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PIK3CA Mutations in Vascular Malformations

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Abstract

Purpose of the review: Recently, it has been discovered that a subset of vascular malformations, of the lymphatic and venous type, are caused by oncogenic mutations in the *PIK3CA* gene. Now, efforts have been focused in the understanding of the molecular and cellular consequences of these mutations and the opportunities for novel targeted therapies for these diseases.

Recent findings: Here, we review the latest findings in the biology of oncogenic *PIK3CA* mutations in the pathogenesis of vascular malformations. We focus on the recent development of *in vitro* and *in vivo* tools for the study of *PIK3CA*-mutant vascular malformations with special interest in preclinical models for drug testing. Also, we review the latest advances in PI3K inhibitors in the clinic and their repurposing for the treatment of lymphatic malformations (LMs) and venous malformations (VMs).

Summary: Oncogenic mutations on *PIK3CA* causing LMs and VMs are also frequently found in epithelial cancer. Thus, fundamental research done in the cancer field during the past decades might be applied to the understanding of LMs and VMs. Likewise, repurposing PI3K pathway inhibitors that are currently in cancer clinical trials can be used as a novel strategy for the treatment of these diseases. Here, we also open a debate for the consideration of LMs and VMs as developmental tumours.

Keywords: PIK3CA, PI3K, venous malformations, lymphatic malformations

Vascular malformations are a heterogeneous group of diseases affecting a large population (1 in 100 individuals) (1) for whom limited treatment options exist. These lesions appear during embryonic development and are manifested at birth (congenital) or throughout the life of affected individuals (2). Vascular malformations are classified according to their histological appearance in different categories that include low-flow lesions: capillary, venous, and lymphatic; and fast-flow arteriovenous malformations. Specifically, venous malformations (VMs) are localized collections of abnormal and tortuous venous channels. VMs have a major impact on the quality of life of patients; they are painful and disfiguring, and many lead to bleeding, recurrent infections, thrombosis, organ dysfunction, and even death. Similarly, lymphatic malformations (LMs) are congenital lesions occurring as a result of a defect in the development of the lymphatic system. These lesions are composed by dilated lymphatic channels and can occur anywhere in the body (3). VMs and LMs do not regress but expand proportionally with the growth of the child. Current standard of care includes compression, surgical excision, and/or sclerotherapy (4). However, these treatment strategies are likely to be insufficient and patients commonly experience a high risk of recurrence and progression. In both types of malformations, the blood and lymphatic vessels are dedifferentiated, immature, and most likely occur as a consequence of an abnormal hyperproliferation of endothelial cells during development.

With the emergence of high-throughput and ultra-deep sequencing technologies, the genetic landscape of vascular malformations has undergone a great progress. A decade ago, it was determined that around half of sporadic VMs are caused by somatic gain-of-function mutations in the *TEK* gene which codes for the endothelial tyrosine-protein kinase receptor TIE-2 (5). In 2015 it was discovered that most LMs were caused by somatic oncogenic mutations in the *PIK3CA* gene (coding for the p110α isoform of PI3K lipid kinases) (6), and, a year later, it was demonstrated that around 25-30% of VMs are also caused by these oncogenic *PIK3CA* mutations (7) (8, 9)*. In most VMs, *PIK3CA* and *TEK* mutations are mutually exclusive, all triggering an overactivation of the PI3K signalling pathway. An unusual case has been recently reported in which both *PIK3CA* and *TEK* mutations have been found in endothelial cells derived from same VMs (10)**. Of note, in this specific case, neither *PIK3CA* nor *TEK* mutations are hotspots mutations of the disease.

Despite the discovery of the genetic cause of venous and lymphatic malformations, it is still unclear how these mutations change the cellular phenotype and how the molecular mechanisms driven by these mutations lead to the pathogenesis of these diseases. Here, we review the most recent advances in the biological effects of *PIK3CA* mutations in VM and LM pathogenesis towards the implementation of new targeted therapies for these diseases. Also, we propose LMs and VMs to be considered as developmental tumours considering their genetics and biology.

PIK3CA signalling in vascular biology

PIK3CA encodes for the p110α lipid kinase protein, one of the four p110 catalytic subunits of the class I phosphoinositide 3-kinases (PI3Ks) (11). In cells, p110 isoforms exist in a heterodimeric complex with a regulatory subunit. Based on the regulatory subunit binding preferences, they are grouped in class IA (p110α, β and δ) and class IB (p110γ). Class IA isoforms bind to a p85 regulatory subunit (there are five variants: p85α, p55α, p50α, p85β, and p55γ), while class IB isoform binds either to p84 or p101 regulatory subunits. The regulatory subunits prevent from p110s protein degradation, but also, the p85 subunits keep p110α, β and δ in an inactive cytosolic state. Activation of class IA PI3Ks can occur by 3 modes, all of which start with binding of a ligand to receptor tyrosine kinases (RTKs). First mode of activation occurs through a direct binding of p85 to RTKs allowing conformation change and activation of the p110 subunits. Another way of activation occurs through adaptor proteins such as the GRB2/GAB complex which bind p85 upon being recruited to RTKs. Finally, the small GTPases may also activate class I PI3Ks by interacting with the so called RAS binding domain (RBD) contained in the p110 catalytic subunits. Additionally, the p110β subunits can be activated by G protein-coupled receptors (12).

Class I PI3Ks activation generates the lipid phosphatidlylinositiol-3,4,5-triphosphate [$PI(3,4,5)P_3$ or PIP_3], which may be metabolised to $PI(3,4)P_2$ by 5-phosphatases such as SHIP. Both, $PI(3,4,5)P_3$ and $PI(3,4)P_2$ act as second messengers by interacting with lipid-binding pleckstrin homology (PH) domains in a variety of protein effectors and regulating their localization and/or activity. The main PI3K effector proteins include protein kinases (being the serine/threonine kinase AKT the most well-known), regulators of GTPases and scaffolding proteins (13). This plethora of downstream mediators allows PI3Ks to regulate cell growth, metabolism, survival and proliferation in physiology and disease (11, 13). The 3-phosphatase PTEN inhibits class I PI3K signalling by dephosphorylating $PI(3,4,5)P_3$ and $PI(3,4)P_2$.

All class I PI3K isoforms produce the same lipid but they regulate distinct physiological functions. This is partially explained by their relative expression levels in different cell types and by their mode of activation by upstream signals (11). In agreement with the presence of activating mutations of *PIK3CA* in VMs and LMs, and not yet identified other mutant PI3K isoforms, p110α is the sole isoform required for blood and lymphatic vascular development (14-16). The catalytic domain of p110α has been reported to be necessary for angiogenesis and lymphangiogenesis (14, 15). In contrast, blocking the ability of p110α to bind RAS impairs

lymphatic vascular development only (16). This indicates that p110α capacity to respond to upstream signals is different in blood and in lymphatic vessels' endothelial cells. Understanding how this occurs might shed light into the pathogenic mechanisms of *PIK3CA* mutations in VMs and LMs.

An interesting observation from mouse gene targeting studies (including ubiquitous and endothelial cell-specific models) is that too much and too little activation of p110α leads to embryonic lethality due to incomplete development of blood vessels (9, 14, 17, 18). This suggests that endothelial cells are extremely sensitive to PIP_3 fluctuations, and that its levels need to be tightly regulated for an adequate formation of the vascular plexus. This might also explain why activating mutations in *PIK3CA* have only been identified somatically and in a mosaic fashion as the presence of activating mutations in the germline is incompatible with life (Figure 1). From a biological perspective, the study of loss-of-function genetic models have unravelled that p110α signalling critically regulates collective cell migration during angiogenesis. Specifically, p110α signalling prevents NUAK1-dependent phosphorylation of the myosin phosphatase targeting-1 (MYPT1) protein. This allows myosin light chain phosphatase (MLCP) activity which reduces actomyosin contractility (19)*. It remains to be unravelled whether these biological processes are also relevant in the pathogenic endothelium upon expression of activating mutations of *PIK3CA*. In line with this, defective cell migration cause capillary-venous malformations when endothelial cells are unable to re-distribute within the vascular network (20). Taken together, it is tempting to speculate that aberrant endothelial cell migration also accounts for the pathogenic mechanism induced by activating mutations in *PIK3CA* in LMs and VMs.

Oncogenic *PIK3CA* mutations in LMs and VMs

PIK3CA mutations occur in most LMs patients (21)* whereas *PIK3CA*-mutations in VMs are less common, being present in 25-30% of the cases (7-9). These discoveries are in line with previous and consequent findings of oncogenic *PIK3CA* mutations in the so-called PROS (*PIK3CA*-Related Overgrowth Spectrum) which also present vascular malformations (22). This suggests that *PIK3CA* mutations appear at different stages during embryonic development affecting different cell types and in a mosaic fashion which leads to the broad spectrum of syndromes caused by these mutations (23). Since isolated LMs and VMs are usually localized lesions, it is tempting to speculate that, in these cases, mutations occur late during development, affecting a single clone of endothelial cells (Figure 1). However, the cell of origin of these malformations is still a mystery; whether they come from an endothelial cell progenitor or an already venous/lymphatic-committed endothelial cell remains unknown. In fact, since lymphatic vessels arise from primitive lymph sacs within veins (24), LMs and VMs might arise from the same cell lineage at different embryonic stages. This is plausible since very often LMs and VMs are mixed malformations with the presence of abnormal lymphatic and blood vessels within the lesion. Furthermore, LMs appear filled with both lymphatic fluids and blood. This speculation highlights that single cell-tracing *in vivo* studies might be of the utmost importance to reveal the cellular origin of LMs and VMs and better understand their pathogenesis.

Like in epithelial cancer, the most common mutations found in LMs and VMs are activating mutations in the helical (E542K, E542K) and kinase (H1047R, H1047L) domains of *PIK3CA* (25). In fact, these are the so-called oncogenic mutations in *PIK3CA*. These two types of mutations lead to a constitutive binding of p110α to the plasma membrane by two different and independent gain-of-function mechanisms both of which trigger an overactivation of the PI3K signalling pathway (26, 27). It is still unclear whether helical or kinase mutations prevail one over the other in these pathologies. Other less studied activating mutations in *PIK3CA* have been also found in LMs and VMs (10, 21, 28) (29)**. To date, there is no well-known correlation between any particular *PIK3CA* mutation and phenotype. However, a recent study including data from sixteen institutions has given valuable information about the genetic landscape of vascular anomalies with broad phenotypic

severity (29). A critical question is whether higher activation (helical and kinase mutations) leads to more aggressive phenotypes in LMs and VMs.

Biology of *PIK3CA* mutations in LMs and VMs

With the discovery of *PIK3CA* mutations in LMs and VMs, the next required step for fundamental researchers in the field is to understand their biological effect in the pathogenesis of these diseases. For this, there has been developed a wide spectrum of *in vitro* and *in vivo* models of LMs and VMs: from cell lines ectopically expressing the mutations to sophisticated genetic mouse models of the diseases (Table 1). By integrating these very recent studies we have gained an extensive molecular and cellular insight into the pathogenesis of LMs and VMs. Importantly, these studies have served as critical proof-of-concept for the use of targeted therapies in these diseases. However, these are still very early days for a comprehensive and deep understanding of the pathogenesis of LMs and VMs and many questions are still unanswered since their genetic cause was only recently discovered. Yet, great advances in the molecular and cellular processes underlying the pathogenesis of LMs and VMs have been made (Figure 2). Most of the fundamental and preclinical studies in LMs have been made using human LM-derived endothelial cells (6, 21, 28, 30-32). These works, apart from discovering *PIK3CA* oncogenic mutations in LMs, have demonstrated that *PIK3CA*-mutant lymphatic endothelial cells show enhanced proliferation and sprouting capacity. Also, *in vitro* preclinical studies reveal that PI3K pathway inhibitors effectively block PI3K pathway overactivation and proliferation. However, the inhibitors tested (mostly pan-PI3K and PI3K downstream pathway inhibitors) are not specifically targeting mutant cells since they also impaired cell growth in normal lymphatic endothelial cells. At the molecular level, these studies assess the expression of lymphangiogenic factors on *PIK3CA*-mutant cells. They show that VEGF-C, VEGFR3, and Neuropilin-2 are overexpressed in these cells, while Angiopoietin-2 (ANG-2) and CXCR4 are downregulated, leading to pro-angiogenic properties of these cells (6, 30). Even though these studies have much helped in the understanding of LM pathogenesis, yet, unbiased, high throughput molecular approaches will allow to decipher new key players in the generation and maintenance of LMs. Moreover, genetically-engineered animal models of this disease (such as mice or zebrafish) will strongly benefit the field by providing highly valuable preclinical tools to test potential molecular therapies for LMs. This will also generate crucial knowledge in its pathogenesis, such as deciphering the cell origin, mutant-cell dynamics or the involvement of other cell types. A very recent study has provided the first *PIK3CA*-driven mouse model of LMs (32). Although this mouse model resembles generalized lymphatic anomaly (GLA), where the LMs are diffuse or multifocal, it serves as a preclinical model of LMs. Indeed, the authors show clinical improvement in these mice when treated with the mTOR inhibitor rapamycin and this was further supported in a pilot clinical assay. Still, this model expresses the mutant form of *Pik3ca* under the *Rosa26* locus which might lead to non-physiological expression levels of Pik3ca. It is possible then that some of the biological effects occurring in these mice are caused by abnormal mutant *Pik3ca* overexpression; this might be overcome by using a mouse model expressing mutant *Pik3ca* under its endogenous promoter.

Biological research on *PIK3CA*-driven VMs is a step further compared to LMs, probably due to the development of xenograft and genetic mouse models of this disease (8-10, 33). Critically, when the transgenic mouse model finely reproduces the aetiology of VMs (*Pik3ca* mutation expressed under its endogenous promoter in a mosaic fashion within the embryonic mesoderm) these lesions fully recapitulate the systemic effects occurring in humans with isolated VMs such as intravascular coagulopathy (high D-dimer levels) or phlebectasias of the main internal veins (8). This highlights the need to use accurate preclinical models in order to assess therapy efficacy. VM mouse models have further demonstrated the exceptional sensitivity of endothelial cells for *Pik3ca* alterations since ubiquitous and non-endothelial specific expression of mutant *Pik3ca* leads to the development of VMs. The cellular phenotype triggered by oncogenic *PIK3CA* mutations in

VMs is similar to LMs, where endothelial cells show enhanced proliferation (8-10, 33). Also, *Pik3ca*-driven VMs in mice show scarce mural cell coverage, a feature typically observed in human VMs (8). At the molecular level, targeted approaches have shown that PDGFB and ANG-2 expression is reduced in *PIK3CA* mutant endothelial cells, which could contribute to the poor mural cell coverage in these lesions (8, 9). Arteriovenous specification markers are also reduced in these mutant cells, supporting the idea of a dedifferentiated or progenitor-like state of these cells. *In vivo* preclinical studies for VMs have demonstrated that rapamycin and the p110α-specific inhibitor BYL719 are effective in reducing VM lesions as well as limiting the systemic effects of these malformations (8, 9). These drugs reduce *PIK3CA*-mutant endothelial cell proliferation; however, for already-established VMs which are low proliferative the mechanism of action of these inhibitors in still unclear.

Are LMs and VMs developmental tumours?

LMs and VMs are a collection of abnormal and non-functional vascular channels in which the vascular vessels are dedifferentiated and immature, and they lack mural cell coverage. LMs and VMs have long been considered "vascular malformations" but not "vascular tumours". This is partially based on the observation that in adults these vascular malformations are relatively static (34, 35); hence these lesions are considered slow proliferative malformations. This is compatible with the fact that the vasculature primarily expands during embryonic development and remains quiescent in adulthood. Furthermore, it suggests that the presence of the mutation in only endothelial cells is not sufficient to result in high proliferative vascular lesions, and thereby growth factor signals are required to promote proliferation. In line with this, endothelial cell hyperproliferation during vasculogenesis has previously been shown to give rise to vessel hyperfusion and to result in dilated, dysfunctional vessels, similar to those found in VMs (36). Yet, it should be taken into consideration that *PIK3CA*-driven LMs and VMs do share most of the typical characteristics of the so-called childhood solid tumours or developmental solid tumours (37, 38). LMs and VMs are rare congenital entities: they arise as a consequence of genetic errors during development. The cell of origin of LMs and VMs is mesodermal, as mainly occurs in paediatric tumours. In paediatric tumours, often, a single, specific driver alteration might promote cancer development in certain cell lineages during a crucial developmental period. In LMs and VMs, a single *PIK3CA* activating mutation is sufficient to develop the lesion when affecting endothelial cells, a cell type that has been proven to be extremely sensitive to *PIK3CA* alterations. In addition, the same *PIK3CA* mutations occurring in LMs and VMs are frequently found in adult epithelial tumours such as breast or colon (13). However, in contrast to LMs and VMs, epithelial adult tumours bearing *PIK3CA* mutations need a multiple-hit process in which genetic alterations accumulate before the onset of the tumour. The current paradigm posits that LMs and VMs do not metastasize which fits with the fact that most of these malformations are presented as isolated lesions. However, occasionally there are patients in which the disease is manifested as multifocal. This has been described for VMs carrying mutations in the *TEK* gene (39), an upstream activator of p110α, and for GLA carrying mutations in *PIK3CA* (32). Yet, it is not known whether these multifocal lesions within the patient have arisen from the same early precursor or the lesions derive from others through dissemination or metastasis. Although the current studies provide valuable insights, much work on clonal evolution is still required to complete this picture. With these premises, the current classification of LMs and VMs calls for a debate.

Future perspectives and therapeutic opportunities

The discovery of oncogenic *PIK3CA* mutations as drivers of LMs and VMs has opened an enormous window of opportunities for the fundamental understanding of the pathogenesis of these malformations as well as of the role of this key gene in the disease. There is no doubt that this has also had an invaluable impact on the way these patients are managed; from diagnostic approaches to therapeutic opportunities. In order to optimize efforts in the path to discover new molecular players and cellular processes driven by *PIK3CA*

mutations in LMs and VMs, we advise to always keep in mind the "oncogenic" nature of these mutations. Indeed, most of the functional research done so far has been focused on assessing the proliferative and growth capacity of mutant cells. In line with this, findings made in the cellular and molecular mechanisms driven by oncogenic *PIK3CA* mutations in a cancer context might be applied to understand the pathogenesis of these vascular malformations. For instance, it has been shown that oncogenic *PIK3CA* leads to centrosome amplification and tolerance to spontaneous genome doubling, which might account for irreversible genetic changes in tumours with these mutations (18). Another example of the biological impact of these mutations in epithelial cancer is the switch on cellular metabolic requirements (40). Might these mechanisms be also applied to *PIK3CA*-driven LMs and VMs? Also, the experimental approaches to assess the molecular mechanisms triggered by *PIK3CA* mutations in LMs and VMs have been mostly biased, based in the canonical PI3K signalling pathway where activation of AKT is at the core of the pathway. Therefore, a holistic approach using a combination of high throughput technologies will be very valuable to decipher new molecular players involved in the pathogenesis of these diseases. This will impact in the development of novel therapies in combination with PI3K inhibitors.

At present, LMs and VMs are often treated with rapamycin or rapamycin analogues such as everolimus or sirolimus with success in the improvement of lesions and quality of life of patients (41). However, these malformations do not significantly regress or disappear upon rapamycin treatment (41). The use of p110α-specific inhibitors, which are currently on clinical trials for cancer (42, 43), might be a better option for LMs and VMs caused by *PIK3CA* mutations. Still these inhibitors are currently used at maximum-tolerated doses in cancer patients making these drugs poorly tolerated by the patients. Also, when used at high concentrations, they induce signalling feedback loops that can block their effect. A recent successful study treating PROS patients with the p110α-specific inhibitor BYL719 has given new hope for VMs and LMs patients (44)**. Thus, long-term treatment with low-dose of p110α inhibitor could normalize aberrant PI3K signalling avoiding systemic toxicity and be effective in reducing or eliminating *PIK3CA*-driven vascular malformations. The efficacy of these drugs at low doses is crucial taking into account that these are congenital diseases and most patients are paediatric. Similar to developmental tumours, LMs and VMs carrying a single mutation might be much more sensitive to targeted therapies than adult tumours which carry multiple genetic alterations (37). It is also important to keep in mind that new fundamental insight in the pathogenesis of LMs and VMs may lead to the use of combined therapies with other molecular inhibitors.

Conclusions

Since the discovery of *PIK3CA* mutations in LMs and VMs, not other genetic alteration has been found in these diseases. Yet, the genetic causes of all LMs and VMs cases have not been identified; thus, the discovery of new genetic events in these diseases will be crucial to apply targeted therapies. In line with this, the comprehensive genetic landscape of LMs and VMs will lead to an accurate stratification of these patients. Likewise, unbiased molecular approaches might shed light on unexpected roles of *PIK3CA* mutations in the pathogenesis of these vascular malformations which might lead to the development of novel therapeutic strategies. Lastly, we open a debate for the consideration of LMs and VMs as developmental tumours; this might have a positive impact in the clinical management of these diseases.

Key points

- Oncogenic *PIK3CA* mutations cause venous and lymphatic vascular malformations.
- *PIK3CA* mutations lead to a hyperproliferative phenotype of endothelial cells, probably as the main mechanisms for the pathogenesis of venous and lymphatic vascular malformations.

- *PIK3CA*-mutant vascular malformations present similar characteristics to developmental tumours.
- Repurposing PI3K inhibitors is an appropriate strategy for the treatment of *PIK3CA*-driven vascular malformations.

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This work develops for the first time a successful pilot clinical trial for the use of the p110α-specific inhibitor BYL719 for PROS patients.

Figure titles and legends

Table 1. Experimental *in vitro* and *in vivo* models of *PIK3CA*-driven LMs and VMs.

Figure 1. Effect of germline and somatic *PIK3CA* mutations during embryonic development and adulthood.

Figure 2. Molecular and cellular mechanisms driven by *PIK3CA* mutations in the pathogenesis of LMs and VMs.

Table 1. Experimental *in vitro* and *in vivo* models of *PIK3CA*-driven LMs and VMs.

Disease	Model	Molecular mechanism	Organismal phenotype and cellular mechanism	Ref.
LM	Lymphatic endothelial cells from human LMs.	Upregulation of VEGFR3 and Neuropilin-2.	Increased cell proliferation.	(6)
LM	Lymphatic endothelial cells from human LMs.	Upregulation of VEGF-C and COX2. Downregulation of ANG-2 and CXCR4.	Increased sprouting; reduced doubling time.	(30)
LM-GLA	Mouse model of adult LM-GLA: lymphatic endothelial cell expression of mutant *Pik3ca.*	na	Hyperplastic lymphatic network. Functional impairment of lymphatic vessels. Increased cell proliferation.	(32)
VM	HUVECs with ectopic expression of mutant *PIK3CA.*	Downregulation of ANG-2 and PDGFB.	Abnormal morphology of endothelial cells. Loss of ECM fibronectin.	(7)
VM	Mouse model of congenital VMs: mosaic expression of mutant *Pik3ca* in the embryonic mesoderm under its endogenous promoter. Mouse model of endothelial-specific expression of mutant *Pik3ca* (postnatal retinas).	Downregulation of Pdfgb and arteriovenous specification markers.	Congenital VMs (cutaneous and internal). Systemic effects: phlebectasia of internal veins, bleedings. Endothelial cell hyperproliferation; loss of mural cell coverage.	(8)
VM	Mouse model of adult VMs: ubiquitous expression of mutant *Pik3ca.* HUVECs with ectopic expression of mutant *PIK3CA.*	Downregulation of ANG-2.	Adult VMs (cutaneous and internal). Systemic effects: increased D-dimer levels. Endothelial cell hyperproliferation. Aberrant tube formation.	(9)
VM	Patient-derived VM-endothelial cell mouse xenograft model.	na	Scarce mural cell coverage.	(10)
VM	Mouse model of VMs: endothelial-specific expression of mutant *Pik3ca.* HUVECs with ectopic expression of mutant *PIK3CA.*	na	Adult internal VMs. Recruitment of inflammatory cells to the VM. Increased cell proliferation and senescence. Enhanced angiogenic sprouting.	(33)

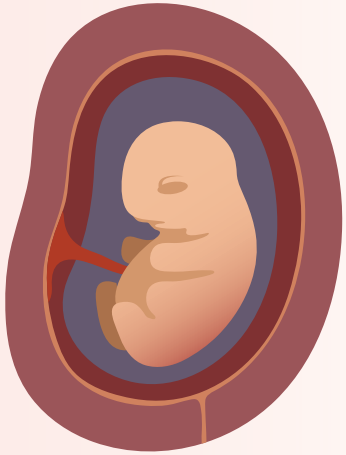
LMs: lymphatic malformations; VMs: venous malformations; GLA: generalized lymphatic anomaly; HUVECs: human umbilical vein endothelial cells. Na: not applicable.

Figure 1

PIK3CA MUTATIONS

A

GERMLINE



embryonic lethality
(vascular defects)

B

SOMATIC MOSAIC

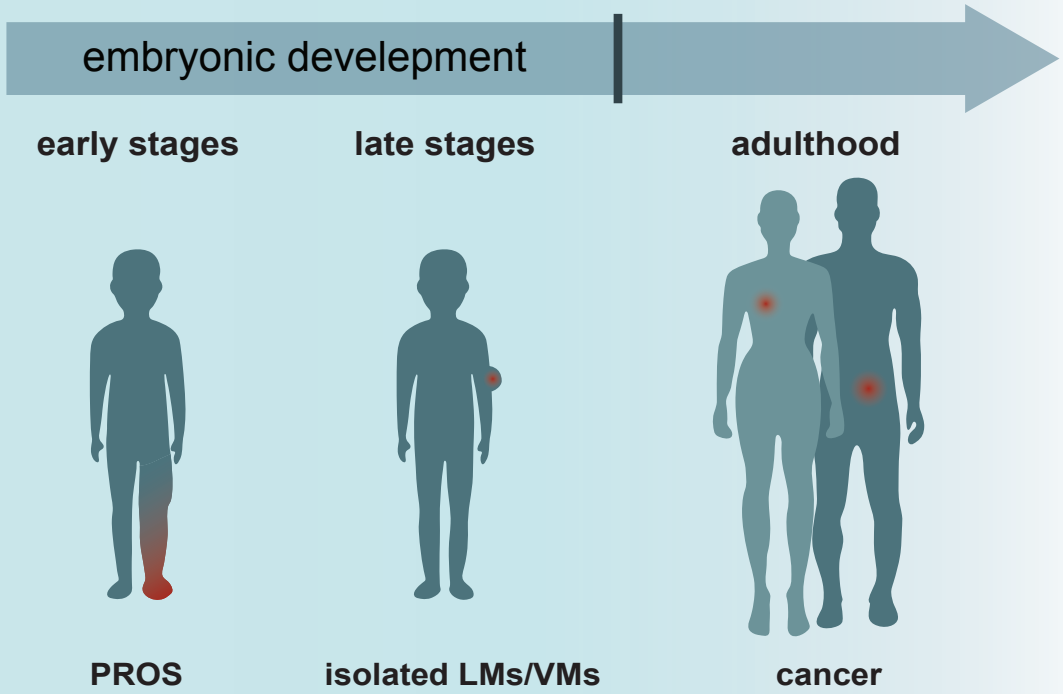
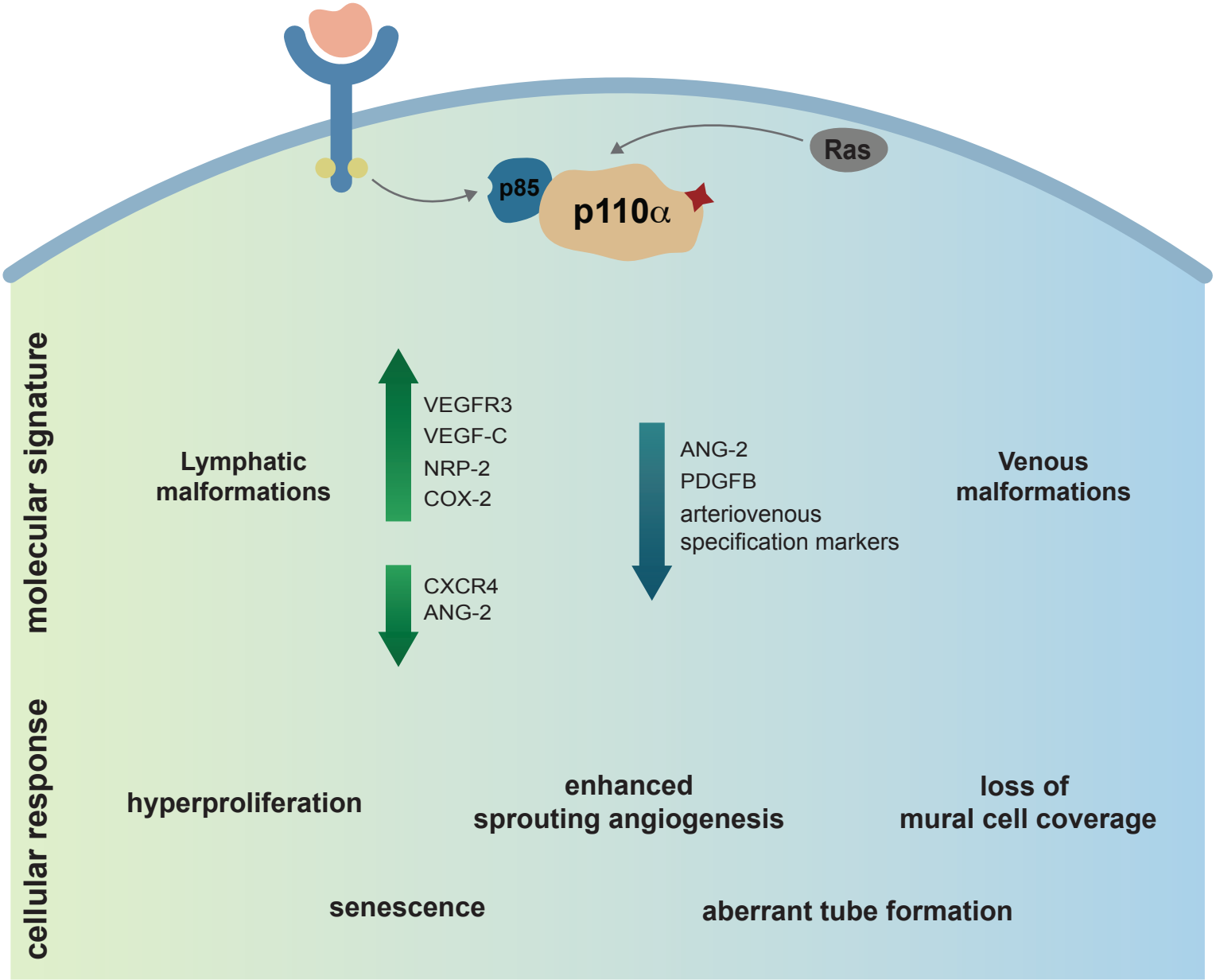


Figure 2


Ras
p85
p110α
molecular signature
Lymphatic malformations
VEGFR3
VEGF-C
NRP-2
COX-2
CXCR4
ANG-2
ANG-2
PDGFB
arteriovenous specification markers
Venous malformations
cellular response
hyperproliferation
enhanced sprouting angiogenesis
loss of mural cell coverage
senescence
aberrant tube formation