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Snail Promotes the Cell-Autonomous Generation of Flk1⁺ Endothelial Cells Through the Repression of the microRNA-200 Family

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Expression of the transcription factor *Snail* is required for normal vasculogenesis in the developing mouse embryo. In addition, tumors expressing *Snail* have been associated with a more malignant phenotype, with both increased invasive properties and angiogenesis. Although the relationship between *Snail* and vasculogenesis has been noted, no mechanistic analysis has been elucidated. Here, we show that in addition to inducing an epithelial mesenchymal transition, *Snail* promotes the cell-autonomous induction of Flk1⁺ endothelial cells in an early subset of differentiating mouse embryonic stem (ES) cells. Cells that become Flk1⁺ in response to *Snail* have a transcriptional profile specific to Gata6⁺ primitive endoderm, but not the early Nanog⁺ epiblast. We further show that *Snail*'s ability to promote Flk1⁺ endothelium depends on fibroblast growth factor signaling as well as the repression of the microRNA-200 (miR-200) family, which directly targets the 3' UTRs of Flk1 and Ets1. Together, our results show that *Snail* is capable of inducing Flk1⁺ lineage commitment in a subset of differentiating ES cells through the down-regulation of the miR-200 family. We hypothesize that this mechanism of *Snail*-induced vasculogenesis may be conserved in both the early developing embryo and malignant cancers.

Introduction

SNAIL HOMOLOG 1 (*Snail*) IS A ZINC finger transcription factor important for the induction of epithelial mesenchymal transition (EMT) and gastrulation in the developing mouse embryo [1]. In Meox2-Cre/*Snail*^{loxP/loxP} mice, where *Snail* is selectively deleted in the embryo proper, gastrulation occurs, but the mice die from several vascular defects [2]. In addition to failed chorionic-allantoic fusion, these embryos display a severe reduction in blood vessels in the embryo proper as detected by VE-cadherin staining [3]. These findings suggest that in addition to its general function in EMT and gastrulation, *Snail* has a specific role in vasculogenesis. How *Snail* might promote vasculogenesis and whether its effects are cell intrinsic or cell extrinsic are currently unknown.

In addition to its role in the embryo, *Snail* has also been associated with blood vessel formation in tumors. Nonsmall cell lung carcinomas overexpressing *Snail* as well as madin darby canine kidney (MDCK)-*Snail* xenograft tumors displayed increased concentrations of blood vessels, which were thought to be a result of cell-extrinsic effects [4,5]. However, expression studies have hinted at the possibility that *Snail* may actually have a cell-intrinsic role in endothelial formation. In addition to being upregulated after vascular endo-

thelial growth factor-A (VEGFA) stimulation [6], *Snail* has been found to be highly expressed in endothelial cells of invasive breast cancer [7], spindle cell carcinoma [8], and oral squamous cell carcinoma [9]. The latter study noted that the expression of *Snail* in endothelial cells was greater in blood vessels close to the tumor, thus suggesting that the newly-formed blood vessels of tumors uniquely express *Snail* as compared with the endothelium of normal tissue.

To better understand the relationship between *Snail* and vasculogenesis, we decided to employ the use of mouse embryonic stem (ES) cells. Mouse ES cells are derived from the inner cell mass (ICM) of embryos, which is a heterogeneous mix of cells destined to become either epiblast or primitive endoderm. We recently used the ES cell model system to demonstrate the role of *Snail* and the microRNA-200 (miR-200) family in the exit and differentiation of cells from the epiblast stage [10]. Here, we show a different role for *Snail* and miR-200 in the specification of cells from a subset of differentiating ES cells that resemble primitive endoderm. We show that *Snail*, in a cell-autonomous manner, induces Flk1⁺ endothelial cells independent of Wnt, bone morphogenetic protein (BMP), and activin signaling. Further, the induction of Flk1⁺ cells depends on *Snail*'s repression of the miR-200 family, which directly targets the 3' UTRs of

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Flk1 and Ets1. Together, these findings suggest that Snail may have a direct role in promoting endothelial generation when expressed in a specific subset of cells.

Materials and Methods

ES cell maintenance and differentiation

A2.Snail and other modified *A2lox* [11] cells were maintained on feeder layers of irradiated mouse embryonic fibroblasts in Iscove's modified Dulbecco's medium (IMDM) with 15% fetal calf serum (FCS), non-essential amino acids (NEAA) (0.1 mM each), L-glutamine (2 mM), sodium pyruvate (1 mM), Pen/Strep (1,000 U/mL), 2-mercaptoethanol (55 μ M), and LIF (EsGro; Chemicon International; 1,000 U/mL). In the passage before differentiation, cells were passaged on gelatin alone in the absence of feeder cells.

To generate inducible cell lines (such as *A2.Snail*, *A2.Er71*, and *A2.miR-200*), we used gene-specific primers (Supplementary Table S1; Supplementary Data are available online at www.liebertonline.com/scd) to amplify individual cDNAs or miRNA sequences. These were cloned from embryoid body RNA or genomic DNA and inserted into the p2lox targeting vector. To generate these inducible cell lines, *A2lox* cells were co-transfected with the appropriate p2lox vector and Cre recombinase as previously described.

In addition, the p2lox. Δ SNAG.*Snail* vector was generated by using around-the-world PCR (See Supplementary Table S1 for primers). To generate vectors for inducible/constitutive microRNA (miRNA) cell lines, miRNA families were cloned from mouse genomic DNA by using primers flanking the outermost family members (see Supplementary Table S1 for primers). This DNA was then cloned into the p2lox targeting vector such that the miRNA family members would be expressed from the doxycycline-inducible promoter (Fig. 3) OR from the CAG promoter-driven GFP 3' UTR (Fig. 4).

For differentiation, ES cells were plated in petri dishes in suspension at 1.5×10^4 cells/mL in IMDM with 10% FCS, NEAA (0.1 mM each), L-glutamine (2 mM), sodium pyruvate (1 mM), Pen/Strep (1,000 U/mL), and 2-mercaptoethanol (55 μ M). Where indicated, cultures were supplemented with DKK1-his (Dickkopf-1, an inhibitor of canonical Wnt signaling) as described [12], SB-431542 (10 μ M; Sigma), rm-Noggin (500 ng/mL; R&D Systems), or SU5402 (10 μ M; Tocris Bioscience). Gene expression was induced by addition of doxycycline (250–500 ng/mL).

Flow cytometry and sorting

Cells were disassociated with trypsin/ethylenediaminetetraacetic acid for 5 min at 37°C and stained with antibodies. Primary antibodies were as follows: biotin α -mE-cadherin (R&D Systems; 1.25 μ g/mL, 1:200), PE α -Flk1 (eBioscience; Avas12a1, 1:200), biotin α -Flk1 (eBioscience; Avas12a1, 1:200), APC α -PDGFR α (eBioscience; APA5, 1:200), α VE-cadherin (eBioscience; eBioBV13, 1:200), α Tie-2 (eBioscience; TEK4, 1:200), biotin α -c-kit (BD Pharmingen; 2B8, 1:200), and APC α -CD34 (eBioscience; RAM34, 1:200). Secondary detection reagents were SA-APC (BD Pharmingen; 1:400) and SA-PE-Cy7 (BD Pharmingen, 1:400). Data were acquired on the FACS Canto II (Becton Dickinson) and analyzed on FlowJo (Tree Star). *A2.Snail* DKK and DKK+dox samples were

sorted as described in the text based on Flk1 expression by using a MoFlo cytometer (Cytomation).

Blood/endothelial assays

For hematopoietic assays, day 3, 4, or 6 *A2.Snail* and *A2.Er71* embryoid bodies were trypsinized, passaged through a 20-gauge needle, and set up in triplicate dishes in methylcellulose plus cytokines (Stem Cell Technologies; 03434) at a concentration of 50,000 cells/mL. For cells plated at day 3, BL-CFCs (blast-colony forming cells, as described in Ref. [13]) were counted in all samples on day 8. For cells plated on day 4 or 6, primitive and/or definitive blood colonies were counted 6–10 days later.

For the hemangioblast assay, the same day 3 *A2.Snail* and *A2.Er71* cells from above were plated at a density of 50,000 cells/mL in 24-well plates in 1% methylcellulose in differentiation media supplemented with kit ligand and D4T (an embryonic endothelial cell line) conditioned media (gifts from KC Choi), and VEGF (5 ng/mL, Peprotech). Images were recorded 4 days later by using a Nikon Eclipse TS100 microscope and Optronics camera.

The endothelial tube formation assay was performed in Matrigel as described [14], and cultures were supplemented with VEGF. Briefly, 0.2 mL of Matrigel (BD Biosciences) was added to each well of a 24-well plate and allowed to solidify at 37°C. After solidification, 0.2 mL of a cell suspension containing 5×10^4 *A2.Snail* cells on day 3 of embryoid body culture (after trypsinization and passage through a 20-gauge needle) were plated on top of the Matrigel in triplicate in differentiation media plus VEGF. The cultures were incubated at 37°C, 5% CO₂ and observed at 24 h for rearrangement of cells into tubular structures and networks. Images were recorded by using a Nikon Eclipse TS100 microscope and Optronics camera.

Acetylated low-density lipoprotein (Ac-LDL)-uptake assays were performed as previously described [15]. Briefly, cells were differentiated as just described with 500 ng/mL of doxycycline added to cultures on day 2. On day 4, cultures were kept in suspension or transferred to Type I collagen dishes, and 10 μ g/mL of AlexaFluor-488-labeled Ac-LDL (Invitrogen; L233–80) was added to the appropriate cultures. Six hours later, cells were washed with PBS and examined by flow cytometry (suspension cultures) or fluorescent microscopy (adherent cultures) to assess for Ac-LDL uptake.

Gene expression analysis

To evaluate expression of individual genes, RNA was isolated with RNeasy kits (Qiagen). Large-scale gene expression analysis was done by using Affymetrix MOE430_2.0 arrays as previously described [16]. Data were normalized and modeled by using DNA-Chip Analyzer (dChip). CEL files and accompanying data were deposited in the NCBI GEO database under accession number GSE26524.

miRNA expression analysis

Total RNA was isolated by using *mirVana* miRNA Isolation kit (Ambion). TaqMan microRNA assays (Applied Biosystems) were performed according to manufacturer's instructions by using a StepOnePlus Real-time PCR system

(Applied Biosystems). Individual miRNA data are expressed relative to a U6 snRNA TaqMan PCR performed on the same sample.

Luciferase assays

pMIR-Report Luciferase plasmids (Ambion) containing Luciferase with the indicated 3'UTRs, CMV-Renilla, and +/- 5 μ M miRNAs were cotransfected into 293T cells with Lipofectamine 2000 (Invitrogen). See Supplementary Table S1 for a list of primers used to generate the 3' UTRs and mutations. Cells were harvested 24 h later, and luciferase activity was measured by using an Optocomp II automated luminometer (MGM Instruments, Inc.). Firefly luciferase activity was normalized to Renilla luciferase activity to account for possible differences in cell density and transfection efficiency.

Western analysis

A2.Snail.CAG.GFP, *A2.Snail.miR200A*, and *A2.Snail.miR200C* ES cell lines were plated in 10 cm gelatinized dishes in ES cell media. Doxycycline was added in select dishes at 250 ng/mL. Cells were harvested in RIPA buffer along with protease inhibitors 24 h later. As a positive control for Snail expression, 10 μ M MG132 was added to a subset of the doxycycline dishes 4 h before harvesting of the protein lysate. Thirty micrograms of protein lysate was added to each well of a 12% polyacrylamide gel electrophoresis. Protein was transferred to a nitrocellulose membrane, blocked, and probed by using an α Snail antibody (Abcam; ab85931, 1:400) with HRP-anti-rabbit secondary (Jackson Immunoresearch; 1:20,000) or α - β -actin antibody (Santa Cruz Biotechnologies; sc-47778, 1:5000) with HRP-anti-mouse secondary (Jackson Immunoresearch; 1:20,000). ECL Plus Detection System (GE Healthcare) was used for detection following the manufacturer's instructions.

Statistical analyses

For methylcellulose assays, cultures were performed in triplicate. As described in the figure legends, shown is the mean number of colonies with error bars indicating standard error of the mean. Paired student's *t*-tests were performed to calculate *P* values and are indicated in the graphs. For statistical analysis of fluorescence-activated cell sorting (FACS) data, shown are mean percentages of the indicated population from 3 or more experiments (error bars = standard error of the mean). Paired student's *t*-tests were used for calculating *P* values.

Generation of Snail +/- ES cells

The targeting vector was constructed in pLNTK by using a 1.5 kb genomic fragment 5' to *Snail* exon 1, and a 3.6 kb genomic fragment 3' of the stop codon. The 5' arm was generated by PCR from genomic DNA with the use of the following oligonucleotides: 5'arm F (GGGTGGGAATCATTCTGCTGTTGC) and 5'arm R (GTCGACGGTAGCAAGGACCAGGGCTAAGGC). The 3' arm was generated by using 3'arm F (GTTGGTGTGGTGACCTCAACTCTG) and 3'arm R (GTCGACGGCTGTGCTGAAAGGGCTCCAT). The 5' and 3' arms were ligated into pLNTK vector, which was linear-

ized with PvuI before electroporation into A2lox ES cells. Positive clones were initially screened by PCR and confirmed by Southern analysis with 5' and 3' DIG-labeled probes (5'probeF: TGTCTTCAGTCCCATGTCCGGG; 5'probeR: CAACAAAATCCATGCAACAAGGCAG; 3'probeF: GACA GTTGGCTTCCTGCCTAAGAACA; 3'probeR: CAGATCCC TGCCTGCTGGGAC). Of the 408 ES cell colonies screened, 1 was correctly targeted.

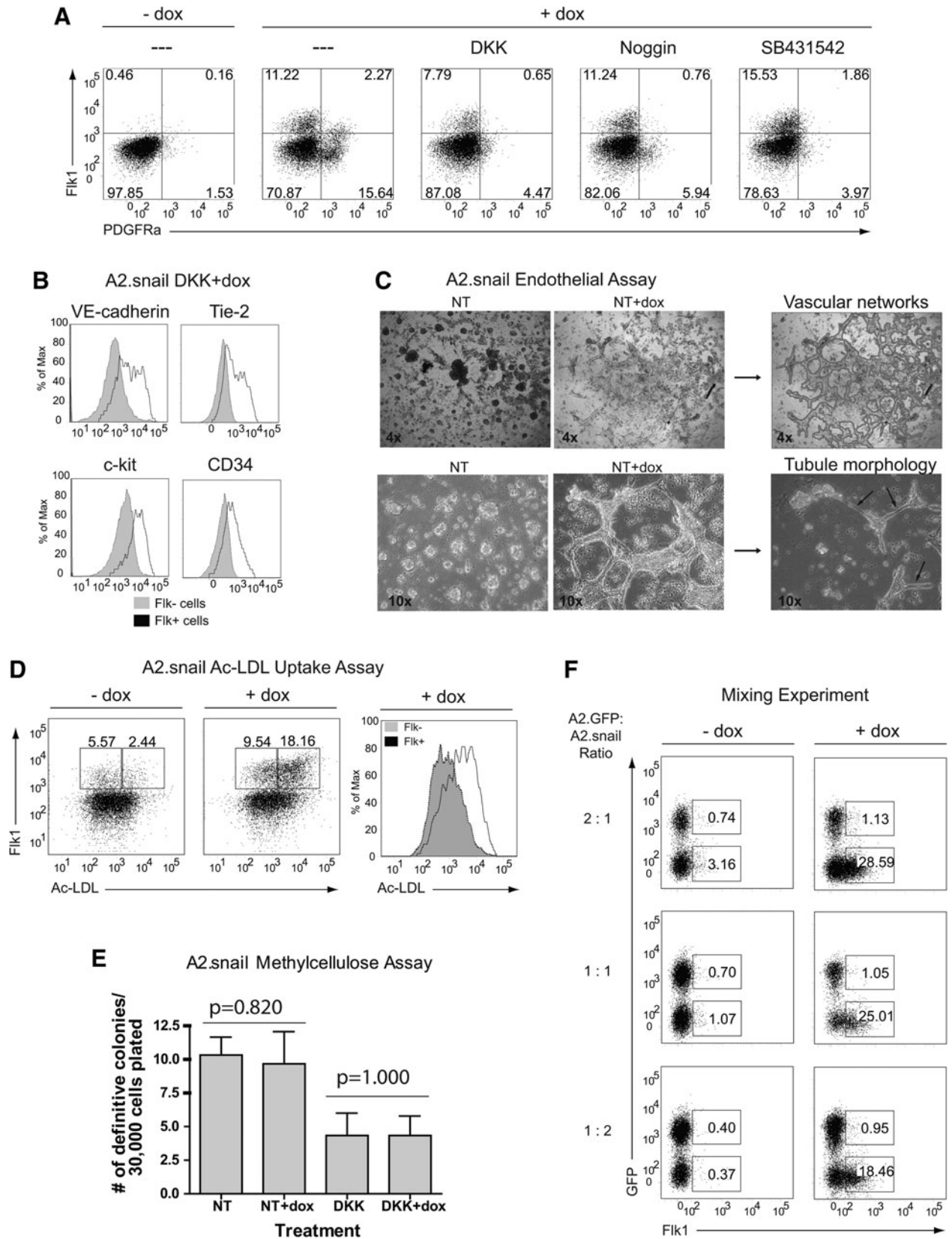
Results

Using an ES cell line that inducibly expresses *Snail* on addition of doxycycline (*A2.Snail*) [16], we previously found that *Snail* could induce EMT and accelerate mesoderm commitment in differentiating ES cells as assessed by the mesoderm markers Flk1 and PDGFR α [10]. To evaluate how *Snail* induces Flk1⁺ and PDGFR α ⁺ cell populations, we analyzed the differentiation of *A2.Snail* ES cells while inhibiting Wnt, Activin, and BMP pathways, which are known to regulate primitive streak formation and patterning. Doxycycline induction of *Snail* on day 1 generated populations expressing PDGFR α and Flk1, corresponding to early paraxial and lateral mesoderm, respectively [17], by day 3 (Fig. 1A). Interestingly, when BMP, Activin, or Wnt pathways were inhibited (by DKK, Noggin, or SB431542 respectively), the PDGFR α Flk1⁺ population was selectively maintained (Fig. 1A, Supplementary Fig. S1A). These results suggested that *Snail* induces the Flk1⁺ single-positive population independently of cell-extrinsic cues known to be important for primitive streak formation.

Since hemangioblasts express Flk1 and arise early in ES cell differentiation [13], we wondered whether *Snail*-induced Flk1⁺ cells might be progenitors of blood and endothelial lineages. We, therefore, assessed the Flk1⁺ PDGFR α - cells induced by *Snail* for expression of other markers associated with endothelial and blood progenitors. VE-cadherin, Tie-2, c-kit, and CD34 were highly expressed in the *Snail*-induced Flk1⁺ population (Fig. 1B). This was true even when the Wnt inhibitor DKK1 was omitted from the culture (Supplementary Fig. S1B).

We next asked whether *Snail*-induced Flk1⁺ cells had the potential to generate blood and endothelial lineages. *A2.Er71* cells, harboring a doxycycline-inducible transcription factor *Er71* that robustly induces hemangioblast differentiation [18], were used as a positive control. To examine the endothelial potential of *A2.Snail* cells, differentiating *A2.Snail* and *A2.Er71* cells that were treated with or without doxycycline on day 1 and on day 3 were trypsinized and replated on Matrigel in the presence of VEGF. After 24 h, doxycycline-treated *A2.Snail* cells formed networks of cells with a tubular, sprouting morphology characteristic of endothelial cell cultures (Fig. 1C), whereas untreated *A2.Snail* cells lacked these features, appearing instead as typical embryoid bodies. *A2.Snail* cells treated with DKK and *A2.Er71* cells showed similar morphological findings depending on the presence or absence of doxycycline (Supplementary Fig. S1C).

To show that these were truly endothelial cells, we then performed a functional assay assessing the ability of these Flk1⁺ cells to endocytose AlexaFluor488-labeled Acetylated-LDL added to the culture. *A2.Snail* cells were treated with and without doxycycline on day 2, and Ac-LDL was added to the culture on day 4. On day 4.25, cells were examined by flow cytometry and fluorescent microscopy to assess uptake of



Ac-LDL. As shown in Fig. 1D, induction of *Snail* induces a Flk1⁺ population, of which the majority are capable of endocytosing Ac-LDL. This uptake of Ac-LDL is enhanced uniquely in the Flk1⁺ cells, as can be seen when comparing the Flk1⁺ cells to Flk1⁻ cells in the same culture (Fig. 1D, right panel). Embryoid bodies plated on Type I Collagen before the uptake assay showed similar results (Supplementary Fig. S1D), where labeled Ac-LDL highlighted the developing vascular networks in the *Snail*-induced cultures.

To assess blood-forming potential, differentiating *A2.Snail* cells from untreated and doxycycline-treated cultures were placed into methylcellulose and cytokines on days 3, 4, and 6. When plated on day 3, *Er71* strongly induced blast colonies as expected, but *Snail* was, at least, 100-fold less efficient in inducing blast colonies under all conditions (Supplementary Fig. S1E). Similar results were seen in additional hemangioblast assays when *Snail* was plated in methylcellulose on day 4 (Fig. 1E) or day 6 (Supplementary Fig. S1F). No improvement in *Snail*'s ability to induce either primitive or definitive colonies was found by plating cells at these additional timepoints. In summary, *Snail* induces a Flk1⁺ population that lacks significant potential to form blood lineages but does form endothelial lineages.

Since *Snail* induced this endothelial-like Flk1⁺ population independently of BMP, Activin, and Wnt signaling, we asked whether it occurred by a cell-intrinsic or cell-extrinsic mechanism (Fig. 1F). We carried out a series of mixing experiments by using various ratios of *A2.Snail* ES cells and *A2lox.GFP*, an ES cell line that constitutively expresses the GFP protein (Supplementary Fig. S1G). Without doxycycline treatment, neither cell line expressed significant Flk1 on day 4 at any ratio of cell mixing (Fig. 1F, left panels). However, doxycycline treatment caused expression of Flk1 only in *A2.Snail* cells under all mixing ratios, whereas *A2.GFP* cells remained negative for Flk1 expression (Fig. 1F, right panels). These results indicate that *Snail* promotes Flk1 expression through a cell-intrinsic mechanism.

Similar to cells of the embryo's ICM, ES cells are heterogeneous with regard to their commitment to primitive endoderm or the epiblast, a process regulated by the transcription factors GATA6 and Nanog, respectively [19,20]. Conceivably, the mixed Flk1/PDGFR α expression pattern in *Snail*-expressing ES cells might be based on differential actions of *Snail* in either GATA6-expressing or Nanog-expressing ES cells.

To reveal potential heterogeneity, we used flow cytometry to purify Flk1⁺ high and Flk1⁻ ES cells 24 h after induction of *Snail* in the presence or absence of DKK (Fig. 2A) and compared their transcriptional profiles by using gene expression microarrays (Fig. 2B). Consistent with our findings just mentioned, *Snail*-induced Flk1⁺ cells expressed numerous factors associated with endothelial progenitors (Supplementary Fig. S2A). In addition, known targets of *Snail*, such as Occludin [21], Claudin 3, and Crumbs homolog 3 [22], were markedly inhibited by induction of *Snail* in both Flk1⁺ and Flk1⁻ populations compared with cells in which *Snail* was not induced, thus indicating that *Snail* was functional in both populations (Fig. 2B, Supplementary Fig. S2B). In contrast, other genes showed differential expression within these 2 subsets. In particular, the epiblast-specific genes Nanog and Oct4 were selective in Flk1⁻ cells (Fig. 2B, middle panel). In contrast, primitive endoderm-related genes such as GATA6, Sox7, and BMP2 were selectively expressed in the Flk1⁺ subset (Fig. 2B, bottom panel). This suggests that promotion of Flk1⁺ cells by *Snail* may be selectively mediated by actions within the GATA6⁺ primitive endoderm subset of an initially heterogeneous ES cell population.

Since fibroblast growth factor (FGF) receptor signaling is required for the formation of primitive endoderm [23], we tested whether inhibition of FGF signaling would diminish *Snail*-induced Flk1 expression. To do this, we used the FGF signaling antagonist SU5402 [24] (Fig. 2C). Treatment of ES cells with SU5402 from day 1 reduced the number of Flk1-expressing cells by approximately 3-fold (Fig. 2C), and also decreased the number of cells able to down-regulate E-cadherin after *Snail* expression (Supplementary Fig. S2C).

Snail is thought to act primarily as a transcriptional repressor through its N-terminal SNAG domain [25], and yet appeared to be promoting increased Flk1 expression. To test whether Flk1 induction required the SNAG domain, we created the *A2. Δ SNAG.Snail* cell line, in which the SNAG domain has been removed from the inducible *Snail* protein (Fig. 3A). As a control, we confirmed that deletion of this domain blocks *Snail*'s ability to inhibit expression of E-cadherin (Supplementary Fig. S3A), a known direct transcriptional target of *Snail* [1]. In addition, this deletion also prevented the induction of Flk1 expression induced by *Snail* in differentiating ES cells (Fig. 3A, Supplementary Fig. S3B). This result indicates that *Snail*'s induction of Flk1 is indirect, and may be mediated through *Snail* repressing a repressor of Flk1 expression.

FIG. 1. *Snail* promotes a population of Flk1⁺ endothelial cells in a cell-autonomous manner independent of Wnt, BMP, and activin signaling. **(A)** *A2.Snail* cells were differentiated with or without the addition of doxycycline and the indicated inhibitors on day 1 of differentiation. Shown is a FACS analysis for Flk1 and PDGFR α on day 3 of differentiation, where plots are gated on live cells. **(B)** *A2.Snail* cells were differentiated and analyzed by FACS on day 3 of differentiation in the presence of DKK + / - doxycycline on day 1. Histograms for VE-cadherin, Tie-2, c-kit, and CD34 are shown for Flk1⁺ and Flk1⁻ gated populations. **(C)** Embryoid bodies from day 3 *A2.Snail* differentiation cultures **(B)** were trypsinized and replated in Matrigel-coated 24-well plates with vascular endothelial growth factor. On day 4, cultures were examined for the formation of networks and tubules. Red lines help delineate the outline of the vascular network, whereas arrows show examples of tubules. **(D)** *A2.Snail* ES cells were differentiated with and without doxycycline addition on day 2. On day 4, A-488 labeled Ac-LDL was added to the culture media, and cells were washed and analyzed by FACS 6 h later on day 4.25. Flk1 staining was performed and shown on the y-axis. **(E)** *A2.Snail* ES cells were differentiated as just described and plated on day 4 in methylcellulose media with hematopoietic cytokines. Shown is the # of definitive colonies that grew in the indicated conditions by day 12. Error bars indicate SEM. **(F)** *A2.CAG.GFP* and *A2.Snail* ES cells were mixed in ratios of 2:1, 1:1, and 1:2 at the onset of differentiation. Doxycycline was added on day 2 of differentiation, and FACS analysis was performed on day 4. Shown is an FACS plot of live cells, examining GFP expression as well as Flk1. Ac-LDL, acetylated-low density lipoprotein; FACS, fluorescence-activated cell sorting; ES, embryonic stem; SEM, standard error of the mean.

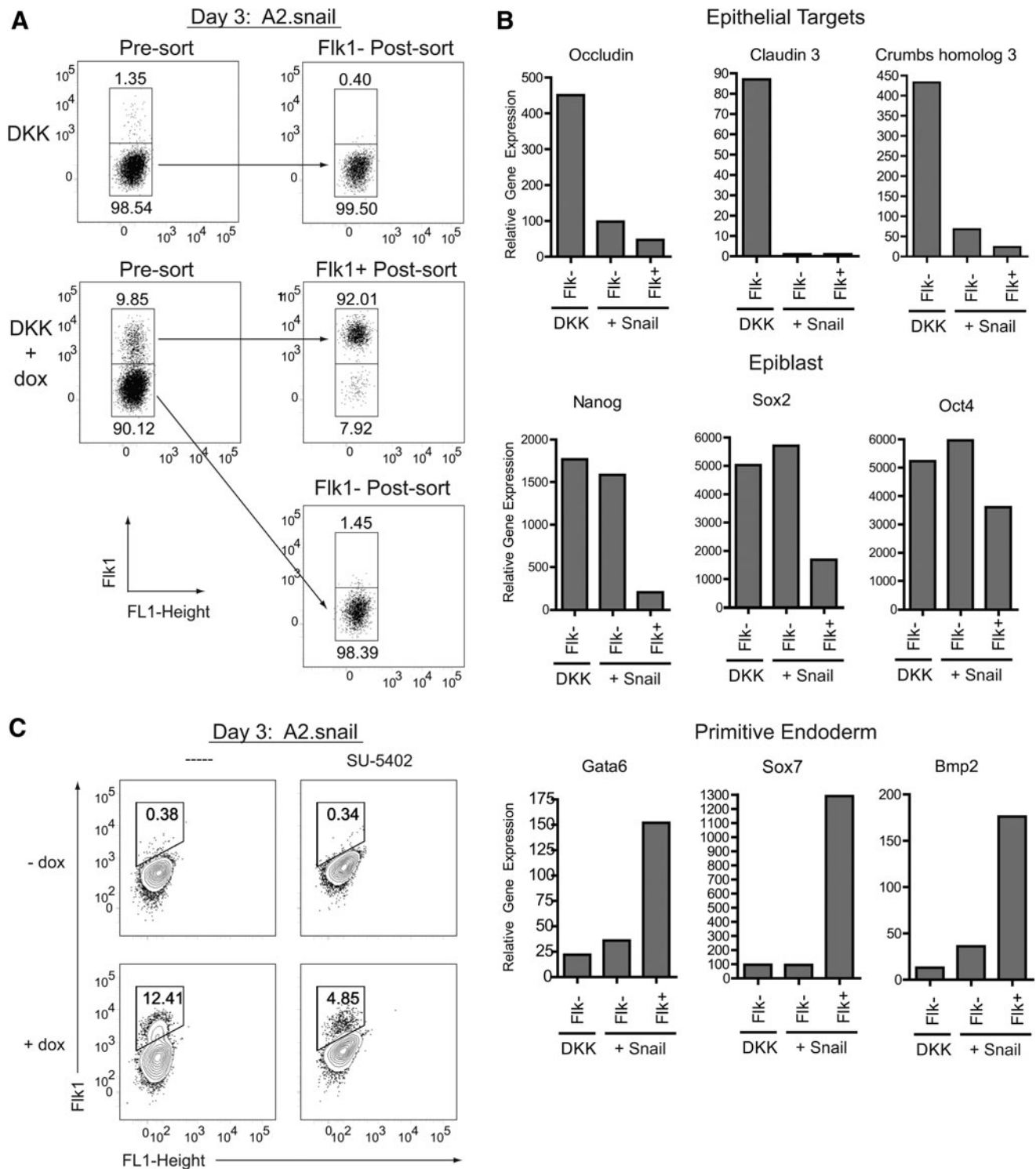


FIG. 2. *Snail*-derived Flk1 + cells appear to arise from a Gata6+ subset of ES cells in an FGF-dependent manner. (A) A2.*Snail* cells were differentiated in DKK, with or without the addition of doxycycline on day 2. On day 3, Flk1+ high and Flk1- cells were sorted, and RNA was isolated from each population. Total RNA was then submitted for gene chip analysis to characterize the separate populations. (B) Relative gene expression of epithelial targets, epiblast, and primitive endoderm markers in the sorted populations. (C) A2.*Snail* ES cells were differentiated with and without the addition of doxycycline and the FGF-inhibitor SU-5402 on day 1. FACS analysis was performed on day 3 for Flk1. Shown are gated live cells. FGF, fibroblast growth factor.

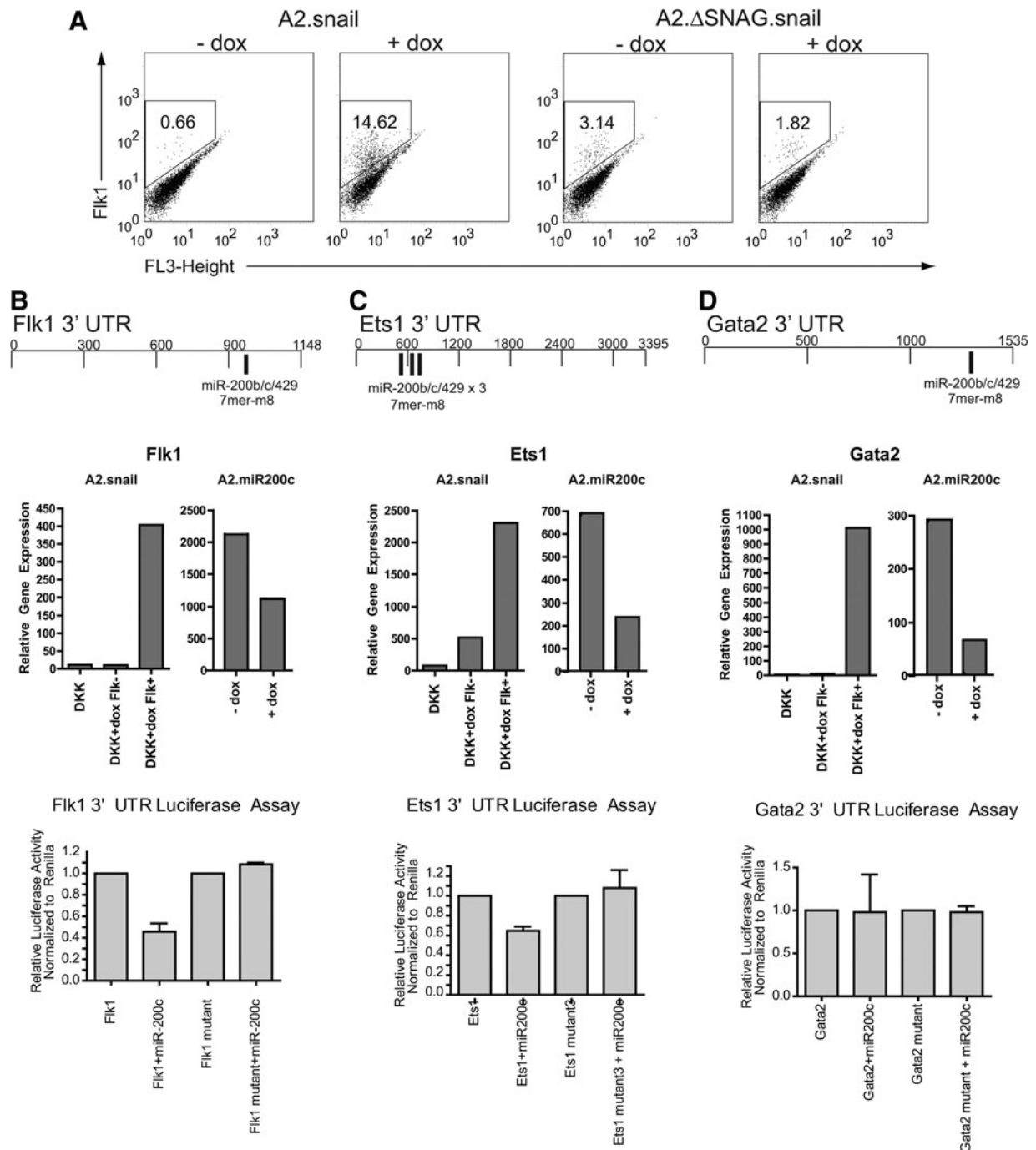


FIG. 3. *Snail* induction of Flk1 is indirect, whereas its downstream target, the miR-200 family, directly targets Flk1 and Ets1 3' UTRs. **(A)** A2.*Snail* and A2.ΔSNAG.*Snail* cells were differentiated as described in the Methods section. Doxycycline was added on day 2 of differentiation, and FACS analysis was performed on day 4. Shown is an FACS plot of live cells, examining Flk1 expression. **(B)** (top) Diagram of predicted miR-200c target sites in the 3' UTR of Flk1. (middle) Relative expression of Flk1 in A2.*Snail* sorted populations from Fig. 2B as well as in day 5 A2.miR200c cells induced or not induced to express miR-200c/141 by doxycycline from day 2. (bottom) Relative luciferase activity of 293T cells transfected with a CMV-luciferase-Flk1 3'UTR construct, with or without addition of a miR-200c mimic, as well as 293T cells transfected with a CMV-luciferase-Flk1 MUTATED 3' UTR construct, with or without addition of a miR-200c mimic. Data was normalized to co-transfected CMV-Renilla activity. **(C, D)** Analogous experiments to Fig. 3B for Ets1 and Gata2.

We previously found several miRNAs, including the miR-200b/c/429 family, that are inhibited by *Snail* during ES cell differentiation [10]. We, therefore, wondered whether *Snail*'s generation of Flk1⁺ cells was mediated by repression of miRNAs that may repress Flk1 or other factors important for

endothelial differentiation. Interestingly, we found that Flk1 itself is predicted to be a target of the miR-200b/c/429 family (Fig. 3B, top panel, Supplementary Fig. S3C). Consistent with this prediction, the induction of miR-200c in A2.miR200c/141 ES cells reduces *Flk1* mRNA and protein [10] expression

(Fig. 3B, middle panel). This regulation of Flk1 expression maps to the 3' UTR of the *Flk1* transcript, which contains a predicted miR-200c target at position 959 (Supplementary Fig. S3C). To test whether miR-200c directly targets Flk1, we generated luciferase reporter constructs containing a CMV-driven luciferase transcript upstream of the Flk1 3' UTR. We transfected these constructs into 293T cells with or without a miR-200c mimic. We found that miR-200c selectively repressed luciferase activity in constructs containing the native Flk1 3' UTR by 50%, but failed to reduce activity of a luciferase construct in which the target sequence at position 959–965 was scrambled (Fig. 3B). Thus, miR-200c appears to directly down-regulate Flk1 expression through the targeting of the Flk1 3' UTR.

We noted that 2 other genes induced by *Snail*, *Ets1* and *Gata2* (Fig. 3C, D), also contained predicted target sequences for miR-200c in their 3' UTR (Supplementary Fig. S3D, E). These genes are also known to be important in vasculogenesis [26,27]. Similar to *Flk1*, *Ets1* and *Gata2* transcripts were both repressed by doxycycline induction of miR-200c/141 in A2.miR200c/141 ES cells (Fig. 3C, D). To test whether miR-200c directly targeted *Ets1* and *Gata2* 3' UTRs, we generated

additional CMV-luciferase reporter constructs upstream of *Ets1* and *Gata2* 3' UTRs. Our data (Fig. 3C) confirmed that the *Ets1* 3' UTR is responsive to miR-200c, as demonstrated by reduced luciferase activity in the presence of miR-200c, as recently reported [28]. We further identified the location of these responsive sites to be at positions 574, 625, and 633 in the *Ets1* 3' UTR transcript. In contrast, miR-200c did not appear to directly repress the *Gata2* 3' UTR (Fig. 3D). In addition to *Flk1* and *Ets1*, *Snail* strongly induced expression of *Flt1* as well as *Neuropilin1* (Supplementary Fig. S2A). Notably, these genes are also known to be targets of miR-200 [29,30]. Together, these data support the hypothesis that miR-200 family miRNAs directly repress the level of several genes important or required for early vasculogenesis.

To determine whether repression of miR-200c/141 is required for *Snail*'s enhancement of Flk1 in differentiating ES cells, we generated A2.*Snail.miR200c* ES cell lines expressing doxycycline-inducible *Snail*, but constitutive expression of miR-200c/141 under the control of the chicken β -actin promoter (Fig. 4A, Supplementary Fig. 4A). We carried out a dose response for doxycycline and examined the induction of Flk1 in these cell lines by using flow cytometry (Fig. 4A).

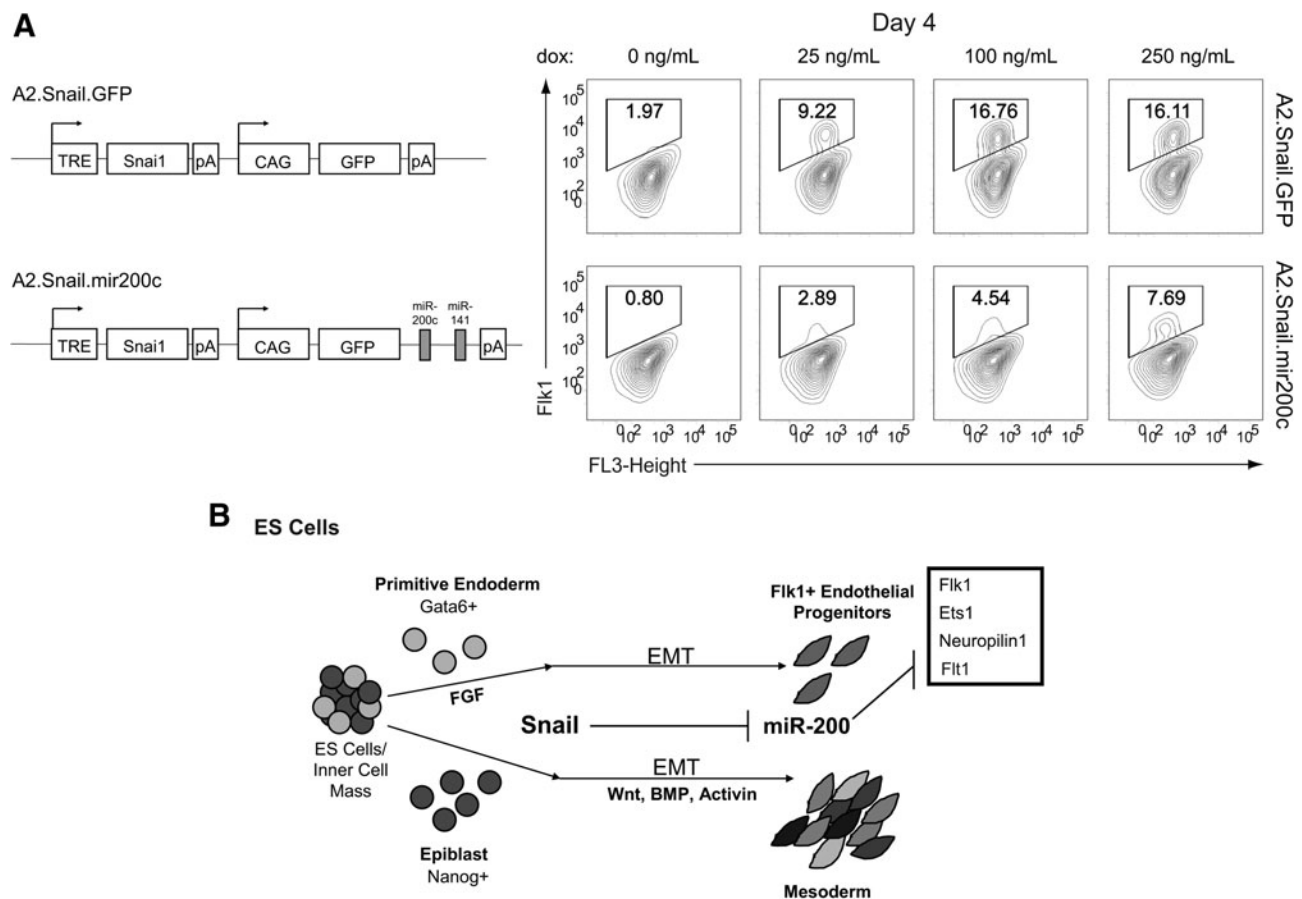


FIG. 4. *Snail* requires the down-regulation of the miR-200 family for efficient generation of Flk1+ endothelial cells. **(A)** Diagram of cell line generated with inducible *Snail* expression with or without constitutive expression of miR-200c/141. A2.*Snail.GFP* (top) and A2.*Snail.miR200c* (bottom) ES cells were differentiated with or without various concentrations of doxycycline induction on day 2. FACS analysis was performed for Flk1 expression on day 4. Shown are gated live cells. **(B)** Proposed model: In differentiating ES cells, *Snail* promotes Flk1+ endothelial progenitors in a cell-intrinsic manner in a subset of primitive endoderm-like cells. This process requires the down-regulation of the miR-200 family that directly targets Flk1 and *Ets1*, as well as *Neuropilin1* and *Flt1*.

Without expression of miR-200c and miR-141, half-maximal induction of Flk1 occurred at 25 ng/mL doxycycline, and maximal Flk1 was achieved at 100 ng/mL (Fig. 4A, upper panels). In *A2.Snaill.miR200c* cells constitutively expressing miR-200c and miR-141, there was a significant inhibition of Flk1 induction throughout the dose range of doxycycline (Fig. 4A, lower panels). Constitutive expression of miR-200a/b/429 family members in an additionally generated *A2.Snaill.miR200a* line also reduced Flk1 induction in response to doxycycline, although not as strongly as seen with miR-200c and miR-141 (Supplementary Fig. S4B, C). Western analysis of protein expression verified that induction of *Snail* was similar across these cell lines (Supplementary Fig. S4D). Notably, *Snail*'s ability to down-regulate E-cadherin was compromised in the cell lines constitutively expressing the miR-200 family (Supplementary Fig. S4E). Since the miR-200 family directly targets *Zeb1* and *Zeb2* expression [31], this may suggest that *Snail* requires their function for efficient E-cadherin down-regulation.

Overall, these data show that induction of Flk1 cells by *Snail* requires the down-regulation of the miR-200 family, which directly target the 3' UTRs of Flk1 itself, *Ets1*, and additional factors important in vasculogenesis (Fig. 4B). Future studies will determine whether other miRNAs regulated by *Snail* might also cooperate in this pathway to promote vascular development.

Discussion

This study broadens the scope of *Snail*'s actions in embryonic differentiation. Earlier, the major focus of *Snail*'s function has centered on its triggering of the EMT and its direct inhibition of E-cadherin. However, deletion of *Snail* under the control of a *Meox2*-driven Cre enzyme led to embryonic lethality due to early vascular defects, presumably due to actions of *Snail* on endothelium [2,3]. However, it was unclear in that study whether *Snail* was acting within the endothelium itself, or alternatively influencing endothelium through indirect effects of *Snail* in nonendothelial tissues.

Our data would imply that *Snail* can directly act within the appropriate precursors to influence the expression of a variety of genes controlling endothelial differentiation, including Flk1 and *Ets1*. Importantly, these endothelial-specific gene targets do not appear to be directly repressed by *Snail*, as in the case of E-cadherin, but are rather indirectly augmented through the *Snail*-dependent inhibition of miR-200 family miRNAs. These results highlight an action of *Snail* in the differentiation of primitive endoderm, rather than actions of *Snail* in cells undergoing EMT within the embryo proper.

These data raise the question of how primitive endoderm-derived Flk1⁺ cells (angioblasts) might be related to the mesoderm-derived Flk1⁺ cells (hemangioblasts) previously described [13]. Since the embryonic yolk sac vasculature is thought to be derived from both primitive endoderm as well as extraembryonic mesoderm [32], we would hypothesize that each of these precursors contribute to their own niche within the yolk sac. Although blood islands may be derived from an extraembryonic-mesoderm derived Flk1⁺ hemangioblast, it is tempting to speculate that the angioblast-derived portions of the yolk sac (ie, the plexi adjacent to the chorion and to the embryo [33]) may arise from *Snail*-

expressing primitive endoderm. Since extraembryonic mesoderm has also been shown to express *Snail*, it is possible that *Snail*/EMT plays a secondary role in directing the bipotential hemangioblast toward the endothelial lineage.

By looking at embryos lacking *Snail* protein (entirely [34] or in the embryo proper [3]), it is clear that *Snail* is required for normal vascular development and efficient endothelial commitment in the developing embryo. Future studies will determine whether *Snail* is also required for generation of endothelial cells in ES cell differentiation. We have generated a haploinsufficient *Snail* +/− ES cell clone that appears to have decreased differentiation of both Flk1 and PDGFR α -expressing cells (Supplementary Fig. S5). Although we are unable to say whether this is due to *Snail* haploinsufficiency or clonal variation, it suggests that *Snail* may also be required for efficient commitment of ES cells to early vascular lineages.

In conclusion, we propose that *Snail* and miR-200c link EMT and vasculogenesis in a subset of ES cells that resemble primitive endoderm. The promotion of endothelial progenitors depends on FGF signaling as well as *Snail*'s down-regulation of the miR-200 family, which directly target Flk1 and *Ets1*. Understanding the mechanism behind *Snail*'s dual functions will be important in future studies of embryogenesis and possible therapeutics aimed at limiting tumor vasculogenesis.

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Author Disclosure Statement

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