senescence (Figure 5a). We also determined the cellular localization of these proteins (Supplementary Figure 6a). Galectin-3 depletion decreased the phosphorylated forms of Rb in the nucleus and cytosol. The nuclear E2F1 levels remained unchanged, whereas an increased Rb level was evident only in the nucleus. The nuclear levels of cyclin D1 and CDK4 were decreased. In addition, Skp2 protein levels decreased in both the nucleus and cytosol, whereas p27^{KIP1} level increased with galectin-3 depletion.

Furthermore, the overexpression of galectin-3 increased the levels of phosphorylated Rb (Ser 780, Ser 807/811) and Skp2, and decreased p27^{KIP1} expression (Figure 5b and Supplementary Figure 5b). The expression of cyclin D1, CDK4, k-Ras, and c-myc increased, whereas that of PTEN was decreased by galectin-3 overexpression. We also detected the cellular localization of these proteins during

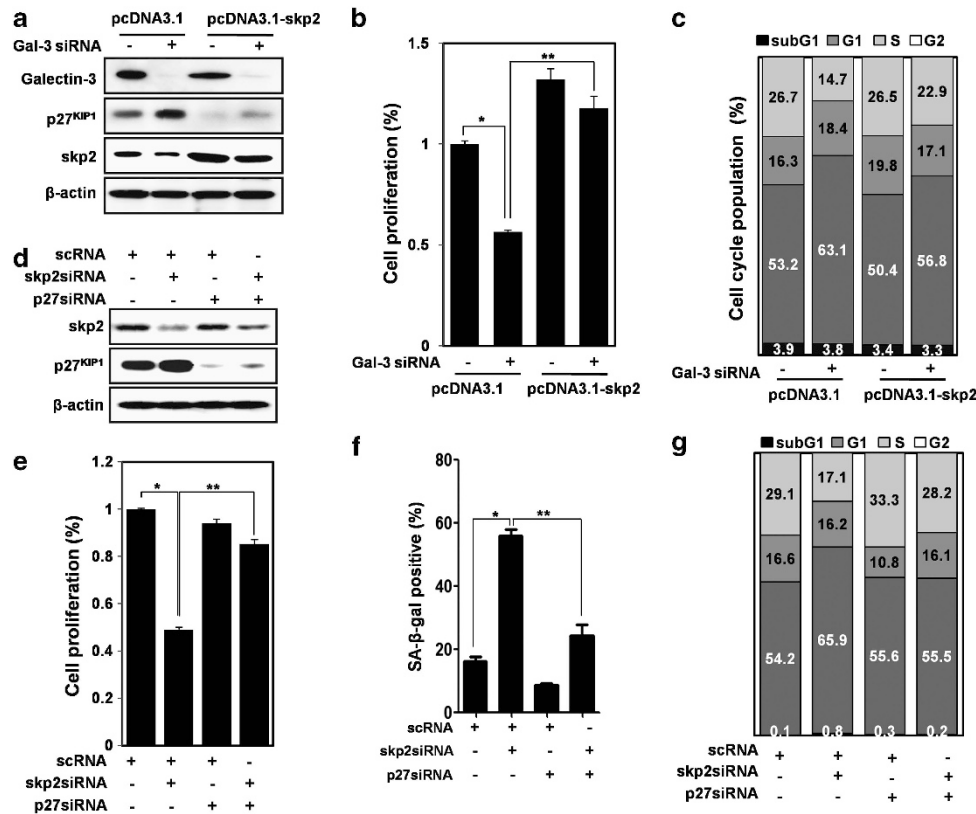


Figure 4 Inhibition of Skp2 reduces cell proliferation and induces premature senescence through an increase of p27^{KIP1} protein expression. (a–c) After transfection with the Skp2 plasmids, galectin-3 siRNA was additionally transfected into the AGS cells. (a) Protein expression, (b) cell proliferation, and (c) cell cycle population analyses were performed by western blotting, Ez-Cytox assay, and PI staining, respectively. The error bars indicate 95% confidence intervals; **P* = 0.0094 using the two-sided *t*-test. AGS cells were transfected with control siRNA, Skp2 siRNA, p27^{KIP1} siRNA, or both siRNAs for 48 h; (d) the protein expression was analyzed by western blotting and (e) cell proliferation was evaluated and the data are presented as a histogram. The error bars indicate 95% confidence intervals; **P* = 0.0005 and ***P* = 0.0156 using the two-sided *t*-test. (f) Premature senescence was detected by β-galactosidase activity. The graph shows the percentage of β-galactosidase-positive cells. The error bars indicate 95% confidence intervals; **P* = 0.0013 and ***P* = 0.0017 using the two-sided *t*-test. (g) The cell cycle populations were analyzed by PI staining

galectin-3 overexpression using the nuclear isolation assay (Supplementary Figure 6b). Taken together, depletion and overexpression of galectin-3 induce a contrasting pattern of expression of these proteins in the nucleus and cytosol (Supplementary Figures 6a and b).

Galectin-3 induces Rb phosphorylation by cyclin D1 and CDK4. Rb phosphorylation induced by galectin-3 overexpression was diminished by the depletion of cyclin D1 and CDK4, without any changes in the Rb expression (Figure 5c). Depletion of cyclin D1 and CDK4 changed the expression of Skp2 and p27^{KIP1}. We prepared several domains of the amino acid sequences of galectin-3 and transiently overexpressed them in the SNU-638 gastric cancer cells (Figure 5d). Although the full-length galectin-3 and 1–110 amino acids (aa) increased Rb phosphorylation and the expression of cyclin D1 and CDK4, the 33–250, 63–250, and 111–250 aa of galectin-3 did not affect the expression of these proteins (Figure 5e). This indicates that 1–32 aa of galectin-3 affect Rb phosphorylation and the expression of cyclin D1 and CDK4.

The N-terminal domain of galectin-3 directly interacts with Rb and the cyclin D1/CDK4 complex. The fact that

1–32 aa of galectin-3 affect Rb phosphorylation prompted us to speculate that the two proteins may physically bind to each other. Using immunoprecipitation, we detected a strong interaction between galectin-3 and Rb and no interaction between galectin-3 and E2F1 (Figure 6a). The cyclin D1/CDK4 complex was also found to interact with galectin-3, suggesting that galectin-3 mediates the phosphorylation of Rb and its interaction with the cyclin D1/CDK4 complex. Overexpression of the full-length galectin-3 and the absence of the carbohydrate-recognition-binding domain (CRD) domain (i.e., 1–110 aa) result in the interaction of galectin-3 with Rb, cyclin D1, and CDK4 (Figure 6b) whereas the overexpression of other domains, that is, in the absence of the N-terminal tail (33–250, 63–250, or 111–250 aa), inhibits the interaction of galectin-3 with these proteins.

Interestingly, the interaction between Rb and E2F1 was strongly detected in the absence of galectin-3, although Rb did not interact with cyclin D1 and CDK4 (Supplementary Figure 7a). The interaction between p27^{KIP1}, cyclin D1, and CDK4 was enhanced by galectin-3 depletion. In addition, overexpression of galectin-3 reduced the interaction between Rb and E2F1 and induced the interaction between Rb and the cyclin D1/CDK4 complex (Supplementary Figure 7b).

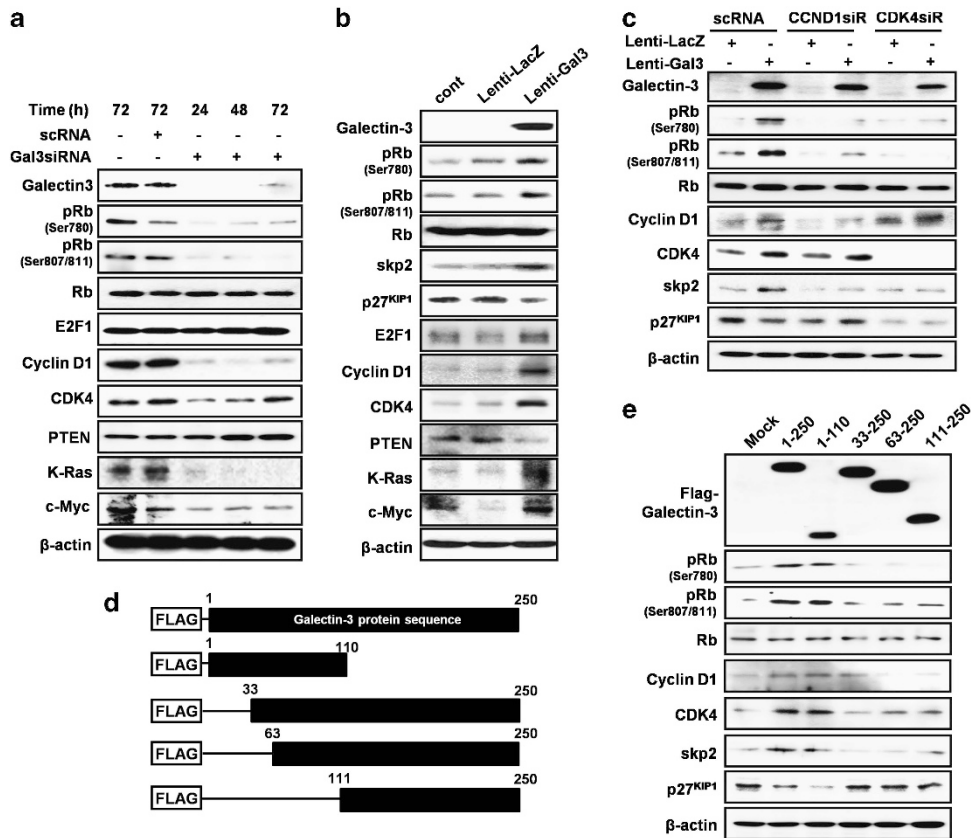


Figure 5 Inhibition or overexpression of galectin-3 regulates the phosphorylation of Rb and expression levels of cyclin D1 and CDK4. (a) Detection of protein levels of ppRb (at Ser 780 and Ser 807/811), Rb, E2F1, cyclin D1, and CDK4 after the depletion of galectin-3 using siRNA transfection in the AGS human gastric cancer cells. (b) Detection of the protein levels of galectin-3, Skp2, Rb, ppRb (at Ser 780 and Ser 807/811), and p27^{KIP1} by western blot analysis in SNU-638 cells infected with a lentiviral construct containing galectin-3 or LacZ as the negative control. (c) Detection of the levels of galectin-3, Skp2, Rb, ppRb (at Ser 780 and Ser 807/811), and p27^{KIP1} by western blot analysis after transfection of cyclin D1 and CDK4 in SNU-638 cells infected with the constructs in (b). (d) Schematic model of Flag-galectin-3 domain (1–250 aa as the full length; 1–110 aa as the N-terminal tail; and 33–250, 63–250, and 111–250 aa as the CRD). (e) Detection of the levels of galectin-3, Skp2, Rb, ppRb (at Ser 780 and Ser 807/811), and p27^{KIP1} by western blot analysis after transfection of the galectin-3 domains in SNU-638 cells. β-Actin was used as the normalization control

The interaction between p27^{KIP1}, cyclin D1, and CDK4 was also decreased by galectin-3 overexpression.

Galectin-3 releases E2F1 to promote its transcriptional activity in a cyclin D1- and CDK4-dependent manner.

We investigated the E2F1 binding motif in the Skp2 promoter by performing the chromatin immunoprecipitation (ChIP) assay (Figure 6c). Galectin-3 depletion was found to suppress the binding of E2F1 to this region. The E2F1 luciferase reporter activity was found to significantly increase by the overexpression of the full-length galectin-3 and the absence of the CRD domain (1–110 aa), but remained unchanged by overexpression of the other domains of galectin-3 (absence of the N-terminal tail of galectin-3 (33–250, 63–250, or 111–250 aa); Figure 6d). Moreover, the E2F1 luciferase activity was decreased by the depletion of galectin-3, cyclin D1, or CDK4 (Figure 6e). Increased E2F1 luciferase activity by galectin-3 overexpression was reversed by cyclin D1 or CDK4 (Figure 6f).

Taken together, our results suggest that galectin-3 directly interacts with Rb and the cyclin D1/CDK4 complex that in turn induces Rb phosphorylation. In addition, galectin-3 releases E2F1 and promotes its transcriptional activity. Increased Skp2

decreases the stability of p27^{KIP1}, thereby promoting cell cycle progression and inhibiting premature senescence (Figure 7d).

Galectin-3 depletion reduces tumor burden that is reversed by the overexpression of Skp2 in mice xenografted with AGS cancer cells.

We prepared stable galectin-3-depleted gastric cancer cells with Skp2 overexpression (Figure 7a) to detect the *in vivo* effects of galectin-3 and Skp2 on gastric tumorigenesis in mice. The gastric tumor size was reduced in galectin-3-depleted xenografted mice; however, Skp2 overexpression restored the tumor growth to the control levels (Figures 7b and c). Immunohistochemical analysis revealed that increased p27^{KIP1} expression and decreased Skp2 expression were observed in the galectin-3-depleted tumor sections, whereas Skp2 overexpression reversed the decrease of p27^{KIP1} expression (Supplementary Figure 8).

Clinicopathological analysis of galectin-3 expression in malignant tissues of gastric cancer patients.

We analyzed the expression of galectin-3 in the malignant tissues of 50 gastric cancer patients using tissue microarrays (Supplementary Tables 1 and 2) and found that galectin-3

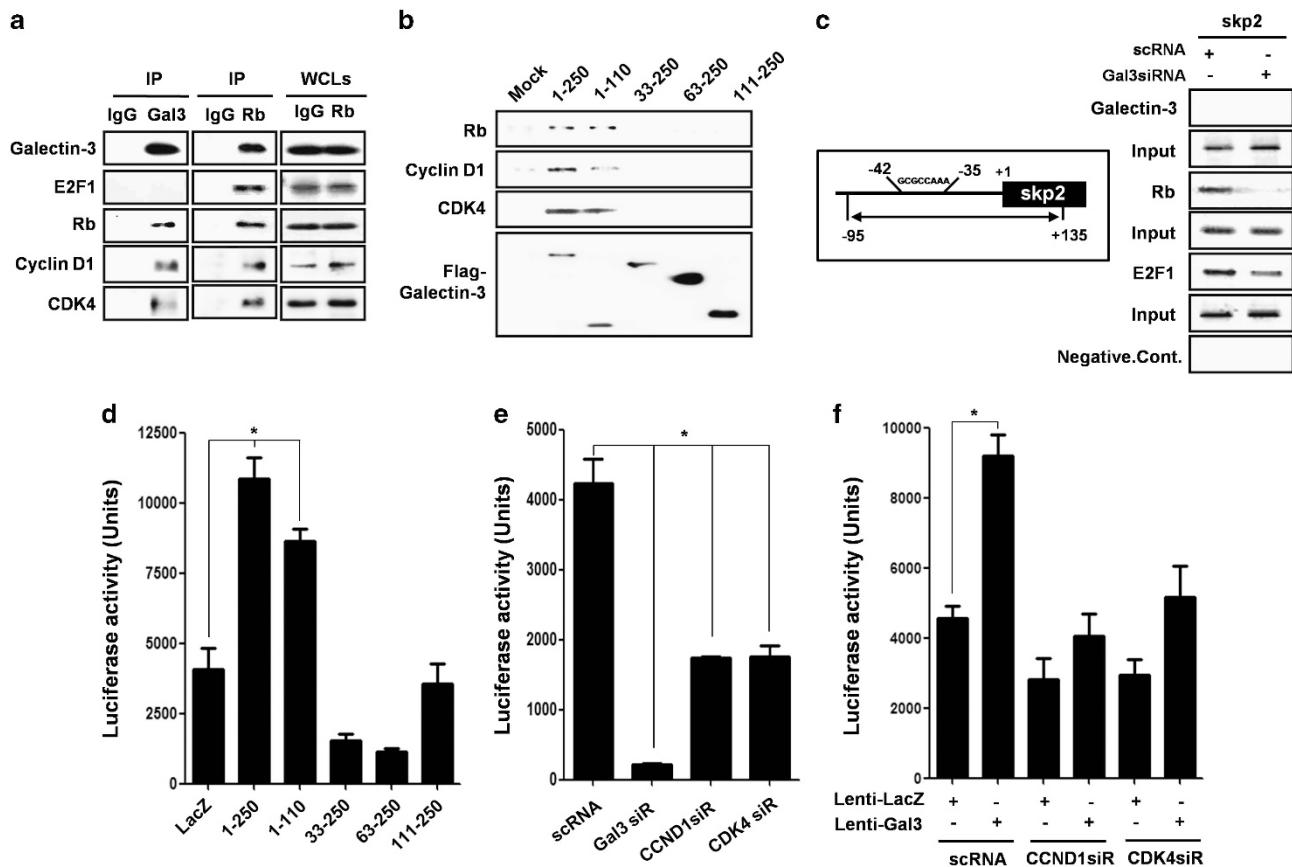


Figure 6 Galectin-3 directly interacts with Rb and regulates the transcriptional activity of E2F1. (a) Immunoprecipitation was performed with antibodies against galectin-3 and Rb to detect the interactions of galectin-3 with E2F1, Rb, cyclin D1, and CDK4 in the AGS cells. Whole cell lysate was used as the positive control. (b) Immunoprecipitation with Flag-galectin-3 domains, Cyclin D1, CDK4, and Rb using transfection of the galectin-3 domains in SNU-638 cells. (c) Schematic model of the interaction of the Skp2 promoter with the E2F1 binding site. ChIP primers were prepared to detect the E2F1 binding site (–42 to –35) from –95 to +135. The ChIP assay was performed using antibodies against galectin-3, Rb, and E2F1 in scRNA- and galectin-3 siRNA-transfected AGS cells. A PCR primer for the Skp2 promoter was used to detect promoter fragments in the immunoprecipitates. The input lane with total genomic DNA was used as a control for the PCR reaction. (d–f) Detection of luciferase activity after transfection of (d) SNU638 cells with an E2F1 consensus plasmid and galectin-3 domains for 48 h, (e) AGS cells with siRNA targeting galectin-3, Cyclin D1, and CDK4, or LacZ as a negative control for 48 h, and (f) galectin-3-overexpressing SNU638 cells with siRNA targeting Cyclin D1 and CDK4 for 48 h. The cells were transfected with 1 μg of the E2F1 consensus plasmid. The error bars indicate 95% confidence intervals; **P*<0.0001 using a two-sided *t*-test. Three independent experiments were performed

expression was statistically correlated with increasing T classification ($P=0.0262$), lymph node metastases ($P=0.001$), and increasing TNM (tumor, node, metastasis) stage ($P=0.0053$). This suggests that increased galectin-3 expression in gastric cancer tissues may indicate poor prognosis.

Positive correlation between the expression of galectin-3 and Skp2 and the significantly decreased probability of survival of gastric cancer patients with tumors showing high galectin-3 and low p27^{KIP1} expression. Initially, we found that compared with normal gastric tissues in 52 gastric cancer patients, the expression of both galectin-3 and Skp2 mRNA was increased in the malignant gastric tissues ($P=0.0001$ and $P=0.0034$, respectively; two-sided χ^2 -test) (Figure 8a and Supplementary Figure 9). Compared with their normal counterparts, malignant tissues showed a 76.9% and 73.1% increase in the expression of galectin-3 and Skp2, respectively (Supplementary Figure 9b), with a combined increase of 77.5%.

Using immunohistochemical analysis of the tissue microarrays, the colocalization of galectin-3 and Skp2 and the inverse relationship between galectin-3 and p27^{KIP1} expression were also evident in the malignant tissues of the gastric cancer patients (Figure 8c). The probability of survival of patients whose tumors showed high expression of galectin-3 and low expression of p27^{KIP1} ($n=24/52$) was significantly lower than that of patients whose tumors showed low expression of galectin-3 and high expression of p27^{KIP1} ($n=6/52$) ($P=0.0417$), as determined by immunohistochemical analysis (Figure 8b). These data support the notion that galectin-3 promotes gastric tumorigenesis by regulating the expression of Skp2 and the stability of p27^{KIP1}.

Discussion

Premature senescence involves the loss of proliferative ability and has been extensively studied with respect to tumorigenesis. It depends on a number of signaling pathways that include tumor suppressors and oncogenes, such as the

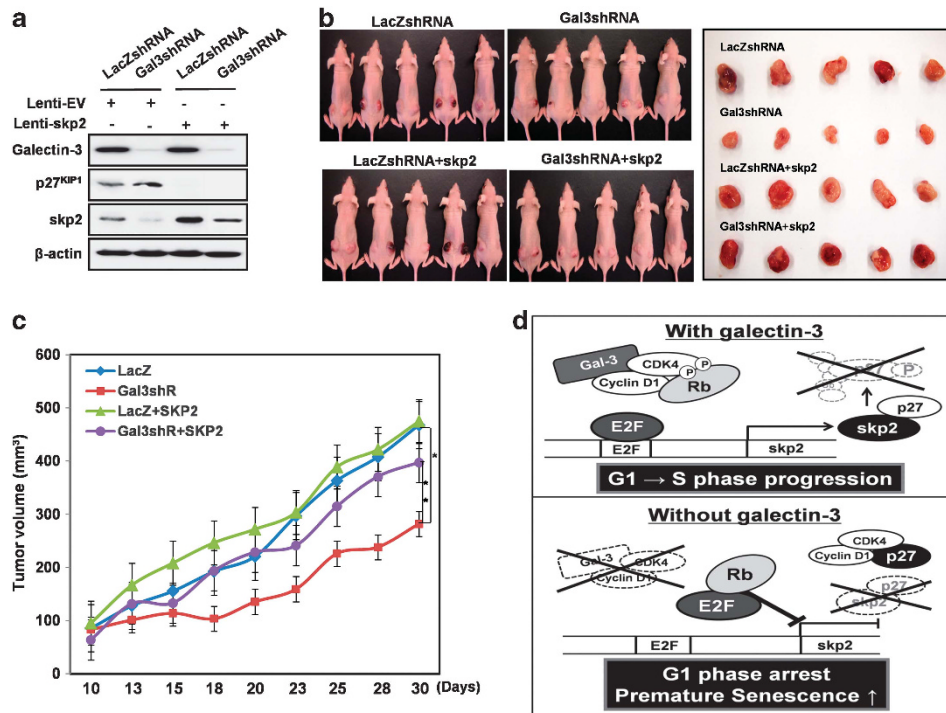


Figure 7 Galectin-3 depletion reduces the tumor burden in gastric cancer cell-xenografted mice and these effects are reversed by overexpression of *skp2*. (a–d) Lentiviruses expressing galectin-3 shRNA and overexpressing *Skp2* were employed to produce stable AGS cell lines. Lentivirus expressing shRNA targeting *LacZ* was used as a control. Mice ($n = 5$ per group) were inoculated subcutaneously into both flanks with 10^6 cells of each of the AGS cell lines. (a) *Skp2* overexpression and galectin-3 depletion were confirmed by western blot analysis. (b) Tumor formation was observed 30 days after inoculation. (c) Tumor formation was quantified by measuring the tumor volume 10 days after inoculation ($n = 5$). The error bars indicate 95% confidence intervals; $*P = 0.001$ and $**P = 0.0015$ using two-sided *t*-test. All statistical tests were two sided. (d) Schematic model of galectin-3-dependent promotion of tumor progression and premature senescence. Galectin-3 interacts with Cyclin D1, CDK4, and Rb and blocks the inhibition of E2F1 transcription. This increases the expression of *Skp2* and reduces the stability of $p27^{KIP1}$ to promote the proliferation of gastric cancer cells. After galectin-3 depletion, Rb interacts with E2F1 to block the transcriptional activity of E2F1, suppress *Skp2* expression, and increase the stability of $p27^{KIP1}$. This results in the suppression of the proliferation of gastric cancer cells and in the induction of premature cellular senescence

$p16^{INK4a}/Rb$ pathway, the ARF/ $p53/p21^{WAF1/Cip1}$ pathway, and the PTEN/ $p27^{KIP1}$ pathway.^{1,19} Oncogenic stress or DNA damage can trigger premature senescence. When the stress exceeds a threshold level, the expression of $p15$ and $p16^{INK4a}$ increases to inhibit cyclin D1-CDK4/6; subsequently, dephosphorylated Rb controls the induction of senescence by repressing the transcriptional activity of E2F.²⁰ ARF is also induced by oncogenic stress and inhibits MDM2, thereby activating $p53$ and $p21^{WAF1/Cip1}$.²¹ In case of the activation of a tumor suppressor, PTEN dephosphorylates AKT and consequently stabilizes $p27^{KIP1}$.¹⁹ Collectively, the induction of cell cycle regulators by oncogenes or tumor suppressors plays a critical role in senescence.

Previously, we showed that galectin-3 regulates the cell cycle in gastric cancer;²² however, the role of galectin-3 in premature senescence is unknown. We demonstrated that galectin-3 depletion caused retarded growth and earlier induction of cellular senescence in galectin-3^{-/-} MEFs than in galectin-3^{+/+} MEFs. Galectin-3 depletion also induces premature senescence in gastric cancer cells. It is worth noting that induction of premature senescence occurs with galectin-3 depletion alone. This finding was unexpected, because galectin-3 is an activator of oncogenes such as Ras or Myc^{23,24} and Akt,²⁵ and galectin-3 increases the levels of $p21^{WAF1/Cip1}$ by direct interaction and stabilization.²⁶ We also confirmed that galectin-3 depletion reduced k-Ras and c-Myc.

Therefore, the mechanism by which galectin-3 regulates premature senescence remains unclear.

In this study, we determined the mechanism of the induction of premature senescence by galectin-3 depletion without oncogenic stimuli. This phenomenon is mediated by galectin-3-induced Rb hyperphosphorylation that is in turn caused by the direct interaction of galectin-3 and the cyclin D1/CDK4 complex. Rb phosphorylation plays an important role in the regulation of Rb activities by CDK complexes.²⁷ Hypophosphorylated Rb actively inhibits cell cycle progression from the G₁ to S phase and also induces premature senescence.²⁸ We show that galectin-3 depletion reduces the expression of both cyclin D1 and CDK4. Previous reports also indicate that galectin-3 augments cyclin D1 expression.²⁹ Therefore, we suggest that galectin-3 increases both the expression and activation of the cyclin D1/CDK4 complex and consequently leads to hyperphosphorylation of Rb.

The Rb tumor suppressor gene is inactivated in many cancers, including gastric cancer.^{30,31} Rb affects tumor progression by regulating the cell cycle, differentiation, and apoptosis. These functions are mediated by the interaction of Rb with other cellular proteins, especially the E2F transcription factors.^{27,32} When galectin-3 hyperphosphorylates Rb through the cyclin D1/CDK4 complex, E2F1 is released from Rb and initiates its transcriptional functions. The direct interaction of galectin-3 with E2F1, a transcription factor of

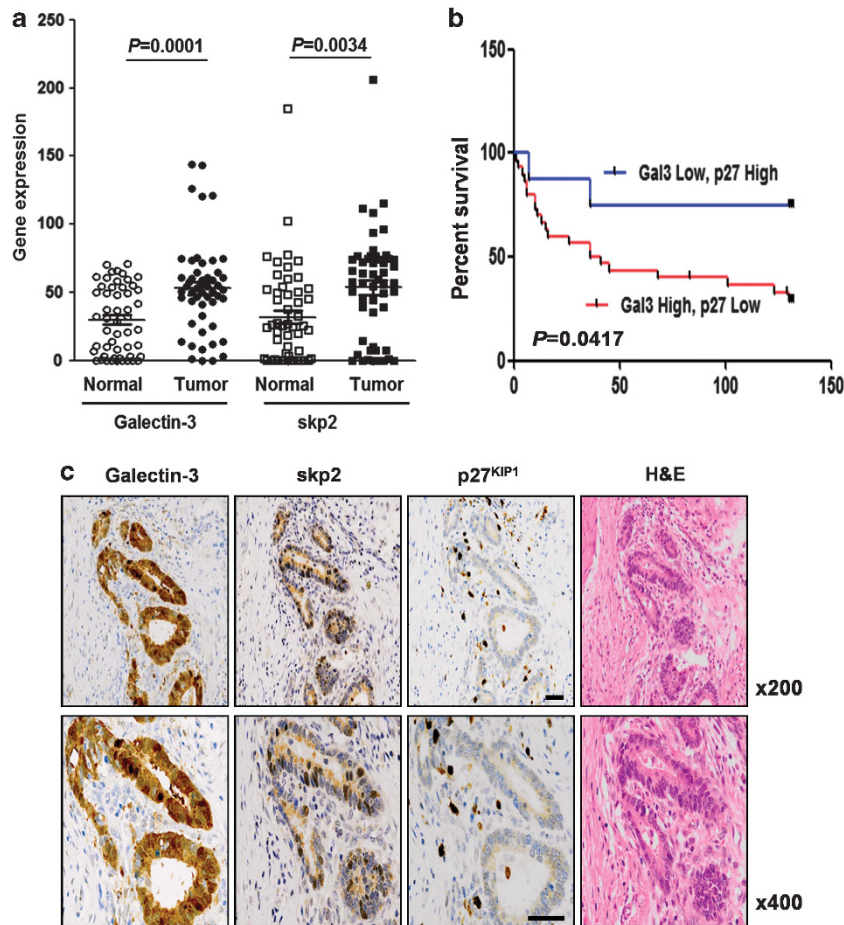


Figure 8 Positive association between galectin-3 and Skp2 expression and negative association between galectin-3 and p27^{KIP1} expression is found in malignant tissues of gastric cancer patients. (a) The correlation between the mRNA expression of galectin-3 and Skp2 in the malignant tissues of 52 gastric cancer patients. The mRNA expression was detected by RT-PCR and quantified by an NIH ImageJ analyzer. GAPDH and β -actin were used as normalization controls. (b) The probability of survival of gastric cancer patients whose tumors show high galectin-3 and low p27^{KIP1} levels is (red line) compared with that of gastric cancer patients whose tumors show low galectin-3 and high p27^{KIP1} levels (blue line) using Kaplan-Meier analysis. Statistical analysis is described in the Materials and Methods section. (c) Protein expression of galectin-3, Skp2, and p27^{KIP1} in the malignant tissues of gastric cancer patients, as shown by immunohistochemical staining (brown) with hematoxylin and eosin (H&E). The stained tissue samples were observed using an inverted light microscope. Magnification: $\times 200$ (top) and $\times 400$ (bottom)

Skp2,¹⁵ is not supported by our data. A direct interaction between Rb and galectin-3 activated E2F1 to generate skp2.

We have shown that galectin-3 regulates the expression of Skp2 and p27^{KIP1} during the induction of premature senescence. Although Skp2/p27^{KIP1} is known to be involved in cellular senescence, it is usually accompanied by the activation of p16^{INK4a} or the tumor suppressor PTEN. Here, galectin-3 directly regulates the expression of Skp2 and p27^{KIP1} without activating p16^{INK4a} or PTEN. This effect seems to be organ specific, and these results are concordant with the clinicopathological data from gastric cancer patients. The levels of galectin-3 were proportional to the progression of the TNM stage and lymph node metastasis, providing strong evidence for the involvement of galectin-3 in the progression and metastasis of gastric cancer. Interestingly, premature senescence is caused by the stabilization of p27^{KIP1} in the absence of galectin-3. In gastric cancer, loss of p27^{KIP1} is associated with advanced stages and decreased survival.^{33,34} We also demonstrate poor survival in gastric cancer patients with high expression of galectin-3 and low

expression of p27^{KIP1}. Although galectin-3 is known to regulate p27^{KIP1} expression,³⁵ the underlying molecular mechanism remains unexplored. Here, we demonstrate that galectin-3 increases the expression of Skp2 and induces degradation of p27^{KIP1} protein, without influencing p27^{KIP1} mRNA levels. Skp2 is known to promote gastric tumorigenesis^{17,36} and block premature senescence.^{18,37} We have demonstrated a parallel expression of galectin-3 and Skp2 in gastric cancer, and our data suggest a molecular mechanism that links galectin-3 and Skp2 to decreased expression of p27^{KIP1} and prevention of senescence.

Taken together, our data suggest that galectin-3 prevents premature senescence and accelerates gastric tumorigenesis. Thus, inhibition of galectin-3 may have therapeutic implications in the treatment of gastric cancer.

Materials and Methods

Cell culture. Twelve human gastric cancer cell lines (AGS, MKN28, YCC-2, KATOIII, SNU-1, SNU-5, SNU-16, SNU-216, SNU-601, SNU-638, SNU-668, and SNU-719) obtained from the Korea Cell Line Bank (KCLB, Seoul, Korea) were

cultured in RPMI-1640 medium supplemented with 5% fetal bovine serum (FBS) and 1% antibiotics. The phenotypes of these cell lines have been authenticated by the KCLB.

Preparation of galectin-3 KO MEFs. C57BL/6 mice with a galectin-3-knockout (KO) allele (male and female mice, homozygous $^{-/-}$), were kindly provided by Dr. FT Liu (UC Davis, CA, USA).³⁸ Female and male N6 galectin-3 $^{+/-}$ mice were crossed to obtain galectin-3 $^{+/+}$ and galectin-3 $^{-/-}$ MEFs. The preparation of MEFs was performed as described previously.³⁹

SA- β -galactosidase assay. The β -galactosidase assay for senescence was performed using a Senescence Detection Kit (#K320-250, BioVision, Mountain View, CA, USA), as previously described.²¹ Briefly, cells were plated in 60-mm dishes, cultured for 2–3 days, washed once with phosphate-buffered saline (PBS), and fixed with a fixation solution for 15 min at room temperature. Cells were washed twice with PBS and incubated with the staining solution overnight at 37°C before microscopic analysis.

RNA isolation and reverse transcription-PCR (RT-PCR). RNA isolation was performed with the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Subsequently, RT-PCR using a Reverse Transcription system (Promega, Madison, WI, USA) was performed as previously described.⁴⁰ The following primers were used: Skp2, forward 5'-CGTGTACAGCACATGGAACCT-3' and reverse 5'-GGGCAAATTCAGAGAA TCCA-3'; LGALS3, forward 5'-ATGGCAGACAATTTTCGCTCC-3' and reverse 5'-ATGTCACCAGAAATCCAGT T-3'; p27^{KIP1}, forward 5'-AGATGCAAACGT GCGAGTG-3' and reverse 5'-TCTCTGCAGTGCTTCTCAA-3'; β -actin, forward 5'-AGCCTCCGCTTTGCCGA-3' and reverse 5'-CTGGTGCTGGGGCG-3'; and GAPDH, forward 5'-GGCTGCTTTAACTCTGGTA-3' and reverse 5'-ACTTGAT TTTGGAGGGATCT-3'. PCR was performed according to the Ex-Taq manual (TaKaRa, Shiga, Japan).

Galectin-3 overexpression: construction of vectors and infection. A galectin-3 overexpression vector (pcDNA3.1/NT-GFP-galectin-3) and lentiviral vectors containing galectin-3 overexpression were constructed by inserting the galectin-3 gene into the lentiviral vector pLL3.7, as described previously.^{5,24}

Western blot analysis and immunoprecipitation. Western blotting was performed using anti-galectin-3, anti-Rb, anti-phospho-Rb (Ser 807/811) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-SKP2, anti-p27^{KIP1}, anti-p21^{WAF1/CIP1}, anti-p15^{INK4B}, anti-cyclin A, anti-cyclin D1, anti-CDK4, anti-E2F1, anti-GAPDH, and anti-lamin A/C antibodies (Cell Signaling, Danvers, MA, USA). The normalization control was anti- β -actin (Sigma, St. Louis, MO, USA). Immunoprecipitation was carried out with A/G agarose beads coated with anti-galectin-3 and anti-Rb (Santa Cruz Biotechnology) antibodies. The proteins were detected by western blot analysis using antibodies against anti-galectin-3, anti-Rb, and anti-E2F1. Mouse/rabbit IgG (Santa Cruz Biotechnology) was used as a negative control.⁴¹

Luciferase assay. The E2F1 luciferase reporter plasmid under the control of 4 E2F1 consensus and negative control SP1 plasmids as a 3x \times SP1 consensus were transfected in galectin-3-depleted or galectin-3-overexpressing cells using the Lipofectamine 2000 Reagent (Invitrogen) as described previously.⁴² After 30–40 h, the cells were harvested and the luciferase activity was estimated using a Luciferase Assay System (Promega). The results were normalized to β -galactosidase.

ChIP assay. ChIP assays were performed using a ChIP Assay Kit (Upstate, Temecula, CA, USA), according to the manufacturer's instructions. Anti-galectin-3, anti-Rb, anti-E2F1, and rabbit IgG antibodies were used to immunoprecipitate the DNA-containing complexes. We prepared a primer pair that anneals to E2F1 in the SKP2 promoter-binding sites: SKP2 promoter (–95) 5'-CTCCCCGCTACC CCGTGG-3'; SKP2 promoter (+135) 5'-CAGACCCGCTAAGCCTAGCAACG-3'; and GAPDH 5'-AGCTCAGGCCTCAAGACCTT-3' and 5'-AAGAAGATGCGGCT GACTGT-3' as the normalization control. PCR was performed according to the Ex-Taq manual (TaKaRa).

Fractionation of cellular extracts. Nuclear and cytoplasmic extracts were prepared from AGS and SNU638 cells after treatment with galectin-3 siRNA

and galectin-3 overexpression vectors. The experiments were performed as described previously.⁵

Crystal violet staining. Cells were transfected with 20 nM of galectin-3, Skp2, or p27^{KIP1} siRNA by a reverse transfection method using Lipofectamine RNAiMax. For crystal violet staining, cells were plated onto 12-well culture dishes and stained with crystal violet at 2 days after Galectin-3, skp2, or p27^{KIP1} siRNA transfection.

Cell proliferation and cell cycle analysis. Inhibition of cell proliferation by galectin-3, Skp2, or p27^{KIP1} siRNA treatment was evaluated using Ez-Cytox Assay Kits. AGS and SNU601 cells were plated in 96-well culture plates (3×10^3 cells per well). After incubation for 24 h, the cells were treated with 20 nM of Galectin-3, Skp2, or p27^{KIP1} siRNA for 48 h. Highly sensitive water-soluble tetrazolium salt (WST) solution (Daeil, Seoul, Korea; the working of the WST solution is based on the cleavage of the tetrazolium salt into a water-soluble formazan by the succinate-tetrazolium reductase system that belongs to the respiratory chain of mitochondria. It is active only in viable cells) was subsequently added to each well. After 1–3 h of additional incubation, the plate was shaken gently. The absorbance was measured on an ELISA reader at a wavelength of 450 nm. In addition, cell cycle distribution after galectin-3, Skp2, and p27^{KIP1} siRNA treatment was measured by PI staining using FACS.

Preparation of galectin-3-depleted AGS gastric cancer cells for xenografting into mice. All the animal experiments were approved by the Institutional Review Board of the Yonsei University College of Medicine and were performed in specific pathogen-free facilities in accordance with the Guidelines for the Care and Use of Laboratory Animals of the University (2013-0018). The preparation of xenograft mice was performed as described previously.⁴³

Microarray analyses of human gastric cancer tissue. For immunohistochemical analysis, biopsy specimens from the core tissue (2 mm in diameter) were obtained from individual paraffin-embedded gastric carcinoma tissues (donor blocks) and arranged in new recipient paraffin blocks (tissue array blocks) using a trephine apparatus (SuperBioChips Laboratories, Seoul, Korea). Immunohistochemical analysis of galectin-3 and p27^{KIP1} was performed as described previously.^{44,45}

Preparation of frozen tissues from gastric cancer patients. Two pairs of biopsy specimens (2 mm in diameter) were obtained from 52 patients with gastric adenocarcinoma during diagnostic endoscopic submucosal dissection. Immediately after biopsy, these tissue samples were frozen in liquid nitrogen and stored in a deep freezer at -70°C until further use. All the participants provided written informed consent. All the experiments were approved by the Institutional Review Board of the National Cancer Center (approval number NCCNSH 03-024).

Statistical analyses. We employed χ^2 -tests to evaluate the relationship between the levels of galectin-3 and p27^{KIP1} in the tissues of the gastric cancer patients. Two tumors per mouse were obtained and the mean tumor volume per mouse was analyzed. Unpaired *t*-tests were used to analyze the mean tumor volume in xenograft mice. All the statistical tests are two sided, and the values are expressed as the mean with 95% confidence intervals (CI). The *P*-values of <0.05 were considered statistically significant. Statistical analyses were performed using GraphPad Prism software (version 6; GraphPad Software Inc., La Jolla, CA, USA).

Conflict of Interest

The authors declare no conflict of interest.

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Author contributions

SJ Kim, HW Lee, HG Kang, and SH La performed the experiments; IJ Choi provided patient samples and clinical information; JY Ro performed the pathological analyses; RS Bresalier and J Song analyzed the data; and KH Chun designed and funded the research.

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