

Hemangioblastomas Share Protein Expression with Embryonal Hemangioblast Progenitor Cell

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Abstract

Hemangioblastomas are central nervous system (CNS) tumors of unknown histogenesis, which can occur sporadically or in von Hippel-Lindau disease. Hemangioblastomas are composed of neoplastic “stromal” cells of unknown origin, accompanied by intensive reactive angiogenesis. Failure to specify the cytologic origin of the stromal cell has precluded the development of nonsurgical therapies and limits understanding of its basic biology. We report that the stromal cells express proteins (Scl, brachyury, Csf-1R, Gata-1, Flk-1, and Tie-2) that characterize embryonic progenitor cells with hemangioblastic differentiation potential and conclude that embryonic progenitors with hemangioblast potential represent a possible cytologic equivalent of the stromal cell. We also identified a new autocrine/paracrine stimulatory loop between the receptor Tie-2 and the hypoxia-inducible factor target Ang-1, which, combined with previous observations, suggests that a variety of autocrine loops may be initiated in hemangioblastomas, depending on the differentiation status of the tumor cells and the extent of HIF downstream activation. Finally, the consistent identification of Scl in the stromal cells may help explain the unique and characteristic topographical distribution of hemangioblastomas within the CNS. (Cancer Res 2006; 66(8): 4167-72)

Introduction

Hemangioblastoma is a highly vascular tumor, which comprises ~3% of all tumors of the central nervous system (CNS; ref. 1). Hemangioblastomas occur as a sporadic entity or as a component tumor of von Hippel-Lindau disease (VHL), an autosomal dominantly inherited disorder. They occur in a strikingly limited subset of CNS locations, including retina, cerebellum, brainstem, and spinal cord (1).

Hemangioblastomas are composed of vascular and “stromal” cells. Loss of heterozygosity analysis of microdissected stromal cells of hemangioblastomas reveals the stromal cells to represent the neoplastic *VHL*-deficient component of the tumor (2). Because a series of immunohistochemical and ultrastructural studies failed to clarify the origin of the stromal cell, hemangioblastomas remain to be tumors “of uncertain histogenesis” (3).

In 1931, Arvid Lindau hypothesized that hemangioblastomas may be derived from a “congenital anlage” and that the histologic picture revealed an “. . . embryological type of the tumor cells” (4).

Stein et al. (5) suggested an angiomesenchymal origin of hemangioblastoma, based on original, developmental biological observations made by Florence Sabin in 1917 (6). Hemangioblastomas are capable of blood island formation with potential extramedullary hematopoiesis analogous to embryonic hemangioblastic stem cells (5, 7, 8). Furthermore, hemangioblastomas express the erythropoietin receptor (Epo-R; ref. 8), which is also observed during early embryonic blood island formation at mouse embryonic days 8.0 to 9.5 (9); the same study showed that the hematopoietic cells in hemangioblastomas are *VHL* deficient (8). It has, thus, been suggested that hemangioblastomas may be primarily comprised of developmentally arrested hemangioblastic stem cells with differentiation potential into primitive vascular structures and RBC in analogy to embryonic angioblastic mesenchyme (8, 10).

The morphologic evolution of embryonic angiomesenchymal tissue into hemangioblasts was shown by Sabin in 1917 (6) and Murray in 1932 (11). However, the hemangioblast remained a “hypothetical” cell until Choi et al. (12) reported detection of a common precursor for hematopoietic and endothelial cells. Generation of hemangioblasts from embryonic stem cells (12) allowed detailed analysis of the sequence of gene and protein expression during embryonic blood and vessel formation. Thus, a series of proteins have recently been identified that are directly associated with prehemangioblastic and hemangioblastic differentiation (13, 14). Among these recently identified growth factors, transcription factors and transmembrane receptors are brachyury (also known as T), Scl, Csf-1R, Gata-1, Flk-1, and Tie-2 (15). We here report that these proteins are consistently expressed in stromal cells of hemangioblastomas and thus provide further evidence that the stromal cell is an embryonic progenitor with hemangioblastic differentiation potential.

Materials and Methods

Tissue. Formalin-fixed, paraffin-embedded tissue samples of 10 hemangioblastomas, resected recently at the NIH from patients with VHL disease, were included in the study. In addition, fresh tissue from 10 additional hemangioblastomas was embedded in ornithine carbamyl transferase (OCT) and snap-frozen for morphologic analysis and subsequent Western blot analysis. Additionally, analyzed were frozen tissue samples obtained from five sporadic hemangioblastomas. For control frozen samples of five gliomas of different WHO grading were included in the study. The study was done in compliance with Institutional Review Board–approved protocols.

Microscopic evaluation and immunohistochemistry. Serial sections were taken from paraffin-embedded or OCT-embedded tissue blocks for histologic and immunohistochemical examinations. For antigen retrieval of paraffin sections, slides were treated with DAKO Target Retrieval Solution (DAKO, Carpinteria, CA) and incubated at 95°C for 20 minutes. Sections were cooled at room temperature and washed thrice in PBS. Frozen sections were fixed in 85% ethanol. Slides were then quenched for 20 minutes in a solution of 3 mL H₂O₂ and 180 mL methanol. After three washes in PBS,

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sections were incubated in 10% horse serum for 1 hour. The primary antibody was diluted in 2% horse serum, and the sections were incubated in a humidified chamber at 4°C overnight. As primary antibodies, anti-human Scl (Active Motif, Inc., Carlsbad, CA), brachyury, Gata-1, Csf-1R, Flk-1, Ang-1, and Tie-2 (Santa Cruz Biotechnology, Santa Cruz, CA) were used. The sections were then incubated with secondary antibody and avidin-biotin-complex for 1 hour each. The reaction product was visualized with diaminobenzidine followed by 3 minutes of counterstaining with Mayer's hematoxylin. Sections were dehydrated by graded ethanol washes of 95% and 100% and washed with xylene before being mounted.

Western blot analysis. Protein extraction of hemangioblastoma and glioma tissue was done by three cycles of quick freezing and thawing of frozen tissue. The concentrations of protein were measured with the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Forty micrograms of extracted protein were subjected to 4% to 20% SDS-polyacrylamide gel (Invitrogen, Carlsbad, CA) electrophoresis, transferred to nitrocellulose (Invitrogen), primarily probed with Scl antiserum (Active Motif) at a 1:200 dilution, brachyury (Santa Cruz Biotechnology) at a 1:200 dilution, or β -actin monoclonal antibody (Sigma, St. Louis, MO) at a 1:500 dilution. Horseradish peroxidase-conjugated second antibody (Santa Cruz Biotechnology) was applied at a 1:20,000 dilution. Signal was detected by enhanced chemiluminescence substrate (Pierce, Rockford, IL). For negative control, normal human brain tissue (cortex and white matter) was used.

Results

Hemangioblastomas express the hemangioblastic proteins Scl and brachyury. Several proteins are expressed by embryonic progenitor cells with hemangioblastic differentiation potential (15).

The *Scl* gene (also known as Tal-1) encodes a basic helix-loop-helix (bHLH) protein, and antisense and overexpression studies have suggested that Scl modulates proliferation and self-renewal of multipotent hematopoietic cells (16) and also acts as a positive regulator of erythroid differentiation (17). The expression of Scl defines and specifies the hemangioblast (13, 14). We investigated 10 VHL disease associated and five sporadic hemangioblastomas for the expression of Scl by Western blot. All tumors revealed bands at the expected molecular weight of 34 kDa (Fig. 1A and F). No Scl protein was detected in human brain control tissue. To clarify which cellular subcomponent of the tumor expressed the Scl protein, we did immunohistochemical analysis of the 10 VHL-associated hemangioblastomas and consistently observed positive nuclear and cytoplasmic immunoreactivity in all 10 tumors (Fig. 1B and C). Positive immunoreactivity was exclusively observed in stromal cells; reactive vascular cells were consistently negative.

Brachyury protein is vital for the formation and differentiation of posterior mesoderm and for axial development in all vertebrates. Huber et al. (15) showed that the hemangioblast is a subpopulation of embryonic cells that coexpresses brachyury and Flk1. The expression of brachyury ceases after the mesoderm stage and has not been reported in any adult tissue. All 10 investigated VHL-associated and all five sporadic hemangioblastomas expressed the protein indicated by a band at 47.4 kDa in the Western blot (Fig. 1D and F). By immunohistochemistry, anti-brachyury was

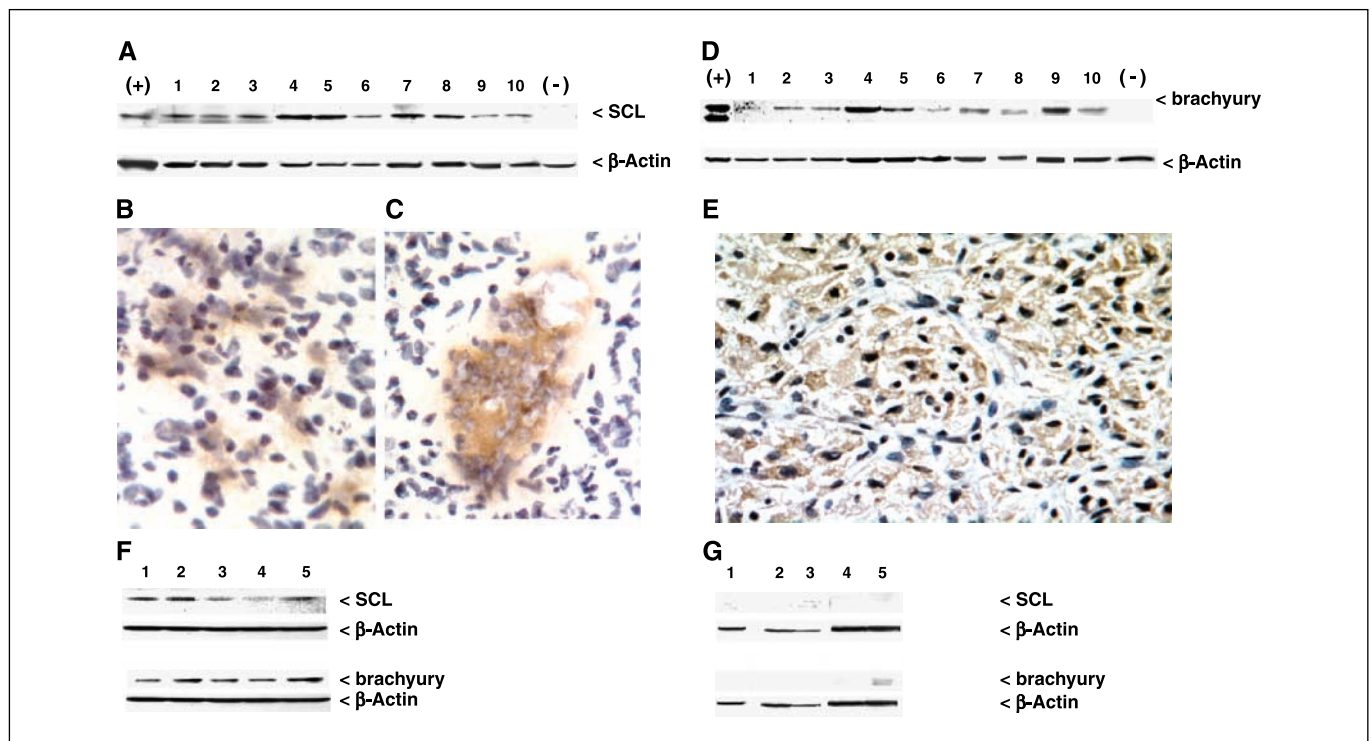


Figure 1. Demonstration of Scl and brachyury protein expression in stromal cells of different hemangioblastomas. A, Western blot of hemangioblastoma for Scl reveals a band in all 10 investigated tumors (lanes 1-10). No Scl protein is detected in human brain control tissue (-). B and C, immunohistochemical detection of Scl and brachyury protein in hemangioblastoma stromal cells. Positive immunoreactivity with the Scl antibody is detected in diffusely scattered stromal cells (B) and in stromal cell clusters (C). D, investigation for brachyury expression in hemangioblastomas reveals a positive band in all six investigated tumors (lanes 1-6). E, immunohistochemistry reveals that it is the stromal cell component of the tumor that expresses brachyury, whereas the reactive vessels are negative. F, investigation of five sporadic hemangioblastomas (lanes 1-5) for expression of Scl and brachyury reveals coexpression of both proteins in all investigated tumors. G, investigation of coexpression of Scl and brachyury in five gliomas of different grades of progression, including astrocytoma WHO II (lane 1), astrocytoma WHO III (lanes 2 and 3), and glioblastoma (lanes 4 and 5). Western blot reveals a weak positive band for brachyury in one glioblastoma (lane 5); expression of Scl is not detected in any of the samples.

exclusively and consistently positive in the stromal cells and negative in the reactive vessels (Fig. 1E).

For control purpose, glioma tissue was analyzed for expression of Scl and brachyury by Western blot. A positive band for brachyury was detected in one of five glioma (WHO IV). None of the gliomas showed expression Scl (Fig. 1G).

Expression of Csf-1R, Gata-1, Flk-1, Tie-2, and Ang-1. Other recently identified proteins associated with hemangioblast development include Csf-1R, a protein tyrosine-kinase transmembrane receptor for Csf-1, which is a macrophage-specific colony-stimulating factor; Gata-1, a tissue-specific transcription factor essential for erythroid and megakaryocyte development; Flk-1, also known as kdr (kinase insert domain receptor), a growth factor receptor tyrosine kinase whose ligand is vascular endothelial growth factor (VEGF), a factor that promotes vascular endothelial cell differentiation; and Tie-2 receptor tyrosine kinase, which plays an important role in angiogenesis, particularly for vascular network formation. We also investigated the ligand Ang-1, which is not known to be associated with hemangioblastic lineage but is a downstream target of hypoxia-inducible factor (HIF; ref. 18). Expression of all these proteins is not limited to the embryonic period and can be found in adult tissues. We, therefore, restricted our investigation to immunohistochemical analysis of these proteins.

We consistently detected positive immunoreactivity for all investigated proteins in each of the 10 VHL-associated hemangioblastomas. For Csf-1R, positive immunoreactivity was confined to the cytoplasm and membrane of the stromal cells (Fig. 2A). There was occasional mild positivity of the reactive vessels. The immunoreactivity of Gata-1 was more confined to the nucleus and the cytoplasm (Fig. 2B). We also observed occasional mild immunoreactivity with the vessels for this protein. Flk-1 revealed a strong cytoplasmic reactivity in the stromal cells and rare mild vascular staining (Fig. 2C). Tie-2 was immunoreactive with the cytoplasm of the stromal cells (Fig. 2D), and staining of the endothelium of reactive vessels was observed. Immunohistochemistry for Ang-1 revealed a positive cytoplasmic signal in the stromal cells (Fig. 2E).

Discussion

A series of immunohistochemical studies has been done to elucidate the unknown origin of the stromal cell resulting in identification of markers that are consistently, frequently, or only occasionally immunoreactive with the stromal cells. Epitopes that seem to be consistently immunoreactive include neuron-specific enolase (19–21), neural cell adhesion molecule (CD56; refs. 3, 22), and vimentin (3, 20, 22, 23). Positive immunoreactivity for S-100 protein is frequently but not always observed (2, 20–22, 24–28). Occasionally, positive results in stromal cells are reported for CD 57 (22), desmin (20, 21), renin (27), keratin (27), synaptophysin (19), substance P (19), neuropeptide Y (19), transthyretin, and transferrin (29). In addition, there is occasional expression of ricinus communis lectin receptor (20, 21) and ulex europeus lectin (20, 21, 23).

Immunohistochemical expression of other proteins, however, is controversial. Factor XIIIa has been reported to be expressed by hemangioblastoma stromal cells (20, 21), whereas other studies found it exclusively expressed by the reactive vascular component (23, 24). Similarly, factor VIII has been found in the stromal cells by some authors (20, 30, 31), whereas others report expression to be limited to vascular cells (24). The expression of glial fibrillary

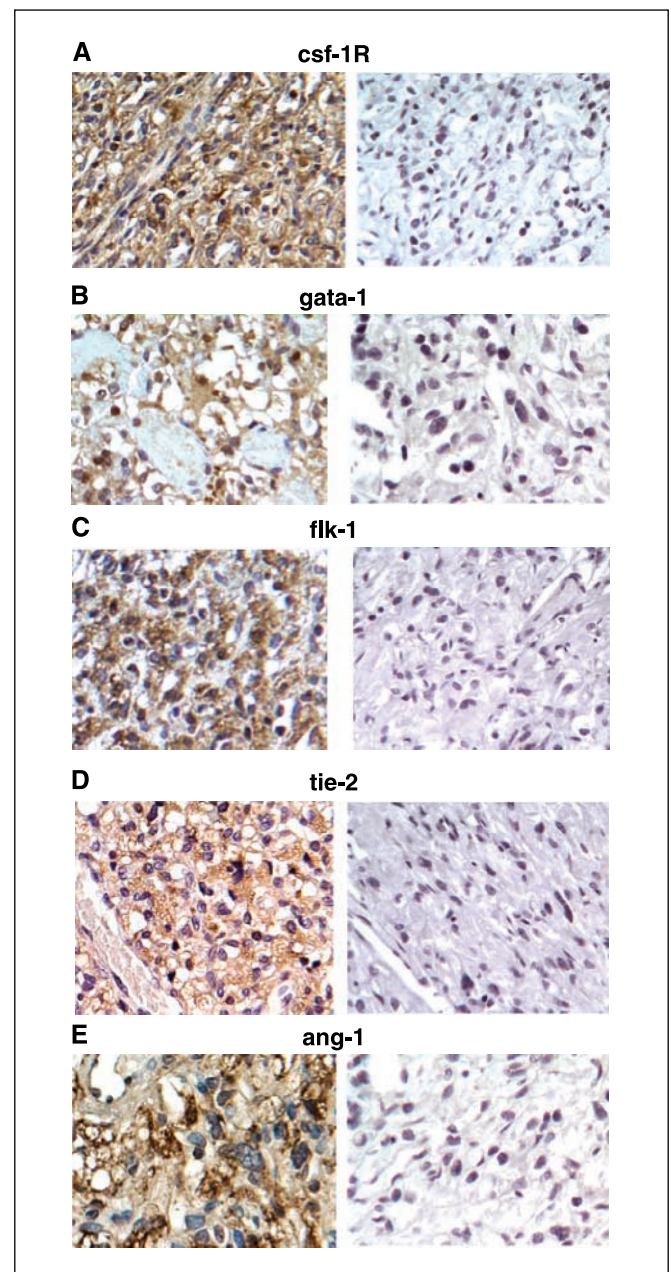


Figure 2. Expression of hemangioblast progenitor proteins Csf-1R, Gata-1, Flk-1, Tie-2, and the ligand for Tie-2, Ang-1, in stromal cells is shown by immunohistochemistry. *Left*, positive immunohistochemistry for or Csf-1R (A), Gata-1 (B), Flk-1 (C), Tie-2 (D) and Ang-1 (E); *right*, respective negative controls.

astrocytic protein (GFAP) in hemangioblastomas is also controversial. Some studies report a frequent positive immunoreactivity (23, 24, 30), whereas others observe only occasional positive cells in hemangioblastomas (20, 22, 25, 26). One study finds GFAP to be consistently negative in hemangioblastoma stromal cells (32). It is also unclear whether scattered GFAP-positive cells represent entrapped reactive astrocytes (23, 25, 28), stromal cells with glial differentiation (22, 23), or stromal cells with intracytoplasmic GFAP from phagocytic activity (30).

It is, therefore, not surprising that this abundance of contradictory observations has led to controversial interpretations about the

histogenesis of the stromal cell. Suggested origins include glial cells, endothelial cells, arachnoid cells, embryonic choroid plexus, neuroendocrine cells, fibrohistiocytic cells, cells of neuroectodermal derivation, or heterogeneous cell populations (reviewed in ref. 2). This has resulted in the current WHO classification of hemangioblastomas as a “neoplasm of uncertain histogenesis” (3). In summary, previous immunohistochemical studies have failed to clearly identify the origin of stromal cells. However, the complexity of the immunohistochemical features in conjunction with the unique morphology of the stromal cells may also indicate that there is no cytologic equivalent in mature brain or any tissue outside the CNS.

Other investigators recognized the remarkable morphologic heterogeneity of hemangioblastomas and tried to identify transitional forms between different cytologic constituents or different architectural features. Ultrastructural studies primarily focused on the cytogenetic relation between stromal and vascular cells. Some studies (30, 33–36) detect evidence of differentiation from stromal cells into vascular cells. Other studies, however, detect no transition from stromal cells into “vasoformative” elements and suggest that vascular and stromal cells are separate cytologic constituents (19, 24, 26, 28). A third group of studies found ultrastructural evidence of Weibel-Palade body formation in the cytoplasm of the stromal cells, which is interpreted as a vascular differentiation potential of the stromal cell (21, 37). Among the stromal cells, Chaudry et al. identified cells with transitional features among stromal cells, endothelial cells, and pericytes. They also found ultrastructural features characteristic for embryonic cells and concluded that stromal cells and vasoformative elements share a common ancestry, most likely angioblastic lineage (38). Therefore, although a synopsis of previous ultrastructural studies is similarly as inconclusive as previous immunohistochemical studies, ultrastructural studies recognized embryonic characteristics in the stromal cells and variable phenotypes of differentiation.

Hemangioblasts have been recently identified and generated from embryonic stem cells, and the capacity of hemangioblasts to differentiate into blood cells and vascular structures was recently documented by Choi et al. (39). Nevertheless, several detailed descriptions of the evolution of hemangioblast formation and differentiation had been provided before Choi's definitive identification. In 1917, Florence Sabin identified blood islands in the chick embryo that subsequently differentiated into vascular and blood cells (6). In 1932, Paul Murray gave a detailed account of vacuolated “wandering” cells, most likely the morphologic equivalent of the stromal cell, that were consistently associated with blood island formation (11). By cell culture experiments with mouse stem cells, Doetschmann et al. observed in 1985 the formation of primitive blood islands with the capacity to differentiate into hemoglobin-containing RBC (40).

A striking resemblance of neoplastic hemangioblastomas with morphologic evolution of the hemangioblastic lineage has been noted previously (5, 7), and the capacity of stromal cells to differentiate into RBC has been experimentally confirmed (8). We, therefore, propose that the recent unequivocal identification of the hemangioblast (39), in conjunction with the established differentiation potential of the stromal cell (8), indicates that an embryonic progenitor cell with hemangioblastic differentiation potential represents the cytologic equivalent of the stromal cell. Several proteins have been recently identified, the expression of which is characteristic of, or specific for, embryonic progenitor cells with hemangioblastic differentiation potential (15). Investigation of

these proteins in the stromal cells of hemangioblastoma is, therefore, a logical step to examine the hypothesis that the stromal cell of hemangioblastoma represents elements of hemangioblastic differentiation lineage.

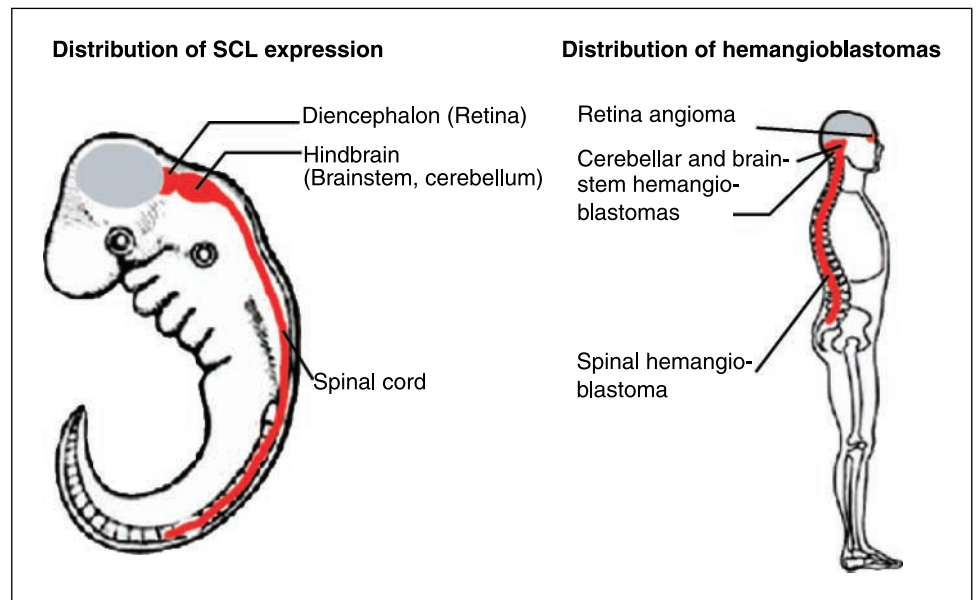
Recently identified proteins expressed during early hemangioblast differentiation include (a) Scl (also known as Tal-1), which modulates proliferation and self-renewal of multipotent hematopoietic cells (16) and also acts as a positive regulator of erythroid differentiation (17) and (b) brachyury protein, which is vital for the formation and differentiation of posterior mesoderm and for axial development in all vertebrates (15). Other proteins related to either vasculogenesis (Flk-1 and Tie-2) or hematopoiesis (Csf-1R and Gata-1) have been recently identified to be associated with hemangioblast development (15). Csf-1R is a protein tyrosine kinase transmembrane receptor for Csf-1, which is a macrophage-specific colony-stimulating factor. Gata-1 is a tissue-specific transcription factor essential for erythroid and megakaryocyte development. Flk-1, also known as kdr, is a growth factor receptor tyrosine kinase whose ligand is VEGF, a protein that promotes vascular endothelial cell differentiation. Tie-2 receptor tyrosine kinase plays an important role in angiogenesis, particularly for vascular network formation. We have shown these proteins to be consistently expressed in the stromal cells of hemangioblastomas, which strongly supports the hypothesis that embryonic progenitor cells with hemangioblastic differentiation potential can be considered the cytologic equivalent for the stromal cell.

A previous study observed coexpression of both Epo and its receptor (Epo-R) in areas of blood island differentiation within hemangioblastomas (8). Epo-R is up-regulated during early blood island differentiation (9), whereas Epo is a HIF target protein and is up-regulated as a result of HIF activation in the VHL-deficient stromal cells. It has, therefore, been suggested that coinciding expression of developmental proteins and HIF target proteins may create autocrine/paracrine loops that would promote tumor growth. After detecting consistent expression of Tie-2 in stromal cells, we further investigated and confirmed coexpression of Ang-1, which is also a HIF target protein (18). Our detection of a potential Tie-2/Ang-1 autocrine/paracrine loop in hemangioblastoma stromal cells is preceded by the demonstration of three other potential autocrine/paracrine loops: (a) transforming growth factor- α , which is another HIF target protein (41), and its receptor (42); (b) Flk-1 and HIF target VEGF (43); and (c) stromal cell-derived factor-1 and CXCR4 (44). Furthermore, the Tie-2/Ang-1 autocrine/paracrine loop has previously been identified in other human neoplasms (45).

In experimental models, HIF and several of its target proteins are up-regulated as a direct effect of VHL inactivation (46–48). However, several other HIF target proteins, including Tie-2, transforming growth factor- α , and Epo, have not been investigated in this context, and it is unknown whether all HIF downstream target proteins are immediately up-regulated or whether up-regulation of some HIF target proteins may be a more protracted process. Autocrine/paracrine loop formation in hemangioblastoma stromal cells may, therefore, be a complex process that depends on (a) the extent of downstream HIF activation and (b) the differentiation status of the individual tumor cells. Thus, autocrine/paracrine loop formation may have stimulatory effects on hemangioblastoma growth, which offers the potential of pharmacologic interaction and therapeutic effects.

Hemangioblastomas occur in a strikingly limited subset of CNS locations, including retina, brainstem, cerebellum, and spinal cord (Fig. 3). This limitation is highly significant and its cause remains

Figure 3. Topographic distribution of Scl expression in the 20 somite stage zebrafish embryo (modified after Gering et al. 13). Scl is expressed within the embryonic CNS at exactly the sites from which hemangioblastomas develop in VHL disease, including the midbrain (retina), hindbrain (cerebellum and brain stem), and spinal cord.



enigmatic to date. With interest, we have noted that the topographical distribution of *Scl* gene expression in the CNS of the zebra fish, quail, and mouse embryo is highly conserved and is confined to midbrain, hindbrain, and spinal cord (13, 14, 49), an anatomic distribution that closely recapitulates the distribution of hemangioblastomas in VHL disease (1). We here show that expression of Scl protein is not only confined to specific tissues (13, 14, 49, 50) and topographical sites within embryonic tissue but is also detectable in tumor tissue known to originate exclusively from those specific tissues and at those sites. Expression of Scl protein in VHL disease-associated hemangioblastomas does not, therefore, merely provide further evidence for the embryologic nature of these tumors but also provides an explanation for the peculiar, although highly consistent, topographical distribution of hemangioblastomas in VHL patients. Scl protein expression in

VHL-deficient hemangioblastomatous growth in retina, spinal cord, brainstem, and cerebellum is, therefore, confined to exactly those areas in which Scl is only transiently expressed during normal embryonic development, specifically the diencephalon, metencephalon, and spinal cord (refs. 13, 14, 49; Fig. 3). The striking topographical analogy between embryonic Scl expression and the sites of tumor development may, therefore, help explain the selective topography of CNS hemangioblastoma.

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