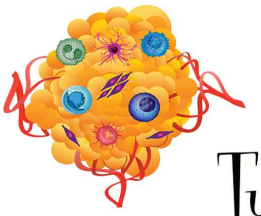
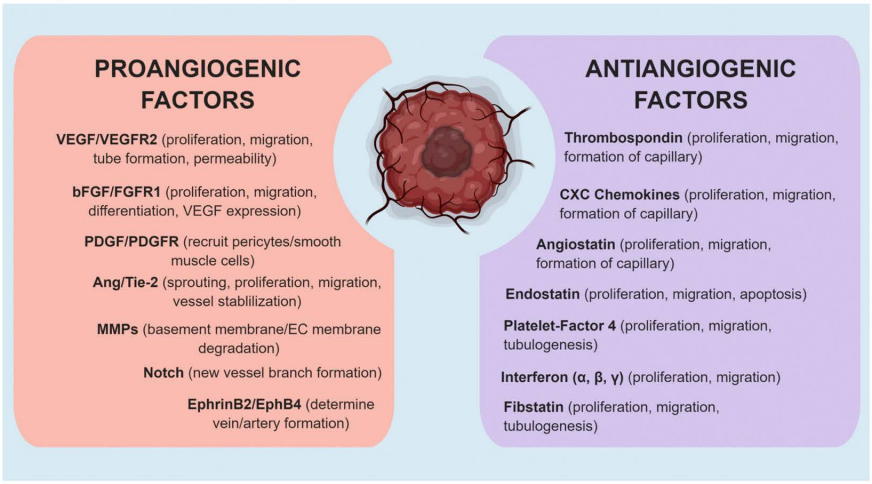


The Tumor Microenvironment 2: Tumor Angiogenesis and Metastasis

A. The Hows and Whys of Angiogenesis

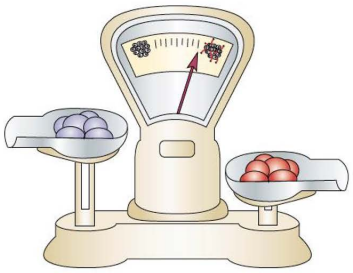
1] **the process of angiogenesis (def. *"a physiological process involving the growth of new blood vessels from pre-existing vessels"*) represents a delicate balance between the expression of pro- and anti-angiogenic proteins in response to changes in the cellular microenvironment; one or more signaling pathways 'notify" the critical cells that conditions have changed, and that new gene expression is required**

M. Yunus, et al. BBA - General Subjects 1863 (2019) 1217–1225



The Angiogenic Switch is triggered by a change in the balance of pro- versus anti-angiogeneic factors.

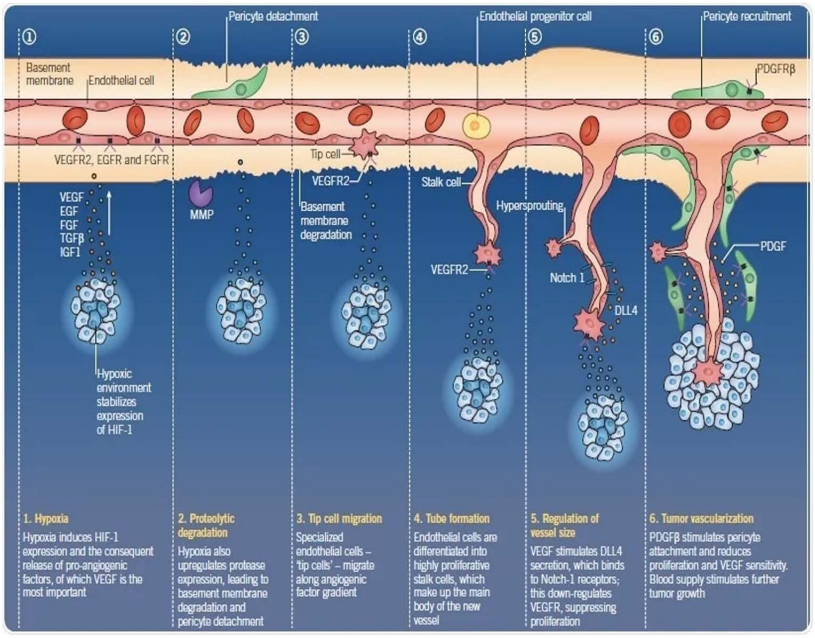
(Tumors typically have WAY more pro- than anti-.)



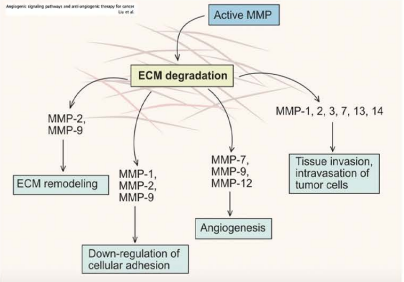
Bergers G et al. Nat Rev. Cancer 2003

a) there are different flavors of angiogenesis, depending on the particular situation...

Sprouting Angiogenesis - the first type of angiogenesis identified, and that occurs in several well-characterized stages. Sprouting can occur at a rate of *several millimeters per day*; ***thought* to be the major kind of angiogenesis in tumors**

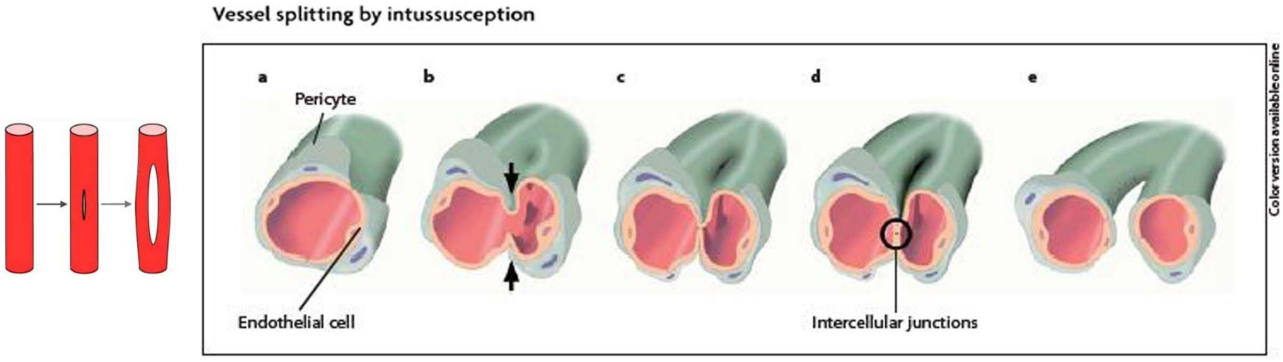


- Gradients of VEGF, PDGF and bFGF from the tumor activate vascular endothelial cells and cause them to release ***matrix metalloproteinases*** (MMPs) that degrade the basement membrane.



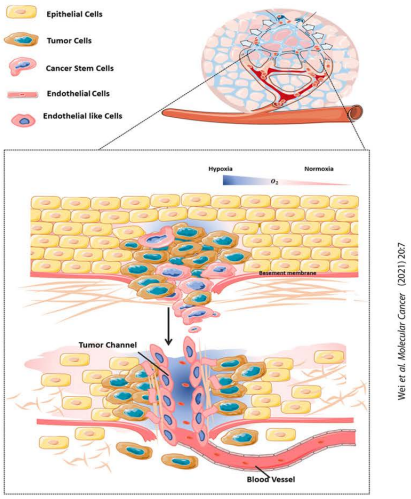
- This frees them to proliferate and migrate toward the tumor mass.

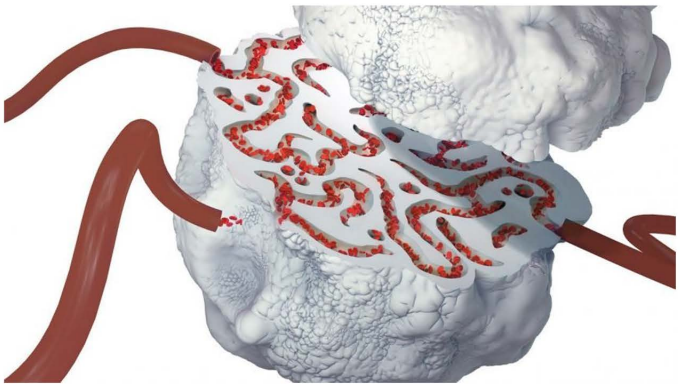
Intussusceptive Angiogenesis - aka "splitting angiogenesis", is especially important during embryonic development because there are not enough resources to create a rich microvasculature with new cells every time a new vessel develops. It allows a vast increase in the number of capillaries without a corresponding increase in the number of endothelial cells. There are four phases of intussusceptive angiogenesis:



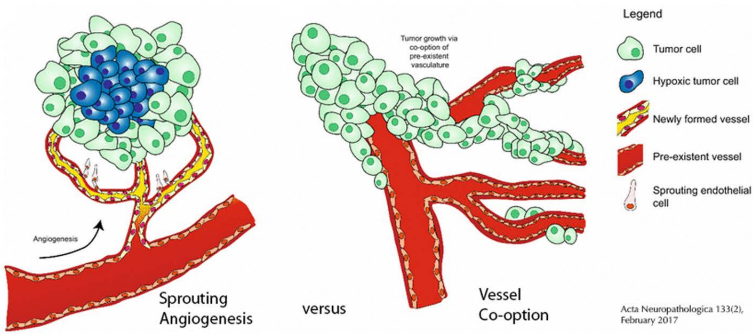
First, the two opposing capillary walls establish a zone of contact. Second, the endothelial cell junctions are reorganized and the vessel bilayer is perforated to allow growth factors and cells to penetrate into the lumen. Third, a core is formed between the two new vessels at the zone of contact that is filled with pericytes and myofibroblasts. These cells begin laying collagen fibers into the core to provide an extracellular matrix for growth of the vessel lumen. Finally, the core is fleshed out with no alterations to the basic structure.

Vasculogenic Mimicry - occurs when tumor cells arrange themselves into tubular channels that mimic blood vessels, and that ultimately connect with actual vessels from the host, allowing blood to flow into the tumor mass

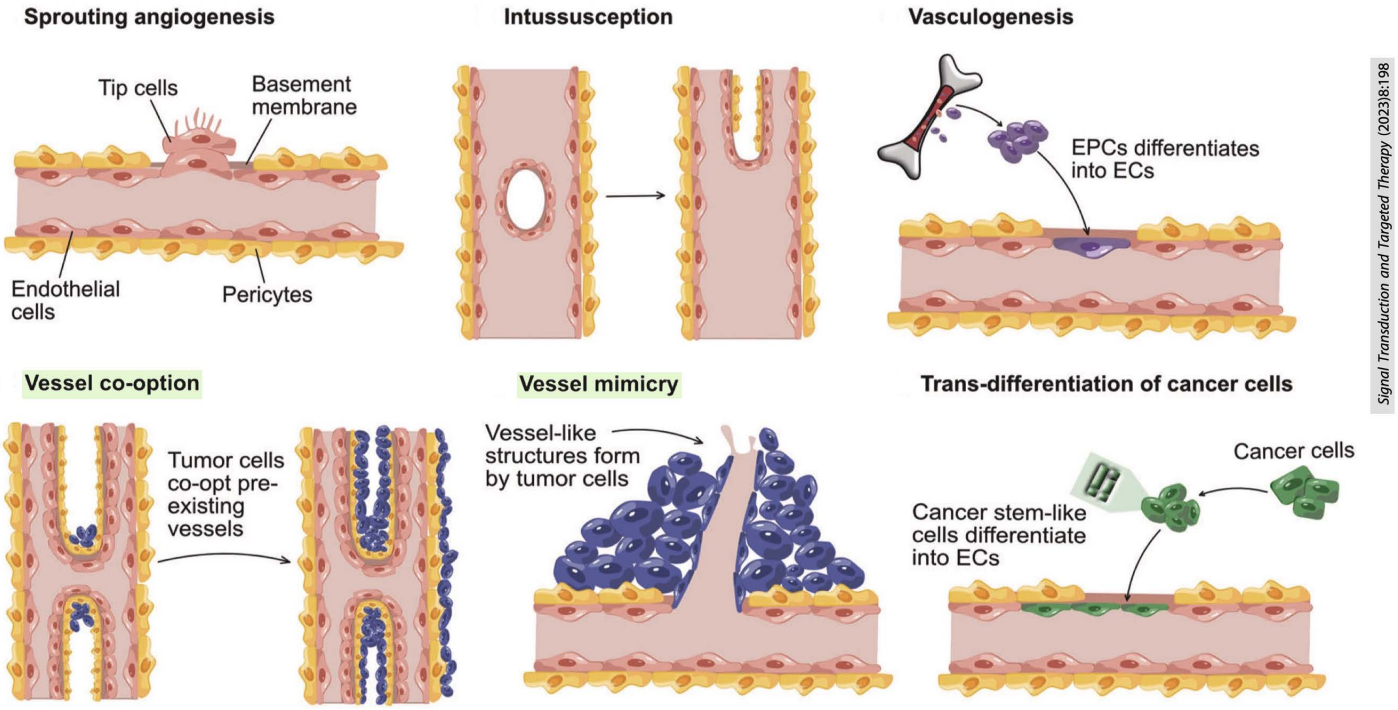




Vessel Co-option - occurs when tumor cells (especially when establishing new metastases) proliferate and surround pre-existing host vessels, and take them over

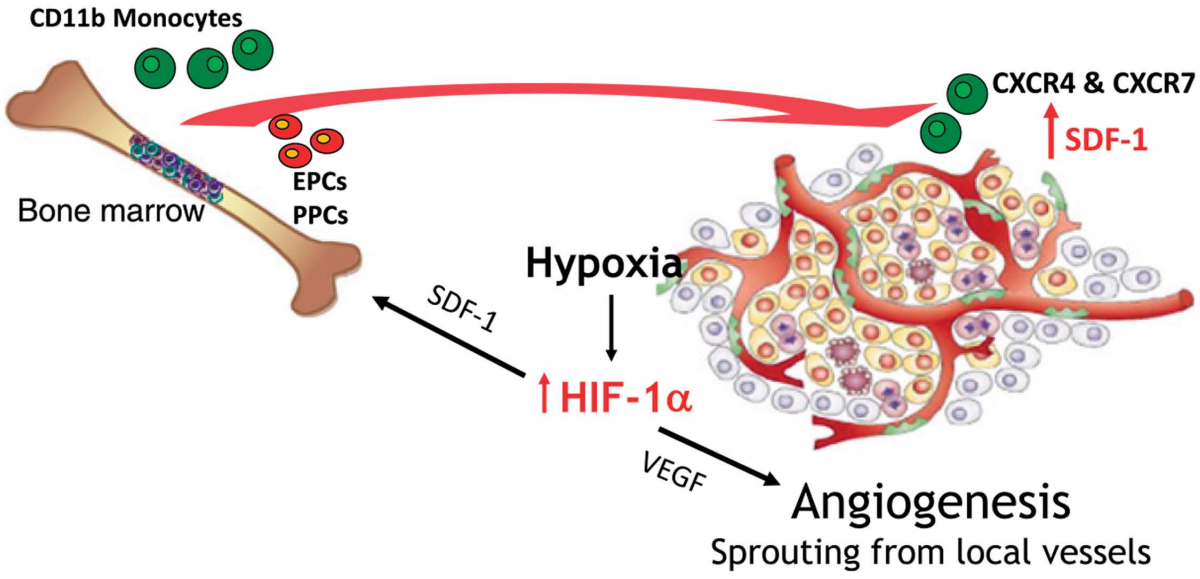


a. Note that *vascular mimicry and vessel co-option can save the tumor some energy and resources because no actual angiogenesis is needed*

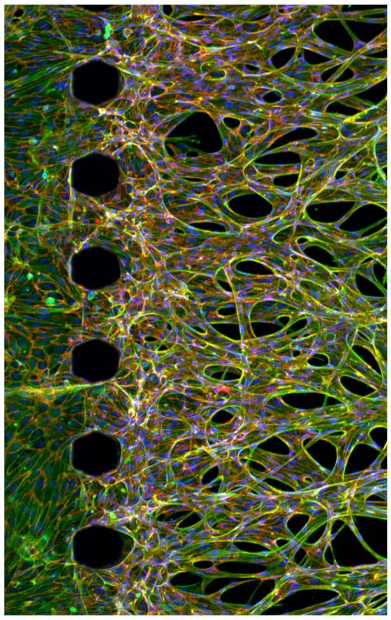


Signal Transduction and Targeted Therapy (2023)8:198

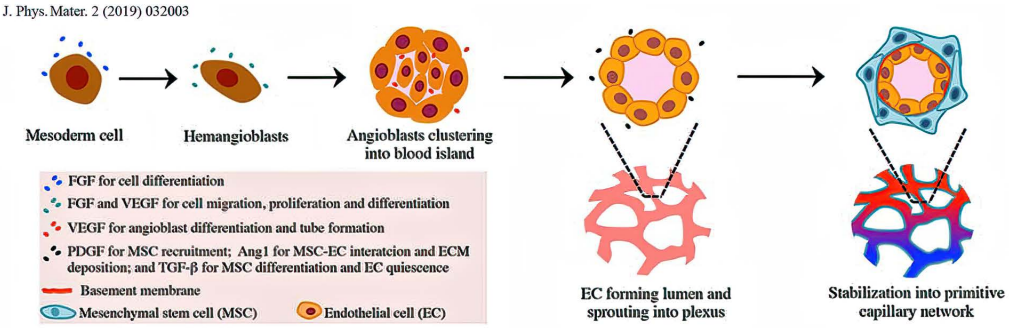
Vasculogenesis - an alternate blood vessel-generating mechanism that occurs either when other methods of angiogenesis are inhibited or when the tumor's vasculature is completely destroyed (such as, after a full course of radiotherapy to a high total dose), ***in which new vessels are made "from scratch" using bone marrow and/or stroma-derived progenitor cells (MSPCs) that then differentiate into the various blood vessel components*** (vascular endothelial cells, pericytes, etc.)



1. vasculogenesis is the process used during embryonic development to create the embryo's vascular system, but it generally isn't needed any longer once "regular" angiogenesis kicks in...however sure enough, tumors have learned how to reactivate this process

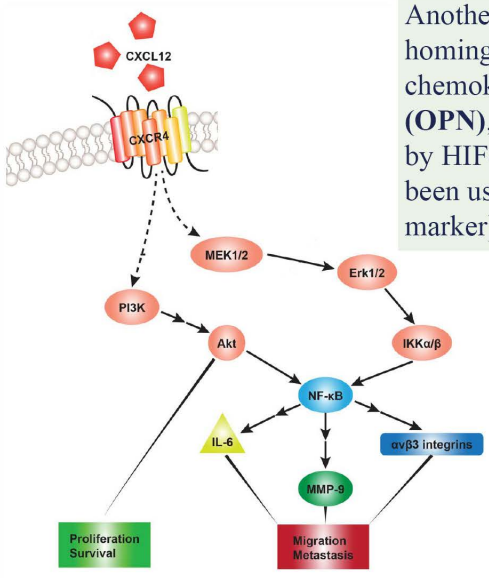


These MSPCs assemble into a primitive vascular plexus that, under the influence of some of the same growth factors that control regular angiogenesis, differentiate into new tumor blood vessels



How do these marrow-derived cells know where the tumor is?

- Answer: They have homing beacons, chemokines produced by the tumor, and whose genes are regulated by HIF-1. Their production ramps up when the resident tumor vasculature is compromised, which results in more hypoxia.
- **Stromal cell derived factor-1 (SDF-1, CXCL12)** is released by tumors into the circulation, and upon reaching the bone marrow, binds to its receptor, **CXCR4**, on the MSPCs. This gives them the ability to leave the bone marrow, survive the migration through the circulation to the tumor site, take up residence, and multiply.

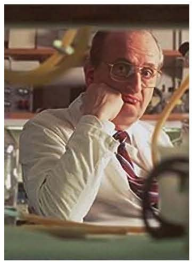


Another common homing beacon is the chemokine **osteopontin (OPN)**, also regulated by HIF-1 (that has also been used as a hypoxia marker)

2. ***vasculogenesis is becoming an increasingly important clinical target, as it is now thought to be one of the main ways tumors are able to recur after radiotherapy despite their original vasculature being completely destroyed***

B. Continuous angiogenesis is one of the hallmarks of cancer...why do tumors need it, what triggers it, and why are the resulting vessels so abnormal?

1] angiogenesis is an absolute requirement for the development of any solid tumor (as well as the "take" of any distant metastasis)

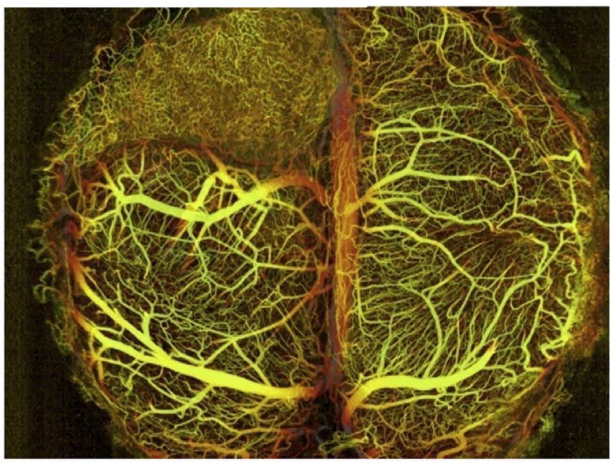


a) ***"no tumor mass would grow beyond about 2 mm in diameter in the absence of angiogenesis"*** claimed the late Dr. Judah Folkman (NEJM 285: 1182, 1971)...just before being laughed out of virtually every scientific forum in which he made this statement (go figure!)

b) ***tumors manage to stimulate angiogenesis by tipping the balance in favor of the (over-production) of pro-angiogenic factors; that being said, the often severe imbalance and need to generate new vaculature quickly and extensively causes the new vasculature to be grossly "immature" and abnormal***

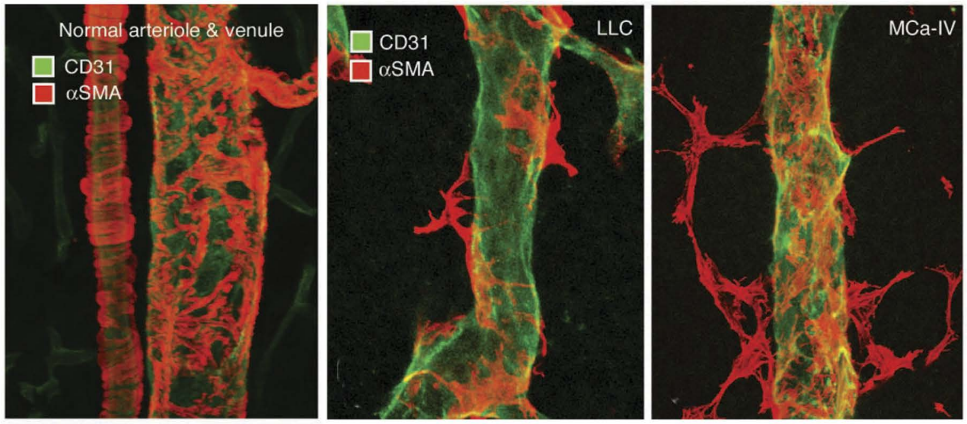
1. among the abnormalities in tumor vasculature:
   - tortuosity, variable lumen size, blind ends, shunts, etc. (leads to hypoxia)
   - leakiness (leads to high interstitial fluid pressure in tumors)
   - lack of innervation and/or smooth muscle cells (can cause paradoxical responses to vasoactive agents)

Based on all the vascular abnormalities, it's not hard to find where the tumor is!



V. Askoxylakis et al. / Advanced Drug Delivery Reviews 119 (2017) 159–174

Vasculature of a brain tumor (upper left quadrant) and the surrounding brain of a mouse. The tumor vessels are abnormal, characterized by tortuousity and hyperpermeability.

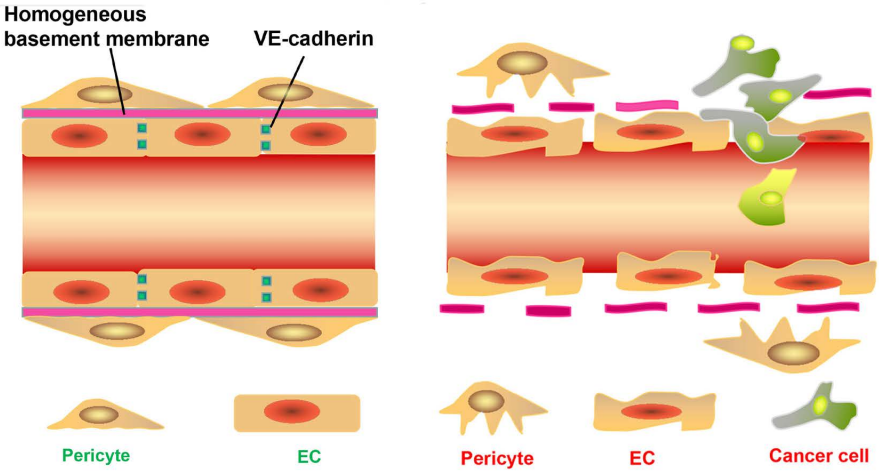


Unlike their normal counterparts, tumor vessels are not lined uniformly by vascular endothelial cells on their interiors and by pericytes on their exteriors. Basement membranes can also contain gaps.

Normal murine vessels (far left) compared to vessels from two mouse tumors (center and right).

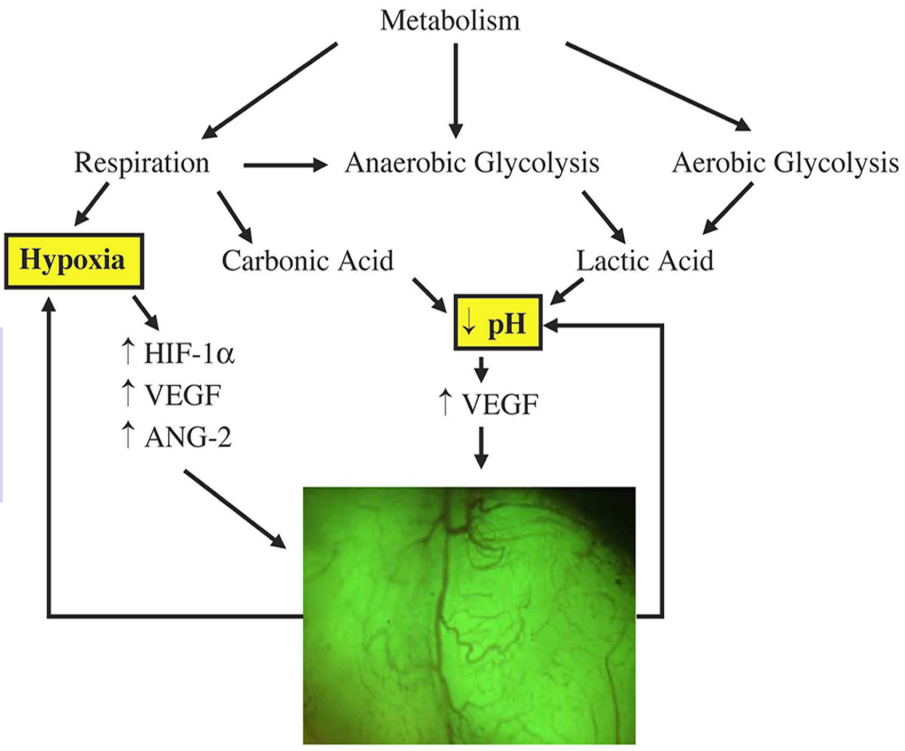
Green stain = vascular endothelial cells and basement membrane

Red stain = pericytes



Closer-up view of a normal (left) versus tumor (right) blood vessel

2] What are the microenvironmental conditions during the (very) early history of primary or metastatic tumors that turn on the ***"angiogenic switch"***?

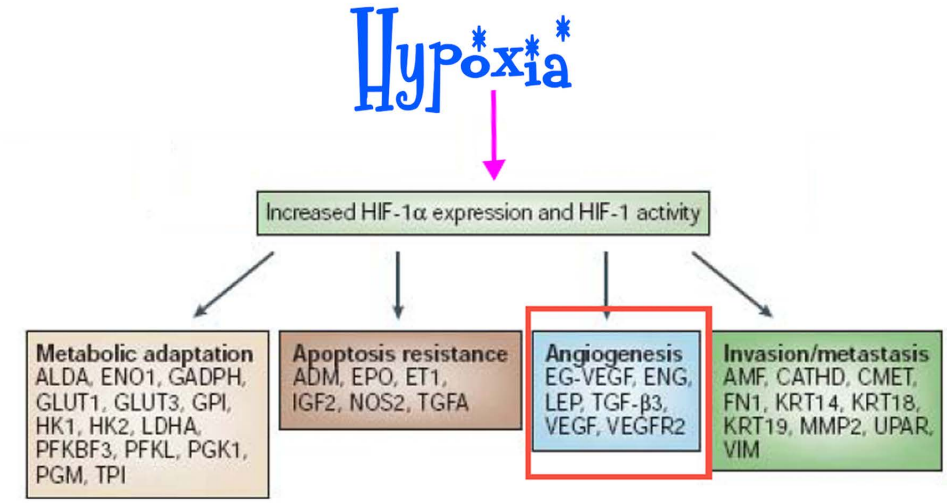


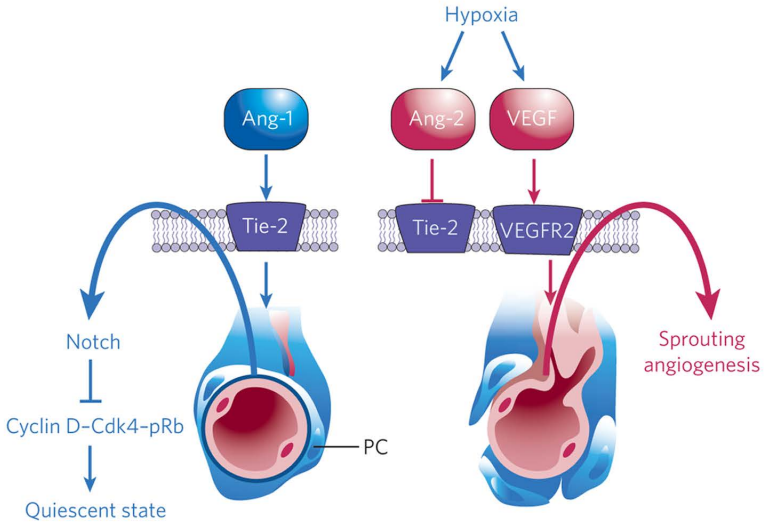
The development of hypoxia and the acidification of the tumor micro-environment are key stimuli that flip the angiogenic switch. Activation of certain oncogenes (e.g., *RAS*) also contribute.

M.W. Dewhirst et al / Hematol Oncol Clin N Am 18 (2004) 973–990

a. molecularly speaking, how does the angiogenic switch trigger?

HIF-1α, the hypoxia sensing transcription factor, is stabilized and activated under hypoxic conditions, and goes on to activate (among other things) many angiogenesis-related genes – especially VEGF – by binding to the hypoxia responsive regulatory regions (HRE) of the target genes.





VEGF-A and angiopoietin-2 are key angiogenic factors induced by hypoxia.

VEGF and Ang-2 released in excess by tumor cells bind to the VEGR and Tie-2 receptors, respectively, on the surface of vascular endothelial cells. The binding triggers multiple signaling cascades that together allow these cells to proliferate, migrate, resist cell death, and degrade the surrounding stroma in order to facilitate new vessel formation.

Normally, this process is kept in check due to Ang-1's binding to the Tie-2 receptors, which keeps the vascular endothelial cells well-differentiated and quiescent.

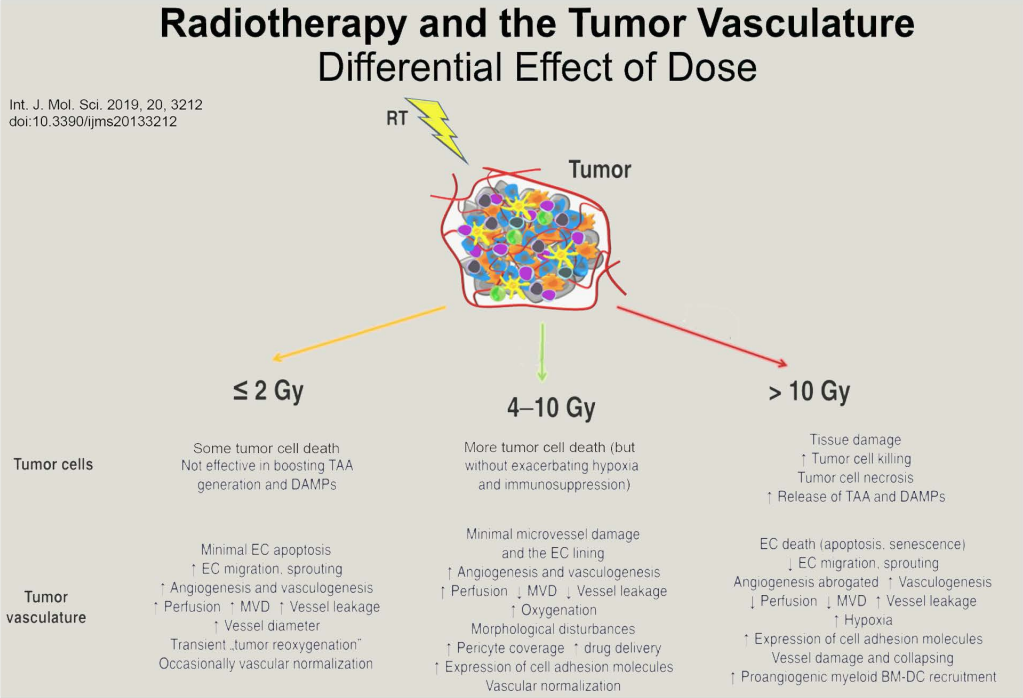
How does tumor vasculature respond to irradiation?

Short Answer: It's complicated

Longer Answer:

A. first, ***it's complicated because tumor vasculature is very dynamic and is constantly remodeling itself over the tumor's lifetime anyway, and that irradiation can perturb this process in multiple ways***

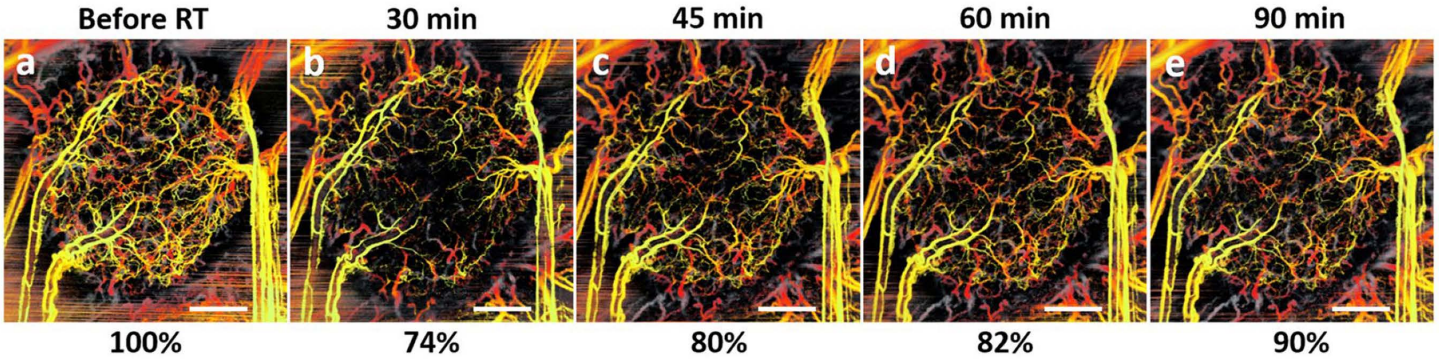
B. and second, ***it's complicated because radiation's effects on tumor vasculature vary depending on the dose***



Especially noteworthy is that for conventional fractionation and mild/moderate hypofractionation, both angiogenesis and vasculogenesis are stimulated, and vessel perfusion increases (i.e., improved oxygenation).

It is only in the case of "extreme" hypofractionation (>10 Gy/fxn) that angiogenesis, but not vasculogenesis, is inhibited, and vessel perfusion decreases (i.e., more hypoxia).

1. in human tumor xenografts, doses per fraction up to about 10 Gy cause a decrease in microvessel density (most apparent for the higher end of this dose range) and reduced vascular perfusion *within 30 minutes of irradiation*

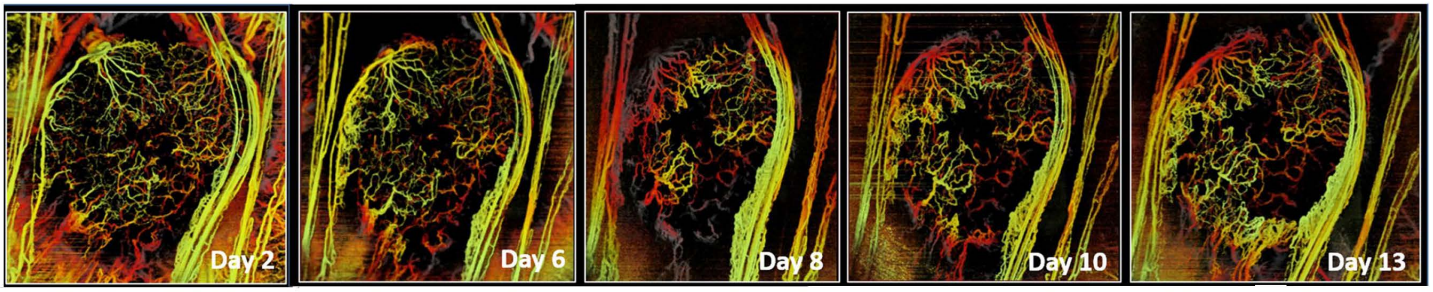


Prompt microvascular response in human pancreatic cancer xenografts irradiated with a large single dose of 10 Gy, resulting in a slight reduction in tumor vascularity/perfusion in first 1.5 hours after irradiation. (Vasculature imaged using non-invasive 3D optical coherence tomography.)

SCIENTIFIC REPORTS | (2018) 8:38 | DOI:10.1038/s41598-017-18635-w

(Reminder: the tumor is human but the vasculature is murine)

2. despite a reduction in vascularity within the first hours to a day or two after irradiation with a large, single dose, vessels regrow fairly rapidly thereafter



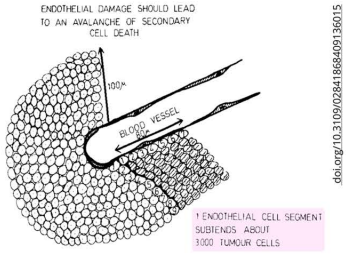
Targeting Tumor Angiogenesis as a Clinical Strategy

A. What is the rationale for doing so?

1) *destroying all the vasculature of an existing (primary) tumor would, in theory, starve it completely of both nutrients and oxygen, leading to massive ischemia, necrosis and cell death*

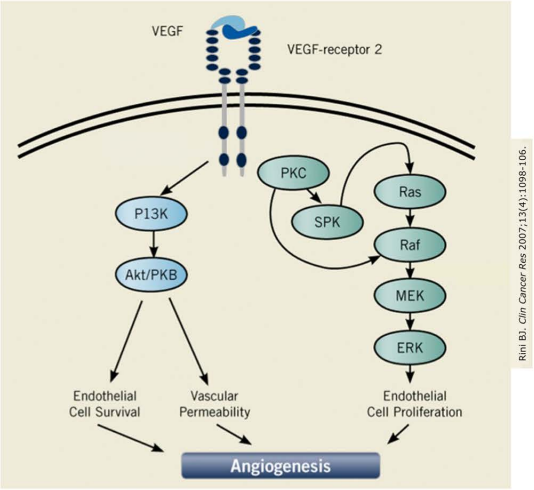
2) *even partial angiogenesis inhibition has the potential to knock out a whole boatload of tumor cells, since any one tumor blood vessel likely supplies around 1,000 tumor cells*

Illustrating how many tumor cells (~1,000!) are potentially fed by a single, small capillary segment... and how many could die if that vessel was lost



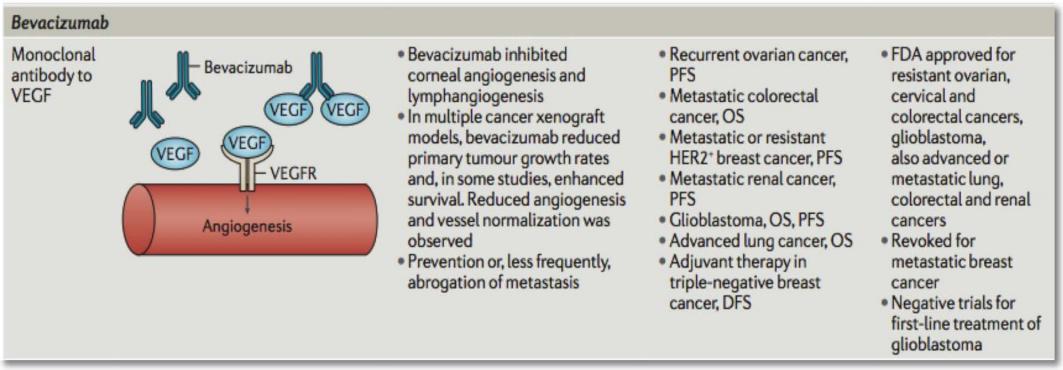
3) angiogenesis inhibition might also be useful in **preventing micro-metastases from recruiting their own blood supply, which at minimum would stop their further growth, if not kill them outright**

B. What approach to take? Review the major signaling pathways involved in angiogenesis, and pick a druggable target...



Receptor tyrosine kinases, like those that bind VEGF, EGFR, HER-2, etc., activate two major signaling pathways – the Ras-Raf-Mek-Erk and PI3K-AKT pathways – that are associated with cell survival, cell cycle regulation, proliferation and protein synthesis, all of which are needed for angiogenesis. (And these are only a few of the properties these pathways govern.)

1. First attempt at inhibiting angiogenesis was using **bevacizumab** *(FDA approved in 2004)*



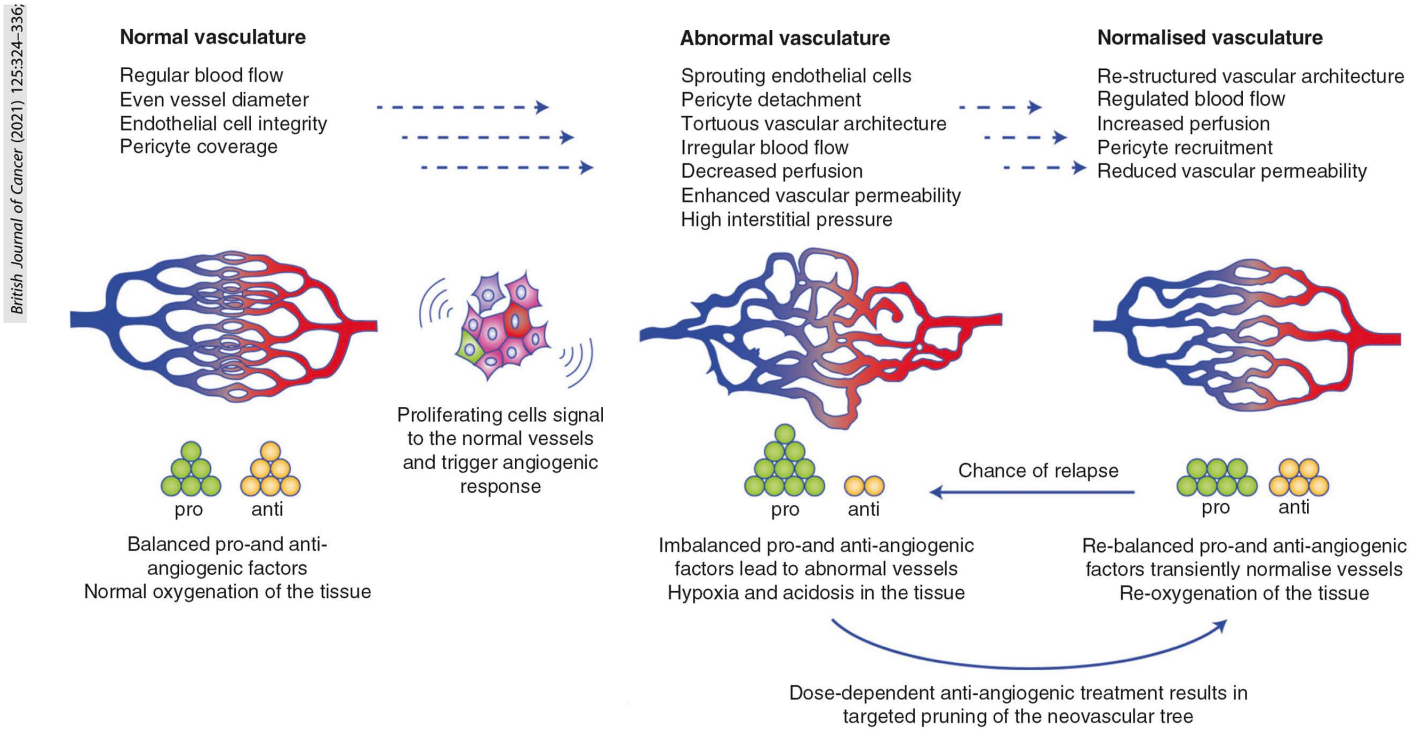
Bevacizumab is a monoclonal antibody that removes VEGF so it can't initiate (or maintain) angiogenesis. *Many other drugs are now available that target different steps in the angiogenesis process.*

a. In the early studies with bevacizumab, ***the assumption was that initially, the tumor would become more resistant to radiation (i.e., more hypoxia) and drugs (i.e., reduced tumor access)***, but that over time, the tumor cells would die of nutrient and oxygen deprivation....

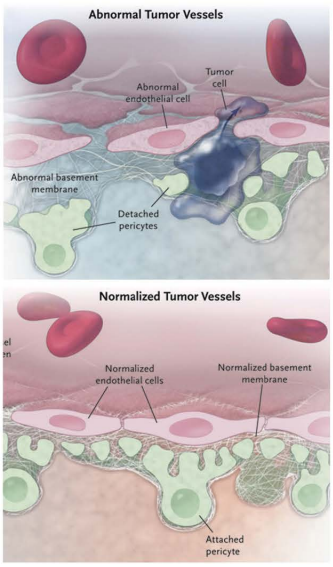
But is that what happened?

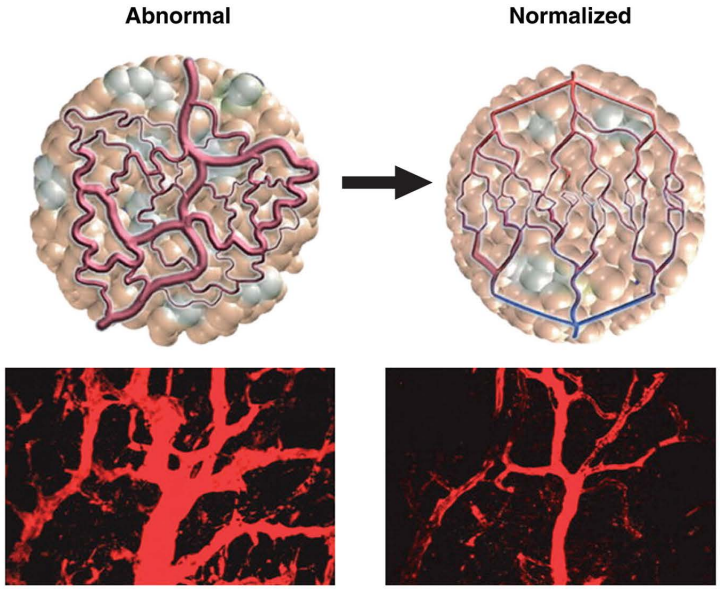
NOPE! What happened was that bevacizumab <u>sensitized</u> tumors instead! Why?????

Answer: **Vascular Normalization**



a) ***Vascular normalization occurs when an anti-angiogenic drug prunes away some of the most aberrant, smaller tumor vessels, allowing the remaining vasculature to assume a more normal conformation and function; molecularly-speaking, this occurs because the drug restores the balance between pro- and antiangiogenic factors***

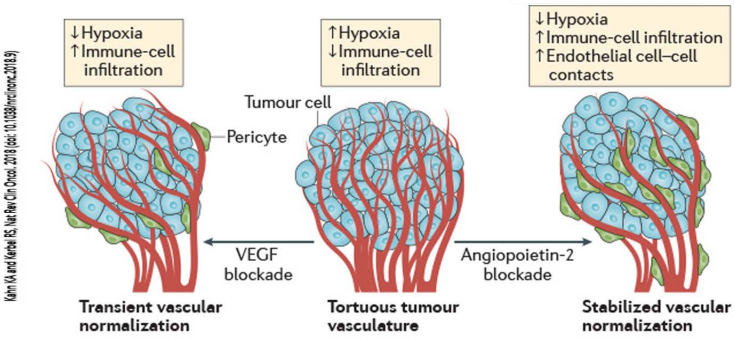




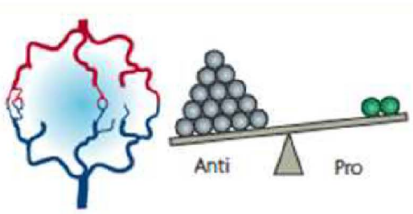
Goel S...Jain RK. Normalization of the vasculature for treatment of cancer and other diseases. *Physiol. Rev.* 2011

2. the bad news is that **vascular normalization is temporary**, and it is possible that the tumor could then compensate by producing even more pro-angiogenic factors, which would only make matters worse

a. *one proposed solution is to add additional anti-angiogenics in sequence, in order either to maintain the vascular normalization effect indefinitely and/or to promote further destruction of the tumor's vasculature*



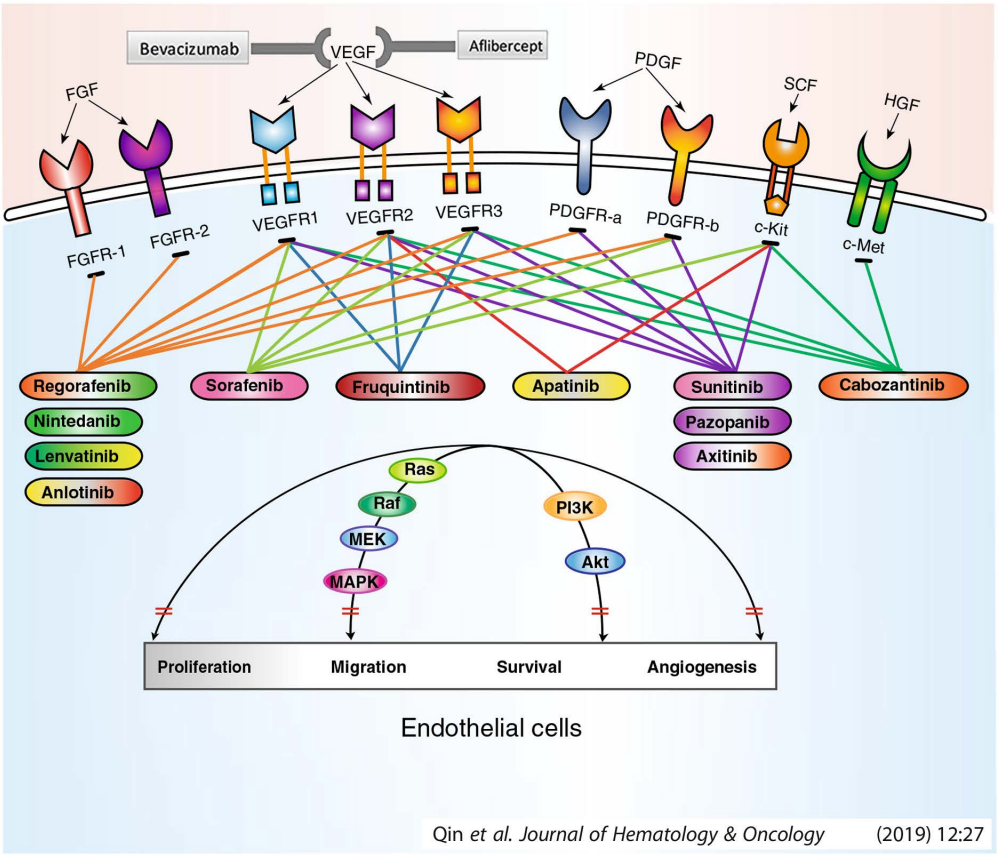
OR



5) and finally, *because we now have a much better understanding of the molecular underpinings of the angiogenesis process, we've realized that there are many possible strategies to inhibit it, and many different targets – alone or in combination – that may be "druggable"*

FDA-approved anti-angiogenic drugs

- Axitinib (Inlyta®)
- Bevacizumab (Avastin®)
- Cabozantinib (Cometriq®)
- Everolimus (Afinitor®)
- Lenalidomide (Revlimid®)
- Lenvatinib mesylate (Lenvima®)
- Pazopanib (Votrient®)
- Ramucirumab (Cyramza®)
- Regorafenib (Stivarga®)
- Sorafenib (Nexavar®)
- Sunitinib (Sutent®)
- Thalidomide (Synovir, Thalomid®)
- Vandetanib (Caprelsa®)
- Ziv-aflibercept (Zaltrap®)



Qin *et al. Journal of Hematology & Oncology* (2019) 12:27

b. **targeting tumor vasculature and strategies for angiogenesis inhibition**

1) vaguely non-specific, physiological methods - these are drugs that technically don't destroy tumor blood vessels or prevent new vessel formation, but rather, either open up existing tumor vessels for easier access of traditional chemotherapy agents and/or to reduce tumor hypoxia, or else shut down tumor blood flow to cause massive ischemia and necrosis

a] some examples:

- Verapamil, Hydralazine, Nitric Oxide, Nicotinamide, Hyperthermia - increase blood flow to tumors (sometimes doing the exact opposite to normal tissues)

- Combretastatin(s) - constrict tumor blood flow by altering the size, shape and reproductive capacity of vascular endothelial cells (through cytoskeletal disruption/depolymerization of microtubules)

2) preventing the production of pro-angiogenic factors (VEGF, bFGF, PDGF) by tumor cells in the first place, and/or adding back anti-angiogenic factors to counterbalance them - might someday be accomplished more convincingly by gene therapy, but in the meantime...

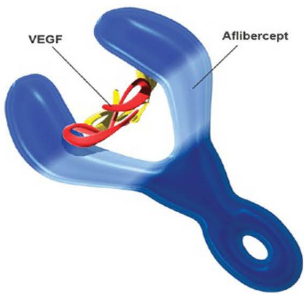
a] some examples:

- Gefitinib (Iressa) and Erlotinib (Tarceva) - both downregulate the production of VEGF and other pro-angiogenic factors in tumors, although this is NOT their main mechanism of action (see below)

- Endostatin - downregulates VEGF and bFGF (among other things), and upregulates thrombospondin 1 and some of the TIMP's ("tissue inhibitors of metalloproteinases"), both of which are anti-angiogenic substances

3) "neutralizing" VEGF and/or other pro-angiogenic factors after they've already been made - the first molecular strategy combating angiogenesis to make a big splash!

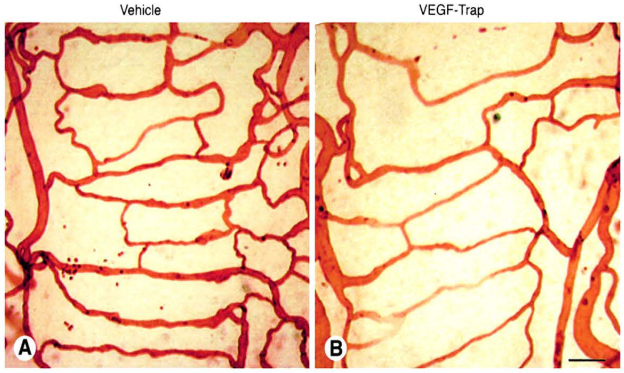
a] the classic example:



- Bevacizumab (Avastin) - a monoclonal antibody raised against VEGF; FDA-approved for certain indications as of 2004, and the first of its kind

b] more recently approved:

- Ziv-aflibercept (Zaltrap) - aka "VEGF Trap", a dummy receptor drug that binds to and ties up VEGF so it never reaches its intended receptor on vascular endothelial cells

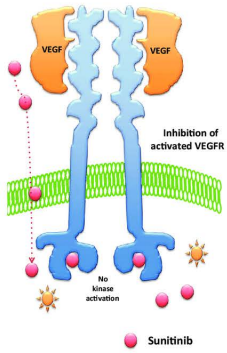


4) block the VEGF receptor (and/or others) on the vascular endothelial cells, so that VEGF can't bind - this idea was borrowed from the comparable research with the HER2 and EGF receptors, and the drugs trastuzumab (Herceptin) and cetuximab (Erbitux) - example: ramucirumab (Cyramza)

5) prevent the VEGF (or other) receptor from "firing" once the VEGF binds - this would have the net effect of the vascular endothelial cell not "sensing" or responding to the signal to proliferate and create new blood vessels

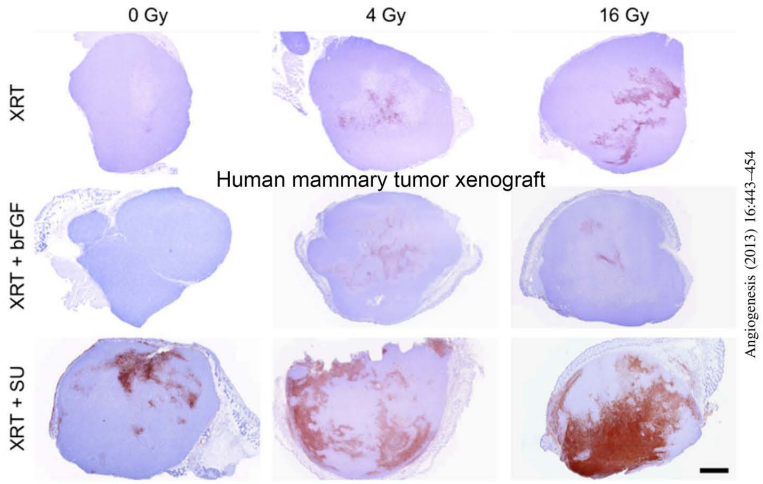
a] many signaling pathways (by no means limited to the ones having to do with angiogenesis) turned on by the binding of a growth factor to its appropriate cell surface receptor begin with the activation of a **receptor tyrosine kinase (RTK)**, so blocking this from happening would terminate the signal

b] some examples:



- Sunitinib (Sutent) - a small molecule RTK inhibitor "tuned" to the VEGF signaling pathway by shutting off the activation of its receptor; also shows some activity against the PDGF receptor as well; approved for the treatment of GIST and advanced kidney cancer as of 2006

- Erlotinib, gefitinib, lapatinib, etc. - also RTK inhibitors, but more "broad spectrum" and shut down RTK's associated with other signaling pathways in addition to the VEGF/VEGFR one (in particular, the HER-2/EGF receptor family)



Representative images of tumor sections (treated with 0, 4 or 16 Gy alone, or in combination with Sunitinib or bFGF) stained with in situ end labelling (ISEL) for cell death. We observed increasing amounts of cell death with increasing radiation doses. There appears to be lesser amounts of ISEL staining when 16 Gy radiation is combined with bFGF than 16 Gy alone. Treatments with Sunitinib alone demonstrate cell death staining equivalent to 16 Gy alone. However, combining Sunitinib with radiation appears to enhance cell death significantly. The *scale bar* represents 1 mm.

More Anti-Angiogenic Approaches *(but downstream from VEGF)*

6) shutting down the genes or inactivating the gene products made by vascular endothelial cells in response to stimulation by pro-angiogenic factors - many genes/products to choose from, including those governing proliferation, degradation of the extracellular matrix, motility and chemotaxis, etc.

a] an example:

- Marimistat - inhibitor of matrix metalloproteinases (MMP's), that help the endothelial cells break down the extracellular matrix to facilitate migration and new vessel development; a bust clinically when tested in SCLC, but research continues

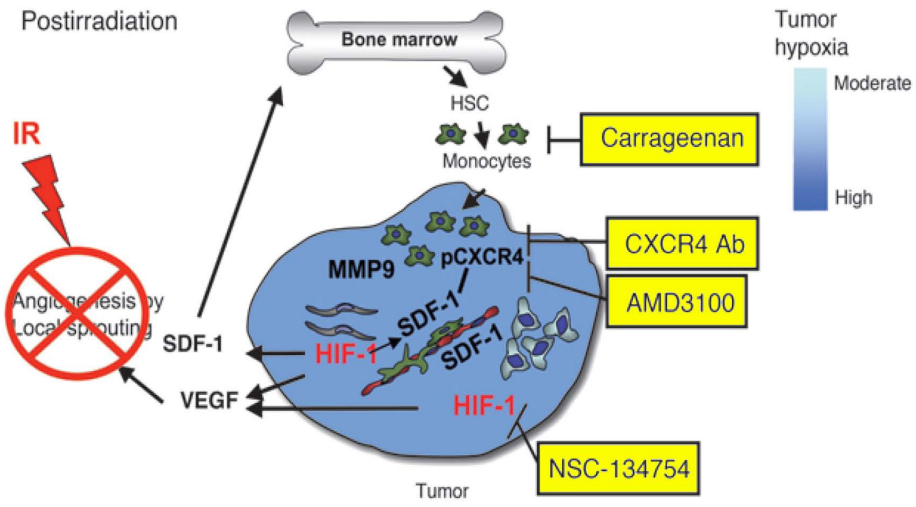
7) all of the above, none of the above, or unknown mechanism of anti-angiogenic action

a] some examples:

- Caplostatin - broad spectrum anti-angiogenic
- Thalidomide - mechanism of action unclear (*possibly* working along the lines of Marimistat?), but definitely has both anti-inflammatory and anti-angiogenic properties; approved for multiple myeloma and some auto-immune diseases, but use is highly regulated

OK, what about inhibiting *vasculogenesis*?

A. In theory, this could go a long way in preventing tumor recurrences after definitive radiotherapy...



J Clin Invest. 2010;120(3):694–705

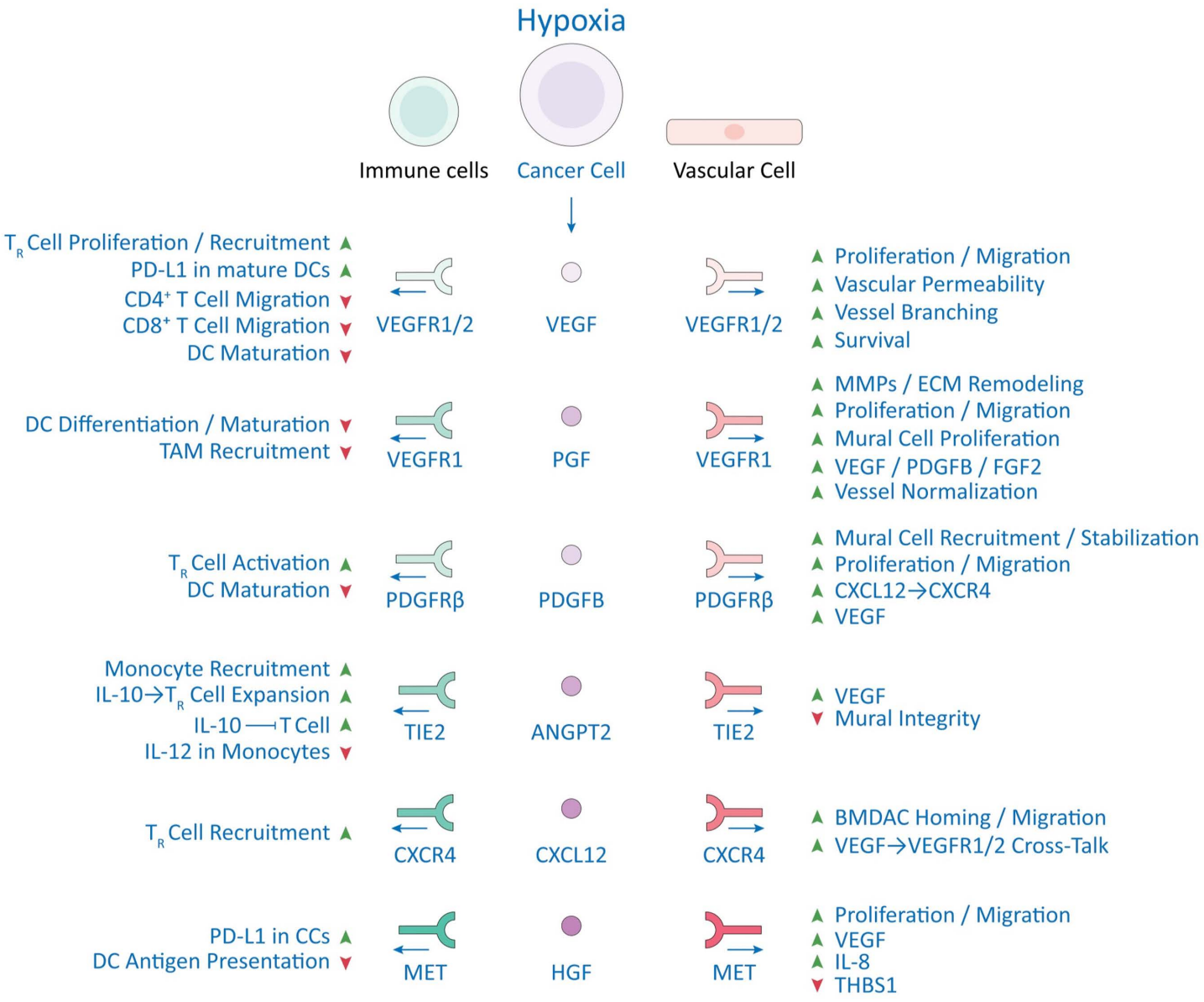
At the end of radiotherapy when the tumor's vasculature has been (presumably) destroyed, any surviving tumor cells will find themselves under increasingly hypoxic conditions. This upregulates HIF-1, which upregulates VEGF, but it can't do anything if there's no remainling vasculature, so this is where SDF-1 comes in to enter the circulation and mobilize bone marrow stem cells to migrate to the tumor. It follows therefore that ***inhibitors of SDF-1, the CXCR-4 receptor on the bone marrow cells, or other agents that prevent the bone marrow cells from migrating to the tumor (including killing them outright) would inhibit vasculogenesis.*** Antibodies and other small molecule inhibitors have already been shown to do this in some animal models.

An interesting thought...

What if one of the reasons why chemo-rads works is that the chemo kills bone marrow cells, including those responsible for vasculogenesis (CD11b+ monocytes/angioblasts in particular)? Hmmmmmmm.

And finally, there's a strong connection between tumor angiogenesis and tumor immune suppression…

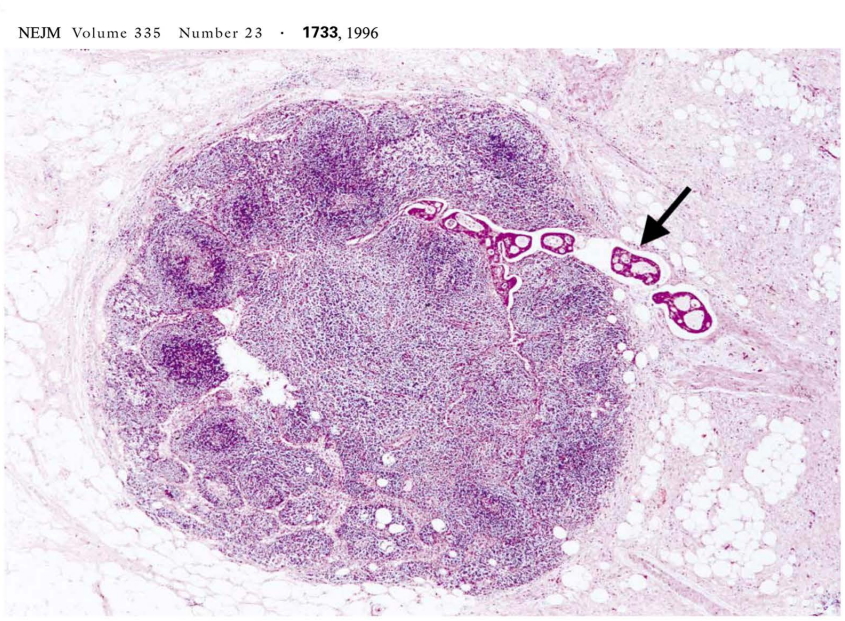
…many, if not most, of the up-regulated (by hypoxia) proteins that support the tumor's creation of vaculature also play roles in the tumor's ability to avoid detection by the host's immune system



Vascular and immunological effects of hypoxia-induced angiogenesis.

C. Hallmarks of Malignant Tumors: Invasion and Metastasis

1) invasiveness and metastatic potential, along with angiogenesis, are among the major hallmarks of cancer

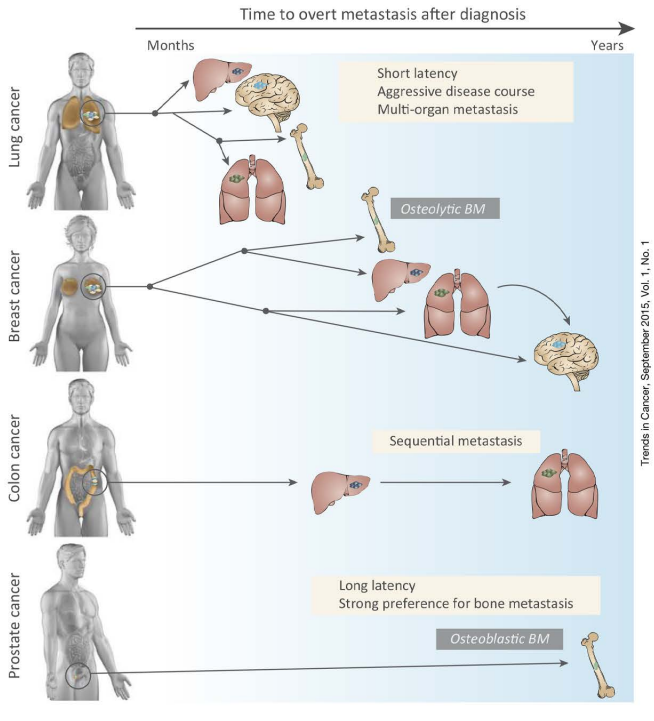


A Metastasis Caught in the Act

2) the process of metastatsis in particular, has intrigued oncologists for more than a century, owing to the sometimes unusual behavior that tumor metastases demonstrate, including:

a. Metastatic "tropism" - that different tumor types have preferred sites of metastatic spread, that may or may not follow based only on blood or lymphatic circulation patterns

Patterns of Metastatic Spread of Solid Tumors



Trends in Cancer, September 2015, Vol. 1, No. 1

Different cancer types exhibit remarkable variability in their metastatic course, reflected in the length of the latency period (months to years), the organs affected (most commonly the liver, lung, bone, and brain) and the type of metastasis (e.g., osteolytic or osteoblastic bone metastasis). Latency period (denoted by the arrow on top of the figure – left: months, right: years after diagnosis): lung cancer metastasis typically occurs within months after initial diagnosis, whereas prostate cancer and some subtypes of breast cancer can produce distant relapse up to decades after initial diagnosis. Lung cancer is the main contributor to brain metastasis, whereas it is a late occurrence in breast cancer. Organ pattern (the most-frequently affected organ is located on the top of each cancer type): lung and breast cancers metastasize to different organs (with a different propensity), whereas colon cancer most frequently metastasizes to liver, and from established liver metastasis secondarily to lung. Prostate cancer typically although not exclusively metastasizes to bone. Different cancer types also vary in the type of metastatic lesions they induce, well illustrated by the development of osteolytic bone metastasis in breast and lung cancer, and osteoblastic bone metastasis in prostate cancer. Abbreviation: BM, bone metastasis.

b. **Variable metastatic phenotypes** - that metastases in particular organs can be of more than one form or type, e.g., bone mets can be *osteolytic* (where normal bone is destroyed by the metastasis) or *osteoblastic* (when the metastasis causes new bone deposition) or a combination of both

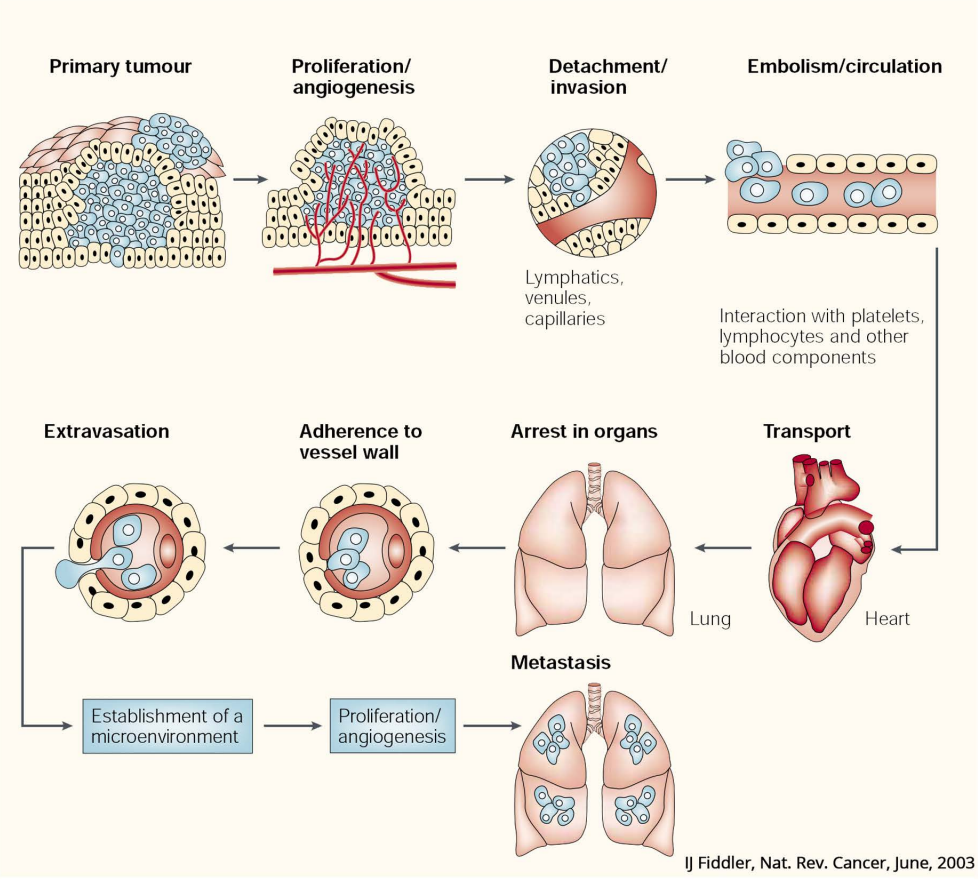
c. **Cross-talk between primary tumors and their metastases** - for example, when treatment (or removal) of the primary tumor causes metastases to suddenly explode or regress

d. **Metastatic "timing"** - recognition that the development of metastases is typically rather late in a tumor's natural history…but not always

e. Growing understanding that **distant metastases are what lead to the vast majority of cancer deaths (~90%)**

1) around 30% of patients already have metastatic disease at diagnosis, and another 30% are thought to harbor occult metastases that emerge later

Metastasis is a "process", termed the METASTATIC CASCADE...



…however, it's a very inefficient one.

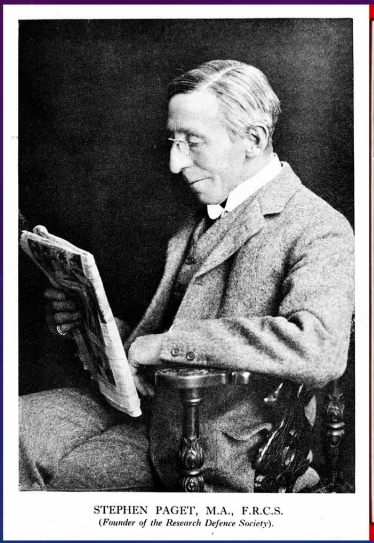
Estimates are that less than about 0.02% of cells that leave the primary tumor mass ever end up as clinically detectable metastases. (Still, given the total number of cells in tumors, this is not an insignificant number.)

The step in the process most likely to kill them is not when they first enter the bloodstream, but once they extravasate out of the bloodstream into the new metastatic site.

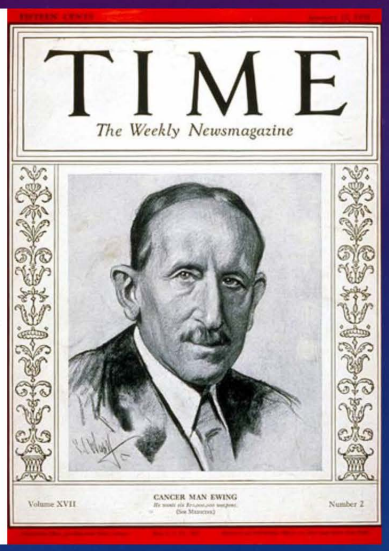
Two main theories of metastatic spread

"Seed and Soil"

- Pattern of metastatic spread based on compatibility of the "seed" (cancer) with its "soil" (secondary organ site)
- NSCLC->Adrenal
- Uveal melanoma->Liver
- Melanoma->Brain
- Gastric Ca->Ovary



STEPHEN PAGET, M.A., F.R.C.S.
(*Founder of the Research Defence Society*).



Mechanical Spread

- Pattern of metastatic spread is higher in the organ that is first encountered by blood flow
- Colorectal Ca->Liver
- Breast Ca->Lung

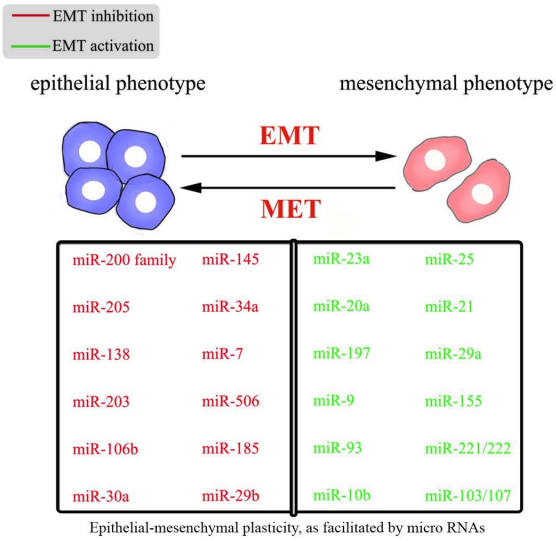
*Both theories are likely correct to a degree. Yet, the "**Seed and Soil**" hypothesis has been preferred to explain the unique metastatic "tropism" patterns that are clinically observed, and highlighted the importance of tissue microenvironments*

Molecularly-speaking, how do cells in the primary tumor acquire the skills to become invasive, and ultimatey, metastatic?

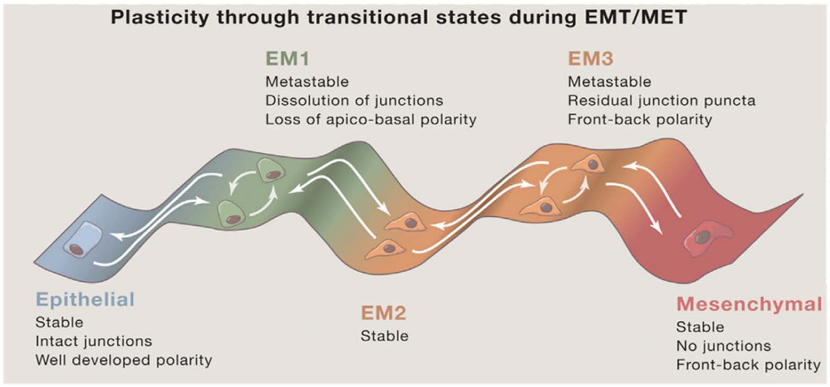
A. Several phenotypic changes are required for a tumor cell to become fully competent to form a metastasis

1) **Cells need to fundamentally alter their molecular "program" to accomplish this, termed the epithelial-to-mesenchymal transition (EMT). The main drivers for this are genomic instability (i.e., mutations), hypoxia and the actions of microRNAs (i.e., epigenetics).**

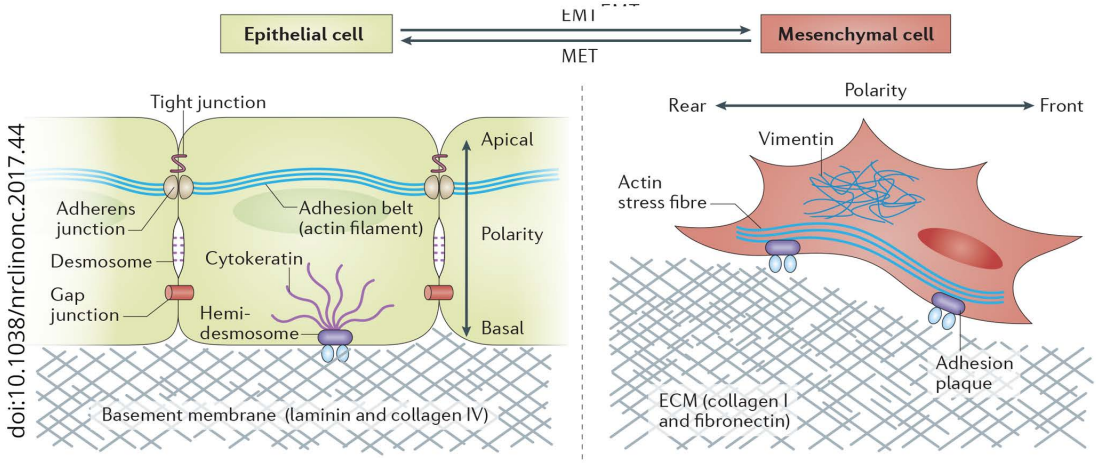
a. ***Emerging science suggests that it's the miRNAs that play the largest role in the EMT, and that changes in their expression accout for the fact that the EMT is "plastic"***, i.e., that cells can move from epthelial-to-mesechymal and back, and also can exist in several intermediate states



Epithelial-mesenchymal plasticity, as facilitated by micro RNAs

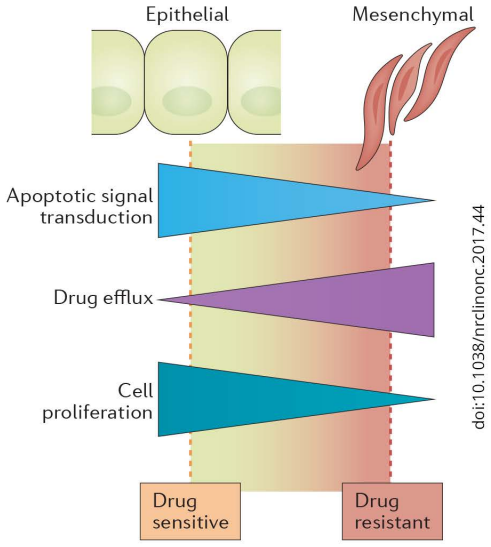


EMT = *"An orchestrated series of molecular events that alters cell-cell and cell-extracellular matrix interactions resulting in the release of epithelial cells from their surrounding tissue, and causing them to acquire a mesenchymal phenotype"*



Tight junctions, adherens junctions, gap junctions, and desmosomes	**Cell–cell junctions**	Minimal
• Actin filaments are aligned with adherens junctions (adhesion belt) • Cytokeratin intermediate filaments	**Cytoskeleton**	• Actin stress fibres • Vimentin intermediate filaments
Via integrin $\alpha_6\beta_4$ at hemidesmosomes; linked to cytokeratins	**Interactions with ECM**	Via β_1-containing or β_3-containing integrins at adhesion plaques; linked to actin stress fibres
Apical–basal polarity	**Polarity**	Front–rear polarity

3) Another (unfortunate) feature of the EMT is that some of the resulting mesenchymal cells acquire stem cell-like characteristics ("stemness") and this in turn is associated with treatment resistance



Compared to epithelial cells, mesenchymal cell are less likely to die by apoptosis, less likely to proliferate, and more likely to have upregulated membrane transporters that efflux drugs. Mesenchymal cells also can develop resistance to EGFR inhibitors. And finally, they express more PD-L1, which facilitates immunosuppression.

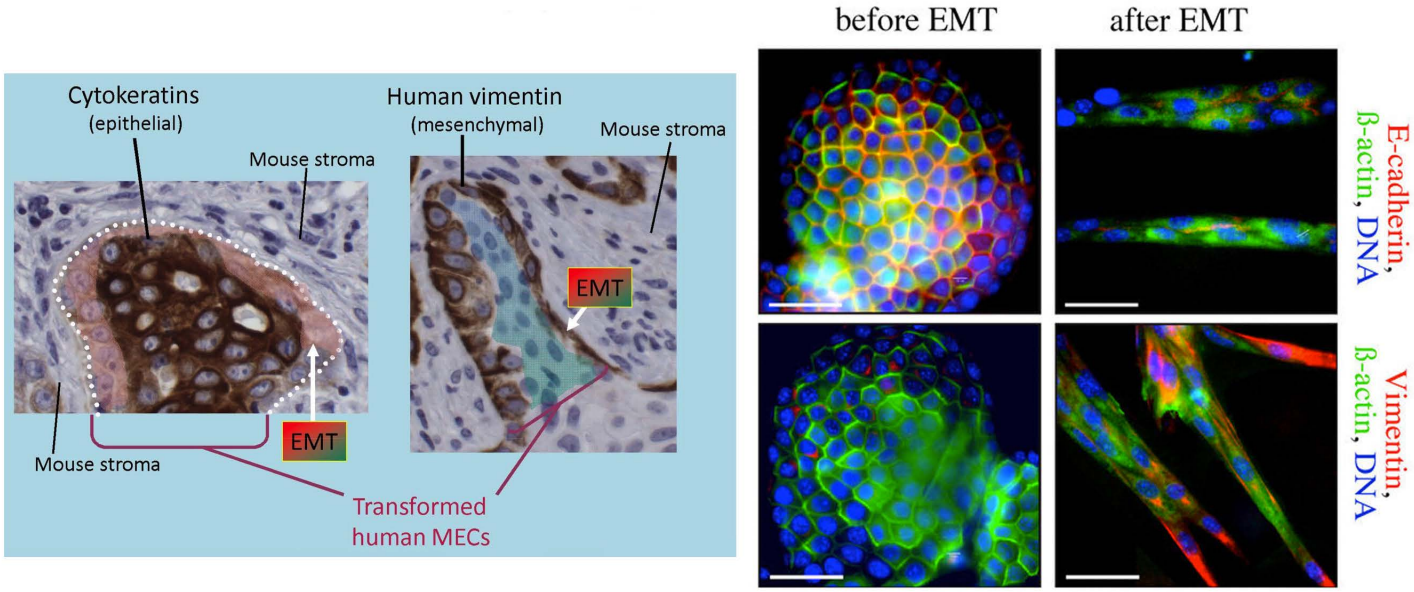
Therapy resistance conferred by epithelial-to-mesenchymal transition (EMT)

Therapeutic agent	Observations
Inhibition of apoptotic signalling	
Cisplatin	Slug blocks p53-mediated transcriptional induction of *PUMA* (also known as *BBC3*, encoding Bcl-2-binding component 3) expression by directly repressing the *PUMA* promoter region; multiple lung adenocarcinoma cell lines acquire cisplatin resistance through this mechanism
Tumour necrosis factor α (TNFα) treatment; γ-irradiation	Snail confers resistance against multiple apoptosis-inducing stimuli, in part by promoting AKT activation, upregulating the expression of the pro-survival protein Bcl-X_L, and delaying cell-cycle progression
TNF-related apoptosis-inducing ligand (TRAIL)	EMT-programme activation diminishes E-cadherin-mediated clustering of the TRAIL receptors DR4 and DR5, thereby making carcinoma cells resistant to TRAIL-induced apoptosis
Enhancement of drug efflux	
Doxorubicin	EMT-programme activation induces the expression of multiple members of the ATP-binding cassette (ABC) transporter family, thereby rendering these cells resistant to doxorubicin.
Protection against molecular targeted agents	
EGFR inhibitors	The activation of EMT and subsequent expression of AXL receptor tyrosine kinase confer resistance to EGFR inhibitors on *EGFR*-mutant non-small-cell lung carcinoma (NSCLC) cells
EGFR inhibitors; PI3K inhibitors	An EMT-associated gene-expression signature predicts the resistance of NSCLC cells to EGFR inhibitors and PI3K inhibitors
Desensitization to immunotherapy	
Dendritic cell (DC)-mediated immunotherapy (intratumoral injection of DCs pulsed with a tumour antigen)	Snail expression in melanoma cells contributes to resistance to DC-mediated and CTL-mediated immunotherapy via enhanced thrombospondin-1 expression and resultant induction of immunosuppressive regulatory T cells within the tumour tissue
Immune-checkpoint inhibition	Zinc finger E-box-binding homeobox (ZEB1)-mediated activation of EMT in NSCLC cells relieves miR-200-mediated repression of programmed cell death 1 ligand 1 (PD-L1) expression, a major inhibitory ligand for the programmed cell death protein 1 (PD-1) immune-checkpoint protein on $CD8^+$ CTLs. This effect sensitizes these cells to immunotherapies targeting the PD-1–PD-L1 axis, while potentially conferring on them resistance to other strategies of activating antitumour immunity, such as the functional blockade of another immune-checkpoint protein, CTLA-4

doi:10.1038/nrclinonc.2017.44

3) a deeper dive into the EMT

a. **Snail, Twist Zeb and Notch/WNT** - involved in the transdifferentiation from the epithelial to mesenchymal phenotype; this causes **the downregulation of E-cadherin and β-catenin**, and **the upregulation of vimentin and N-cadherin**

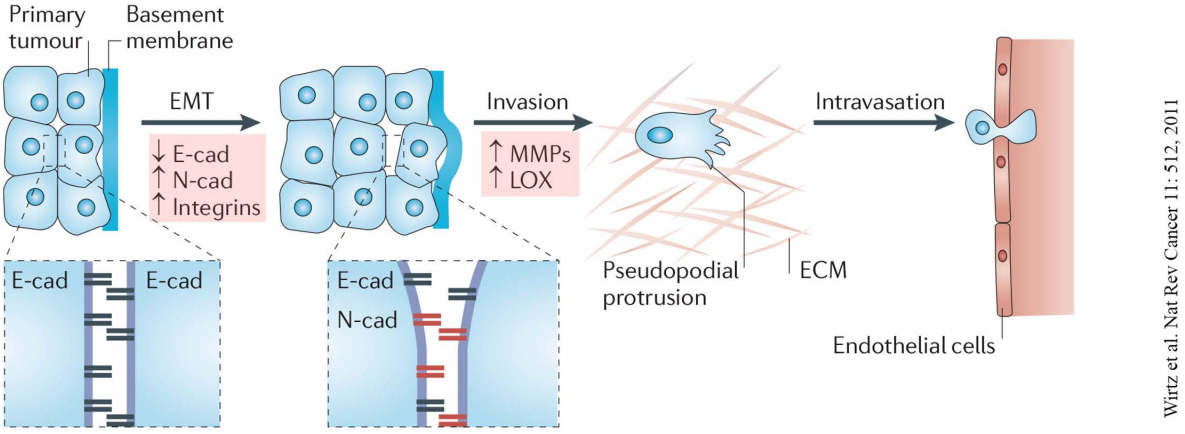


1. as a result, the attachment of cells to each other and to basement membranes (e.g., tight junctions, gap junctions, desmosomes) is lost

b. **RhoA** - facilitates cellular motility

c. **Matrix Metalloproteinases (MMPs)** – allow the cells to degrade the ECM, which aids in motility and intravasation of tumor cells into blood and lymph vessels

d. **Lysyl oxidase (LOX)** – helps "stiffen" the collagen matrix to allow tumor cells to track along it (LOX has also been used as a hypoxia marker)



Wirtz et al. Nat Rev Cancer 11: 512, 2011

The physics of invasion and intravasation. The epithelial-to-mesenchymal transition (EMT) is associated with a loss of adhesion through downregulation of E-cadherin (E-cad) and a change in morphology. Invasion by tumour cells of the surrounding tissue and subsequent motion is dictated by the physicochemical properties of the extracellular matrix (ECM).

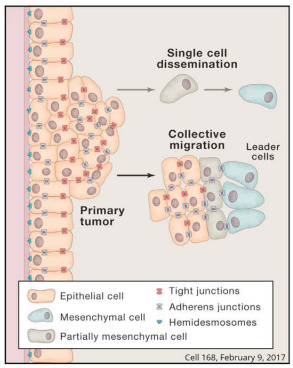
4) Once the metastatic cells leave the primary tumor mass, how do they survive the harsh conditions out in the circulation?

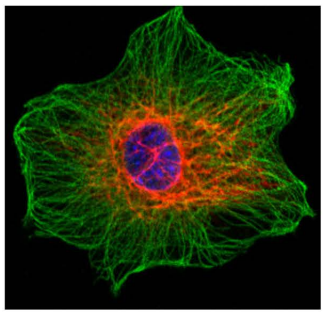
a. most don't, but among those that do, the following strategies are used:

1. **downregulate anoikis** (apoptosis secondary to loss of cell-cell attachment), if not already absent, which it usually is

2. **avoid the shear forces** by beefing up the cell's cytoskeleton - vimentin helps with this, as can a coating of platelets, or the tumor cells traveling in clumps rather than as single cells

Safety in numbers by tumor cells traveling in clumps once entering the circulation. Note that some may not even undergo the EMT, provided at least a few "leader cells" do.



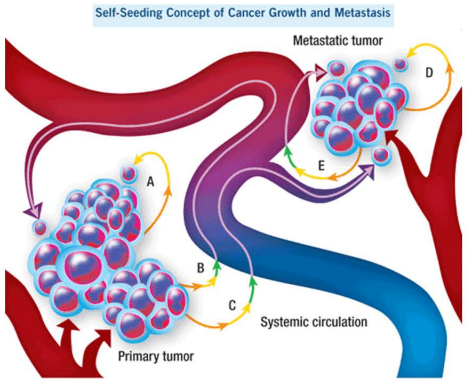


Nucleus = purple
Vimentin = red
Tubulin = green

Vimentin forms a cage around the cell's nucleus to protect it

3. **evade detection and destruction by immune cells** - the circulating tumor cells protect themselves from the immune system by coating themselves in platelets, expressing immunosuppresive ligands on their cell surface and/or secreting immunosuppressive cytokines (e.g., TGF-β)

b. some of the circulating tumor cells don't end up at metastatic sites, but instead circle back and return to the primary tumor; this is termed **reseeding**, and is thought to be a strategy for re-enriching the primary tumor with more aggressive cells that have been "hardened" by their time spent in the circulation

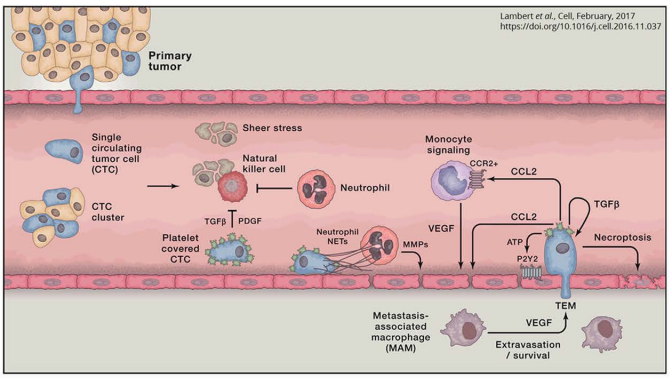


Self-seeding may take place along the following paths: (A) dislodging and reattachment of a primary tumor cell at the primary site; (B) dislodging, intravasation, circulation, then extravasation back to the primary site; (C) dislodging, intravasation, circulation, then extravasation to a metastatic site; (D or E) self-seeding from a metastatic site following path A or B.

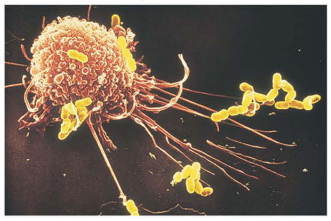
SOURCE: Reprinted by permission from Macmillan Publishers Ltd: *Nature Medicine*. Norton L, Massagué J. **Is cancer a disease of self-seeding?** *Nat Med* 2006;12(8):875-8, copyright 2006.

5) Once the circulating tumor cells reach their destination, how do they get out of the circulation and into the metastatic site?

a. in some cases, neutrophils in the circulation can grab them in micro-nets and pull them through the wall of the blood vessel - normally, neutrophils use this method to snag pathogens and kill them, but tumor cells have learned how to evade the "kill them" part

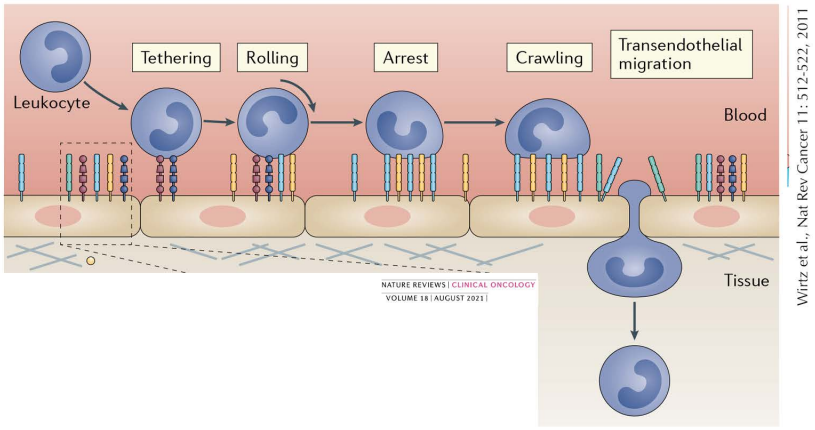


Neutrophil Net



b. they can also use the "roll, grab, arrest and extravasate" method, which is normally used by monocytes/macrophages that move between the circulation and tissues; this is facilitated by microtentacles and adhesion molecules

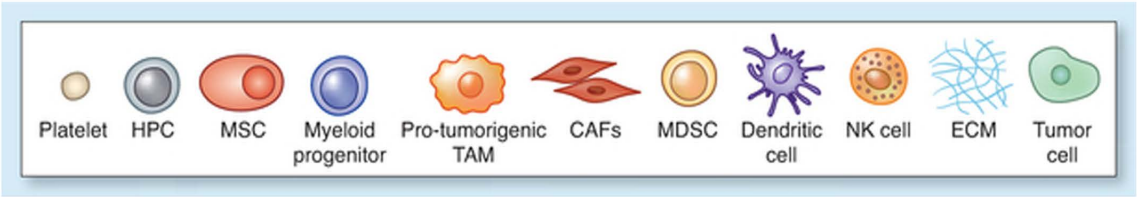
Rolling Capture



6) How do the circulating tumor cells know where they're supposed to go to establish a new metastasis?

a) *as is the case for vasculogenesis when bone marrow stem cells are activated and mobilized to the primary tumor, circulating tumor cells are also following chemokine gradients (SDF-1 and others) to the metastatic site*

1. How? Because **the future metastatic site has already been populated by a variety of cells from the primary tumor, many of which are normal cells whose activities have been coopted by the tumor:**

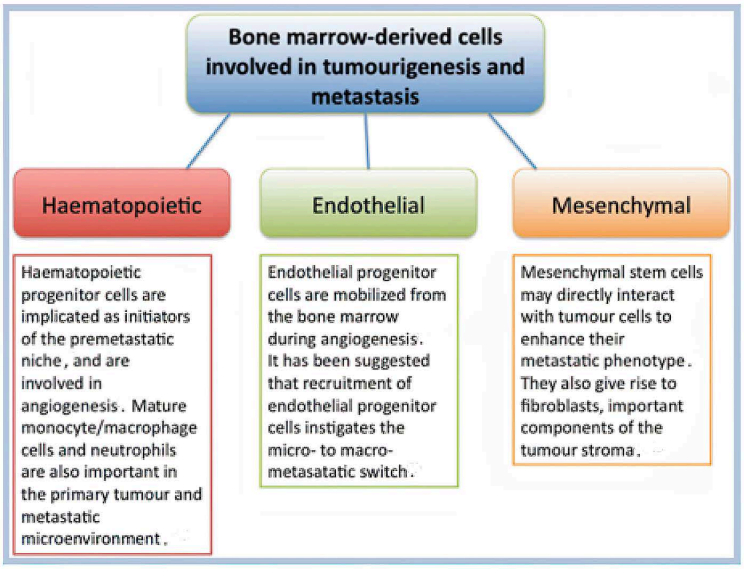


These cells – including various bone marrow and stromal stem/progenitor cells, inhibitory immune cells, tumor-associated macrophages (TAMs) and cancer-associated fibroblasts (CAFs) – together comprise what is termed the PREMETASTATIC NICHE.

2. When do these premetastatic niches form?

Answer: Not completely clear at present, although ***a growing body of evidence suggests that they first form VERY early in the primary tumor's natural history***, and then just wait around until the primary is larger and/or when enough tumor cells have undergone the EMT to strike out on their own by entering the circulation

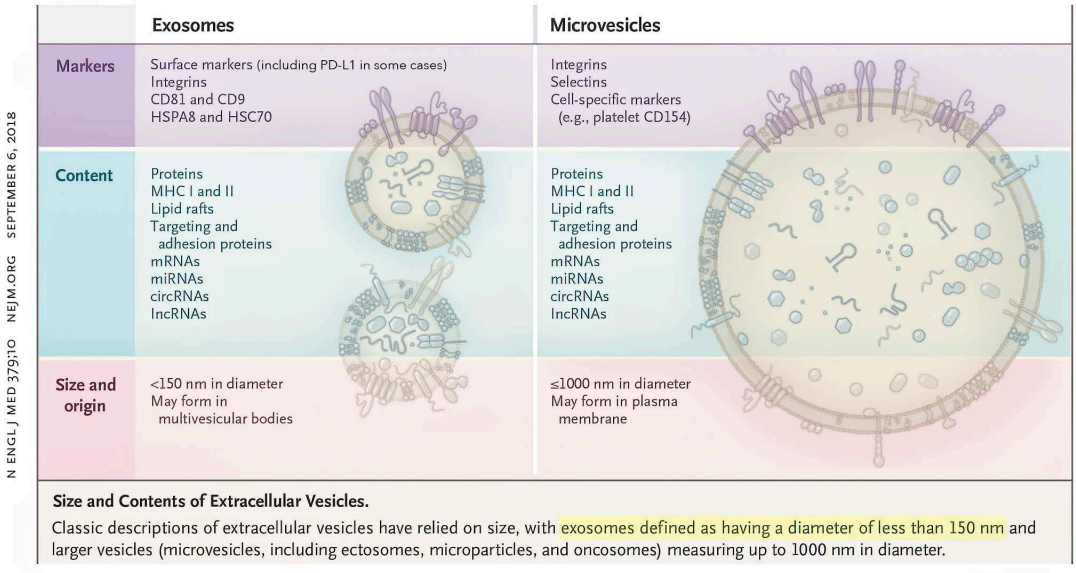
More about (pre-) metastatic niches



2. What else is in this niche in addition to actual cells?

- Inflammatory cytokines and chemokines
- Extracellular matrix components
- DNA, RNA (miRNAs in particular), lipids, proteins

(a) these acellular materials are delivered to the niche by **EXOSOMES**, small membrane-enclosed vesicles extruded from tumor cells in the primary site

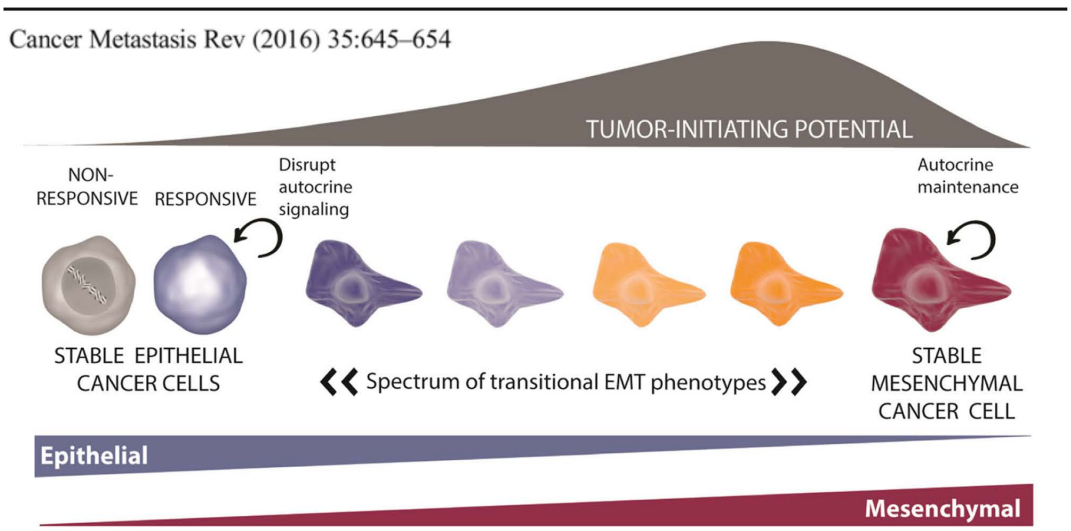


Clinical Correlate:

This is why there's interest in using liquid biopsy techniques to isolate and characterize circulating vesicles released from tumor cells, i.e., to look for biomarkers that can gauge the status of (pre-) metastatic niches

7) Once the metastatic cells arrive at the niche and settle in, then what happens?

a. cells gradually revert back to more epithelial phenotypes, although not necessarily all the way back, given that the EMT/MET is a continuum

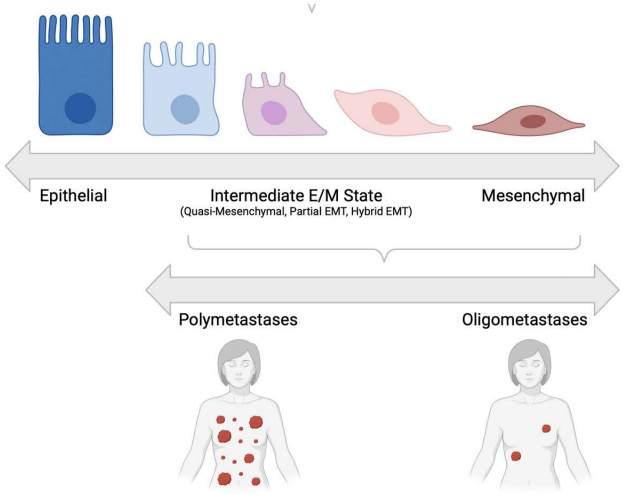


Clinical Correlate (Maybe):

We all know what "oligometastatic disease" is, right? That is, a mostly operationally-defined clinical state characterized by a limited number of metastatic sites and extent of disease that is amenable to metastasis-directed therapy (that might even be curative in some cases).

But does the oligometastatic state have a biological underpinning(s)? One theory is that because the EMT exists along a spectrum that evolves over time, maybe oligomets are made up of tumor cells that still have less aggressive, more treatable, EMT phenotypes?

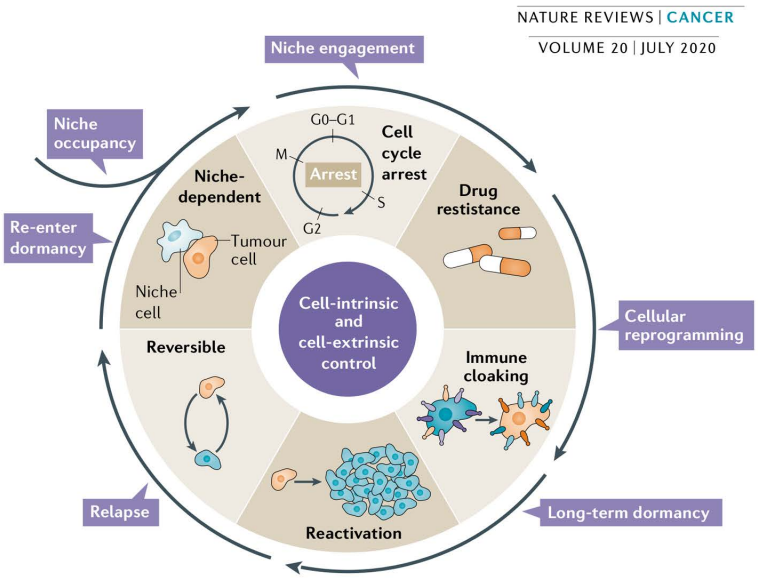
Probably much more complicated than that, but still, an interesting idea...



b. the (small?) subset of tumor cells that have acquired "stem-ness" as part of the EMT are the ones capable of dividing and colonizing the new site

c. activate angiogenesis (once the new tumor mass begins to become hypoxic), and further reinforce an immunosuppressive tumor microenvironment

Another possible option, if conditions aren't quite right in the metastatic niche, signals from the primary tumor are conflicting and/or that some immune response occurs...**DORMANCY**



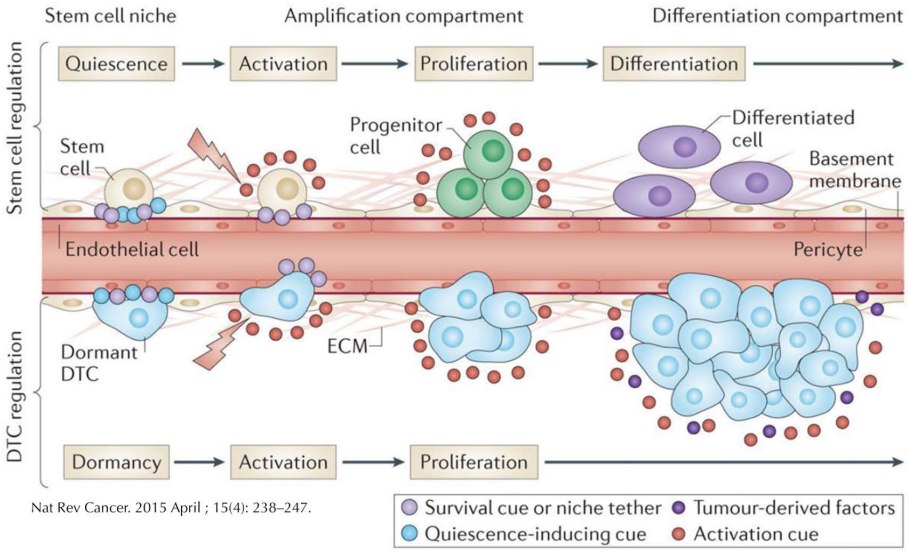
Dormant cancer cell life cycle and the hallmarks of cancer cell dormancy. Dormant cancer cells are subject to both cell-intrinsic control and cell-extrinsic control by the niche.

What are the characteristics of dormant tumor (stem) cells in metastatic niches?

Answer: pretty much the opposite of what we'd like.

1) how did these cells - and their niches - "learn" how to become dormant?

Answer: *they took some of their cues from (normal) stem cell niches, where normal stem cells exist in a state akin to dormancy until they receive signals to proliferate,* i.e., in response to a tissue injury



Nat Rev Cancer. 2015 April ; 15(4): 238–247.

Parallels between stem cell- and dormant disseminated tumor cell (DTC)-regulation by the perivascular niche
Stem cell survival and growth-arrest are maintained in a number of tissues by the perivascular niche. Upon activation of the niche (e.g., by wounding), growth-arrest cues are lost and/or overcome by proliferation-inducing signals, some of which are derived from endothelium. Stem cells give rise to rapidly dividing tissue-specific progenitors, which are ultimately steered towards differentiated cell types found in that tissue. DTCs are regulated in a similar fashion. Stable microvasculature maintains DTCs in a quiescent state; upon disruption of tissue homeostasis, activation cues (e.g., those derived from nascent, sprouting endothelium) spark DTC proliferation. Here, however, proliferating DTCs ignore differentiation cues and secrete a number of factors that lure resident- and circulating-cells to aid metastatic progression.

1. another strategy to maintain dormancy in the metastatic niche is to keep it (at least somewhat) hypoxic

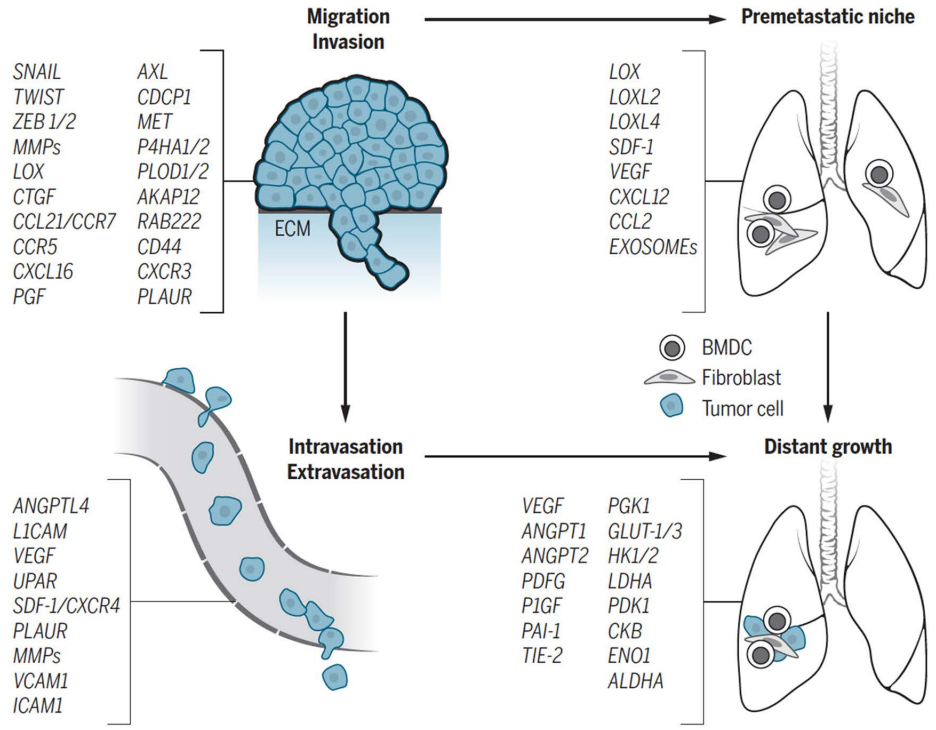
a) ***hypoxia likewise would prevent any metastatic stem cells from continuing to go through the cell cycle, and unfortunately, would also render them radiation- and possibly chemoresistant***

Targeting the Metastatic Process as a Clinical Strategy?

1. Many of the anti-angiogenic/anti-vasculogenic approaches might also be effective against metastases

2. Reduce or eliminate tumor hypoxia, as it is a major driver of the metastatic cascade

HIF Signaling Regulates Multiple Metastatic Steps



Rankin & Giaccia, Science 2016 Apr 8;352(6282):175-80

3. Target cancer stem cells, as at least some of them end up at metastatic sites and presumably are responsible for colonization

Signal Transduction and Targeted Therapy (2021)6:62

Emerging agents targeting CSC-associated pathways

Drug class/mechanism	Agent	Experimental research	Suggested patient population	Notes	Phase
Agents targeting the Sonic Hedgehog pathway					
SMO antagonists	Vismodegib (GDC-0449)	GDC-0449 could inhibit stemness209 and reverse erlotinib resistance, radiation and carboplatin resistance	Multiple basel-cell carcinomas (MIKIE)	Good activity in long-term regimens of MIKIE	2
			TNBC	Downregulates CSC markers expression and sensitizes tumors to docetaxel	1
			Myelofibrosis	Not improved any of the efficacy outcome	1b
	Sonidegib (LDE225)	LDE225 could destroy CSCs niche and reverse docetaxel resistance	TNBC	No drug-to-drug interactions between sonidegib and docetaxel were found in the PK assessment	1b
			mBCC	Sonidegib continued to demonstrate long-term efficacy and safety in mBCC.	2
SMO inhibitors	Glasdegib (PF-04449913)		Myelofibrosis	Further study of glasdegib in combination with JAKi in a MF population may be warranted	1b/2
	Taladegib (LY2940680)		Advanced solid tumors	Taladegib doses of 100 mg and 200 mg were well tolerated in this population of Japanese patients with advanced solid tumors.	1
			BCC	LY2940680 treatment resulted in an acceptable safety profile in patients with advanced/ metastatic cancer	1
	Saridegib (IPI-926)		Advanced Pancreatic Adenocarcinoma	The study closed early	1
Agents targeting Notch pathway					
γ-secretase inhibition (GSI)	MK-0752		Pancreatic cancer	Tumor response evaluation was available in 19 of 33	1
	RO4929097	RO4929097 reverse antiandrogen resistance, radiation resistance,and tamoxifen resistance mediated by CSCs	Recurrent Malignant Glioma	Combination of antiangiogenic and notch signaling inhibitors should be considered	1
			Glioma	A specific decrease in the $CD133^+$ CSC population	0/1
	PF-03084014	PF-03084014 reverse docetaxel resistance in CSCs	Advanced TNBC	16% of 25 response-evaluable patients achieved a confirmed partial response	1
			Desmoid Fibromatosis	Objective response rate of 71.4%	1
			Aggressive Fibromatosis	PF-03084014 was well tolerated and demonstrated promising clinical benefit in patients	1
DLL4 inhibitors	Demcizumab (OMP-21M18)		Metastatic Non-Squamous NSCLC	50% had objective tumor responses	1b
Agents targeting Wnt/β-catenin pathway					
Ligand sequestration	OMP-54F28 (FZD8-Fc)		Advanced solid tumors	Agent was well tolerated	1
			Recurrent platinum-sensitive ovarian cancer	75.7% of overall response rate	1b
Inhibitors of β-catenin	PRI-724	PRI-724 could downregulate expression of SOX2, CD44 and reverse cisplatin resistance in CSCs	Hepatitis C Virus-related Cirrhosis	Liver injury may be a possible related serious adverse event	1
	CWP232291	CWP232291 could reverse castration resistance in CSCs	NCT03055286	Recommended Phase 2 dose	1b
Agents targeting NF-κB pathway					
Nuclear export protein exportin 1 inhibitor	Selinexor	Selinexor could reverse paclitaxel resistance mediated by CSCs	Triple-class refractory multiple myeloma	Approved by FDA	

4. Disrupt the EMT

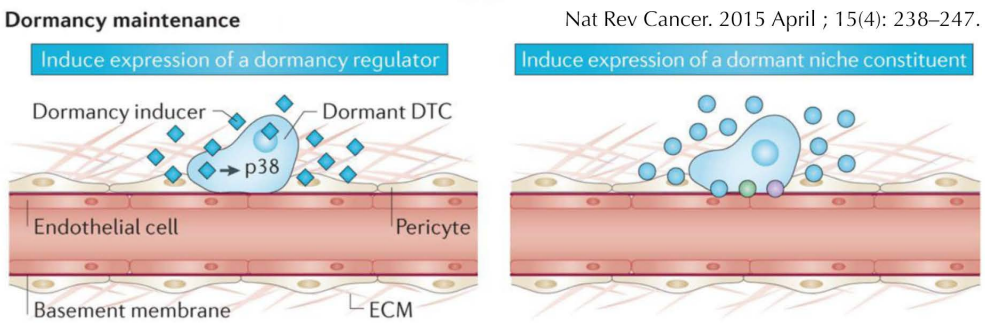
Small molecule inhibitors of epithelial–mesenchymal transition (EMT) and their functions.

Drugs	Target Genes	Function	Cancer
Curcumin	BMI1, SUZ12 and EZH2	Inhibits EMT and reverses 5-fluorouracil resistance	Colorectal cancer
Mocetinostat	HDAC	Induces sensitivity against chemotherapy	Pancreatic cancer
Zidovudine	Akt-GSK3 beta-Snail pathway	Inhibits EMT and reverses gemcitabine resistance	Pancreatic cancer
Evodiamine	WNT pathway	Inhibits EMT and reverses oxaliplatin resistance	Gastric cancer
Pyrvinium pamoate	WNT pathway	Inhibits EMT	Breast cancer
Moscatilin	Vimentin, Slug, and Snail	Inhibits EMT and sensitizes anoikis	Lung cancer
Metformin	ZEB1, Slug, Twist and Vimentin	Inhibits EMT	Breast cancer Ovarian cancer
Palbociclib	c-Jun/COX-2	Inhibits EMT	Breast cancer
Icaritin	PTEN/Akt/HIF-1α pathway	Inhibits EMT	Glioblastoma
Disulfiram	ERK/NF-kappa B/Snail pathway	Inhibits EMT and stem cell-like features	Breast cancer
Zerumbone	TGFβ pathway	Inhibits EMT	Non-small cell lung cancer
Bufalin	TGFβ pathway	Inhibits EMT	Lung cancer

Molecules **2016**, *21*, 965

5. Disrupt the metastatic niche - possibly could restore drug (and radiation?) sensitivity of dormant tumor cells (DTCs) and/or cancer stem cells (CSCs)

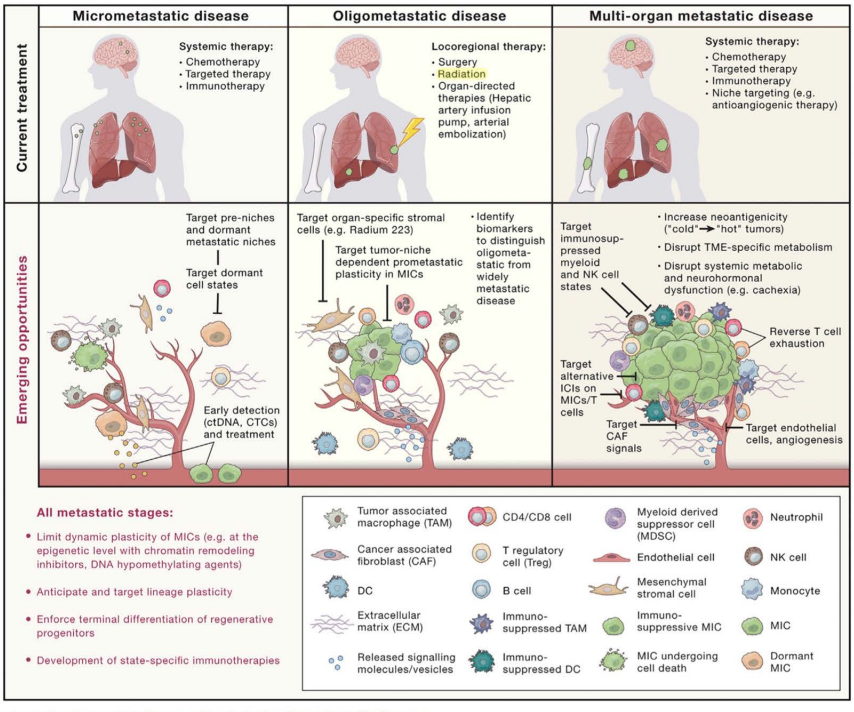
6. Promote dormancy instead of colonization - keep metastatic cells “asleep”, hopefully permanently



Nat Rev Cancer. 2015 April ; 15(4): 238–247.

7. Ablate oligometastases in an effort to produce a significant delay in tumor progression...or maybe even cure a select few patients

Seems clear that future directions in the treatment of metastatic disease in general – not just oligometastases – will be informed by our growing understanding of tumor biology and the tumor microenvironment.



Current and emerging therapeutic strategies for metastatic disease