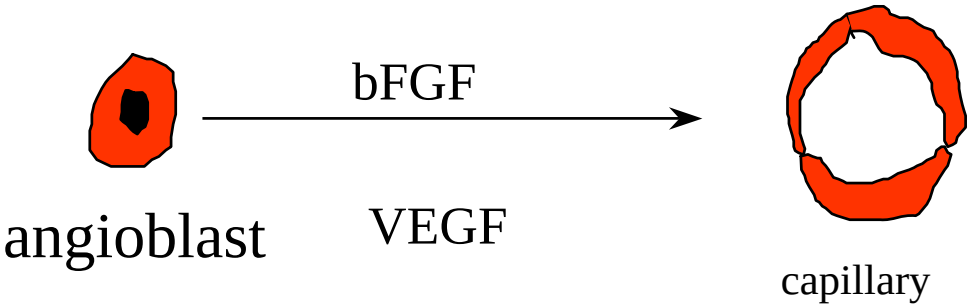
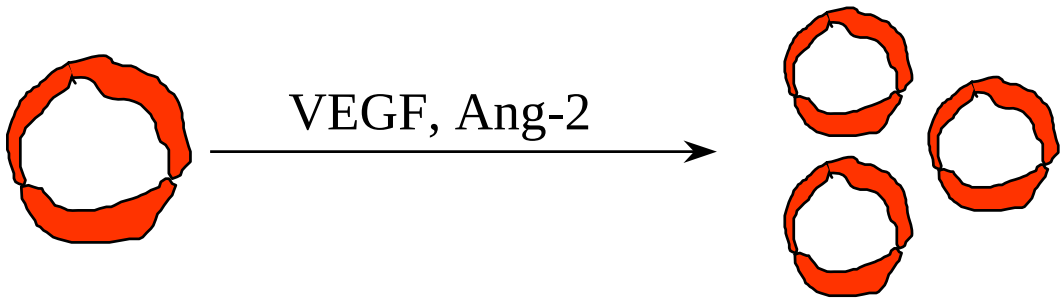
Molecular mechanisms of angiogenesis

Three ways of formation of blood vessels



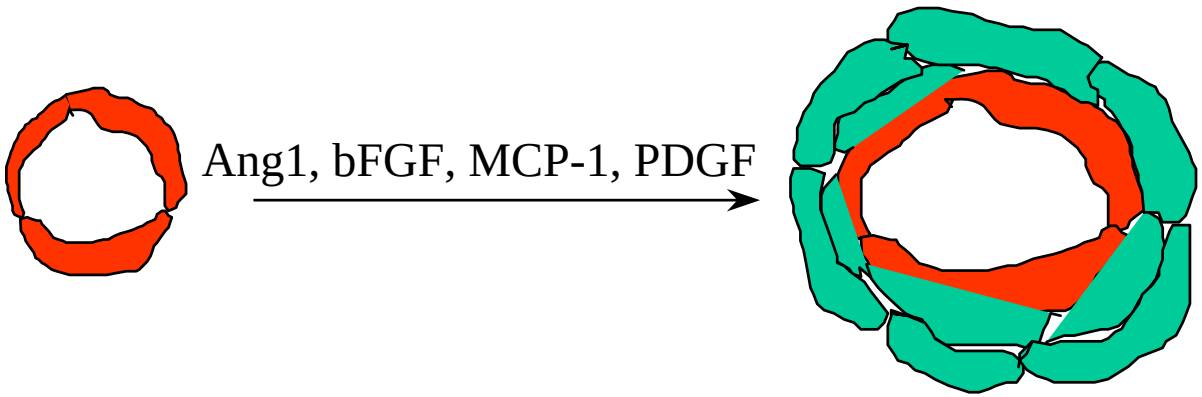
Vasculogenesis

capillaries are formed from vascular progenitor cells



Angiogenesis

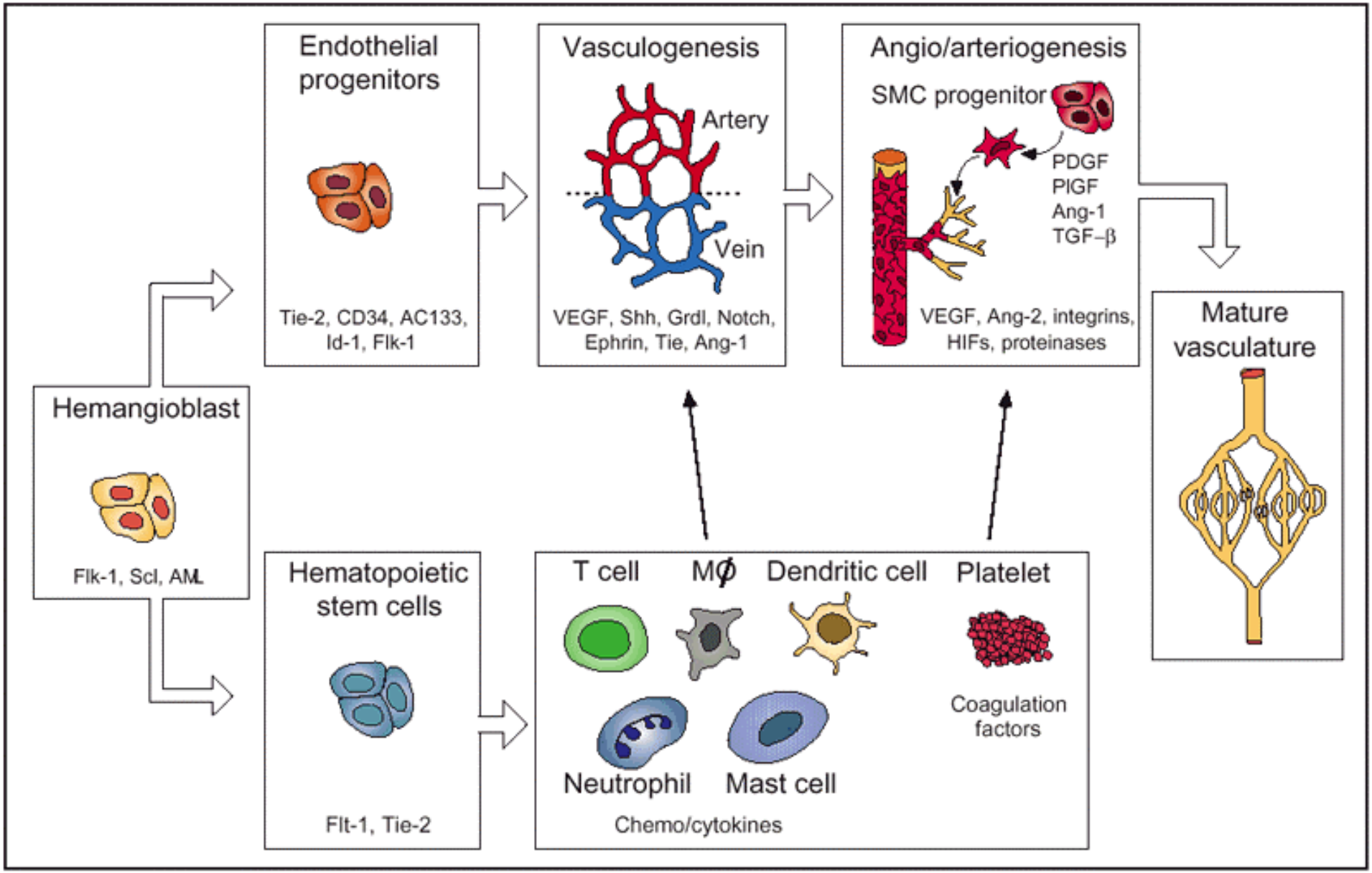
formation of new blood vessels from pre-existing vessels



Arteriogenesis

formation of mature blood vessels; differentiation into veins and arteries

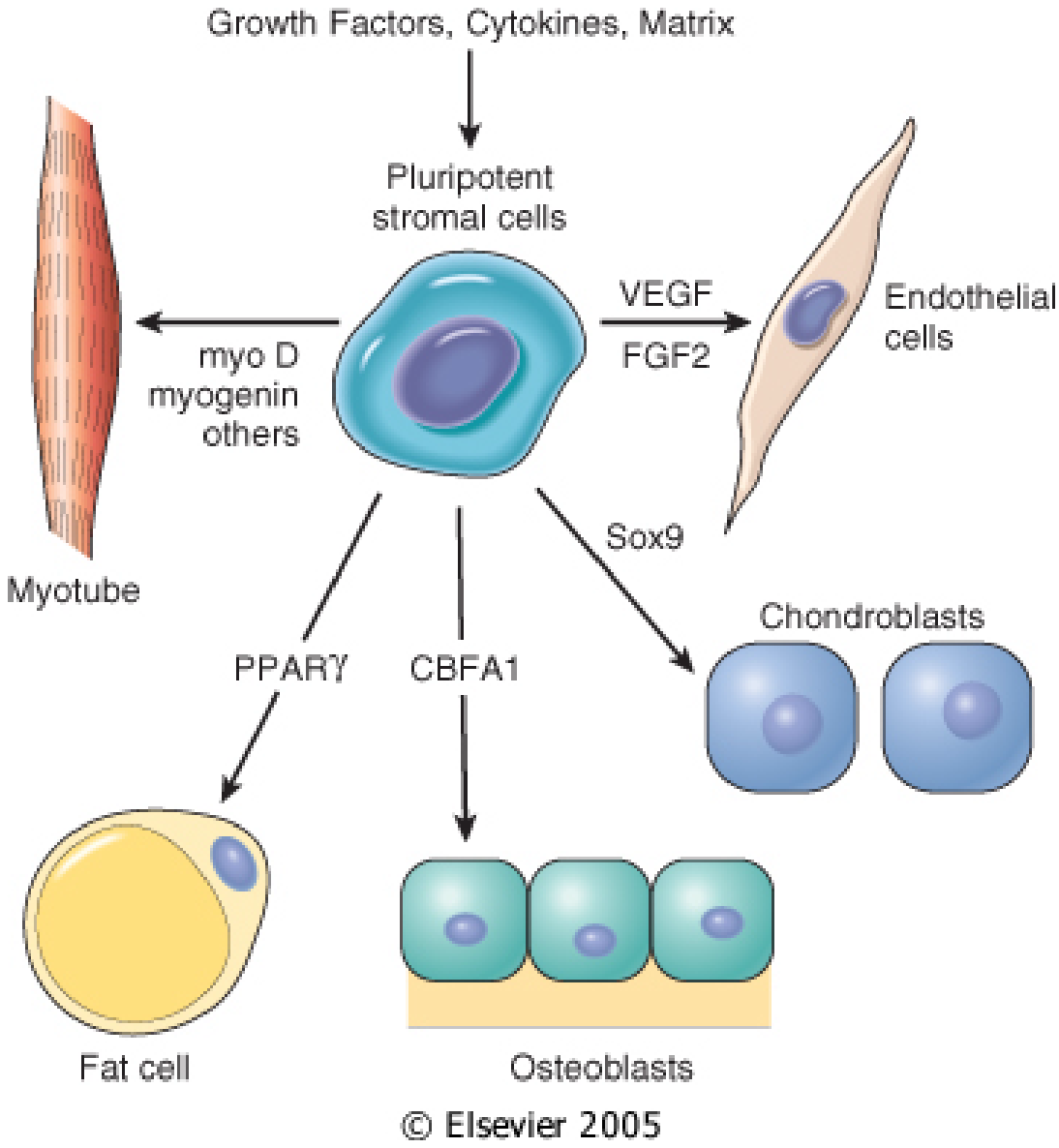
Formation of a vascular network



Carmeliet P., Nature Med. 2003

Vasculogenesis in adult

Differentiation patwhays for pluripotent bone marrow stromal cells

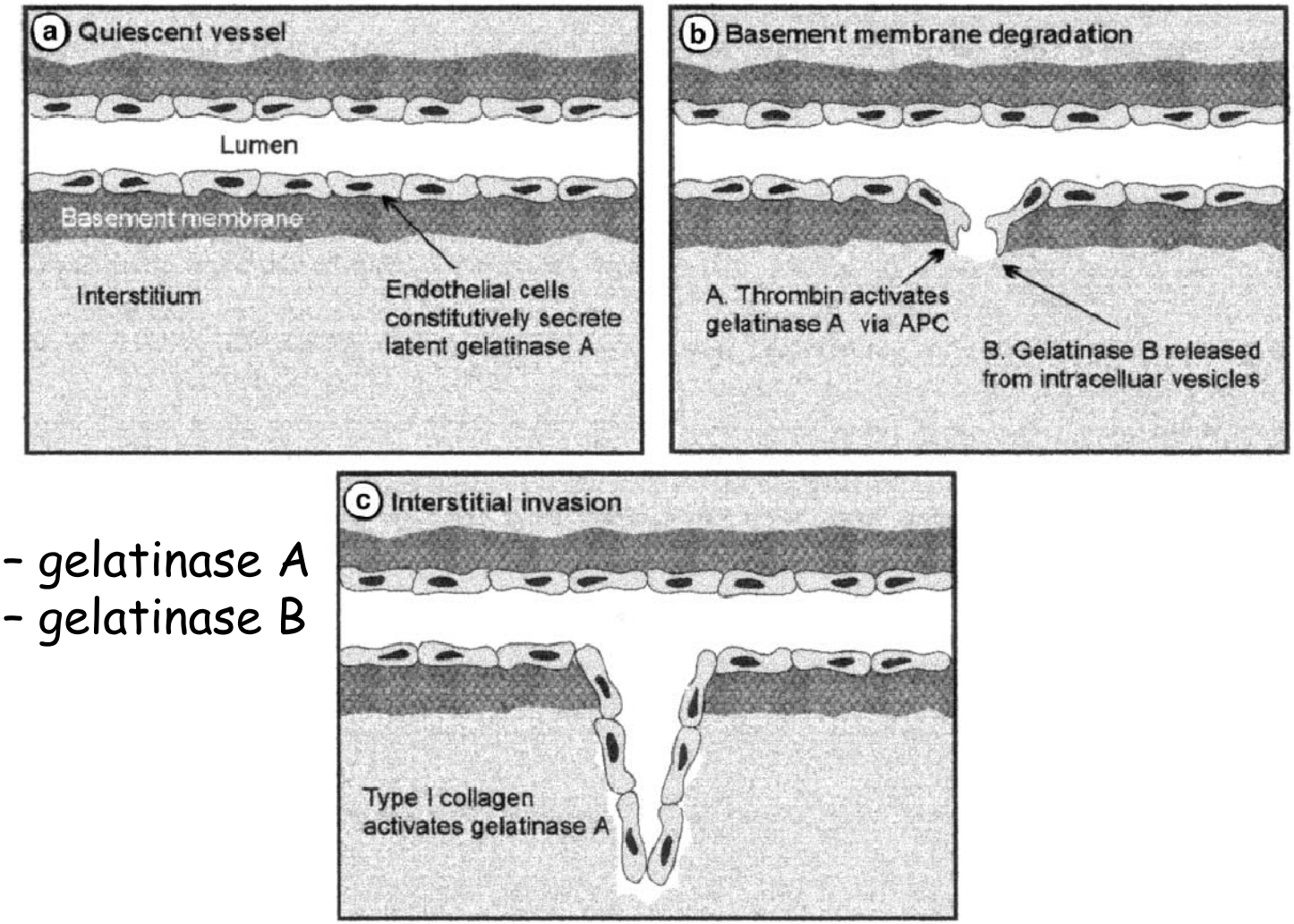


Stages of angiogenesis

- increase in vessel permeability and thrombin deposition
- loosening of pericyte contact
- proteinase release from endothelial cells
- digestion of basement membrane and extracellular matrix
- migration and proliferation of endothelial cells
- formation of vascular structures
- fusion of new vessels
- initiation of blood flow
  - inhibition of endothelial cell proliferation
  - inhibition of the migration of endothelial cells
- formation of basement membrane

Crucial role of metalloproteinases in angiogenesis

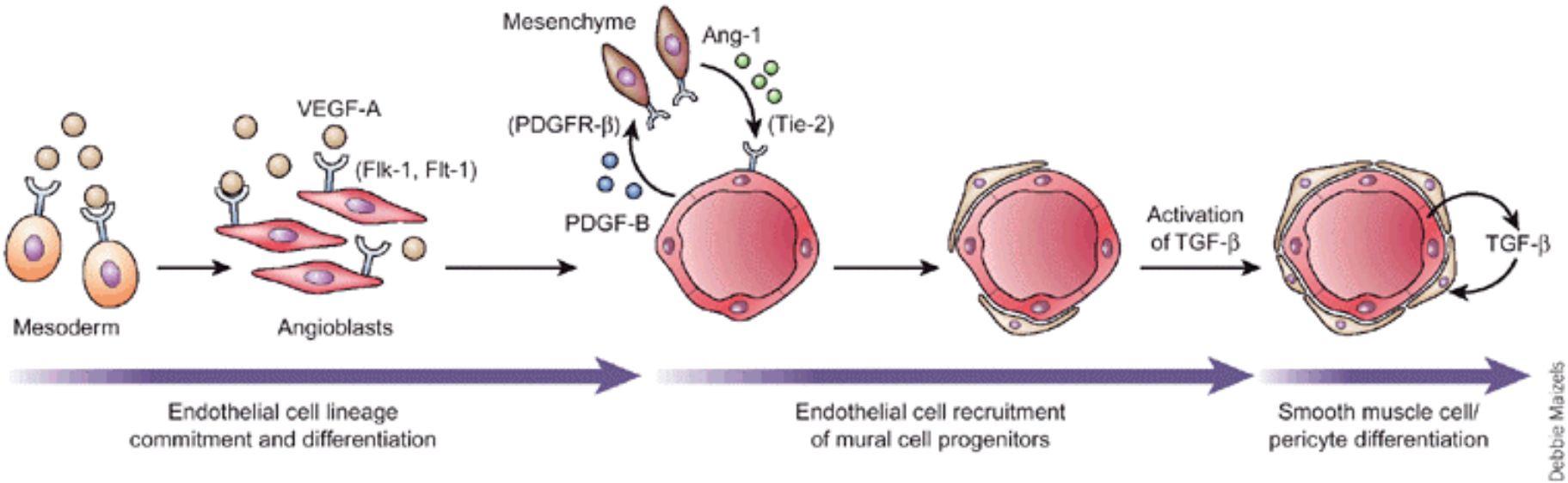
DMB



MMP-2 – gelatinase A
MMP-9 – gelatinase B

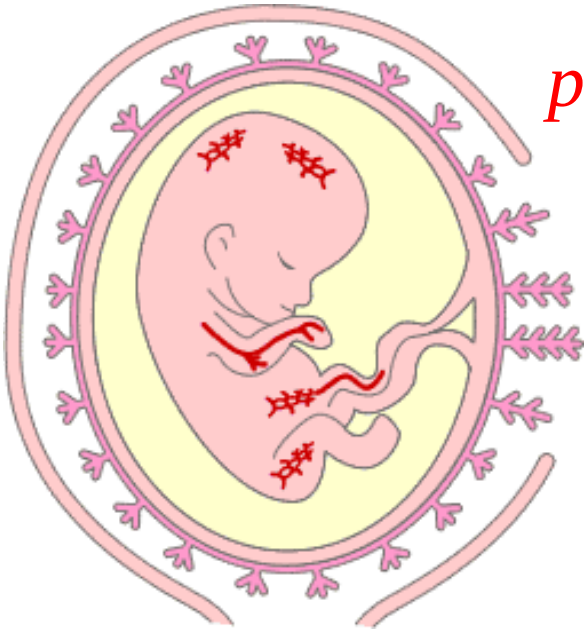
Fig. 2. Role of gelatinases during angiogeneis: (a) Gelatinase A is constitutively secreted by endothelial cells; (b) Thrombin, which is elevated in angiogenic situations, activates protein C on the endothelial surface. The resultant APC rapidly activates gelatinase A allowing the endothelial cells to degrade the basement membrane. Active gelatinase B may be released from intracellular storage vesicles of endothelial cells to assist basement membrane degradation; (c) Once the endothelial cells have penetrated the basement membrane they contact the interstitium. Type I collagen further activates gelatinase A for sustained periods of time.

Vessel wall assembly

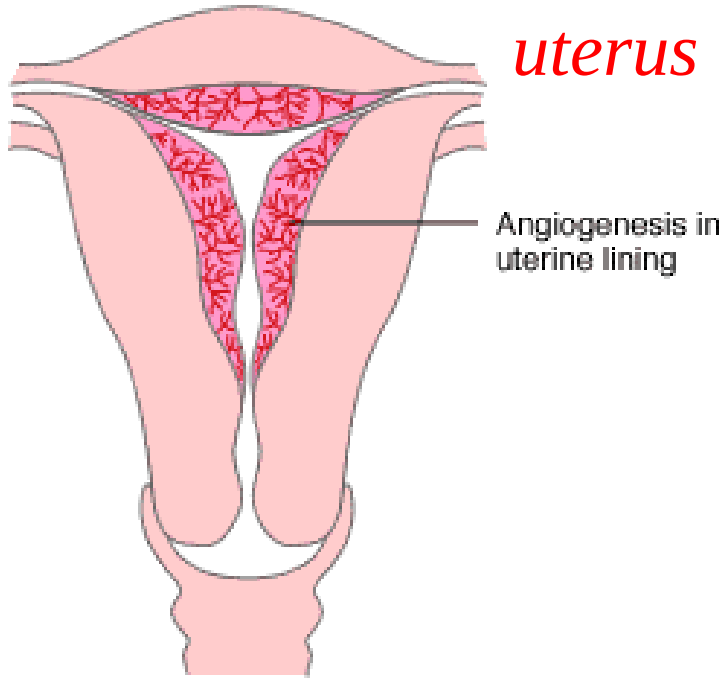


Cleaver O & Melton DA, Nature Med. June 2003

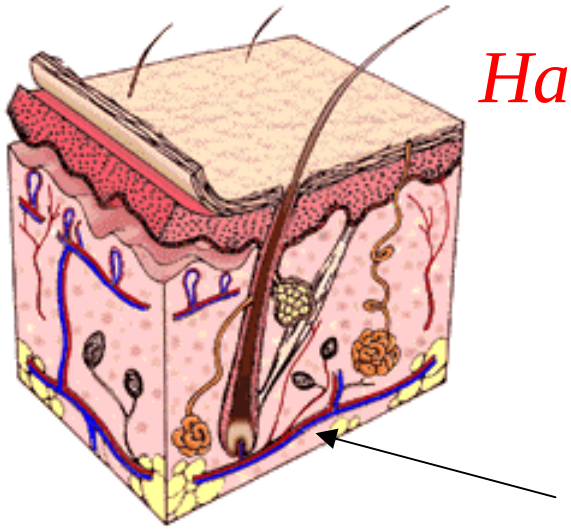
Physiological angiogenesis in adults is restricted



placenta

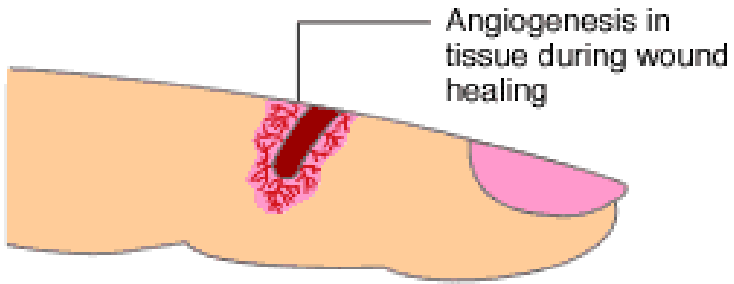


uterus



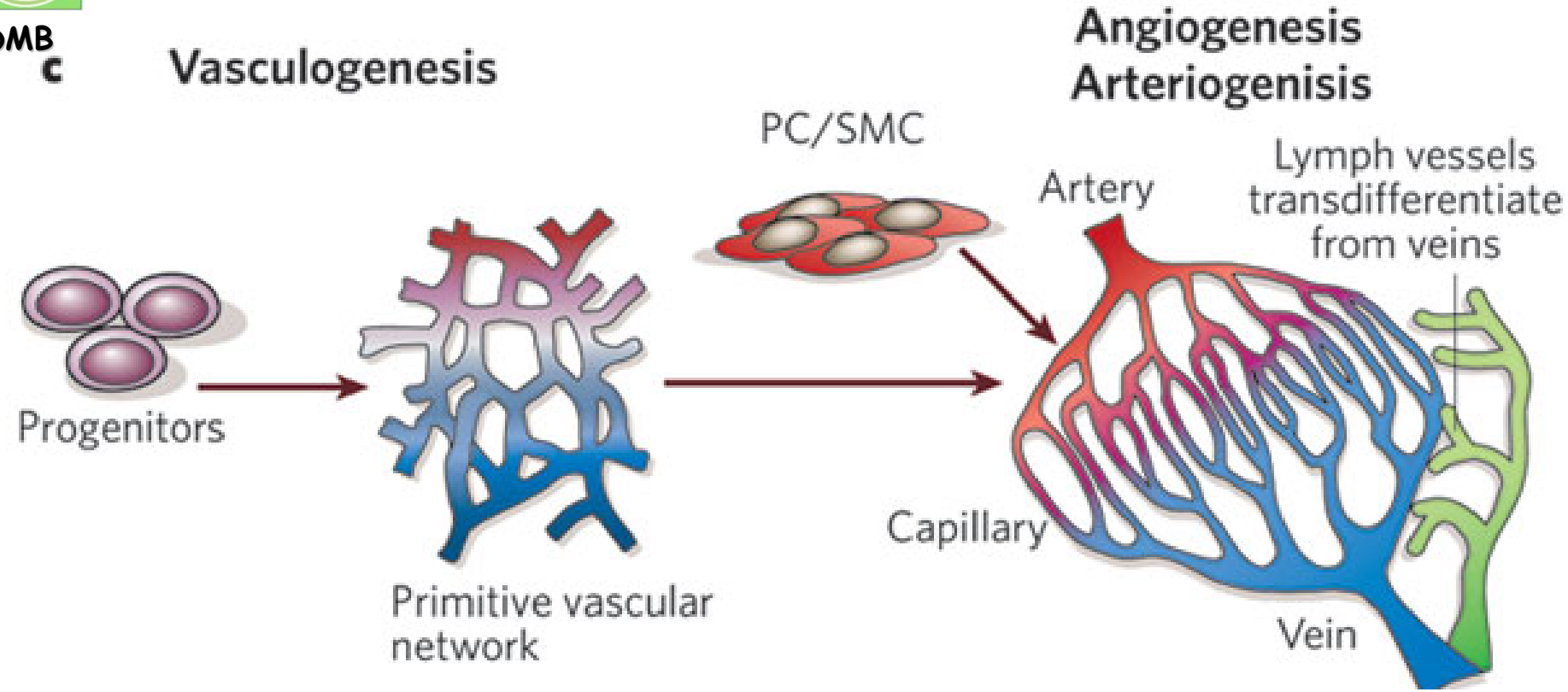
Hair growth

Wound healing



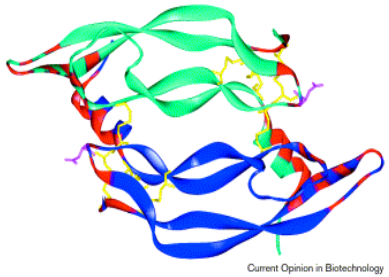
Blood vessel formation – various ways

DMB c



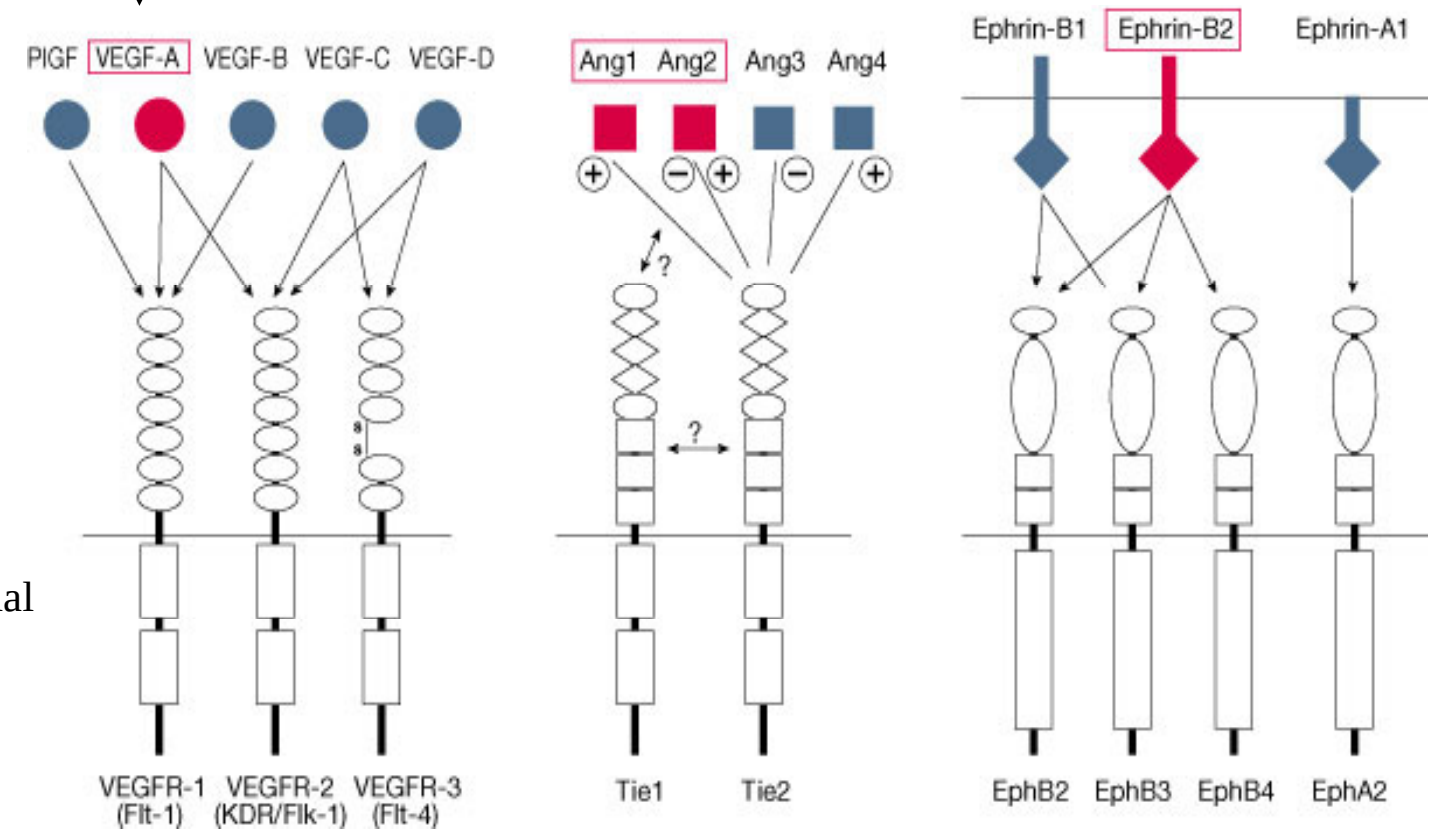
Endothelial Cell Differentiation Proliferation Tube Formation	Vascular Branching and Remodeling	Pericyte Recruitment	Maintenance of Mature Vessel	Ischememia-Induced Angiogenesis
VEGF FGF2 TGF-ß1	VEGF FGF ANG1	VEGF ANG1 PDGF-BB	ANG1	VEGF FGF2 ANG2 PDGF-BB PLGF

Vascular endothelial growth factor-A (VEGF)



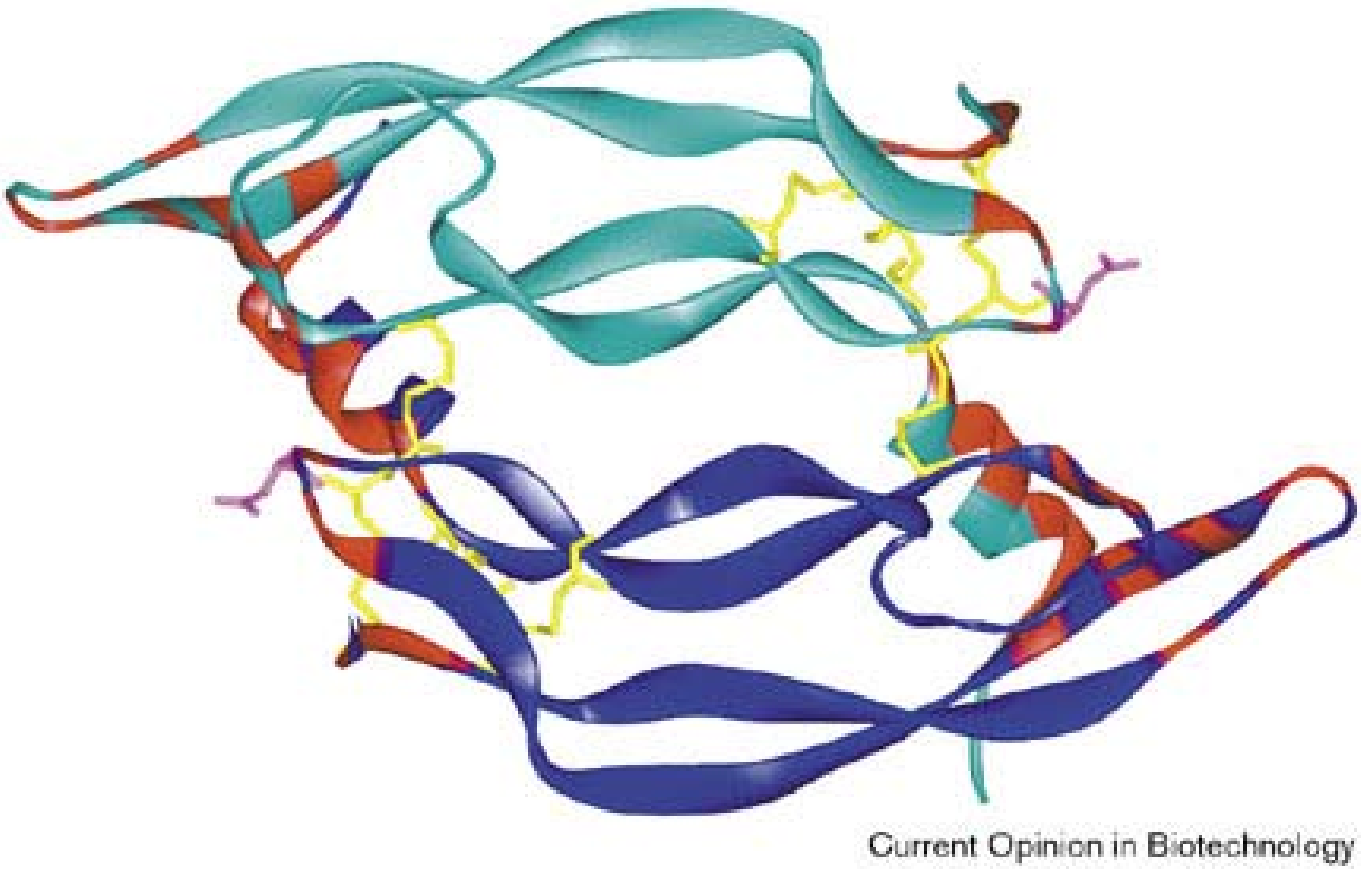
VEGF-A is a major angiogenic growth factor. It acts on endothelial cells, being produced by numerous cell types, including vascular smooth muscle cells (VSMC), fibroblasts or tumor cells.

Receptors on endothelial cells



Yancopoulos, Science 2000

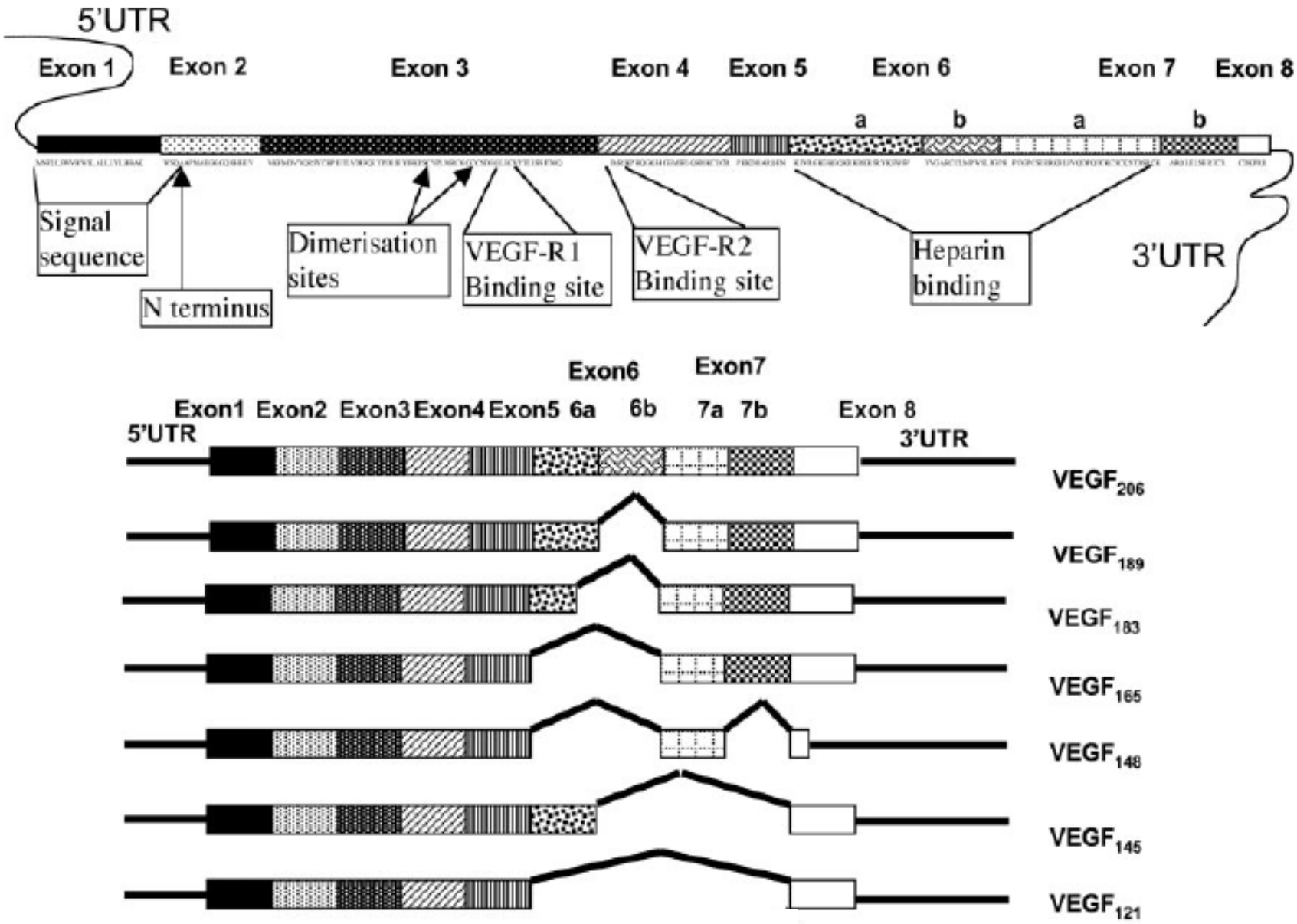
VEGF, VEGF-A



-a dimeric glycoprotein
-belongs to a so-called cysteine-knot superfamily of growth factors
- one interchain disulfide bond

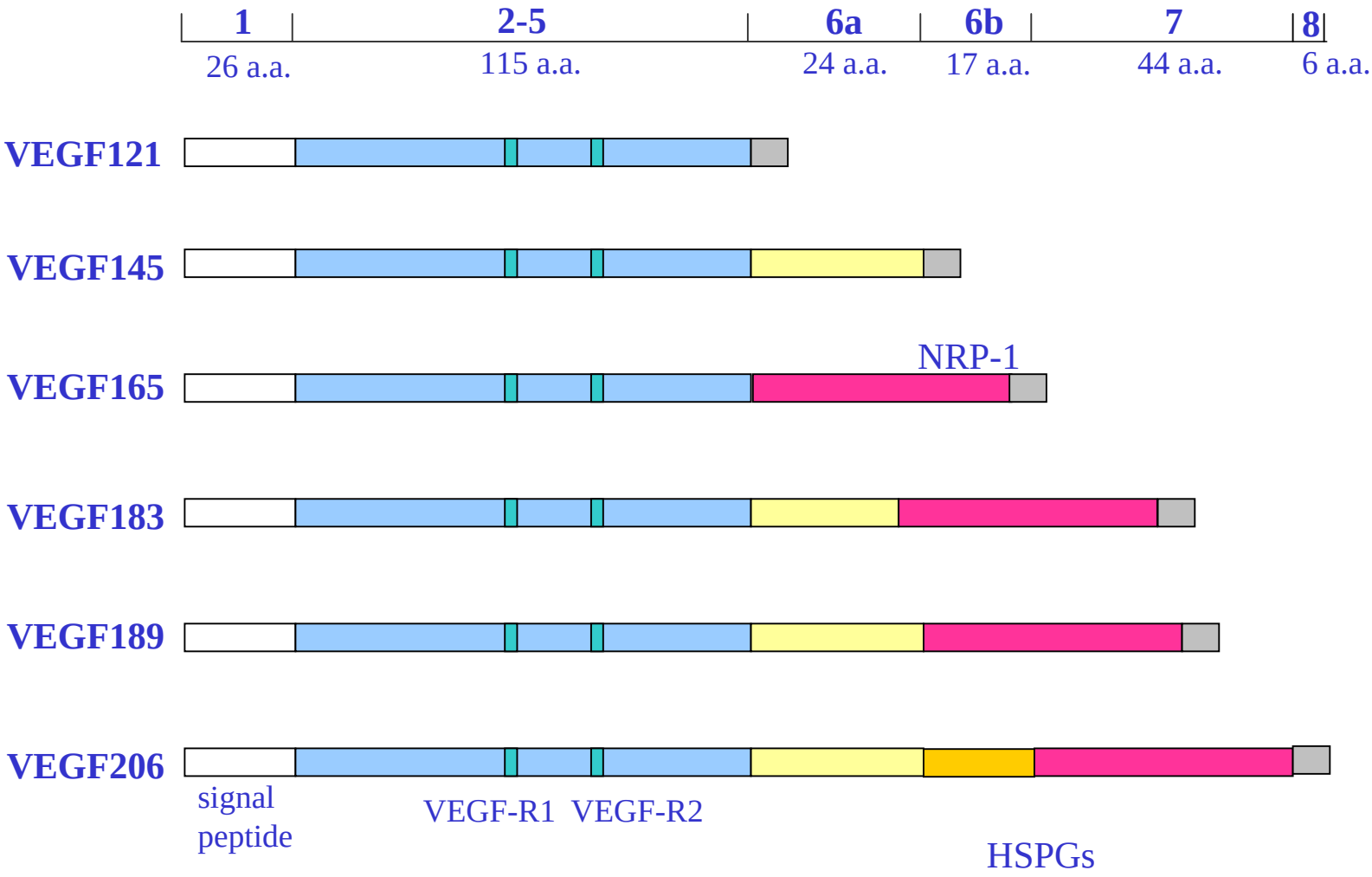
Oloffson et al., 2000

Organisation of VEGF gene and VEGF isoforms



Bates et al., Cancer Res 2004

Splice variants of human VEGF



After Robinson and Stringer 2001, J Cell Science 114:853-65

Properties of VEGF isoforms

$VEGF_{121}$ is a soluble acid polypeptide

$VEGF_{189}$ and $VEGF_{206}$ are highly basic and bind very strongly to heparin, thus they are completely sequestred in extra-cellular matrix (ECM)

$VEGF_{165}$ has intermediate properties: it is secreted, but significant fractions remains bound to cell surface and ECM

Proteolytic processing of VEGF-A

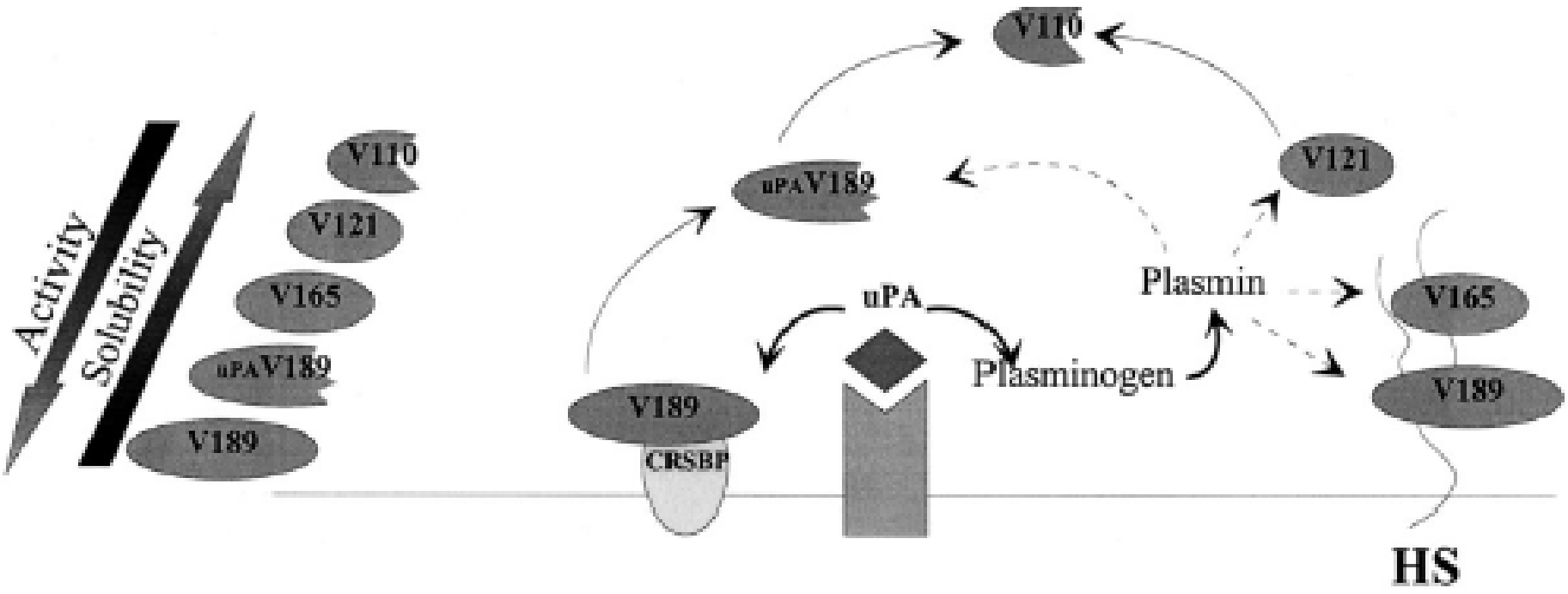


Figure 5. *VEGF storage in the extracellular matrix and protease processing.* The binding of V189 to both proteoheparansulfates and retention sites (HS and CRS BP) induces its sequestration into the extracellular matrix. The disruption of its interaction with either storage site allows urokinase to cleave VEGF 189 into 2 soluble fragments of 140 aa and 49 aa. When urokinase converts plasminogen into plasmin, all the VEGF isoforms are processed in a very soluble 110 aa isoform.

Receptors for VEGF-A

Main receptors:

VEGFR-1 (flt-1)

VEGFR-2 (Flk1;KDR)

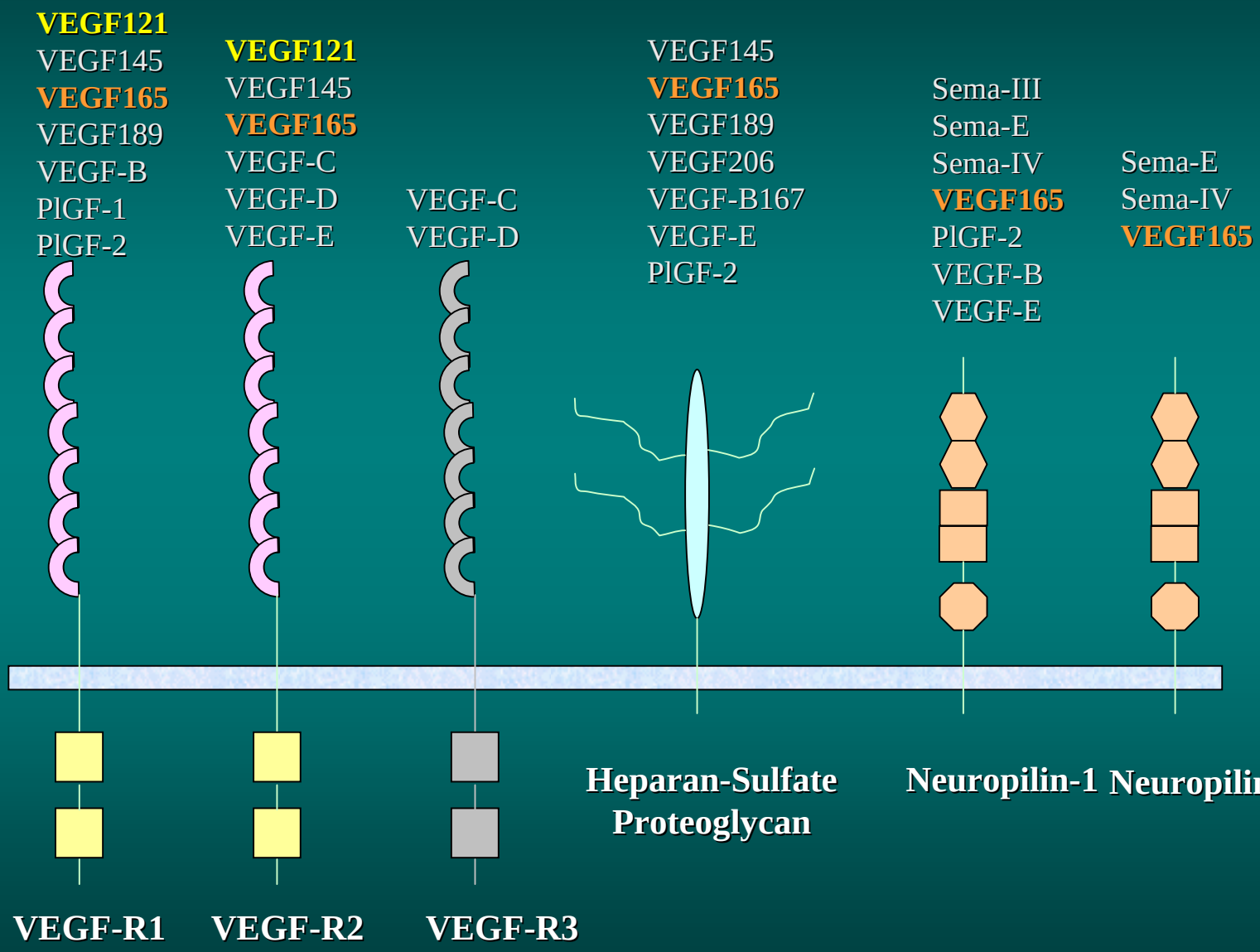
Accessory receptors

Neuropilin 1 (NRP1)

Neuropilin 2 (NRP2)

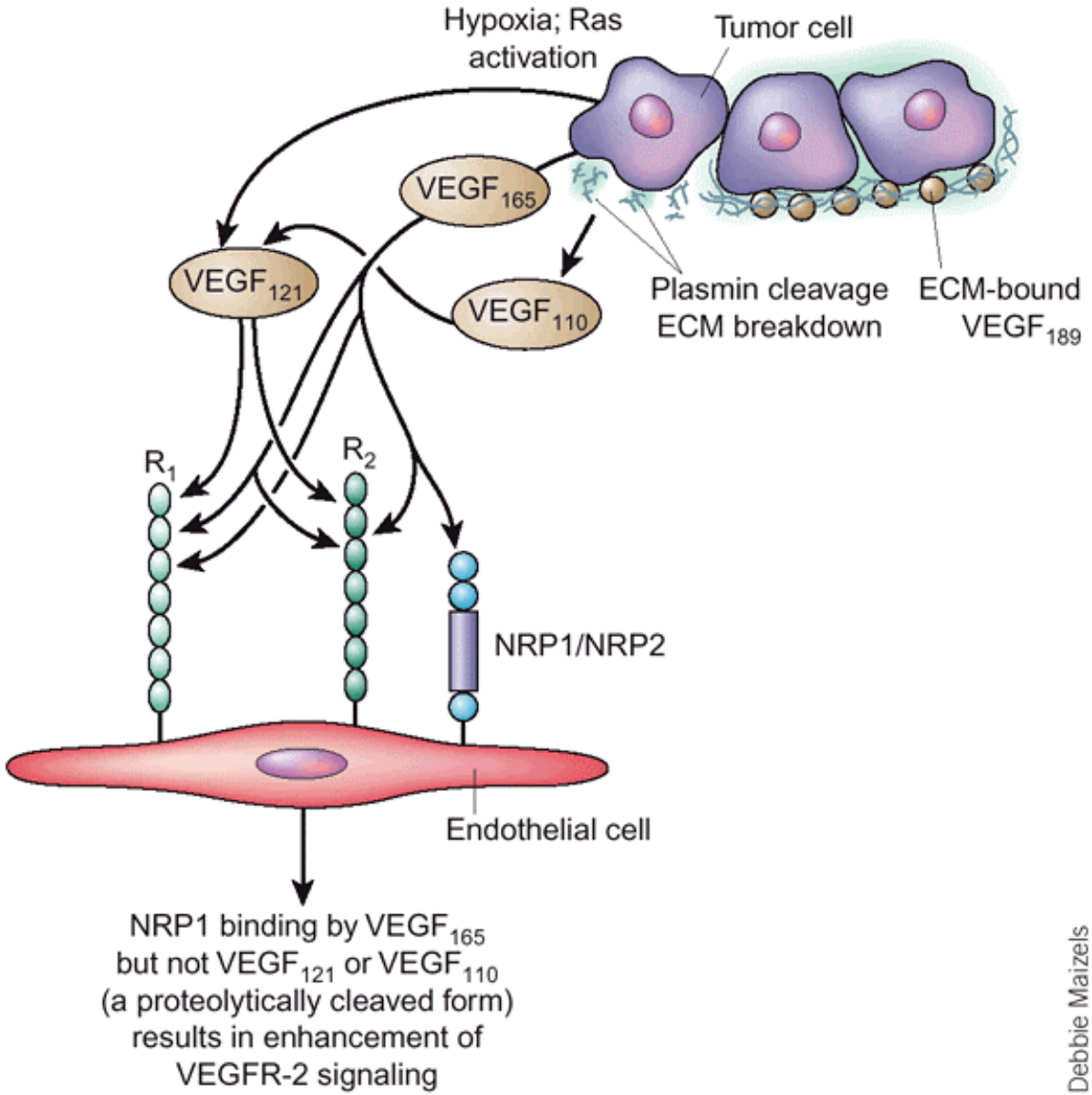
Storage
heparan sulphate proteoglycans

Growth factors and receptors of the VEGF family



After Neufeld et al.. 1999, FASEB J 13:9-22

Interactions of VEGF isoforms with the receptors



Ferrara N et al., Nature Med., June 2003

Expression of VEGF isoforms

- Most VEGF-producing cells express $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and often $VEGF_{183}$. $VEGF_{145}$ and $VEGF_{206}$ are seemingly restricted to cells of placental origin.

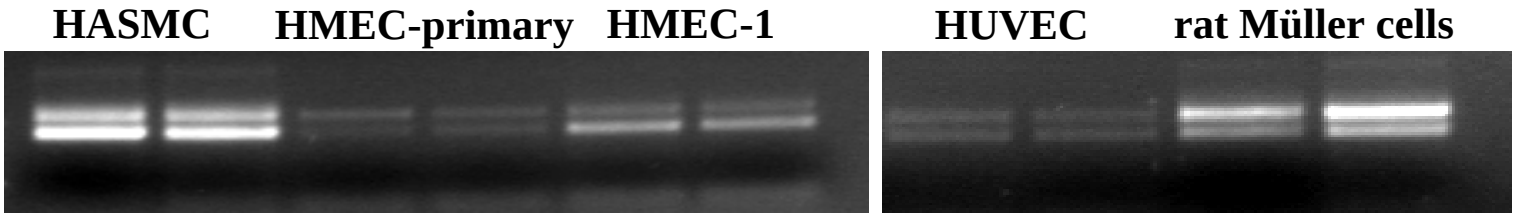
- $VEGF_{165}$ is most abundantly expressed, but $VEGF_{189}$ is a major isoform in lungs, and both $VEGF_{165}$ and $VEGF_{189}$ predominate in heart. Furthermore, the relative levels of VEGF isoforms may vary during development or in response to cytokine stimulation.

- $VEGF_{121}$, $VEGF_{145}$ and $VEGF_{165}$ induce proliferation and migration of endothelial cells.

Not every cells express the same amounts of VEGF

VEGF isoforms in several cell lines - intact cells

(24 h incubation)



Significance of VEGF and VEGF receptors has been recognized by targeting disruption of those genes in mice

Effect of knockouts of VEGF receptors

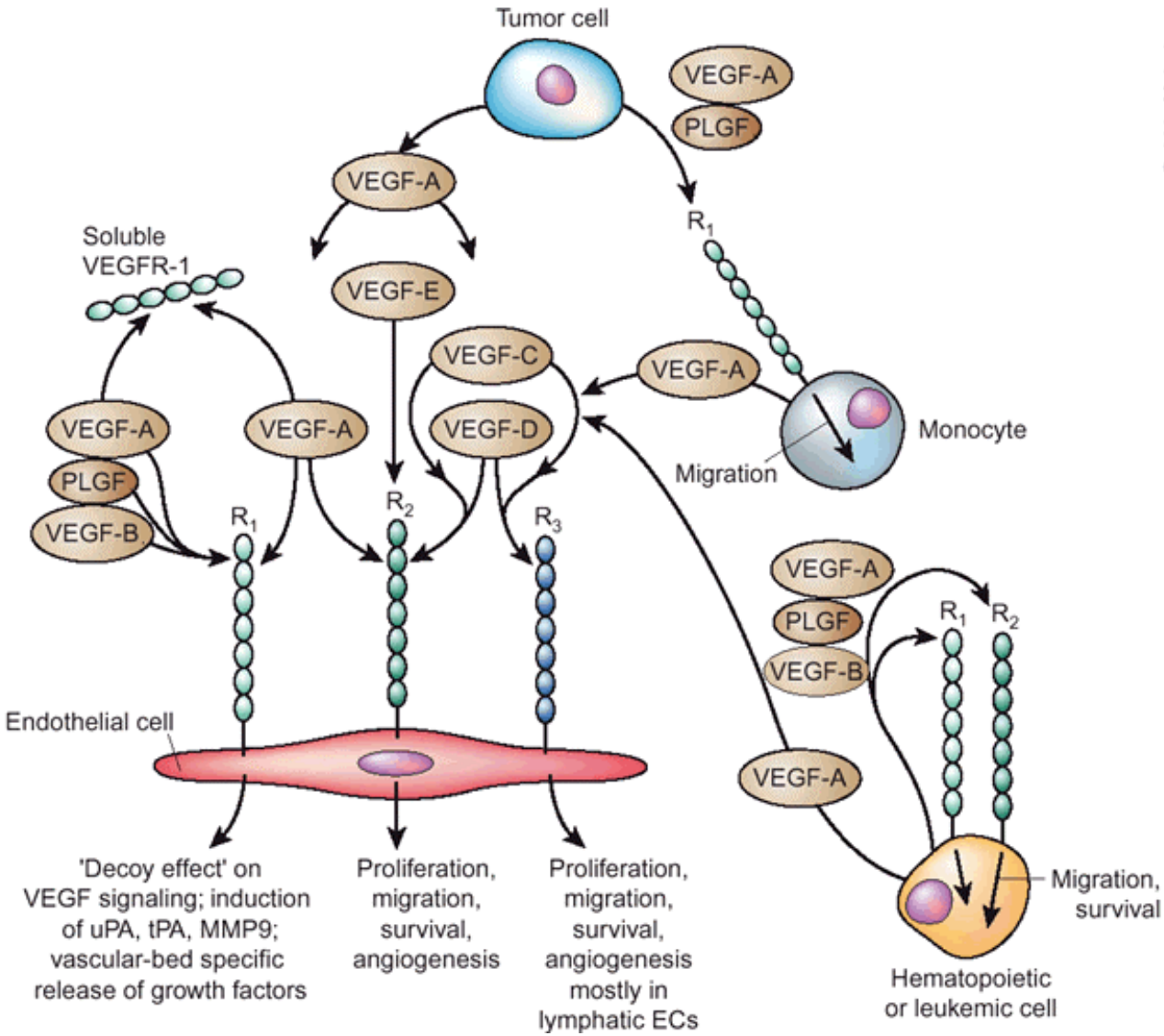
VEGFR-1

Flt1-/- mice die in utero between days 8.5 and 9.5

- EC develop but do not organize into vascular chanels
- excessive proliferation of angioblasts

Thus, at least during early development, VEGFR-1 is a negative regulator of VEGF action

Functions of VEGF receptors



Ferrara N et al., Nature Med., June 2003

Effect of knockouts of VEGF receptors

VEGFR-2

Flk1-null mice die between day 8.5 and 9.5

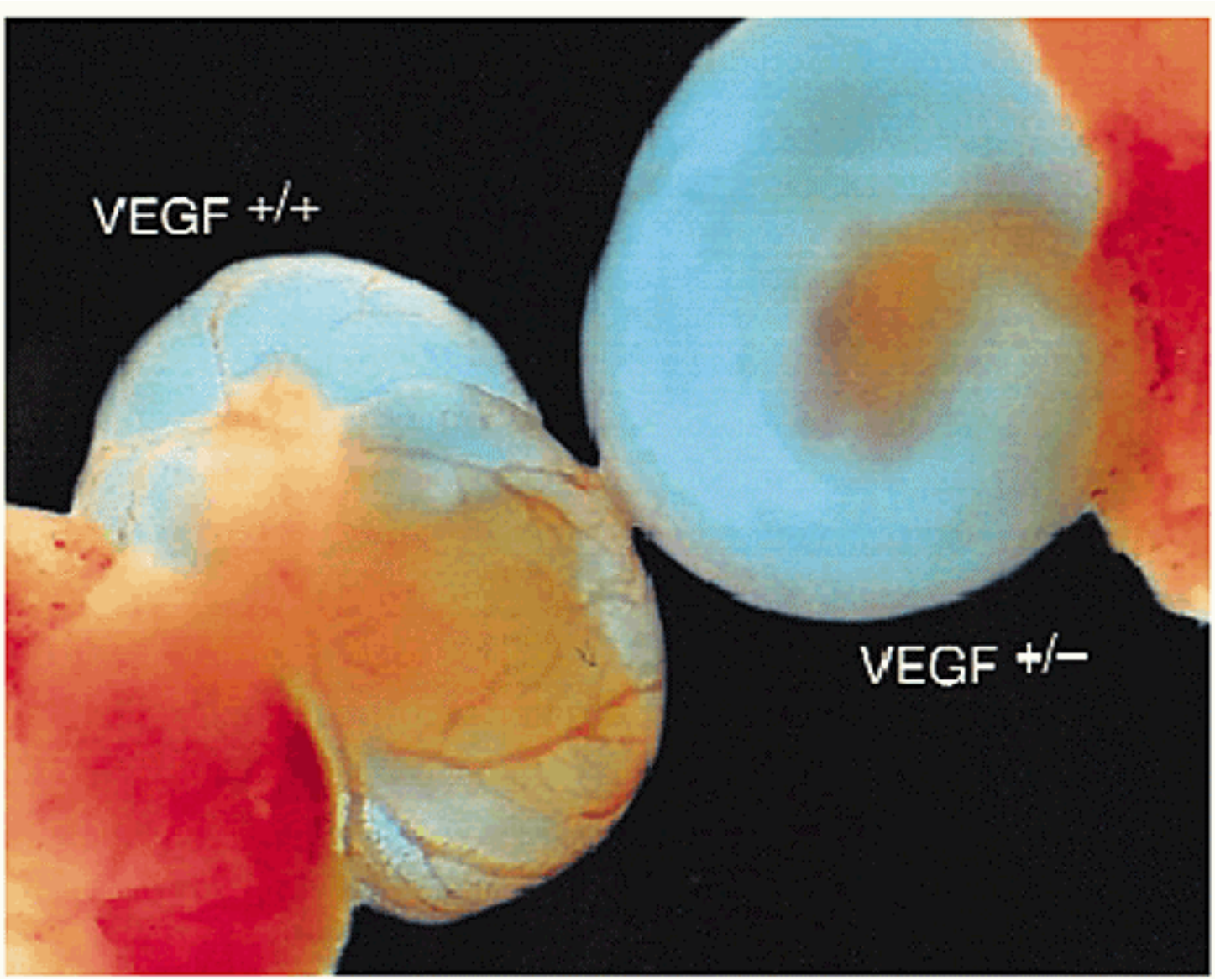
Lack of vasculogenesis and failure to develop blood islands and organized blood vessels

VEGFR-2 is the key receptor for VEGF-A-induced angiogenesis.

It signals mitogenic, chemotactic and pro-survival effects

Lethal effects of VEGF gene knockout

Knockout of VEGF is lethal in heterozygous form



Ferrara &Alitalo, Nature Med. 2000

Lethal effects of VEGF and VEGFRs knockouts

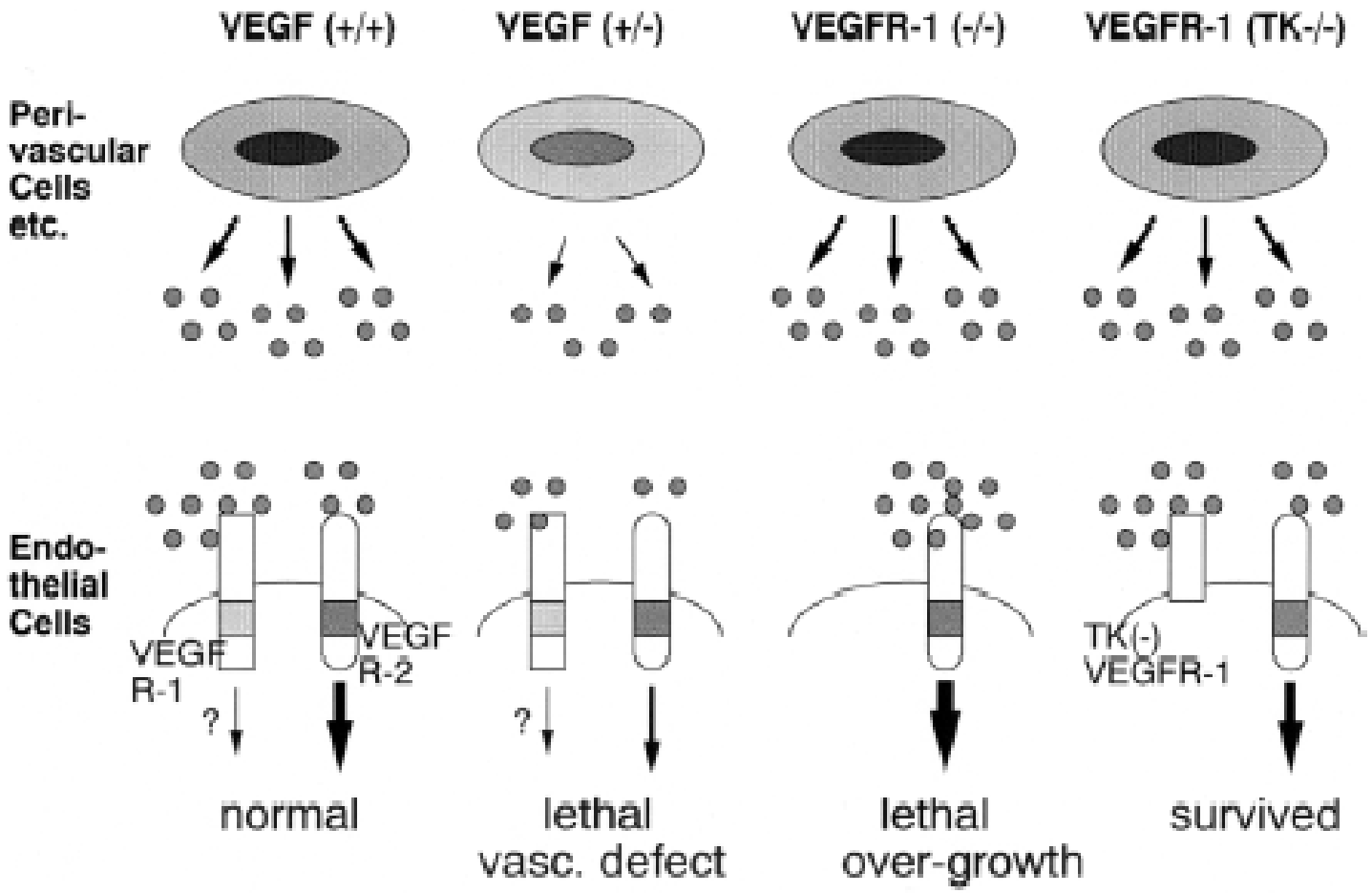


Fig. 3. Models for *VEGF* and *VEGFR-1 (Flt-1)* gene targeted mice. The *VEGFR-1 (Flt-1)* gene null mutation is lethal due to abnormal over-growth of endothelial-like cells. The extracellular domain of the *VEGFR-1 (Flt-1)* gene rescues most of the null mutant defects, possibly by trapping VEGF and decreasing the levels of VEGF.

Expression of VEGF receptors

-endothelial cells: VEGFR-1, VEGFR-2, co-receptors

- other cells:
    - monocytes
    - vascular smooth muscle cells?
    - tumor cells?
    - hematopoietic stem cells

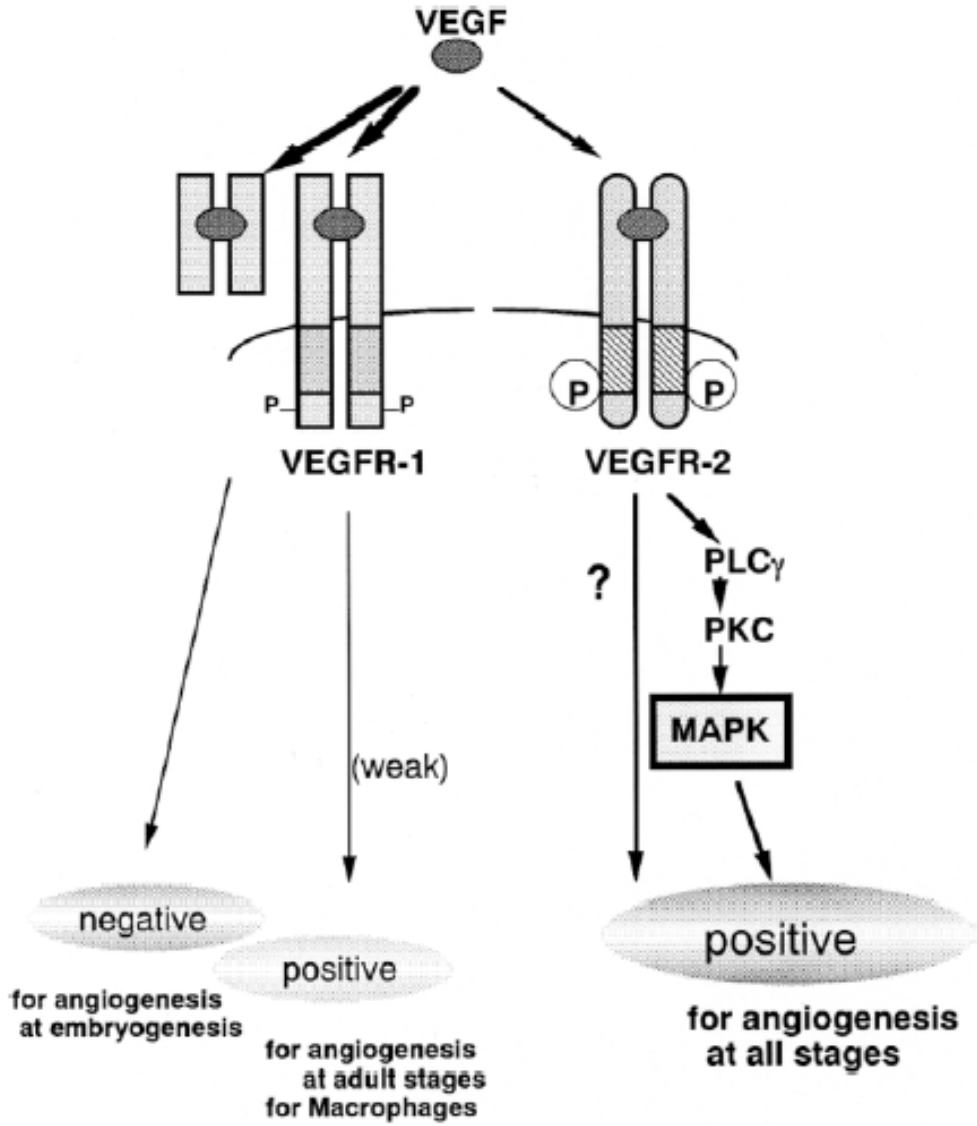


Fig. 4. A model for function and signal transduction from two VEGFRs (Flt-1 and KDR/Flk-1). VEGFR-1 (Flt-1) has functions in either a negative or positive manner depending on the biological system or developmental stage.

Semaphorin receptors – Np-1 and Np-2

- form complexes with type A plexins
- complexes serves as signaling receptors for class-3 semaphorins
- involved in axonal guidance

Np-1 and Np-2 in angiogenesis

- binds VEGF165, VEGF-B, PlGF-2
- knockout of Np.-1 – lethal at E12.5
- overexpression of Np1- excessive capillary formation, dilated blood vessels extensive hemorrhage
- no discernible abnormalities in Np.-2 knockout mice, but Np-2-/-Np1+/- are lethal
- double knockouts Np.-1-/-Np.-2-/- - died in uter at E8.5, completely avascular yolk sacs

Angiogenic and vasculoprotective functions of VEGF

- vascular permeability functions

-endothelial cells survival factor

-Endothelial cell proliferation

-Endothelial cell migration

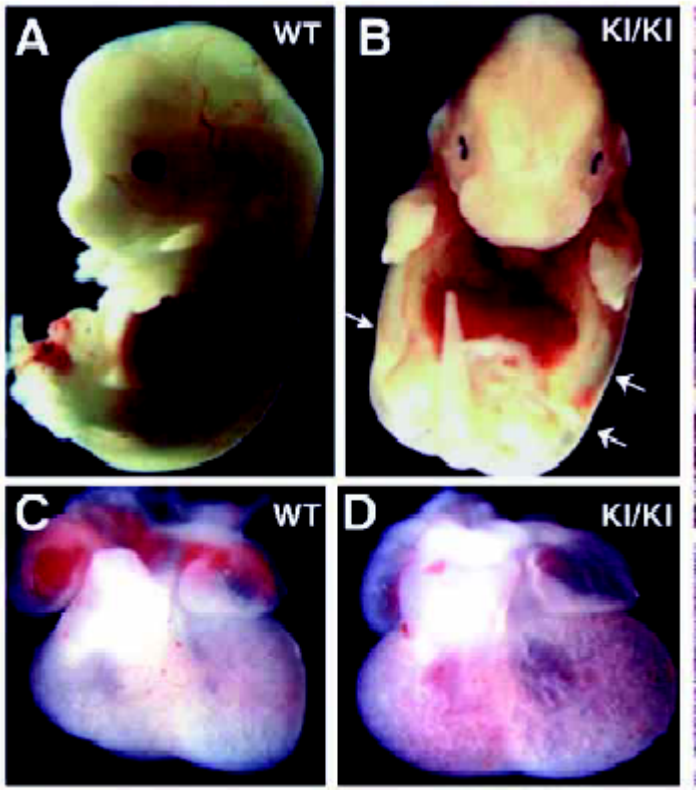
- inhibition of thrombosis

VEGF level has to be tightly regulated during development

Embryonic development is disrupted by modest increases in VEGF gene expression

Miquerol L, Langille BL, Nagy A.
Development, 2000: 127:3941-6

2-3 fold overexpression is deletorious to embryonic development

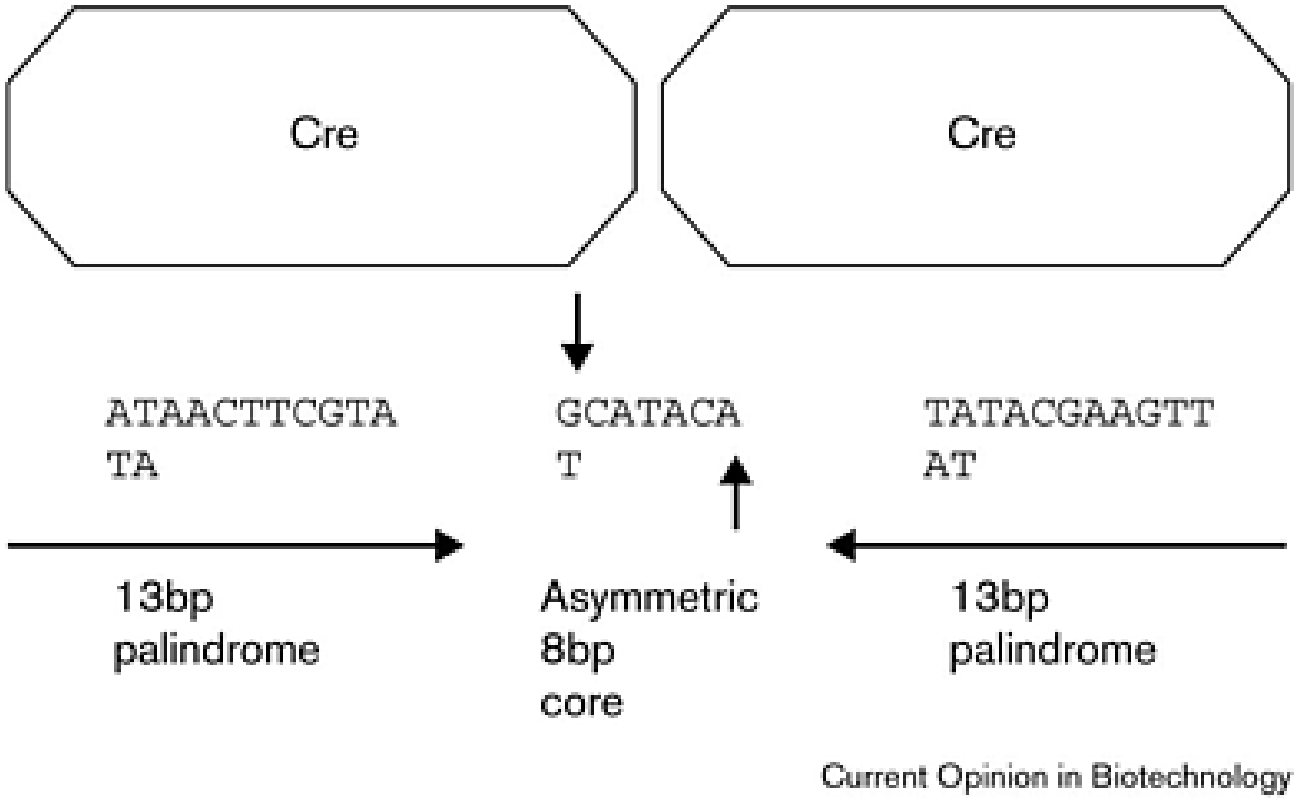


Enlarged hearts

Embryos died between E12.5 and E14.5

Conditional knockouts of genes

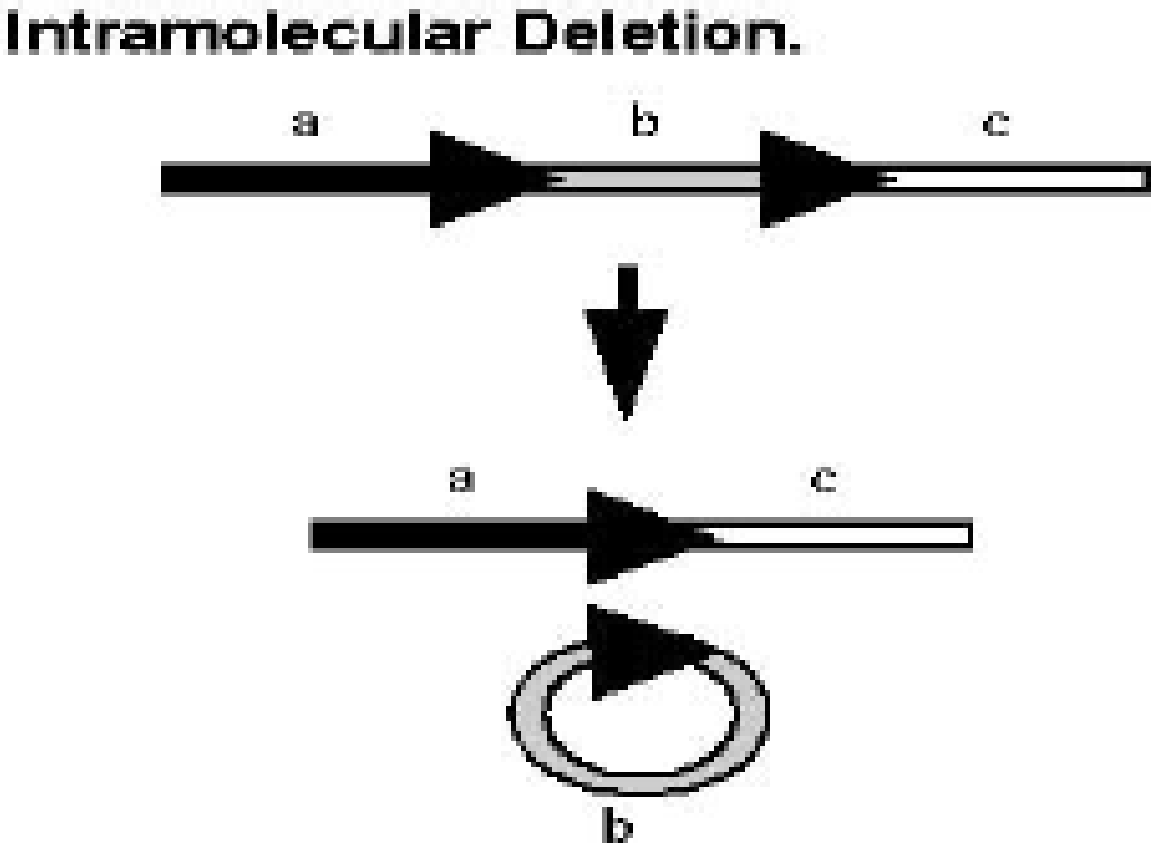
Use of Cre recombinase for conditional knockouts



DNA recognition site for recombinase enzymes. The DNA recombinases have a similar basic recognition site, as shown here for the Cre enzyme. Two palindromic sequences (loxP sites for the enzyme Cre) are separated by a DNA core. The core sequence can vary, whereas the palindromic sequences must contain a subset of the nucleotides shown to support integration.

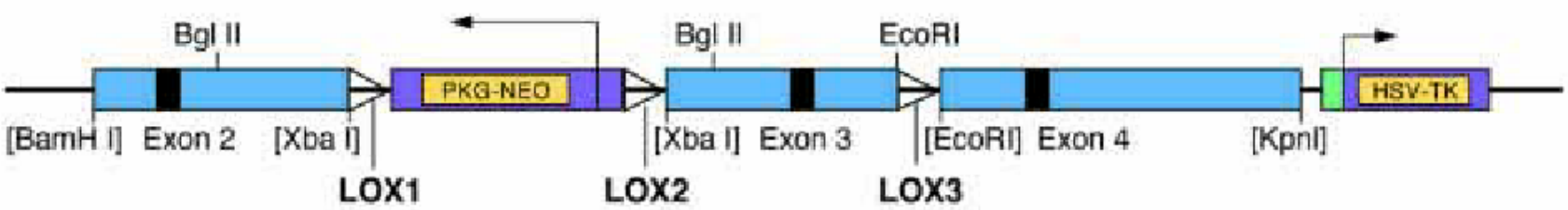
Gorman, Curr Opinion Biotech 2000

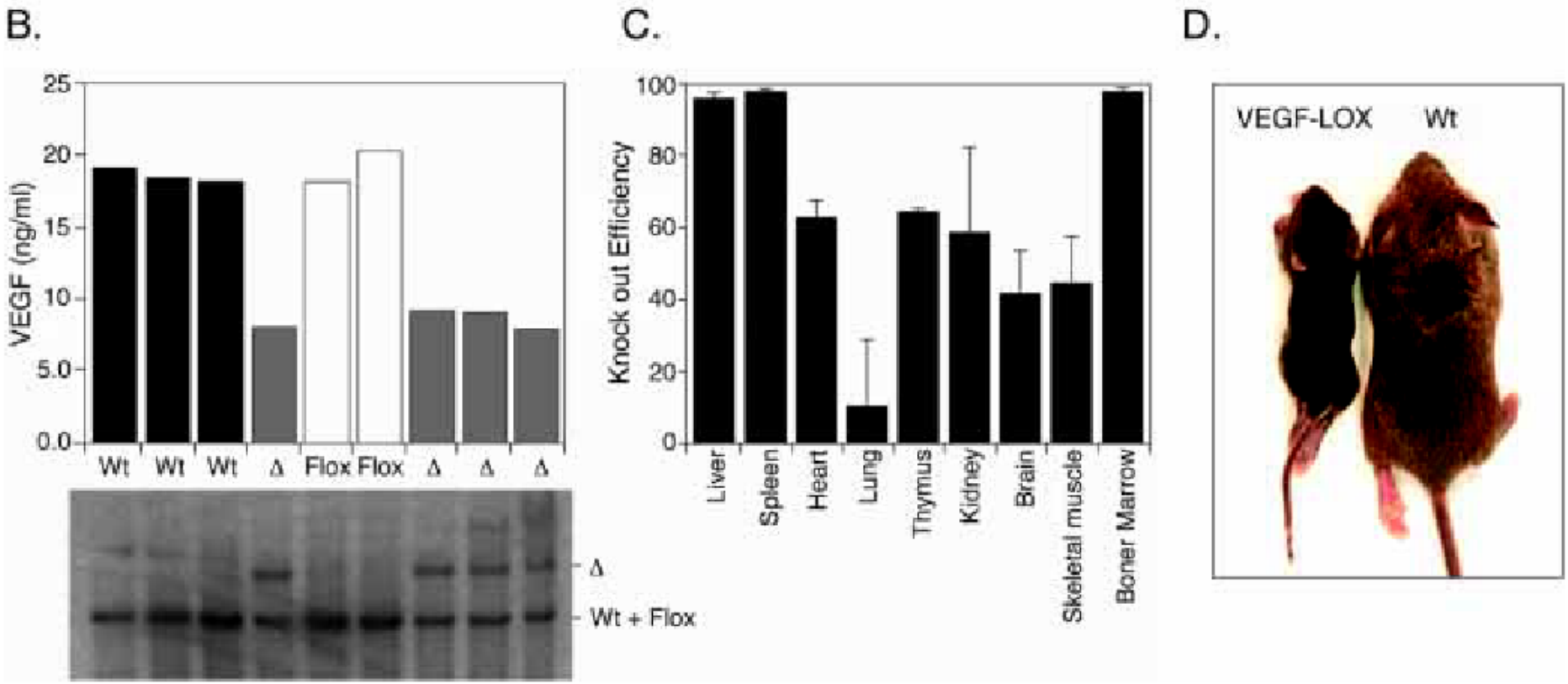
Cre recombinase mediated deletion



ADS Ryding et al., J Endocrinol 2001

VEGF is required for growth and survival in neonatal mice





Gerber et al., 1999

Table 1. Analysis of growth retardation and mortality of newborn mice of different genotype

Genotype	% Mortality at day 7	% Growth retarded survivors*	% Animals with normal body mass at day 27
VEGF *loxP*(+/+)			
Cre+ (*n*=21)	38	52	10
VEGF *loxP*(+/–)			
Cre+ (*n*=6)	0	33	66
VEGF *loxP*(+/+)			
Cre– (*n*=10)	0	0	100

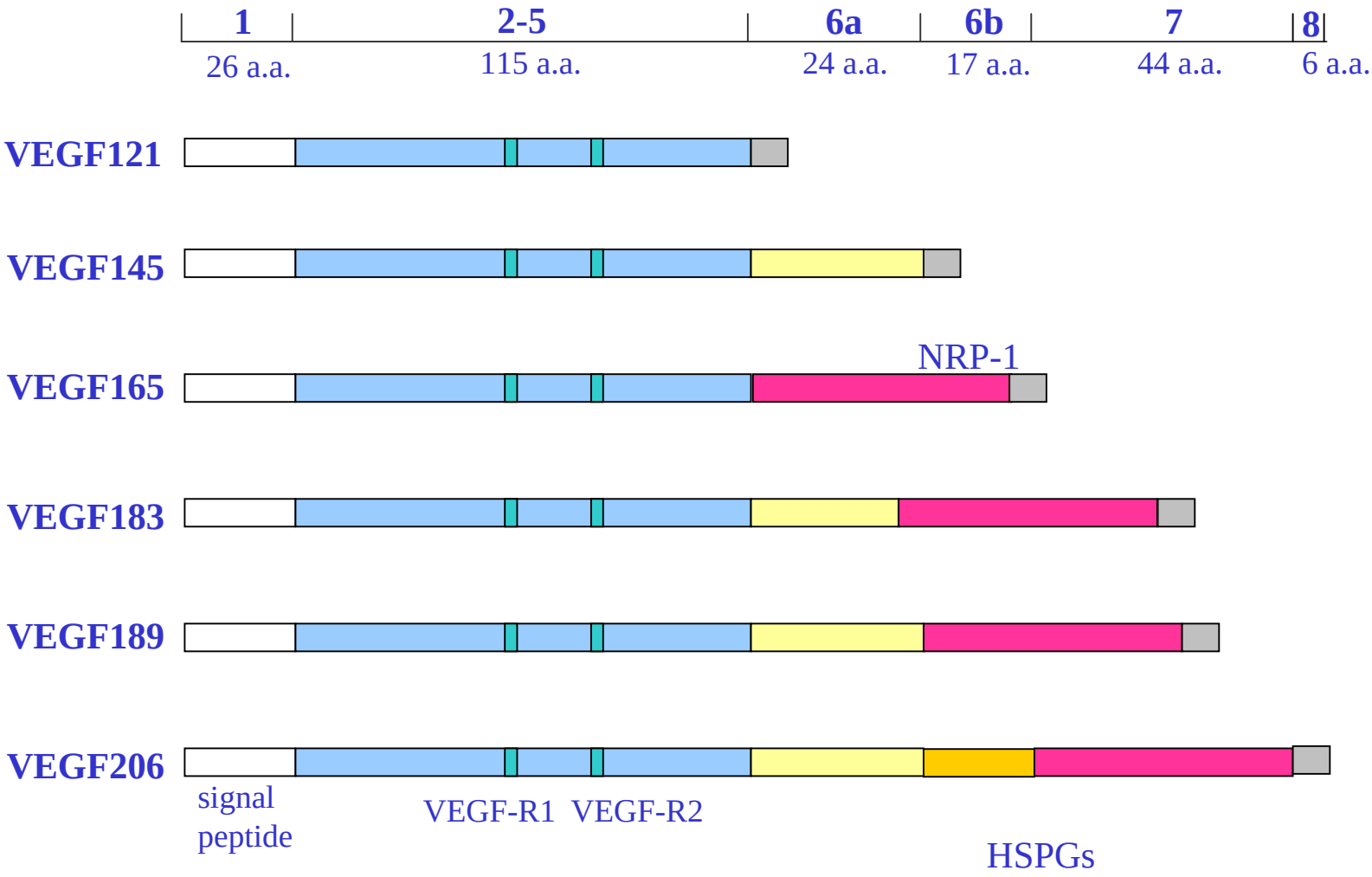
All groups were treated with 10,000 i.u. of IFN-α at days 3, 5 and 7 postnatally.

*Values >20% compared to control littermates were counted.

VEGF is required for growth and survival in neonatal mice

1. 38% mortality at day 7 in mice without VEGF (its synthesis was blocked from day 3);
2. Liver changes - smaller hepatocytes, immature sinusoids, increased extramedullary hematopoiesis and almost complete absence of Flk-1 positive endothelial cells;
3. Similar effects as after targeted knockouting of VEGF were obtained when mice were treated with anti-VEGF antibodies

Splice variants of human VEGF



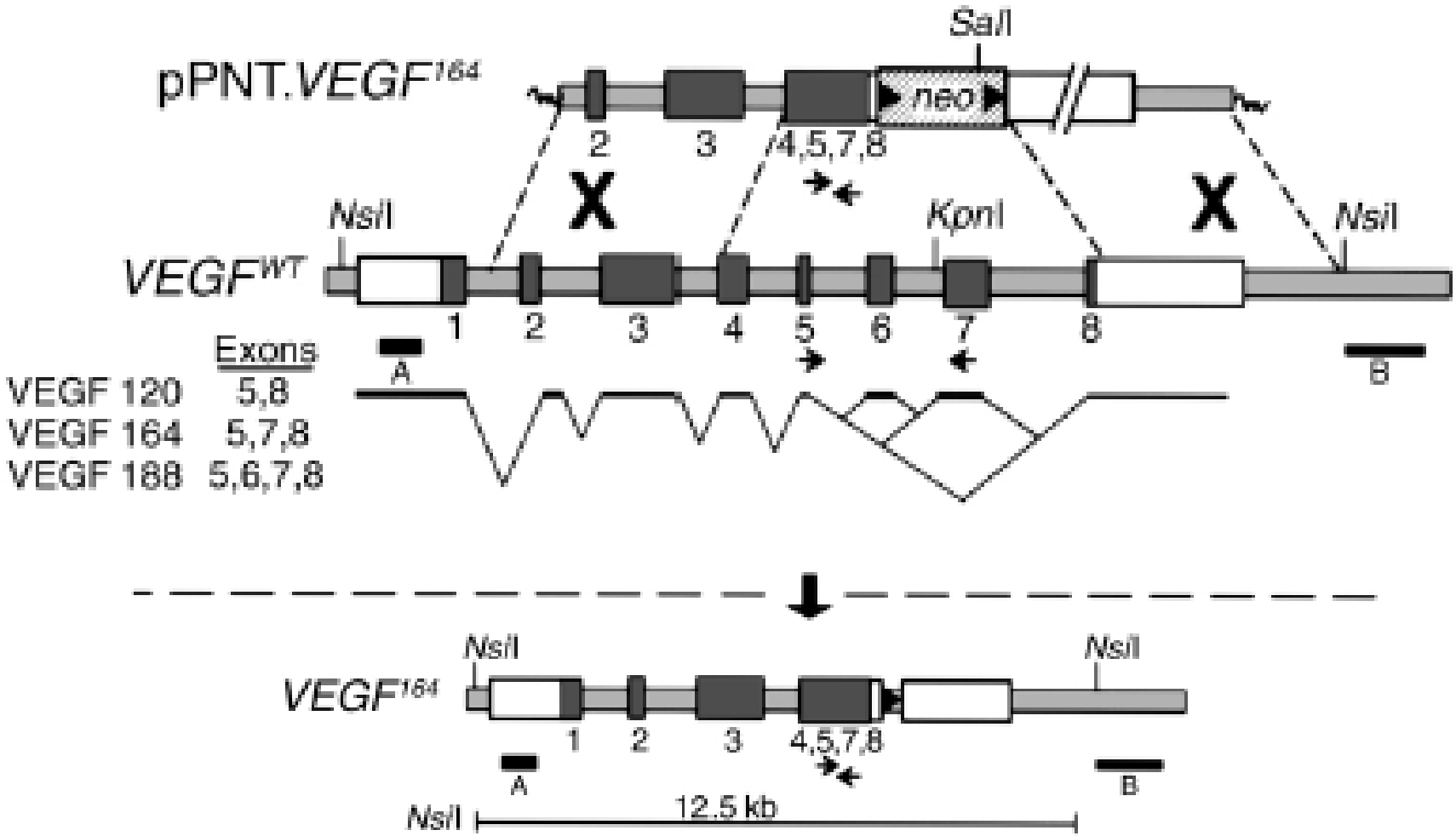
After Robinson and Stringer 2001, J Cell Science 114:853-65

Differential role of VEGF isoforms

VEGF164 is the crucial isoform

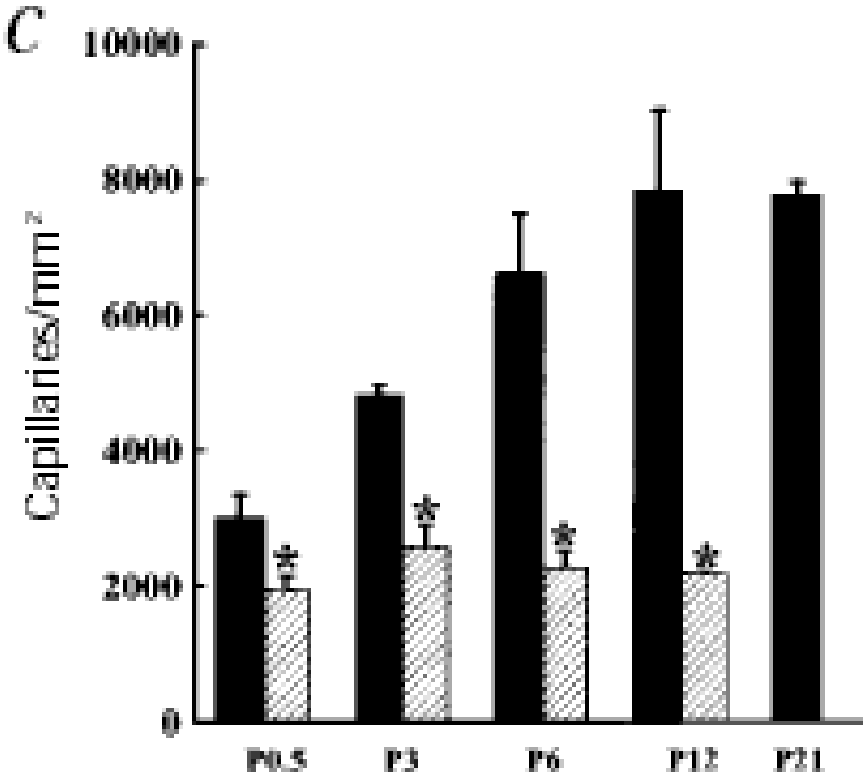
How to assess the role of different VEGF isoforms, if the knockout of the gene is lethal?

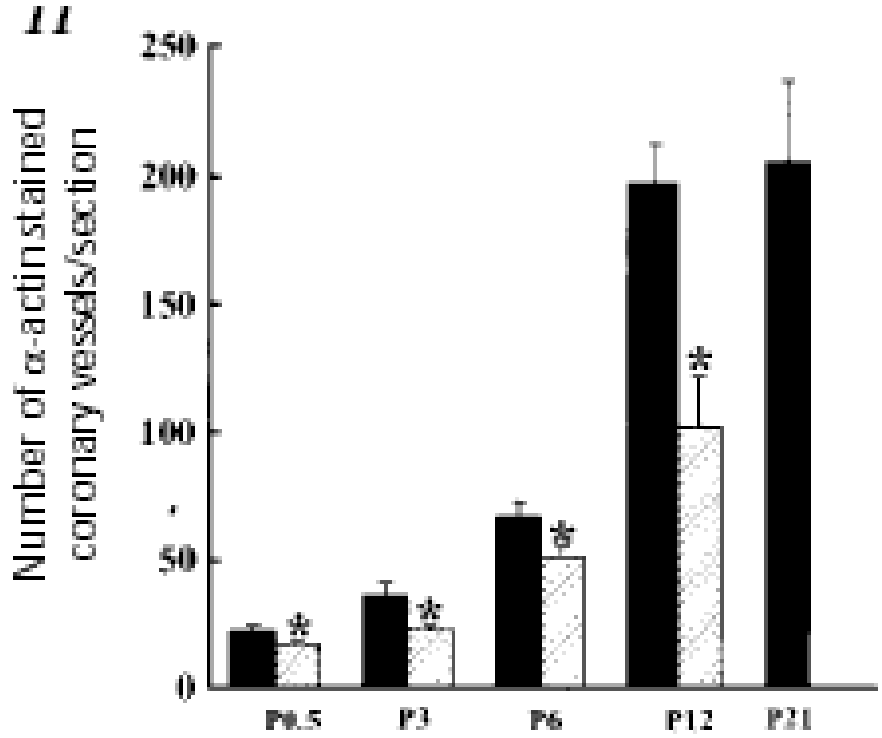
Targeting of VEGF isoform-specific alleles

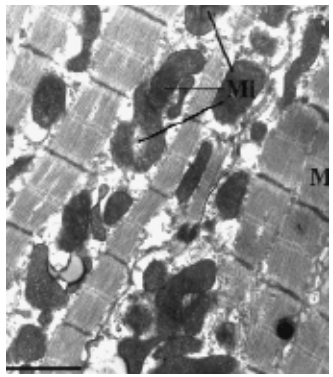


Stalmans et al., JCI 2002

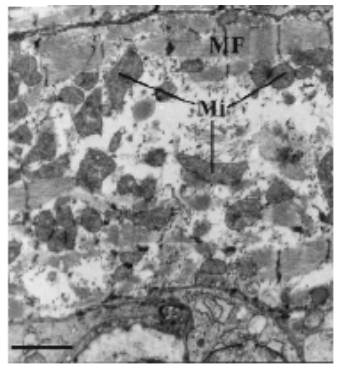
Effect of conditional knockout of VEGF164 on myocardial angiogenesis







WT



VEGF$^{120/120}$

Carmeliet et al., 1999

Impaired myocardial angiogenesis and ischemic cardiomyopathy in mice lacking the vascular endothelial growth factor isoforms $VEGF_{164}$ and $VEGF_{188}$

Peter Carmeliet[1], Yin-Shan Ng[2], Dieter Nuyens[1], Gregor Theilmeier[1], Koen Brusselmans[1], Ivo Cornelissen[1], Elisabeth Ehler[3], Vijay V. Kakkar[4], Ingeborg Stalmans[1], Virginie Mattot[1], Jean-Claude Perriard[3], Mieke Dewerchin[1], Willem Flameng[5], Andras Nagy[6], Florea Lupu[4], Lieve Moons[1], Désiré Collen[1], Patricia A. D'Amore[2] & David T. Shima[2,7]

About half the $VEGF^{120/120}$ neonates died within a few hours after birth because of bleeding in several organs (data not shown). The $VEGF^{120/120}$ neonates that survived this perinatal period had normal body weights at birth, but failed to gain weight.

They all died before postantal day 14

Viability of VEGF-isoform mice

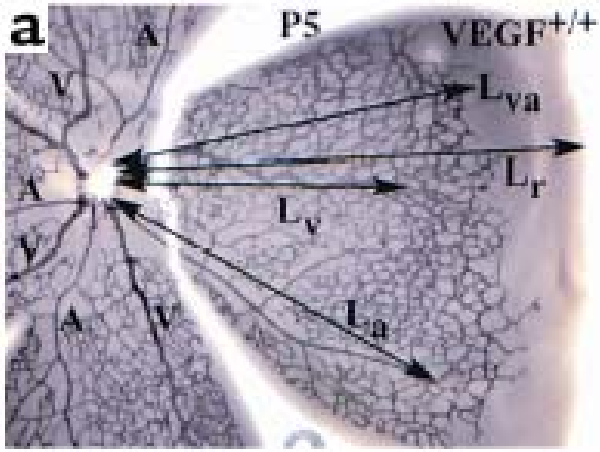
$VEGF^{120/120}$ – half neonates died shortly after births because of cardiorespiratory distress; the other died within 2 weeks after birth, in part due to impaired myocardial angiogenesis resulting in cardiac failure

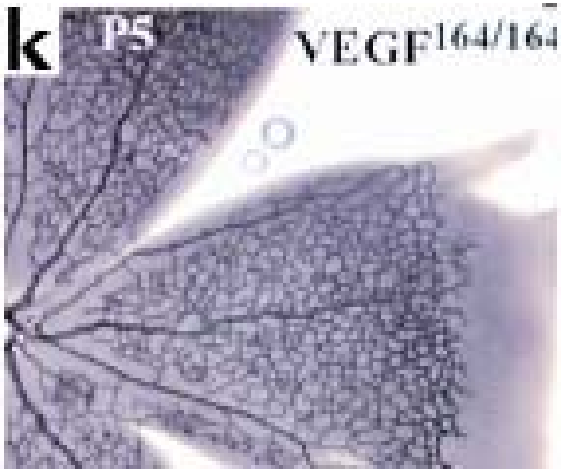
$VEGF^{164/164}$ – were normal

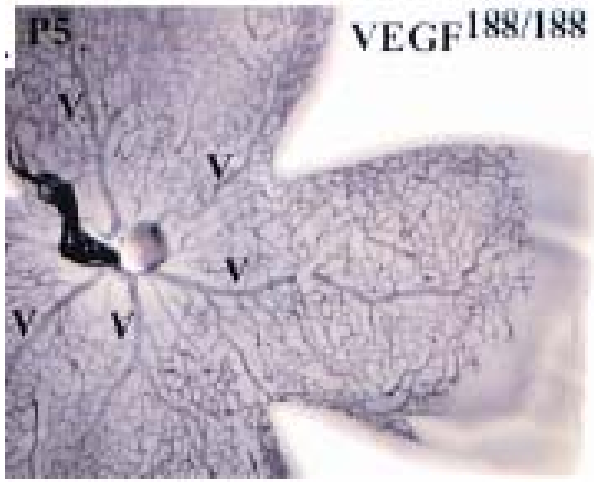
$VEGF^{188/188}$ – half of embryos died in utero

- surviving gain less weigth, were less fertile and had smaller litter size

Impaired retinal vascular development in $VEGF^{120/120}$ and $VEGF^{188/188}$ mice







Stalmans et al., JCI 2002

Role of VEGF in arteriogenesis

Stalmans et al., JCI 2002

The murine *VEGF* gene is alternatively transcribed to yield the $VEGF_{120}$, $VEGF_{164}$, and $VEGF_{188}$ isoforms, which differ in their potential to bind to heparan sulfate and neuropilin-1 and to stimulate endothelial growth. Here, their role in retinal vascular development was studied in mice selectively expressing single isoforms. $VEGF^{164/164}$ mice were normal, healthy, and had normal retinal angiogenesis. In contrast, $VEGF^{120/120}$ mice exhibited severe defects in vascular outgrowth and patterning, whereas $VEGF^{188/188}$ mice displayed normal venular outgrowth but impaired arterial development. It is noteworthy that neuropilin-1, a receptor for $VEGF_{164}$, was predominantly expressed in retinal arterioles. These findings reveal distinct roles of the various VEGF isoforms in vascular patterning and arterial development in the retina.

J. Clin. Invest. **109**:327–336 (2002). DOI:10.1172/JCI200214362.

VEGF120 – not sufficient for venular and even less so for arteriolar

VEGF188 – allows venular development only

VEGF164 – sufficient for both

Angiogenic and vasculoprotective functions of VEGF

- vascular permeability functions

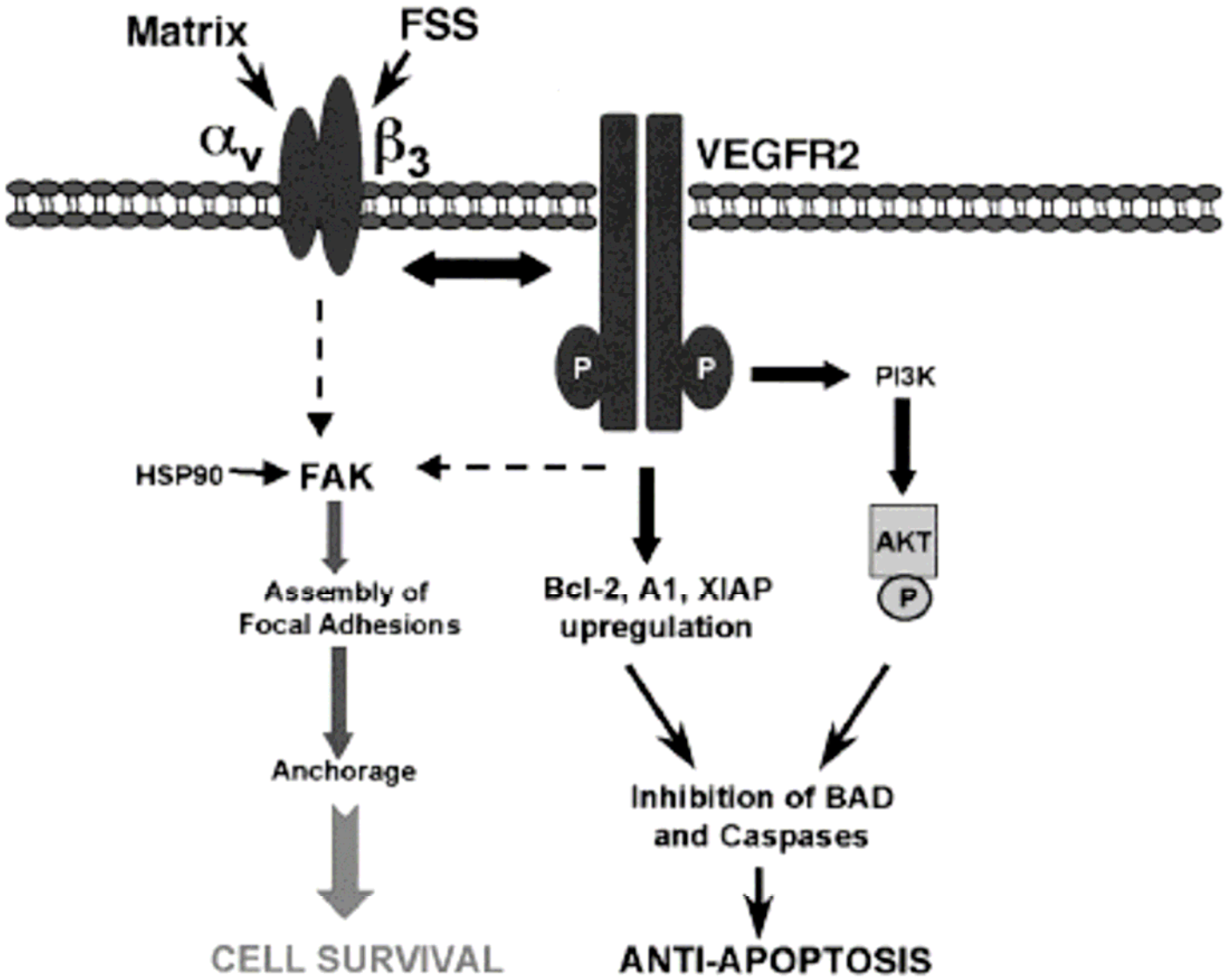
-endothelial cells survival factor

-Endothelial cell proliferation

-Endothelial cell migration

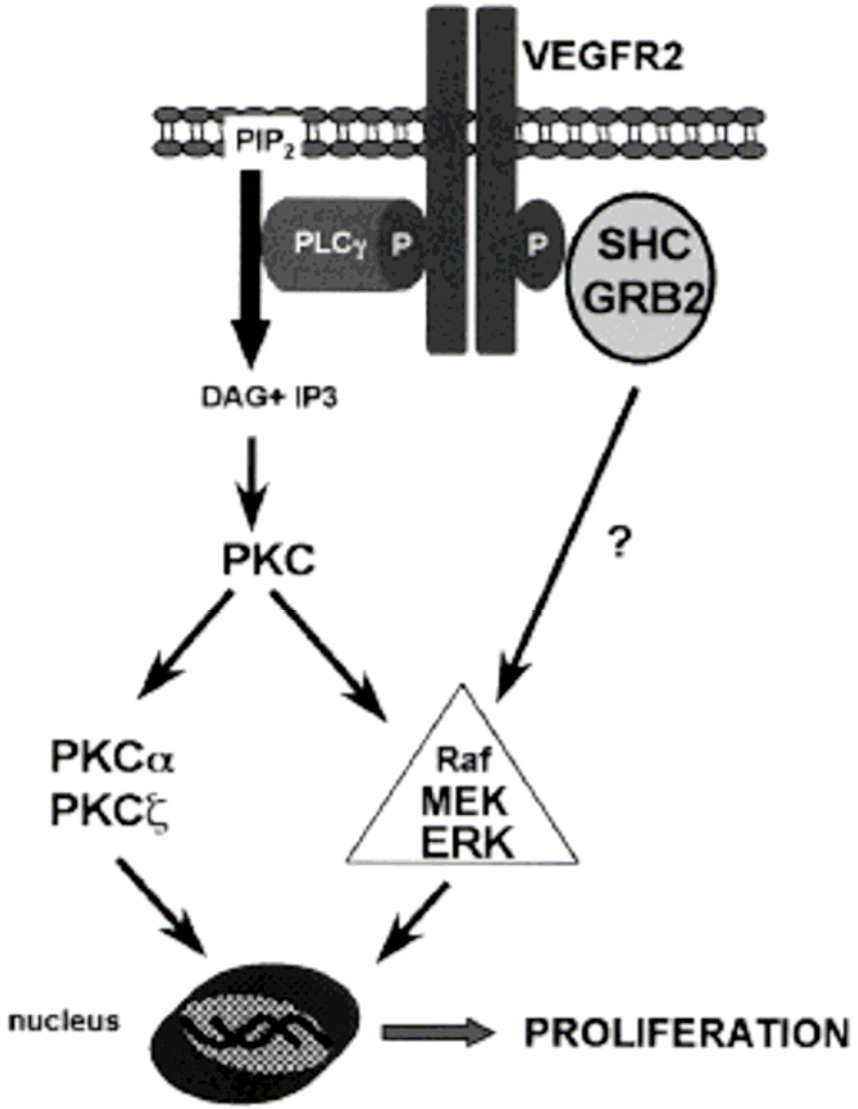
- inhibition of thrombosis

Mechanisms of anti-apoptotic VEGF signaling



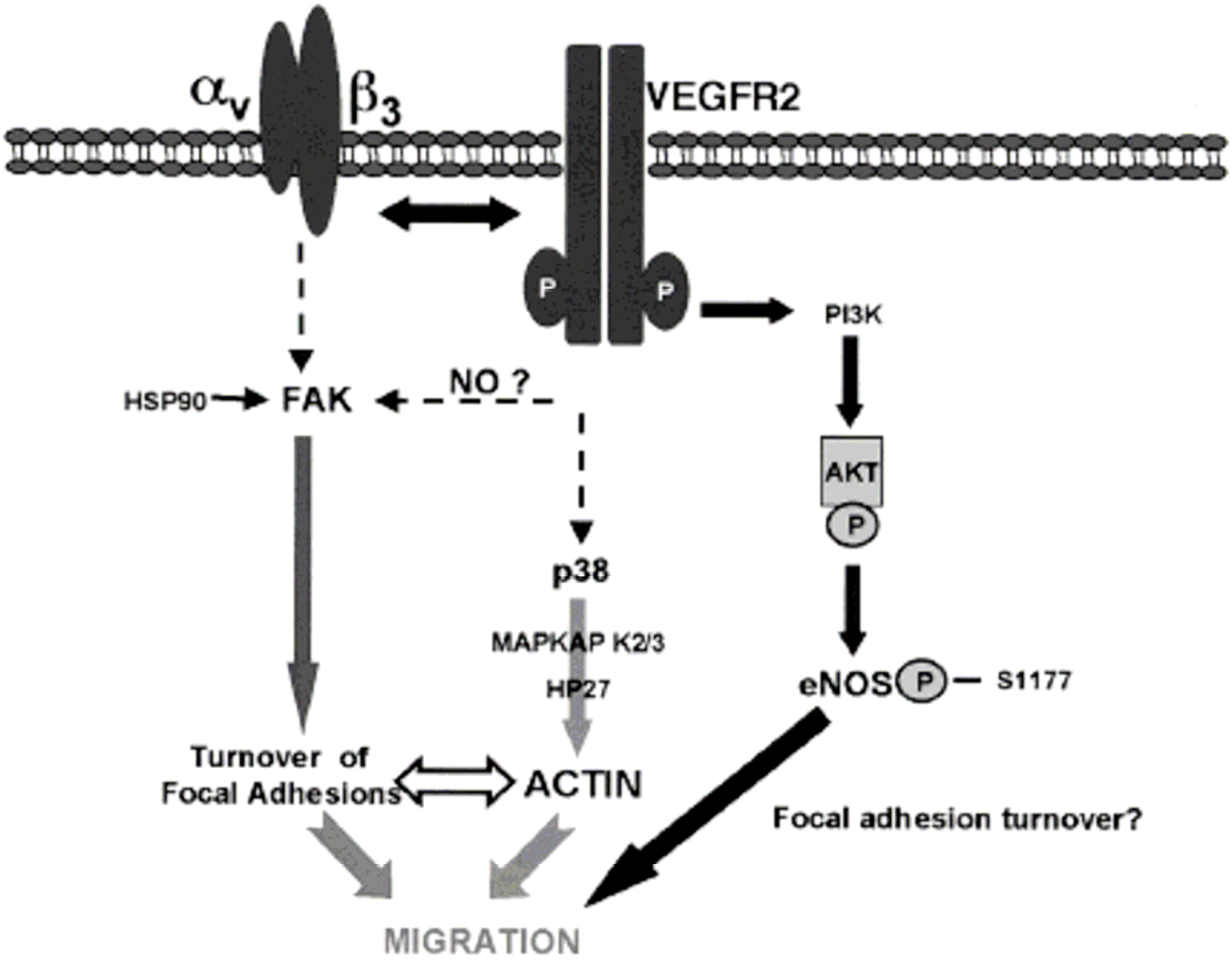
Zachary, Cardiovasc Res 2001

Mechanisms of mitogenic VEGF signaling



Zachary, Cardiovasc Res 2001

Mechanisms of chemotactic VEGF signaling



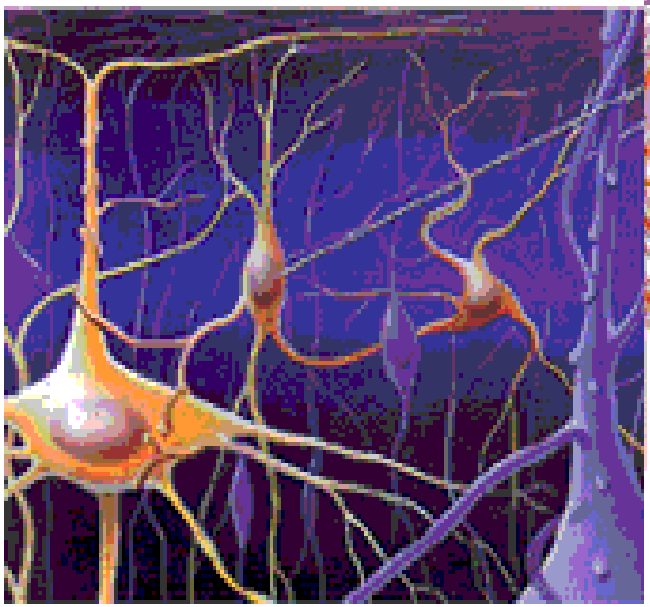
Zachary, Cardiovasc Res 2001

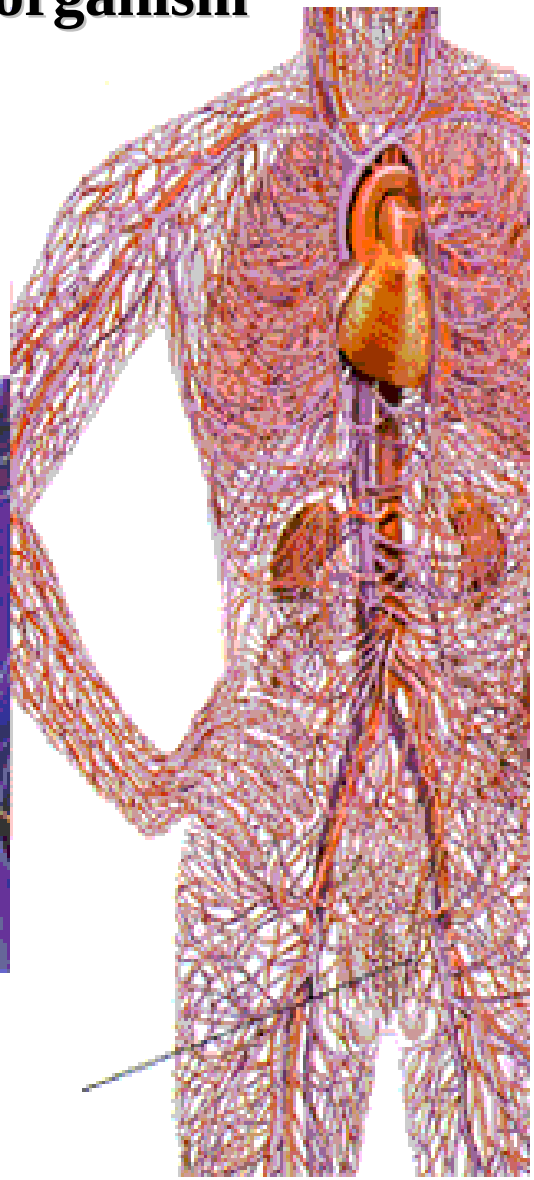
Why different VEGF isoforms have different angiogenic potentials?

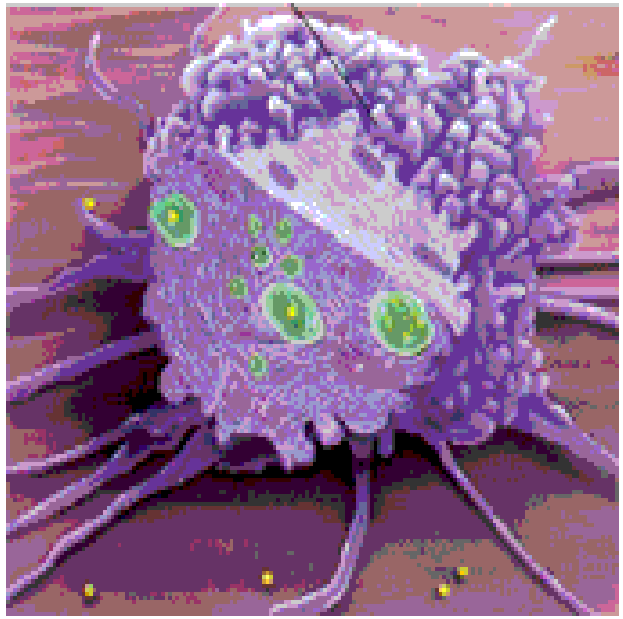
Is there a role of some downstream meadiators in those differences?

Nitric oxide as a mediator of VEGF signaling

Sources of NO in the organism



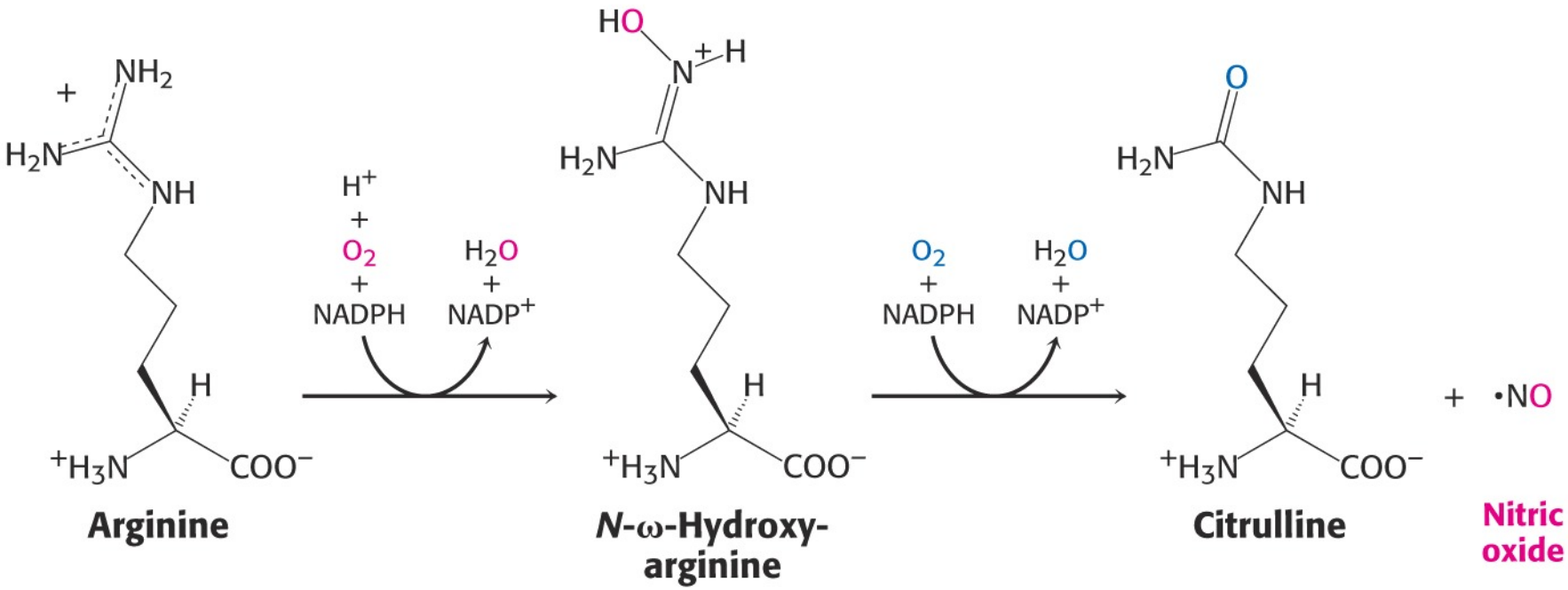


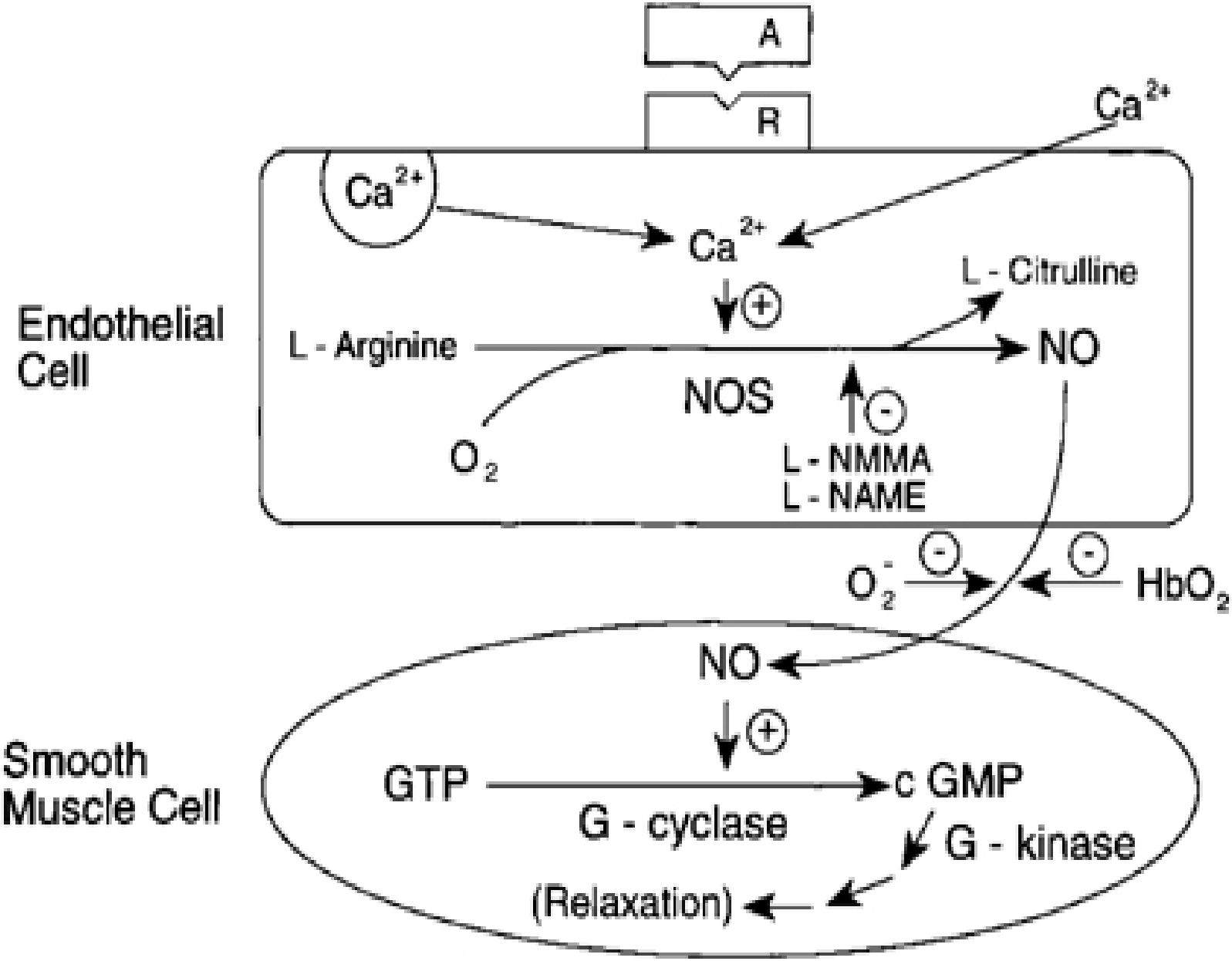


Nitric oxide synthases

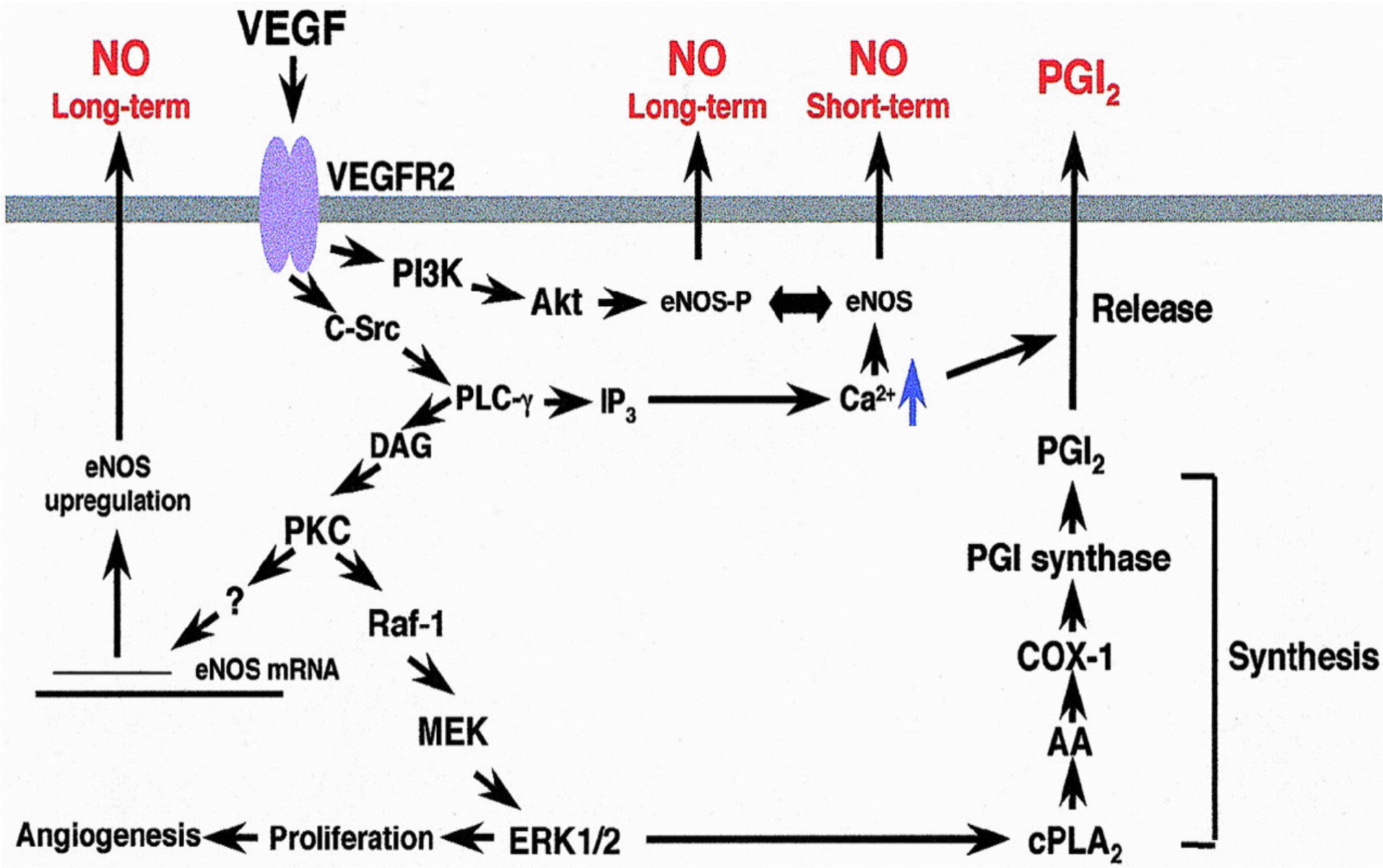
eNOS - endothelial (constitutive) NOS (NOS III)
nNOS - neuronal (constitutive) NOS (NOS I)
iNOS - inducible (NOS II)

Nitric oxide is produced from L-arginine by nitric oxide synthases



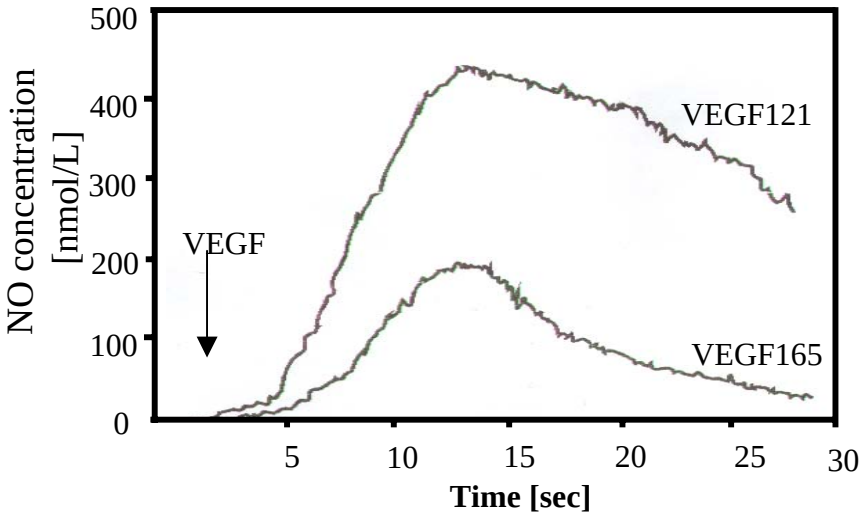

A
R
Ca2+
Ca2+
Ca2+
Endothelial Cell
L - Citrulline
L - Arginine
NO
NOS
O2
L - NMMA
L - NAME
O2-
HbO2
NO
Smooth Muscle Cell
GTP
c GMP
G - cyclase
G - kinase
(Relaxation)

VEGF-induced signaling in endothelial cells



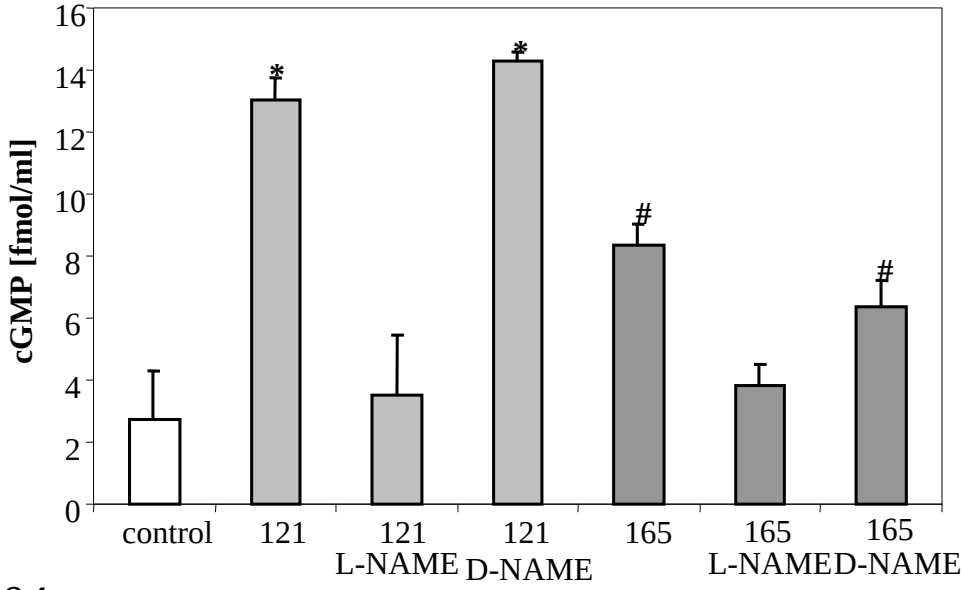
Zachary, Cardiovasc Res 2001

Involvement of nitric oxide in angiogenic activities of VEGF isoforms



Release of NO by VEGF-stimulated endothelial cells is stronger in case of $VEGF_{121}$ isoform

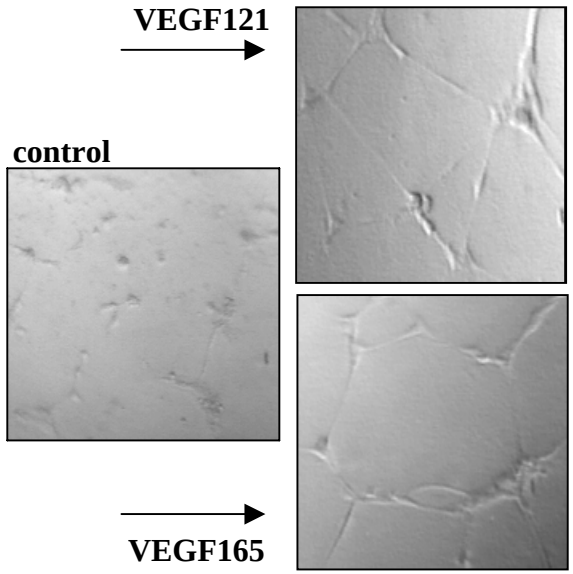
Synthesis of cGMP by VEGF-stimulated endothelial cells is higher in case of $VEGF_{121}$ isoform



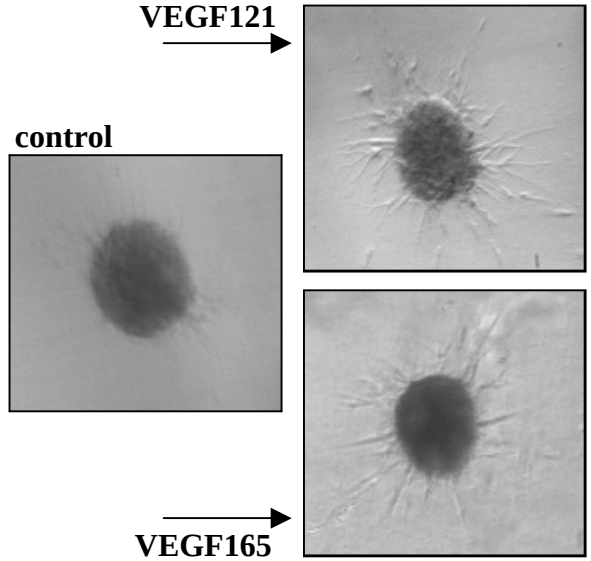
Józkowicz, Dulak et al., Growth Factor, 2004

Involvement of nitric oxide in angiogenic activities of VEGF isoforms

No difference in angiogenic potentials of various VEGF isoforms

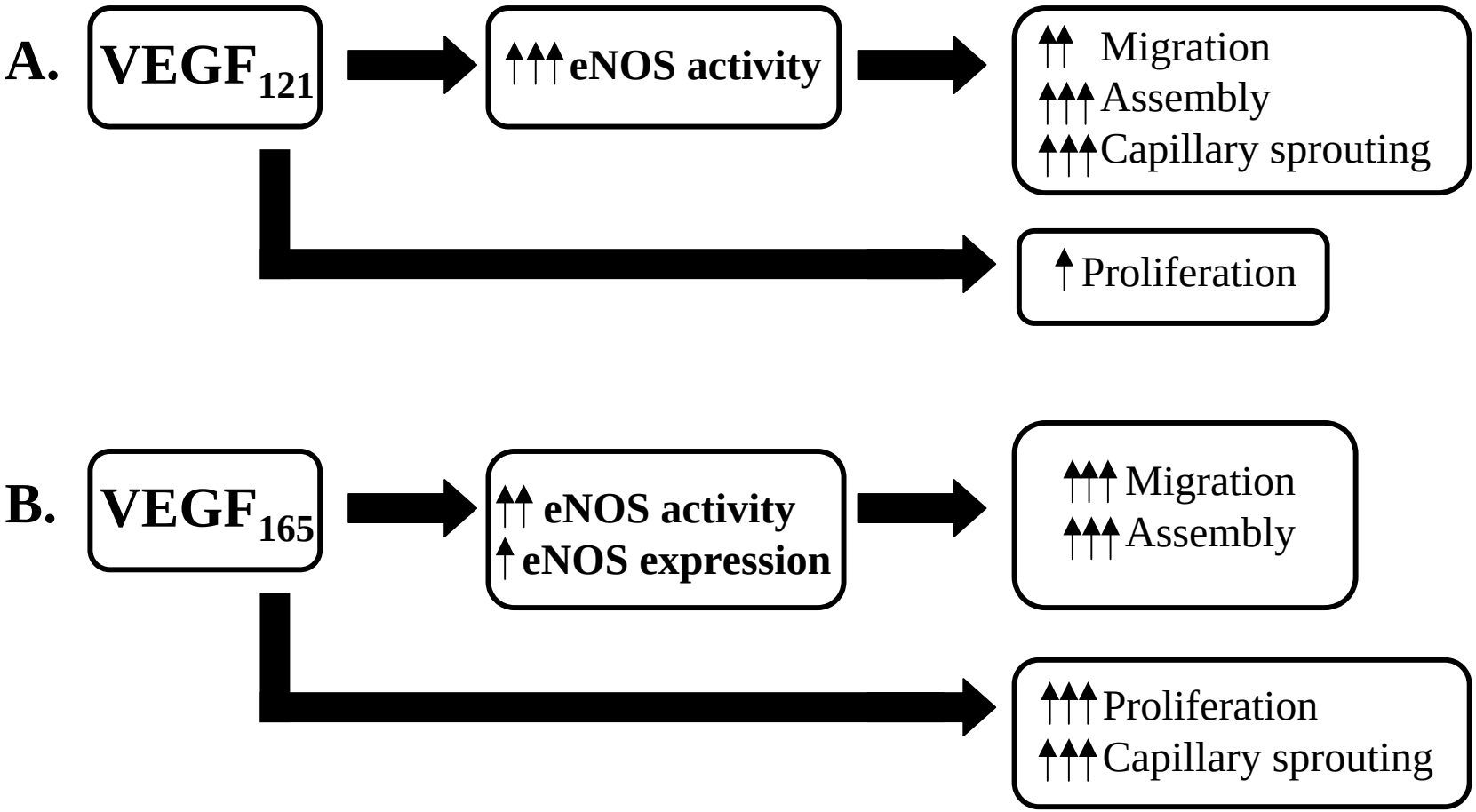


Matrigel assay

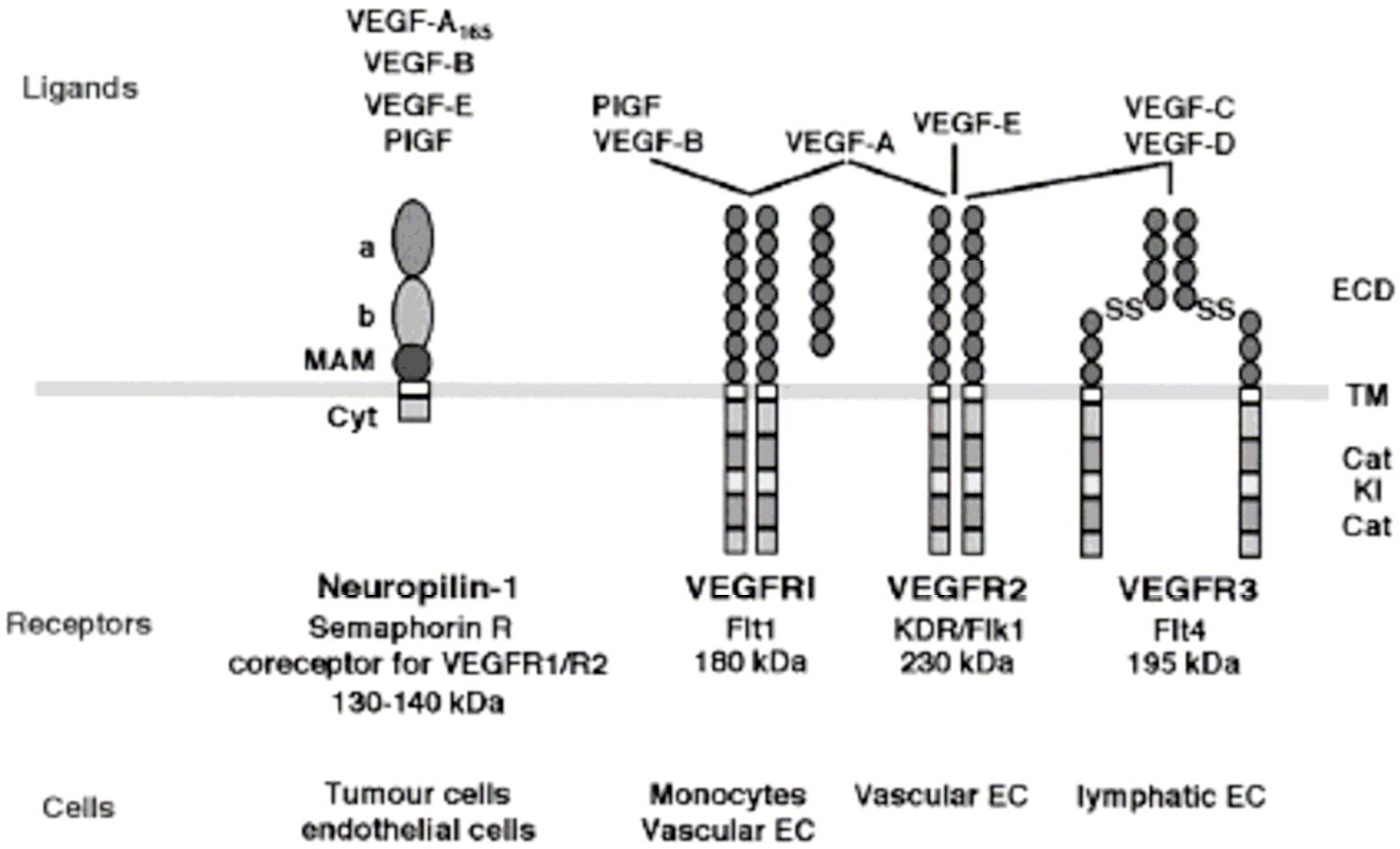


Spheroid assay

Properties of $VEGF_{121}$ and $VEGF_{165}$ isoforms



Expression of VEGF receptors is not restricted to endothelial cells



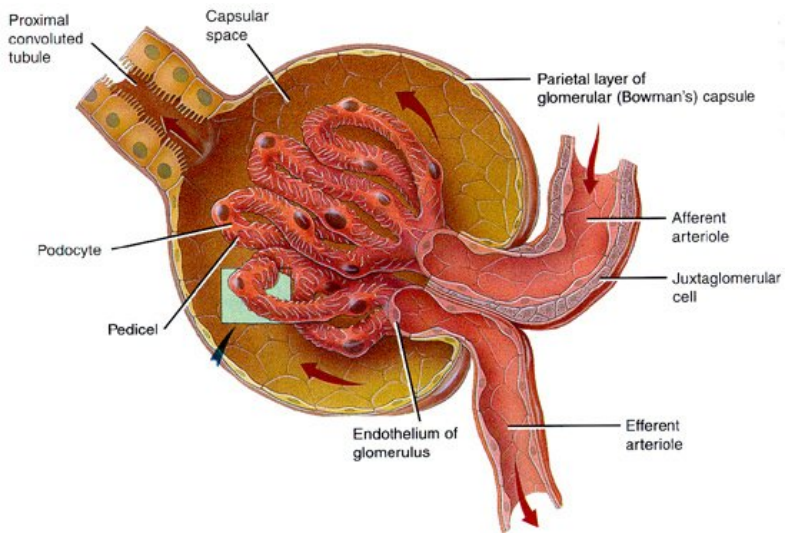
Zachary et al., 2003

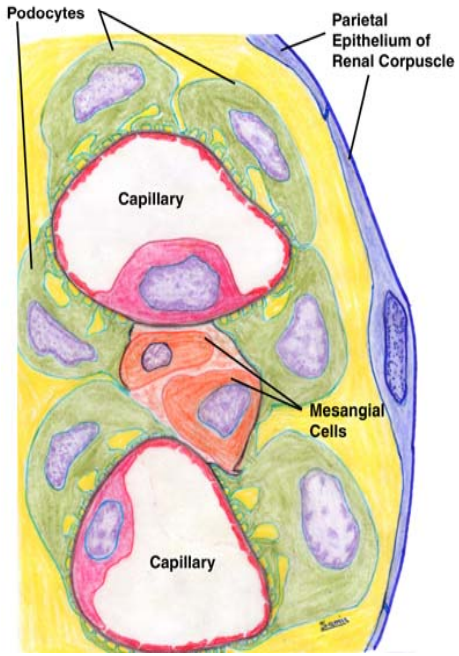
Physiological roles of VEGF in adult organism

1. Filtration in kidney glomerulus
2. Maturation of oocytes and corpus luteum formation
3. Vascularisation of uterine lining
4. Skeletal growth and endochordal bone formation

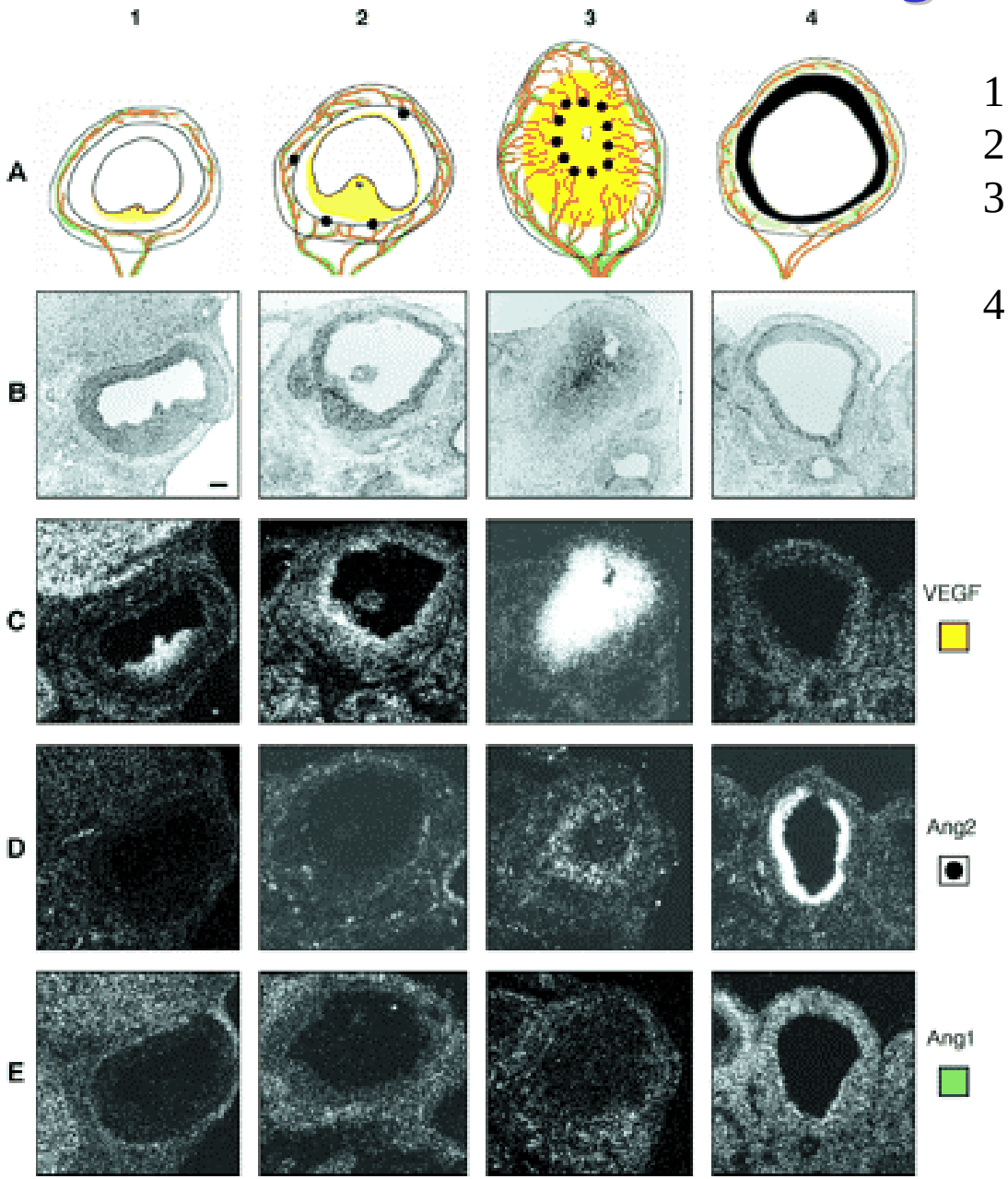
Role of VEGF in filtration in kidney glomerulus

Selective VEGF deletion in podocytes leads to glomerular diseases. Heterozygotes mice develop renal disease, characterized by proteinuria and endotheliosis by 2.5 weeks of age. Homozygosity resulted in perinatal lethality.





Vascular remodeling in the rat ovary



1 – small vesicular follicle
2 – large preovulatory follicle
3 – developing corpus luteum
(~ 8 hours after ovulation)
4 - non-productive follicle undergoing atretic regression

At maturation, the follicle ruptures, expels the ovum, and then undergoes reorganization into a cell-dense secretory structure known as the corpus luteum. This process includes a wave of vascular sprouting and ingrowth that hypervascularizes the corpus luteum; these vessels eventually regress as the corpus luteum ages.

Maisonpierre et al, Science 277: 55-60, 1997

Role of VEGF in endochordal ossification

Strong VEGFR1 expression detected also on osteoblasts and in the cells at the cartilage-bone junction.

Strong VEGFR2 on the cells at the cartilage-bone junction, but weak on osteoblasts

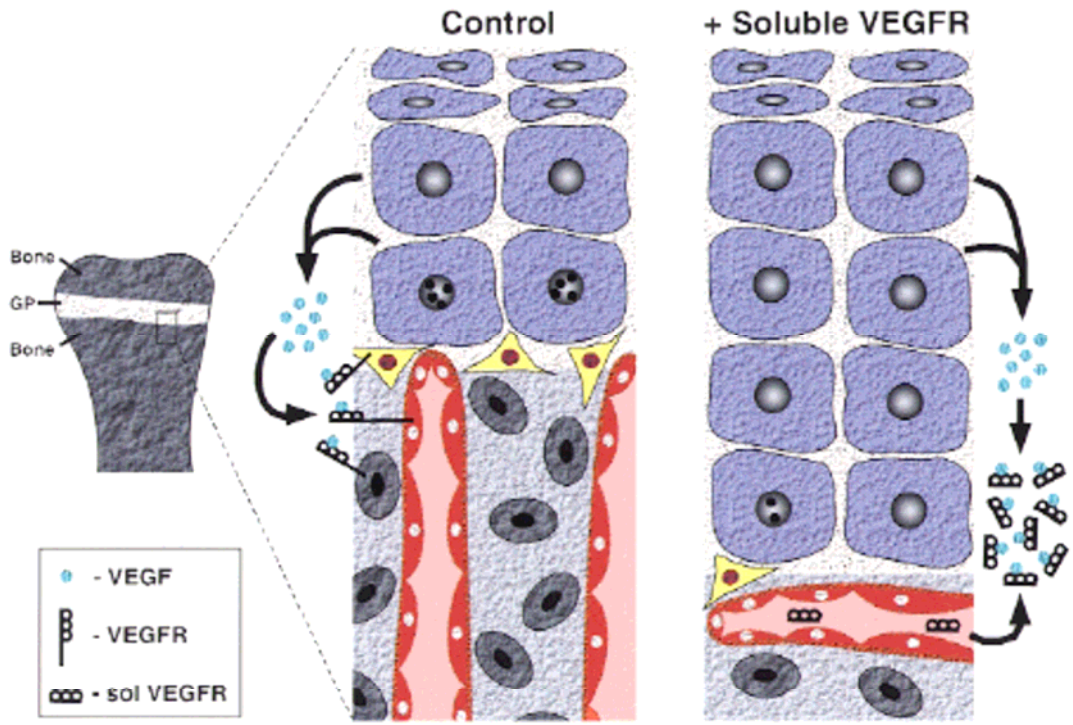


Fig. The long bones grow as the expanding cartilage of the growth plate (GP) is replaced by advancing bone through endochondral ossification. In control animals, endochondral ossification occurs as the chondrocytes (blue) of the GP mature into a final hypertrophic state. These hypertrophic chondrocytes secrete VEGF which induces vascular invasion (red) into the GP. These cells then undergo apoptosis (speckled nuclei). Chondroclasts (yellow) aid in the resorption of the hypertrophic cartilage, paving the way for osteoblasts (gray) to migrate in and deposit bone matrix. These events are mediated by VEGF which binds to VEGF receptors (VEGFR) expressed by these cells. In animals treated intravenously with soluble VEGFR, the zone of hypertrophic chondrocytes is expanded, blood vessel invasion into the GP is inhibited, apoptosis of the hypertrophic chondrocytes is delayed, and recruitment/differentiation of chondroclasts is delayed. This occurs as soluble VEGFR sequesters the VEGF secreted by hypertrophic chondrocytes, thereby impairing VEGF signaling.

Harper & Klagsbrun, 1999 (comment to Gerber et al,

Non-vascular effects of VEGF

Neuroprotective activity:motor neuron survival

Lung development

Bone growth/skeletal development

Hematopoiesis: direct effect on hematopoietic stem cells

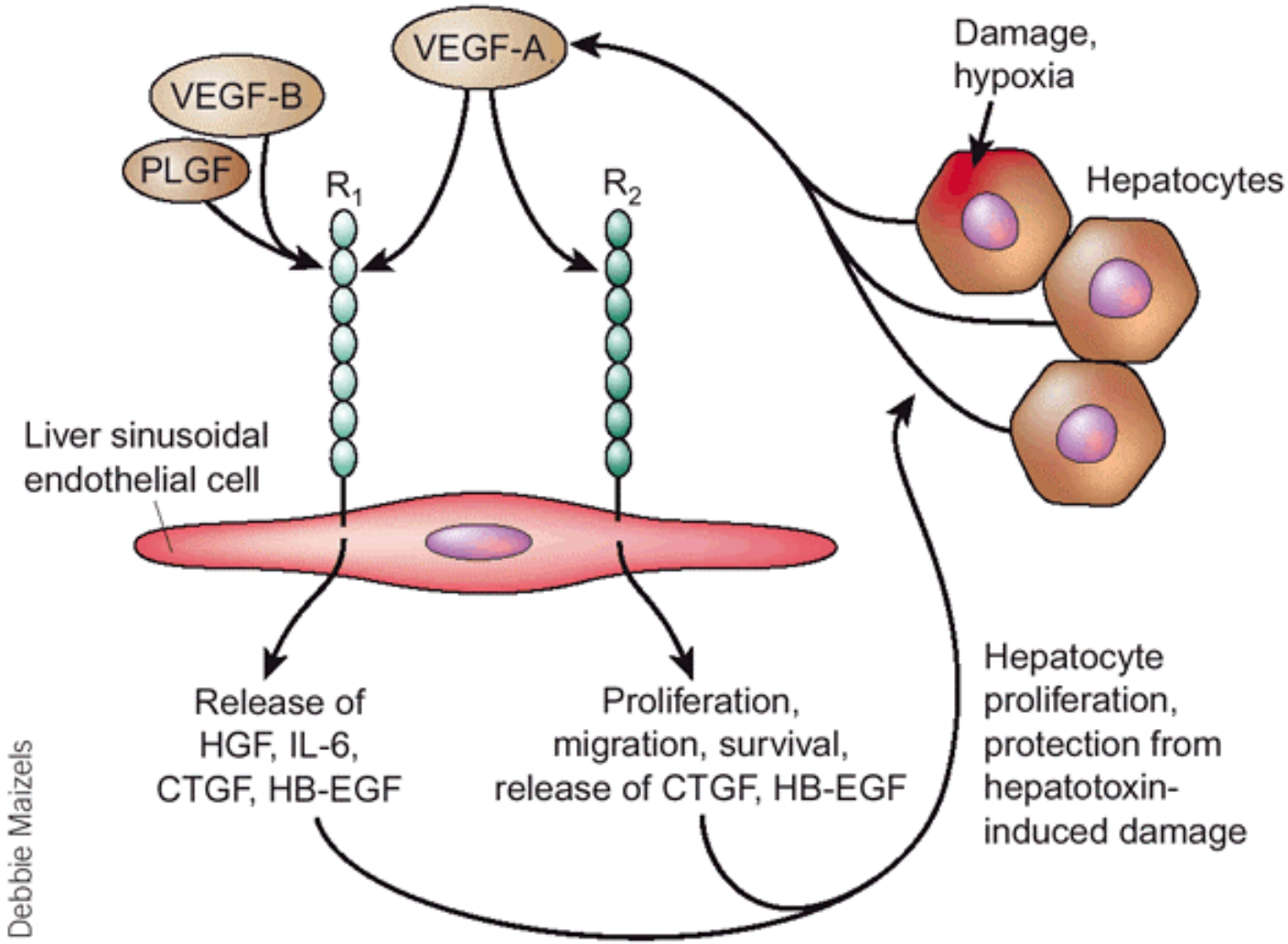
Renal homeostasis

Reproductive homeostasis: oocyte fertilization,

Indirect effects of VEGF

By stimulation of endothelial cells VEGF may induce production of mediators, which affect functions of other cells

VEGF and hepatocytes



Ferrara N et al., Nature Med., June 2003

Take-home messagess

VEGF (VEGF-A) is a key mediator of vasculogenesis, angiogenesis and arteriogenes

VEGF is generated in the form of several isforms, being the results of alternative splicing

The most common and the most active and crucial isoform is $VEGF_{165}$

VEGF exerts its activity by binding to its receptors: VEGFR1, VEGFR2 and co-receptors: neuropilin 1 & 2.

VEGFR2 is the key receptor, mediating the majority of actions of VEGF.

VEGFR1 is a decoy receptor, playing important role in modulating VEGF activity during development