

Developmentally arrested structures preceding cerebellar tumors in von Hippel–Lindau disease

Sharon B Shively^{1,2,*}, Eric A Falke^{3,*}, Jie Li³, Maxine GB Tran⁴, Eli R Thompson¹, Patrick H Maxwell⁵, Erich Roessler⁶, Edward H Oldfield⁷, Russell R Lonser¹ and Alexander O Vortmeyer³

¹*Surgical Neurology Branch, National Institute of Neurological Disorders and Stroke, NIH, Bethesda, MD, USA;* ²*Department of Molecular Medicine, Institute for Biomedical Sciences, George Washington University, Washington, DC, USA;* ³*Department of Pathology, Yale School of Medicine, New Haven, CT, USA;* ⁴*CRUK Uro-Oncology Research Group, Cancer Research UK, Li Ka Shing Centre, Cambridge Research Institute, Cambridge, UK;* ⁵*Division of Medicine, University College London, London, UK;* ⁶*Medical Genetics Branch, National Human Genome Research Institute, NIH, Bethesda, MD, USA* and ⁷*Department of Neurological Surgery, University of Virginia Health System, Charlottesville, VA, USA*

There is increasing evidence that suggests that knockout of tumor-suppressor gene function causes developmental arrest and protraction of cellular differentiation. In the peripheral nervous system of patients with the tumor-suppressor gene disorder, von Hippel–Lindau disease, we have demonstrated developmentally arrested structural elements composed of hemangioblast progenitor cells. Some developmentally arrested structural elements progress to a frank tumor, hemangioblastoma. However, in von Hippel–Lindau disease, hemangioblastomas are frequently observed in the cerebellum, suggesting an origin in the central nervous system. We performed a structural and topographic analysis of cerebellar tissues obtained from von Hippel–Lindau disease patients to identify and characterize developmentally arrested structural elements in the central nervous system. We examined the entire cerebella of five tumor-free von Hippel–Lindau disease patients and of three non-von Hippel–Lindau disease controls. In all, 9 cerebellar developmentally arrested structural elements were detected and topographically mapped in 385 blocks of von Hippel–Lindau disease cerebella. No developmentally arrested structural elements were seen in 214 blocks from control cerebella. Developmentally arrested structural elements are composed of poorly differentiated cells that express hypoxia-inducible factor (HIF)2 α , but not HIF1 α or brachyury, and preferentially involve the molecular layer of the dorsum cerebelli. For the first time, we identify and characterize developmentally arrested structural elements in the central nervous system of von Hippel–Lindau patients. We provide evidence that developmentally arrested structural elements in the cerebellum are composed of developmentally arrested hemangioblast progenitor cells in the molecular layer of the dorsum cerebelli.

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Correspondence: Dr AO Vortmeyer, MD, PhD, Department of Pathology, Yale University School of Medicine, 416A Lauder Hall, 310 Cedar Street, New Haven, CT 06520, USA.
E-mail: alexander.vortmeyer@yale.edu

*These authors contributed equally to this work.
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Von Hippel–Lindau disease is a tumor-suppressor gene syndrome, characterized by the occurrence of a set of characteristic tumors.¹ The most consistently occurring tumors in patients with von Hippel–Lindau disease are nervous system hemangioblastomas and clear cell renal carcinomas.²

Hemangioblastomas are composed of von Hippel–Lindau tumor-suppressor (*vhl*)-deficient tumor cells with a hemangioblastic phenotype.^{3–6} Hemangioblastomas in von Hippel–Lindau disease are not uniformly distributed, but are strongly associated with a limited number of nervous system regions including the dorsal half of the spinal cord, the obex region of the brainstem, the cerebellum, and the retina.⁷ Among these vulnerable nervous system areas, the cerebellum and the spinal cord are most often involved.⁷ Hemangioblastomas can cause significant morbidity and mortality. Before routine magnetic resonance imaging screening and neurosurgical resection, the cerebellar hemangioblastoma was the leading cause of death in von Hippel–Lindau patients.^{8,9} Emerging evidence suggests that von Hippel–Lindau patients' increased risk of hemangioblastoma is due to the loss of the *vhl*'s protein's function during nervous system development.^{10–13}

Vhl encodes a multifunctional tumor-suppressor protein critical for cell differentiation during both development and adult life.^{11,12} During mouse fetal development, homozygous deletion of *vhl* results in mid-gestational lethality due to defects in placental vasculogenesis and cardiac malformations.¹¹ Tissue-specific inactivation of *vhl* in mice generally results in abnormal differentiation, often associated with decreased proliferation. Abnormal differentiation has been noted in several tissues, including neurons, mammary and kidney epithelial cells, and bone.^{10,11} In embryonic stem cells *in vitro*, loss of the *vhl* function causes differentiation block or delay. *Vhl*^{–/–} embryonic stem cells rarely or never give rise to fully differentiated adult tissues in a primary culture system.¹⁰

A link between developmental arrest and loss of *vhl* function is provided by its role as a negative regulator of the two α -subunits of hypoxia-inducible factor (HIF), namely HIF1 α and HIF2 α .¹⁴ Both of these HIF α proteins dimerize with the constitutively expressed HIF β subunit to form transcription activators.^{15,16} HIF-regulated genes include vascular endothelial growth factor.

A (*VEGFA*) and carbonic anhydrase IX (*CA9*).¹⁷ Cells with a loss of *vhl*-mediated HIF degradation express higher levels of HIF α proteins, VEGFA, and CA9.^{18–20} Although the transcriptional consequences and regulation of HIF1 α and HIF2 α do not completely overlap, both HIF α subunits have crucial roles in cell determination and their dysregulation leads to developmental arrest.^{14,21–24} In particular, increased HIF2 α expression, like the loss of *vhl* function, causes developmental arrest in embryonic stem cells and dysregulated hematopoiesis.^{14,23} Therefore, the loss of *vhl* function can lead to developmental arrest of cell determination by dysregulation of HIF α proteins.

Similarly, after structural and molecular analyses of tissues obtained from von Hippel–Lindau patients, we previously demonstrated that neoplastic growth in von Hippel–Lindau disease is associated

with developmental arrest^{25,26} and that nervous system tumorigenesis can be characterized as a process of protracted hemangioblastic differentiation caused by the loss of normal *vhl* function and increased expression of HIF2 α .²⁷ Disrupted differentiation during development is not unique to von Hippel–Lindau disease. Most other tumor-suppressor gene disorders not only produce frank tumors but also developmentally aberrant hamartomatous structures,^{26,28–33} which are defined as mature tissues that have been 'wrongly assembled in the course of development.'³⁴

We recently applied a detailed, primarily structural approach to examine the developmental effects of von Hippel–Lindau disease. It was our hypothesis that affected organ systems in von Hippel–Lindau disease would demonstrate structural evidence of hamartomatous maldevelopment after detailed analyses. In various tissues obtained from patients with von Hippel–Lindau disease, including nerve roots,^{27,35} epididymis,^{36,37} and endolymphatic sac,³⁸ we detected various microscopic-sized atypical non-tumorous structures. As these structures appeared structurally similar, but cytologically distinct from previously observed hamartomas, we have used descriptive terminology in earlier publications, such as 'microscopic atypical structures,' 'mesenchymal tumorlet,' or 'maldeveloped mesonephric material,' and others.^{27,35,37} Subsequent studies have revealed these structures to be fundamentally different from 'classic' hamartomas. First, 'microscopic atypical structures' are composed of immature cells,^{27,35,37} whereas hamartoma cells are defined as 'mature.'^{34,39–42} Second, a small subset of 'atypical structures' has the capacity to undergo morphological and molecular transitions into tumor,^{27,35,37} whereas hamartomas are not recognized as precursor structures for neoplasia.^{34,39–42} For simplification, here, we propose to use the term 'developmentally arrested structural element' for minute structural events in tissues that escape classic definitions of either 'hamartoma' or 'tumor' in tumor-suppressor gene syndromes.

In the nervous system of von Hippel–Lindau patients, developmentally arrested structural elements have the potential to progress to tumor, but up to now, have only been observed in the peripheral nervous system (PNS).^{27,35} However, the topographic distribution of central nervous system tumors in von Hippel–Lindau disease is strongly indicative of tumor initiation in the central nervous system proper. The purpose of this analysis was to identify and characterize cerebellar developmentally arrested structural elements, and to clarify their site of origin.

Materials and methods

Patients

All experiments with human tissues were conducted in accordance with IRB guidelines at the

Table 1 Age and gender of investigated patients

Patient	Age (years)	Gender
Control cerebellum no. 1	28	Female
Control cerebellum no. 2	48	Female
Control cerebellum no. 3	49	Male
<i>vhl</i> cerebellum no. 1	54	Male
<i>vhl</i> cerebellum no. 2	17	Female
<i>vhl</i> cerebellum no. 3	39	Male
<i>vhl</i> cerebellum no. 4	47	Male
<i>vhl</i> cerebellum no. 5	26	Female

National Institutes of Health. The entire cerebella were collected at the time of autopsy from five patients with von Hippel–Lindau disease and from three non-von Hippel–Lindau disease controls (Table 1). Control patients showed no evidence of stigmata of von Hippel–Lindau disease. All five von Hippel–Lindau disease patients had a documented germline mutation in the *vhl* gene. Upon careful gross examination, none of the cerebellar tissues revealed detectable tumors.

Tissues

Cerebellar tissues were initially sectioned parasagittally into 3-mm slices. Each slice was then sectioned into coronal segments for processing in standard 25 × 30 mm² histology cassettes yielding an average of 75 cassettes per case. A total of 599 cassettes were processed from the 8 cerebella. Each segment was fixed in formalin and then embedded in a paraffin block after tissue exposure to increasing concentrations of ethanol and xylene. A section was cut from each paraffin block, stained with hematoxylin–eosin (HE) and then screened for developmentally arrested structural elements under a light microscope at high power. When microscopic developmentally arrested structural elements were detected histologically, the paraffin block containing the developmentally arrested structural element material and the block coronally *en face* to it were serially sectioned at 6 μm. Numerous serial sections (at least each tenth section) were then stained with HE and evaluated microscopically. Immunohistochemical labeling was performed on unstained interval tissue sections. Immunohistochemistry for CD34, CD31, CA9, brachyury, HIF1α, and HIF2α was performed as reported previously;^{6,35} for comparison, immunohistochemistry was also applied to hemangioblastoma tumor tissues.

Results

The von Hippel–Lindau Cerebellum Contains Microscopic Developmentally Arrested Structural Elements

We have previously demonstrated that at least a subset of hemangioblastomas originates from

Table 2 Number of developmentally arrested structural elements detected in von Hippel–Lindau disease patients and control tissues

Patients	No. cerebellar paraffin blocks	No. cerebellar DASEs
Control cerebellum no. 1	73	0
Control cerebellum no. 2	66	0
Control cerebellum no. 3	75	0
<i>vhl</i> cerebellum no. 1	82	4
<i>vhl</i> cerebellum no. 2	87	3
<i>vhl</i> cerebellum no. 3	78	1
<i>vhl</i> cerebellum no. 4	73	1
<i>vhl</i> cerebellum no. 5	65	0

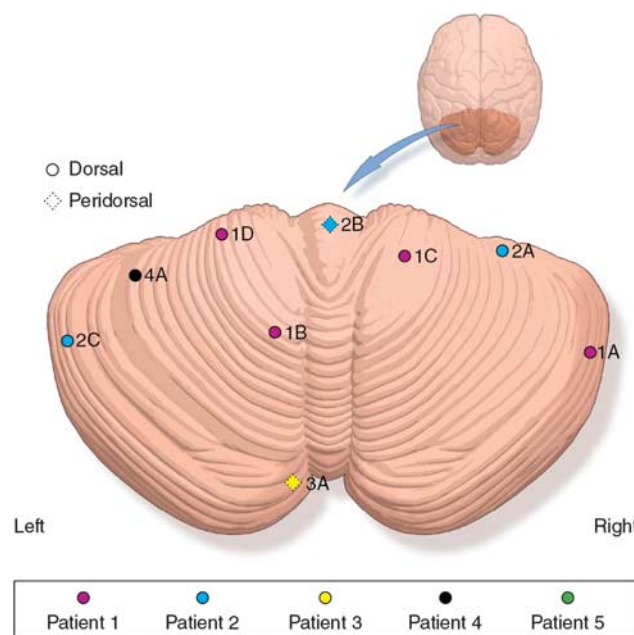


Figure 1 Topographical distribution of nine developmentally arrested structural elements identified in the cerebella of five von Hippel–Lindau patients. The developmentally arrested structural elements are located in the dorsal (circle) and peridorsal (dotted diamond) cerebellum.

microscopic developmentally arrested structural elements found abundantly in tumor-free PNS tissues from von Hippel–Lindau patients.²⁷ Here, we sought to determine whether developmentally arrested structural elements similarly exist in the cerebellum. No developmentally arrested structural elements were detected in 214 samples from 3 control cerebella. In all, 9 cerebellar developmentally arrested structural elements were detected in the 385 samples of 5 von Hippel–Lindau cerebella (Table 2) and topographically mapped (Figure 1). Developmentally arrested structural elements were present in four out of the five von Hippel–Lindau cerebella (Table 2). On HE staining, all nine

cerebellar developmentally arrested structural elements closely resembled previously described nerve root developmentally arrested structural elements.²⁷ Developmentally arrested structural elements measured between 150 μm and <1 mm in diameter. After serial sectioning of the blocks, developmentally arrested structural elements structures were exhausted after 10–90 sections (60–540- μm deep in the block).

Developmentally Arrested Structural Elements Preferentially Involve the Molecular Layer of the Dorsal Cerebellum

Each developmentally arrested structural element was mapped by serially sectioning both the block in which the developmentally arrested structural element was found and the *en face* block at 6 μm . Numerous serial sections (at least each tenth section) were then stained with HE and evaluated microscopically. Developmentally arrested structural elements were located primarily on the dorsal aspect of the cerebellum. Seven out of nine developmentally arrested structural elements were located in the dorsal cerebellum, whereas two developmentally arrested structural elements were identified close to the dorsal surface (Figure 1). All nine developmentally arrested structural elements involved the molecular layer of the cerebellar cortex. Three developmentally arrested structural elements were located exclusively in the molecular layer, whereas no developmentally arrested structural elements were found exclusively in the granular cell layer or the deep cerebellar white matter (Figure 2). Six developmentally arrested structural elements extended to the granular cell layer from the molecular layer.

Cerebellar Developmentally Arrested Structural Elements are Composed of Immature Cells with Activation of HIF2 α

Increased expression of HIF α subunits and CA9 are associated with *vhl* inactivation. Developmentally arrested structural elements are composed of poorly differentiated cells that express CA9, HIF2 α , but not HIF1 α (Figure 2). In contrast, hemangioblastoma tumor cells are known to be immunopositive for both HIF2 α and HIF1 α ,^{43,44} as well as CA9.³⁵ For peripheral nerve tissue, we recently showed in autopsy materials³⁵ and in surgically resected materials²⁷ that developmentally arrested structural elements have the potential to progress from CA9+/HIF2 α + /HIF1 α - to the well-known CA9+/HIF2 α + /HIF1 α + hemangioblastoma phenotype. Surrounding cerebellar tissues never showed expression of CA9, HIF2 α , or HIF1 α (Figure 2, result for CA9 not shown). Consistent with their immature phenotype, poorly differentiated cells did not express brachyury, a developmental marker of

hemangioblasts,^{6,45} whereas hemangioblastoma tumor cells are immunopositive for brachyury as reported previously.⁶ Immunohistochemical staining with the vascular antibodies CD34 and CD31 showed identical results indicative of abundant reactive vascularization in developmentally arrested structural elements (Figure 2).

Discussion

Searching for hamartomatous maldevelopment, in this study and in other previous studies, we performed detailed analyses of tumor-free organ tissues of von Hippel–Lindau patients.^{25,35–38} All investigated organs contained significant numbers of microscopic, developmentally arrested structures that were, however, different from classic hamartomas.^{25,36–38} First, we demonstrated that these microscopic structures were in part composed of immature cells;^{25,37} second, we demonstrated that a subset of these microscopic structures could progress to tumors.^{27,35,37} We suggest that these developmentally arrested structures should be understood as distinct from hamartomas and propose that these structures be distinguished from hamartomas by designating them as ‘developmentally arrested structural elements.’ The presence of developmentally arrested structural elements in von Hippel–Lindau disease is consistent with recent observations suggesting that the loss of *vhl* function leads to differentiation arrest during the development of multiple organ systems.^{10,35–37} It remains to be shown whether equivalents for developmentally arrested structural elements exist in other tumor-suppressor gene syndromes, as well as whether some of the ‘hamartomatous structures’ in other tumor-suppressor gene syndromes are also tumor precursor structures that arise because of developmental arrest.

Here, we provide the first detailed analysis of the effects of *vhl* germline mutation in brain tissues. Our results were obtained after sectioning 5 cerebella from von Hippel–Lindau patients into a total of 385 segments and submitting all of them for histopathological evaluation. Our search resulted in the identification of nine developmentally arrested structural elements. No developmentally arrested structural elements were found in 214 segments of cerebella from 3 control patients. Importantly, our numeric developmentally arrested structural element counts reflect the number of developmentally arrested structural elements at 3-mm intervals within the cerebellar tissue, and the actual number of cerebellar developmentally arrested structural elements may be significantly higher. However, given that the same screening procedure was previously used for nerve root tissue adjacent to spinal cord,³⁵ the number of cerebellar developmentally arrested structural elements appears small compared with that observed in the

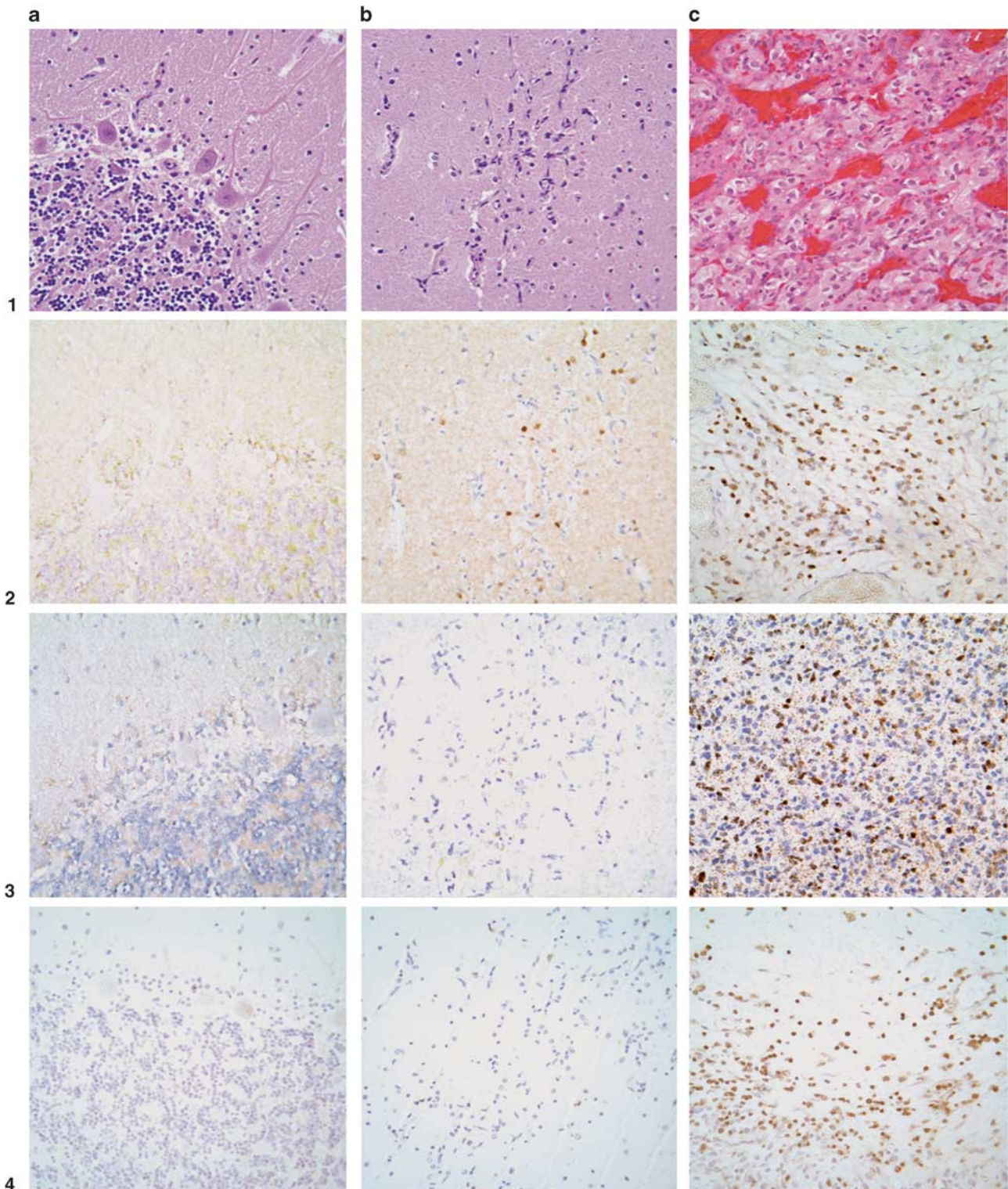


Figure 2 Cerebellar developmentally arrested structural elements are composed of immature progenitor cells with activation of HIF2 α and intense reactive angiogenesis. **(a)** Normal cerebellum, HE stain (1a). No activation of HIF2 α (2a), HIF1 α (3a), or brachyury (4a); immunohistochemistry for CD34 shows regular vascularization (5a). **(b)** Microscopic-sized developmentally arrested structural elements in molecular layer (HE stain, 1b) shows HIF2 α activation in immature progenitor cells (2b) and the absence of HIF1 α activation (3b); developmentally arrested structural element cells do not express brachyury (4b); immunohistochemistry for CD34 shows abundant vascularization (5b). **(c)** Frank tumor, hemangioblastoma (HE stain, 1c), reveals activation of both HIF2 α (2c) and HIF1 α (3c); expression of brachyury (4c); immunohistochemistry for CD34 shows abundant vascularization (5c).

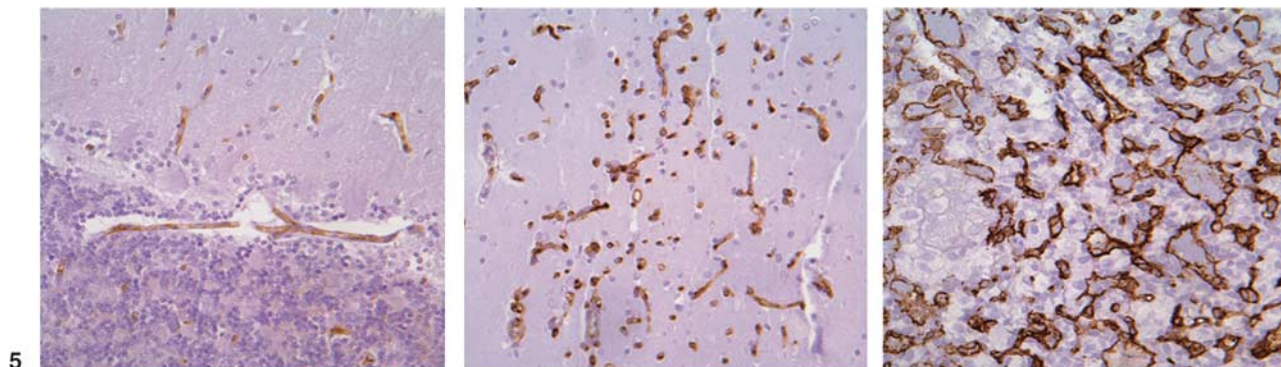


Figure 2 Continued.

nerve root tissue in von Hippel–Lindau disease. At first glance, this seems to contradict earlier studies that report cerebellar hemangioblastomas to occur most frequently in the cerebellum;^{9,46–48} however, after the use of more sensitive imaging techniques, the incidence of spinal hemangioblastomas has been reported as more frequent compared with cerebellar tumors in von Hippel–Lindau disease.^{49,50}

Consistent with a developmental origin, cerebellar developmentally arrested structural elements were preferentially located in the molecular layer of the dorsal cerebellum. A similar pattern of preferential distribution was previously noted in the vicinity of the spinal cord with dorsal nerve roots being far more frequently affected compared with anterior nerve roots.^{25,35} Lindau⁴ also noted a third prominent von Hippel–Lindau syndrome tumor distribution pattern when he identified the obex as the predilection site for hemangioblastomas of the brainstem.

Cerebellar developmentally arrested structural elements strikingly resemble those previously observed in the nerve root tissue.²⁷ The smallest cerebellar developmentally arrested structural elements consist only of few scattered immature cells and are confined to the molecular layer. Immature cells show exclusive activation of HIF2 α (Figure 2), in contrast to frank cerebellar tumors that show activation of both HIF2 α and HIF1 α (Figure 2). In developmentally arrested structural elements, activation of HIF2 α upregulates VEGFA,^{24,51} resulting in intense secondary angiogenesis.³⁷ Similarly, others have shown activation of HIF2 α , but not of HIF1 α , in embryonic stem cells before further differentiation commitment.¹⁰ Through the transcription factor OCT4, HIF2 α maintains cell pluripotency in multiple stem and progenitor cells during development, including in embryonic stem cells.^{14,23} Consistent with this, HIF2 α knockout embryos display severe developmental patterning defects.¹⁴

We conclude that developmentally arrested structural elements in the von Hippel–Lindau cerebellum are composed of developmentally arrested hemangioblast progenitor cells in the molecular layer, with

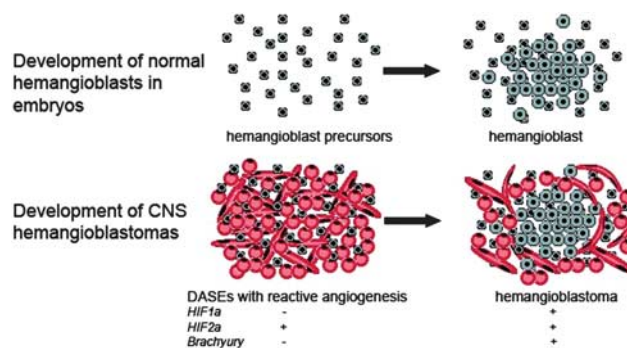


Figure 3 Proposed model for the progression of developmentally arrested structural elements (DASEs) to a frank tumor, hemangioblastoma. Developmentally arrested structural element cells undergo structural and molecular changes analogous to those seen in the differentiation of hemangioblast progenitors into hemangioblasts. Consistent with this, developmentally arrested structural element cells appear morphologically similar to mesenchymal hemangioblast progenitors. Progression to the frank tumor is associated with acquisition of hemangioblast morphology and activation/expression of HIF1 α and brachyury.

activation of HIF2 α , but not of HIF1 α . In contrast, frank tumors acquire a hemangioblastic phenotype with additional activation of HIF1 α and expression of brachyury, among other markers of hemangioblastic differentiation. Our findings suggest that in von Hippel–Lindau disease, the loss of *vhl*-mediated degradation of HIF2 α causes multifocal accumulation of developmentally arrested hemangioblast progenitor cells that serve as potential precursor material for hemangioblastic tumors (Figure 3).

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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