

# The Basic Helix-Loop-Helix Transcription Factor HESR1 Regulates Endothelial Cell Tube Formation\*

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**Human endothelial cells can be induced to form capillary-like tubular networks in collagen gels. We have used this *in vitro* model and representational difference analysis to identify genes involved in the formation of new blood vessels. HESR1 (HEY-1/HRT-1/CHF-2/gridlock), a basic helix-loop-helix protein related to the hairy/enhancer of split/HES family, is absent in migrating and proliferating cultures of endothelial cells but is rapidly induced during capillary-like network formation. HESR1 is detectable in all adult tissues and at high levels in well vascularized organs such as heart and brain. Its expression is also enriched in aorta and purified capillaries. Overexpression of HESR1 in endothelial cells down-regulates vascular endothelial cell growth factor receptor-2 (VEGFR2) mRNA levels and blocks proliferation, migration, and network formation. Interestingly, reduction of expression of HESR1 by antisense oligonucleotides also blocks endothelial cell network formation *in vitro*. Finally, HESR1 expression is altered in several breast, lung, and kidney tumors. These data are consistent with a temporal model for HESR1 action where down-regulation at the initiation of new vessel budding is required to allow VEGFR2-mediated migration and proliferation, but re-expression of HESR1 is necessary for induction of tubular network formation and continued maintenance of the mature, quiescent vessel.**

The formation of new blood vessels by angiogenesis is critical to development of normal tissues as well as growth of solid tumors (1, 2). Angiogenesis is a multistep sequence of distinct cellular processes beginning with degradation of extracellular matrix, then proliferation, and migration of endothelial cells (EC),<sup>1</sup> followed by lumen formation and functional maturation

(3, 4). Currently, two families of EC-specific growth factors are known to regulate these steps. The vascular endothelial growth factors (VEGFs), together with the more widely expressed and pleiotropic fibroblast growth factors (FGFs), promote EC migration, proliferation, and tube formation (5–9). The second family is composed of angiopoietins (Ang) 1–5, of which Ang-1 and Ang-2, acting through the Tie-2 receptor tyrosine kinase, are known to be critical for the later processes of vessel maturation and stabilization (10–12). The VEGFs, which can drive all of the early stages of angiogenesis, act through two tyrosine kinase receptors, VEGFR1 (*flt-1*) (13) and VEGFR2 (*KDR/flk-1*) (14). EC also express neuropilin-1 and neuropilin-2, which only bind the VEGF<sub>165</sub> isoform (15).

Although transcription factors such as HIF and Tfeb are known to mediate EC responses to specific angiogenic inducers (hypoxia and placental growth, respectively (16, 17)), downstream events coordinating EC responses to general angiogenic growth factors remain unknown. In particular, it is not clear how sequential cellular processes can be triggered by continued or repeated exposure to the same stimulus, although a model for reiterative signaling has been proposed to explain FGF-induced branching morphogenesis in lung development (18).

To aid in further understanding the process of vessel formation, we wish to identify genes up-regulated in cultured EC induced to differentiate into capillary-like tubular networks. As a first step we have used the well characterized system of cultured EC forming networks in collagen gels (three-dimensional cultures) (19–22) and compared these to EC growing on top of collagen (two-dimensional cultures) using the PCR-based subtractive hybridization technique of representational difference analysis (RDA). This system models the temporally regulated angiogenic processes of migration, alignment, and tube formation and likely involves many of the same genes. This screen yielded the novel bHLH transcription factor HESR1, which has recently been identified as one of a new 3-member family of Hairy and E(spl)-related bHLH transcription factors, and is variously called HESR1 (23), HRT-1 (24), HEY-1 (25), and CHF-2 (26). The gene will be referred to as *HESR1* in this report.

HESR1 is widely expressed in the developing vasculature as well as in the presomitic mesoderm, brain, and limbs (23–25, 27). It is specifically expressed in atrial precursors, in the cardiac outflow tract, in aortic arch arteries, and in the dorsal aorta (24, 25). In adult tissues HESR1 has been detected in heart, brain, and lung but was not localized to particular cells (24). Recently, the gene responsible for the gridlock mutation in zebrafish was cloned and shown to be identical to Hey-2 (Hrt-2, CHF-1), the second member of this family. Mutation of gridlock disrupts caudal blood flow due to failure of the anterior lateral dorsal aortae to merge into the single midline aorta (27).

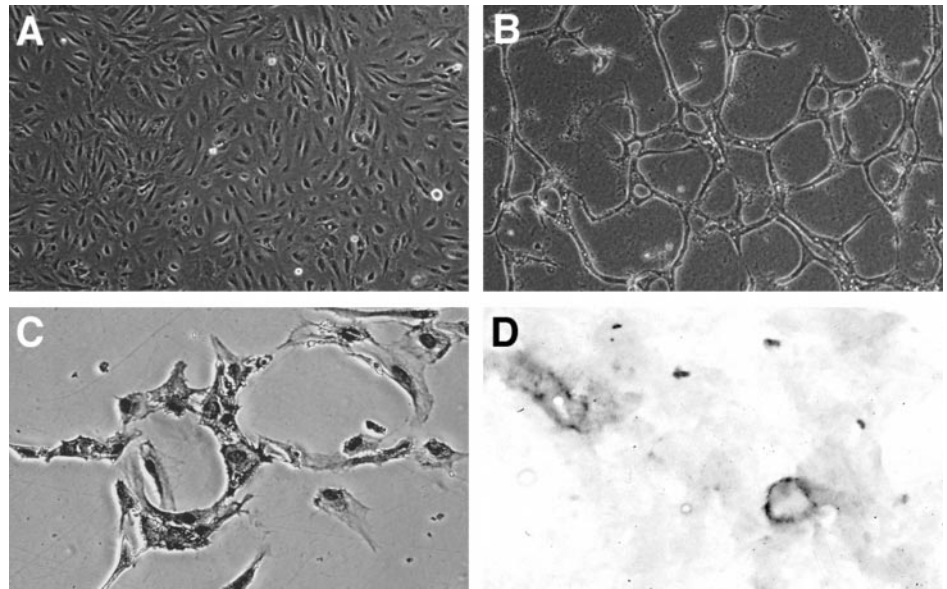
There is a high degree of homology in the bHLH domain

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<sup>1</sup> The abbreviations used are: EC, endothelial cell(s); RDA, representational difference analysis; HUVEC, human umbilical vein endothelial cells; HUCE, human capillary endothelial cells; bHLH, basic helix-loop-helix; RT-PCR, reverse transcriptase-polymerase chain reaction; GFP, green fluorescent protein; EGFP, enhanced GFP; VEGFR, vascular endothelial cell growth factor receptor; VEGFs, vascular endothelial growth factors; bFGF, basic fibroblast growth factor; bp, base pair; FACS, fluorescence-activated cell sorter; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FBS, fetal bovine serum; 7AAD, 7-aminoactinomycinD; XTT, (2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide); kb, kilobase pair; ARNT, aryl hydrocarbon receptor nuclear translocator.

**FIG. 1. Endothelial cells form tubes in collagen gels.** *A*, cultured ECs grow to form a monolayer when plated on top of collagen gels. *B*, ECs form capillary-like tubes 18 h after seeding into three-dimensional collagen gels. *C*, tube-forming cells continue to express EC-specific markers as demonstrated by staining with an anti-CD34 antibody. Staining with an isotype-matched control antibody was negative. *D*, ECs form capillary-like structures with patent lumens, demonstrated by staining with a polyclonal antibody to the EC-associated extracellular matrix protein  $\beta$ ig3. Staining with nonimmune serum was negative. All cultures contained 25 ng/ml bFGF and VEGF.



between HESR1, HES, and Hairy; however, homology outside of the bHLH is low until the C terminus. HESR1 has a C-terminal tetrapeptide YRPW sequence homologous to the WRPW motif in the Hairy/HES family and the WRPY motif in the Runt family (28). This domain interacts with groucho in flies (29) and the TLE family in mammals (30) and is necessary for transcriptional repression of downstream targets such as achaete-scute in flies and MASH in mammals.

We have now analyzed the function of HESR1 in cultured EC and show that expression is essential for capillary-like network formation.

#### EXPERIMENTAL PROCEDURES

**Cell Culture**—Human capillary endothelial (HUCE) cells were prepared from liposuction adipose tissue using anti-CD31-coated magnetic beads exactly as described (31). Tissue was obtained under protocols approved by the appropriate IRB committees. To induce capillary formation, HUCE cells were seeded onto fibronectin-coated rat tail type I collagen, rested for 1 h, and then overlaid with a second layer of collagen (1.5 mg/ml) to provide a three-dimensional matrix. Cells were grown in Medium 199 with 20% FBS, 25 ng/ml recombinant human vascular endothelial cell growth factor (VEGF, Genzyme), and 25 ng/ml recombinant human basic fibroblast growth factor (bFGF, R & D Systems). Two-dimensional cultures were identical except for the absence of the second layer of collagen. For the sorting experiments cells were resuspended in collagen, and 100- $\mu$ l drops were added to bacteriological grade 100-mm dishes on ice. After 15 min to allow cells to settle, the plates were moved to a 37 °C incubator to allow gelling. In some experiments early passage (p1–2) human umbilical vein EC (HUVEC) were used (32). Results were identical to those obtained with HUCE.

**Representational Difference Analysis**—For RDA, HUCE cultures were harvested after 18 h. Monolayers were lysed *in situ* using RNA isolation kits (Stratagene). Cells in collagen gels were harvested by digestion of the gel with 0.4% collagenase I (Worthington), and RNA was prepared as for monolayer cultures. Poly(A)<sup>+</sup> mRNA was purified over oligo(dT) columns (Stratagene). RDA was performed exactly as described (33). mRNA from monolayer EC (Driver) and tube-forming EC (Tester) was reverse-transcribed, and double-stranded cDNAs were digested with *DpnII*, ligated to linkers, amplified, and subtracted. Three rounds of subtractive hybridization were performed with increasing ratios of tester to driver: DP1, 1:100; DP2, 1:800; and DP3, 1:400,000. DP3 was cloned into pBluescript II-KS, and transformants were selected for analysis. IMAGE clones corresponding to the human (R60704) and mouse (AA980080) A21 genes (HESR1) were obtained and sequenced.

**RT-PCR and Northern Blotting**—Poly(A)<sup>+</sup> mRNA was prepared as above and resolved on standard formaldehyde gels. After cross-linking to nylon membranes, blots were hybridized to Psoralen-Biotin labeled probes (Ambion). After hybridization, blots were washed and developed according to the manufacturer's instructions. For detection of VEGFR2,

total RNA from control (pcDNA3) or pcDNA3-HESR1-transfected EC was resolved and blotted as above. Membranes were hybridized with a [ $\alpha$ -<sup>32</sup>P]dATP-labeled, random-primed probe, generated by PCR. Blots were washed and exposed either to film for 12–18 h or to PhosphorImager screens (Molecular Dynamics). A multiple tissue tumor blot was obtained from CLONTECH and probed according to the manufacturer's instructions. For RT-PCR, 2  $\mu$ g of DNase I-treated total RNA isolated from cells or mouse tissues was primed with random hexamers and reverse-transcribed using SuperScript II (Life Technologies, Inc.). Tissue was obtained under IACUC-approved protocols. 2–5  $\mu$ l of cDNA reaction was subjected to PCR amplification using gene-specific primers. The following primer pairs were used: GAPDH, sense 5' ACCACAGTCCATGCCATCAC 3' and antisense 5' TCCACCACCTGTTGCTGTA 3' (product size, 450 bp, annealing temperature, 67 °C, 25 cycles); human HESR1, sense 5' GGAGAGGCGCCGCTGTAGTTA 3' and antisense 5' CAAGGGCGTGCGCGTCAAAGTA 3' (product size, 429 bp, annealing temperature, 63.5 °C, 30 cycles); and mouse HESR1, sense 5' AGGGTGGGATCAGTGTGC 3' and antisense 5' TGCTTCTCAAAGGCACTG 3' (product size, 355 bp, annealing temperature, 56 °C, 30 cycles). The amplification profile was 94 °C, 5 min for hot start, followed by denaturation at 94 °C, 30 s; annealing as indicated, extension at 72 °C, 1 min for the indicated number of cycles with a final extension at 72 °C for 10 min. Amplifications were run on a PTC-200 (MJ Research). To confirm the absence of contaminating genomic DNA, the RT step was omitted as indicated.

**Plasmids**—The full-length coding sequence of HESR1 was cloned into either pcDNA3 (human gene, clone A21) or pIRES2-EGFP (mouse gene) using standard protocols. In experiments where pcDNA3-HESR1 was used, pEGFPN-1 was used to monitor transfection efficiency.

**Transfections and Cell Sorting**—Transfection of EC was performed using LipofectAMINE reagent in Opti-MEM (Life Technologies, Inc.). Three-day confluent (synchronized) EC were plated into 6-well plates 1 day before transfection. After incubation with DNA/liposome for 1.5 h, medium was changed. Transfection efficiency was monitored by FACS analysis. We routinely obtained 10–30% of cells expressing GFP. In some experiments transfected cells coexpressing GFP were sorted on a Cytomation Mo-Flo and then cultured for 24 h to allow recovery. Cells were then replated in 100- $\mu$ l collagen gels as indicated.

**Migration and Proliferation Assays**—To monitor EC migration, control or HESR1-transfected cells were plated onto gelatin-coated Transwell filters (PET membrane, 24-well, Falcon) with a pore size of 8  $\mu$ m. Medium M199 + 5% FBS was added to the Transwell and M199, 5% FBS + VEGF and bFGF at 25 ng/ml was added to the lower chamber. After 24 h cells were fixed in 3.7% formaldehyde and stained with crystal violet (0.5% in 25% methanol). Cells on the upper surface were then removed with a cotton swab, and the remaining dye (from transmigrated cells) was solubilized in 0.1 M citric acid in 50% ethanol, and the absorbance was read at 590 nm. Readings were normalized to the total number of cells plated by assaying membranes from parallel cultures that did not have the cells on the upper surface removed. Proliferation was measured by direct counting of replicate cultures in

24-well plates or by XTT assay (Sigma) with 10–12 replicates for each time point in 96-well plates (34).

**7AAD**—HUVEC were transfected with pcDNA-3 control and pEGFPN-1 or pcDNA3-HESR1 and pEGFPN-1 and stained 2 days later with 20  $\mu\text{g}/\text{ml}$  7AAD (Sigma) for 20 min at 4 °C in the dark (35). Cells were then analyzed by FACS.

**Immunostaining**—Cells growing in collagen gels were fixed in 4% paraformaldehyde and stained with either CD34 monoclonal antibody (BioGenex, San Ramon, CA) or anti- $\beta\text{hgh3}$  (gift of Tony Purchio, Hepatix, San Diego, CA). Detection was by peroxidase-conjugated secondary antibody (Biorad) and 3,3'-diaminobenzidine (DAKO).

**In Vitro Transcription/Translation**—2  $\mu\text{g}$  of plasmid DNA was used in the TNT T3/T7 coupled transcription/translation system (Promega) according to the manufacturer's instructions. After a 90-min reaction at 30 °C,  $^{35}\text{S}$ -labeled translated products were resolved by SDS-polyacrylamide gel electrophoresis. Dried gels were exposed to film overnight.

**Quantitation of Tube Formation**—Photomicrographs of the developing tubular networks were skeletonized using a digitizing tablet by an observer blinded to the experimental conditions. A number of branch points were then enumerated, and the average interbranch distance was calculated. Three to five randomized fields for each gel were quantitated.

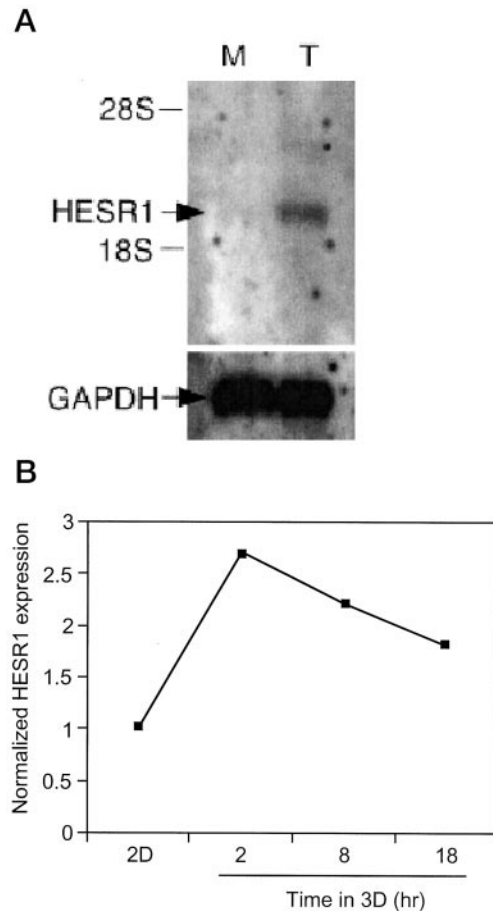
**Antisense Treatment**—The following sequence-specific S-oligonucleotides were used: HESR1 A1, 5' TCCGCACTCTCCTTCTCC 3'; A2, 5' TCGTCGGCGCTTCTCAAT 3'; A3, 5' TCCCGAAATCCCAAAC 3'; nonsense control, 5' CCCTCCCTGTACTTACTCC 3', and VEGFR2, positive control, 5'CGGACTCAGAACCACATC 3'. EC were loaded with antisense oligonucleotides using Influx pinocytic cell-loading reagent (Molecular Probes). After loading, cells were allowed to recover for at least 10 min before plating in collagen gels. Assays were performed in triplicate. Cultures were scored 24 h after plating, by two individuals blinded to the experimental set up. The degree of tube formation was graded in 5 levels from 0 to +++. For RT-PCR analysis of antisense action, cells were harvested after 24 h in collagen.

## RESULTS

Human EC, seeded into three-dimensional collagen gels, formed capillary-like networks within 18–24 h (Fig. 1B), whereas cells growing on top of collagen continued to proliferate and eventually formed a monolayer (Fig. 1A). Network-forming cells retain their EC phenotype as shown by their continued expression of CD34 (Fig. 1C), a commonly used immunohistochemical blood vessel marker. The networks are composed of tubes as evidenced by the presence of lumens (Fig. 1D), outlined by staining for the extracellular matrix protein  $\beta\text{hgh3}$  (36). Significantly, when the gels containing the neovessels are transplanted into a skin pocket on a SCID mouse, the human vessels anastomose with ingrowing mouse vessels and are perfused with blood, indicating that the cultured cells are fully competent to form mature vascular networks<sup>2</sup> (37).

Genes differentially expressed between these two culture geometries were identified by RDA. Several well characterized "angiogenic" genes were obtained, including the  $\alpha_v$  integrin and plasminogen activator inhibitor, suggesting overlap in gene expression between tube-forming cells *in vitro* and *in vivo*.<sup>3</sup> A highly represented fragment (27/138 clones) we obtained after three rounds of subtraction showed homology to human and *Drosophila* Hairy protein and mammalian *hes* genes and was further characterized. The A21 cDNA contained a single large open reading frame coding for a protein of 304 amino acids and was recently independently named HESR1 (23). We also cloned and sequenced the mouse homologue, which has been variously named HEY-1 (25), HRT-1 (24), and CHF-2 (26).

To confirm the differential expression of HESR1 in EC-forming tubes compared with migrating and proliferating EC growing in two dimensions, we isolated poly(A)<sup>+</sup> mRNA from three- and two-dimensional cultures and used this for Northern blotting. A single species of ~2.0–2.5 kb was detected in the three-



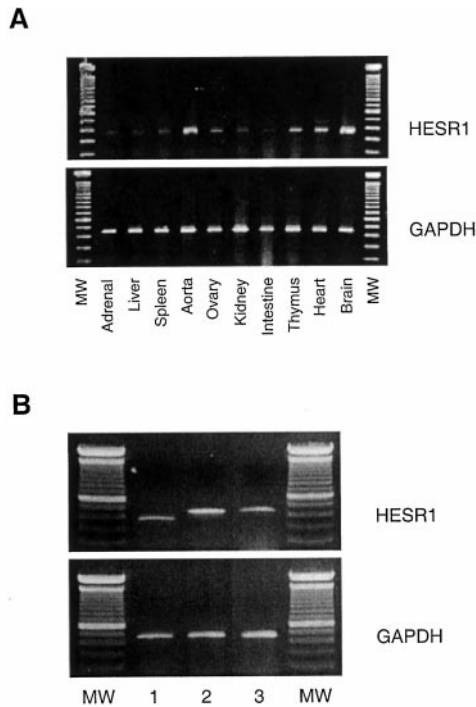
**FIG. 2. Expression of HESR1.** A, Northern blot of poly(A)<sup>+</sup> mRNA (1  $\mu\text{g}$ ) from tube-forming (T) and migrating/proliferating (M) EC hybridized with psoralen-biotin-labeled HESR1. A single species of ~2.5 kb, corresponding to the predicted HESR1 mRNA, was present in the tube forming EC but absent in migrating/proliferating EC. Equal loading was demonstrated using a GAPDH probe (bottom panel). B, time course of HESR1 expression. RNA was harvested from migrating/proliferating cells (two-dimensional) and from network-forming cells at the indicated times after plating into collagen gels (three-dimensional). RT-PCR was performed for HESR1, and this was normalized to GAPDH expression. Data are plotted in arbitrary units normalized to two-dimensional culture. One of four similar experiments is shown.

dimensional tube but not in the two-dimensional cultures (Fig. 2A) consistent with the cDNA size of 2.2 kb and the reported transcript size for mouse of ~2.3 kb (24). To analyze the time course of HESR1 expression during network formation, we harvested RNA and performed RT-PCR (Fig. 2B). In migrating and proliferating cells (two-dimensional cultures) HESR1 was absent or present at very low levels. Induction was rapid when the cells were embedded in collagen gels (three-dimensional) with expression peaking by 2 h. Expression then fell slowly over the next 15–18 h to a level still considerably higher than that seen in two-dimensional cultures.

To investigate tissue distribution in the adult, we used RT-PCR on mRNA prepared from several adult mouse tissues. A single band of the predicted size was detected (Fig. 3A), and its identity as mouse HESR1 was confirmed by sequencing. No bands were present when the RT step was omitted, confirming the absence of genomic contamination (data not shown). As a semi-quantitative estimate of relative abundance, we normalized HESR1 band intensities to those of GAPDH amplified in parallel and compared these to the tissue expressing the lowest level of HESR1 (kidney). This revealed a dramatic enrichment of HESR1 transcripts in well vascularized tissues such as brain (7.5 times) and heart (4.8 times), as well as high levels in aorta

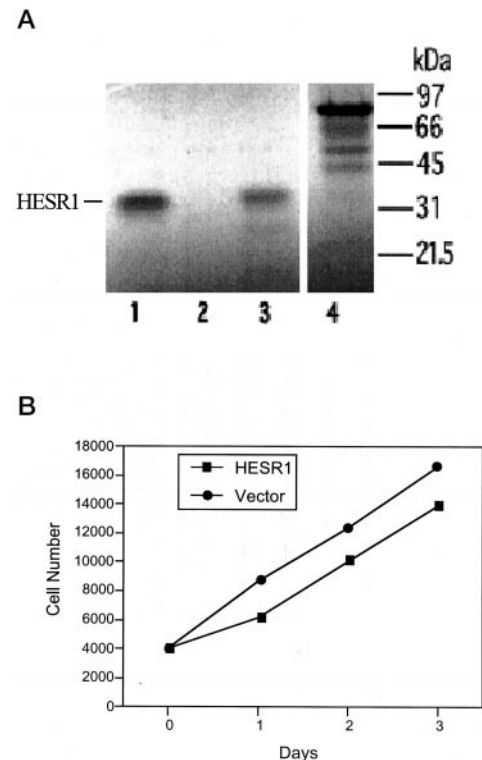
<sup>2</sup> J. Mestas and C. C. W. Hughes, unpublished observations.

<sup>3</sup> M. Aitkenhead, S.-J. Wang, J. Mestas, C. Heard, and C. C. W. Hughes, submitted for publication.



**FIG. 3. Expression pattern of HESR1 in different adult tissues.** A, RT-PCR performed on cDNA derived from various mouse tissues, using HESR1-specific primers, amplified a 355-bp fragment (upper panel). Expression of HESR1 was highest in brain, heart, thymus, and aorta and detectable in all organs tested. Amplification of a GAPDH fragment was used to confirm equal target cDNA from each of the organs (lower panel). The MW lanes contain a 100-bp ladder, where the double intensity band is 600 bp long. B, RT-PCR demonstrating HESR1 expression in purified human capillaries. Lane 1, mouse brain (355 bp); lane 2, human capillaries (429 bp); lane 3, human EC-forming tubes *in vitro* (429 bp). Amplification of a GAPDH fragment was used to confirm equal target cDNA in each of the samples (lower panel). MW marker lanes contain a 100-bp ladder, where the double intensity band is 600 bp long. One of three similar experiments is shown.

(4.5 times). These data are consistent with recently published data showing expression in brain and heart by multiple tissue Northern blot. The high level of sensitivity provided by RT-PCR allowed us to detect transcripts in other tissues not revealed by Northern blotting. So far we have been unable to detect HESR1 mRNA in adult tissues by *in situ* hybridization, presumably due to low levels of transcript. All previous *in situ* hybridization studies have been on embryos. As an alternative strategy to confirm vascular expression of HESR1 *in vivo*, we used RT-PCR on mRNA prepared from highly purified, freshly isolated adipose-derived capillaries (Fig. 3B). We obtained a strong signal indicating that HESR1 is indeed expressed in capillaries *in vivo*, most likely by EC; however, expression in both EC and pericytes cannot be ruled out by our data. No signal was obtained in the absence of the RT step (data not shown). We also wished to determine whether levels of HESR1 are altered in the vasculature of tumors, but again *in situ* hybridization proved too insensitive. We therefore analyzed HESR1 expression in a multiple tissue tumor blot spotted with amplified cDNA from matched normal and tumor tissue. A potential problem with this kind of analysis is that up- or down-regulation of a gene may only be occurring in a discrete subset of cells and at discrete time points. In an analysis of bulk RNA, therefore, a signal may be masked by background noise. We believe this to be the case for HESR1 as we saw up-regulation in 3 of 3 lung tumors but down-regulation in 11 of 15 kidney tumors and 5/9 breast tumors (data not shown). This variation may reflect site of sampling (angiogenic edge or quiescent middle of the tumor), state of the tumor (rapidly growing



**FIG. 4. Overexpression of HESR1 suppresses proliferation.** A, *in vitro* transcription/translation of HESR1 confirms the predicted protein size. Autoradiograph of translated products from different plasmids resolved by SDS-polyacrylamide gel electrophoresis. Lane 1, HESR1 in pcDNA 3.1(+); lane 2, reverse orientation HESR1 in pcDNA 3.1 (-); lane 3, HESR1 in pBSII KS(+); lane 4, luciferase control. A band corresponding to a 31–33-kDa protein was detected in lanes 1 and 3 but was absent in lane 2. B, EC were transfected with pcDNA3 control or pcDNA3-HESR1 and plated in 96-well plates. Transfection efficiency was assessed by cotransfection of pEGFPN-1 and for the experiment shown was 9%. At the indicated times cell number was assessed by XTT assay against a standard curve. Each data point is the mean of 12 replicate wells, and error bars fall within the symbol. One of three similar experiments is shown.

compared with static or slow growing), or may reflect tissue-specific effects. More sensitive *in situ* hybridization will help resolve this question.

Our *in vitro* and *in vivo* data suggest that in the adult HESR1 is expressed in mature vessels in all tissues, is decreased during migration and proliferation (two-dimensional cultures), and is re-expressed during the tube-forming stage of new vessel formation (three-dimensional cultures). This temporal expression pattern suggests that the level of HESR1 may regulate the phenotype of the EC, high levels acting to maintain quiescence while reduced levels may allow migration and proliferation. To test this hypothesis we constructed a HESR1 expression vector and used this to test the effect of HESR1 overexpression on EC phenotype. As a test of the plasmid, we performed *in vitro* transcription/translation of the pcDNA3-HESR1 plasmid, which revealed a single band of 31–33 kDa, consistent with the predicted size of 32,627 kDa for the conceptually translated protein (Fig. 4A). To examine the effect of HESR1 on cell proliferation, ECs were transfected with pcDNA3-HESR1 or pcDNA3 control, along with pEGFPN-1 to monitor transfection efficiency, and cell number was monitored over 3 days by direct counting or XTT assay. By both assays there was a reproducible decrease in cell proliferation at all time points. In the XTT assay shown (Fig. 4B) the decrease in proliferation was consistent with the transfection efficiency for this experiment of ~10%. To confirm that HESR1 overexpression was not killing cells by inducing apoptosis, we stained

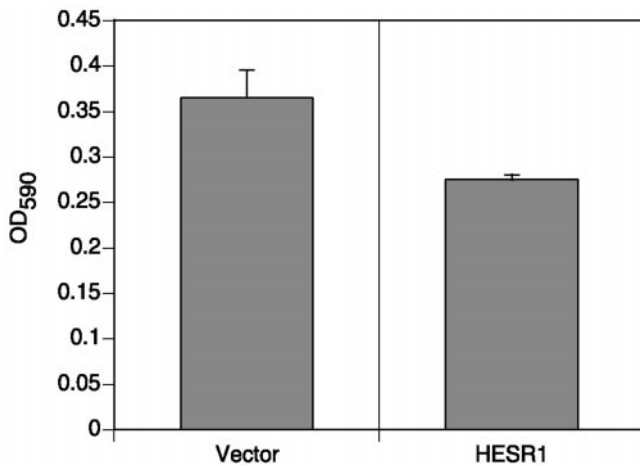


FIG. 5. **Overexpression of HESR1 suppresses migration.** EC were transfected with pcDNA3 control or pcDNA3-HESR1 and plated into transwells as described under “Experimental Procedures.” Transfection efficiency was assessed by cotransfection of pEGFPN-1 and for the experiment shown was 13%. After 24 h the number of transmigrated cells was assessed by staining with crystal violet. Mean and S.D. of three replicate wells is shown. One of three similar experiments is shown.

HESR1 and control-transfected cells with 7AAD (35). By FACS, transfected and apoptotic cells appear GFP bright and 7AAD bright, respectively. This population represented 2.9% of the total in control-transfected cells and 2.1% of the HESR1-transfected cells (data not shown). Salicylate-induced apoptosis was used as a positive control and yielded 27% positive cells. These data indicate that there is no increase in apoptosis in response to HESR1 expression. Finally, we counted floating (dead) cells directly, and again there was no significant difference at 3 days between control and HESR1-transfected cells (data not shown).

To examine migration of EC-overexpressing HESR1, we plated transfected cells onto the upper chamber of 8- $\mu$ m pore size transwells and measured transmigration in response to VEGF and bFGF after 24 h. As shown in Fig. 5, HESR1 overexpression decreased migration by 25%. Taken with the effect on proliferation, these data suggest that HESR1 maintains EC quiescence and that HESR1 down-regulation may be essential to allow expression of the angiogenic phenotype.

We next asked whether overexpression of HESR1 would promote or disrupt capillary-like network formation. Our prediction was that, given the importance of EC migration in the early stages of angiogenesis, preventing migration by overexpression of HESR1 would block migration and proper alignment of the EC and therefore disrupt formation of the network. This indeed was the case. EC were transfected with control or pIRES2-EGFP-HESR1 and then sorted for GFP expression. When compared with EC transfected with a control plasmid, HESR1-overexpressing cells survived but were unable to generate extensive branching networks (Table I). There appeared to be a failure of “islands” of cells to interconnect, due to a lack of budding and branching. When the cultures were quantitated the data revealed an ~50% decrease in the number of branch points in HESR1-transfected cells (Table I).

Both EC migration and proliferation are driven by VEGF acting through VEGFR2, and consequently the receptor has a critical role in vasculogenesis and angiogenesis. Moreover, the promoter of VEGFR2 contains several E boxes, including one at position -175 that has been suggested to bind an EC-specific factor (38). We wondered, therefore, whether VEGFR2 is a downstream target of HESR1 and whether HESR1 overexpression might down-regulate VEGFR2 expression. To test this, ECs were transfected with control vector or pcDNA3-HESR1

expression vector and harvested for RNA isolation 24 h later. Northern analysis revealed that overexpression of HESR1 in EC led to a dramatic down-regulation of VEGFR2 mRNA levels (Fig. 6). Preliminary experiments suggest that HESR1 is acting at the transcriptional level as cotransfection of pcDNA3-HESR1 into EC blocks VEGFR2 promoter-driven luciferase expression. A similar effect is seen with the VEGFR1 promoter.<sup>4</sup> HESR1, like Hairy, HES, and E(spl), therefore appears to have a role in negative regulation of gene expression. These data suggest that HESR1-mediated inhibition of EC migration and proliferation is due, at least in part, to down-regulation of VEGFR2 and loss of responsiveness to VEGF. This interpretation is supported by our antisense experiments (see below) where we used a VEGFR2 antisense oligonucleotide as a positive control to block network formation.

By having determined that HESR1 is expressed in mature, quiescent vessels and that its down-regulation is necessary for the early stages of angiogenesis, namely migration and proliferation, we next wanted to determine whether re-expression of HESR1 is required for the later stages of EC alignment, tube formation, and network maturation. We designed several antisense oligonucleotides to HESR1 and tested these in the three-dimensional culture model. Based on lack of sequence homology, none of the oligonucleotides are predicted to cross-react with other family members. The positive control sequence from the VEGFR2 gene blocked network formation in a dose-dependent manner to a maximum of 88% at 3  $\mu$ M (Fig. 7A). Two of the HESR1 oligonucleotides also blocked network formation. A1 was most potent, blocking by 75% at 0.3  $\mu$ M and greater than 95% at 3  $\mu$ M; A3 was less effective, only showing significant blocking at 3  $\mu$ M; A2 did not block. Variable effectiveness between different oligonucleotides is a common occurrence when using antisense to block gene expression. Matched nonsense oligonucleotides were ineffective at blocking. Neither the antisense nor the nonsense oligonucleotides had any effect on the morphology of monolayer cultures (data not shown). To rule out a nonspecific action of the antisense compounds, we analyzed HESR1 mRNA levels in cells treated with antisense or nonsense oligonucleotides. In the presence of 3  $\mu$ M A1 oligonucleotide, no HESR1 message was detectable, whereas levels of GAPDH message were unaffected (Fig. 7B). Nonsense oligonucleotides had no effect on expression of either gene. These experiments indicate that HESR1 expression is necessary for establishment of the mature EC network.

#### DISCUSSION

In a screen to isolate EC genes up-regulated during *in vitro* capillary-like network formation, we identified the bHLH transcription factor HESR1 (23). This gene is homologous to the Hairy/HES family of genes that act downstream of Notch to regulate neuronal development in *Drosophila* and mice (39, 40) and has recently been independently identified and named HEY-1 (Hairy, E(spl) related with YRPW) (25), HRT-1 (Hairy-related transcription factor), and CHF-2 (cardiovascular helix-loop-helix factor 2) (26). There are two other members of the family so far identified (24, 25, 27), with similar structure and overlapping expression patterns. To date, expression of HESR1 has been examined mostly in embryos, by whole-mount *in situ* hybridization. HESR1 is observed in the atrium of the heart, in the cardiac outflow tract, in the aorta, and in the somitic mesoderm, several regions of the central nervous system, and in limbs.

We found HESR1 expression in all adult tissues examined including aorta and purified capillaries, indicating that it might be expressed by EC throughout the body. Expression was down-regulated in migrating and proliferating EC in culture

<sup>4</sup> A. M. Henderson and C. C. W. Hughes, unpublished observations.

TABLE I  
HESR1 overexpression suppresses network formation

EC were transfected with either pIRES2-EGFP control or pIRES2-EGFP-HESR1 and sorted for GFP expression. After resting for 24 h cells were plated into collagen gels and photographed 18 h later. Five random fields were then quantitated as described under "Experimental Procedures." Two independent experiments, performed on different days, are shown.

	Branch points		Interbranch distance	
	Vector	HESR1	Vector	HESR1
Exp. 1	41 ± 8.6 <sup>a</sup>	21 ± 5.5	11.7 ± 1.0 <sup>b</sup>	12.0 ± 2.4
Exp. 2	40 ± 4.0	21.8 ± 2.4	11.1 ± 0.6	8.3 ± 0.4

<sup>a</sup> Mean ± S.E. of buds/branch points per field.

<sup>b</sup> Mean ± S.E. of interbranch distance in arbitrary units.

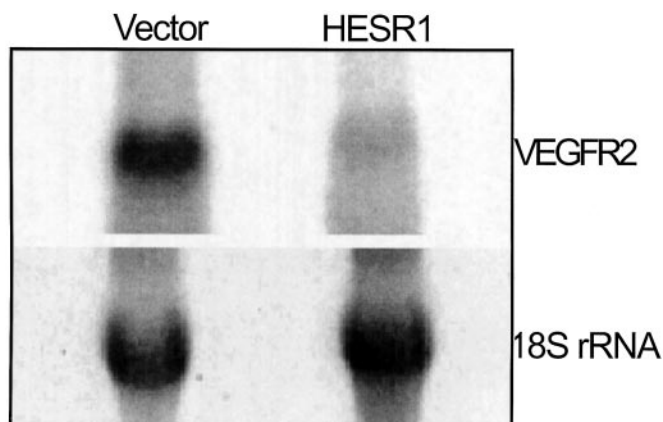


FIG. 6. HESR1 expression down-regulates VEGFR2 expression. Total RNA was isolated from HUVEC transfected with either control (pcDNA3) or HESR1 expression vector (pcDNA3-HESR1) and cultured for 24 h. The blot was probed with a fragment of the VEGFR2 gene generated by PCR. Intensity of the 18 S rRNA bands confirms approximately equal loading in each lane. One of two similar experiments is shown.

suggesting that HESR1 may be involved in induction and/or maintenance of the mature, quiescent vascular phenotypes. test this hypothesis we overexpressed HESR1 in EC and examined migration and proliferation, as well as expression of VEGFR2. This gene, in contrast to VEGFR1, delivers a proliferative signal to EC (5) and has been demonstrated in the proliferating EC of vascular sprouts in the brain (8). Moreover, VEGFR2 expression was shown to be dramatically down-regulated in adult brain where no angiogenesis is occurring, compared with the embryo where all new vessels in the brain are generated by angiogenesis. When we overexpressed HESR1 in our system, to mimic quiescent vessels, it down-regulated VEGFR2 expression and slowed migration and proliferation of EC. Furthermore, expression of HESR1 blocked network formation in three-dimensional gels, presumably due to suppressed EC migration. Adding further support to this interpretation is the finding that antisense to VEGFR2 likewise prevented network formation. Thus, down-regulation of VEGFR2 by HESR1 may be a switch that regulates the transition from the proliferating, migrating phenotype to the network-forming and vessel maturation phenotype.

Interestingly, antisense inhibition of HESR1 expression also inhibits network formation, demonstrating that the gene is essential for this process. Our interpretation is that while HESR1 down-regulation is essential in the early stages of migration, presumably to allow VEGFR2 expression, re-expression of HESR1 is required at a later stage to allow alignment, tube formation and vessel maturation. Antisense knockout of HESR1, therefore, prevents this stage in network formation.

To summarize, we hypothesize that HESR1 is required to induce and maintain the mature vascular phenotype, in part by preventing EC proliferation and migration (Fig. 8). During the

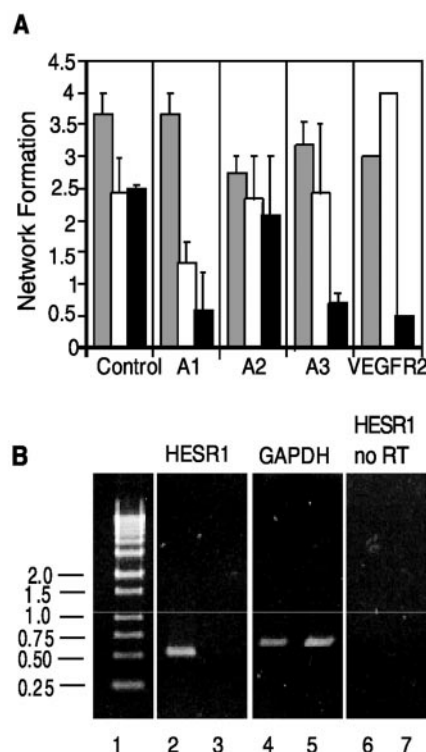


FIG. 7. Antisense inhibition of HESR1 expression in EC causes disruption of network formation. A, three antisense oligonucleotides (A1, A2, and A3) were selected from nonoverlapping regions of the HESR1 cDNA sequence and incorporated into EC by hypo-osmotic shock. Cells were cultured in three-dimensional collagen gels for 24 h. The ability of treated EC to form networks was assessed (see "Experimental Procedures") and plotted on an arbitrary scale. A VEGFR2 oligonucleotide was used as a positive control, and a nonsense oligonucleotide served as a negative control (Control). The mean and S.E. of three experiments is shown. The VEGFR2 data are from a single experiment. All experiments were performed with three concentrations of oligonucleotides: 0.03 μM (gray); 0.3 μM (white); 3 μM (black). B, to confirm the specificity of antisense action, RNA was isolated from A1-treated and nonsense control-treated cells and RT-PCR performed with primers specific to HESR1 and GAPDH. Lane 1, molecular weight ladder (kb); lanes 2 and 4, nonsense oligonucleotide-treated cell cDNA, amplified with HESR1 and GAPDH primers, respectively. Lanes 3 and 5, A1 antisense-treated cell cDNA, amplified with HESR1 and GAPDH primers, respectively. Lanes 6 and 7, same as lanes 2 and 3 but no reverse transcription. The absence of a band in lane 3 confirms the effectiveness and specificity of the A1 oligonucleotide.

early stages of angiogenesis HESR1 expression would be expected to fall, allowing cells to migrate away from the parent vessel, express VEGFR2, and proliferate. Later, re-expression of HESR1 would be required for down-regulation of VEGFR2, leading to cessation of proliferation, and for tube formation and re-establishment of the mature vessel phenotype. Our data are consistent with this model in that HESR1 is expressed in mature blood vessels (Fig. 3); its expression is suppressed in migrating and proliferating cells, and it is rapidly reinduced in

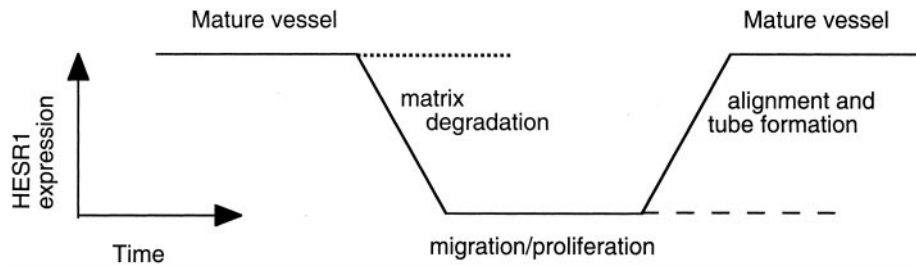


FIG. 8. **Proposed model for HESR1 action.** HESR1 is expressed in mature vessels but disappears during the early stages of angiogenesis when ECs migrate and proliferate. Later, as alignment and tube formation begins, HESR1 is re-expressed and inhibits further EC proliferation. Overexpression of HESR1 (dotted line) inhibits migration and proliferation. Inhibition of HESR1 expression (dashed line) disrupts the processes of alignment and tube formation and re-establishment of the mature vessel phenotype.

network-forming cells (Fig. 2). Overexpression of HESR1 prevents VEGFR2-mediated migration and proliferation (Figs. 4 and 5), whereas antisense reduction of HESR1 expression prevents the switch from the migrating and proliferating phenotype of cells in two dimensions to the tube-forming phenotype in three-dimensions (Fig. 7). As HESR1 overexpression does not induce tube formation directly in two-dimensional culture, we propose that re-expression of HESR1 is permissive for network formation and vessel maturation, rather than instructive. Although this model is somewhat speculative, it does provide a framework for future experiments.

Most bHLH proteins bind to DNA as either homo- or heterodimers. Most bind to the canonical E box (CANNTG), although Hairy proteins bind a variant called the N box (CACNAG) due to a conserved proline in the basic region. HESR1 has a glycine at this position and is predicted to bind to an E box. HESR1 is likely acting in our system by dimerizing with other bHLH proteins. Indeed, a recent report identified CHF-1 and CHF-2 (HESR1) in a two-hybrid screen using as bait the bHLH domain of the aryl hydrocarbon receptor nuclear translocator (ARNT) (26). ARNT has been shown to regulate VEGF expression by binding to the HIF-1 site in the VEGF promoter. CHF-1 was shown to displace ARNT from this site and down-regulate VEGF promoter activity. CHF-2 was not tested. It is not possible to predict, based on sequence alone, whether HESR1 will positively or negatively regulate other target genes, or whether it will interact with corepressors such as groucho/TLE (29, 30). By analogy with the Hairy family, and based on the presence of the YRPW motif, a role in negative regulation in mature vessels may be expected, possibly of Achaete-Scute complex homologues (41), potentially of other proangiogenic genes such as matrix metalloproteinases and angiopoietin-2. Clearly, our data on VEGFR2 expression are consistent with this hypothesis. Interestingly, however, there is good evidence that the Runt family of genes, which contain the WRPY motif, are capable of mediating both negative and positive regulation of transcription (42, 43). HESR1 may behave similarly as it also carries a variant of the WRPW motif, namely YRPW. Binding of groucho to the WRPY motif of Runt appears to be regulated, in contrast to the constitutive binding of groucho to the WRPW motif found in the Hairy/HES family. Context-dependent binding of groucho to target promoters appears to determine whether positive or negative regulation occurs.

Hairy/HES genes act downstream of Notch to determine cell fate decisions in neurons by regulating the expression of the pro-neural achaete-scute complex genes (39). Notch has also been implicated in angiogenesis; Zimrin *et al.* (44) demonstrated that inhibiting expression of the Notch ligand Jagged-1 potentiated the ability of EC to form capillary-like networks in culture. The direct demonstration in mice that HESR1 is downstream of Notch in presomitic mesoderm (23) suggests that HESR1 may be downstream of Notch in EC, further supporting a role for HESR1 in angiogenesis. Interestingly, mutations in

the human Jagged-1 ligand are reported to be responsible for Alagille syndrome, which manifests as cerebral vascular hemorrhaging (45), whereas a number of the notch and notch ligand knock-out mice show vascular phenotypes (46). We are currently investigating the expression of Notch proteins in human EC.

In conclusion, our findings suggest that HESR1 may be a genetic switch, the control of which may regulate EC phenotype and the multiple stages of angiogenesis.

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