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FOXC1 regulates the functions of human basal-like breast cancer cells by activating NF-κB signaling

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Abstract

Human basal-like breast cancer (BLBC) is an enigmatic and aggressive malignancy with a poor prognosis. There is an urgent need to identify therapeutic targets for BLBC because current treatment modalities are limited and not effective. The forkhead box transcription factor FOXC1 has recently been identified as a critical functional biomarker for BLBC. However, how it orchestrates BLBC cells was not clear. Here we show that FOXC1 activates the transcription factor NF-κB in BLBC cells by increasing p65/RelA protein stability. High NF-κB activity has been associated with estrogen receptor-negative breast cancer, particularly BLBC. The effect of FOXC1 on p65/RelA protein stability is mediated by increased expression of Pin1, a peptidyl-prolyl isomerase. FOXC1 requires NF-κB for its regulation of cell proliferation, migration, and invasion. Notably, FOXC1 overexpression renders breast cancer cells more susceptible to pharmacologic inhibition of NF-κB. These results suggest that BLBC cells may rely on FOXC1-driven NF-κB signaling. Interventions of this pathway may provide modalities for the treatment of BLBC.

Keywords

basal-like breast cancer; FOXC1; NF-κB; p65/RelA; Pin1; protein stability

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Conflict of Interest

Dr. Wang, Dr. Ray, Dr. Bagaria, and Dr. Cui are named inventors on patent applications regarding the role of FOXC1 in cancer.

Introduction

Although first reported more than 20 years ago on the basis of immunohistochemical (IHC) detection of high molecular weight basal cytokeratins (CKs) in a small subgroup of breast cancers (1, 2), the basal-like subtype again became notable after global gene expression analysis confirmed it as a distinct molecular entity within breast cancer (3). Basal-like breast cancers (BLBCs) express genes characteristic of basal/myoepithelial cells in the normal mammary gland and comprise up to 25% of all breast cancers (4, 5). They underexpress estrogen receptor (ERα), progesterone receptor (PR), and HER2, and are associated with high histological grade and aggressive clinical behavior (6). However, BLBC is not synonymous with the ER-/PR-/HER2-triple-negative phenotype (TNP). Whereas ERα and HER2 guide targeted treatment of luminal and HER2-positive breast cancers, respectively, chemotherapy is still the only modality of systemic therapy for BLBC. The high mortality of BLBC reflects its rapid growth rate (7, 8) and aggressive migration/invasion (9). Perhaps not surprisingly, BLBC is overrepresented among the interval breast cancers arising between mammograms (10).

Recently, the forkhead box transcription factor FOXC1 has been identified as a potential pivotal biomarker for BLBC, and high expression correlates with poor overall survival in breast cancer (11–13). Forkhead box transcription factors, characterized by a common 100-amino acid winged-helix DNA-binding domain, play important roles in cell growth, survival, differentiation, migration, and longevity (14). FOXC1 has been postulated to control the development of embryonic mesenchymal tissue (15). FOXC1 is mutated in the autosomal dominant disease Axenfeld-Rieger syndrome (AR) (16), which is characterized by ocular defects, cardiac disease, and cranio-facial abnormalities. FOXC1 homozygous knockout mice die at birth with hydrocephalus, skeletal, and eye defects (17). Further examination of FOXC1 heterozygous knockout mice showed that these mice have anterior eye segment malformations similar to those found in human patients with FOXC1 mutations (18). Mechanistically, how FOXC1 exerts these effects is not well understood.

Previously, it was shown that ectopic overexpression of FOXC1 in breast cancer cells induced aggressive phenotypes such as epithelial-mesenchymal transition, and increased cell proliferation, migration, and invasion (12). Knockdown of FOXC1 using shRNA in breast cancer cells with high endogenous levels of FOXC1 demonstrated the opposite effects with loss of aggressive phenotypic features. However, the mechanism underlying the role of FOXC1 in BLBC cells is not clear. To address this question, this study was designed to determine how FOXC1 enlists or interacts with other basal-like tumor-associated signaling pathways to control cancer cell functions. By characterizing the molecular actions of FOXC1, our results demonstrate that FOXC1 orchestrates BLBC-associated phenotypes by regulating Pin1/NF-κB signaling.

Results and Discussion

To date, the genetic profile and biologic basis of BLBC are poorly understood. Recent studies have implicated several signaling pathways such as MEK/PI3K (19, 20), integrin (21), Notch pathways (22), and NF-κB (23) in BLBC development and progression. To identify BLBC-specific signaling features and to confirm the relevance of these and other pathways reported to impact breast cancer, we used the Ingenuity IPA platform and publicly available cDNA microarray datasets to conduct unbiased, systematic screening analysis of signaling networks in human breast cancer. As illustrated in Figure 1A, NF-κB was revealed as one of the most distinctive pathways in basal-like tumors. This is consistent with previous reports that sustained NF-κB activation exists mostly in human BLBC cell lines (23, 24) and ER-negative breast cancer (25) with its highest activity found in triple-negative tumors (26).

NF-κB activity was also found to be essential for the proliferation and survival of BLBC cells (23).

An interesting and axiomatic issue arising from these findings is whether FOXC1 coordinates specific BLBC-associated signaling pathways. Because some Fox transcription factors reportedly modulate NF-κB in non-malignant conditions (27), we postulated that FOXC1 might modulate NF-κB function in BLBC cells. Using immunoblotting, we found that the NF-κB p65 subunit and its phosphorylation at Ser-546 (by IκB kinase [IKK]) were markedly induced by FOXC1 overexpression in basal-like MDA-MB-231 breast cancer cells (Figure 1B) and luminal MCF-7 breast cancer cells (Supplemental Figure 1A), which harbor low and undetectable endogenous FOXC1 levels, respectively (12). Notably, levels of the NF-κB inhibitor IκBα were moderately but consistently reduced, which could be attributed to modestly higher IKK levels in these cells (data not shown). Conversely, knockdown of FOXC1 by its shRNA, which reduced FOXC1 levels by > 90%, suppressed p65 expression in basal-like BT-549 (human, Figure 1C) and 4T1 (mouse, Supplemental Figure 1B) breast cancer cells, both of which possess high endogenous FOXC1 levels (12).

Next we examined the nuclear localization of p65, an indicator of activated NF-κB. Immunoblotting with nuclear extracts of vector- and FOXC1-overexpressing MDA-MB-231 and MCF-7 cells indicated that FOXC1 promoted p65 translocation into the nucleus (Figure 1D and Supplemental Figure 1C). This was corroborated by increased immunofluorescence staining of nuclear p65 in FOXC1-overexpressing cells (Figure 1E). In agreement with this result, TransAM ELISA with oligonucleotides comprising consensus NF-κB-binding sequences showed that increase of FOXC1 expression potentiated the DNA-binding activity of p65 without affecting that of p50 in breast cancer cells (Supplemental Figure 1D). Conversely, FOXC1 knockdown by its shRNAs reduced p65 DNA-binding activity (Supplemental Figure 1E). To further substantiate that FOXC1 enhances NF-κB activity, we used a NF-κB-responsive luciferase reporter construct. As expected, FOXC1 overexpression robustly increased NF-κB-driven luciferase activity in MDA-MB-231 in (Figure 1F) and MCF-7 cells (Supplemental Figure 1F), and ectopic overexpression of the IκBα S32A/S36A super-repressor (IκBα-SR) abolished this FOXC1 effect. Supporting the above findings, FOXC1 overexpression upregulated NF-κB-inducible interleukin-6 (IL-6) expression in MDA-MB-231 (Supplemental Figure 1G), whereas FOXC1 knockdown downregulated IL-6 expression in BT-549 and 4T1 cells (Supplemental Figure 1H). Taken together, these results demonstrate that FOXC1 is a potent inducer of NF-κB activity in breast cancer cells and provides clues to why NF-κB is hyperactive in BLBC.

We then explored the potential mechanisms underlying FOXC1-mediated upregulation of p65. Whereas p65 mRNA levels were similar across breast cancer subgroups in microarray analysis (data not shown) and were not altered by FOXC1 overexpression, IκBα transcription was enhanced (Supplemental Figure 2), reflecting a known negative feedback mechanism for regulating increased NF-κB activity. Thus we postulated that p65 expression might be regulated at the protein level. Pin1, a peptidyl-prolyl isomerase that binds to and isomerizes specific phosphorylated Ser or Thr, was of particular interest due to its pivotal role in the control of p65 protein stability and activity (28) and its involvement in cancer development (29). It binds to p65 and thereby blocks its association with IκBα and SOCS-1, a ubiquitin ligase for p65, leading to inhibition of p65 proteolysis. Indeed, immunoblotting and quantitative RT-PCR showed that Pin1 was upregulated by FOXC1 overexpression in MDA-MB-231 cells (Figure 2A and B), while downregulated by FOXC1 shRNAs in BT-549 cells (Supplemental Figure 3). In addition, FOXC1 potentiated the luciferase reporter activity driven by a Pin1 promoter (Figure 2C).

To examine whether FOXC1 enhances the association between Pin1 and p65, we performed immunoprecipitation with an anti-p65 antibody followed by immunoblotting. As presented in Figure 2D, FOXC1 increased the binding of p65 to Pin1, but decreased its binding to IκBα and SOCS-1. As SOCS-1 facilitates p65 degradation (28), we reasoned that ubiquitin-mediated proteolysis is involved in the FOXC1 effect on p65 protein levels. To address this question, we immunoprecipitated p65 and performed immunoblotting of ubiquitin after treating MDA-MB-231 cells with the proteasome inhibitor MG-132. As illustrated in Figure 2E, FOXC1 overexpression attenuated the ubiquitination of p65. Similar results were obtained when ubiquitinated proteins were immunoprecipitated, followed by immunoblotting of p65 (Supplemental Figure 4). To corroborate these results, we treated the same cells with the translation inhibitor cycloheximide for different time periods. Immunoblot analysis of cells with inhibited de novo protein synthesis demonstrated that FOXC1 enhanced p65 protein stability (Figure 2F). In line with these findings, knockdown of Pin1 by its siRNA reduced NF-κB-responsive luciferase reporter activity in parental MDA-MB-231 cells and abolished FOXC1-induced increase of NF-κB-responsive luciferase activity and p65 levels (Supplemental Figure 5). Collectively, these data indicate that Pin1 is involved in the activation of NF-κB by FOXC1.

Consistent with a previous study showing a trend towards a correlation of Pin1 protein levels with ER-negative breast cancer (30), analysis of two cDNA microarray datasets of 51 human breast cancer cell lines revealed that Pin1 mRNA levels were significantly higher in BLBC cells than in luminal cells (31, 32) (Supplemental Figure 6A), which was confirmed by immunoblotting (Supplemental Figure 6B). Surprisingly, we did not find a statistically significant association between Pin1 mRNA levels and tumors of the basal-like subgroup in cDNA microarray analysis (data not shown), although high Pin1 expression correlated with worse recurrence-free survival (Supplemental Figure 6C) and higher tumor grade, which are commonly associated with basal-like tumors. We also observed a trend towards decreased overall survival in breast cancer patients with high Pin1 levels (Supplemental Figure 7). Because Pin1 is essential for breast cancer development, its overexpression across all subgroups of breast cancer might mask detection of higher Pin1 levels in BLBC. Nevertheless, our findings may implicate Pin1 in the regulation of BLBC. How Pin1 expression is controlled by FOXC1 remains to be determined.

Because of a critical role of NF-κB in cancer cell functions, there has been great interest in targeting NF-κB for development of anticancer therapy. To determine whether NF-κB blockade impairs FOXC1-induced cell phenotypes, we treated vector- and FOXC1-overexpressing MDA-MB-231 cells with small-molecule NF-κB inhibitors. As illustrated in Figure 3A, increase of FOXC1 sensitized MDA-MB-231 cells to pharmacologic inhibition of NF-κB by the IKK inhibitor BMS-345541 in cell proliferation, migration, and invasion assays. Similar results were found with other NF-κB inhibitors such as BAY-117082 (data not shown). Coupled with previous findings that FOXC1 is critically involved in BLBC cell functions (12), these data suggest that FOXC1 exploits NF-κB to promote aggressive cellular traits commonly associated with BLBC (7, 9). To further corroborate that NF-κB is involved in the effects of FOXC1, we overexpressed FOXC1 in IKKα/IKKβ double-knockout and p65 knockout mouse embryonic fibroblasts (MEFs). MTT assays showed that increased FOXC1 did lead to enhanced cell proliferation in wildtype MEFs, but not in IKKα/IKKβ-null (Figure 3B) and p65-null MEFs (Supplemental Figure 8). Consistent with these results, FOXC1 overexpression increased levels of the NF-κB and Pin1 target cyclin D1 and the percentage of cells in S phase of the cell cycle in wildtype MEFs but not in IKKα/IKKβ-null MEFs (Figure 3C). Taken together, these data suggest that the effect of FOXC1 requires intact NF-κB activity.

In summary, these findings uncover a functional link between FOXC1 expression and NF-κB signaling in BLBC cells. The FOXC1-NF-κB pathway, which involves increased expression of Pin1 and possibly IKK, might be key for acquisition of aggressive cellular traits of BLBC, and targets on this pathway might serve as the basis for therapeutic interventions in BLBC. Further studies will determine whether FOXC1 also contributes to activation or mediation of other BLBC-associated pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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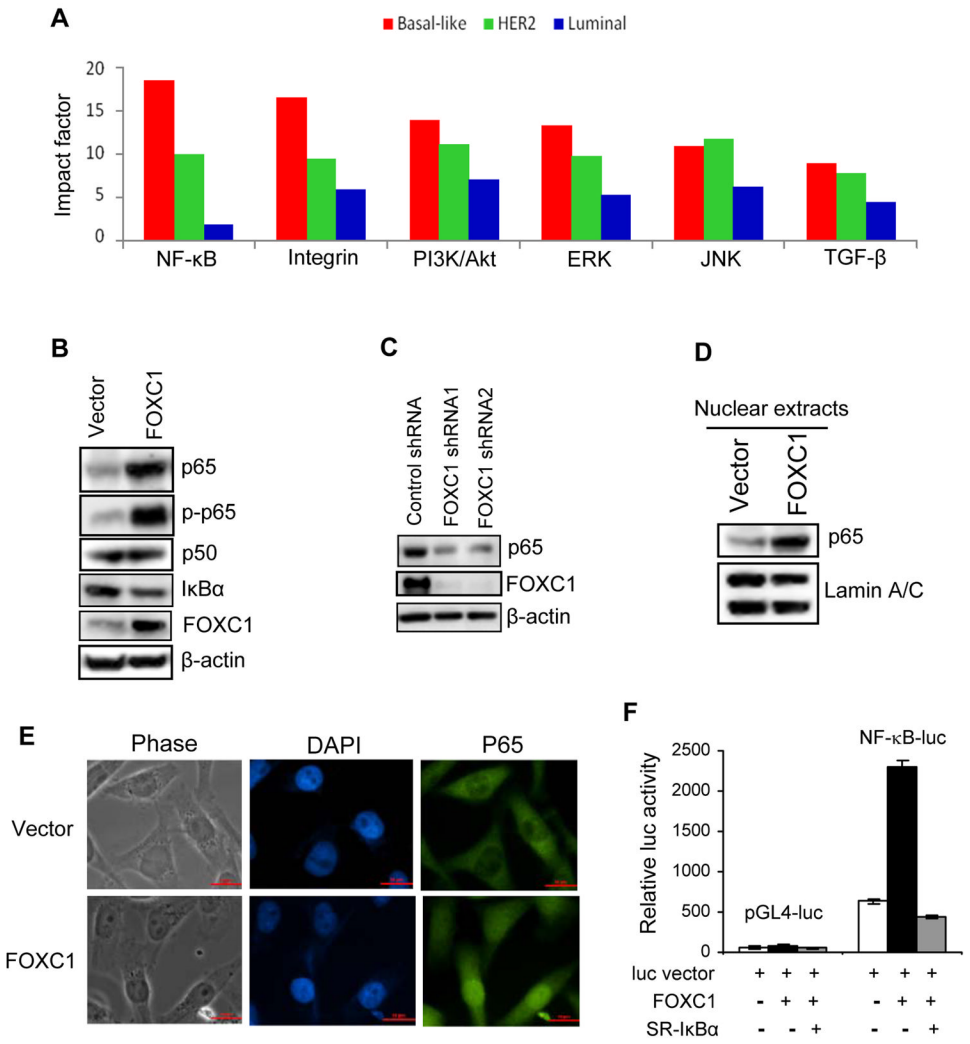

Figure 1. FOXC1 induces NF-κB activity in breast cancer cells

(**A**) Significant canonical signaling pathways in basal-like (*red*), HER2 (*green*) and luminal (*blue*) breast cancers from the Richardson et al. dataset were identified using Ingenuity Pathway Analysis and ranked by the impact factor (see Supplementary Information for detailed methods). (**B**) Immunoblotting of NF-κB components in MDA-MB-231 cells overexpressing FOXC1 or the vector. (**C**) Immunoblotting of p65 in control or FOXC1 shRNA-expressing BT-549 breast cancer cells. (**D**) Nuclear proteins were isolated from vector- or FOXC1-overexpressing MDA-MB-231 cells, followed by immunoblotting of p65 and a nuclear marker Lamin A/C. (**E**) Nuclear localization of p65 protein was visualized by fluorescence microscopy (*green*, p65; *blue*, nuclear DNA staining by DAPI). (**F**) MDA-MB-231 cells were transiently transfected with NF-κB-luc or the vector pGL4-luc, FOXC1, and IκBα-SR. NF-κB activity was assessed by luciferase assays. Datarepresent mean ± SD (n = 3).

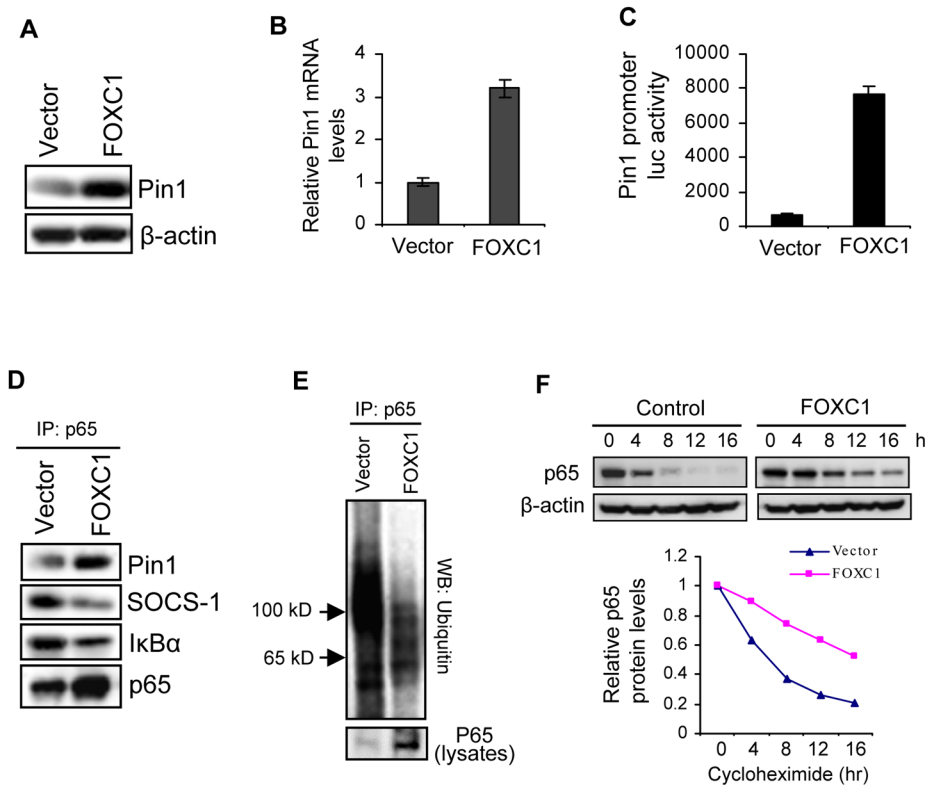

Figure 2. FOXC1 increases p65 protein stability by upregulating Pin1 in breast cancer cells

(**A**) Immunoblotting of Pin1 in MDA-MB-231 cells overexpressing FOXC1 or the vector. (**B**) Real-time RT-PCR analysis of Pin1 mRNA in the same cells. The Pin primers are 5′-TGGGTGCCTTCAGCAGAGGTCAG-3′ and 5′-CCGGAATCCGTGAACACGGGC-3′ (see Supplementary Information for detailed methods). (**C**) A 2.3 kb Pin1 promoter-luciferase reporter construct was co-transfected into MDA-MB-231 cells with FOXC1 or the vector, followed by luciferase assays. (**D**) Lysates from FOXC1- or vector-overexpressing MDA-MB-231 cells were immunoprecipitated with an anti-p65 antibody, followed by immunoblotting of IκBα, Pin1, and SOCS-1. (**E**) Same cells were transfected with a ubiquitin construct, treated with 10 μM MG-132, and subjected to immunoprecipitation with an anti-p65 antibody, followed by immunoblotting of ubiquitin. (**F**) Same cells were treated with cycloheximide (10 μg/ml) and harvested at the indicated time points, followed by immunoblotting and densitometry of protein bands. Band intensities were normalized to that of actin, then normalized to the t = 0 controls.

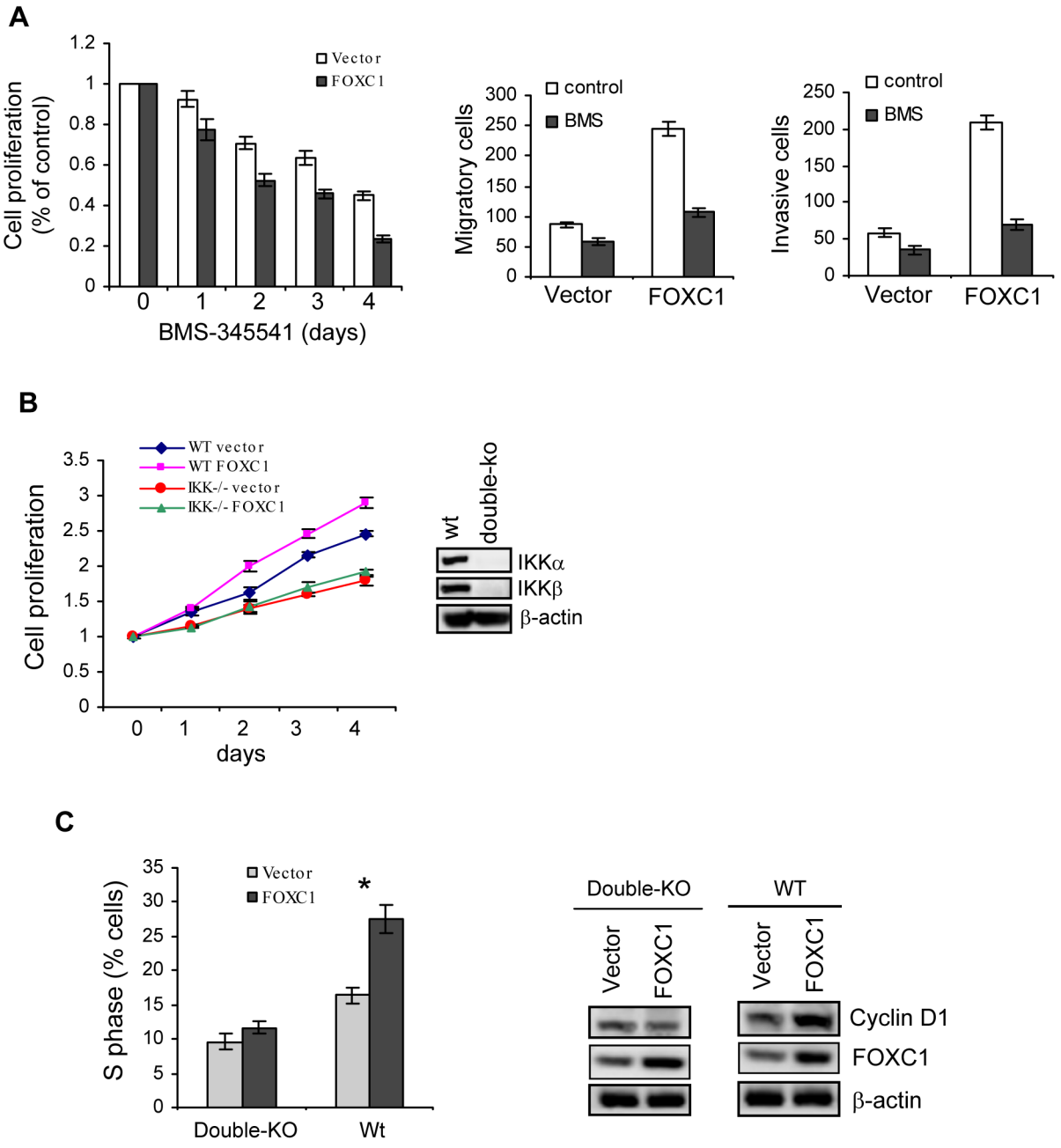

Figure 3. NF-κB mediates the effects of FOXC1 on cell proliferation, migration, and invasion

(**A**) FOXC1- or vector-overexpressing MDA-MB-231 cells were treated with the NF-κB inhibitor BMS-345541 (2 μM), followed by MTT assays (left), transwell migration assays (middle), and transwell invasion assays (right). Data represent mean ± SD (n = 3). (**B**) Wild-type (wt) and IKKα/IKKβ-null MEFs were transfected with FOXC1 or the vector, followed by cell proliferation MTT assays at the indicated time points (left). Deficiency of IKK expression in knockout MEFs is shown by immunoblotting (right). (**C**) Wild-type (wt) and IKKα/IKKβ-null MEFs were transfected with FOXC1 or the vector, followed by cell cycle analysis using flow cytometry (left). *, $P < 0.05$. CyclinD1 expression was assessed by immunoblotting (right).