

Non-angiogenic tumours and their influence on cancer biology

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ABSTRACT

Solid tumours need a blood supply and a large body of evidence had previously suggested that they can only grow if they recruit a blood supply by inducing the development of new blood vessels, known as tumour angiogenesis. Based on this hypothesis, it was proposed that anti-angiogenic drugs should be able to suppress the growth of all solid tumours. However, based on clinical experience with anti-angiogenic agents, we now know that this is not always the case. Reports of tumours growing without the formation of new vessels can be found in the literature dating back to the 1800s, yet no formal recognition, description and demonstration of their special biological status was made until recently. In 1996, we formally recognised and described ‘non-angiogenic tumours’ in lungs where the only blood vessels present are those originating from the normal lung. This is far from an isolated scenario, since non-angiogenic tumour growth has now been observed in tumours of many different organs, in both humans and preclinical animal models. In this Opinion article, we summarise how these tumours were discovered, and discuss what we know so far about their biology and the potential implications of this knowledge for cancer treatment.

[H1] Introduction [Au: Please ignore the [H] symbols throughout as this is a styling note for our production editors.]

“It is a common failing—and one that I have myself suffered from—to fall in love with a hypothesis and to be unwilling to take no for an answer. A love affair with a pet hypothesis can waste years of precious time. There is very often no finally decisive yes, though quite often there can be a decisive no. “

Peter B Medawar. *Advice to a Young Scientist*. Page 73

Basic Books. Perseus Book Group. 1979.

The paradigm that all tumours are angiogenesis-dependent ¹ has not held the test of time, as some advanced, aggressive cancers have been discovered which can grow without angiogenesis, by exploiting pre-existing vessels (**Supplementary Information Table 1**) Therefore, the interaction of cancer cells with blood vessels is more complex than originally thought.

The relationship between blood vessels and tumours has intrigued people for centuries and awareness that cancers contain blood vessels goes back to the dawn of medicine. The concept of new vessel growth or neovascularisation in tumours was formally introduced as far back as 1787, although the word “angiogenesis” was not coined until 1903 ² In 1971, Folkman put forward the idea that “the growth of a solid neoplasm is always accompanied by neo-vascularization” ³.

Angiogenesis is defined as the outgrowth of new blood vessels from pre-existing blood vessels. Several types of angiogenesis have been described in cancer; ‘classic’ sprouting angiogenesis, intussusceptive microvascular growth (IMG; where pre-existing vessels are longitudinally split into two) and glomeruloid microvascular proliferation (GMP; which are small vascular structures reminiscent of kidney glomeruli) ⁴⁻⁶ . In addition, intra-tumour vessels can be formed by post-natal vasculogenesis, in which tumour vessels are derived from circulating endothelial progenitor cells (EPCs) (Figure 1) ⁷.

Sprouting angiogenesis, the most common form of angiogenesis is frequently observed in many tumours ⁸⁻¹¹ and occurs in response to the hypoxic microenvironment that develops within a growing tumour mass ¹². Yet it is emerging that a minority of tumours can grow in a purely non-angiogenic manner despite being hypoxic, whilst others may contain a mixture of both angiogenic and non-angiogenic areas ^{13, 14}.

Reports of non-angiogenic tumours in diverse organs have been appearing increasingly in the literature ¹⁷⁻²⁰ since the observations in the mid 1990s of non-angiogenic tumour growth in

primary and secondary lung cancer^{15, 16} (Supplementary Information **Table 1**)^{15, .} The organs most commonly described to host non-angiogenic tumours, both for primary tumours and metastatic lesions are the lung, liver and the brain. Several independent studies in mice and in humans (Supplementary Information **Table 1**) have confirmed the substantial contribution of non-angiogenic tumour growth to tumour progression⁵, and more recently, comprehensive pre-clinical and clinical studies have confirmed the hypothesis²¹ that non-angiogenic tumours are one of the causes of resistance to anti-angiogenic drugs, revealing the importance of targeting both angiogenic and non-angiogenic vessels^{13, 14, 22-30}. Hence, the study of non-angiogenic tumours has opened a new field in cancer biology.

Cancer blood vessels have been investigated for more than a century and one may be surprised that non-angiogenic tumour growth has been overlooked. Histological descriptions of these tumours were reported but their biological significance as ‘non-angiogenic’ was not appreciated. This is well illustrated by the literature describing brain and lung tumours. In 1994 Wesseling *et al.*¹⁶ suggested that many areas of glioblastomas “may not be overtly angiogenesis dependent” as the authors noted that, in such areas, the vascular density and morphology were comparable to that of normal brain¹⁶. Furthermore, what we now call non-angiogenic tumour growth had been previously described in successful textbooks of modern pathology. In Florey’s General Pathology (1962)³⁶, the author states that: “...a tumour will supplement or replace the stroma by making use of pre-existing structures. For example, occasional tumours in the lung grow around the alveoli using the alveolar walls in place of stroma.”

Interestingly, the early studies leading to the conclusion that tumour growth relies on sprouting angiogenesis were largely based on *in vitro* experiments and animal models³⁷ where the experiments were conducted in relatively avascular sites, such as the cornea and the ear of the rabbit^{38, 39}. Despite the use of these non-physiological settings as most cancers arise in well vascularised tissues, these experiments were regarded as a convincing proof of concept. In contrast, the observations of non-angiogenic growth of cancer have primarily arisen from histopathological studies of tissue samples from patients.

In this Opinion article, we will discuss the evidence that tumours can grow without angiogenesis. We will also describe how non-angiogenic cancer cells co-opt normal blood vessels, what is known of their biology and discuss the different histological patterns of non-angiogenic tumour growth. Finally, we will touch upon the potential clinical implications of non-angiogenic tumours and summarise why their investigation is fundamental to our understanding of tumour biology.

[H1] Non-angiogenic tumour growth

By definition, non-angiogenic tumours grow in the absence of formation of new vessels. In this tumours no new vessels are seen but there is persistence of a vasculature with the structure of the pre-existing vessels and their topographic distribution. Non-angiogenic tumour growth can be achieved by two main mechanisms; one such means is by cancer cells infiltrating normal tissues to exploit pre-existing vessels, known as vascular co-option or vessel co-option. The other method relies on a phenomenon known as vasculogenic mimicry where cancer cells differentiate into structures that act like blood vessels. (**Box 1**).

[H3] Evidence from human studies

Four distinct patterns of growth and vascularization have been described in primary and secondary lung cancer²¹ (**Figure 2A**). Three of these histological growth patterns (HGP; named ‘diffuse’, ‘papillary’ and ‘basal’) destroy normal lung architecture and induce angiogenesis [ref 15, 21](#). The fourth is a non-angiogenic pattern (‘alveolar’), and can be identified by immunostaining for endothelial and lung epithelial markers²¹, which highlight the alveolar vessels, entrapped by the neoplastic cells filling the alveolar spaces; these cancer cells then grow by hijacking the existing vessels. Anthracotic pigment-containing macrophages are commonly observed alongside these vessels indicating that they pre-date the appearance of the tumour [ref 21](#). Two further non-angiogenic patterns (**Figure 2A**) have been described in lung cancer but only in metastatic lesions; in the ‘interstitial’ pattern, tumour cells infiltrate the alveolar walls, using local capillaries, but without entering the alveolar space, and in the ‘perivascular cuffing’ pattern cancer cells grow by surrounding larger vessels. Out of the 164 lung metastatic lesions examined at least one of these non-angiogenic patterns, whether alone or in association with angiogenic areas, was found to be present in more than 80% of the evaluated metastases¹³. Primary and secondary lung tumours containing both angiogenic and non-angiogenic areas are very commonly seen [Ref 13, 21](#).

In the studies described above, immunohistochemical characterization confirmed that only pre-existing alveolar vessels were present in these tumours [Ref 41](#). These vessels stained positive for the normal alveolar wall vascular basement membrane marker LH39, whilst staining for the angiogenic marker $\alpha v\beta 3$ -integrin on endothelial cells was weak or absent⁴¹ in a fashion comparable to that of normal lung vessels. The non-angiogenic nature of some lung tumours was further confirmed by a three dimensional reconstruction: tumour vasculature was indistinguishable

from that of normal lung, while a ‘chaotic’ network of vessels was observed in angiogenic tumours ⁴².

In the liver, three patterns of metastatic growth have been described: two angiogenic, termed ‘desmoplastic’ where cancer cells are separated from the adjacent liver parenchyma by a rim of desmoplastic stroma and ‘pushing’ in which the plates of are pushed away by the tumour, which is separated from the liver parenchyma only by a thin layer of reticulum fibers, and a third, non-angiogenic pattern, called ‘replacement’ ²⁰ (**Figure 2B**). In the replacement growth pattern the neoplastic cells replace the hepatocytes without any disturbance of the normal liver architecture. Unlike the angiogenic patterns of liver metastasis, in the replacement pattern the tumour grows by co-opting the sinusoidal blood vessels ^{20, 43, 44}.

The non-angiogenic growth of gliomas via a mechanism of perivascular cuffing is now well recognised [Ref 29, 62](#) (**Figure 2C**). Other patterns of primary and secondary brain tumour growth have been described and are illustrated in **Figure 2C**. Some have been clearly identified as non-angiogenic while others are so far only postulated to be angiogenic ⁴⁵⁻⁴⁹. Non-angiogenic metastases have also been described in lymph nodes ^{19, 23}. Because of the rich native vascularity of lymph nodes, non-angiogenic metastases can develop in this site although also angiogenic angiogenic metastases are observed ⁵⁰.

In all of tissue sites, non-angiogenic tumours tend to have the overarching morphological characteristic of preserving the vascular architecture of the original tissue in patterns that are distinct from the chaotic and tortuous organization of dilated angiogenic blood vessels.

All of these studies were performed on tumour samples not treated previously with anti-angiogenic drugs and therefore the observed patterns cannot be attributed to the phenomenon of ‘vascular normalization’, which follows anti-vascular endothelial growth factor (VEGF) treatment and induces changes in the newly formed vessels towards a more mature phenotype ⁵¹. For this reason, following anti-VEGFA treatment of tumours with extensive angiogenesis, the differentiation between trapped pre-existing vessels and normalised vessels may not be possible. Equally, in untreated angiogenic tumours it is not possible to be sure whether a very mature intra-tumour vessel is a pre-existing one or a newly formed one that has had the time to acquire a mature phenotype. Presently, specific biomarkers distinguishing these two possibilities of tumour vessel are not available. Therefore, while the identification of a purely non-angiogenic tumour area can be done according to the previously discussed criteria, it is not yet possible to establish, within an overtly angiogenic tumour, what is the contribution, if any, of trapped pre-existing vessels.

[H3] Evidence from animal models.

Observations in human tumours inspired a range of animal studies^{18, 27, 29, 47, 52-67}, many of which are discussed in more detail in the following sections. Both orthotopic and heterotopic transplant animal models have been used. One may speculate that an orthotopic model is superior and indeed, it is necessary to investigate how primary or metastatic non-angiogenic tumours develop in their organ of origin. However, models in which ectopic sites are used, more commonly the subcutaneous tissue, are also necessary to understand the cues which instruct a cancer cell to behave in either an angiogenic or non-angiogenic fashion; this is because the anatomical site in which the cell lines are injected seems to determine the behaviour of the cancer cell. For example, the subcutaneous tissue appears to dictate an angiogenic behaviour whereas the lung directs a non-angiogenic behaviour¹³. By extension, this also means that a cell line investigated *in vitro* cannot be classified as either angiogenic or non-angiogenic. Chick embryo chorio-allantoic membrane (CAM) models have also been used [Ref 52](#). The CAM is a vascular membrane formed during avian development by the fusion of the mesodermal layers of the allantois and the chorion. By opening a window in the shell of a fertilised egg, it is possible to seed cells on the CAM and observe their behaviour for several days as the embryo remains viable and is preserved in a more physiological environment ([REF: Tufan A.C., Satioglu-Tufan N.L.](#)

[The Chick Embryo Chorioallantoic Membrane as a Model System for the Study of Tumor Angiogenesis, Invasion and Development of Anti-Angiogenic Agents](#)
[Current Cancer Drug Targets 5: 249-266, 2005](#))

[H1] Vessel co-option

The term vessel co-option was first used by Holash *et al.*⁵⁸ to describe how tumour cells, in certain preclinical models of glioma and lung metastasis, were observed to utilise pre-existing vessels instead of inducing angiogenesis.

Vessel co-option in cancer may have parallels with other physiological phenomenon; for example when plasma cells line up along the vessels in the bone marrow⁷¹ although the functional implications of this event are unknown. With respect to embryological development, a close functional relationship between vessels and surrounding tissue is well known⁷² [ref \(Ding et al 2016. Angiocrine functions of organ-specific endothelial cells. PMID: 26791722\)](#) but whether these events are comparable to what we broadly call vessel co-option is as of yet undetermined. As we

view co-option as a process in which cancer cells thrive and grow by establishing contact with pre-existing vessels, evidence so far suggests that this is an active mechanism and it is reasonable to raise the hypothesis that angiocrine mechanisms could have a role.

[H3] *Primary tumours.* Much of our understanding of how vessels are co-opted comes from studies of primary brain tumours which have given insight into the molecular mechanisms that drive angiogenic versus non-angiogenic tumour growth, attract cancer cells towards pre-existing vessels and allow attachment of cancer cells to co-opted vessels.

Both in a mouse orthotopic model and a CAM model of glioblastoma, inositol-requiring enzyme 1 (IRE1; also known as ERN1), a proximal endoplasmic reticulum (ER) stress sensor and a central mediator of the unfolded protein response (UPR), was shown to be important in dictating the type of blood supply used by tumours⁵². U87 wild type (WT) glioma cells expressing IRE-1 grew as a discrete, highly angiogenic mass, while IRE1-silenced U87 cells developed as non-angiogenic, infiltrative tumours, co-opting pre-existing vessels⁵². IRE1 activity was mediated by interleukin-6 (IL-6), whose levels diminished following IRE-1 silencing. Transfection of IRE1-silenced U87 cells with IL-6 restored the angiogenic phenotype, blocking co-option, but did not reverse tumour infiltration. These IRE1-depleted U87 cells expressed fewer proangiogenic genes as discussed later. IRE1 missense mutations have been described in gastric adenocarcinomas, glioblastomas, lung adenocarcinomas, ovarian serous carcinomas and renal cell carcinomas⁷³ although their functional effects have not been analysed. Furthermore, there are currently no studies of human tumours correlating IRE1 mutations and/or levels of expression to angiogenic patterns.

For successful co-option to occur, tumour cells need to travel toward the vessel (**Figure 3A**). With an *ex vivo* model using slices of rat brain cortex maintained in a viable state in a bath of artificial Cerebro Spinal Fluid and human glioma cell lines *in vitro*, it was shown that B2 bradykinin receptor (B2R) an activator of matrix metalloproteinases (MMPs), is expressed on the glioma cells, and that a bradykinin gradient acts as a chemoattractant to guide glioma cells toward blood vessels⁷⁴. Accordingly, the number of glioma cells associated with vessels decreased with B2R inhibition, both pharmacological and by mRNA silencing.

Caspani *et al.*⁵⁷ investigated the mechanisms by which glioblastoma cells interact with pre-existing vessels (**Figure 3A**). Seeding of glioblastoma cells on mouse brain explants resulted in vascular co-option within 15 hours. Two-photon and confocal imaging revealed that tumour cells developed actin-based cytoplasmic extensions, named ‘flectopodia’, which contact target pericytes. CDC42, a RHO GTPase regulating actin-dependent cytoplasm extensions and CD44, a

fusogenic protein are both concentrated in the flectopodia; CDC42 is crucial for flectopodia motility, and CD44 is enriched at the point of contact between flectopodia and pericytes. Silencing of the expression of CDC42 and/or CD44, indicated that these molecules synergistically support vessel co-option. The authors concluded that fusion occurs and a new hybrid cell, which they named GDH (GBM cell/ Dextran Labelled Pericytes (DLP) Hybrid), is formed which loses glioblastoma cell markers CD44 and nestin, but maintains high levels of smooth muscle actin (SMA), a marker of smooth muscle cells, supporting co-option ⁵⁷. By inhibiting functions of CDC42 and CD44, vessel co-option is impaired and fusion does not occur ⁵⁷.

Finally, Watkins *et al.* ⁷⁵ described that glioblastoma cells can insert under astrocyte endfeet and interrupt coupling of astrocytes to blood vessels. Moreover, the glioma cells can take over the ability of astrocytes to influence vascular tone. Under these conditions, drugs that are able to induce vasodilation in normal brain tissue, cause vascular constriction. Whether the glioblastoma cells replacing the astrocytes also fuse with brain pericytes was not described in this study.

[H3] *Secondary tumours.* Vessel co-option may mediate early steps of metastatic seeding (**Figure 3B**): based on multiphoton laser scanning microscopy, Kienast *et al.* ⁶⁰ observed that brain metastasis, could only proliferate if able to co-opt vessels. Once again CDC42 may be relevant to this process: in the model described by Reymond *et al.* metastatic cancer cells extravasate from blood vessels by first adhering to endothelial cells, then opening their junctions, inducing endothelial retraction, and finally, inserting themselves into the endothelial monolayer between endothelial cells, a process named intercalation ⁷⁶. Using an experimental lung metastatic model in SCID mice, the authors demonstrated that CDC42 is critical for extravasation and subsequent interaction and abluminal spreading of metastatic prostate PC3 and DU145, and breast MDA-MB-231 cancer cell lines with the sub-endothelial extracellular matrix (ECM) ⁷⁶. CDC42 increased levels of serum response factor (SRF), which induced transcription of $\beta 1$ integrin required for metastatic cancer cells to adhere to endothelial cells. After intercalation, it is again necessary for the cancer cell to protrude and adhere to the basement membrane to complete extravasation and remain attached to the abluminal surface of the vessel ⁷⁶. Direct blockade of $\beta 1$ integrin subunit prevented adhesion of tumour cells to the sub endothelial basement membrane, thereby impairing colonization and therefore the possibility of metastases ⁷⁶. The same observation was made by blocking CDC42 [Ref 76](#).

Another active mechanism related to metastatic co-option of brain blood vessels is

mediated by plasmin [REF 66](#) (Figure 3B). Neurons secrete plasminogen, which is cleaved to plasmin by the astrocyte-secreted tissue (tPA) and urokinase plasminogen activator (uPA). Plasmin has two effects: inactivation of axon pathfinding molecule neural L1 cell adhesion molecule (L1CAM) on metastatic cancer cells, and cleavage of membrane-associated FAS ligand (FASL) present mostly on the astrocytes to its soluble form sFASL, which triggers apoptosis of cancer cells ⁶⁶. Valiente *et al.* [REF 66](#) described in their model that, if the extravasated cancer cells secrete neuroserpin (also known as PI12) or serpin B2 (also known as PAI2), PA is inactivated. Therefore, no apoptosis is triggered and, if the metastatic cells express L1CAM, they can adhere to the basal membrane, co-opt and grow into a metastatic lesion ⁶⁶. By protecting cancer cells from death signals and promoting vessel co-option, PA-inhibiting serpins provide a unifying mechanism for the initiation of some brain metastases ⁶⁶.

[H3] Extravascular migratory metastases. Another phenomenon has been described in which cancer cells adhere to the abluminal surface of vessels, forming what the authors defined as an ‘angio-tumoral complex’, and then move along the vessels as a means to spread through the body ⁶⁹. Subsequently the adhesion of cancer cells to the abluminal vessel surface has also been linked to tumour dormancy, survival and drug resistance ⁷⁰. Therefore, it remains open as to whether the term vessel co-option should define only tumour growth around vessels or, more broadly, any situation in which a cancer cell adheres to the abluminal surface of a pre-existing vessel.

If vessel co-option is considered to include the interaction of cancer cells with the surfaces of vessels, it might also play a role in metastatic spreading: histological observations showed that melanoma cells migrate along vessels, so called angiotropism ⁷⁷, possibly contributing to metastatic cells spreading through the body which has been termed extravascular migratory metastasis ⁷⁸. Perivascular migration of tumour cells at an average speed of 0.3 micron/minute has been demonstrated ⁷⁸. Since neural crest cells migrate on the abluminal surface of vessels in the chick embryo in a similar way, melanoma cells may thus be mimicking embryonic features ([ref 77](#)). Transcriptomic studies from patient samples of melanoma identified a group of 15 genes associated with embryonic features divided into four functional groups: neural crest migration, neural crest-derived tumour migration, general migration and neurotropism. As the melanoma cells replace the pericytes on the abluminal surfaces, they move along the vessels without immediately growing into a metastases but instead spreading throughout the body: this ability of the single cancer cell to take a position on the abluminal surface of a vessel and move along it has been named pericytic mimicry ⁷⁷.

Interchanging vascular status

During tumour progression, metastatic lesions can have a vascularisation mechanism that is different from that of the primary tumour ^{13, 26, 28}. For example, non-angiogenic NSCLC may relapse as an angiogenic brain metastasis, while angiogenic breast cancer can progress to non-angiogenic lung metastasis ^{17, 79}. A recent human study, showed that non-angiogenic patterns of tumour growth (**Figure 2A**) are common in lung metastases from breast, colorectal and renal cancers ¹³. Out of the 164 lung metastases investigated, 86 (53%) were reported as non-angiogenic, 28 (17%) as angiogenic and 50 (30%) as mixed (i.e. with angiogenic and non-angiogenic areas). Therefore, it is apparent that cancer cells can go on to become either angiogenic or non-angiogenic metastatic lesions, independently of the angiogenic status of the primary lesion. Whether this is due to a single cancer cell being able to change status according to the microenvironment or due to the selection of clones with different abilities, e.g. angiogenic clones growing preferentially in a primary breast tumour but non-angiogenic clones growing more when neoplastic cells reach the lung, is yet to be determined. Bridgeman *et al.* ¹³ demonstrated that the mouse mammary carcinoma cell line 4T1 and the mouse colon carcinoma cell line C26 are angiogenic when transplanted into the subcutaneous tissue but non-angiogenic when seeded in the lung through the tail vein. Furthermore, in a mouse model, it was observed that an angiogenic hepatocellular carcinoma could switch to a non-angiogenic phenotype (by co-opting vessels in the previously healthy surrounding liver tissue) during treatment with an inhibitor of sprouting angiogenesis (sorafenib, an inhibitor of RAF–MEK, VEGFR2 and platelet-derived growth factor receptor (PDGFR) kinases) ²⁶. However, after discontinuation of the drug angiogenesis resumed in the tumour highlighting how a therapeutic selection pressure can change the tumour vascular status.

It should also be stressed that in a large number of tumours, both angiogenic and non-angiogenic areas are present in the same lesion. For example, early studies in primary lung cancer have shown it is frequent to have a non-angiogenic periphery while deeper in the lesion, vascular remodelling and angiogenesis are triggered [Ref 21](#). Once one area of a tumour has become angiogenic, it will maintain that feature ^{21, 41}. This change may occur possibly because in some tumours the cells are behaving as non angiogenic as long as they are on the edge of the lesions. As new layers of cells are produced, enlarging the lesions, the cells which found themselves under layers of newly produced cells could switch to angiogenesis, very likely because of hypoxia.

Hence, a common view has been that a non-angiogenic phenotype may predominate in micrometastases whilst angiogenesis could be of pivotal importance when the tumour grows larger⁵⁸. However, this was not demonstrated to be the case for all of the primary lung tumours investigated so far as some can grow whilst remaining non-angiogenic^{21, 41}. Bridgeman *et al.*¹³ also observed that larger metastases (greater than 1cm) can remain non-angiogenic. Additionally, this view is complicated further by observations that synchronous metastases, one angiogenic and one non-angiogenic, have been observed in patients with colorectal cancer liver metastases⁸⁰.

An experimental example highlighting how angiogenic and non-angiogenic lesions can appear at different times during the progression of cancer has been described in the work of Sakariassen *et al.*⁸¹ who transplanted xenografts of human glioblastoma biopsies into the brains of immunodeficient nude rats. Initially the lesions produced were non-angiogenic. However, following serial passages in rats, the lesions switched to a high-grade angiogenic behaviour. Transcriptomic studies showed that cells from the non-angiogenic tumours that were initially grafted in the rats displayed some neuronal stem cell markers, such as Musashi1 (*MSH1*), nestin and vimentin and so the authors concluded that this ability to change the type of growth, when re-implanted in other animals, was possibly related to a cancer stem cells phenotypes. However, as also stated by the authors, it is not clear whether these glioblastoma cells with cancer stem cell properties are derived from transformed neural stem cells, from stem cell fusion events, or from otherwise restricted subpopulations within the tumour [REF 81](#). Together these studies indicate a dynamic switching and variation in vascular phenotype during tumour development and spread, although the underlying mechanisms are yet to be fully understood.

[H1] Non-angiogenic tumour biology

The biology of non-angiogenic tumours has begun to be investigated with histology, protein expression, transcriptome and cell biology studies to gain insight into relevant molecular mechanisms (**Figure 4**).

Histological and immunohistochemical studies of primary lung carcinomas revealed that chronic inflammation and fibrosis is characteristic of angiogenic tumours, but interestingly, no major differences between the degree of tumour cell necrosis and apoptosis, and microvessel density, were seen between angiogenic and non-angiogenic tumours⁸⁵. Comparable observations were made between angiogenic and non-angiogenic liver metastases⁴³. Contrary to our expectations, immunostaining (and later transcriptomics studies) did not demonstrate any major difference in the expression of angiogenesis and hypoxia related markers in primary lung cancers, suggesting that non-angiogenic

tumours sense hypoxia and activate the relevant pathways, but fail to trigger angiogenesis. These observations support the hypothesis that activation of classical hypoxia pathways is not sufficient to induce angiogenesis. Following the publication that IRE1 upregulation was associated with induction of angiogenesis⁸⁷, Auf *et al.*⁵² compared the U87 WT glioma cell line, which induces angiogenesis in the CAM assay, with its non-angiogenic IRE1-silenced counterpart and showed that IRE1 upregulates VEGFA. As a result of this data along with other studies, IRE1 up-regulates VEGF⁵² an initial model of the angiogenic cell is emerging when putting together Auf data with those from other studies (**Figure 4**) After VEGFA binding to VEGFR2, the PI3K–AKT pathway is upregulated.^{21, 52, 86, 88} The activation of PI3K/AKT is also sustained by the high levels of Integrines, the action of the latter is further stimulated when activated KDR is present (add ref : Goel H.L., Mercurion A.A.M. VEGF targets the tumour cell. *Nature Reviews Cancer* 13:871- 882 2013)

We independently analysed the lists of differentially regulated genes from both our lung study Ref 88 and the U87 cells paper Ref 52 through a publicly available tool (<http://amp.pharm.mssm.edu/Enrichr/>): in the context of these reanalysed data, it appears that in the angiogenic cells, the PI3K pathway is associated with angiogenesis and stromal remodelling, the activation of integrins, which promote cell adhesion, and the inhibition of actin activation, which as a consequence leads to inhibition of motility^{21, 86, 88}. In the non-angiogenic cells, PI3K–AKT is also emerging as having a role but in this context, alongside the canonical and non-canonical WNT pathways, these signalling pathways are associated with changes in cell motility and invasion (as described below) rather than angiogenesis and stromal remodelling⁵².

[H3] *Stromal inflammation and remodelling.* The role of the stroma, and of the frequently present chronic inflammation in angiogenic tumours is an intriguing one. In some angiogenic carcinomas, the inflamed stroma appears to be the only site in which angiogenesis develops, while in others the stroma has a more ‘chaotic’ distribution and vessels are not necessarily associated with it. Finally, in a sub-group of lung tumours with the papillary pattern (**Figure 2A**) angiogenesis occurs in the absence of stroma formation²¹. It is likely that in different tumours, even from the same site, stroma has a different role and that there could be an interplay between stromal and inflammatory cells.

One of the striking differences between angiogenic and non-angiogenic tumor growth is that angiogenic tumours usually produce destruction of entire pre-existing normal components of the infiltrated organ which is completely replaced by tumour²¹.

Following the publication that IRE1 upregulation was associated with induction of angiogenesis⁸⁷, Auf et al⁵² compared the U87wt glioma cell line, which induce angiogenesis in the CAM assay, with its non-angiogenic IRE1-silenced counterpart⁵². As IRE1 up-regulates VEGF⁵² a model of the angiogenic cell is emerging when putting together Auf data with those from other studies (**Figure 4**)^{21, 86, 88}. After VEGF binding to KDR, the PI3K/Akt pathway is upregulated alongside the integrin pathway. We independently analysed the lists of differentially regulated genes from both the lung and the U87 cells studies through a publicly available tool (<http://amp.pharm.mssm.edu/Enrichr/>): in the context of these data, the angiogenic cells PI3K pathway is associated with angiogenesis, stroma remodelling and inhibition of actin activation (with consequent inhibition of motility) pathways, and with activation of integrins pathway, which promotes cell adhesion^{21, 86, 88}. In the non-angiogenic cells, PI3K/AKT is also emerging as a player but alongside WNT canonical and non-canonical pathways and are associated with changes involved in motility and invasion, as later described, rather than angiogenesis and stroma remodelling⁵².

In both the non-angiogenic IRE-depleted U87 cells⁵² and angiogenic lung cancers⁸⁸, TSP-1 was found to be up-regulated. This finding could appear contradictory: while the high levels of TSP1 in non-angiogenic tumours is easily interpretable as TSP-1 has an antiangiogenic activity, its increased expression in angiogenic neoplasms is not so easily explained. However, angiogenesis is accompanied by vascular remodelling which involves blocking the formation of some of the new branches. It is in this context that TSP-1 exercises its anti-angiogenic activity, as the TSP1 upregulation in angiogenic lung tumours was demonstrated to be in the stromal compartment rather than in the neoplastic cells themselves⁸⁶. Therefore, in the case of TSP1 it is the differential localization of the protein within the tumour mass and surrounding microenvironment, rather than its level of expression in the tumour cells, which is important.

In angiogenic tumours, the stroma is characterised by inflammation; genes associated with a variety of inflammation related pathways, in particular cytokine and interleukin signalling, have been found **Ref 52, 88**. Tumour associated stromal remodelling and/or inflammation could be one of the steps necessary to trigger and/or maintain angiogenesis in some, but not all, angiogenic tumours.

[H3] Motility and invasion. How cancer cells interact with stroma is tightly associated with their ability to move and invade and there is now increasing evidence that non-angiogenic cancer cells have a highly motile and invasive phenotype. In 1997, we reported the intriguing observation that patients with non-angiogenic NSCLC at follow up developed more metastases than their angiogenic counterparts, and had, if anything, a poorer outcome²¹. These data suggest that, paradoxically, the absence of angiogenesis could actually be

associated with a more metastatic phenotype. Subsequent investigations in preclinical tumour models have shown that inhibition of the VEGF pathway can result in a switch to non-angiogenic tumours in which the neoplastic cells are more motile and/or invasive^{18, 27, 29, 89}.

Gene expression profiling analyses of angiogenic and non-angiogenic human NSCLC biopsy samples and of human brain tumour xenografts in nude rats^{81, 88} demonstrated the association of the PI3K–AKT and WNT pathways with the non-angiogenic phenotype. In non-angiogenic tumours, the PI3K–AKT pathway appears to be activated through MET following decreases in VEGF levels^{90, 91}. Total and phosphorylated cMet and hypoxic markers HIF1, GLUT1 and CAIX were also increased, while T- and E-cadherins levels were diminished. The inverse relationship observed between the levels of VEGF and cMET was demonstrated to be due to an inhibitory effect of VEGF on cMET signalling [Ref 90, 91](#). VEGF increases the interaction of protein tyrosine phosphatase 1B (PTBP1B) with cMET^{Tet}, allowing PTBP1B to dephosphorylate cMET. However, inhibition of VEGF signalling, by knocking down VEGF gene [Ref 90](#) and using an anti mouse VEGF antibody [Ref 91](#), removed this inhibitory signalling, allowing cMET to activate PI3K pathway [Ref 90, 91](#). Importantly, by specifically blocking both the cMET^{Tet} and VEGF signalling pathways, the non-angiogenic tumour phenotype was reversed from an invasive to a non-invasive phenotype^{90, 91}. PI3K/Akt is therefore involved in both angiogenic and non-angiogenic cancer cells: however, as illustrated in **Figure 4**, it is activated through different means and is associated with other different pathways and as consequence, has different roles. Total and phosphorylated cMET^{Tet}, and hypoxic hypoxia-associated markers, hypoxia inducible factor 1 (**HIF1 α**), the glucose transporter GLUT1 and carbonic anhydrase IX (CAIX) were also increased, while levels of T-cadherins and E-cadherins were diminished making the cell less adherent and more likely to invade [Ref 90, 91](#).

Both PI3K and canonical WNT pathways appear to be associated with non-angiogenic tumour cells possibly leading to the stabilization of β -catenin [Ref 81](#). PI3K signalling is also associated with the focal adhesion pathway turnover, suggesting dynamic changes in the adhesion status, while WNT signalling induces actin polymerization and activation of an axon guidance pathway, which could collectively cause increased motility and migration in non-angiogenic tumour cells [Ref 83](#). Furthermore, the activation of the canonical WNT pathway, leads to inhibition of the glycogen synthase kinase 3 β GSK3 β and as a consequence increases the stability of the transcription factor SNAIL, which has been shown to contribute to the epithelial-to-mesenchymal transition (EMT) phenotype⁹². This suggests that in non-angiogenic tumour cells, increased WNT signalling could induce EMT, potentially leading to the loss of adhesion to the ECM and the observed invasive phenotype. In addition, the axon guidance pathway induces expression of the EMT-associated transcription factor ZEB2 adding to the ZEB2 increase which happens when VEGF levels are

diminished as VEGF is an inhibitor of ZEB2 Ref 93.. ZEB2 has been shown to be a transcriptional repressor of EphrinB2 resulting in increased motility and invasion of tumour cells in preclinical models of glioblastoma⁹³ in line with the finding of a more metastatic phenotype for non-angiogenic tumours . Furthermore increased levels of ZEB2 and ZEB1, also contribute to the acquisition of an EMT phenotype in cancer cells through increased expression of SNAIL, N-cadherin and vimentin, which promotes invasion^{90, 91}.

Whilst investigating the effect of the anti-angiogenic drug sorafenib on orthotopic human liver cancer xenografts in mice, using the cell line Hep3B-hCG, Kuczynski *et al.* reported that resistant tumours were more infiltrative and non-angiogenic than drug-sensitive tumours²⁶. Analysis of the miRNA profile present in cells from non-angiogenic, resistant tumours indicated an upregulation of pathways involved with cell motility and invasion, namely axonal guidance, EMT, and signal transducer and activator of transcription 3 (STAT3) and WNT- β -catenin signalling. Using RTPCR they also detected an upregulation of mRNA for vimentin, and ZEB1 and ZEB2.

Finally, we found a higher incidence of cytoplasmic p53 in non-angiogenic lung cancers⁸⁶ and a pilot study also demonstrated a higher incidence of p53 mutations in non-angiogenic (five mutated cases out of seven) than in angiogenic (seven mutated out of twenty five cases) lung cancers⁸⁶. Cytoplasmic p53 activates the transcription co-factor junction-mediating and -regulatory protein (JMY) Ref 94 , which is also highly expressed in non-angiogenic lung cancer cells⁸⁶, and JMY induces actin nucleation through activation of the actin-related protein 2/3 (ARP2/3) complex⁹⁴. We showed¹⁴ that silencing ARP2/3 in colorectal cancer cells prevented the motility of these cells *in vitro* and blocked their ability to co-opt vessels when injected directly into the livers of mice in an orthotopic model of advanced liver metastases¹⁴. Notably, when migration was suppressed by silencing Arp2/3 *in vivo*, the tumours switched to an angiogenic growth pattern¹⁴. It is therefore possible that in non-angiogenic cells, accumulation of cytoplasmic p53 (possibly because of mutation disabling the ability of p53 to traffic into the nucleus) triggers the JMY-ARP2/3 pathway leading to increased motility (Figure 4C and 4D).

[H3] Metabolic reprogramming. Another emerging difference between angiogenic and non-angiogenic cells is the regulation of energy metabolism. Higher levels of mRNA coding for proteins associated with oxidative phosphorylation and mitochondrial biogenesis were detected in non-angiogenic lung tumours, which was confirmed at the protein level⁸⁸. This suggests a metabolic switch in non-angiogenic tumours toward higher mitochondrial activity, although this remains to be validated using functional approaches. Of interest is that the pathways linked to metabolic reprogramming identified in NSCLC tissue⁸⁸ were not identified as differentially regulated between angiogenic U87

cells and non-angiogenic IRE1-silenced U87 cells⁵². Therefore, the relevance of metabolic reprogramming could well be limited to non-angiogenic tumours in certain organs.

[H1] Clinical implications

The prognostic impact of non-angiogenic tumour growth is a matter of debate, as the results of early reports were conflicting⁵. Even within NSCLC, non-angiogenic tumour growth was reported to be both a positive and a negative prognostic factor for survival in separate studies^{40, 95}. However, in liver metastases (Figure 2B), overall survival is typically shown to be increased in the angiogenic desmoplastic HGP when compared to the non-angiogenic replacement HGP or angiogenic pushing HGP subgroups [Ref 14](#). Guidelines for the assessment of liver metastases HGPs have now been proposed⁹⁶ so they can be applied to clinical practice. [UPDATE CITATION: Van Dam PJ et al. International consensus guidelines for scoring the histopathological growth patterns of liver metastasis. British Journal of Cancer 117:1427-1441, 2017](#) Nevertheless, the impact of non-angiogenic tumour growth on patient survival seems to be highly context dependent.

Many mechanisms have been proposed to explain resistance to anti-angiogenic therapy and non-angiogenic growth is emerging as one of them^{9, 97-99}. Several mouse studies have implicated a non-angiogenic phenotype as a potential intrinsic or acquired mechanism for resistance to anti-angiogenic therapies^{29, 90, 100-102}, which has been further confirmed in more recent clinical studies^{13, 14, 23, 26, 30}. For example, in an orthotopic human HCC mouse model, tumours were observed to switch from utilization of a vasculature provided primarily by angiogenesis, to a vasculature provided almost entirely by co-opted liver blood vessels during the development of acquired resistance to sorafenib²⁶. Furthermore, in a cohort of patients with metastatic colorectal cancer receiving preoperative anti-VEGF monoclonal antibody bevacizumab in combination with chemotherapy, patients with a non-angiogenic replacement HGP responded poorly compared to those with the angiogenic desmoplastic HGP¹⁴. In addition, the majority of breast cancer liver metastases showed a non-angiogenic HGP, which is important because the combination of bevacizumab and chemotherapy has thus far failed to provide a survival benefit in metastatic breast cancer¹⁴.

In mouse models of lung cancer, the anti-angiogenic drug sunitinib suppressed the growth of subcutaneously implanted angiogenic tumours, while non-angiogenic lung metastases were resistant to the same treatment¹³. In addition, in one of the preclinical lung metastasis models utilised, sunitinib induced a switch from angiogenic to non-angiogenic tumour growth leading to acquired resistance¹³. Importantly, non-angiogenic tumours were observed more frequently in

lung metastasis from human breast cancer than from human renal cancer. This may also be an indication why treatment with sunitinib has been more effective in renal cancer¹⁰³ compared to breast cancer in phase III clinical trials⁹⁹. Taken together, these data support the involvement of vessel co-option as one of the causes for intrinsic and acquired anti-angiogenic therapy resistance²⁶.

A common conclusion from most of these studies is that a dual anti-vascular and anti-angiogenic approach, targeting both angiogenic and non-angiogenic growth patterns is pivotal. CD276, a highly conserved cell-surface protein, is found broadly overexpressed by multiple malignancies on both cancer cells and blood vessels³⁰. Notably, CD276 was expressed on both newly formed and pre-existing vessels present inside tumours but not in normal vessels outside the neoplastic mass and not during physiological angiogenesis (e.g. regenerating liver tissue)³⁰. In a mouse model, an antibody-drug conjugate pyrrobenzodiazepine-conjugated CD276, eradicated both large established tumours and metastases and improved long-term overall survival potentially as a result of targeting both angiogenic and non-angiogenic tumour blood vessels³⁰. However, as these observations are based on animal studies, work is necessary to confirm whether this would be an effective therapy for angiogenic and non-angiogenic human cancers.

Perspectives

As summarised in **Figure 1**, the relationship between tumour cells and blood vessels is more complex and diverse than originally thought, as angiogenesis, though important and widely present, is not the only mechanism by which a tumour can acquire a vasculature. The discovery of non-angiogenic neoplastic growth has opened a new field in cancer biology that has just begun to be explored. However, some firm points have been established from the studies performed so far. First of all, the recognition that tumours can also grow in the absence of neo-angiogenesis and are characterised rather by the ability to exploit blood vessels, the body pre-existing normal vessels and/or the newly sprouted, rather than by the need to induce angiogenesis¹. Second, while a relatively small number of tumours can be purely non-angiogenic, many others will contain both angiogenic and non-angiogenic areas. Furthermore, it is clear that tumours can switch between angiogenic and non-angiogenic tumour growth during progression, with respect to their anatomical location or in response to treatment. Third, the mechanisms of non-angiogenic growth appear to be influenced more by the organ in which the tumour grows than the type of tumour. Fourth, non-angiogenic neoplastic cells are associated with increased motility, an ability to infiltrate surrounding tissues and are highly metastatic. Fifth, non-angiogenic growth is one of the

many ways in which tumours can escape anti-angiogenic treatment.

However, the outstanding questions still outnumber the answers. The first key question is what makes a tumour choose to grow in an angiogenic or a non-angiogenic fashion? By acquiring more data on the biology of angiogenesis and by investigating the pathways associated with either angiogenic or non-angiogenic tumours, candidate genes are emerging for their ability to be a decision-maker. One example is ⁵² IRE-1, which is potentially important to the switch between the two states, and has provided evidences that it could indeed be involved in the cellular decision making process. Metabolic reprogramming could also be involved in some non-angiogenic tumours. However, further work will be needed to establish its incidence and whether metabolic reprogramming is the cause or consequence of non-angiogenic growth.

We need to further extend our understanding of vessel co-option: some very useful preclinical models have been already established in several malignancies ^{13, 26, 105, 106}. Work to understand the molecular mechanisms of vessel co-option are most advanced in mouse models of brain cancer, where the data show that vessel co-option is an active process driven by distinct molecular mechanisms. As different organs have different vascular characteristics, each one will need to be individually investigated to understand how co-option happens through the body. This leads to another fundamental question, so far overlooked: is there a general molecular mechanism active across tissue sites necessary for the interaction between cancer cells and newly formed vessels and, if yes, could this be another target for treatment?

Blood and lymphatic endothelial cells secrete a number of molecules called angiocrine factors that target cancer cells and, similarly in angiogenic tumours, a number of factors target the endothelial cells, including tumour-secreted angiogenic factors ⁸². Given the well-established cross talk between cancer cells and the endothelium of newly formed vessels, could there be cross-talk between cancer cells and the endothelium of pre-existing co-opted vessels? There has been minimal investigation into this field so far, but understanding the role of the endothelium in the co-opted vessels could shed more light on the behaviour of non-angiogenic tumours. It could be speculated that the unique angiocrine signatures reported in the endothelium of different organs ⁸⁴ could affect the behaviour of cancer cells and their response to treatment but this possibility remains to be thoroughly investigated.

Another challenge will be to develop a more precise correlation between radiology and pathology in order to establish suitable imaging techniques that can enable us to visualise the type of vascularization in a tumour in a clinical setting. Can we improve imaging techniques or find appropriate biomarkers to better differentiate angiogenic and non-angiogenic tumours? As treatment protocols, such as anti-angiogenic therapies, should be used in a more personalized

approach, it will be useful to determine the type of neoplastic vascularization within each patient before making decisions on therapeutic regimens. Another example of the need for such imaging tools is the follow up of patients treated with anti-angiogenic drugs: it is thought that such a treatment can sometimes “normalize” the vessel architecture and the actual benefit of the treatment is an enhanced tumour blood flow improving the delivery of chemotherapy agents or increase the efficacy of radiotherapy.

As it is well-known from the study of angiogenic tumours that the vascular status has a large impact on the delivery of drugs ¹⁰⁷, a greater understanding of the biological characteristics of endothelium in both newly formed and pre-existing vessels could also help to improve drug delivery to cancer cells. Taken together, we think that these research directions have the potential to produce new anti-vascular strategies that can give rise to improvements in cancer treatment.

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Author Contributions

[Au: Please note that we have recently revised our publication policies to ensure transparency regarding author contributions. Authors are now requested to declare their authorship contribution during the submission process; please ensure this is completed when you submit the next revision of your article. A statement of these contributions will be published in the final article. Here is an example of a typical format for the statement: E.G.F. researched the data for the article. K.E.W. provided a substantial contribution to discussions of the content. E.G.F. and K.E.W. contributed equally to writing the article and to review and/or editing of the manuscript before submission.] **TO BE DONE**

Competing Interests

The authors declare no competing financial interests.

Display Items:

[b1] box 1 – Vasculogenic mimicry

Vasculogenic mimicry is another way for tumours to receive oxygen and nutrients in the absence of both angiogenesis and the exploitation of normal blood vessels. It is less commonly observed in tumours than vessel co-option but a formal comparison of the respective frequencies has not been performed. The functional channels that form are not vessels, but are made up from tumour cells mimicking, to various degrees, the normal endothelium. Vasculogenic mimicry was first observed and then further characterised in uveal melanoma, but has since been observed in carcinomas of prostate, kidney, bladder, lung, breast, and in gliomas and sarcomas¹⁰⁸. Vasculogenic mimicry is associated with high tumour grade, invasion, metastasis and decreased survival of patients^{109, 110}.

Vasculogenic mimicry appears to recapitulate post-natal vasculogenesis as neoplastic cells potentially revert to an embryonic-like phenotype and therefore become competent at mimicking endothelial cells. The molecular mechanisms of vasculogenic mimicry are partly unclear. However, it is known that vascular endothelial (VE) cadherin, which is the main adhesion receptor in endothelial adherent junctions, seems to play an important role as its up-regulation by hypoxia inducible factor 2 (HIF2 α) triggers signalling pathways regulating vasculogenic mimicry^{109, 110}. VE-cadherin co-localises with, and phosphorylates ephrin A2 (EphA2) which activates PI3K both directly and through the focal adhesion kinase (FAK)–ERK1 and/or ERK2 pathway Ref 110. Active PI3K cleaves pro-membrane type 1- matrix metalloproteinase (MT1- MMP) and pro-MMP2 into their active forms, which in turn cleave laminin5 γ 2 into the pro-motility fragments 5 γ 2' and 5 γ 2x in the extracellular matrix and guide the reorganization of cancer cells into vessel-like channels¹¹⁰. This pathway can be inhibited by cAMP, which blocks ERK1 and/or ERK2 activity¹¹⁰. However, cAMP can also sustain vasculogenic mimicry by positively regulating VE-Cadherin expression through the NODAL–NOTCH1 and/or NOTCH4 pathway^{110, 111}.

The nuclear localization of the transcription factor TWIST1¹¹², and HER2 were shown to up-regulate tumour cell VE-cadherin in preclinical models of hepatocellular carcinoma and breast cancer, respectively, contributing to vasculogenic mimicry¹¹³. In small cell lung cancer (SCLC), circulating tumour cells have subpopulations co-expressing VE-cadherin and cytokeratins consistent with the potential to undergo vasculogenic mimicry¹¹⁴. VE-cadherin is required for vasculogenic mimicry in SCLC xenografts in mice, where the occurrence of vasculogenic mimicry decreases latency of tumour growth and cisplatin

efficacy¹¹⁴. In addition, knockdown of either MMP2 or VE-cadherin hampered vasculogenic mimicry formation in melanomas *in vivo*¹¹⁰. However, more knowledge on the functional significance of vasculogenic mimicry is warranted to provide new targets for therapeutic intervention.

Figure legends

Figure 1. Classification of the different mechanisms of tumour vascularization.

As primary and metastatic tumours grow they experience hypoxia which leads to the secretion of a number of growth factors, which are able to trigger the formation of new vessels through the process of angiogenesis (with sprouting of new vessels from the pre-existing vasculature being the most common) or post-natal vasculogenesis (where vessels are formed from stem cells). However, in some tumours for reasons still unknown, the formation of new vessels does not occur and the tumour can grow by either exploiting pre-existing vessels or, in a minority of cases, by vasculogenic mimicry in which tumour cells form channels in which blood can flow. It remains unclear why tumour growth is typically supported by one mechanism over the other. While a minority of tumours can be completely non-angiogenic, in many tumours it is common to have both angiogenic and non-angiogenic areas. Of particular interest is the position of Vasculogenic mimicry (VM) which is described as the ability of tumour cells to form vessel-like networks: another way for tumours to receive blood in the absence of angiogenesis [Ref: MANIOTIS, A. J., FOLBERG, R., HESS, A., SEFTOR, E. A., GARDNER, L. M., PE'ER, J., TRENT, J. M., MELTZER, P. S. & HENDRIX, M. J. 1999. Vascular channel formation by human melanoma cells in vivo and in vitro: vasculogenic mimicry. *Am J Pathol*, 155, 739-52..](#) However, it has subsequently been discovered that vasculogenic mimicry is due to cancer cells "stem cell" capacity to differentiate towards a vascular phenotype. This ability is very variable from cancer cell to cancer cell. The results is the generation of channels with a wide spectrum of appearance: from crude channels made up by clearly neoplastic cells up to normal looking vessels, which still are lined by neoplastic cells, as the endothelium contains genomic alterations like the other cancer cells. As it is now emerging that in some instances vasculogenic mimicry appears to be driven by angiogenic pathways ([Ref SEFTOR, R. E., HESS, A. R., SEFTOR, E. A., KIRSCHMANN, D. A., HARDY, K. M., MARGARYAN, N. V. & HENDRIX, M. J. 2012. Tumor cell vasculogenic mimicry: from controversy to therapeutic promise. *Am J Pathol*, 181, 1115-25.](#)) it is well possible that some tumours containing vasculogenic mimicry are actually angiogenic but trigger new vessels formation from cancer cells rather than from pre-existing vessels or normal stem cells.

These patterns of growth are represented in **Figure 2**. Question marks (?) indicate a process or mechanism that is currently unknown.

Figure 2. Patterns of tumour growth in relation to blood vessels.

A) Schematic representations of tumour growth patterns in the lung. In the normal alveolar parenchyma, the lung alveolar spaces are filled by air and are delimited by the alveolar walls. Inside the alveolar walls are blood vessels surrounded by a thin layer of extra cellular matrix. Type I and type II pneumocytes line the alveolar walls and separate the vessel from the air space. Three basic angiogenic patterns of tumour growth are observed in the lung, which can be found both in primary and metastatic lesions: i) *diffuse*, in which the tumour grows without any identifiable architectural structure by replacing normal parenchyma; ii) *basal*, in which nests of neoplastic cells are surrounded by stroma containing vessels and inflammation, which replaces normal parenchyma and; iii) *papillary*, in which papillae made up by a thin fibrous stalk, containing the new vessel, are lined by neoplastic cells to form the tumour. This latter growth pattern is mostly associated with the primary lung bronchioalveolar carcinoma, and some residual normal lung structure can remain visible in some areas of the tumour.

Three non-angiogenic patterns of tumour growth can also be observed: i) *alveolar*, the most common type of non-angiogenic malignant growth in the lung, in both primary and secondary tumours in which neoplastic cells fill the alveolar spaces. Subsequently the pneumocytes disappear: it could be argued that in the initial phase the tumour cells co-opt the epithelium rather than the vessel. ii) *interstitial*, which is seen only with metastatic lesions in the lung, in which the metastatic cells extravasate and grow along the alveolar vessels but do not enter the alveolar spaces and iii) *perivascular cuffing*, also only seen in metastases, in which the cells grow along a medium size artery.

B) Schematic representations of tumour growth patterns in the liver. In the liver, blood flows from the portal vein and from branches of the hepatic artery, located in the portal spaces, through the sinusoids reaching the centrolobular vein. Liver sinusoids are vascular channels lined by specialized endothelial cells containing numerous pores (fenestrae) and scattered cells with phagocytic ability, known as Kupffer cells. Between the endothelium and the hepatic cells is the space of Disse, in which fluids can carry molecules from the hepatocytes to the blood flow and vice versa. Two angiogenic types of tumour growth exist: i) desmoplastic in which cancer cells are surrounded by stroma which separate them from the normal liver. The reticulin pattern of the normal liver is not preserved and; ii) pushing, in which a tumour containing new vessels “pushes” directly against the normal hepatic tissue; again the reticulin pattern of the normal liver is not preserved. In the non-angiogenic replacement type of tumour growth, observed in both primary

and secondary liver tumours, the neoplastic cells effectively replace the hepatocytes but preserve the liver architecture.

C) Schematic representations of tumour growth patterns in the brain. Two growth patterns exist, which contain a tumour core consistent with angiogenesis, and a clear non-angiogenic periphery in a mixed pattern of growth: in the *single cell pattern*, single cells or small clusters spread into the brain parenchyma, without coming into contact with the pre-existing vessels and without inducing formation of new vessels; in the vascular co-option pattern the cancer cells on the edge of the lesion, or even away from the main lesion, advance by co-opting the normal vessels (perivascular cuffing), whilst deeper in the lesion itself angiogenesis is triggered. Two other growth patterns are completely angiogenic: a non-infiltrating or displacing pattern with a discrete tumour-parenchyma border while the other pattern named *infiltrating* has irregular edges with irregular columns of cells infiltrating the parenchyma and inducing new vessel formation. All these patterns can be seen in both primary and metastatic brain tumours.

Figure 3 - Mechanisms of vascular co-option.

Schematics shown are based on studies of brain tumours. The mechanisms driving vessel co-option are highlighted in **A)** primary tumours of the central nervous system and **B)** metastatic lesions to the brain.

A) In glioblastoma, the neoplastic cells move toward the blood vessels along a concentration gradient of bradykinin. Once the cancer cells reach the vessels, two possible events have been described. In one case, the neoplastic cells produce pseudopodia and in a process mediated by CDC42, fuse with pericytes creating a hybrid cell type. If CDC42 activity is blocked, the fusion does not occur and some pericytes can mobilise and acquire an anti-tumour activity [Ref .57](#) In the second mechanism, neoplastic cells place themselves between astrocytes and pericytes, blocking their physiological interaction and altering the function of the pericytes.

B) The first step for cancer cells metastasizing to the brain is extravasation, which is also mediated by CDC42. In the model illustrated, the non-angiogenic metastatic cell infiltrating the brain tissue, is characterised by secretion of neuroserpin and expression of the L1 cell adhesion molecule (L1CAM). Neuroserpin blocks the plasminogen activator, preventing the formation of plasmin. A lack of plasmin release into the microenvironment prevents both the death of the cancer cell (through FAS ligand (FASL) induced apoptosis) and the block of the L1CAM. In this way the

metastatic cell, protected from cell death and maintaining L1CAM expression, can co-opt the vessel through L1CAM.

Figure 4. A schematic identikit of the biological and phenotypic differences between the angiogenic and non-angiogenic cancer cell.

For each type of cancer cell, proposed signalling molecules and pathways, which are up-regulated are shown. The schematics have been constructed using a variety of sources, including different *in vitro* or *in vivo* models, data from human tumours, mRNA and proteins detected with high throughput expression screens or immunohistochemistry, in an attempt to illustrate the differences so far described. However, it should be noted that the pathways shown are not exhaustive. It is likely that differences between angiogenic and non-angiogenic tumors vary according to the preclinical model investigated and the organ involved.

A) shows how an angiogenic cancer cell can be activated by a large number of growth factors, including vascular endothelial growth factor (VEGF), which activate the PI3K and the MAPK pathways, regulating proliferation and apoptosis and inducing angiogenesis and stromal remodelling. Over-expression of the inositol-requiring enzyme 1 (IRE1) gene suppresses motility through inhibition of actin expression REF 52, while overexpression of integrins maintain cell-cell adhesion. Activation of cytokines and interleukins induces inflammation, which contributes to angiogenesis and stromal remodelling. Hypoxia induces hypoxia inducible factor 1 (HIF-1) expression that, in this context, is associated with increased VEGFA production.

B) The phenotypic features of angiogenic cancer cells include increased adhesion, between cells and with the basal membrane and induction of stroma formation, inflammation and angiogenesis.

C) In some cancer cells, PI3K-AKT, MET and the canonical and non-canonical WNT pathways may be involved in their non-angiogenic growth. Proliferation is maintained through β -catenin stabilization but the most striking feature is the large signalling network inducing motility and invasion. Epithelial-to-mesenchymal transition (EMT) is activated through the canonical WNT pathway, as glycogen synthase kinase 3 β (GSK3 β) is inactivated, which subsequently stabilizes the EMT-transcription factor SNAIL. Through the non-canonical WNT pathway, the axon guidance pathway is activated, which through the transcription factor ZEB2 also activates EMT. Increased motility is also triggered by a number of other events including activation of the non-canonical WNT pathway, which induces actin polymerization and inhibits cell-cell adhesion by blocking ephrin 2 (EPH2), and accumulation of cytoplasmic p53 activating junction-mediating

and -regulatory protein (JMY), which induces Arp2/3 activation with consequent actin nucleation. The number of cell-matrix adhesions is also affected by activating the turnover of the focal adhesion pathway. In addition, energy metabolism is affected by changes in the levels of the glucose transporter GLUT1 and components involved in oxidative phosphorylation. Although the hypoxia response is present and it is possible that HIF1 induces some VEGF production; however, no angiogenesis is triggered.

D) The phenotypic features of non-angiogenic cancer cells include reduced adhesion to each other and to the extracellular matrix and increased motility and invasion. The tumour cells are attracted and migrate towards pre-existing vessels via a bradykinin gradient.

CA9, carbonic anhydrase; EGFR, epithelial growth factor receptor; ER, endoplasmic reticulum; FGFR1, fibroblast growth factor receptor 1; IL-6, interleukin-6; L1CAM, L1 cell adhesion molecule; MMP, matrix metalloproteinase; PDGFR, platelet-derived growth factor receptor; TGF β , transforming growth factor β ; TSP1, thrombospondin 1; VEGFR, VEGF receptor; XBP1, X-box binding protein 1.

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Table of Contents Summary

In this Opinion article, Donnem *et al.* outline the evidence for non-angiogenic tumours which use pre-existing blood vessels to support tumour growth, and discuss the studies that are beginning to define their unique biology.

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