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TAL1/SCL Relieves the E2-2-Mediated Repression of VEGFR2 Promoter Activity

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The basic helix-loop-helix (bHLH) protein TAL1/SCL is essential for embryonic-vascular development. TAL1/SCL regulates the activation of endothelial cells by binding directly or indirectly to DNA sequences in critical target genes. We recently demonstrated that E-box protein E2-2 blocks endothelial cell activation via perturbation of VEGFR2 promoter activity. Herein, we report that TAL1/SCL interacts with E2-2 and inhibits E2-2-mediated effects on reporter activity. Mutational analysis revealed that the HLH domain of TAL1/SCL, but not its basic region, is required for interaction with E2-2. Importantly, TAL1/SCL relieves the E2-2-mediated repression of VEGFR2 reporter activity in endothelial cells. Our data elaborate on the bHLH protein interactions that regulate endothelial cell activation.

Key words: angiogenesis, E2-2, endothelial cell, SCL/TAL1, VEGFR2.

Abbreviations: GATA-1, GATA binding protein 1; HEB, human B-HLH factor; Herp2, HES-related repressor protein 2; Id1, inhibitor of DNA binding 1; LMO2, LIM domain only 2; VEGFR2, vascular endothelial growth factor receptor 2.

Angiogenesis involves the formation of new vessels from pre-existing vessels. This process occurs during embryonic development, wound healing, endometrial proliferation and pregnancy. Thus, angiogenesis plays an important role in maintaining homeostasis in the body. However, angiogenesis also takes place during chronic inflammation, tumour growth, age-related macular degeneration, diabetic retinopathy and neovascular glaucoma (*1*).

Angiogenesis encompasses two phases: activation and resolution. The activation phase starts when endothelial cell proliferation is triggered. In this phase, vascular permeability increases and extracellular matrix proteins are degraded, enabling endothelial cells to migrate and sprout new capillaries. In the resolution phase, endothelial cells halt proliferation and migration, and the basement membrane is reconstituted. Endothelial cells become stabilized by the recruitment of mesenchymal cells that differentiate into pericytes. In larger vessels, several layers of smooth muscle cells surround newly formed vessels. The transition from activation to resolution phases, and *vice versa*, is referred as the angiogenic switch, and is determined by an intrinsically regulated balance between inducers and inhibitors of angiogenesis (*2*).

T-cell acute lymphocytic leukemia 1/stem cell leukemia hematopoietic transcription factor (TAL1/SCL; alternatively termed TCL5), which belongs to the basic helix-loop-helix (bHLH) family of proteins, was initially identified as a molecule involved in T-cell acute lymphoblastic leukemia (T-ALL). Activation of the TAL1/SCL gene through chromosomal translocation occurs in up to 25% of patients with T-ALL (*3–5*). TAL1/SCL expression is limited to hematopoietic progenitors, endothelial cells and the central nervous system (*6*). Furthermore, TAL1/SCL is required for the formation of erythroid and megakaryocytic cell lineages (*7*). Indeed, TAL1/SCL is essential not only for the development of all blood lineages but also for angiogenesis in the yolk sac in mice (*8*).

TAL1/SCL is known to form a heterodimer with E-proteins that bind to the E-box consensus sequence (CANNTG) (*9*). The E-protein family consists of three members in mammals: E12/E47, HEB and E2-2. TAL1/SCL can either activate or repress transcription depending on its association with other essential hematopoietic factors such as GATA-1 or LMO2 (*10*, *11*). Recently, TAL1/SCL, together with E47, was reported to up-regulate expression of VE-cadherin in endothelial cells (*12*), whereas TAL1/SCL was found to repress E47/HEB target genes in thymocytes (*13*). Thus, TAL1/SCL regulates transcription of target genes in a context-dependent manner. Furthermore, TAL1/SCL performs its hematopoietic and angiopoietic functions in DNA binding-dependent and -independent fashions (*14*, *15*). Recently, we found that Id1 forms a complex with E2-2 to rescue E2-2-mediated endothelial cell inactivation (Tanaka *et al*., submitted), prompting us to investigate whether TAL1/SCL influences E2-2-mediated responses in endothelial cells.

To determine whether E2-2 can interact with TAL1/SCL in COS7 cells, we carried out immunoprecipitation and western blotting using cell lysates. We also investigated the interaction of E2-2 with other proteins known to be involved in angiogenesis. We found that TAL1/SCL interacted with E2-2, LMO2 and Id1 (albeit weakly), but not with Herp2 (Fig. 1A). Although LMO2 does not contain an HLH domain, TAL1/SCL has been reported to associate with LMO2 *via* its second helix domain to form an active transcription complex in early hematopoietic

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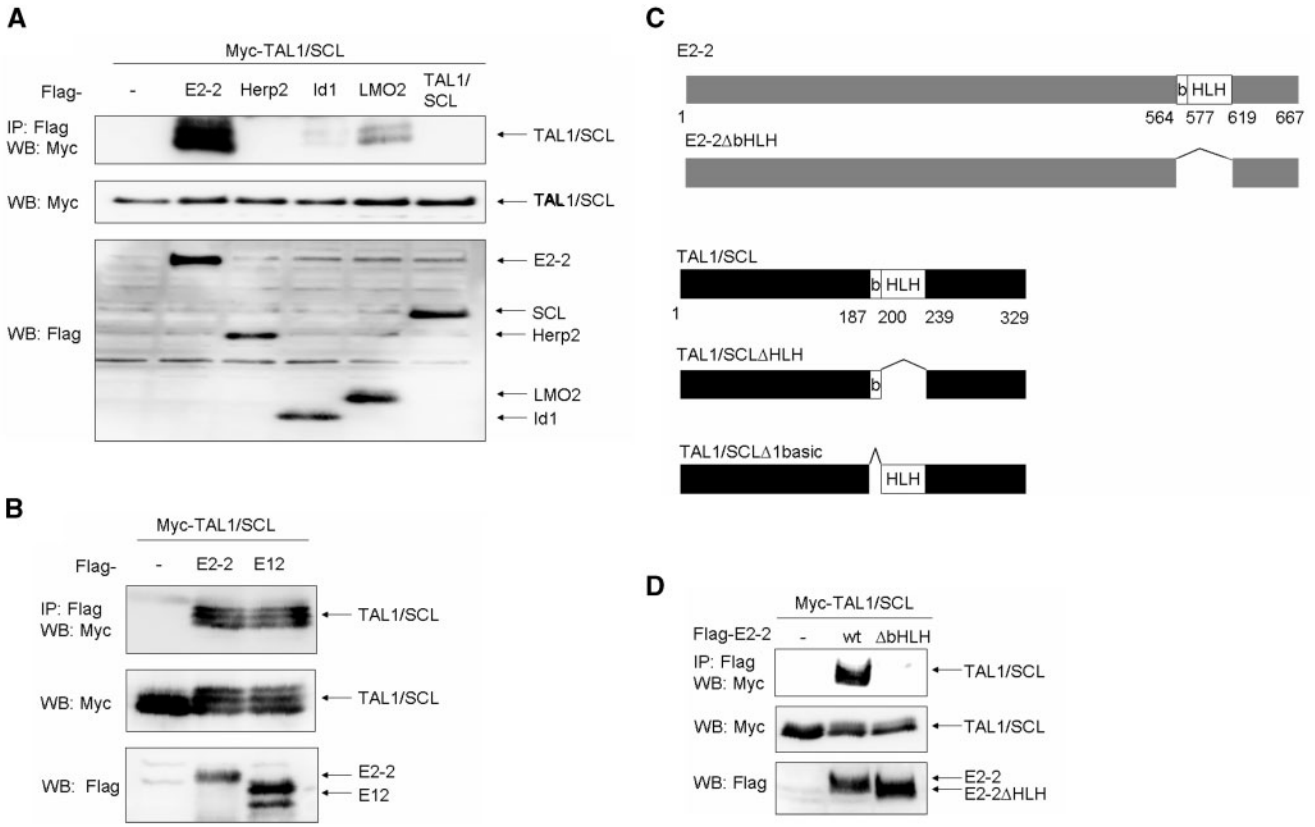

Fig. 1. **Interaction and colocalization between E2-2 and TAL1/SCL.** (A) TAL1/SCL interacts with E2-2. COS7 cells were co-transfected with Myc-TAL1/SCL and Flag-E2-2, Flag-Herp2, Flag-Id1, Flag-LMO2 or Flag-TAL1/SCL. Cell lysates were immunoprecipitated with anti-Flag M5 antibody, followed by western blot analysis using anti-Myc9E10 antibody (upper panel). Protein expression was detected with anti-Myc9E10 (middle panel) and anti-Flag M5 antibodies (lower panel). (B) TAL1/SCL interacts with E12. COS7 cells were co-transfected with Myc-TAL1/SCL and Flag-E12 or Flag-E2-2. The experiment was performed in a similar manner to (A). Interaction of Myc-TAL1/SCL with either Flag-E2-2 or Flag-E12 (upper panel). Expression of Myc-TAL1/SCL and Flag-tagged proteins were checked using anti-Myc9E10 (middle panel) and anti-Flag M5 antibodies (lower panel). (C) Structure of E2-2ΔbHLH, TAL1/SCLΔHLH and TAL1/SCLΔbasic. b, basic region; HLH, helix-loop-helix region. (D) HLH domain of E2-2 is required for interaction with TAL1/SCL. The experiment was performed in a similar manner to (A). Heterodimer formation between E2-2 and TAL1/SCL (upper panel). Expression of Myc-TAL1/SCL was detected with anti-Myc9E10 antibody (middle panel). Expression of Flag-E2-2 and Flag-E2-2ΔbHLH was detected using anti-Flag M5 antibody (lower panel). (E) HLH domain of TAL1/SCL is required for interaction with E2-2. The experiment was carried out as described in Fig. 1A. E2-2 co-immunoprecipitated with TAL1/SCL (upper panel). Expression of Myc-E2-2 was detected using anti-Myc9E10 antibody (middle panel). Expression of Flag-TAL1/SCL and Flag-TAL1/SCLΔHLH was detected with anti-Flag M5 antibody (lower panel). (F) Basic region in TAL1/SCL is not required for heterodimer formation between TAL1/SCL and E2-2. The experiment was carried out as described in (A). Detection of E2-2 co-immunoprecipitated with TAL1/SCL or TAL1/SCLΔbasic (upper panel). Expression of Flag-TAL1/SCL or Flag-TAL1/SCLΔbasic was detected using anti-Flag M5 antibody (middle panel). Expression of Myc-E2-2 was detected with anti-Myc antibody (lower panel). (G) E2-2 colocalizes with TAL1/SCL in the nucleus. Mouse embryonic endothelial cells (MEECs) (*22*) were transiently transfected with Myc-E2-2 and Flag-TAL1/SCL. Myc-E2-2 and Flag-TAL1/SCL were visualized with red and green colors, respectively, as described in the text. Colocalization of E2-2 with TAL1/SCL appears as yellow. Nuclei were stained with DAPI.

cells (*16*). In contrast to previous *in vitro* findings (*17*), and unlike E2-2, Id1 and Herp2, we did not observe TAL1/SCL homodimer formation (Fig. 1A and data not shown). In addition, E12, a closely related protein to E2-2, was also found to interact with TAL1/SCL (Fig. 1B).

The HLH domain in TAL1/SCL has been shown to be critical for hematopoietic development using embryonic stem cells lacking the TAL1/SCL gene (*16*). Furthermore, *in vitro* studies have reported that TAL1/SCL selectively interacts with bHLH family proteins (*17*, *18*). Thus, we tested whether TAL1/SCL interacts with E2-2 through its HLH domain (Fig. 1C), and *vice versa*, in mammalian cells. As expected, TAL1/SCL did not interact with E2-2ΔbHLH (Fig. 1D). Similarly, E2-2 did not interact with TAL1/SCLΔHLH (Fig. 1E). On the other hand, TAL1/SCL lacking the basic region (TAL1/SCLΔbasic) kept the ability to interact with E2-2 (Fig. 1F). These results indicate that the HLH domain of both proteins is required for their interaction. We examined whether TAL1/SCL co-localizes with E2-2 in endothelial cells using immunofluorescence microscopy. After co-transfection of Flag-TAL1/SCL and Myc-E2-2, cells were fixed and stained with mouse anti-Flag M5 and rabbit anti-Myc antibodies. FITC-conjugated goat anti-mouse IgG and TexRed-conjugated goat anti-rabbit antibodies were used for visualization of TAL1/SCL and E2-2, respectively.

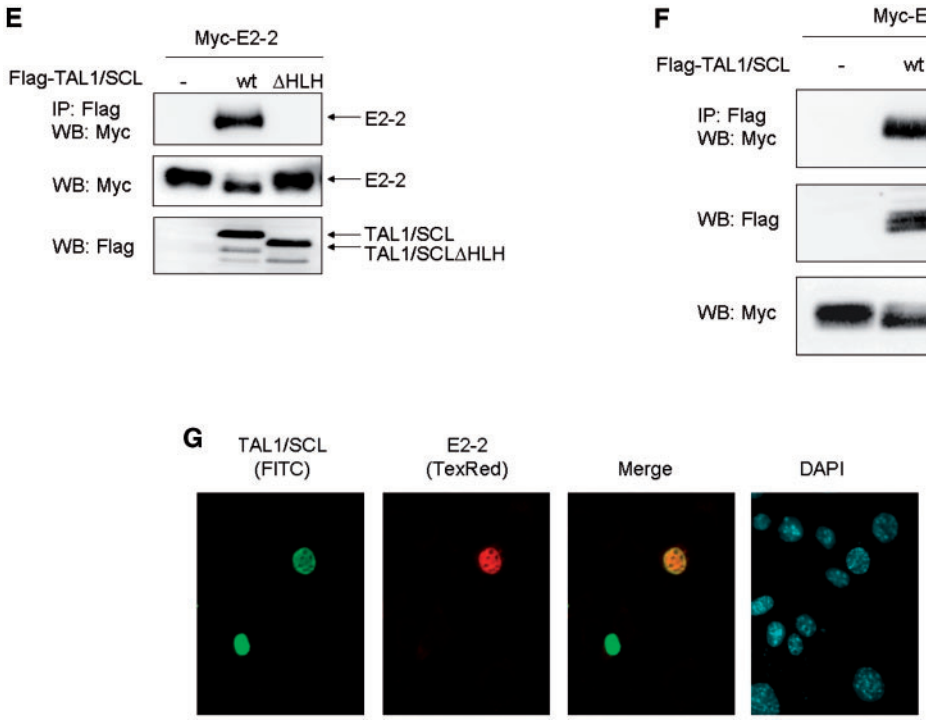

Fig. 1. Continued.

As shown in Fig. 1G, TAL1/SCL colocalized with E2-2 in nuclei. Collectively, these results indicate that TAL1/SCL forms a complex with E2-2 in cells.

To examine the effect of the E2-2/TAL1 interaction on transcription, we carried out reporter assays using a MCKpfos-luc reporter construct containing four E-boxes from the immunoglobulin λ5 promoter/enhancer (*19*). E2-2 enhanced reporter activity, whereas other proteins, including TAL1/SCL, did not (Fig. 2A). Consistent with results for the interaction between E2-2 and TAL1/SCL or TAL1/SCLΔbasic, E2-2-induced MCKpfos-luc reporter activity was blocked by TAL1/SCL or TAL1/SCLΔbasic in a dose-dependent manner (Fig. 2B and C). In contrast, TAL1/SCLΔHLH, which cannot heterodimerize with E-proteins, could not block this activity (Fig. 2D). TAL1/SCL also perturbed the transcriptional activation of MCKpfos-luc by the E2-2-related protein, E12 (Fig. 2E). E2-2 is known to bind to E-boxes either as a homodimer or heterodimer with other proteins. Since TAL1/SCL blocked E2-2-mediated MCKpfos-luc reporter activity, we suspected that TAL1/SCL could disrupt E2-2 homodimer formation. To explore this possibility, we transfected COS7 cells with Myc-E2-2 and Flag-E2-2 in the absence or presence of Myc-TAL1/SCL. Immunoprecipitation of cell extracts revealed that TAL1/SCL blocked E2-2 homodimer formation (Fig. 2F). These results indicate that TAL1/SCL can counteract the activating function of E2-2 by associating with E2-2. To further investigate the effect of TAL1/SCL on function of E2-2, we performed the gel shift assay using the nuclear extracts from COS7 cells transfected with either E2-2 or TAL1/SCL (Fig. 2G and H). E2-2 alone could show specific binding to the 4× E-box probe (Fig. 2G, lane 3), whereas the addition of TAL1/SCL decreased the intensity of these specific shifted bands (Fig. 2G, lane 4). Of note, TAL1/SCL alone could not bind to the 4× E-box probe (Fig. 2G, lane 5). These results indicated that TAL1/SCL can disrupt the interaction between E2-2 and the E-box sequence. However, whether TAL1/SCL can preferentially disrupt E2-2 homodimer formation remains veiled.

To investigate whether the antagonistic action of TAL1/SCL on E2-2 involves target genes implicated in angiogenesis, we performed a reporter assay using two different VEGFR2-luc reporters (Fig. 3A). Using these reporters, we have already demonstrated that E2-2 suppresses the activity of the VEGFR2 promoter (Tanaka *et al*., submitted). As shown in Fig. 3B and C, E2-2 reduced the activity of both reporter constructs, whereas TAL1/SCL relieved this E2-2-mediated repression. Next, we examined if TAL1/SCLΔbasic can rescue E2-2-mediated repression of the VEGFR2 promoter activity. As expected, the VEGFR2 promoter activity inhibited by E2-2 recovered in the presence of TAL1/SCLΔbasic (Fig. 3D). Since pGL2b-VEGFR2-luc(−80/+5) does not contain typical E-boxes, we speculated that E2-2 can modulate the reporter activity without binding to typical E-boxes. Id1, a HLH protein lacking a DNA binding domain, is a crucial target gene of BMP signalling involved in the activation of endothelial cells (*20*). Like Id1, TAL1/SCL may not need to bind directly to the VEGFR2 promoter in order to counter E2-2-mediated transcriptional repression because the basic region in TAL1/SCL is not required for TAL1/SCL to perturb E2-2-mediated repression of the VEGFR2 promoter activity (Fig. 3D). However, further experimentation is needed to confirm this hypothesis.

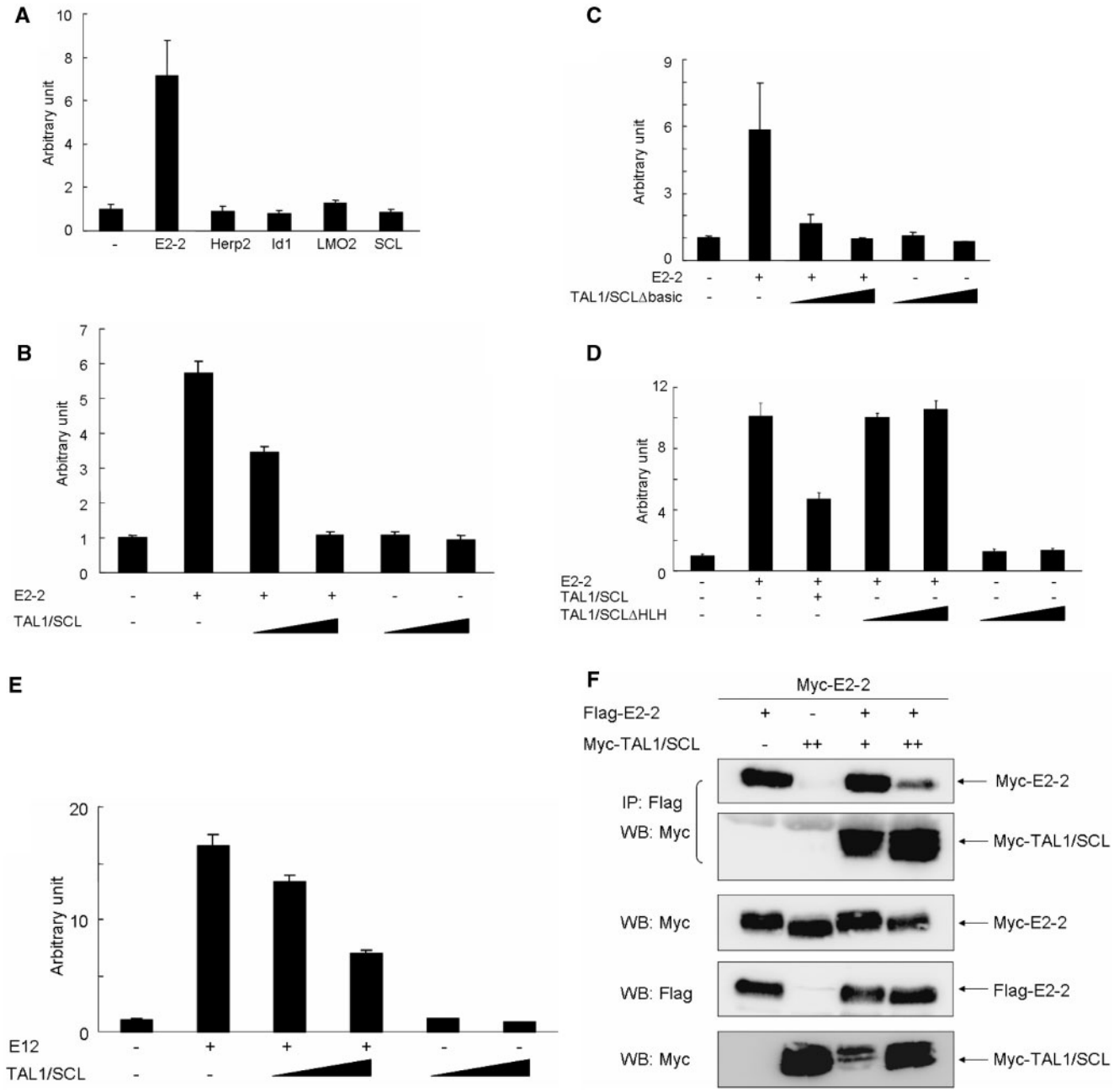

Fig. 2. **TAL1/SCL inhibits E2-2-induced luciferase activity.** (A) E2-2 induces MCKpfos-luc activity. MEECs were transfected with MCKpfos-luc and the indicated plasmids. (B) TAL1/SCL inhibits E2-2-induced MCKpfos-luc activity. MEECs were transfected with MCKpfos-luc, E2-2 and different amounts of TAL1/SCL. (C) TAL1/SCLΔbasic perturbs E2-2-induced MCKpfos-luc activity. MEECs were transfected with MCKpfos-luc, E2-2 and different amounts of TAL1/SCLΔbasic. (D) HLH domain of TAL1/SCL is required to counter E2-2-induced MCKpfos-luc activity. MEECs were transfected with MCKpfos-luc, E2-2, and different amounts of TAL1/SCL or its ΔHLH mutant. Data in A, B, C and D represent mean ± SD. ($n = 3$). (E) TAL1/SCL inhibits E12-induced MCKpfos-luc activity. MEECs were transfected with MCKpfos-luc, E12 and different amounts of TAL1/SCL. (F) TAL1/SCL disrupts E2-2 homodimer formation. The experiment was performed as described in Fig. 1A. E2-2 homodimer (upper panel) and heterodimer formation between E2-2 and TAL1/SCL (second panel) are shown. Expression of Myc-E2-2 (third panel) and Myc-TAL1/SCL (lower panel) was detected with anti-Myc9E10 antibody. Expression of Flag-E2-2 was detected using anti-Flag M5 antibody (fourth panel). (G) TAL1/SCL disrupts binding of E2-2 to E-box sequence. After nuclear extracts from COS7 cells transfected with either E2-2 or TAL1/SCL were prepared, E2-2 alone, mixture of E2-2 with TAL1/SCL or TAL1/SCL alone were incubated with a ^{32}P-labelled 4× E-box probe. Gel shift assay was performed as previously described (*23*). Lane 1, no protein added; lane 2, nuclear extract from COS7 cells transfected with pcDNA3; Lane 3, nuclear extract from COS7 cells transfected with Flag-E2-2; lane 4, nuclear extract from COS7 cells transfected with Flag-E2-2 plus nuclear extract from COS7 cells transfected with Flag-TAL1/SCL; lane 5, nuclear extract from COS7 cells transfected with Flag-TAL1/SCL. Arrows indicate E2-2-DNA complexes. (H) Expression of Flag-E2-2 or Flag-TAL1/SCL in nuclear extracts. Each nuclear extract was loaded on SDS–PAGE. After blotting the proteins on the membrane, it was probed with anti-Flag M5 antibody.

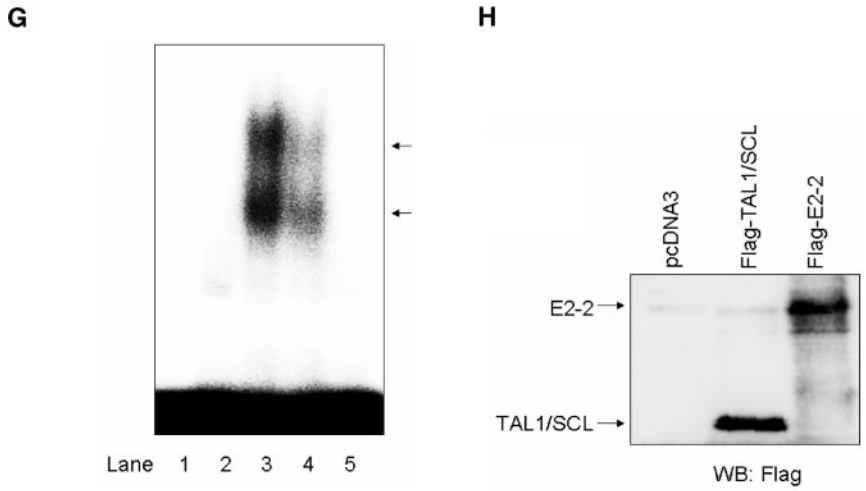

Fig. 2. Continued.

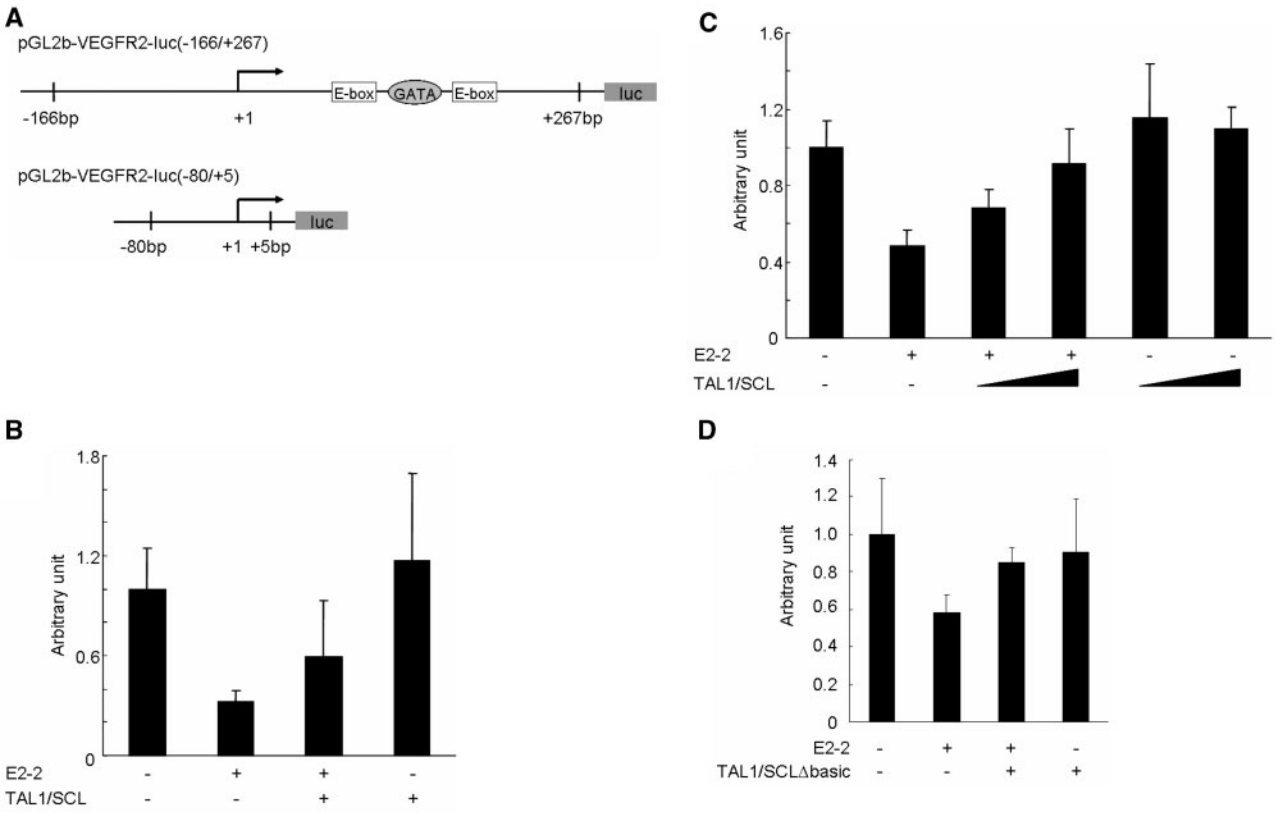

Fig. 3. **TAL1/SCL relieves E2-2-mediated suppression of VEGFR2 reporter.** (A) Schematic representations of the VEGFR2-luc reporter constructs used in experiments. (B) TAL1/SCL rescues E2-2-mediated suppression of pGL2b-VEGFR2-luc (−166/+267) activity. MEECs were transfected with pGL2b-VEGFR2-luc (−166/+267), TAL1/SCL and E2-2. Data represent mean ± SD. ($n = 3$). (C) TAL1/SCL relieves E2-2-mediated suppression of pGL2b-VEGFR2-luc (−80/+5) activity. MEECs were transfected with pGL2b-VEGFR2-luc (−80/+5), TAL1/SCL and E2-2. Data represent mean ± SD. ($n = 3$). (D) TAL1/SCLΔbasic rescues E2-2-mediated suppression of pGL2b-VEGFR2-luc (−80/+5) activity. The experiment was carried out as described in (C).

In conclusion, we found that TAL1/SCL interacts with E2-2 to inhibit E2-2-mediated transcriptional activities. This function of TAL1/SCL may not require its DNA-binding ability because TAL1/SCLΔbasic could inhibit the effect of E2-2 on MCKpfos-luc and pGL2b-VEGFR2-luc(−80/+5) activities comparable to TAL1/SCL. We propose two possible mechanisms by which TAL1/SCL may relieve the repression of VEGFR2 transcription by

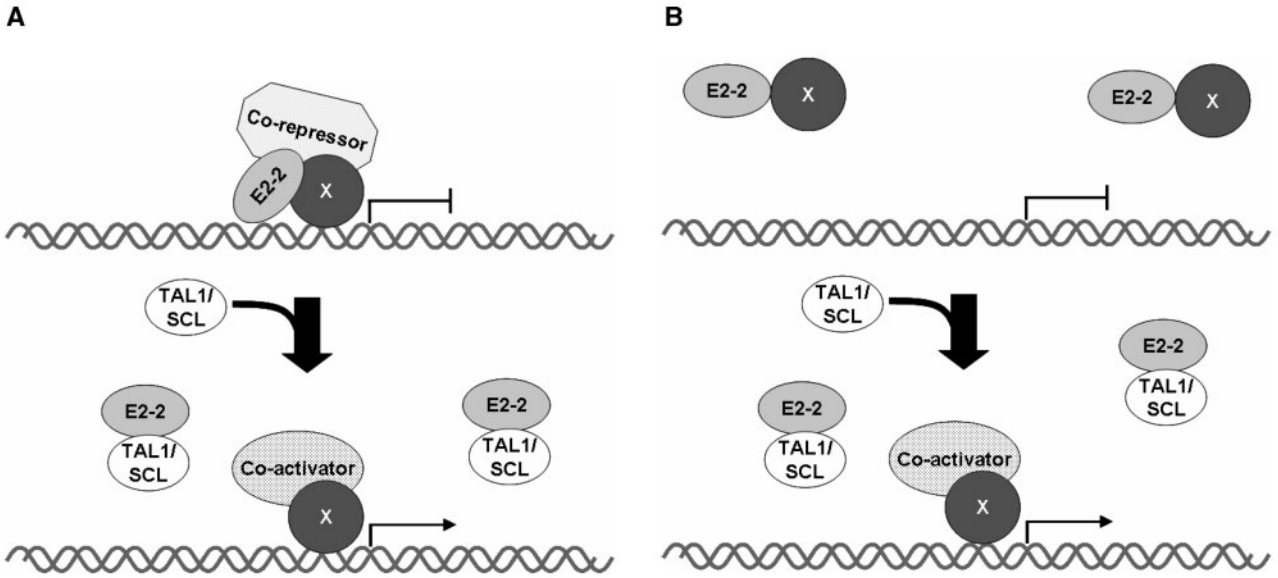

Fig. 4. **Two proposed models for how TAL1/SCL relieves the E2-2-mediated down-regulation of VEGFR2 expression.** (A) In the absence of TAL1/SCL, E2-2 dimerizes with 'X' to attenuate transcription of VEGFR2 gene. TAL1/SCL competes with 'X' for E2-2 binding, thereby dissociating E2-2 from DNA. Consequently, VEGFR2 transcription is activated. (B) In the absence of TAL1/SCL, 'X', which is required for VEGFR2 gene activation, interacts with E2-2, thereby blocking transcription. TAL1/SCL disrupts the interaction between E2-2 and 'X' allowing 'X' to bind the VEGFR2 promoter and activate transcription.

E2-2 (Fig. 4A and B). In brief, E2-2 dimerizes with 'X' to attenuate transcription of VEGFR2 gene in the absence of TAL1/SCL. In the presence of TAL1/SCL, TAL1/SCL competes with 'X' for E2-2 binding, thereby dissociating E2-2 from DNA. Consequently, VEGFR2 transcription is activated (Fig. 4A). As another possibility, 'X', which is required for VEGFR2 gene activation, interacts with E2-2 in the absence of TAL1/SCL, thereby blocking transcription. TAL1/SCL disrupts the interaction between E2-2 and 'X' allowing 'X' to bind the VEGFR2 promoter and activate transcription (Fig. 4B). Recently, TFII-I was found to enhance VEGFR2 transcription in an inr-dependent fashion (*21*). E2-2 might displace TFII-I from inr sequences or interact with TFII-I on inr sequences to prevent TFII-I from activating transcription. Current evidence indicates that interplay between bHLH proteins regulates the activation status of endothelial cells. We are continuing to investigate the importance of the E2-2/TAL1 interaction in this regulation.

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CONFLICT OF INTEREST

None declared.

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