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Gene Expression Profile Signatures Indicate a Role for Wnt Signaling in Endothelial Commitment From Embryonic Stem Cells

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Abstract—We have used global gene expression analysis to establish a comprehensive list of candidate genes in the developing vasculature during embryonic (ES) cell differentiation in vitro. A large set of genes, including growth factors, cell surface molecules, transcriptional factors, and members of several signal transduction pathways that are known to be involved in vasculogenesis or angiogenesis, were found to have expression patterns as expected. Some unknown or functionally uncharacterized genes were differentially regulated in flk1$^+$ cells compared with flk1$^-$ cells, suggesting possible roles for these genes in vascular commitment. Particularly, multiple components of the Wnt signaling pathway were differentially regulated in flk1$^+$ cells, including Wnt proteins, their receptors, downstream transcriptional factors, and other components belonging to this pathway. Activation of the Wnt signal was able to expand vascular progenitor populations whereas suppression of Wnt activity reduced flk1$^+$ populations. Suppression of Wnt signaling also inhibited the formation of matured vascular capillary-like structures during late stages of embryoid body differentiation. These data indicate a requisite and ongoing role for Wnt activity during vascular development, and the gene expression profiles identify candidate components of this pathway that participate in vascular cell differentiation. (***Circ Res*****. 2006;98:1331-1339.)**

Key Words: hemangioblast ■ angiogenesis ■ microarray ■ signaling

The vascular system is a complex network of vessels that perfuses all organs and tissues and that is universally required for their normal development and function. Vascular anomalies are found in many congenital and acquired diseases such as cardiovascular disorders, hypertension, diabetes, and neoplasms. Therefore, much effort has focused on understanding the mechanisms of vascular development for possible therapeutic applications. Unfortunately, our knowledge of endothelial progenitors and particularly of the transcriptional programs that regulate their development is still underdeveloped, and many gaps exist in our understanding of the molecular players responsible for the initiation of stem cell differentiation and subsequent commitment to a blood vessel phenotype.

Studies of vascular development have been hampered by difficulties in accessing the embryo before establishment of blood islands and by the limited number of cells available at this stage. The in vitro embryonic stem (ES) cell differentiation system provides an alternate approach.[1] This system is a powerful model system to determine the cellular and molecular mechanisms of vascular development.[2,3] ES cells can differentiate spontaneously, resulting in the formation of embryo-like structures called embryoid bodies (EBs) that have the potential to generate a variety of embryonic cell lineages. Blood cells and endothelial cells develop within EBs in a manner that faithfully follows developmental progression in vivo.[4,5] Many aspects of normal endothelial growth and development, up to and including the formation of vascular channels, have been reported in this system.[1,6] In addition, a number of intermediate cell populations, such as precursors of endothelial cells, smooth muscle cells (SMC), and blood cells, have been identified.[7–9] In vitro ES cell differentiation experiments have shown that flk1-expressing cells have developmental potential uniquely restricted to hematopoietic and endothelial lineages,[5,8,9] which makes flk1 a useful marker for understanding early steps in vascular development. In this study, we used the in vitro ES cell-derived EB system to establish global gene expression profiles of endothelial differentiation. Among other observations, we have used this dataset to define a critical role for Wnt signaling in endothelial cell differentiation, suggesting that modification of Wnt activity may be a potential tool to regulate vascular patterning in vivo.

Materials and Methods

ES Cell Culture

R1 mouse ES cells were maintained on gelatin-coated plates with conditioned medium from 5637 cells as a source of leukemia inhibitory factor (LIF). In vitro differentiation was induced as

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From the Carolina Cardiovascular Biology Center (H.W., P.C.C., Y.W., R.R., X.P., M.M., V.B., C. Patterson) and Departments of Medicine (C. Patterson), Biology (V.B.), and Genetics (C. Perou), University of North Carolina, Chapel Hill; and Laboratory of Cellular and Molecular Biology, National Cancer Institute (M.B.-K., J.S.R.), Bethesda, Md.

Correspondence to Cam Patterson, MD, Director, Division of Cardiology and Carolina Cardiovascular Biology Center, 8200 Medical Biomolecular Research Building, Chapel Hill, NC 27599-7126. E-mail cpatters@med.unc.edu

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previously described.[10] Differentiation cultures were fed every other day, and in some cases the medium was supplemented with LiCl (10 mmol/L), sFRP-1 (10 μg/mL), or conditioned media as indicated from the initiation of differentiation of EBs.

Fluorescence-Activated Cell Sorting and RT-PCR

For fluorescence-activated cell sorting (FACS) analysis of flk1 expression, EBs were dissociated with trypsin and stained with phycoerythrin-conjugated anti-flk1 antibody before analysis on a FACScan (Becton Dickinson). Cells were sorted using a MoFlo (Cytomation) and reanalyzed on a FACScan. Specific primers used for RT-PCR are indicated in Table I of the online data supplement available at http://circres.ahajournals.org.

Microarray Hybridization and Data Analysis

Total RNA was isolated from undifferentiated ES cells and from differentiated EBs at 72 hours, 84 hours, and 95 hours and 8 days and from sorted flk1$^+$ and flk1$^-$ cells at 84 hours, 95 hours, and 8 days. Microarray hybridization and data analysis are described in detail in the text of the online data supplement.

Production of Conditioned Media and Detection of Activity of Wnt Protein in Conditioned Media

The Wnt3a-producing L cell line and control L cell line were from the American Type Culture Collection. We also used a *Drosophila* S2 cell line expressing Wnt2 and a control S2 cell line, which were generous gifts from Roel Nusse (Stanford University). Wnt2 S2 cells were cultured in Schneider's media supplemented with 10% FBS under selection with hygromycin (125 μg/mL) for 2 to 3 passages before collection of media.

Assays for Proliferation and Apoptosis

EBs treated with Wnt2-CM or S2-CM were fixed and triple-labeled with rabbit anti-phosphohistone H3, rat anti-PECAM, and the DNA-binding dye DAPI. Triple-labeled images were analyzed as previously described.[11] Endothelial mitotic indices were calculated by dividing the number of PECAM$^+$/phosphohistone H3$^+$ cells by the total number of PECAM$^+$ cells. Nonendothelial mitotic indices were also calculated on a per field basis by dividing the number of PECAM$^+$/phosphohistone H3$^+$ cells by the total number of PECAM$^-$ cells.

Results

Identifying Genes Within the Developmental Vascular Niche by Gene Expression Profiling

We used an unbiased analysis to identify patterns of gene expression during the earliest stages of endothelial cell differentiation using flk1 as a marker, as we have previously described.[10] We hypothesized that a global understanding of vascular cell gene expression profiles can identify unanticipated molecular and cellular events in vascular development. In initial experiments, we found that differentiated ES cells did not express detectable flk1 by flow cytometry until approximately 84 hours after differentiation (Figure 1). The expression of flk1 peaked at 95 hours and was maintained in 30% of the culture until at least 8 days, when a mature vascular phenotype with capillary-like structures is fully developed.[12] Based on this temporal pattern, EBs were sampled into flk1$^+$ and flk1$^-$ populations after the onset of flk1 expression at the key times during differentiation (84 hours, 95 hours, and 8 days). RNAs were hybridized after amplification to oligonucleotide arrays that contain more than 20 000 mouse genes related to development. Figure 1 summarizes the experimental design and indicates the outflow of millions of total data points. Validation of this data set is described in text of the online data supplement and supplemental Table I and supplemental Figures I and II.

Differential Gene Expression Within the flk1$^+$ Lineage

Overall, in comparing flk1$^+$ with flk1$^-$ cells, there were 802 differentially regulated mRNAs at 84 hours, 486 at 95 hours, and 270 at day 8 days (supplemental Figure III). Seventy mRNAs were consistently differentially expressed at all time points, including flk1 itself, as expected (Table). Among these, more genes were downregulated than were upregulated in flk1$^+$ cells, which likely indicates that flk1$^-$ cells are a more heterogeneous population. In addition, there were 1134 probe sets that were differentially regulated in flk1$^+$ cells in comparison with flk1$^-$ cells at any of the 3 time points. One thousand forty-three of these have Unigene identifiers and actually correspond to 993 unique genes. Among this broader group, a number of genes associated with the endothelial lineage—coding for proteins such as GATA2, CXCR4, neuropilin-1, and endoglin as well as components of the angiopoietin, ephrin, and notch pathways—were preferentially expressed in flk1$^+$ cells at 1 or more time points. It is also remarkable that genes associated with the cardiomyocyte lineage—including GATA4, myosin light chain 2a, and Mesp1—appear in the early flk1$^+$ populations.

Using hierarchical clustering and TreeView analysis, we compared the expression pattern of those genes differentially expressed in flk1$^+$ cells compared with flk1$^-$ cells during stem cell differentiation (Figure 2). Unsupervised hierarchical clustering of the gene expression pattern from individual samples produced groupings consistent with their development stage and flk1 expression level. Later stage samples (8 days) were grouped together, apart from early stage samples (84 and 95 hours), and flk1$^+$ samples clustered separately from flk1$^-$ samples. Three clusters were selected for further analysis. Cluster A includes flk1 and represents genes mostly upregulated in flk1$^+$ cells independent of time, such as Wnt2, Wnt5a, Nkd1 (Naked Cuticle 1, a Wnt signaling antagonist) and ccnd2 (cyclin D2, a Wnt target gene), cardiomyocyte lineage-associated genes (Hand1, GATA6, GATA4) and transforming growth factor family genes (Tgfb2 and Bmp4). Cluster B contains genes upregulated in early (84 and 95 hours) flk1$^+$ cells but not during the later time points. Again, Wnt signaling associated genes such as Frzb, Cdh2 (N-cadherin), Msx2, Ccnd1 (Cyclin D1), and Dkk1 (Dickkopf 1) are present in this cluster, as are Notch1, GATA2, GATA3, Tgfb1, Bmp7, and Smad1. Cluster C contains genes downregulated in flk1$^+$ cells relative to flk1$^-$ cells. Representative genes in this cluster include E-cadherin and transcriptional factors Sox2, Foxo1, and Foxa2, which collective are associated with endoderm- and ectoderm-derived tissues.[13,14] Notably, E-cadherin and Sox2 are negative regulators of Wnt signaling.[15] The presence of multiple clusters of genes showing temporally coordinated patterns of expression during a critical development stage suggests a common mechanism of transcriptional regulation (see online data supplement).

Differential Expression of Genes Within the Wnt Signaling Pathway

The Wnt signaling pathway has a well-defined role in development. Although not investigated systematically, a

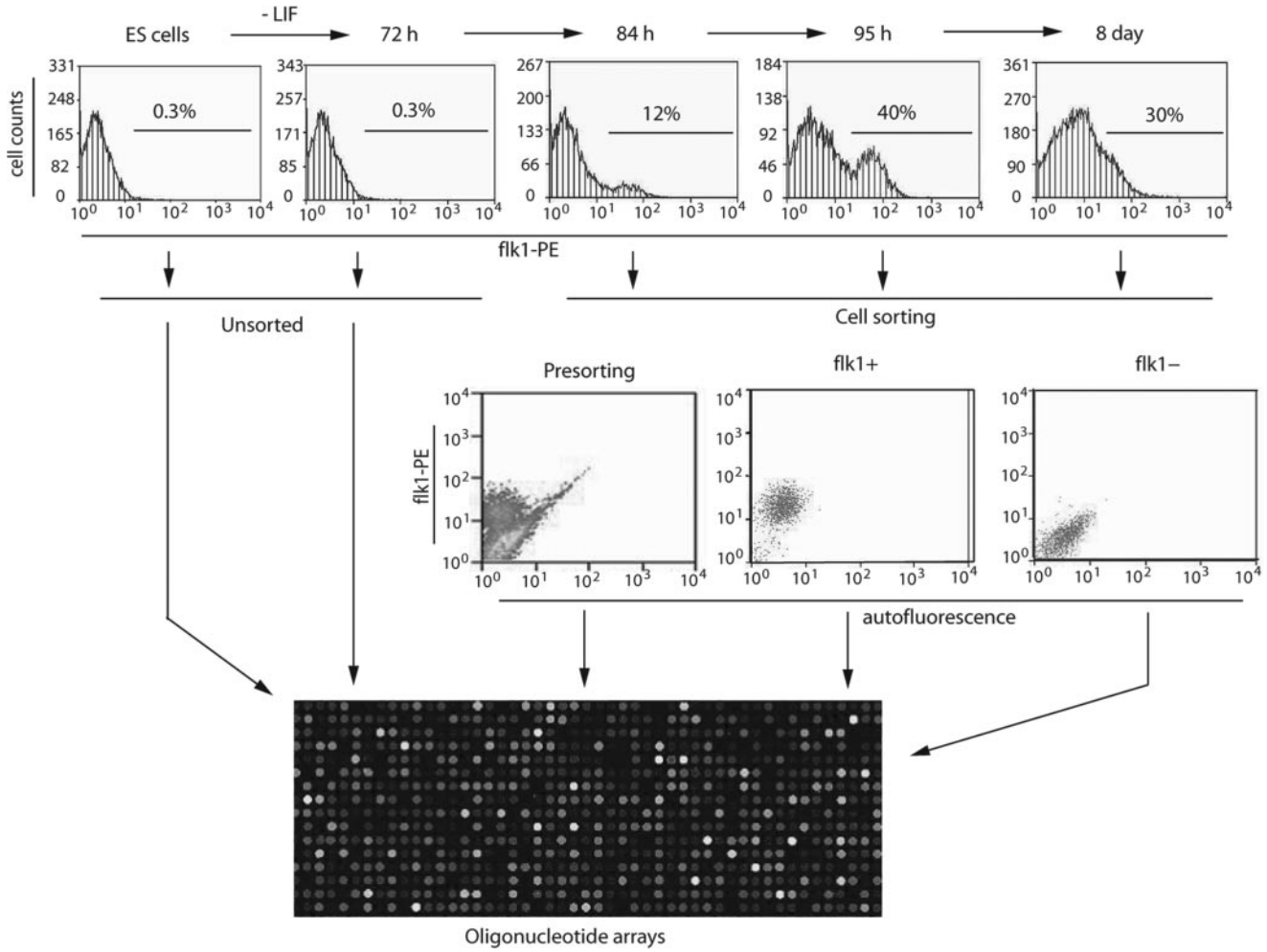

Figure 1. Expression profiling of endothelial differentiation in EBs. Cells were sorted into flk1$^+$ and flk1$^-$ populations after the onset of flk1 expression (at 84 hours, 95 hours, and 8 days) after withdrawal of leukemia inhibitory factor (LIF). Unsorted cell populations at these same times as well as undifferentiated ES cells and differentiated cells at 72 hours were also included. Total RNA was isolated and hybridized to oligonucleotide arrays after amplification. PE indicates phycoerythrin.

few recent studies have suggested a role for Wnt signaling in angiogenesis, but the exact Wnts involved and their roles have not been well delineated. Canonical Wnt signaling is initiated when Wnts bind to a coreceptor complex containing a Frizzled receptor and lipoprotein receptor-related proteins 5 or 6 (LRP-5/6) (Figure 3). β-Catenin is the key effector of the canonical Wnt signaling pathway. In the absence of Wnt, cytosolic β-catenin is phosphorylated by a protein complex containing glycogen synthase kinase-3β, axin, and adenomatous polyposis coli and is degraded rapidly by the ubiquitin–proteasome pathway. Activation of Wnt signaling inhibits β-catenin phosphorylation via disheveled (Dsh). This results in accumulation of cytosolic β-catenin, which then translocates to the nucleus and binds the LEF-1/TCF family of transcriptional factors and induces transcriptional activation of Wnt target genes. There are also several negative regulators of Wnt signaling, including Frizzled-related protein, Dickkopf 1, Naked Cuticle, and the Sox family transcription factors. Cadherins may also act as negative regulators by binding to β-catenin and reducing the availability of cytosolic β-catenin.[15] In our array data, multiple components of the Wnt signaling pathway were differentially regulated in flk1$^+$ cells during differentiation, including Wnt2, Wnt5a, Fzd7, Lef1, Frzb (FRP-3), Dickkopf 1, Nkd1, Sox17, Sox2, and N-cadherin (each of which are marked with stars in Figure 3). Known Wnt target genes including Msx1, Msx2, fibronectin, cyclin D1, cyclin D2, and Myc[16,17] were also specifically upregulated in flk1$^+$ cells, indicating enhanced Wnt activity in these cells. This pattern suggested to us a significant role for Wnt signaling in vascular development. Activation of both positive and negative regulators indicates the importance of tight control of Wnt signaling during this process.

To confirm the microarray data, we performed RT-PCR for selected genes in the Wnt signaling pathway, including Wnt2, Wnt5a, frizzled-receptor 7 (Fzd7), frizzled-receptor 5 (Fzd5), Lef1, Frzb, Nkd1, Cdh2 (N-cadherin), Msx2, and β-catenin. Fzd5 was not printed on the Agilent mouse array, bur we included it in the RT-PCR analysis because Fzd5$^{-/-}$ mice have defects in yolk sac angiogenesis.[18] Again, RT-PCR results correlated closely with our array data (Figure 4). Wnt2 transcripts were first detected by 84 hours of differentiation with peak levels at day 8. Wnt2, which signals via the

Seventy Genes Differentially Regulated in flk1$^+$ Versus flk1$^-$ Cells at All Time Points

Gene Name	GenBank Accession No.	Fold Change 84 Hours	95 Hours	8 Days
Upregulated genes				
Asb4	Mm0.229503	8.98	5.19	2.73
K0115H01-3	Mm0.86699	6.17	4.40	1.62
Kdr	BC020530.1	6.11	7.69	3.69
Wnt2	BC026373.1	5.90	3.68	2.24
Asb4	XM_162997.1	4.10	3.28	1.56
Tnfrsf1a	BC004599.1	2.93	2.24	1.51
Odz4	XM_166254.2	2.79	2.47	1.81
R75022	D87034.1	2.65	2.06	1.62
C330025N11Rik	NM_172731.1	1.61	1.76	2.02
LOC224093	XM_148156.1	1.58	1.69	1.84
C530046K17Rik	NM_146090.1	1.55	1.53	1.68
C1-ten	NM_153533.1	1.54	1.59	1.50
Genes inconsistently regulated over time				
Tbx3	Mm0.23467	5.33	4.02	−1.52
Gata4	AB075549.1	3.01	1.93	−1.58
2600016F06Rik	AK011429.1	2.95	2.00	−1.63
AI256817	XM_149800.2	2.16	2.14	−1.69
Mylc2a	XM_122161.1	2.14	2.75	−1.64
Gpr56	NM_018882.1	−1.63	−2.22	2.08
Klf2	NM_008452.1	−3.93	−3.25	1.75
AL023051	Mm0.181064	−1.61	−1.72	1.55
Downregulated genes				
Sox2	U31967.1	−8.39	−6.27	−1.97
LOC234574	XM_150141.2	−4.63	−3.60	−2.63
Tdh	AY116662.1	−4.25	−3.42	−1.52
Foxa2	NM_010446.1	−3.97	−5.17	−3.21
Cdh1	NM_009864.1	−3.79	−3.64	−2.15
Mt1	BC036990.1	−3.66	−2.04	−1.88
D15Ertd417e	BC030919.1	−3.61	−2.59	−1.52
Stella-pending	NM_139218.1	−3.36	−3.08	−1.56
Irs4	AL832136.1	−3.24	−2.82	−1.72
L0544B11-3	Mm0.216823	−3.15	−3.08	−3.87
LOC224912	XM_128707.2	−3.11	−3.35	−1.83
H3022G12-3	Mm0.25647	−3.00	−3.00	−1.76
zfp339	NM_026924.2	−2.95	−2.75	−1.74
Tacstd1	NM_008532.1	−2.80	−3.20	−2.22
St14	NM_011176.2	−2.76	−2.71	−1.89
1810015C04Rik	NM_019000.2	−2.76	−1.79	−2.01
J0460B01-3	AB010829.1	−2.75	−3.16	−1.90
2210413P12Rik	NM_145977.1	−2.70	−2.00	−1.65
Hook1-pending	BC030877.1	−2.68	−2.48	−1.50
Crtr1-pending	XM_123496.1	−2.56	−1.99	−2.10
Rex3	NM_009052.1	−2.52	−1.92	−1.99
LOC269424	BC026471.1	−2.47	−2.42	−1.90
Gtpat12	XM_125875.2	−2.41	−1.61	−1.50
Lama5	XM_130768.1	−2.32	−2.01	−1.63
Cldn4	NM_009903.1	−2.29	−2.68	−2.41
AI647528	XM_131060.1	−2.26	−1.98	−1.79
Zfp42	NM_009556.1	−2.26	−2.14	−1.53
Nme7	NM_138314.1	−2.26	−1.89	−2.12
Igfbp2	BC012724.1	−2.21	−2.06	−1.75
LOC226352	BC003937.1	−2.12	−2.04	−1.70
Spint2	XM_133318.1	−2.11	−2.27	−1.84
Prg	NM_011157.1	−2.11	−2.52	−3.64
Kif21a	XM_040211.6	−2.03	−1.89	−1.94
Gcnt2	Mm0.26734	−1.99	−1.73	−1.54
Ocln	NM_008756.1	−1.97	−1.80	−1.67
2410003J06Rik	XM_135953.2	−1.95	−2.65	−1.59
Krt2-7	NM_033073.1	−1.93	−1.54	−1.76
Pla2g7	BC010726.1	−1.93	−1.85	−1.72
Plod2	NM_011961.1	−1.88	−1.83	−1.58
Ctgf	NM_010217.1	−1.84	−3.19	−1.87
Tmprss2	BC038393.1	−1.80	−2.90	−2.31
AI563590	XM_131396.2	−1.74	−1.67	−1.57
Ell2	NM_138953.1	−1.74	−1.56	−1.75
AA536730	XM_125841.2	−1.74	−1.59	−1.64
Pem	NM_008818.1	−1.73	−1.90	−2.02
Zfp42	NM_009556.1	−1.73	−1.74	−2.34
Stx3	Mm0.203928	−1.70	−1.73	−1.79
Stx7	NM_016797.1	−1.63	−1.51	−1.52
5730456K23Rik	XM_125830.1	−1.58	−1.69	−1.75
Psx1	NM_008955.1	−1.56	−2.07	−1.89

canonical Wnt pathway, was upregulated in flk1$^+$ cells at all 3 time points and was not detected in flk1$^-$ cells at 84 and 95 hours. It is notable that mice lacking Wnt2 have placental angiogenic defects, although a thorough evaluation of vascular development in these mice has not been reported.[19] Expression of Wnt5a, which typically activates the noncanonical Wnt pathway, was detected in undifferentiated ES cells with a gradual elevation following differentiation until day 8 and was preferentially expressed in flk1$^+$ cells at 84 and 95 hours. Transcripts of other Wnt-associated genes were also upregulated in early flk1$^+$ cells, including receptor Fzd7, nuclear effector Lef1, negative regulators Frzb and Nkd1, N-cadherin, and Wnt target gene Msx2. Fzd5 was expressed at higher levels in flk1$^-$ cells than in flk1$^+$ cells. Remarkably, it has been suggested that both Wnt2 and Wnt5a are possible ligands of Fzd5.[18,20]

Role of the Wnt Signaling Pathway in Endothelial Differentiation

Based on the consistent and highly specific expression pattern of components of the Wnt signaling pathway within the flk1$^+$ population, we tested the hypothesis that Wnt activation regulates endothelial cell maturation. We focused on canon-

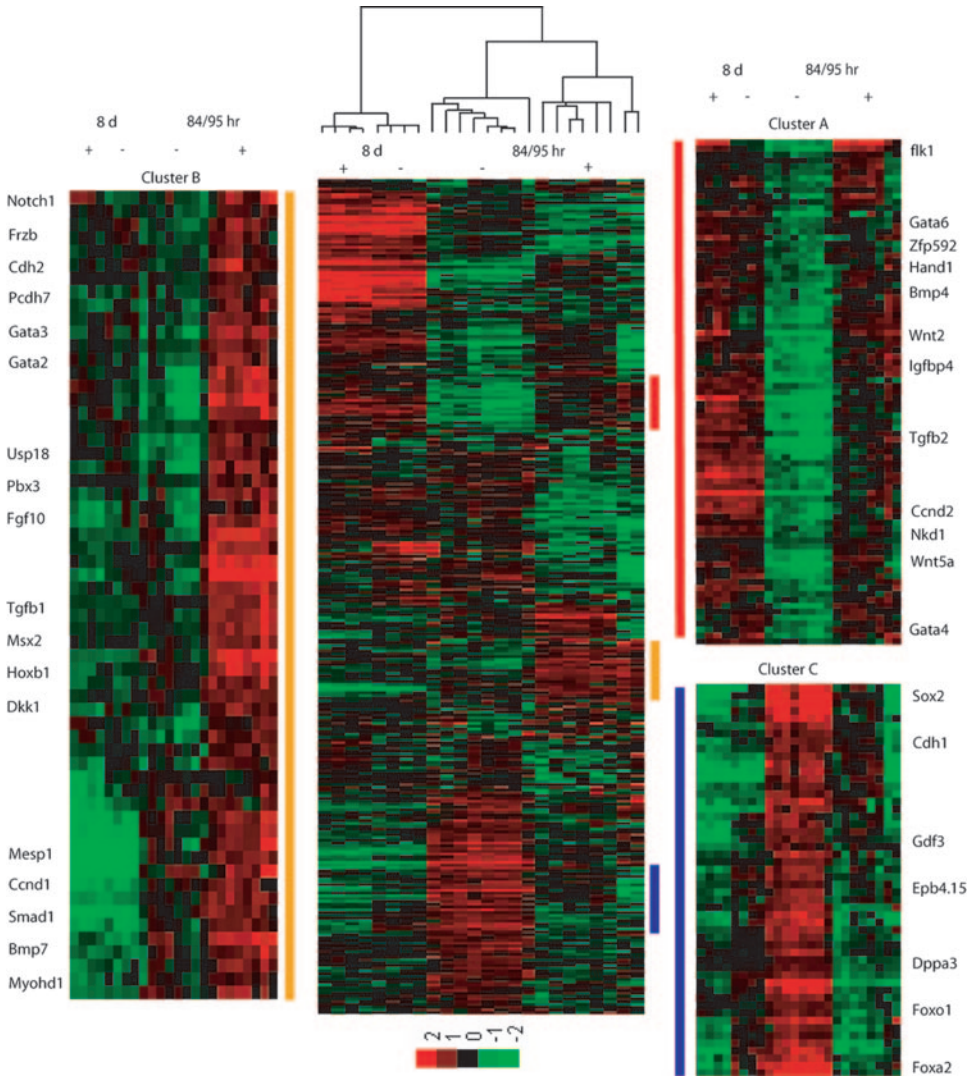

Figure 2. Hierarchical clustering of differentially expressed genes over time during ES cell in vitro differentiation. The color scale ranges from green for log ratio −2 to red for log ratio +2, as indicated. Three individual clusters (A, B, and C) are indicated by colored bars (red, yellow, and blue), and each of these clusters is enlarged to show representative genes, some of which are indicated by name. The complete data set is available online through the Gene Expression Omnibus (Series Record GSE3757, http://www.ncbi.nlm.nih.gov/geo) and through the University of North Carolina Microarray Database (http://genome.unc.edu).

ical Wnt signaling based on the impressive upregulation of Wnt2, which operates through this pathway. The absence of specific antibodies and recombinant proteins as reagents makes analysis of Wnt signaling cascades notoriously difficult. We therefore used lithium (to selectively inhibit GSK-3β activity, which in turn mimics activation of the Wnt pathway[21]) and sFRP-1 (to bind secreted Wnts and suppress Wnt receptor activation[22]) as tools to examine the effect of Wnt signaling on vascular progenitors using the in vitro ES cell differentiation system. Addition of lithium (10 mmol/L) to the differentiation medium moderately but significantly expanded the flk1$^+$ cell population as detected by flow cytometry after 96 hours of differentiation (Figure 5A and 5B). On the contrary, treatment of EBs with sFRP-1 (10 μg/mL) almost completely depleted the flk1$^+$ cell population following 96 hours of differentiation. In both cases, overall morphologies of EB cultures were grossly normal and no evident increase in cell death was noted.

To extend these observations, we also examined the direct effects of Wnt proteins on vascular progenitors in the same model using conditioned media to circumvent the challenges associated with purifying Wnts that retain biological activity. In preliminary experiments, both Wnt3a-CM and Wnt2-CM led to stabilization of β-catenin in L cells (data not shown), indicating that the secreted Wnts retained activity in conditioned medium. Similar to the effects of lithium, both Wnt3a and Wnt2 (which activate the same canonical pathway) were able to expand the flk1$^+$ cell population (Figure 5C and 5D). The absolute percentage of flk1$^+$ cells in the Wnt2 treatment group was lower than after Wnt3a treatment, but this is attributable to the different cell types producing the conditioned medium. Even so, in comparison with S2-CM, Wnt2-CM was still able to significantly increase the percentage of flk1$^+$ cells. Both sFRP-1 and Wnt3a are modulating canonical Wnt signaling in these assays, as indicated by the ability of sFRP1 in increase, and

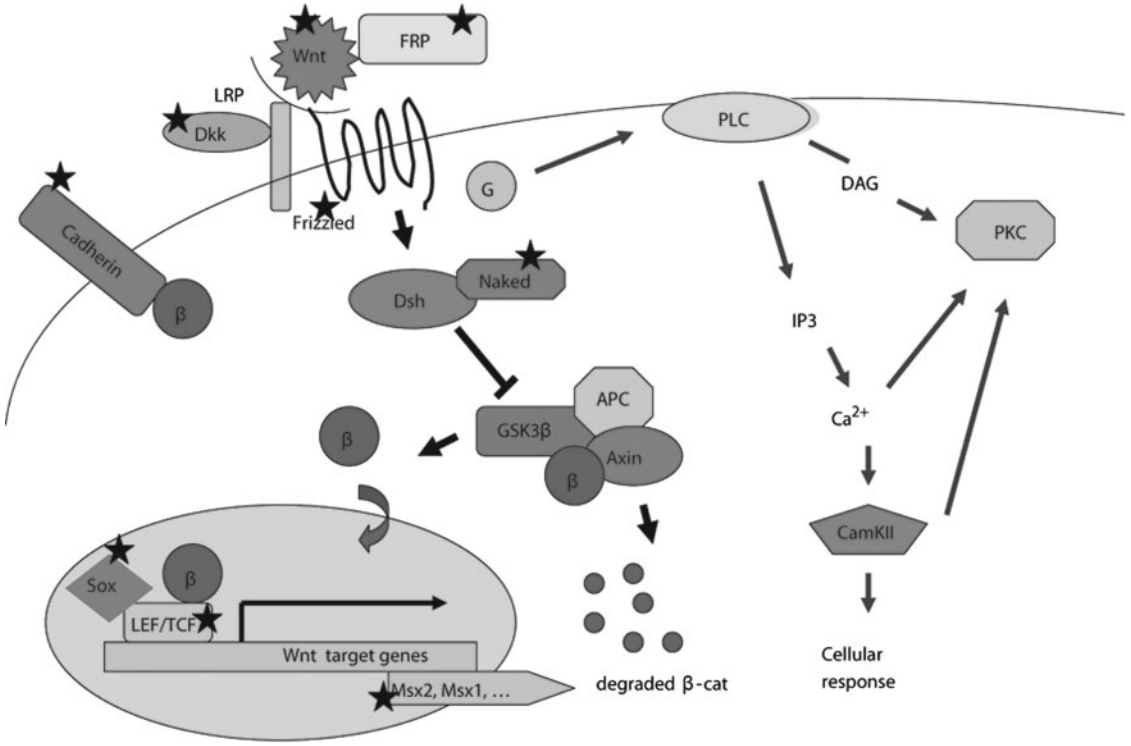

Figure 3. Identification of differentially expressed genes within the Wnt signaling pathway during endothelial differentiation. Members of the Frizzled family and lipoprotein-related proteins (LRPs) 5 and 6 are receptors that transduce Wnt signals to Disheveled (Dsh). Activated Disheveled inhibits GSK3β and prevents degradation of β-catenin by GSK3β, APC, and axin, resulting in accumulation of cytosolic β-catenin. β-Catenin then translocates to the nucleus and binds to members of the LEF-1/TCF transcriptional factor family and induces transcriptional expression of Wnt target genes. Frizzled related protein (FRP), Dickkopf (Dkk), Naked cuticle (Nkd), Sox, and Cadherin are negative effectors of the Wnt pathway. In parallel, Wnt activation results in intracellular Ca^{2+} release and activation of Ca^{2+}-camodulin–dependent protein kinase II (CamKII) and protein kinase C (PKC) in a β-catenin–independent fashion. Multiple components in this pathway (indicated with a star) were differentially regulated in flk1$^+$ cells during differentiation.

Wnt3a to decrease, the ratio of phosphorylated to total GSK-3β (see supplemental Figure IV).

There were several possible explanations for the role of Wnt signaling on expansion of vascular progenitors, including the following: transcriptional activation of flk1, induction of differentiation to vascular progenitors, and/or increase of proliferation of vascular progenitors. Treatment of EBs with sFRP-1 was able to reduce the flk1 transcriptional level as examined by RT-PCR at 96 hour (Figure 6A). However, flk1 transcripts were still detectable at low levels, which is not entirely consistent with complete depletion of flk1$^+$ cells with sFRP-1 treatment at this time point (Figure 5). We also did not find that Wnt activation altered the kinetics of endothelial differentiation in EBs (see supplemental Figure V). To further elucidate the mechanism of the effects of Wnt activity, we treated EBs with sFRP-1 until day 8 and then stained them with the endothelial marker PECAM. With or without sFRP-1, cultures were healthy and EBs differentiated appropriately and appeared grossly normal, as indicated by phase contrast microscopy (Figure 6B). Without sFRP-1, PECAM$^+$ capillary-like structures readily formed, as we have previously described.[10,12] However, vascular networks never formed after sFRP-1 treatment, although rare, isolated PECAM$^+$ endothelial cells were seen.

We next analyzed the possible contribution of several cellular parameters by Wnt signaling to vascular network formation in the same model. First, we labeled day 8 EBs

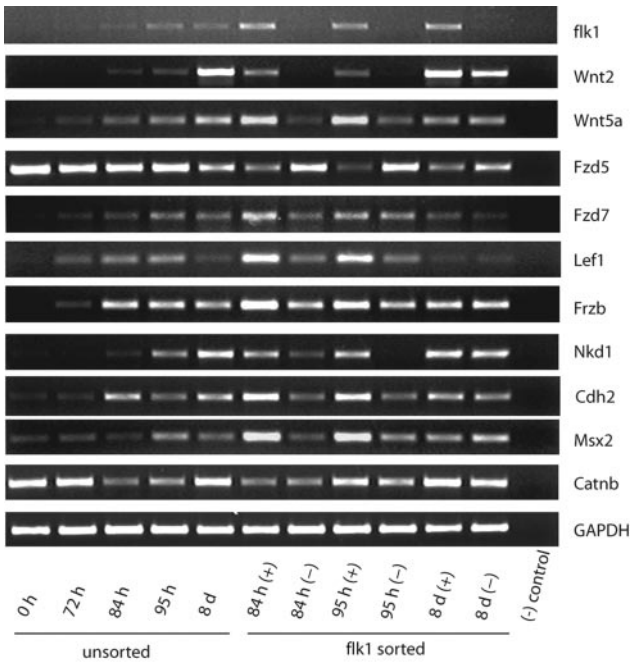

Figure 4. Analysis of mRNA expression of selected mRNA species within the Wnt signaling pathway. RT-PCR was performed to quantify expression of selected genes in the Wnt signaling pathway or Wnt target genes during EB differentiation and in flk1$^+$ and flk1$^-$ cells at 84 hours, 95 hours, and 8 days after onset of differentiation.

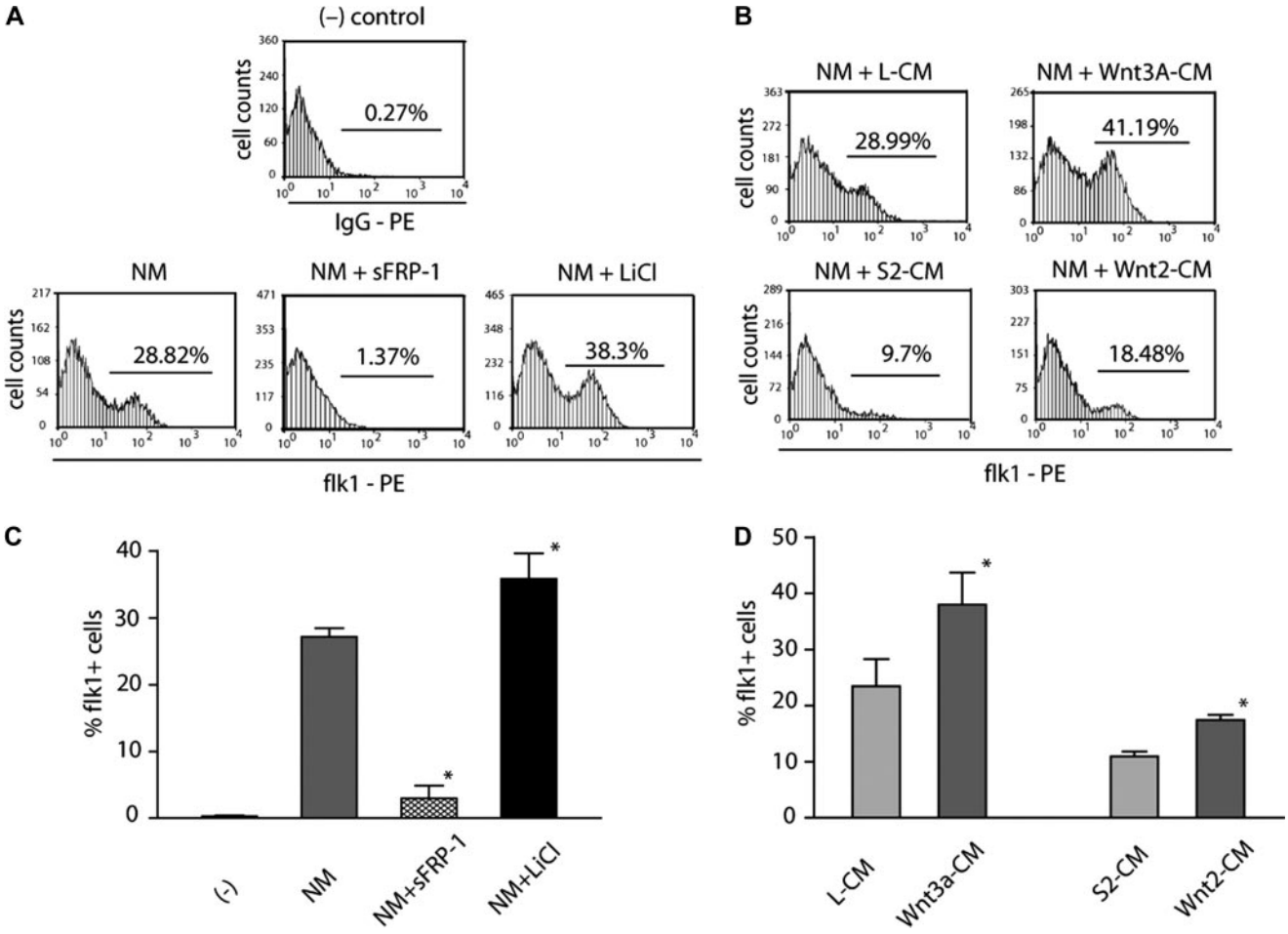

Figure 5. The effect of Wnt signaling on the generation of flk1-expressing cells. A, FACS analysis of differentiated ES cells at day 4 during EB differentiation. EBs were cultured in normal differentiation medium (NM) or supplemented with sFRP-1 (10 μg/mL) or LiCl (10 mmol/L) at day 2 of differentiation. B, Quantitative results of 3 independent experiments performed as described in A are shown. C, FACS analysis of differentiated ES cells at day 4 during EB differentiation. EBs were cultured in L-CM, Wnt3A-CM, S2-CM, or Wnt2-CM as indicated. D, Quantitative results of 3 independent experiments performed as described in C are shown. *$P<0.05$ compared with control.

treated with or without sFRP-1 with antibody to PECAM and to M30 CytoDEATH antibody, which recognizes caspase-cleaved cytokeratin 18 and detects early apoptotic cells. However, no change in positive apoptotic signals were noted (data not shown), which suggests that inhibition of vascular network formation by sFRP-1 is not caused by induction of apoptosis. We did find that Wnt3a enhanced migratory responses of mouse embryonic endothelial cells in Boyden chamber assays (see supplemental Figure VI). To address the contributions of proliferation, EBs were labeled with antibodies to the endothelial marker PECAM and to the mitotic marker phosphohistone H3 (marking cells in the G_2–M phase) and then stained with DAPI (Figure 7). (Experiments with sFRP-1 were not informative for proliferation assays because

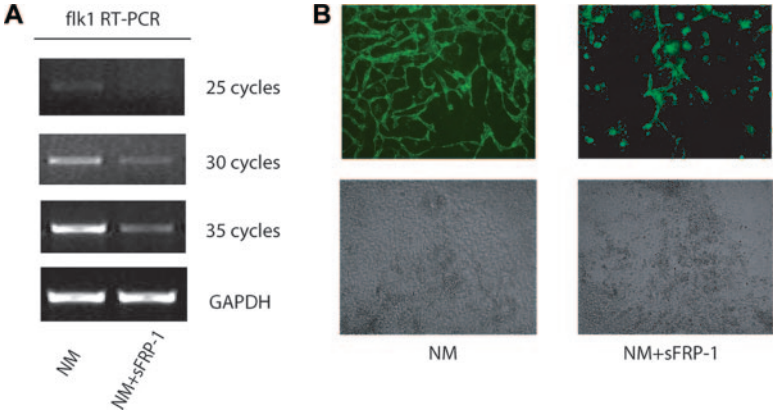

Figure 6. Effect of inhibition of Wnt signaling by sFRP-1 on endothelial differentiation. A, RT-PCR analysis of flk1 mRNA in EBs at day 4 of differentiation. sFRP-1 (10 μg/mL) was added at day 2. B, PECAM immunostaining (top panels) and phase contrast microscopy (bottom panels) of EBs at day 8 of differentiation. EBs were cultured in normal differentiation medium (NM) or supplemented with sFRP-1 from days 2 to 8.

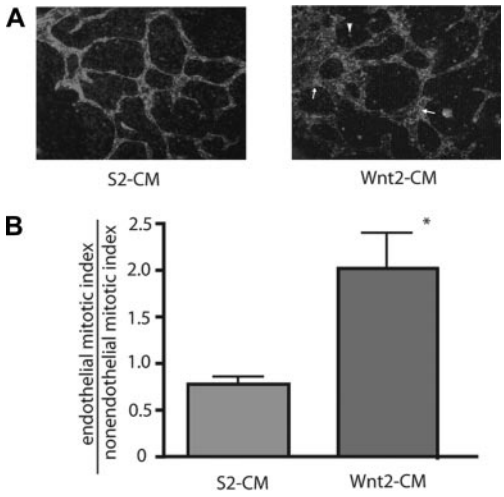

Figure 7. Elevated mitotic indices in ES cell cultures treated with Wnt protein. A, Representative field from day 8 EBs treated with Wnt2-CM or S2-CM that were labeled with antibodies to PECAM (green), phosphohistone H3 (red) and DAPI (blue). The arrowhead indicates a mitotic nonendothelial cell (phosphohistone $H3^+/PECAM^-$), and the arrows point to mitotic endothelial cells (phosphohistone $H3^+/PECAM^+$). B, Triple-labeled images were used to calculate nonendothelial and endothelial mitotic indices. The nonendothelial index was used as a baseline for comparison of endothelial mitotic indices. *$P<0.01$ compared with control.

so few cells are PECAM positive after sFRP-1 treatment.) Visual observation indicated that EBs treated with Wnt2-CM had more robust vascular structures and had more $PECAM^+$ cells that were colabeled with the anti-phosphohistone H3 antibody compared with EBs treated with S2-CM. Digital images from multiple wells in 3 separate experiments were processed and used to calculate the endothelial mitotic indices. There was no difference in nonendothelial cell mitotic indices between Wnt2-CM and S2-CM treatment (data not shown). However, day 8 EBs treated with Wnt2-CM had endothelial mitotic indices (normalized to corresponding nonendothelial cell mitotic indices) that were more than 2-fold higher than EBs treated with S2-CM. These results indicate that the canonical Wnt pathway enhances proliferation of endothelial precursors with specificity and without large effects on viability and that these effects account at least in part for the expansion of the endothelial compartment by Wnt activation in our studies.

Discussion

Differentiation of embryonic stem cells in vitro has proven to be a reliable and reproducible tool for understanding cellular events and signaling responses that occur during embryonic development and is particularly well adapted for characterization of hematovascular lineages,[5,12,23,24] which are otherwise difficult to characterize in whole embryos. Our focus in the studies presented here is on the transcriptome of cells bearing the surface marker flk1 at different times during embryoid body differentiation. This receptor for VEGF was first considered a specific marker of the vascular endothelium in adults,[25,26] and further analyses—including studies using differentiating embryonic stem cells—have indicated that flk1 marks a common progenitor for hematopoietic and endothelial lineages called the hemangioblast.[5,24] Our studies are consistent with the general dogma that, at their first appearance, cells expressing flk1 have primitive characteristics and that over time they develop molecular signatures of mature endothelium that are concordant with the adoption of vascular features at the cellular level.

The patterns of expression of a number of classical endothelium-restricted genes (eg, PECAM, ICAM2, VE-cadherin, flk1 itself) within our microarray dataset are as predicted. However, it is striking that many of the mRNA species that are enriched within $flk1^+$ cell populations are incompletely characterized and/or have not been associated with hematovascular development. The hierarchical clustering analysis suggests that these genes may participate in regulatory networks involving multiple signaling pathways that are required for appropriate maturation of $flk1^+$ cells during the developmental plan, and thus additional analysis of the genes identified within these transcriptional clusters may provide new insights into the molecular events in blood vessel development. Remarkably, we found that a number of genes within the canonical Wnt signaling pathway were preferentially expressed in ES cell-derived $flk1^+$ cells (Figure 3). Both positive and negative regulators of Wnt signaling were identified, suggesting that this signaling pathway requires tight control during the vascular developmental program, and the upregulation of known Wnt target genes (Msx1, Msx2, and Ccnd1) is consistent with activation of canonical Wnt signaling within the $flk1^+$ cellular niche.

Several lines of evidence point to a role for signaling via Wnt family members in vascular development. A transgenic Lef/tcf:lacZ reporter mouse strain experiment has demonstrated activation of canonical Wnt signaling in endothelial cells during intraembryonic angiogenesis.[27] Loss of Fzd5, a Wnt receptor, during mouse development severely impairs yolk sac vascularization and causes intrauterine demise soon after the onset of vascular development,[18] and $Wnt2^{-/-}$ mice have defects in placental angiogenesis.[19] Endothelial cell-specific inactivation of β-catenin disrupts intercellular junctions and enhances vascular fragility,[28] although it is not possible to determine whether this phenotype results from dysregulation of Wnt signaling or from effects of β-catenin on cell–cell adhesions via interactions with members of the cadherin family. We therefore used a systematic approach of activation and inhibition of Wnt signaling to better define a role for the canonical Wnt signaling pathway in regulating endothelial differentiation in ES cell cultures. We find that Wnt activation is both necessary and sufficient for endothelial cell differentiation and assembly into vascular-like structures in embryoid bodies, in part through regulation of differentiation and proliferation of endothelial progenitors. Wnt proteins have been linked to enhanced proliferation in numerous settings,[29] including stem cell systems.[30,31]

The studies presented here provide further support for a role of Wnt signaling and identify for the first time a specific requirement for the canonical Wnt signaling pathway in endothelial cell differentiation and maturation. It is notable that recent evidence points toward a critical role for Wnt activity in hematopoietic stem cell renewal,[31] and the studies presented here suggest that a larger role may exist for Wnts

during hematovascular differentiation. The observations here also raise the broader possibility that Wnts may have a general role in the regulation of angiogenesis under physiological and pathological conditions in adulthood. However, many new questions are also raised by our studies, chief among these being the specific players within the canonical Wnt signaling pathway that are operative during vascular development. Additional studies of the candidates identified through our gene expression profiles are likely to develop a clearer picture of the role of Wnts and their downstream targets in the development of the vascular system and in adult angiogenesis in general.

Acknowledgments

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Supplemental Data

Gene expression profile signatures indicate a role for Wnt signaling in endothelial commitment from embryonic stem cells

Hong Wang*, Peter C. Charles*, Yaxu Wu*, Rongqin Ren*, Xinchun Pi*, Martin Moser*, Michal Barshishat-Kupper‡, Jeffrey S. Rubin‡, Charles PerouΩ, Victoria Bautch*†, and Cam Patterson*¶

Carolina Cardiovascular Biology Center* and Departments of Medicine¶, Biology†, and GeneticsΩ, University of North Carolina, Chapel Hill, NC, and Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, MD‡

Address correspondence and inquiries to:

Cam Patterson, M.D.

Director, Division of Cardiology and Carolina Cardiovascular Biology Center

8200 Medical Biomolecular Research Building

Chapel Hill, NC 27599-7126

Telephone: 919-843-6477

Fax: 919-843-4585

e-mail: cpatters@med.unc.edu

Supplemental Text

Microarray hybridization and data analysis. Total RNA was isolated from undifferentiated ES cells and from differentiated EBs at 72 h, 84 h, 95 h and 8 days, and from sorted flk1+ and flk1– cells at 84 h, 95 h and 8 days. Total RNA (2.5 μg) from each sample was amplified and labeled with Cy5 using the Fluorescent Linear Amplification Kit (Agilent Technologies, CA, USA). Reference RNA (universal mouse reference RNA) was amplified and labeled with Cy3 [1]. Amplified cRNA was hybridized to Mouse Development Oligonucleotide Arrays (Agilent Technologies). Arrays were scanned on an Axon Genepix 4000B Scanner System (Axon Instruments) and analyzed with GenePix Pro 5.0 software (Axon Instruments). Each experiment was performed independently in quadruplicate biological replications. Figure 1 summarizes the overall experimental design. The raw data from each array was normalized using a non-linear modified quantile normalization method [2]. To identify genes that show significant variations in expression between flk1+ cells and flk1– cells over time, and between undifferentiated ES cells and differentiated cells over time, paired *t* tests (Microsoft Excel software, Microsoft, Seattle, WA) on normalized intensities with a p value ≤ 0.05 followed by ratio change (ratio of normalized intensity ≥ 1.5 or ≤ 0.67) were used. Hierarchical clustering with unweighted average linkage clustering was performed as described [3]. The Gene Expression Omnibus accession number for this dataset is GSE3757.

Minimizing systemic and biological variations are crucial to obtain reliable data when analyzing systematic gene expression profiles. In order to increase reliability, we performed our experiments independently in quadruplicate biological replications [4]. Overall similarity in expression profile among replicates was evaluated by calculating Pearson correlation coefficients. The correlation coefficients among replicates ranged from 0.97 to 0.99 for reference RNA, and 0.96 to 0.99 for sample RNA (see Supplemental Table 2). These values indicate the high

reproducibility of the components of our repeated experiments, including sample collection, RNA preparation, cDNA amplification and array hybridization.

To confirm the validity of our dataset independently, we examined whether the expression patterns of well-characterized mRNA species were accurately predicted by our gene expression profiles. We chose Brachyury, flk1 itself, and E-cadherin for these analyses. Brachyury is a T-box transcriptional factor that is a marker of early mesodermal tissue and that is downregulated when these tissues undergo patterning and specification [5, 6]. Consistent with these observations and with previous reports using the *in vitro* EB system [7], expression of Brachyury was detected from 72 h to 95 h after onset of differentiation, and was then reduced to undetectable levels by day 8 (Supplemental Figure 1*A*). No differences were detected in Brachyury expression between flk1+ cells and flk1– cells, as expected. We also examined the expression of flk1 mRNA in our dataset. As anticipated, its expression rose sharply after 72 h of EB differentiation and was restricted to the flk1+ population. Lastly, we examined expression of E-cadherin, which is required for epithelial biogenesis and is downregulated in cells destined to become mesoderm [8, 9]. Consistent with these observations, the E-cadherin transcript was significantly suppressed (>3-fold at 84 h and 95 h, >2-fold at 8 days) in flk1+ cells that are known to be of mesodermal origin. In each case, the expression of these mRNAs was also tested by RT-PCR in comparison with the housekeeping gene GAPDH (Supplemental Figure 1*B*). The gene expression patterns for these genes were remarkably consistent whether analyzed using our microarray dataset or by RT-PCR.

Similar deductive analyses indicated that specific markers of the mature endothelial phenotype, VE-cadherin [10], CD34 [11], ICAM-2 [12], and VE-cadherin 2 [13], demonstrated significant increases in expression only in the flk1+ cells at 8 d (See Supplemental Figure 2*A*), which supports previous reports that flk1+ cells at day 8 have characteristics of mature endothelia, whereas flk1+ at earlier time points are more representative of precursor populations [14]. Because reports indicate that SMC can be derived from flk1+ cells in EBs, we examined the expression of definitive

markers of the SMC lineage: SM α-actin, SM myosin heavy chain, calponin 1 and smoothelin [15]. Of these factors, SM α-actin and calponin 1 were consistently upregulated after EB differentiation until at least day 8; however, there were no changes over time in expression of smoothelin and SM myosin heavy chain (See Supplemental Figure 2*B*). Moreover, we did not see increases in expression for any of these SMC marker genes in flk1+ cells compared with flk1– cells, and the calponin 1 transcript was even higher in flk1– cells than in flk1+ cells. If flk1+ cells give rise to SMC in EBs under the conditions of our experiments, then they must lose flk1 expression rapidly prior to or immediately after differentiation to mural cells, whereas endothelial cells maintain flk1 expression. No intermediate cell populations that share flk1 and mural cell markers were identified in our studies, although we cannot formally exclude their existence [16].

Studies predicated on the restricted potential of flk1+ cells to assume hematopoietic or endothelial phenotypes have proven remarkably informative and have even suggested therapeutic potential for bone marrow-derived cells bearing this marker [17]. However, fate-mapping studies of cells marked for flk1 expression by Cre recombinase have suggested that some cardiomyocytes and skeletal muscle cells derive from flk1-expressing progenitors [18]. In addition, flk1-expressing cells can develop smooth muscle-associated phenotypes when cultured under appropriate conditions [16], suggesting that flk1+ cells may, at least transiently, have a broader potential to contribute to cardiovascular lineages than has been previously suspected. Our observation that transcripts for cardiomyocyte-restricted genes such as GATA4 and myosin light chain 2a are enriched for a transient period in flk1-expressing cells provides additional support for a model in which some cardiomyogenic cells derive from flk1+ progenitors. In contrast, we did not find enrichment of transcripts characteristic of smooth muscle markers in microarray analyses of flk1-expressing cells. Our results are consistent with recent studies that detect flk1 expression in multipotential mesodermal progenitors for cardiac and skeletal muscle, but not smooth muscle, using a flk1-lacZ allele [19]. The contribution of flk1+ cells to cardiogenesis remains an open

question at this time, and additional microarray profiling may provide a means to understand this question.

The presence of multiple clusters of genes showing temporally coordinated patterns of expression during a critical development stage suggests a common mechanism of transcriptional regulation. A shared promoter motif present within the upstream, regulatory region of these genes is one likely means of accomplishing this. While a variety of search engines allow the scanning of sequences of DNA to search for the presence of putative transcription factor binding sites (BLAST, etc), none of these are particularly well suited for the rapid comparison of multiple binding sites for different transcription factors in lists of genes. Recently, a technique for accomplishing this has been described [20]. Preliminary analysis of genes down-regulated in flk1+ cells (Cluster C, Figure 2), identified 8 unique modules of transcription factor binding sites within the 5000 bp 5' to the initiation of transcription for the genes that define this cluster. The 4 most highly significant modules contained canonical binding sites recognized by transcription factors such as Myc, NFAT, MEF2, and NKX family members, all with p-values of less than 0.03. These over-represented modules may form the basis for a promoter motif that allows the transcriptional control of the genes in this cluster—in this case, perhaps to downregulate expression of genes that should not be expressed within the flk1+ lineage. Further studies will be required to demonstrate unambiguously that the sites are functional within the promoter regions of these genes, but this bioinformatic technique represents a first step towards understanding developmentally regulated expression within a dataset such as ours and provides a rational framework from which to begin the study of this regulation.

Supplemental Methods

For cell migration, Boyden chamber assays were performed in a 48-well microchemotaxis chamber (Neuro Probe, Gaithersburg, Md) with collagen-coated polycarbonate filters (pore size,

8 μm; Neuropore Corp, Pleasanton, Calif) using mouse intraembryonic endothelial cells (MEC). MECs (8,000 per well) were placed in the upper chamber, and the lower chamber contained L-CM, Wnt3a-CM, purified Wnt3a protein (R&D Systems) or normal culture media as control. After 6 hours, cells on the bottom of the filters were fixed with methanol and stained with Diff-Quick staining solution (Baxter, Deerfield, Ill). Migration was measured as the number of cells per high-power field (400×) that migrated across the membrane between the upper and lower chambers.

Supplemental Data Legends

Supplemental Figure 1. Independent confirmation of array results by RT-PCR. *A*. Expression pattern of selected genes (brachyury, flk1, E-cadherin and GAPDH) based on microarray results (expressed as log ratio) at the indicated time points in unsorted or flk1-sorted (+ or –) cells. The expression level of undifferentiated ES cells (0 h) was defined as 0 for reference. *B*. RT-PCR analysis of the same genes over time after the onset of differentiation. The last lane is an RT-PCR reaction without RNA as a negative control.

Supplemental Figure 2. Gene expression profiles of cell type-specific mRNAs. *A*. Expression pattern of selected endothelial-associated genes (VE-cadherin, VE-cadherin 2, CD34, and ICAM-2) based on microarray results (expressed as log ratio) at the indicated time points in unsorted or flk1-sorted (+ or –) cells. The expression level of undifferentiated ES cells (0 h) was defined as 0 for reference. *B*. Similar analyses of smooth muscle-associated genes (smooth muscle α-actin, smooth muscle myosin heavy chain, calponin I, smoothelin).

Supplemental Figure 3. Overlap among genes differentially expressed. Venn diagram of the number of genes differentially expressed at different times (84 h, 95 h and 8 d after the onset of

differentiation) during EB differentiation in flk1-positive cells compared with flk1-negative cells, and their overlap. The total number of differentially regulated genes is indicated outside the circles and the division of these genes among each population is indicated within the diagram.

Supplemental Figure 4. Effect of Wnt signaling on phospho-GSK3β levels in EBs. *A*. EBs were treated with sFRP-1 (10 μg/ml) for 48 hours at day 2 of differentiation or treated with L-CM or Wnt3a-CM for 2 hours followed by immunoblot analysis for GSK3β and phospho-GSK3β. A representative blot is shown. *B*. For densitometric analysis of phosphorylated form of GSK3β, three blots were analyzed and normalized to the total GSK3β level. Data are expressed as mean ± SEM (*, sFRP-1 treatment vs control, $p<0.05$; **, Wnt3a-CM vs L-CM, $p<0.05$).

Supplemental Figure 5. Analysis of mRNA expression of selected endothelial-specific genes in EBs treated with L-CM or Wnt3a-CM. RT-PCR was performed to quantify expression of selected genes specific for endothelial cells during EB differentiation with L-CM or Wnt3a-CM treatment. Selected genes examined were flk1, VE-cadherin, Tie2, PECAM, VEGF, flt1 and Endoglin. Hprt was used as a control.

Supplemental Figure 6. Wnt3a promotes migration of mouse embryonic endothelial cells (MEC). MECs were subjected to Boyden chamber assay with Wnt3a-CM (or L-CM) or purified Wnt3a (20 ng/ml and 40 ng/ml) as a stimulant. Data are presented as mean ± SEM of cell numbers per field of view (a total of 10 fields were counted for each well). All comparisons are significant at $p < 0.05$ (*, Wnt3a-CM vs L-CM; **, Wnt3a protein vs control).

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Supplementary Table 1

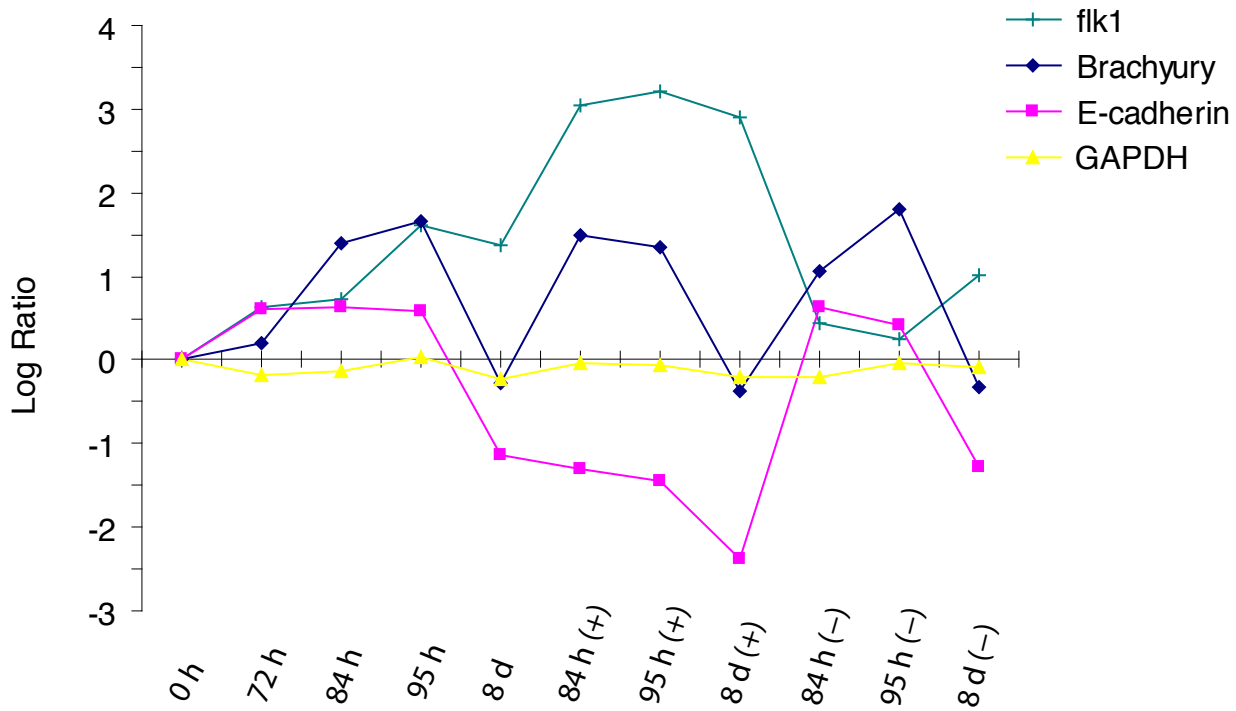
	sense	**antisense**
flk1	5’ GGAACCTGACTATCCGCAGG 3’	5’ CCTCAACAAAGCCTGAGCTGG 3’
Brachyury	5’ CATGTACTCTTTCTTGCTGG 3’	5’ GGTCTCGGGAAAGCAGTGGC 3’
E-cadherin	5' AAGTGACCGATGATGATGCC 3'	5' CTTCTCTGTCCATCTCAGCG 3'
Wnt2	5’ GCCTTTGTTTACGCCATCTC 3’	5’ CGGGAAGTCAAGTTGCACAC 3’
Wnt5a	5’ CCTGTAAGTGTCATGGAGTGT3’	5’ GGTCTGCACTGTCTTAAACTGG 3’
Nkd1	5’ GAGAGACTGAGCGAACCTGG 3’	5’ GTCAAGGAGGTGGAAGGAGC 3’
Frzb	5’ GCCCGGATCTTCTCTTCTTC 3’	5’ CCTCTTTAACTTTAGCCCGG 3’
Fzd5	5’ GACGCGGAGGTTCTGTGTAT 3’	5’ CAGGGCCGGTAGTCTCATAG 3’
Fzd7	5’ CTACTTTTATGAGCAGGCCTTCC 3’	5’ CCAAATAACTTCTCACTTCCAGG 3’
Lef1	5’ CACAACTGGCATCCCTCATC 3’	5’ CATTCTGGGACCTGTACCTG 3’
Msx2	5’ GAGCACCGTGGATACAGGAG 3’	5’ CCGTATATGGATGCTGCTTG 3’
β-catenin	5’ GCACAACCTTTCTCACCACC 3’	5’ CTTCCATCCCTTCCTGCTTAG 3’
N-cadherin	5’ GGCGGAGACCTGTGAAACT 3’	5’ GCCGTTTCATCCATACCAC 3’
GAPDH	5‘ ACCACAGTCCATGCCATCAC 3’	5‘ TCCACCACCCTGTTGCTGTA 3’
PECAM	5’ GTCATGGCCATGGTCGAGTA 3’	5’ CTCCTCGGCGATCTTGCTGAA 3’
Tie2	5’ CCTTCCTACCTGCTA 3’	5’ CCACTACACCTTTCTTTACA 3’
VE-cadherin	5’ GGATGCAGAGGCTCACAGAG 3’	5’ CTGGCGGTTCACGTTGGACT 3’

Supplemental Table 2: Pairwise correlation coefficient, number of replicates = 4

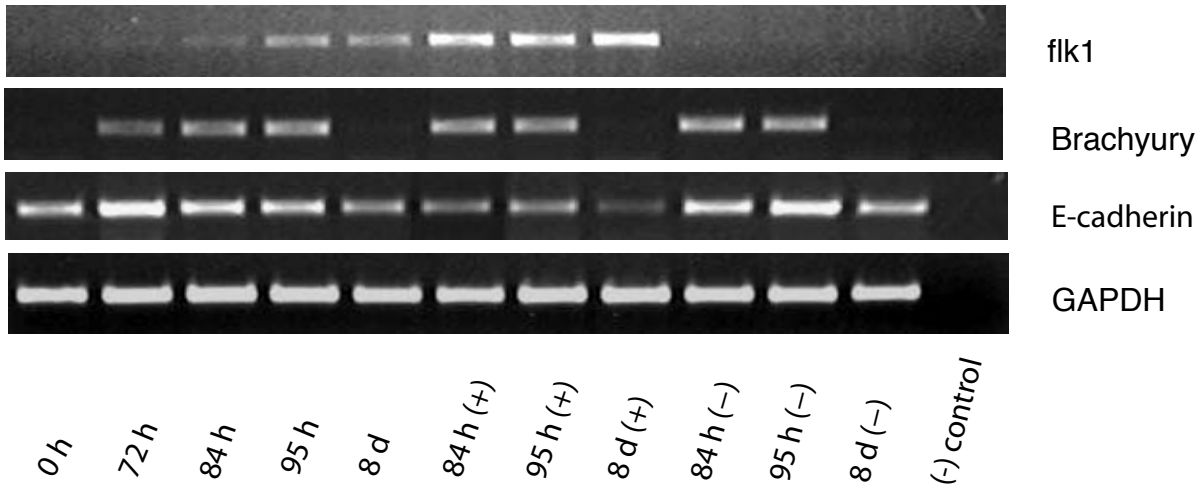
arrays	sample		UMRR‡	
	average value	SD†	average value	SD
undiff *	0.982	0.003	0.980	0.010
pre-72 h	0.991	0.003	0.994	0.001
pre-84 h	0.987	0.004	0.993	0.005
pre-95 h	0.965	0.015	0.986	0.006
pre-8 d	0.972	0.025	0.974	0.026
flk1(+)-84 h	0.984	0.011	0.992	0.003
flk1(+)-95 h	0.964	0.018	0.985	0.006
flk1(+)-8 d	0.987	0.003	0.990	0.006
flk1(–)-84 h	0.987	0.007	0.994	0.003
flk1(–)-95 h	0.971	0.014	0.990	0.002
flk1(–)-8 d	0.989	0.001	0.993	0.002

* undifferentiated ES cells
† standard deviation
‡ universal mouse reference RNA

A

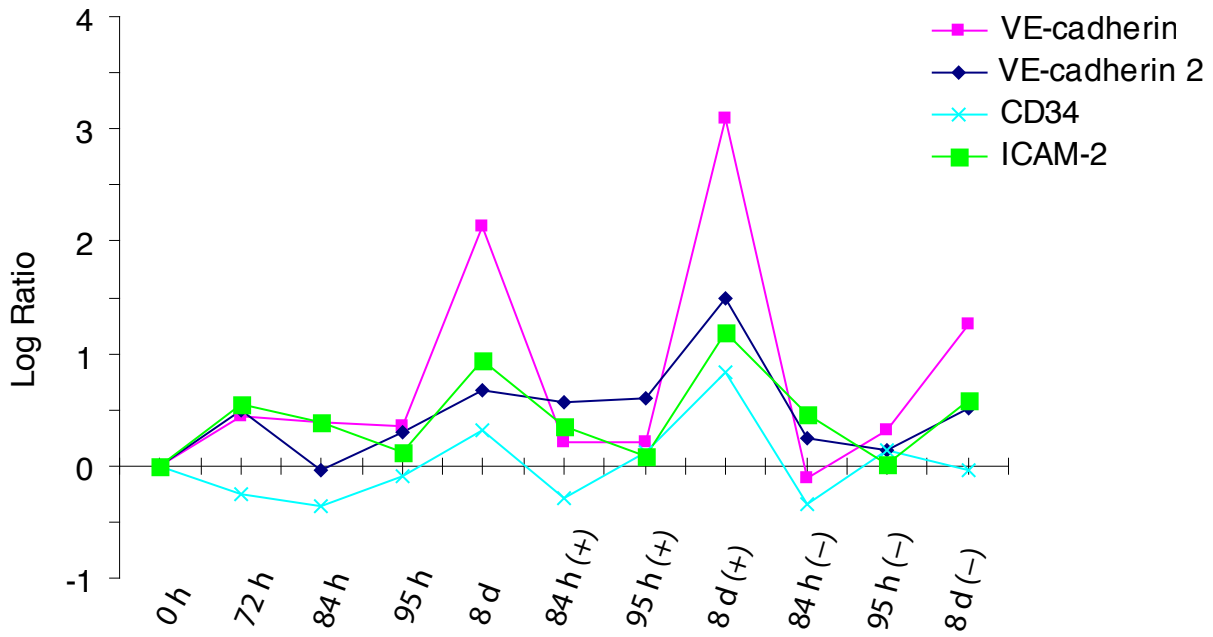


B



Supp Figure 1

A



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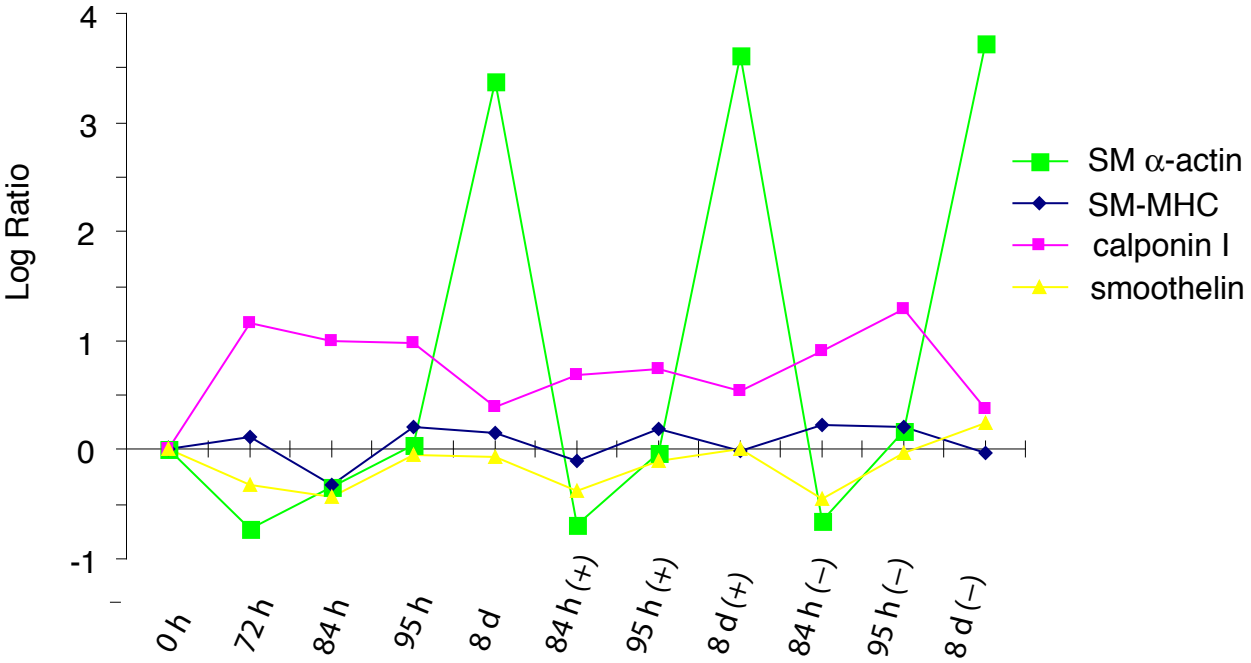
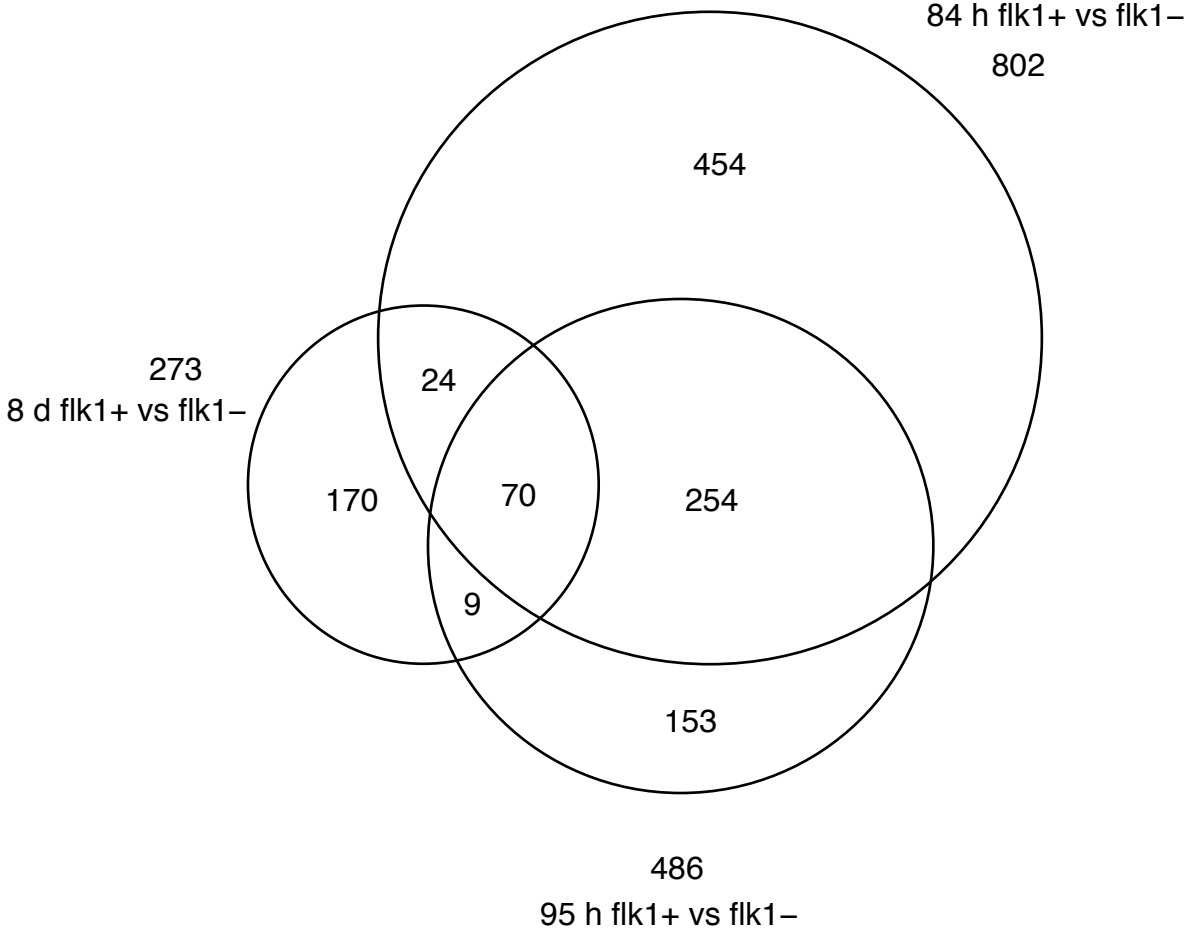
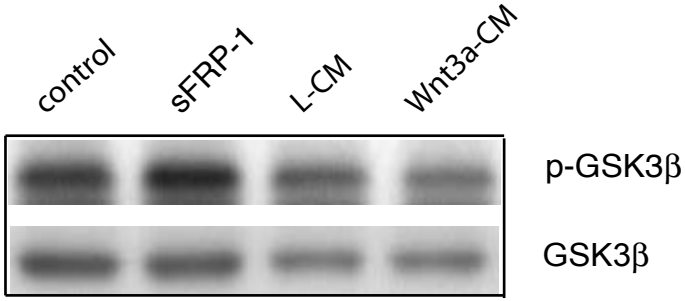


Supp Figure 2



Supp Figure 3

A



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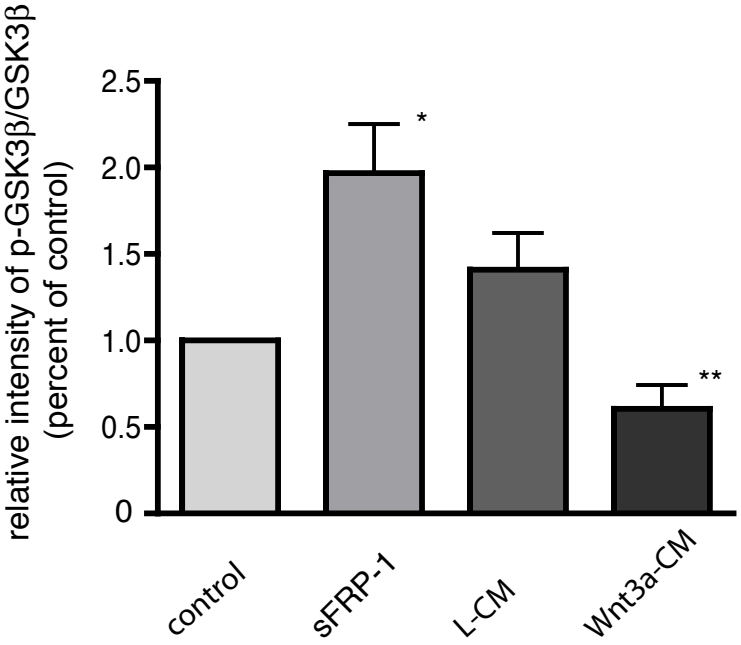
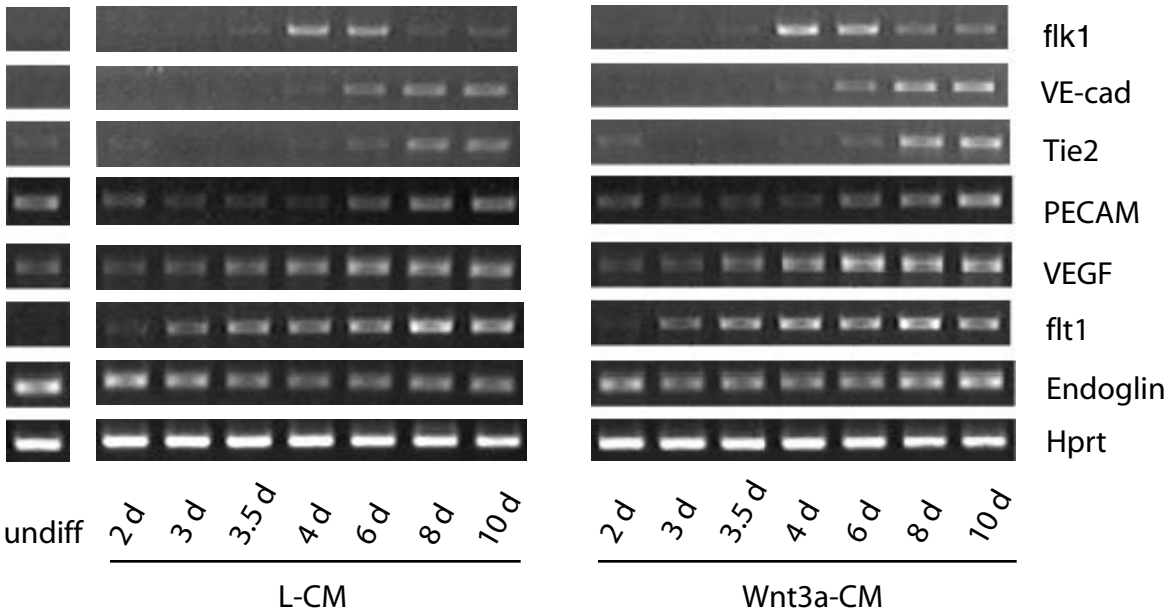
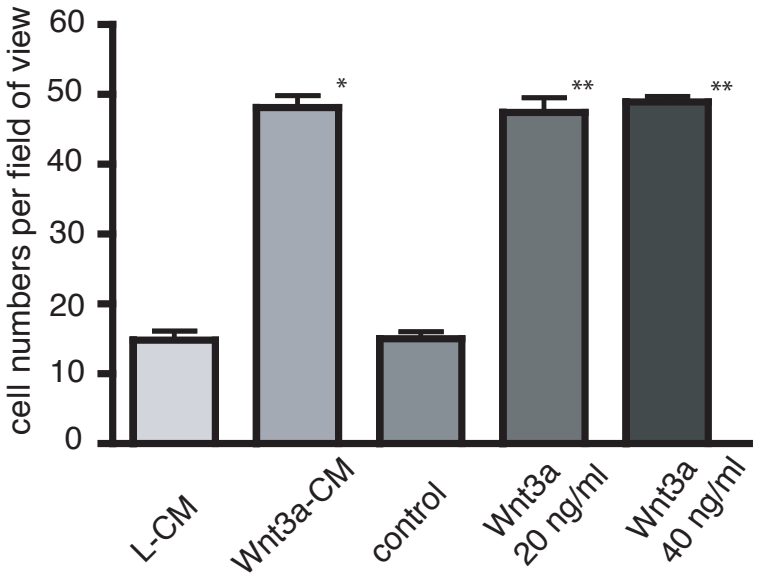


Supp Figure 4



Supp Figure 5



Supp Figure 6