Philosophy of the Vascular Tree

17

A number of aspects stem from the study of vascular development that are suited to a historical and conceptual analysis and are at present poorly investigated. Such an analysis could show how this scientific domain has developed and what meaning research conducted in this field has for science in general and for the development of scientific ideas or methodologies. We have already discussed some of these issues, and they are revisited in this chapter. However, I give herein a more conceptual perspective and my personal view on the subject. I focus on selected issues, including the analysis of paradigm changes, conceptual categories, cross-fertilization of fields, technological advances and impact on angiogenesis, evolutionary considerations, molecular proximity of different systems, and methodological considerations.

17.1 Changing Paradigms

According to Thomas Kuhn, a scientific paradigm is "*a universal recognized scientific achievement that, for a time, provides model for solutions of problems for a community of practitioners*" [338]. Kuhn makes the distinction between normal science that progresses by accumulation of data and knowledge, and scientific revolutions [338, 339]. Scientific revolutions occur when abnormalities in a research field are encountered that demand a full reconsideration of the conceptual framework in which science is conducted at a given time. There is a general tone of scientific relativism in the Kuhnian philosophy, but one can easily accept the Kuhnian scheme for scientific "progress" without falling into a relativistic posture. In Kuhn's view, a paradigm is the result of a radical transformation of a scientific field, which results in a new paradigm that conflicts with previous paradigms. In my opinion, one can adopt a less restrictive notion of *paradigm* and broaden its meaning. In this view, a paradigm is a sort of framework in which the working scientist carries out normal problem-solving science to fill up what is predicated by the paradigm. I would formulate the concept of micro- and macro-paradigms in this respect. Indeed,

A. Bikfalvi, *A Brief History of Blood and Lymphatic Vessels*,
https://doi.org/10.1007/978-3-319-74376-9_17

"revolutions" occur much more frequently on a smaller scale (micro-revolutions) and, in my opinion, they can be considered as micro-paradigms. These micro-revolutions do not affect the whole theoretical edifice of a scientific discipline but shed new light on and solidify the whole conceptual structure of a scientific theory.

Regarding vascularization, our knowledge has undergone a number of micro- and macro-revolutions leading to new micro- and macro-paradigms. A non-exhaustive list of these micro- and macro-revolutions is given in Table 17.1. It should be noted that these conceptual leaps have occurred over a period of more than 2,000 years!

The discovery of the circulatory system is seen as a macro-revolution that completely changed how we viewed the organization of living systems. The cell theory applied to the vasculature is another one. Yet another is the fact that vascularization is dependent on soluble factors produced by normal and pathological tissues, which led to the identification of these factors. Regarding micro-revolutions, I list some in the following: existence of attractive and repulsive factors, the postulate of the specificity of angiogenic factors, the discovery of Vascular endothelial growth factor (VEGF), the discovery of lymphangiogenesis factors, and the concept of guide cells ("tip").

I now briefly discuss in more detail the changing ideas about the significance of tumor angiogenesis. We already described the sequence of discoveries from a historical perspective in this book. Thus, historically speaking, the concept of the vascular Tumor microenvironment (TME) underwent three steps of conceptual modification: nourishing (Thiersch) → host defense (Goldmann) → tumor promotion (Folkman) → promotion and defense (present view) ("dialectic" progression) (Fig. 17.1).

It is important to note that Goldmann coined the notions of bodily reaction and host defense that are provided by the vasculature; this seems to be one of the first reports of inter-dependency between the vasculature and the immune system. As already mentioned, the tumor vasculature—immune interdependency, in the perspective of an anti-tumor response, was validated only recently by the identification of specialized vessels called high endothelial venules (HEV) in tumors, albeit prior reports indicate that immune cells report immune infiltration through the vasculature. These HEVs are present in some solid tumors, such as mammary carcinomas, and trigger an antitumor immune response by allowing the influx of Th1 cells, cytotoxic effector T cells, and naïve and central memory T cells into the tumor. It would be important to elucidate mechanistically how the number of these vessels can be stimulated to increase the therapeutic efficacy of immunotherapy, which has only be recently attempted by the Berger laboratory. This concept is a total conceptual inversion of the Folkman paradigm, because in this case tumor development is limited by increasing blood vessel growth (even if these are a specialized subtype of vessel) and thus therapeutic strategies must be developed to promote the growth of these vessels.

Another second conceptual inversion from the Folkman paradigm is the normalization concept as already mentioned in a previous chapter. As already discussed, in this concept, anti-angiogenic treatment is destined to kill aberrant vasculature in tumors and to normalize the morphology and functionality of the remaining tumor

Table 17.1 Non-exhaustive list of micro- and macro-paradigms related to vascular biology

	Reference and year	Micro- or macro-paradigms
1	Ibn Nafis (1242), Colombus (1559), Servetus (1553), Harvey (1628)	Existence of the pulmonary circulation. Nutrition and respiration are not independent in vertebrates but localized in the same circulation
2	**Harvey (1628)**	**The blood circulation in vertebrates is a closed circulation**
3	Harvey (1628)	It is not the diastole that attracts the blood, but the contraction of the heart (systole) that actively propagates the blood through the organism
4	**Schwann (1845, 1847), His (1865)**	**Vessels, in vertebrates, are composed of different layers and exhibits cells that are in direct contact with the blood (endothelial cells)**
5	**Hunter (1794), Goldmann (1907, 1908)**	**Vascularization is an active process in tissues**
6	Goldmann (1907, 1908)	The vasculature in tumors is part of a host defense mechanism
7	**Greenblatt and Shubik (1968), Ehrmann and Knoth (1968)**	**Soluble morphogenic factors are required for vascularization in tissues (tumor-derived factors)**
8	**Folkman (1971)**	**Vascularization is the prime ingredient of the integrated tumor ecosystem and essential for tumor growth**
9	**Gimbrone and Folkman (1972)**	**Blockade of angiogenesis halts tumor growth**
10	Ferrara (1989)	Angiogenic factors have vascular specificity
11	Keshet (1993)	Vascular morphogens act as gradients
12	Folkman (1971), Adams (2001), Eichmann (2004)	There are four types of factors: stimulatory, inhibitory, attractive, and repulsive
13	**Folkman and Hanahan (1996)**	**Tumors undergo an angiogenic switch to activate angiogenesis**
14	**Alitalo (1996)**	**The lymphatic circulation is independent of blood vascularization and has its own molecular mechanisms (lymphangiogenesis emerged as sub-field)**
15	Betsholtz (1999)	Mechanisms of pericyte recruitment to blood vessels
16	Gerhard and Betsholtz (2003)	Specific cells atop the nascent vessel guide the growth of the vascular tube (tip cells)
17	**Jain (2001, 2003)**	**Anti-vascular therapy normalizes the vasculature to allow better perfusion which improves the efficacy of chemotherapy**
18	Weinstein (2006), Affolter (2008), Lammert (2009)	Vessel lumen formation is an active process which requires at least one endothelial cell
19	Carmeliet (2004)	Angiogenic factors have extra-vascular properties

(continued)

Table 17.1 (continued)

	Reference and year	Micro- or macro-paradigms
20	Carmeliet (2013)	Vessel sprouting requires a specific metabolism
21	**Girard (2011)**	**Vessels may exhibit anti-tumor properties via the immune system**
22	Lammert and Melton (2001), Mastumoto and Zaret (2001), Keshet (2011), Rafii (2011)	Vessels have perfusion-independent roles by providing instructive signals to tissues

Macro-paradigms are indicated in bold

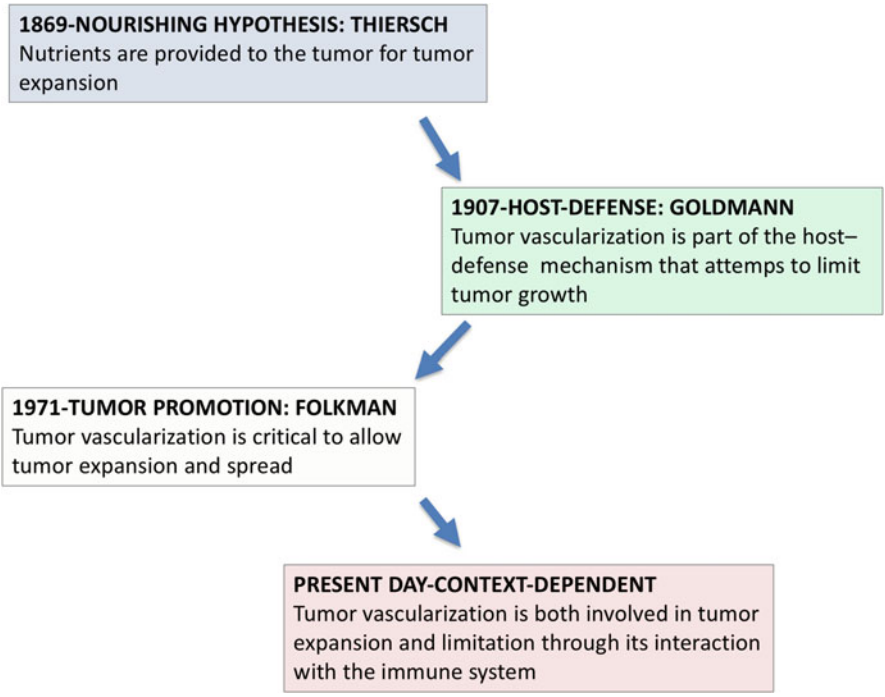

Fig. 17.1 Evolution of the concept of the vascular TME

vasculature. This has the consequence that blood flow in tumors is transitorily increased and this transitorily increases tumor growth but allows anti-tumor therapy to reach the tumor better and kill tumor cells.

A third conceptual modification is the fact that vessels not only seem to be channels for conducting nutrients and oxygen to normal and pathological tissues but also have a morphogenic function of their own by providing angiocrine signaling (see the Sect. 10.9 for more details).

Taken together, this places the vasculature at the center of multiple functions (Fig. 17.2).

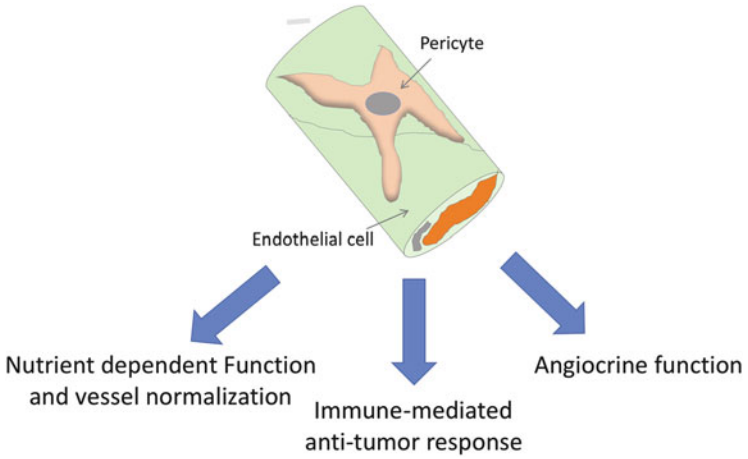

Fig. 17.2 The vessel is at the center of multiple functions. The function of the vasculature from the nutrient-dependent has been expanded to the normalization concept, immune-mediated antitumor response, and angiocrine function

17.2 Angiogenic Factors in Question

With regard to the concept of angiogenesis factors, there are two points to discuss – their discovery and their claimed specificity. Until the twentieth century it was elusive to think about diffusible factors that could control vascular morphogenesis. Vascular morphogenesis was instead viewed more to be the unfolding of an internal program within the vasculature itself. Several critical steps had to be taken in the discovery of morphogenic factors. The first was to demonstrate the existence of soluble and diffusible mediators of vascular development. The second was the characterization of the nature of these factors. The third was to gain mechanistical insights of how vessel development is regulated and the fourth was to attribute, if possible, a hierarchical and qualitative position to the various factors discovered. We have dealt with these in previous chapters of the book.

The discovery of soluble TAFs and growth factors raised the question about the quality or properties of vascular stimulating factors with regard to their multiple functions. These include growth promotion (vascular growth factors), migration (migratory factors), inhibition (negative regulators), guidance (guidance cues), and induction of permeability (permeability factors). These attributes have been determined "a priori," or can come from experimental evidence, or were imported from other scientific domains. "A priori" attributes are such as "if there are positive regulators there must be negative ones." Such a hypothesis may then lead to the search and discovery of these kinds of factors. Another way is experimental evidence where, for instance, a conditioned medium stimulates endothelial cell proliferation or induces vascular permeability, which leads to the identification of such

stimulating factors. Yet another possibility is the import of a concept stemming from another field. The attribute "guidance cue" is such an example. Guidance cue is derived initially from developmental neurobiology and was later introduced into vascular biology (see Sect. 10.1 "Tip Cells").

It should be mentioned that discoveries made initially within different conceptual frameworks may lead to converging findings, as is the case for VEGF and VPF (Vascular permeability factor), which are identical molecules. The discovery of VEGF came from the idea to discover a factor that stimulated specifically the growth of endothelial cells. The discovery of VPF was derived from the idea to discover a factor that regulated vascular permeability.

It was believed at one time that angiogenic factors such as VEGF were specific for the vasculature and their only role was therefore to stimulate angiogenesis. The thinking was that a pleiotropic factor (i.e., that could interact with other cells outside the vasculature) was unlikely to play a predominant role in the morphogenic process. The concept that specificity equals importance was supported by in vitro studies and genetic experiments in mice where, for instance, VEGF knockout causes a very severe abnormal vascular phenotype. Thus, the wrong belief in the veracity of a concept can be reinforced by valid experiments and models. It is my view that such a kind of reductionist stand has been in the core of vascular biologists at the time and is still for many. Indeed, further studies discovered various other functions outside of the vasculature for vascular regulators. For example, VEGF has roles in the nervous system and the reproductive system. Thus, the quest for a "specific" factor, even if the assumption proved to be wrong, paradoxically significantly promoted research and allowed the discovery of VEGF and other angiogenic factors. This is an example of a conceptual error that can be, paradoxically, beneficial for research in a particular field of science. In my opinion, if one had made the initial correct assumption that no vascular specific factors exist, then already existing alternatives at hand would have satisfied scientist and hindered or slowed down the discovery of major vascular regulators. Fibroblast growth factors (FGFs), which are very potent angioregulatory molecules, were already discovered at that time. However, as the late Werner Risau pointed out at the time of the discovery of VEGF, FGFs were broad-range regulators and had not the "correct" spatial and temporal expression and hypoxia regulation, which, of course, VEGF possessed. To me, this was also the reason that pushed scientists to look for vascular specific factors.

17.3 Conceptual Categories Shaping Vascular Development Research

When focusing on vascular development, we can already define, in my view, several conceptual categories (Fig. 17.3) that include soluble angiogenic factors (module 1), vessel stability and maturation (module 2), sprouting and guidance (module 3), tumor/pathological angiogenesis (module 4), and effects of the vasculature on organ development (module 5).

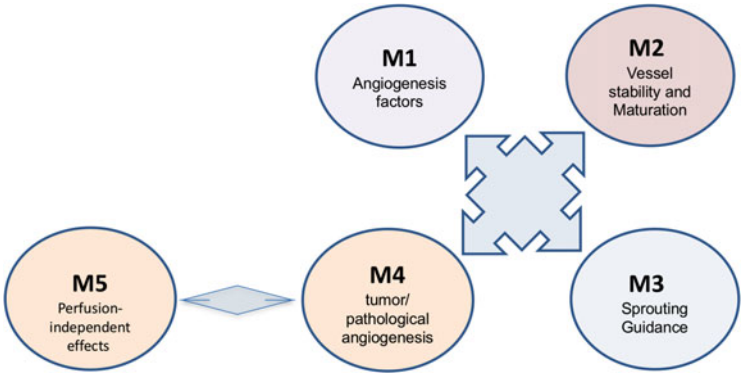

Fig. 17.3 Five conceptual categories (modules) in vascular development. There is interaction between four modules. Module 5 seems only indirectly conceptually connected

Module 1 is related to the discovery of soluble angiogenic factors. In this case, a first paradigm is represented by the contention that vascularization is an active process. The following sequence can be envisioned: vascularization is an active process → soluble factors are required for vascularization → vascular morphogens are specific → vascular morphogens act as gradients.

In module 2, the sequence is the following: vessels are composed of different cell layers → pericytes are present on capillary vessels → pericytes contribute to barrier function → pericytes stabilize capillary vessels and contribute to the response to angiogenesis factors or inhibitors.

For module 3 the sequence is: vessels are formed by sprouting → sprouting is dependent on morphogenic gradients → sprouting is dependent on tip cells → the orientation/direction of the vascular sprout is regulated by guidance factors.

For module 4 the sequence is: tumors are embedded into an integrated ecosystem → tumors are vascularized → vascularization is an active process in tumors with branches to go either toward a protumor (a) or antitumor (b) effect of the vasculature:

(a) Blockade of angiogenesis halts tumor growth → vascularization is dependent on diffusible factors → blockade of angiogenic factors halts tumor growth
(b) Vessels interact with immune cells → some tumor vessels have anti-tumor properties by promoting anti-tumor immunity → promoting these vessels inhibits tumor growth.

For Module 5, which is related to organ effects of the vasculature, perfusion dependent effects precede perfusion independent effects (perfusion dependent effects of the vasculature → perfusion independent effects of the vasculature).

It is important to remember that the progression of each of the paradigms is dependent on two factors of variable weight: the preexisting knowledge and the technological development at a given time.

Conceptual categories may also interact with each other (Fig. 17.2). For example, module 2 (pericyte coverage) and module 4 (tumor/pathological angiogenesis) interact because vascularization in tumors is immature and destabilized with a defect in pericyte coverage. There is interaction between modules 1, 3 and 4 because sprouting is aberrant in tumors and morphogenic gradients are abnormal. Module 5 is a stand-alone module that may only be indirectly connected to module 4 because emerging evidence connects this module to tumor angiogenesis. Thus, conceptual categories that depict different layers of knowledge have strong connections with each other and may contribute to the formulation of new hypotheses and paradigms.

17.4 Interactions Between Different Scientific Fields ("Cross-Fertilization of Fields")

17.4.1 Vasculature, the Central Ingredient of the Integrated Ecosystem in Tumors

It is surprising that cancer research opened the gate for a molecular explanation of vascularization. Oncology at the time of the pioneering work of Judah Folkman was under the influence of an exclusively tumor cell-driven explanation of cancer. Cancer research was mainly focused on changes taking place inside the tumor cell, such as alterations of the cell cycle or the study of oncogenes. The microenvironment was considered to be a non-active participant playing no role in the development of tumors. However, following the pioneering work of Judah Folkman and other researchers, it could not be ignored that the vascularization played a role in the regulation of tumor development. Folkman further coined the term "integrated ecosystem" where, in his view, the vasculature had the central role. This concept was instrumental in discovering many factors, receptors, and regulatory circuits that not only apply to the tumor context but also exhibit a general role in the vasculature. The concept of the ecosystem came from ecology and was introduced by Clapham in 1930 but it was Tansley who defined the concept fully and used it in 1935 in a publication [340]. Tansley devised the concept to draw attention to the importance of transfer of materials between organisms and their environment. Applied to cancer, this would mean that organs and tissues are seen as ecosystems in homoeostasis that tumor cells disrupt. However, this is not the meaning implied by Folkman when he speaks about tumors as integrated ecosystems. In his definition, tumors create their own ecosystem by re-educating the host and establishing privileged interactions with components of the micro-environment, among which, in Folkman's mind, the vasculature was playing the major role. This means that the tumor ecosystem disturbs the body's own homeostatic ecosystem by redirecting promoting signals to the tumor and by blocking inhibitory input from the body's ecosystem.

Why did Folkman call this an integrated ecosystem? Because he did not give any more details about this, one can only speculate about the reason. I give here my interpretation. The TME is composed of several entities that are metabolically active

and have, in a certain sense, an autonomous component. In an ecosystem there is nutrient demand, waste production, and interaction of its constituents. Integrated may mean that all parts of it work together toward a common goal which, in the case of the TME, is to promote tumor expansion and spread. In Folkman's opinion, the vasculature is the essential ingredient of the integrated ecosystem because the vasculature is a crucial element in nutrition and waste exchange and this allows the passage of the tumor from a dormant stage to an actively growing and invading stage.

17.4.2 Angiogenic Factors Have Extravascular Properties and Vice-Versa

The history of VEGF's discovery is a striking example in this context. First, considered as a tumor angiogenesis factor, VEGF conquered developmental biology, cardiology, ophthalmology, neurology, and neuroscience, to cite only a few examples.

A number of molecules and receptors that were largely studied in other fields of the medical and biological sciences have been found to play crucial roles in the vasculature. As such, ephrins or netrins that play an important role in the development of the nervous system were discovered to exhibit important functions in the vasculature. These factors are not only molecules for axonal guidance but also instrumental for vessel guidance. Indeed, both vascular and nervous systems have an afferent and efferent system (arteries/veins, motor pathways/sensory pathways) and use similar molecular guidance systems. In the vascular system, there is the cell "tip" in the nervous system and the growth cone (see Sect. 10.1 "Tip Cells"). Furthermore, both systems seem interconnected at two levels. Vessels attract peripheral nerves and nerves attract vessels. In the central nervous system, a strong interconnection is found at the level of the blood-brain barrier. Hinman and Davidson proposed the term "Kernel" to designate a molecular regulatory circuit that is similar in different species but that comes in various flavors [341]. It may be not only the result of mutations or gene duplication – it may be composed of gene products, which have nothing to do with each other on a structural level. Moreover, this Kernel can function by transposition of a regulator assembly in another cell and tissue context. What is important in the Kernel is that its interrelations of the various molecular components are preserved.

In this context, we could eventually introduce the concept of micro- and macro-Kernel. A micro-Kernel could be the implementation of a specific function (such as the transposition FGF-Notch Delta in the tracheal system to the VEGF-Notch Delta in the endothelium) and the macro-Kernel could be a set of nodes which converge in a common functional purpose.

17.5 Technological Advances and Impact on Vascular Biology

One can identify different technological leaps that have accompanied the development of research in the vascular biology and angiogenesis field (Table 17.2). There are many techno-logical leaps such as, in the early days, injection of specific dyes to visualize the vasculature by John Hunter for the collateral circulation or the visualization of the capillary structure by Theodore Schwann. Another technology used in the early twentieth century was X-ray imaging that helps to visualize tumor vascularization in humans after bismuth oil administration, as done by Goldman in 1907.

One of the most important technological advances, in my opinion, is heparin-sepharose chromatography as many morphogens have a strong affinity for heparin. The interesting feature of this chromatographic method is that it allows the elution of a variety of factors using different ionic strengths. FGFs have a very high heparin-binding capacity whereas VEGF has a much lower affinity for heparin. Ferrara and collaborators used this method to purify VEGF, which started by the observation that a mitogenic activity was eluted from the heparin-sepharose column at a much lower ionic strength than FGF. This led ultimately to the purification and identification of VEGF. It should be noted that Dvorak and collaborators also used heparin-sepharose chromatography to purify VPF, which ultimately turned out to be identical to VEGF. Had this technology not been invented, it would have significantly delayed the discovery of vascular stimulating factors and slowed down the research of the entire field.

Table 17.2 Technological leaps in angiogenesis research

Technological leap	Reference	Consequences
Injection of dyes, etc.	Hunter (1794)	Visualization of in vivo angiogenesis
Microscopy	Schwann (1845, 1847), His (1865)	Visualization of vessel structure
Improvement of biochemical techniques (heparin-sepharose chromatography)	Singh and Klagsbrun (1984)	Purification of angiogenesis factors
Culture of vascular cells in vitro	Jaffe (1973), Gimbrone (1973), Buzney and Robison (1975), Campbell, Chameley-Campbell (1971, 1979)	Study of endothelial cell or vascular smooth muscle/pericyte phenotypes in vitro, elucidation of signaling mechanisms
Vascular specific animal models and gene deletions of vascular genes	Carmeliet (1996), Ferrara (1996)	Validation of an in vivo role of angiogenesis regulators, receptors, etc.
Molecular biology	Ferrara (1989), Ferrara and Williams (1992), Betsholtz (2016)	Cloning of angiogenic factors and receptors, micro-analysis of tissues at the gene level, single cell sequencing, etc.
Advanced imaging techniques	Jain (1987, 1992), Gerhardt (2010)	Visualization of fine morphogenic events (dynamically)

Additional important advances have been made by successfully isolating endothelial cells and other vascular cells from various sources and by successfully culturing them in vitro. This has allowed the investigation of cell phenotypes and behavior as well as cell signaling. Other no less important discoveries include vascular specific animal models that allowed specific deletions of vascular genes as well as molecular biology techniques that led to the cloning of vascular morphogens and receptors, as well as micro-analysis of tissues at the gene level. It is to emphasize that single cell sequence technologies are now applied to gain insights into the heterogeneity of the vasculature. Furthermore, recent microscopic techniques using two/multiphoton live imaging were instrumental in analyzing precise vascular morphogenic processes dynamically, such as tip and stalk cell organization in the nascent vascular tube.

17.6 Evolutionary Considerations and Principles

We have already dealt with this in a previous chapter of the book. However, I believe that it is useful to discuss some of the aspects here again.

The vasculature comes in different flavors in the animal kingdom. In invertebrates, open and closed vascular systems are encountered whereas in vertebrates only closed circulatory systems exist. In invertebrates such as *Drosophila*, the oxygenation system is constituted by the tracheal system, which is separated from the vascular system ("dual" mode). A comparison of the vascular system of *Drosophila* and vertebrates provides valuable insights into how the vasculature is organized. In *Drosophila*, there are coelomic cavities segmented by the dorsal parts of the mesoderm. The vascular system consists of a central contractile vessel ("heart") that receives hemolymph in the anterior part to be ejected at the posterior part. The wall of the vessel consists of mesothelial cells (also called myoepithelial cells because of their contractile ability) and matrix ("basal"). It should be noted that the matrix is located in the vessel lumen and is therefore exposed to the blood.

In vertebrates, the morpho-functional situation is quite different. First, the system is closed and allows the recirculation of blood through the venous and arterial systems. Second, an endothelium covers the internal surface of the vessel and is therefore in contact with the blood. In a pathological situation, the absence of the endothelium (because of damage caused by atherosclerosis, for example) is the initiator of adhesion and activation of platelets. In *Drosophila* this does not apply. In insects there are, most likely, circulating anticoagulant factors that prevent coagulation and maintain blood fluidity. Third, in vertebrates there is convergence of the functions of nutrition and respiration (oxygen). In *Drosophila*, the oxygenation system is constituted by the tracheal system, which is separated from the vascular system ("dual" mode). It should be noted that the hypoxia-inducible system in *Drosophila*, which involves HIFs, is located in the tracheal system and regulates FGFs. In vertebrates, the HIF system is located in blood vessels and surrounding tissue and is dependent on VEGFs. Altogether, these morpho-functional differences between invertebrates/insects and vertebrates show that, during

evolution, a "shift" has occurred with regard to this function from the tracheal to the vascular system. As described previously, the term Kernel was proposed to designate control modules found in various biological systems, which exhibit different components, albeit having the same relationships with each other. Further insights can be gained from the study of *Botryllus schlosseri*, a marine invertebrate. As we have already seen in Chap. 3 "Evolution of Vascular System", in *Botryllus*, the endothelium is absent, but *Botryllus* has the ability to form external vascular tubular structures composed of epithelial cells. Surprisingly, these vascular structures express a homologue of VEGF receptor.

This raises the question of what the origin of the endothelium at an evolutionary level might be. Theoretically, both cell types, hemocytes and epithelial cells, could claim this role. Hemocytes are equipped with a homologue of VEGF receptor and are stimulated by VEGF counterparts. This suggests that hemocytes have acquired the ability to become/function as endothelial cells during evolution. This process must be accompanied by acquiring genetic elements of hypoxic regulation. We can infer from these observations that the function of the VEGF system was initially only aimed at controlling the movement of blood cells to the tissues. This function was hijacked during evolution to acquire a new morphogenic and structural role. It was no longer sufficient to convey the blood cells to tissues by an exclusive action on blood cells, but there was also the need to form the channels to achieve this effectively. When put into context, epithelial cells of *Botryllus schlosseri*, for instance, have gradually lost their dependence on VEGF, the role taken over by endothelial cells probably derived from hemocytes, which already have a dependency on VEGF for inducing cell migration. The alternative explanation is that the endothelium is derived from mesothelial or myoepithelial cells by trans-differentiation. This is not completely excluded because, as mentioned before, they may express receptors for vascular growth factors. However, this explanation seems unlikely because such a functional shift would imply a myoepithelial–endothelial transition. Furthermore, myoepithelial cells have a contractile capacity, which makes them cousins of pericytes and smooth muscle cells.

Do invertebrates have angiogenesis, strictly speaking? In vertebrates, angiogenesis is related primarily to the endothelial cells. They are the ones that initiate the cascade of events leading to the formation of the vascular tube. In invertebrates, these cells are absent. However, as we have seen, VEGF receptors are present in some invertebrates and this implies that VEGF is implicated in the formation of tubular structures. As already discussed, *Botryllus* have an internal and external circulatory system where epithelial morphogenesis is controlled by VEGF. However, the regulation of VEGF expression appears to be independent of hypoxia. Munoz and colleagues proposed the term "non-endothelial" angiogenesis for invertebrates [342]. However, more appropriate seems the formation of vessels in invertebrates as "vascular tubulogenesis" to differentiate specifically this phenomenon from "angiogenesis" which is associated with the endothelial organizing principle.

In summary, two important leaps occurred for the vascular tree during evolution: (1) a passage from the organizing principle "epithelium/myoepithelium" to

"endothelium" and (2) a re-contextualization of regulatory mechanisms with the integration of the hypoxia control system into the endothelium and the surrounding tissue.

Related to this discussion is the concept of vascular mimicry, which may represent an ancient form of tube formation. It has been proposed that tumors, in some cases, produce vascular channels devoid of endothelial cells and only lined by tumor cells (see a more detailed discussion in the previous chapter).

17.7 Model Organisms and Angiogenesis

Angiogenesis uses a number of model organisms in research. These include the rodent model (mouse, hamster), the chicken embryo model, and the zebrafish model. In addition, research is carried out using the *Xenopus* (frog) embryo model. The model of corneal angiogenesis in rabbits was used to test the effect of activating and inhibitory molecules of angiogenesis.

Obviously, one may wonder how the results obtained in this way can be generalized. A simple test is to evaluate and compare the results obtained in different models and see whether they are consistent. In other words, this means that the results obtained on one model are the same in another model (or, better, in several other models). If, for example, we look at the VEGF family and its receptors, it has been clearly shown that they have very similar roles in the mouse, zebrafish, chicken embryo, and *Xenopus* models. In all these models the VEGF system is crucial to vascular development because extinction of the gene or its receptor(s) leads to a disturbance of angiogenesis with a defect in vessel sprouting. However, this rule must be applied with caution because in some cases it does not apply. During phylogeny, the molecular repertoire becomes more complex, and some molecules that play a role in mice and humans are not present in phylogenetically inferior organisms. An extreme example of this situation is represented by the variant-1 of Platelet Factor-4 (PF4v1), which is present only in humans, monkeys, and chimpanzees. It is therefore difficult to apprehend its true function because it would mean that its gene must be silenced in man or at least in the other two species. In this case, only the artificial expression of this gene in different organisms is possible and can indirectly inform us about its potential function.

Another situation is represented when two organisms have similar molecular systems but when the target tissue is different. Can we, in this case, deduce at least one common function? We have previously seen that the system regulated by hypoxia (together with the Notch and Delta systems) is involved in the formation of the tracheal tree in *Drosophila* and in the formation of the vascular system in vertebrates. However, in these two cases, these systems are involved in tracheal and vascular sprouting and therefore have similar morphogenic effects, even if some differences exist.

Another aspect is represented by the experimental approaches used in the different model organisms. Can they be transposed easily from one organization to another? For some approaches this is certainly the case. Developmental observation

is common to any model organism. In addition, hypoxia conditions can be induced in mice and zebrafish, for example. Tumor angiogenesis has been studied in mice, chick embryo, and zebrafish. Other experimental approaches are rather specific. For example, if we want to study the role of the immune system in angiogenesis, it is preferable to use an immunocompetent and not an immunodeficient mouse model.

17.8 Scientific Methodology in Vascular Biology

The reductionist approach in the life sciences has produced spectacular results for the knowledge of living systems and has led to the development of treatments that have entered routine clinical practice.

Philosophers have extensively thought of how knowledge is acquired during the scientific enterprise and different theoretical frameworks have been formulated, which include inductive and deductive elements in varying proportions. These are inductive inference, deductive inference from hypothesis, the Bayesian approach, error-statistical approach, and by inference to the best explanation (for details on this topic see the excellent article by Marcel Weber (http://plato.stanford.edu/entries/biology-experiment/ with an extensive bibliography). These principles are, of course, general to experimental biology, but the reader may find it useful to discuss them in the present context.

In vascular biology, the principal method is inductive inference. Inductive inference obeys the following criteria: (1) simultaneous moment of occurrence of two events (if at any time an event takes place and at another time it does not and has all the conditions in common except one that exists only in the first event, the condition that is different between these two events is the cause or a necessary part of the cause of the observed phenomenon); (2) the two compared events must be uniform with agent/causal mechanism and the induced response need only be present in one but not the other situation; (3) other causes that can induce the response should not be present at the time of the experiment. Inductive inference is a key approach in vascular biology and angiogenesis research. It extrapolates from temporal occurrences a causal connection between the molecular alterations and the observed phenotype.

The following examples of inductive inference in vascular biology can be cited: discovery of VEGF and other vascular morphogens, stimulatory or inhibitory effects of factors that modify cellular phenotype or signaling, identification of vascular inhibitory factors, and loss-of function or gain-of function experiments for vascular morphogens, receptors or signaling molecules, etc.

In biology, there is not, strictly speaking, a similar theoretical framework as in physics, but there is a set of observations/assertions from which hypotheses can be deduced. As such, deductive inference is present in vascular biology, although this is not the "hypothetico-deductivism" which operates in other branches of science. An example of deductive inference is the angiocrine role of the vasculature. This is derived from the hypothesis that, besides the role in tissue oxygenation and nutrition, the vasculature and specifically the endothelium has a supporting role in normal and

malignant tissue by producing growth factors, cytokines, or other cell-bound or soluble mediators. Another example is guidance (and guidance molecules), a concept derived from neurobiology with the formulation of the hypothesis that guidance also represents an important mechanism for directional growth of the nascent vascular tube.

It should be noted that inductive and deductive inference are closely linked and may flip back and forth. One can, for instance, start with a hypothesis and test the hypothesis by deductive inference, which may be followed by inductive inference, which can lead to new hypothesis that are again experimentally tested, and so on.

The Bayesian approach is a statistical approach that assigns a probability to an event related to another condition. Back in fashion, it found some interest in biology and in particular the analysis of genomic data, which is obviously important in angiogenesis research.

Another approach is the error-statistical approach. In this case, there is no probability assigned to hypotheses but the likelihood with which a hypothesis is tested is evaluated. In this case, the chance is evaluated that a negative hypothesis ("null hypothesis") passes the test. One can only suspect that this approach was inspired by Karl Popper and his falsification criterion [343]. In this case, a theory seems more solid if it has a significant risk of being falsified but after evaluation it is not. This has very general meaning and applies to all areas of vascular biology, but we can cite one example. The hypothesis that angiogenesis factors were specific for the vasculature had a high chance of being falsified, but it was not for a time because cell biology and genetics did not invalidate the hypothesis and, on the contrary, reinforced it. Thus, for a time it could be regarded as a very solid hypothesis. However, the hypothesis was ultimately refuted because more data were produced that demonstrated the contrary.

Inference to the best explanation (also called IBE) is an interesting approach for vascular biology. This approach bridges the gap between inductive inference, which is exclusively based on temporary occurrences to establish a causal link and mechanistic explanations, which involve concepts such as topology, structure, and feedback. How is IBE related to angiogenesis research? One example can be given here. By analyzing the structure of the VEGF promoter, the information derived from its structure identifies potential sites that are responsible for the binding of specific transcription factors, the role of which can then be functionally investigated through inductive or deductive inference.

We cannot follow the Popperian argument that the "queen" of scientific methods to acquire knowledge is the deductive method and the falsification procedure. Any researcher who has carried out experimental science knows that the process of knowledge generation in science involves a combination of induction, deduction, verification, and falsification. This is clearly illustrated by research in the angiogenesis field. Careful observation of the various morphogenic processes allowed the formulation of hypotheses, which were then validated by subsequent experiments. We must, however, be careful how much weight we give either to hypotheses or to experimental data. If a hypothesis is not verifiable or falsifiable, it can either be abrogated or included in what is called an auxiliary hypothesis that restricts the scope

of the hypothesis and bring it into line with the experiments. This is a tricky situation because one can either over-evaluate the hypothesis or over-evaluate the experiment. However, it is only science when one can attribute the respective values (to hypothesis or experiment) and this can lead both to an enrichment of knowledge (if subsequent experiments by others are convincing and go in the same direction), and to drifts not conforming to the reality of the facts. A danger is always confirmation bias that can touch even entire research domains.

We have seen that, as with any research field, angiogenesis research has often taken tortuous paths, and is sometimes exposed to antagonistic concepts and encounters failures. We have encountered in the preceding chapters a number of these antagonisms and failures, to mention only angiostatin and endostatin in this respect. However, even failures can be beneficial, and all of this research contributes to the progress of science. There must be no church in science, no firm dogma, and every researcher, even the most reputable, must be able to be criticized and his theories tested, and this by the most novice scientists. This distinguishes it from the beliefs and ideologies for which full accession is required which cannot be questioned.

As in all human activities, science is not spared from certain drifts and can be contaminated by ideology and by using it in an indirect way in order to obtain a prestigious position or to increase its own power status. On the other hand, it sometimes happens that science undergoes what Irvine Langmuir called "pathological science" (https://www.cs.princeton.edu/~ken/Langmuir/langmuir.html). In a presentation to the Knolls Research Laboratory in December 1953, Langmuir describes the characteristics and symptoms of a pathological science which, to quote Langmuir in the text, are as follows:

> *The maximum effect is observed by a causative agent of low intensity and the magnitude of the effect is independent of the intensity of the causative agent. Intensity, and the magnitude of the effect are substantially independent of the intensity of the cause.*
>
> *The effect is of a magnitude that remains close to the limit of detectability because of the low statistical significance of the results.*
>
> *Claims are of great accuracy.*
>
> *Theories are always fanciful and contrary to experience.*
>
> *Criticisms are met by ad hoc excuses thought up on the spur of the moment.*
>
> *The ratio of supporters to critics is up to somewhere near 50% and then falls gradually to oblivion.*

In the scientific literature, a certain number of scientific works fall precisely into this category. Langmuir, who was a physicist, cites the Allison effect, the Davis-Barnes effect, N and mitogenic rays, extrasensory perception, and flying saucers. In the biological literature, the case of water memory was particularly revealing. Jacques Benveniste had published an article in the prestigious journal Nature

which describes an experiment attempting to prove the principle of homeopathy [344]. This work had aroused much controversy and is now discredited, although Luc Montagnier, Nobel Prize in Medicine, who took up the torch of water memory, persists in this direction and affirms the opposite. There is even a recent publication on this subject in which the transmission of information by electromagnetic signals emitted by water, which had been previously in contact with the DNA, has been reported [345]! There is also a recent publication in a relatively modest toxicology journal by Seralini et al. [346] saying that tumors in rats can be induced by foods containing Genetically modified organisms (GMOs). This article had made a great impact in the media and the French newspaper "Le Nouvel Observateur" had published a long article with a title page concerning this work. I am not opposed to audacious ideas or a particular advocate of GMOs, but these works correspond to all the criteria Langmuir set out in his lecture and therefore reflects what he called "pathological science."

To quote the American astronomer Carl Sagan: "*Extraordinary claims require extraordinary evidence!*" Not everybody can call himself a convincing heretic with solid claims!

What about research on blood vessels? Can we detect signs of a pathological science? I have not found indications of a true pathological science in the various writings I have consulted. There are times when overly broad statements or erroneous conclusions based on biased experimentation have been made to cite only the angiogenin, angiostatin, and endostatin episodes that made a great deal of noise in the press of the time, or the role of endothelial cell progenitors in tumors.

However, everything that comes to mind does not detract from the fact that the basis of scientific activity is critical reason and not blind adherence to any postulate, and this makes science one of the noblest activities of the human mind.

Ill-intentioned minds may say that science does not need philosophy and can do very well by itself. However, conceptual research seems useful and can even enrich scientific research, as was shown by the philosopher Thomas Pradeu in his collaboration with the physician-biologist Éric Vivier on the immune system [347]. Such approaches deserve to be extended to other areas of biology, such as vascular biology or cancer.

17.9 Summary and Concluding Remarks on Conceptual Issues

We have identified several areas from the vascular development field that can be subjected to a conceptual analysis (Summary Box). These include paradigm changes, cross-fertilization of domains, technological development and the impact on discovery and knowledge building, evolutionary biology issues, and methods for scientific discovery in the vascular biology field. Paradigm shifts, as discussed, take place on a smaller (micro-paradigms) or larger scale (macro-paradigms). It is noteworthy that "false" paradigms can have a positive effect on the discovery path of a given scientific domain, such as the assumed specificity of vascular growth factors. One interesting aspect is how vascular development in tumors was viewed

during history: first a nourishing tissue, then a host-defense mechanism, and finally a tumor promoter. Vascular biology has been cross-fertilized by other domains such as cancer biology or neurobiology. Cancer research has allowed the discovery of vascular trophic factors, and neurobiology has introduced concepts such as guidance and tip cells into vascular biology. Thus, concept transposition, such as seen for the notion of tip cells and guidance, has been/is a valuable device to fuel research in vascular biology and the angiogenesis field. Another important aspect is technology development and methods that are available at a given time, and how they impact on the formulation of concepts and theories. As shown, a simple biochemical method such as heparin-sepharose chromatography has led to the discovery of vascular development factors, confirming the anticipated "belief" of the existence of such trophic factors. This has then further spurred not only the vascular biology field but also many other fields, such as tumor biology and oncology. Finally, analysis of scientific methodology still shows a preponderance of inductive inference, even if deductive inference or methods such as IBE are also in use.

An important question is now where the field is heading and what might be the landscape in the next 20 years. I mention a few examples that, to me, seem important. At present, one important direction that is taken is the study of relation of the vascular system with immunology. The idea of Ernest Goldmann at the beginning of the last century has gained attention recently and, in this respect, recent work by two laboratories stresses the importance of HEVs to trigger anti-tumor immunity. It is important to stimulate HEVs in tumor cells in order to optimize immunotherapy. A step into this direction has been taken by demonstrating the combination of anti-angiogenic therapy and immunotherapy using anti-PDL1 can stimulate the number of these vessels in tumors. The effort in this direction must be enhanced.

Another area of investigation is how larger vessels are construed during development. Where do the endothelial cells that are incorporated into larger vessels come from? Is there retrograde trafficking of endothelial cells from capillaries and smaller vessels?

Important questions are also related to the molecular heterogeneity of vascular cells during development and pathology. Are expression profiles (such as obtained by RNAseq) of endothelial cells in one territory heterogeneous? What is the situation in pathology?

Computational biology has been used by a number of investigators to model vessel formation [348]. However, there is no convincing understanding unless a multilayer approach is developed with the integration of morphological and molecular data. This should be tackled in the future and the inclusion of new molecular data as indicated above can help the push in this direction. A step in this direction has been taken by the development of an open Library for Spatial Modeling of Vascularized Tissues (the microvessel Chaste) [349].

On the translational side, anti-angiogenesis therapy in cancer has deceived clinicians but, at the same time, was very successful in ophthalmology. Might anti-angiogenesis therapy in cancer still be used or abandoned in the near future? Industry is not investing additional efforts in the development of anti-angiogenic

compounds in cancer. It is mandatory that new venues are explored, such as mentioned above with immunotherapy, for anti-angiogenesis therapy in cancer to survive.

Summary Box: Conceptual Issues

- Paradigm shifts occur at a micro- and macro-scale in vascular development research
- False paradigms may be useful in the discovery path and may lead to progress (i.e., VEGF specificity)
- Historically, concepts undergo transformations which may lead to opposing/conflicting views (i.e., the significance of tumor angiogenesis) with possible later resolution
- Cross-fertilization from other fields occurs. This happens at two levels, at a morphological level (i.e., the concept of tip cells and guidance from neurobiology) and at a molecular level (i.e., Netrin as a vascular guidance factor)
- Technology development of simple methods may have a major impact on the development of the entire field (i.e., heparin-sepharose chromatography)
- During evolution, the role of the various components of the vessel wall underwent modifications and the organizing principles were changed (i.e., myoepithelium → endothelium)
- Micro-and macro-Kernels can be defined. A micro-Kernel is related to the implementation of a specific function (i.e., transposition FGF-Notch Delta in the tracheal system to the VEGF-Notch Delta in the endothelium), whereas the macro-Kernel is related to set of nodes, which converge in a common functional purpose
- Analysis of scientific methodology still shows a preponderance of inductive inference, even if deductive inference or methods such as IBE are also in use. Inferences may flip back and forth (i.e., deductive ↔ inductive inference)