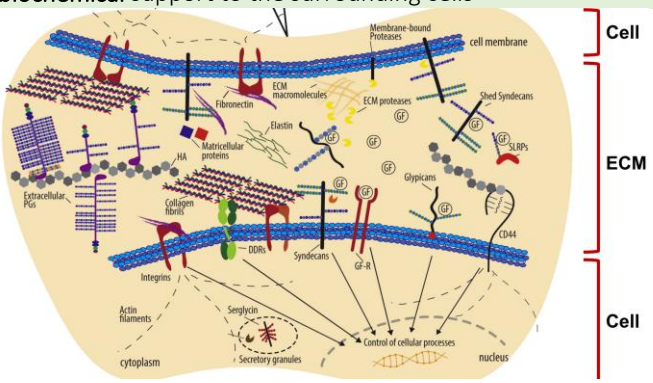
# BORDOLI AND WERNER

## EXTRACELLULAR MATRIX

**Extracellular matrix (ECM)** is defined as the **non-cellular component** present in all tissues and organs

= everything you find between cells

The extracellular matrix (ECM) is a collection of **extracellular molecules secreted by cells** that **provides structural and biochemical** support to the surrounding cells



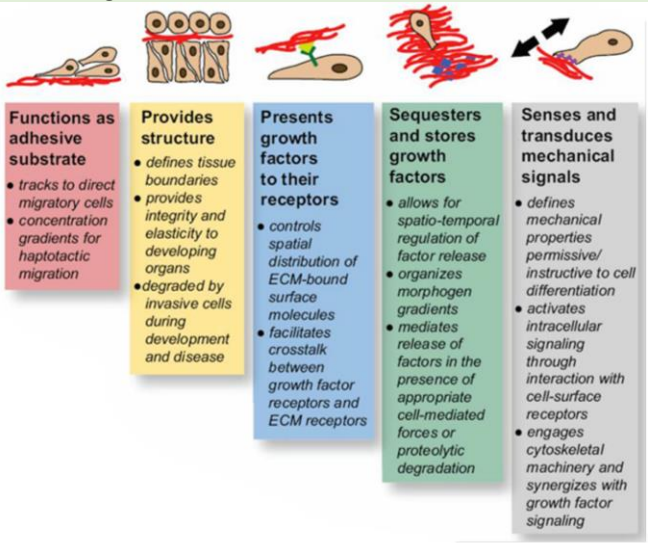

- ECM is a very complex dynamic environment

## PHYSIOLOGICAL FUNCTIONS OF THE ECM

The ECM largely determines how tissue looks and function

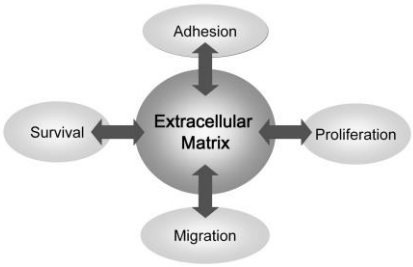
- Provides **scaffold** and **structural support** to the surrounding cells
- Provides **biochemical support**
- **Segregates** tissues from each other
- Regulates **intercellular communication** (cell-to-cell communication/signaling) and **differentiation**
- **Cell adhesion**
- ECM is important in tissue and **organ development**, **homeostasis and repair**

ECM plays fundamental roles in health and disease not only by providing a **structural scaffold** but also by directly influencing **cellular behavior, crosstalk** and **homeostasis**




## CELL-ECM CROSSTALK

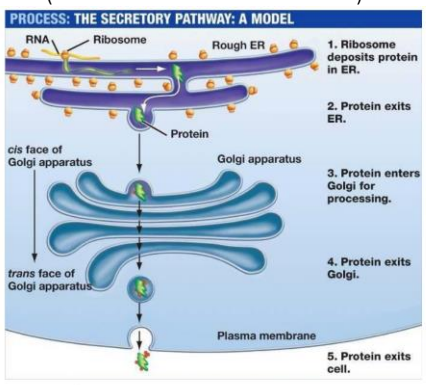
- Cells secrete factors that **modify the surrounding matrix**
- Signals coming from the matrix influence all aspects of **cell behavior**
- Cellular homeostasis relies on a **close interaction** and a **continuous crosstalk** between the **cells** and their **surrounding matrix**




  - specific signals originating from the matrix influence a variety of cellular processes, including **adhesion**, **migration, survival** or **proliferation**
  - A broad array of factors secreted from cells actively and dynamically modify the surrounding matrix

## SECRETION OF ECM COMPONENTS

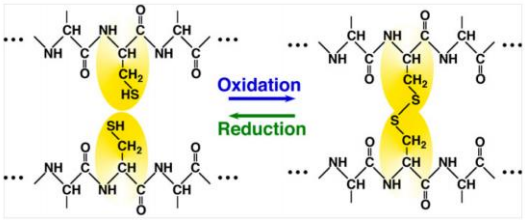
- Secretory pathway: Ribosome (first 20-25 amino acids of the protein are a special signal) → **ER** → **Golgi** (processing) → vesicular transport → Protein secretion (in extracellular environment)





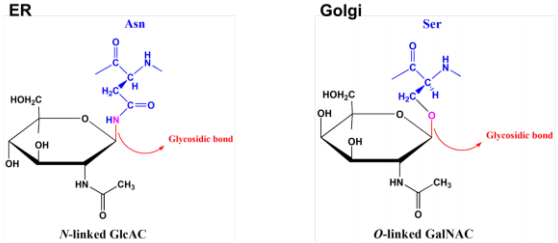
## ESSENTIAL PROTEIN MODIFICATIONS IN THE SECRETORY PATHWAY

### 1. DISULFIDE BOND FORMATION IN THE ER




- formation of intra- or inter- protein disulfide bonds
- stabilize tertiary or quaternary structure of secreted proteins

### 2. PROTEIN GLYCOSYLATION IN THE ER AND GOLGI




- Supports proper **protein folding** and **transport**
- **Limits protease accessibility** providing **resistance** to digestion
- Regulatory role in signaling

# MAIN COMPONENTS OF THE ECM

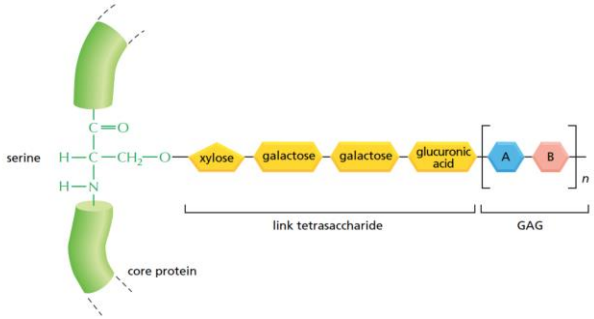
THE **THREE MAJOR COMPONENTS** OF THE ECM:

1. **Highly viscous proteoglycans** → form high viscosity and hydrated matrices (aggrecan, perlecan, syndecan, …)
2. **Insoluble collagen fibers** → provide strength, support and flexibility
3. **Multiadhesive ECM proteins** (fibronectin, laminins, ...)

## PROTEOGLYCANS

### STRUCTURE

- Proteins covalently linked to glycosaminoglycans (GAGs) of variable length
- Carbohydrates make up ~ 95% of its weight

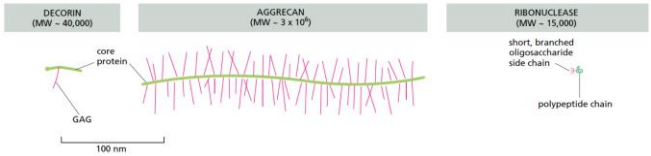



### FUNCTIONS:

- Occupy space between cells and collagens
- Form a high viscosity environment (e.g. lubricating fluid in the joints)
- Formation of network by linking collagen fibers
- Organize water molecules
- Form hydrated matrices
- Function as **sponge/schock absorber**
- Assist migration and adhesion of cells by providing **scaffold**
- Bind cytokines and chemokines to **protect them from proteolysis**

### GLYCOPROTEIN VS. PROTEOGLYCAN

- Both: Protein and Sugar component




**Glycoprotein** *(Ribonuclease, right)*

- 1-60 % carbohydrates by weight (usually few percent)
- Numerous, short, branched oligosaccharides

**Proteoglycan** *(Decorin and Aggrecan, structures left)*

- As much as 95% carbohydrates by weight
- Long, unbranched glycosaminoglycan chains

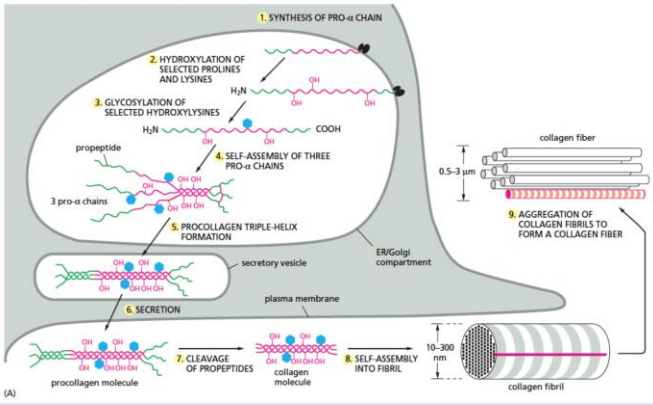
## COLLAGENS

### STRUCTURE

- Highly organized structure

### FUNCTIONS

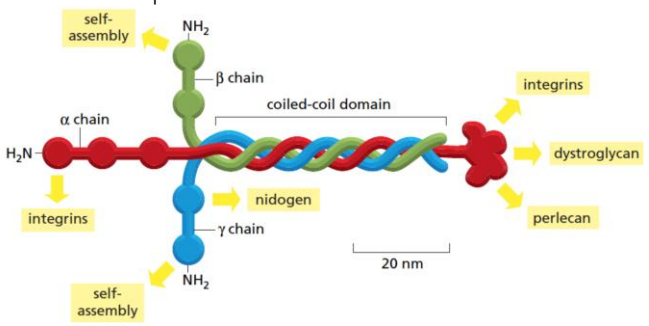
- Collagen is the **most abundant protein** in the body (**~30%** of the total protein mass)
- Main structural protein in connective tissue
- Provides **strength, support, flexibility** and **shape** to tissues
- Proper alignment of cells for proliferation and differentiation
- Sequestering of soluble ECM cytokines
- Activation of signaling cascades




## MULTIADHESIVE ECM PROTEINS

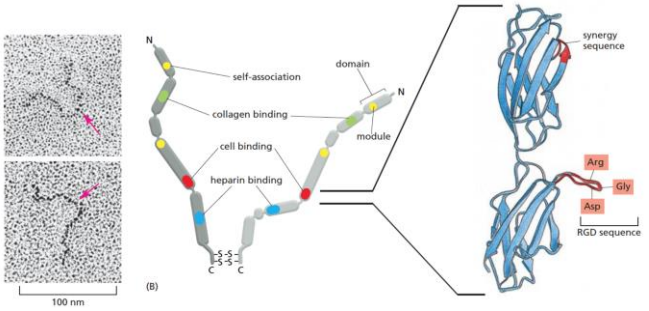
### LAMININ

- Formed by **three disulfide-bonded polypeptide chains**
- **Several specialized binding motifs** for other proteins
- Main component of the basal lamina




### FIBRONECTIN

- **Two polypeptide chains** joined by **disulfide bonds**
- Each chain subdivided into domains
- Individual domains contain specialized modules
  - A lot of integrins bind the RGD sequence




## MINOR/ OTHER ECM COMPONENTS

### MATRICELLULAR PROTEINS

- **Dynamically expressed**, non-structural matrix proteins with regulatory roles

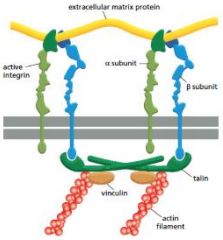
OSTEOPONTIN
TENASCIN C
CCN
THROMBOSPONDINS
…

### GROWTH FACTORS

- Autocrine and paracrine signaling
- Elicit intracellular signaling cascades

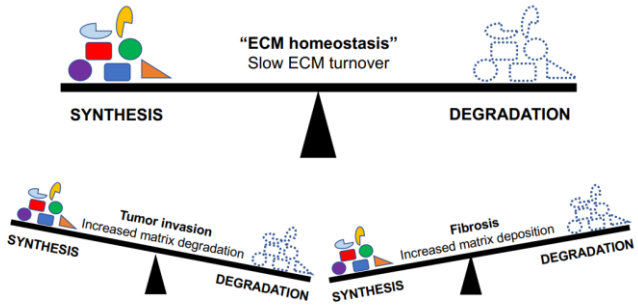

### EXTRACELLULAR PORTION OF TRANSMEMBRANE RECEPTORS

- Involved in extra-/intracellular signaling and binding to extracellular components



## ECM HOMEOSTASIS AND REMODELING

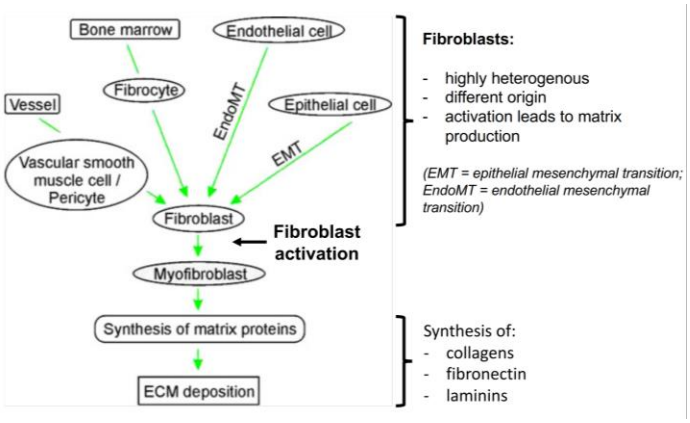
ECM remodeling as a **fine tuned process** between ECM production and degradation




- Aberrant ECM remodelling leads to **disease conditions**
- Dynamic matrix rearrangements are a driving force in several physiological and pathophysiological processes

## MATRIX PRODUCTION:

### FIBROBLASTS ARE THE MAJOR MATRIX "PRODUCERS"

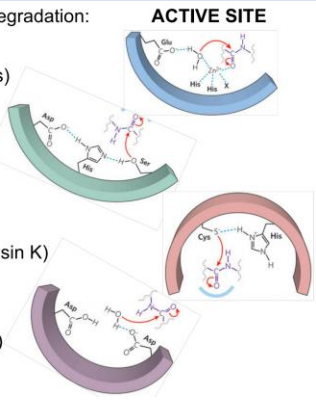



- Continuous/constitutive fibroblast activation to myofibroblast leads to a massive amount of matrix deposition

## ECM DEGRADATION AND REMODELING

Proteolytic enzymes capable of ECM degradation:

1. **Matrix metalloproteinases** (MMPs)
2. **Serine proteinases** (e.g. plasmin)
3. **Cysteine proteinases** (e.g. cathepsin K)
4. **Aspartic proteinases** (e.g. pepsin)

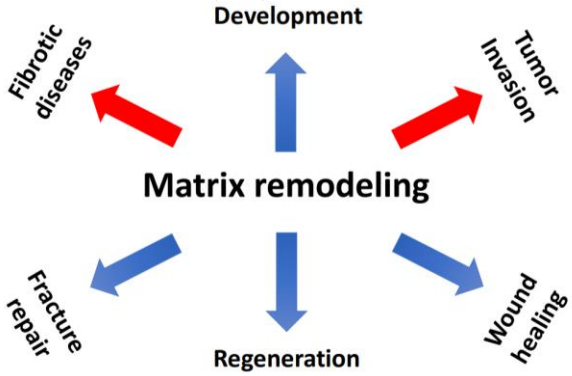



*Difference: How they do it (depending on their active site)*

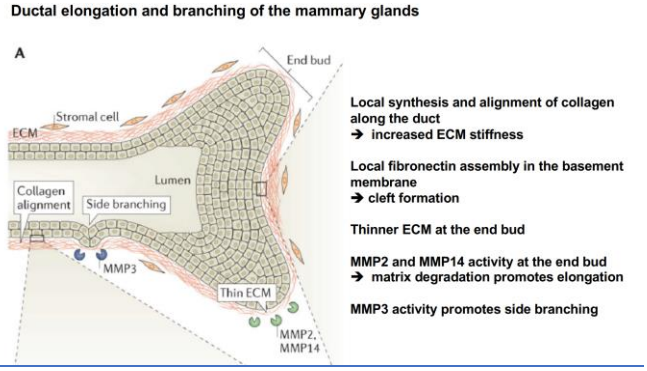
## PROCESSES CHARACTERIZED BY **ECM REMODELLING**

**ECM rearrangement** plays a key role in a **variety of physiological processes**

- Matrix can be quite dynamic → highly regulated process
- Dynamic ECM remodelling takes place
  - In **embryonic development**
  - In **organogenesis**
  - As a characteristic feature of **wound healing** and other **tissue repair processes** (during adulthood)

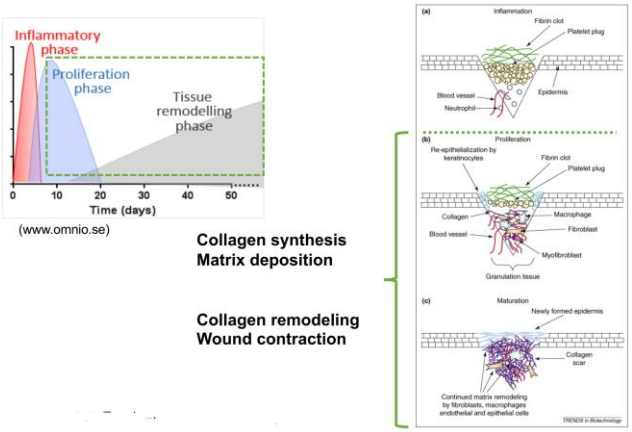



### ECM REMODELING DURING **DEVELOPMENT**: BRANCHING MORPHOGENESIS

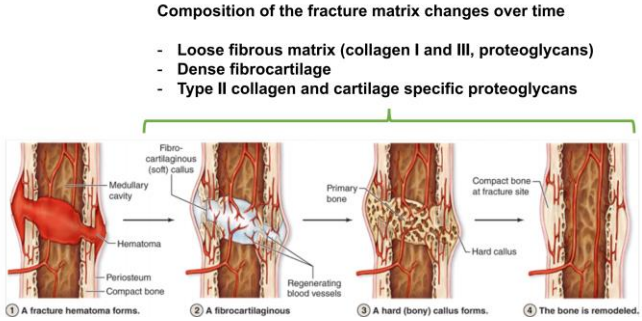



### ECM REMODELLING DURING **WOUND HEALING**

- Wound healing has 3 phases




### ECM REMODELING DURING **FRACTURE REPAIR**



## ECM DYNAMICS IN DISEASE

Dynamic matrix rearrangements are a **driving force in several physiological and pathophysiological processes → deregulated ECM remodeling** is a striking feature of **several pathologies**, including **fibrotic diseases** and **cancer**

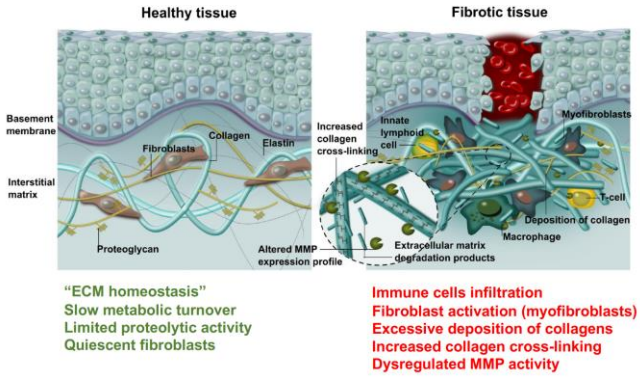
## FIBROPROLIFERATIVE DISEASES

Fibro-proliferative diseases are characterized by **abnormal matrix remodeling and deposition**

- **Excessive ECM production/deposition** and **deregulated remodeling** leads to **impairment of organ function**
- Can affect **every single tissue** in our body
- 45% of all deaths worldwide are associated with fibroproliferative diseases (huge clinical problem, today there are no cure yet)

Conditions characterized by (**2 major factors**):

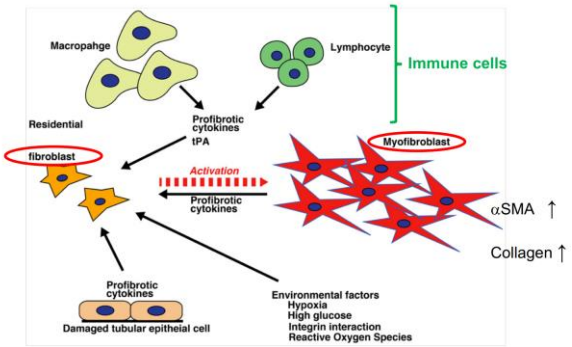
- Excessive ECM (collagen) deposition
- Aberrant ECM remodelling (remodelling is not absent, but dysregulated)




- Healthy tissue: ECM homeostasis, ECM dynamics are characterized by a **very slow metabolic turn-over of ECM proteins** and **limited proteolytic activity**, quiescent fibroblasts
- Fibrogenesis:
  - **Infiltration of immune cells** leads to sustained **inflammatory conditions**
  - **Fibroblast activation and differentiation to myofibroblasts** contributes to **excessive matrix production** → excessive **deposition of collagen**
  - pro-fibrotic conditions lead to **enhanced collagen cross-linking** (tissue stiffness → loss of function of tissue/organ) and **matrix degradation is impaired** heavily (there is degradation, but activity is not controlled/regulated anymore) → **affecting the ECM structure** and consequently **organ function**

### FIBROBLAST ACTIVATION IN FIBROTIC DISEASES

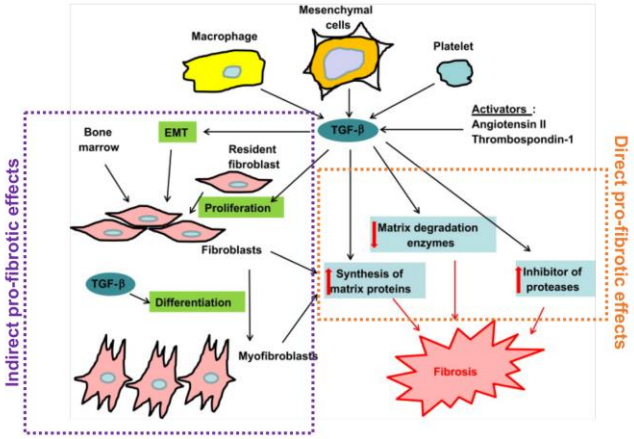
- Pro-fibrotic cytokines drive the transition from quiescent to activated fibroblasts




- Immunostaining for activated Myofibroblasts: αSMA

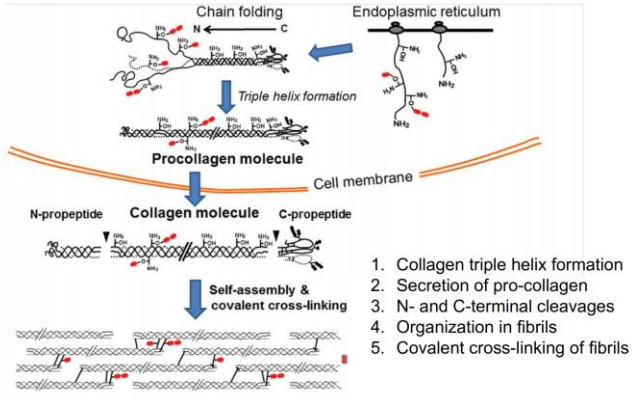
### TGF-B

*How can **immune cells infiltrate**?* (which then cause activation of fibroblasts) → due to pro-fibrogenic TGF-B signalling (All results in fibroblastic proliferation)

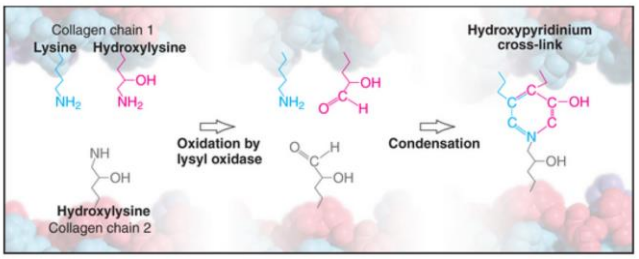



- TGF-B is **released by many cell types** (Macrophages, Mesenchymal cells, Platelet, …)
- TGF-β can **induce the synthesis of matrix proteins** and **downregulate the expression of degradating proteins**
  - **Direct effects**: inhibits matrix degradation enzymes, enhances inhibitors of proteases, enhances synthesis of matrix proteins
  - Also causes **proliferation of fibroblasts** and their **differentiation to myofibroblasts** (these cells secrete a lot of matrix proteins)

### INCREASED COLLAGEN DEPOSITION IN FIBROTIC TISSUE



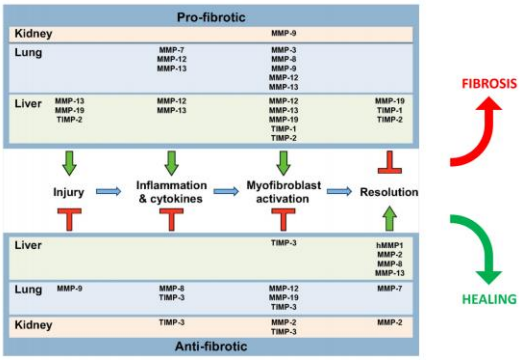

Increased collagen cross-linking in fibrotic tissue results in increased stiffness




1. Lysyl oxidase deaminates lysine or hydroxylysine residues in adjacent collagen fibrils
2. Resulting reactive aldehydes condense with neighboring lysines or hydroxylysines
3. Mature collagen fibers are covalently linked

## DYSREGULATED MMP EXPRESSION AND ACTIVITY CONTRIBUTE TO FIBROSIS

- Different MMPs are expressed in different organs
- Are expressed at different timepoints
- Different MMPs can be fibrotic in one organ, others in another



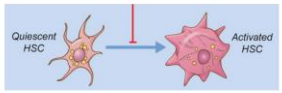

*Don't learn this table by heart!*

## TREATMENT APPROACHES FOR FIBROSIS

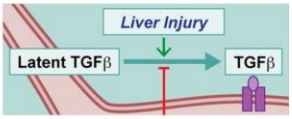
- There exist a lot of causes resulting in fibro-proliferative disease → hard to find a treatment/an approach to control this condition

**1. Control or cure the primary disease**

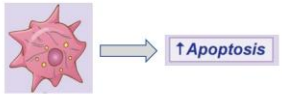

**2. Block cellular differentiation (e.g. fibroblast activation)**




**3. Inhibit fibrogenesis**




**4. Promote resolution of fibrosis**

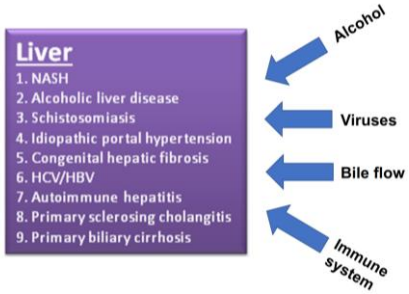



1. <u>Control or cure the primary disease</u> → Try to block the infection (Problem: People realize their injury to late)
2. <u>Try to block the cellular differentiation</u> (e.g. fibroblast activation) if you already have this condition (Problem: Most patients show up with a very advanced disease)
3. <u>Inhibit fibrogenesis</u> → inhibit TGF-B signaling (on a molecular level, not that promising approach)
4. <u>Promote resolution of fibrosis</u> → Adaptosis (most promising approach since most of the patients already have an advanced state of disease → try to reverse it)

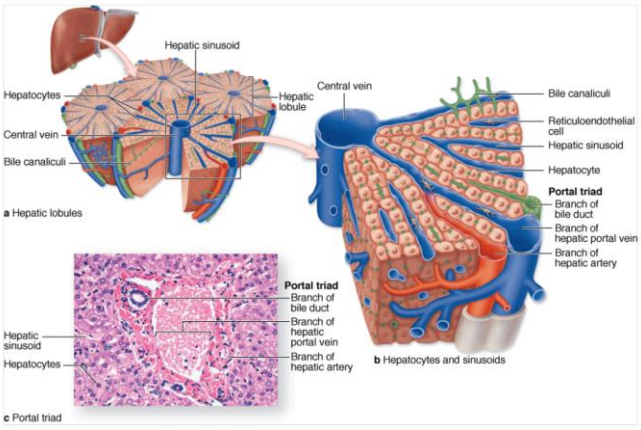
→ At the moment there is no cure (only possibility to slow down course of the disease)

## LIVER FIBROSIS

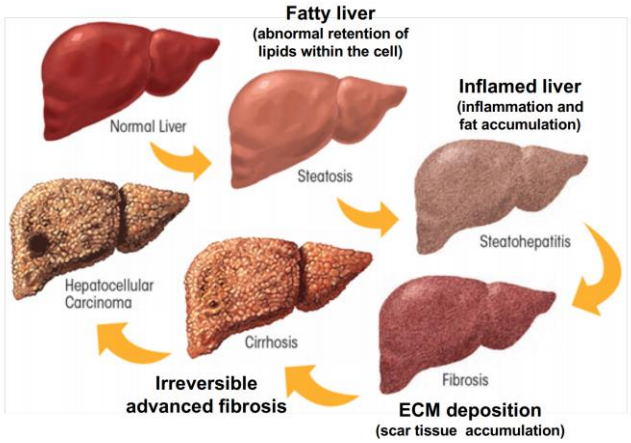
- A common denominator in a multitude of liver diseases




## LIVER MORPHOLOGY AND STRUCTURE

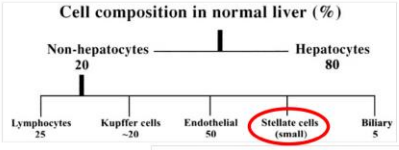


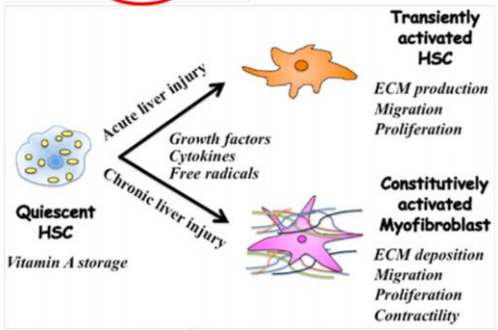

## FROM NORMAL TO DAMAGED LIVER




- Cirrhosis → Irreversible → point of no return → patient needs a liver transplantation

## HEPATIC STELLATE CELL: KEY PLAYER IN LIVER FIBROSIS

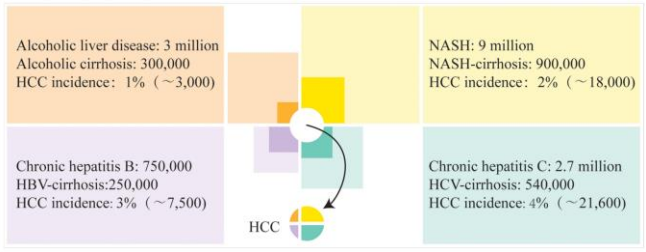



Stellate cells:

- Are normally in a quiescent stage
- In an acute injury they get transiently activated
- Problematic if they get consistently active

## INCIDENCE OF LIVER DISEASES

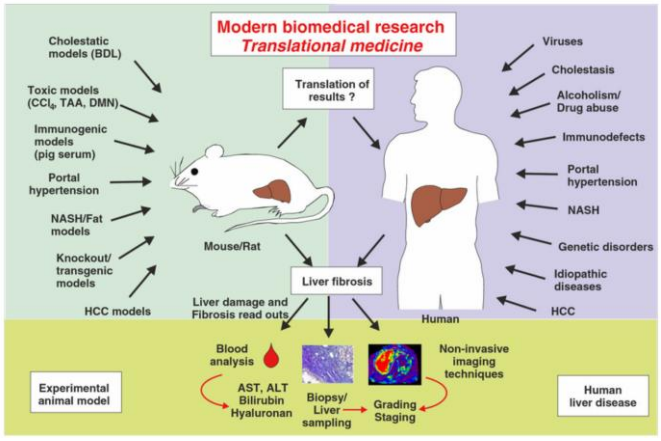



*Individuals with chronic liver disease in any given year in the USA*

- Prevalence of NAFLD worldwide is between 15-23%

## HOW TO STUDY FIBROSIS

*How good do studies of mice result to human conditions?*
Problem: There is no animal model that really mimics the human disease → no good model to study this condition




## USE OF ANIMAL MODELS IN RESEARCH

Choice of the model depends on the process to be analysed

- not all animal models are suited for a certain experimental question
- choice also depends on the number of animals needed
  - Costs etc.
  - Use of the right animal model and the right number of animals is crucial → number needs to provide statistical relevance
  - Always aim for 3R: **reduce, refine, replace**!

## ADVANTAGES OF ANIMAL MODELS

*(In the context of liver fibrosis research)*

Compared to clinical studies:

- Collection of **multiple samples** at **different time-points** → Possibility to realize sequential studies
- The disease develops in a **relative short time**
- **Control and reduction of variables** that cannot be closely followed in humans
- Potential use of **genetically modified animals** → Study of specific genes/signaling pathways

Compared to in vitro approaches:

- Study of the **complete organ** (i.e. liver)
- **Intact** and **dynamic** cell-cell and cell-matrix **interactions**
- **Cross-talk** with immune system/circulatory system, etc.

## DISADVANTAGES/DRAWBACKS OF ANIMAL MODELS

*(In the context of liver fibrosis research)*

- Animals **do not develop human diseases**
- Animals **react differently** to various noxious agents
  - Rodents (Nager) have a total aversion to alcohol
- Some hepatic diseases **do not exist** in rodents
  - HCV only infects humans
- Different timing in onset and progression of diseases
  - Biliary cirrhosis develops in only a few weeks in rodents
- Difficult/impossible to reproduce multifactor diseases
  - No animal model recapitulates all complex hepatic and extrahepatic features typical of a human condition

## MOUSE MODELS TO STUDY LIVER FIBROSIS

### 1. *Hepatotoxin-induced liver fibrosis*

- Models of post-necrotic fibrosis (**killing of hepatocytes**)
- Repeated injections of TAA, $CCl_4$, DMN, DEN, … toxins

### 2. *Biliary fibrosis*

- Interruption of bile flow (common bile duct ligation)

### 3. *Autoimmune fibrosis*

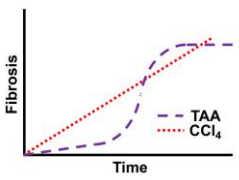
- Challenge lies in **breaking immune tolerance** and long-term maintenance of **immune alterations**
- Use of **genetic models** (Alb-HA/CL4-TCR)

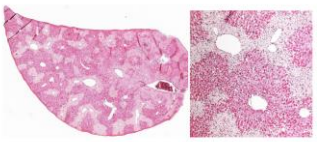
### 4. *NASH-associated fibrosis*

- Dietary (hypercaloric), genetic (PTEN deletion in hepatocytes) or combined models

## CHOICE OF THE BEST MOUSE MODEL: TAA VS. $CCL_4$ HEPATOTOXIN MODELS

- Both toxins are used as models of post-necrotic fibrosis
- Toxins have different kinetics of fibrosis onset
- TAA but not $CCl_4$ is hepato- and cholangiocellular carcinogenic
- TAA induces rapid progression from periportal fibrosis to “human-like” cirrhosis



(H&E mouse liver, 48 hrs post $CCl_4$; *courtesy of S. Pantasis, unpublished data*)

## HOW TO STUDY ALCOHOL-INDUCED LIVER FIBROSIS?


➢ Alcoholic liver disease is a major health issue in developed countries

➔ first cause of advanced liver disease in Europe

Animal models are of **limited benefit**:

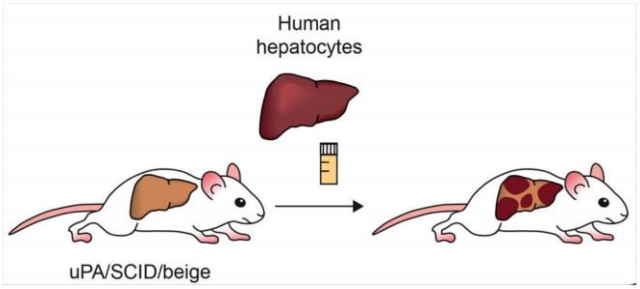
- Rodents have a total aversion (*Abneigung*) to alcohol
- **Absence of addictive behaviour**
- Low alcohol blood levels due to rapid alcohol metabolism

***Lieber-De Carli model***: Continuous administration of alcohol-containing liquid diet. Mild steatosis but no fibrosis

***Intragastric feeding model***: Causes steatosis, inflammation and fibrosis. Implantation and maintenance of an intragastric cannula are technically very challenging

## FUTURE DIRECTIONS IN LIVER FIBROSIS RESEARCH

- HBV and HCV viruses only infect human hepatocytes → no suitable mouse model available




*Chimeric mouse with humanized liver*

- Up to 99% chimerism when human hepatocytes are transplanted into immunodeficient mice with induced liver injury
- FRG mouse: hepatotoxicity by NTBC withdrawal (accumulation of toxic metabolites due to failure in tyrosine breakdown), 42% chimerism

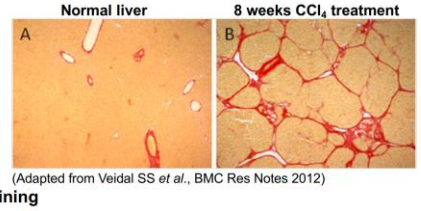
### DRAWBACKS OF THE CHIMERIC LIVER MODEL

- No progression to fibrosis or HCC commonly seen in human livers infected by HBV or HCV was observed in the chimeric mouse models
- Cross-talk and interactions between human engrafted hepatocytes and resident murine non-parenchymal cells is suboptimal
- Missing immune system (i.e. immunodeficient mice), which normally plays a fundamental role in progression of the infection/disease
  - Generation of double engrafted mice (human immune system and human hepatocytes

## DETECTION OF FIBROTIC TISSUE

- **Sirius Red staining**
  - Collagen staining (red)

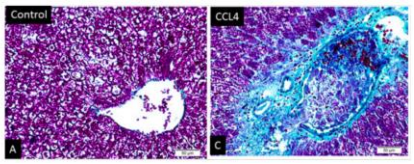
Used for diagnosis of fibrosis




(Adapted from Veidal SS *et al.*, BMC Res Notes 2012)

- **Masson’s thrichrome staining**
  - Collagen green to blue
  - Keratin red
  - Cell nuclei brown to black

Used for the study of cardiac, muscular, renal and hepatic pathologies




## SUMMARY

**Matrix components**

- Proteoglycans, collagens, multiadhesive proteins
- Matricellular proteins, growth factors, proteases

**Fibroblast activation**

- Reversible vs. constitutive activation

**ECM remodeling**

- Balance between matrix synthesis and degradation

**ECM deposition in fibroproliferative diseases**

- Chronic injury, constitutive fibroblast activation, continuous matrix synthesis

**Animal models of liver fibrosis**

- Choice of animal model is crucial
- Several levels of decision

## MOLECULAR REGULATION OF MATRIX REMODELING

- Basic molecular mechanisms **regulating the dynamic rearrangements** of the ECM **are not yet understood**
- Focus of current studies: Understand how ECM dynamics are regulated in health and disease and how these processes could be exploited and modulated for future therapeutic purposes

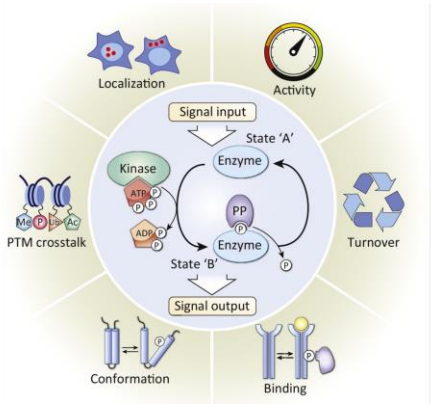
## POTENTIAL REGULATION OF ECM DYNAMICS BY EXTRACELLULAR PHOSPHORYLATION

Hypothesis: **Extracellular tyrosine phosphorylation** might be a major player in regulation of matrix dynamics *(but its biological relevance is completely unknown)*

### INTRA- VS. EXTRACELLULAR PHOSPHORYLATION

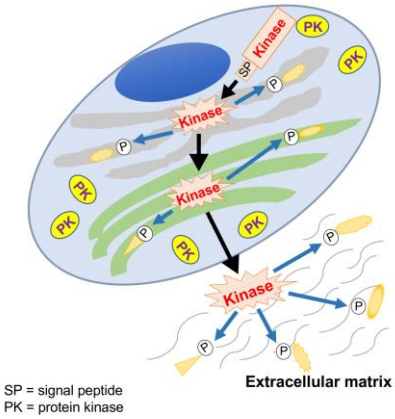
| Intracellular phosphorylation | Extracellular phosphorylation |
|---|---|
| - Well understood | - Mostly uncharacterized |
| - Many kinases reported | - Only two known kinases |
| - Fundamental biological process | - Largely unknown biological significance |
| - Reversible process | - Not known if reversible |
| - Takes place in the cytoplasm | - Not known when/where it takes place |
| - Known ATP optima | - Unknown ATP conc. |
| - Kinases as major drug targets | - Experimental protocols to be established |
| - Well optimized experimental protocols | |

- Intracellular protein phosphorylation is a fundamental biological regulatory mechanism




Surprisingly, to date, the biological significance of **extracellular (tyrosine) phosphorylation** is still completely unknown!

## CHARACTERISTICS OF SECRETED KINASES



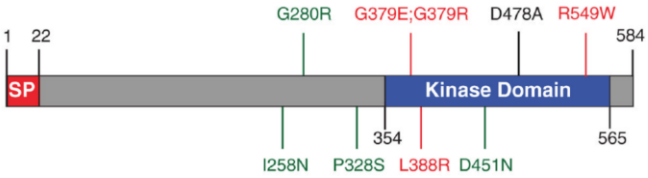

SP = signal peptide
PK = protein kinase

*We are looking for:* A protein with **"kinase features"** (conservation of specific residues necessary for **kinase activity**) with a **canonical signal sequence** which **localizes to the secretory pathway** and potentially **outside the cell**

## FAM20C IS SECRETED

- FAM20C localizes to the **Golgi** and is **secreted outside the cell**
- FAM20C **phosphorylates casein**
- FAM20C in disease: Raine syndrome
  → Rare autosomal recessive congenital disorder, usually lethal within a few hrs of birth

FAM20C mutations associated with Raine syndrome; **lethal** and **non-lethal**



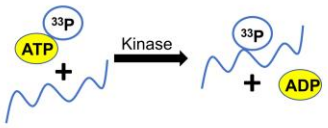

- Tyrosine phosphorylation of secreted proteins is widespread (1/3 of all secreted proteins have been reported to be tyrosine phosphorylated in unbiased LC-MS/MS studies)
- Extracellular phosphorylations occur in structurally conserved domains

## IDENTIFICATION OF THE FIRST SECRETED TYROSINE KINASE: VLK

**Vertebrate lonesome kinase (VLK, PKDCC)**

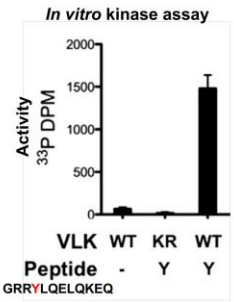
- VLK shows kinase activity

***In vitro* kinase assay principle**



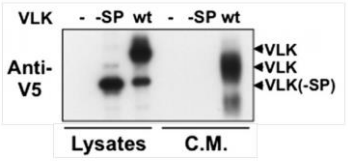

Transfer of a radioactively labelled P-group from ATP to a substrate

***In vitro* kinase assay**




- VLK has a functional signal peptide

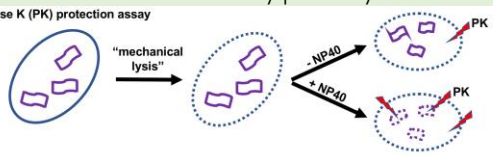
SP = signal peptide




1. Expression of V5-tagged VLK or VLK(-SP)
2. Immunoprecipitation from lysate and media
3. Detection by western blotting

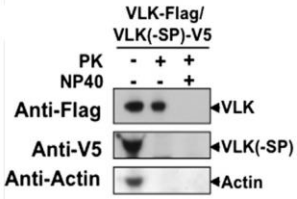
- A signal peptide is required for secretion of VLK
- VLK localizes to the secretory pathway

Proteinase K (PK) protection assay



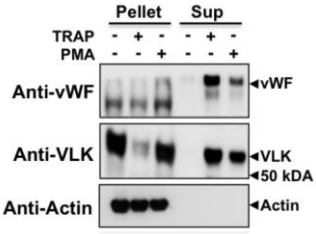

**Principle:**

1. Destruction of outer membrane only
2. Degradation of accessible proteins by PK
3. Lysis of internal membranes by addition of detergent
4. Degradation of accessible proteins by PK




- **Platelets** release VLK following degranulation
  - Platelets are key **regulators of matrix remodelling**
  - Platelets release their **secretory granules** content in response to physiological stimuli
  - Platelets locally release **high amounts of ATP**
  - VLK was localized to **platelets alpha-granules**

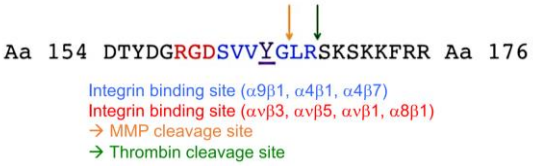
- TRAP/PMA leads to secretion of granules in platelets




- VLK knock-out mice die at birth (are smaller and die due to respiratory failure)
- But till now no connection to a known disease
- VLK phosphorylates secreted proteins
- **ER-resident proteins**
  - (Endoplasmic reticulum resident protein 29 Y(365)
- **Golgi transmembrane proteins**
  - Golgi integral membrane protein 4 Y(9)
- **Secreted proteins**
  - Matrix metalloproteinase 13 Y(25)
  - Collagen alpha-1(I) chain
  - Insulin-like growth factor-binding protein 7
- p-Tyr at conserved functional sites in secreted proteins

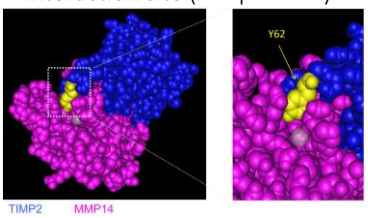
Example 1: **Osteopontin**

- VLK-dependent phosphorylation in the middle of an **integrin binding site**



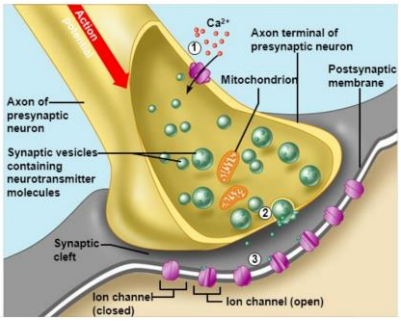

Example 2: **TIMP2**

- VLK-dependent phosphorylation in the middle of the **interaction site** (will probably affect binding)

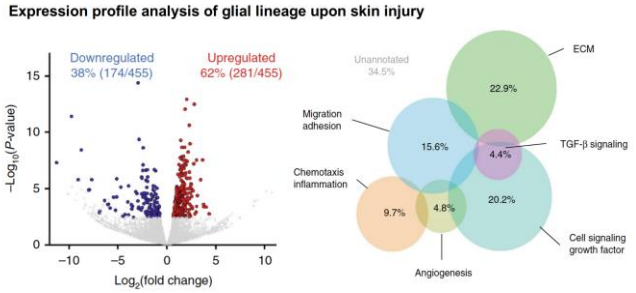



## OUTLOOK: SIGNIFICANCE OF VLK STUDIES

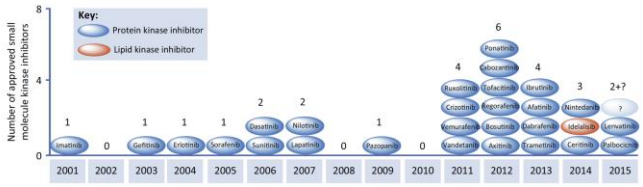
- **Peptide hormones** of the endocrine system are often **phosphorylated**
  - **Biological relevance** of these phosphorylations is currently **unknown**
  - Interesting for further investigation
- Several **neuropeptides** have been reported phosphorylated (POMC, Neuropeptide Y, Neuromedin U, Orexin, …)
  - ATP and phosphorylated neuropeptides are released at the synaptic cleft




- Secretome involvement in ECM remodelling: Products of several of these identified genes have been reported phosphorylated (SEMA4F, AREG, MMP14, MEGF10, WISP1, ASPN, TNC, TNN, FGF5, …)




## IMPORTANCE OF KINASES AS DRUG TARGETS




*Could in the future extracellular kinases become attractive drug targets too?*

## CURRENT ONGOING RESEARCH IN WERNERS GROUP

*Question:* How is matrix remodeling regulated at the molecular level?

*Hypothesis:* extracellular tyrosine phosphorylation as a novel regulatory mechanism of ECM remodeling

Project 1:

**Role of VLK in liver regeneration following acute or chronic injury** (Sophia Pantasis)

Project 2:

**Role of VLK in lung development and lung fibrosis** (Salome Brütsch)

*Selected techniques*

Tissue specific KO mice, (immuno-) histochemistry, (phospho-) proteomics, metabolomics, isolation and culture of primary cells, protein biochemistry, basic molecular and cell biology techniques

# SABINE WERNER

## GROWTH FACTORS

### DISCOVERY OF GROWTH FACTORS

**1908**: Discovery of **erythropoietin**: Component of the blood that stimulates the production of red blood cells

**1940**: Discovery of substances that **induce the innervation of tumors**: Isolation of nerve growth factor and epidermal growth factor from salivary glands

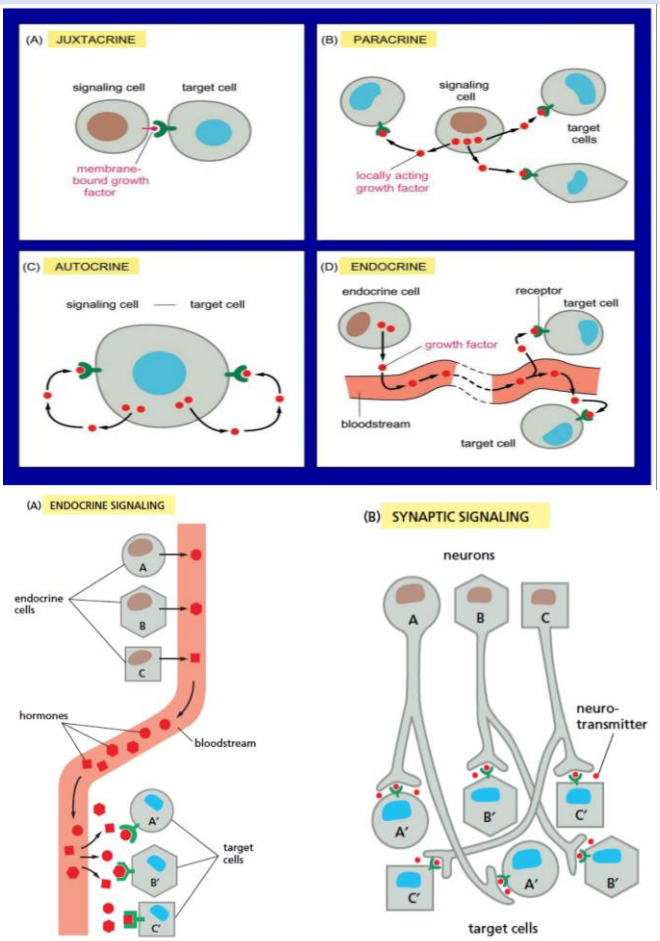
Culture of mammalian cells:

Important observation: Cells grow in medium with serum; without serum they remain in the G0 phase of the cell cycle

Hypothesis: Cells require growth factors that are present in serum

**1975**: Purification of 100µg of a growth factor from platelet extracts of 200l human blood: Platelet-derived growth factor (PDGF)

### MECHANISMS OF INTERCELLULAR SIGNALING




### GROWTH FACTORS

- Produced by **almost every cell type**
- Usually **small, secreted peptides**
- Every cell requires the presence of a certain set of growth factors
- Growth factors act on cells that have the appropriate **receptor** (requires binding of the ligand to a receptor)
- Growth factors are active in nanomolar or picomolar concentrations (**very low concentration**)

### GROWTH FACTOR FUNCTIONS

- Regulate **proliferation**, **differentiation**, **survival**, **migration**
- Are **always required**, in particular during **development**, **tissue repair** and **tumorigenesis**
- Cellular responses depend on **ligand**, **receptor** and **cell-type** as well as on the **combination of ligands**

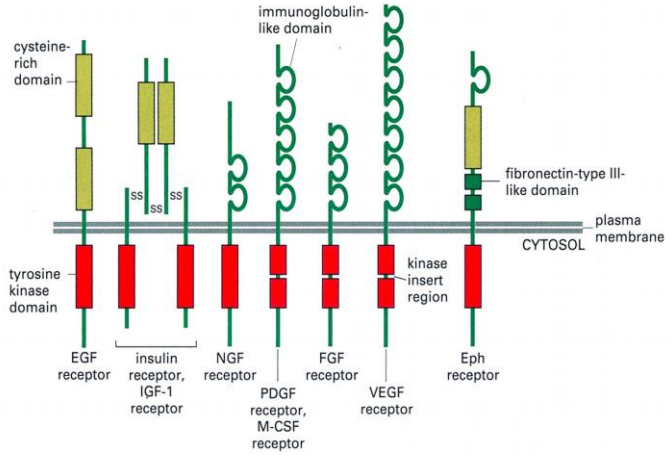
### CYTOKINES

- Larger family of signalling molecules
  → includes the normal growth factors

***Old definition**: Proteins that regulate proliferation and differentiation of haematopoietic cells*

New definition: Molecules that **regulate proliferation, differentiation, survival** and **other cellular functions** at extremely **low concentrations** (includes the "classical" growth factors)

### RECEPTOR TYROSINE KINASES




- Extracellular portion: Ligand binds
- Intracellular part: Tyrosine kinase domain

### STRUCTURAL COMPONENTS OF RTKS

Inactive form:

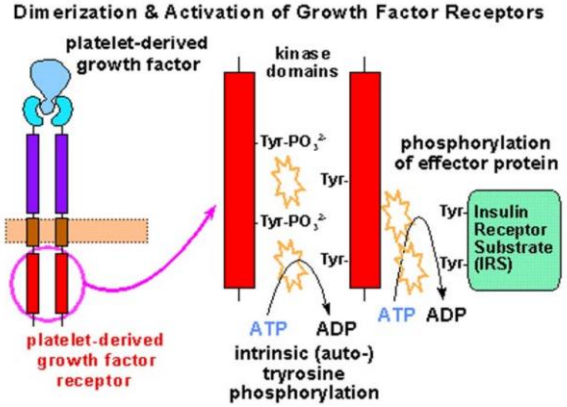
- Intracellular tyrosine-kinase (tyr-/Y-kinase) domain
- Mostly monomers (unless pre-formed as a dimer)
- Extracellular Ig-like or cysteine-rich domains (very frequent)

Active form:

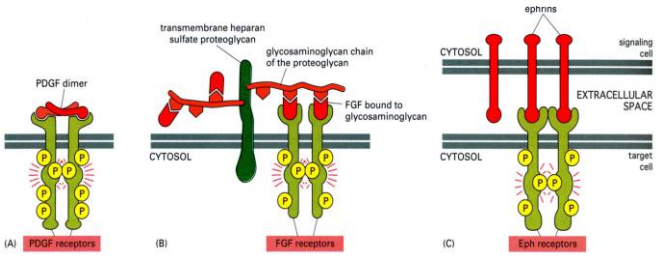
- Di-/Polymers
- Requires binding of ligand(-s) by 2 receptors
- Y-kinase domains transphosphorylate (ATP-dependent)
  →can then phosphorylate downstream signalling molecules

## RECEPTOR **DIMERIZATION** AND **ACTIVATION**

- Ligand-binding leads to **dimerization** of the receptor
- Dimerization **activates** the receptor → Kinase domain **cross-phosphorylate** each other (→ Conformational change → Access to cytosolic phosphorylation sites)→ Binding of signalling molecules to phosphorylated tyrosine → **Phosphorylation/ activation of signal molecules** → Signal is transmitted downstream



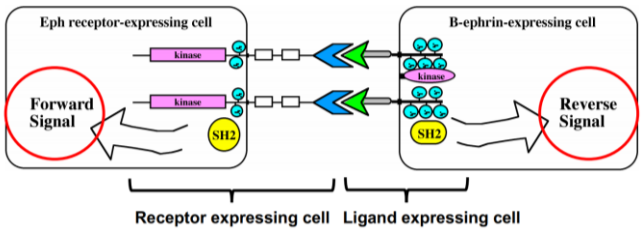

## **DIMERIZATION** OF RECEPTOR TYROSINE KINASES BY EXTRACELLULAR LIGANDS




A) Ligand itself is a dimer: Brings 2 kinases together (PDGF)
B) Ligand bound by proteoglycans (FGF)
C) Ligand is cell-surface bound (Eph, Juxtracrine)

### EPHRIN SIGNALING:

- **Ephrins** and **Eph** receptors can simultaneously act as ligands or receptors
- Needs the **contact** between the signalling and the targeting cell
- Signal goes in both directions → **Bidirectional signalling** → Behaviour of both, signalling and target cell is altered

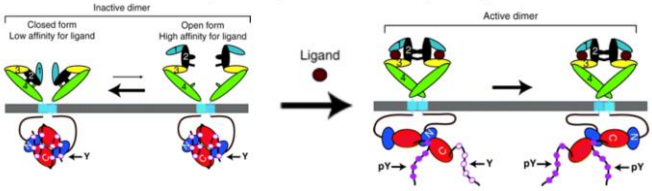



- During development they prevent group of cells to mix with each other

## LIGAND-DEPENDENT **RECEPTOR ACTIVATION**:

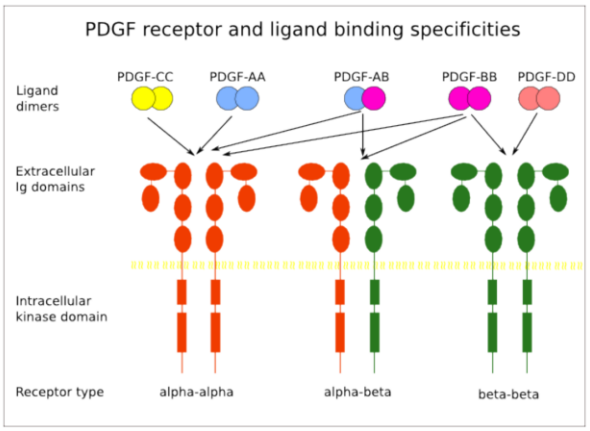
<u>Conformational change of pre-formed dimers:</u>

- Shown for EGF receptors (monomeric ligands)



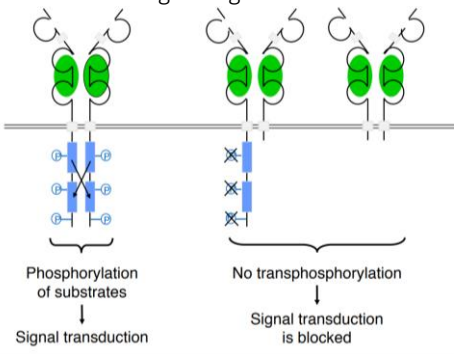

## SOME RECEPTORS CAN FORM **HETERODIMERS**

- Heterodimerization is possible for growth factor receptors that belong to the **same family**
- Each heterodimer binds a specific set of ligands
- Examples: ***PDGF*** *receptor family,* ***EGF*** *receptor family,* ***FGF*** *receptor family*

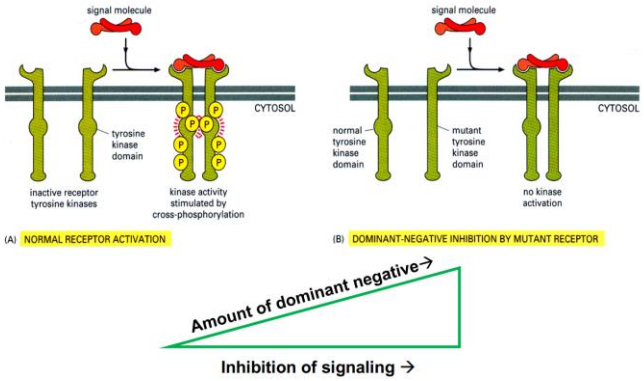



## THE PRINCIPLE OF **DOMINANT-NEGATIVE RECEPTOR MUTANTS**

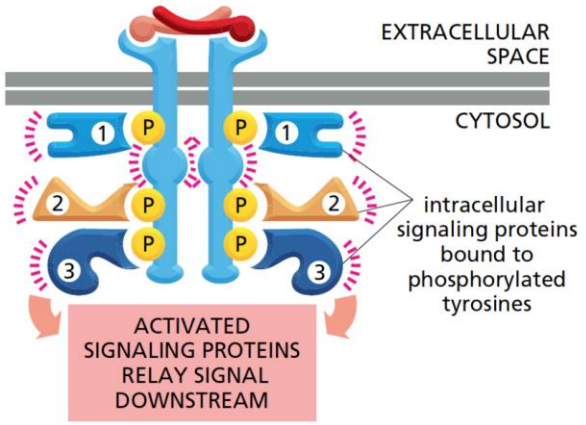
- Normal: Signal is transmitted by the kinase domain in the intracellular domain → This makes it easy to modulate the signalling:



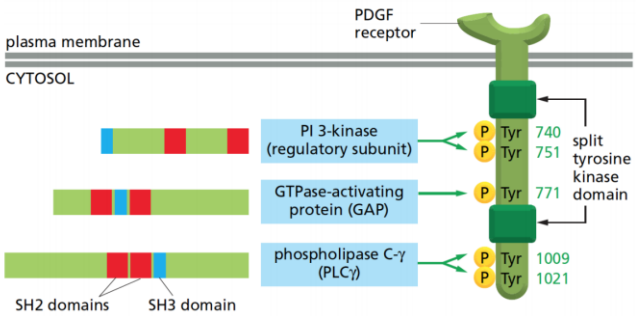

- Dominant-negative FGF receptors: Receptor that does not have any intracellular kinase domain (or not intact or no intracellular domain at all) → Mutant of the kinase domain will **not have any signalling downstream**! → Block signalling at the level of the receptor



## SIGNAL TRANSDUCTION BY RECEPTOR TYROSINE KINASES




- Phosphorylation outside the kinase domain provide docking sites for other proteins
- Transautophosphorylation acts as a switch to trigger the assembly of a transient intracellular signaling complex
- Intracellular proteins bind on specific motifs on the receptor (only when the receptor is phosphorylated)




- A variety of proteins bind phosphorylated tyrosines on RTKs
- Common highly specific p-Tyr binding domains (e.g. SH2 or TB)

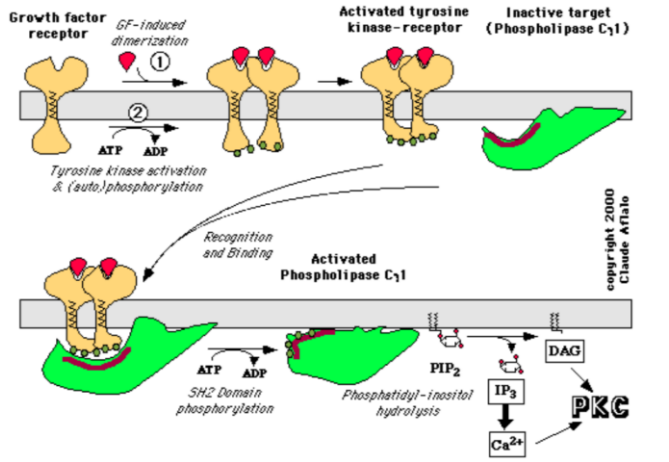
### ACTIVATION OF SIGNALLING MOLECULES BY RECEPTOR TYROSINE KINASES

- Signalling proteins bind to phosphorylated receptors via **SH2 domains or PTB domains**
  - **SH2**: src homology domain 2:
    Approximately 100 amino acids that recognize phosphorylated tyrosines and their close vicinity
    Bind with high affinity (10-9M)
  - **PTB**: Phosphotyrosine binding domain
- Activation of signal molecules:
  - By **tyrosine phosphorylation**
  - By **conformational change** without tyrosine phosphorylation
  - Through **recruitment to the membrane**: Signaling molecule is closer to their substrates

Signalling proteins that are activated by receptor tyrosine kinases:

- **Enzymes**: PLC-g, Src (cytoplasmic tyrosine kinase), c-cbl (Ubiquitin ligase), PI3-Kinase
- **Structural proteins**
- **Adaptor proteins** (Grb-2, Shc, Eps15, Epsin)

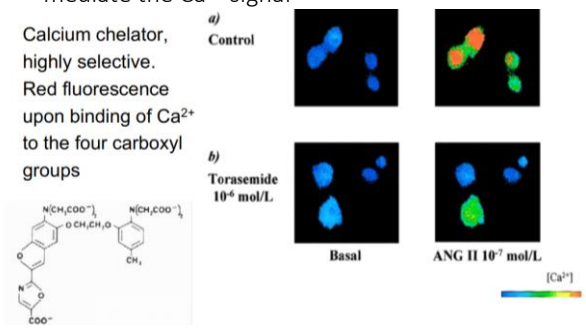
## ACTIVATION OF **PHOSPHOLIPASE C-γ** BY RECEPTOR TYROSINE KINASES




GF → activated RTK → PLC-γ gets phosphorylated → cleaves $PIP_2$ → DAG + $IP_3$ → $IP_3$ induces **increase in intracellular calcium levels** → $Ca^{2+}$ + DAG activate PKC

### $CA^{2+}$ AS SECOND MESSENGER

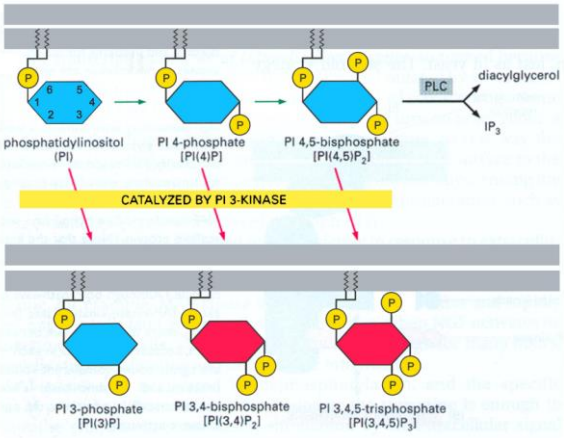
- **Cytosolic** [$Ca^{2+}$]: **$10^{-7}$M**
- **Extracellular** [$Ca^{2+}$]: **$10^{-3}$M** (also in the **lumen of the ER**)
- Opening of $Ca^{2+}$- channels after growth factor stimulation: **10-12-fold increase** in the cytosolic $Ca^{2+}$-concentrations
- $Ca^{2+}$ binds to different proteins (e.g. calmodulin) that mediate the $Ca^{2+}$ signal




*Determination of intracellular $Ca^{2+}$ by **FURA-2** (= cell-permeable $Ca^{2+}$ dye, chelates calcium)*

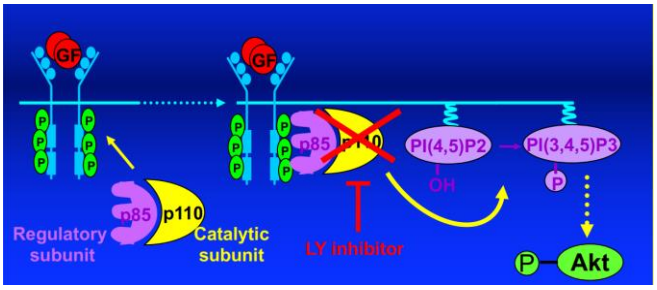
### PHOSPHATIDYLINOSITIDE-3-KINASE

- Consists of a **regulatory and a catalytic subunit**
  - Regulatory subunit binds to phosphorylated receptor tyrosine kinase: Recruitment of the enzyme to the membrane where its substrates are located
  - Catalytic subunit phosphorylates **phosphoinositol-4,5-biphosphate** at the plasma membrane at position 3




*Substrates and Products of PI3-Kinase*

## ACTIVATION OF THE PI3K SIGNALLING PATHWAY

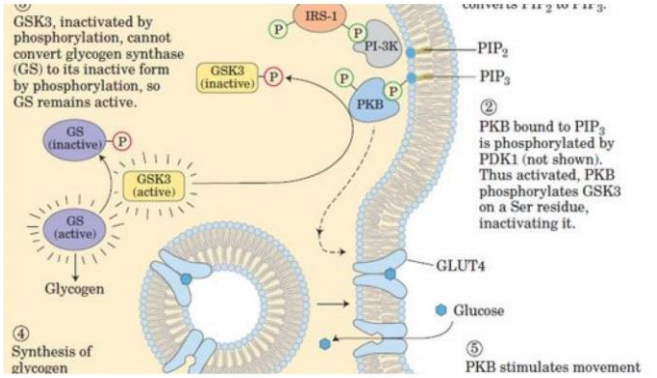



RTK activation → PI3K activation → PIP3 → PKB and PDK1 bind to PIP3 → PDK1 phosphorylates PKB → p-PKB phosphorylates translation initiation factor (TIF) → Phosphorylated TIF induces translation

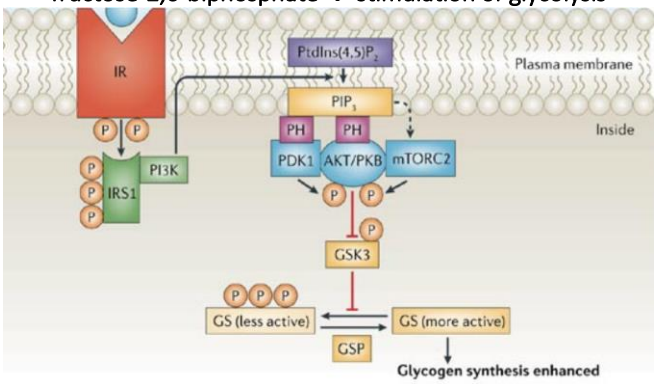
## BIOLOGICAL FUNCTIONS OF PKB

- Phosphorylates **translation initiation factor** → stimulation of translation
- **Prevents apoptosis** via different mechanisms: Increased cell survival under stress conditions
- Involved in tumorigenesis: Often overexpressed in malignant tumors (Warburg effect)
- PKB **increases the glucose metabolism**

## EFFECT OF PKB ON GLUCOSE METABOLISM:



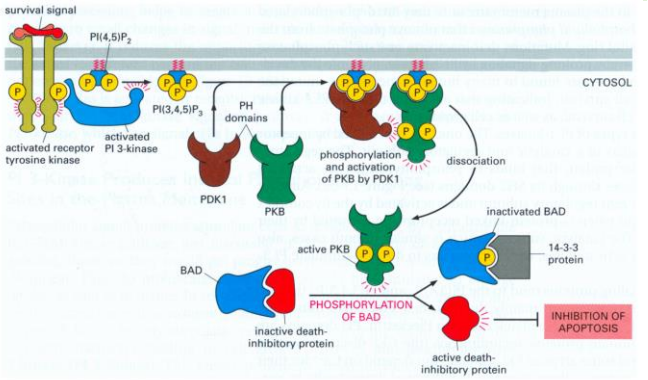

- Gets glucose **in the cell** → Induces translocation of GLUT transporter to the membrane)
- Gets glucose through **glycolysis** → Phosphorylates **phosphofructokinas-2** → increases production of **fructose-2,6-biphosphate** → **stimulation of glycolysis**




- Stores glucose if glycolysis is saturated → **glycogen production** → phosphorylates **glycogen synthase kinase 3** and thus inactivates it → reduces phosphorylation of glycogen synthase → **Enhanced production of glycogen**

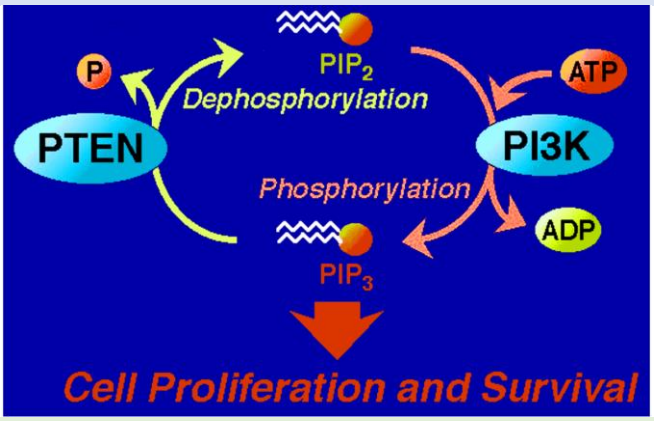
## EFFECT OF PKB ON APOPTOSIS

PKB activation **inhibits apoptosis**

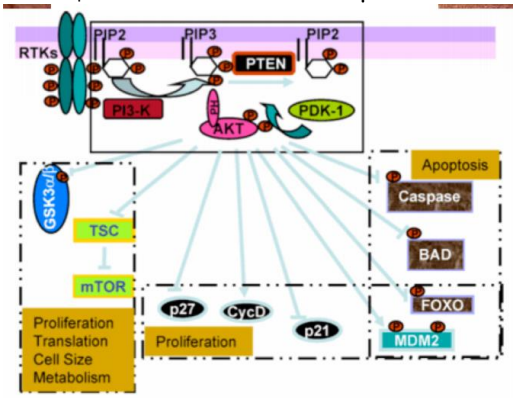



- Aktive PKB phosphorylates BAD → inactivation of BAD and release of active death-inhibitory proteins

## PTEN: A PIP3 PHOSPHATASE




- PTEN is a **phosphatase** and a TENsin homologue deleted from chromosome 10
- **Dephosphorylates PIP3** on position 3
- Is an antagonist of PI3K
- Classical **tumor suppressor**: Somatic mutations in many human tumors (in particular prostate, thyroid endometrium), causing **increased PKB activity**
- Consequence: Mutated cells **escape senescence**

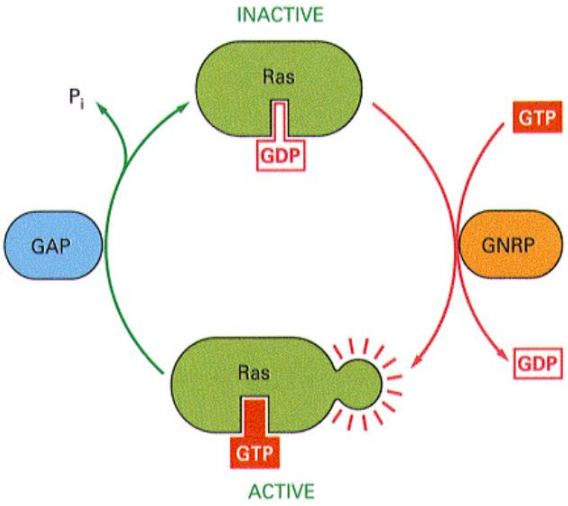



- Knockout mice die before birth
- Heterozygous mice have an **increased tumor incidence**
- High breast cancer risk
- Cowden disease: Genetic disease characterized by formation of multiple tumors: Germ-line mutations in the PTEN gene; patients have multiple skin lesions and develop polyps and adenomas in the gut and thyroid

## ADAPTOR PROTEIN BINDING RTKS

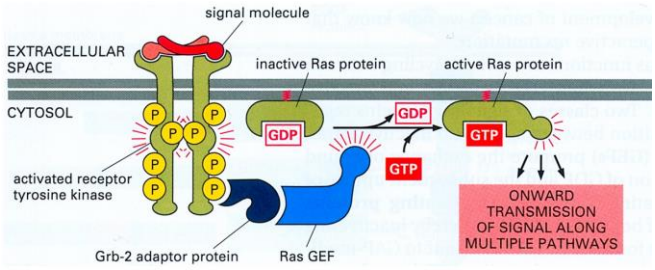
- **Lack a catalytical domain**
- Have at least one SH2 domain or PTB domain as well as domains that interact with other proteins (e.g. SH3)
- Examples: *CRK, NCK, GRB-2, SHC*

## RAS




- **Ras**: monomeric G-Protein
- Is activated by **GNRP**
  - ***GNRP*** *= Guanine nucleotide exchange factor*
- Is inactivated by **GAP** by dephosphorylation
  - ***GAP*** *= GTPase activating protein*
- Active RAS activates the **MAP kinase signaling pathway**
- **Oncogenic Ras** → Unable to hydrolyse GTP → Hyperactive

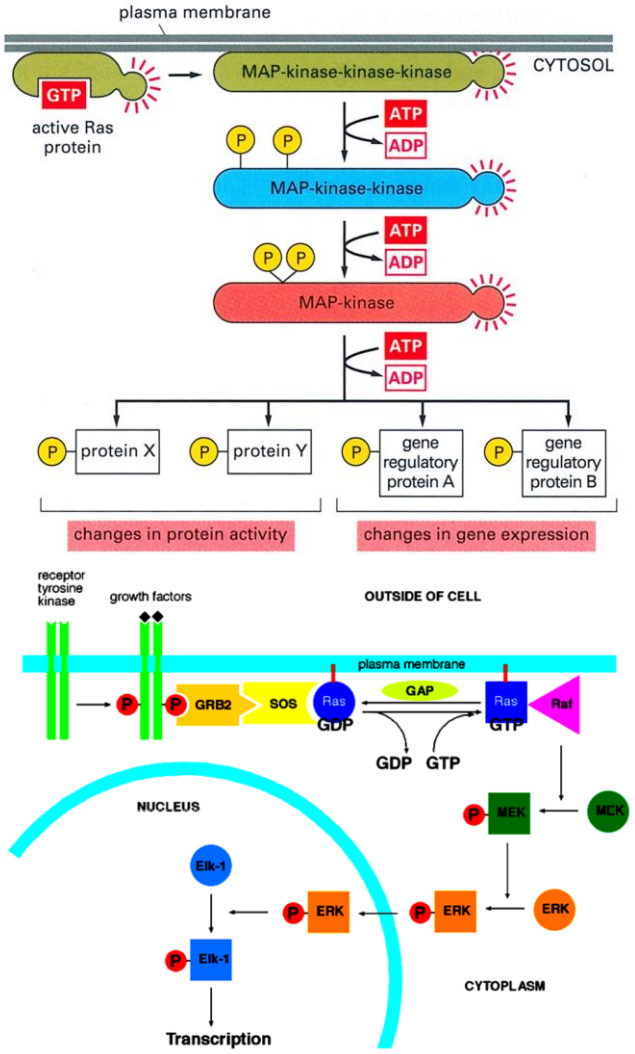
## ACTIVATION OF RAS THROUGH RECEPTOR TYROSINE KINASES




*Grb-2 = growth factor receptor binding protein 2*
*GEF: Guanylate exchange factor*

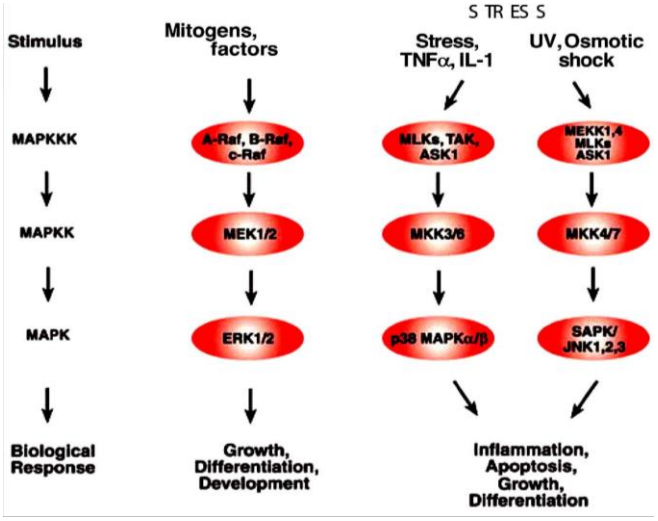
1. RTK activation
2. Grb2 binds to RTK
3. GEF (SOS) binds to Grb-2
4. SOS is active and activates RAS

## THE MAP KINASE SIGNALLING PATHWAY




- MAP kinase translocases to the nucleus after mitogen stimulation

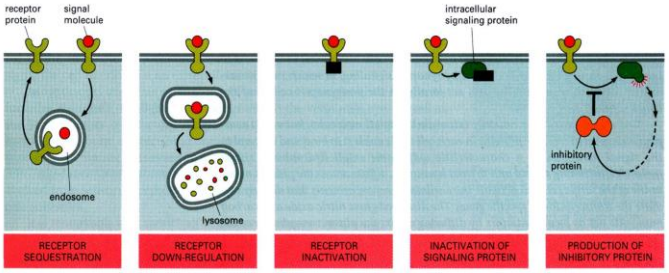
## 3 DIFFERENT MAP KINASE CASCADES



## ADAPTATION/DESENSITATION

- Prolonged exposure to a growth factor **reduces the cellular response** (is a reversible process)
- Principle: Delayed negative feedback
- +) Cells can respond to changes in growth factor levels

### TYPES OF ADAPTION MECHANISMS

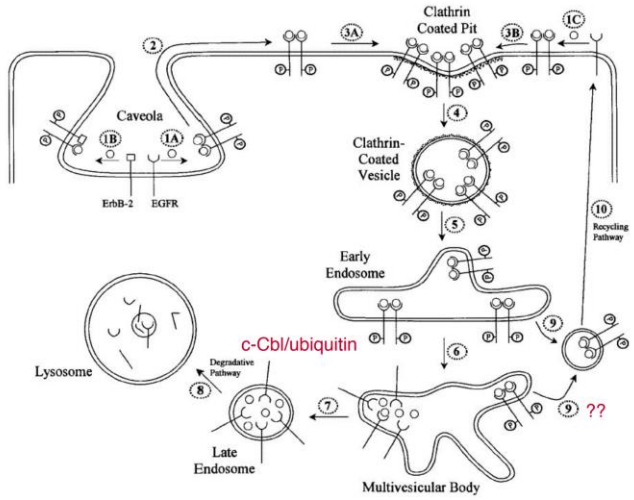



- **Receptor sequestration** (lasts few hours, recycling)
- **Receptor downregulation** (lasts ca. 12h, new transcription and production)
- **Receptor inactivation** (nearly total blockage)
- **Inactivation of signaling protein** (nearly total blockage)
- Production of **inhibitory protein** (nearly total blockage, but needs more time)

## C-CBL

- C-cbl is an E3 ubiquitin ligase
- Substrate of phosphorylated receptor tyrosine kinase
- Catalyses monoubiquitinylation of RTK → Ubiquitinylated receptor is targeted to lysosomes for degradation
- By targeting to degradation, C-cbl inhibits receptor recycling to the plasma membrane and activity of RTK in endosomes
- Inhibition of C-Cbl leads to receptor recycling (used in tumor cells)

### GENERAL PRINCIPLES OF RECEPTOR TRAFFICKING




## ENDOCYTOSIS OF RECEPTOR-BOUND GF

- **RTK** are endocytosed via clathrin-coated pits
- Activation of the MAP-kinase pathway requires endocytosis
- Signaling complexes are formed on clathrin-coated vesicles
- Receptor endocytosis and subsequent degradation are important for signal attenuation

## METHODS TO ANALYSE GROWTH FACTOR FUNCTION

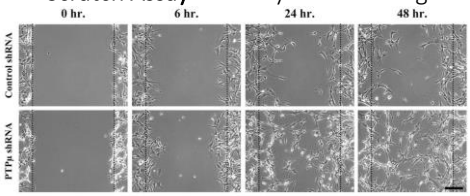
- **Molecular level**:
  - Analysis of intracellular signalling cascades
- **Transcriptional level**:
  - Analysis of growth factor regulated gene expression
- **Phenotypic level:**
  - Analysis of cell proliferation
  - Analysis of cell migration
  - Analysis of cell survival
- **Generation of transgenic or knockout mice**

### METHODS TO DETERMINE CELL PROLIFERATION

- Seed equal number of cells and **count cells at different time points**
- Incorporation of **$^{3}$H-thymidine**: Measure incorporated radioactivity or identify labelled cells by autoradiography
- Incorporation of **5-bromo-2`-deoxyuridine (BrdU**; nucleotide analogon): Identify labelled cells with an antibody directed against BrdU
- **Ki67** staining to detect proliferating cells: KI-67 is a nuclear protein that is associated with and may be **necessary for cellular proliferation**

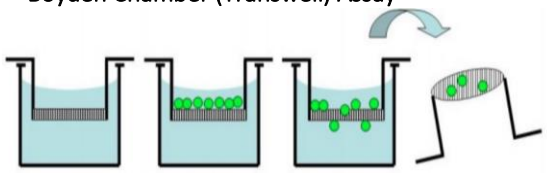
### METHOD TO DETERMINE CELL MIGRATION

- **Scratch Assay** → Analysis of cell migration



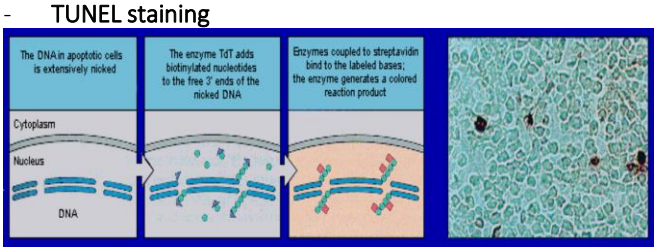

### METHOD TO ANALYZE CHEMOTAXIS

- **Boyden Chamber (Transwell) Assay**



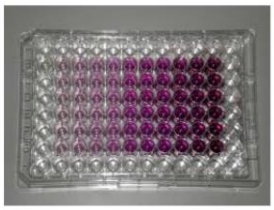
### METHODS FOR DETECTION OF APOPTOTIC CELLS

- **TUNEL staining**




- Staining for **Cleaved Caspase-3**
- **MTT Assay:** Quantitative colorimetric assay for cell survival and proliferation
  - Based on reduction of MTT (yellow) to a formazan product (violet) in mitochondria

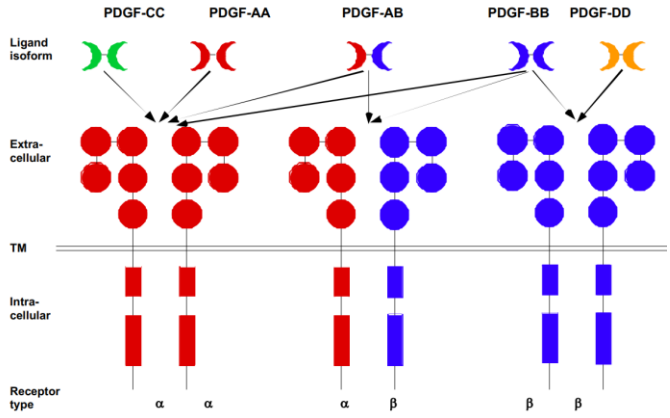
➢ Experimental procedure
- ➢Seed cells in 96 well plates
- ➢Serum starvation
- ➢Incubation with menadione
- ➢Addition of MTT
- ➢Photometric analysis



The MTT assay is a colorimetric assay for assessing cell **metabolic activity** → reflect number of **viable** cells present

# PDGFS

## PDGFS AND THEIR RECEPTORS




## IN VIVO FUNCTION OF PDGF

- **Embryonic development**:
  - Kidneys (mesangial cells)
  - Blood vessels (smooth muscle cells, pericytes)
  - Lungs (alveolar smooth muscle cells)
  - CNS (oligodendrocytes)
- Stimulation of **wound healing**

## PDGF IN DISEASE

- **Fibrotic conditions**
  - Lung fibrosis
  - Glomerulonephritis
  - Liver cirrhosis
  - Myelofibrosis
- **Atherosclerosis**
- **Cancer**
  - Autocrine stimulation (glioblastoma, sarcoma)
  - Paracrine stimulation (carcinomas: produced by cancer cells, stimulates stromal cells)

## DERMATOFIBROSARCOMA PROTUBERANS (DFSP)

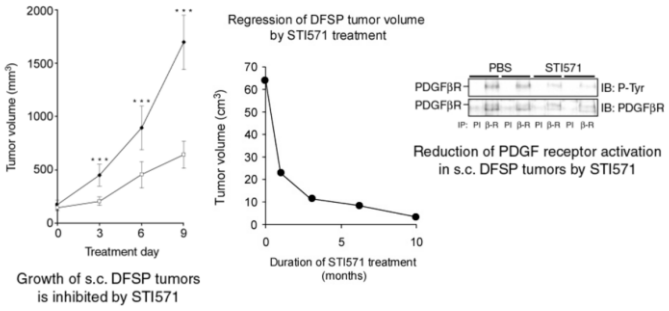
- **Skin tumor** derived from **fibroblasts** of intermediate malignancy
- Treated with surgery, high recurrence rate
- Genetically characterized by **collagen 1A1/PDGF-B gene fusion** → Fusion gene encodes precursor, which is processed to PDGF-BB: High levels of the growth factor are produced in tumors → **Autocrine stimulation**

## PDGF ANTAGONISTS

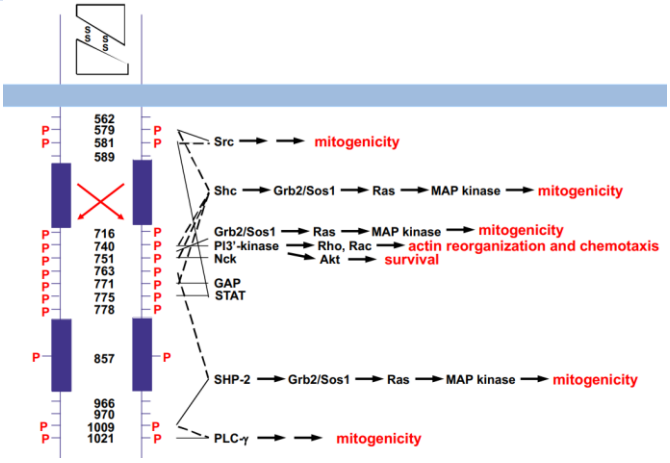
- PDGF aptamer
  - DNA molecule, binds PDGF B-chain with high affinity
  - Specific
  - expensive, inconvenient to administer
- STI571 (Gleevec)
  - Low molecular weight inhibitor of PDGF receptors, c-Kit, Bcr-Abl
  - Not absolutely specific
  - Inexpensive, easy to administer

## TREATMENT OF HUMAN DFSP WITH STI571

- **STI571** reduces growth of subcutaneous DFSP tumors and blocks **PDGF** receptor activation in tumors

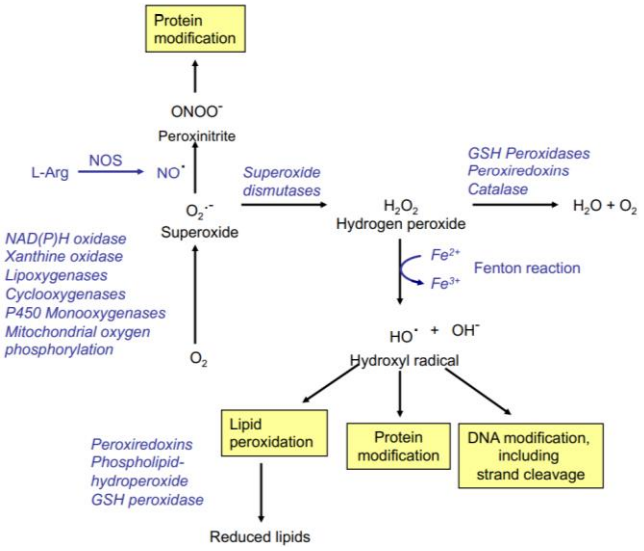



## SIGNALLING BY THE PDGF-B RECEPTOR



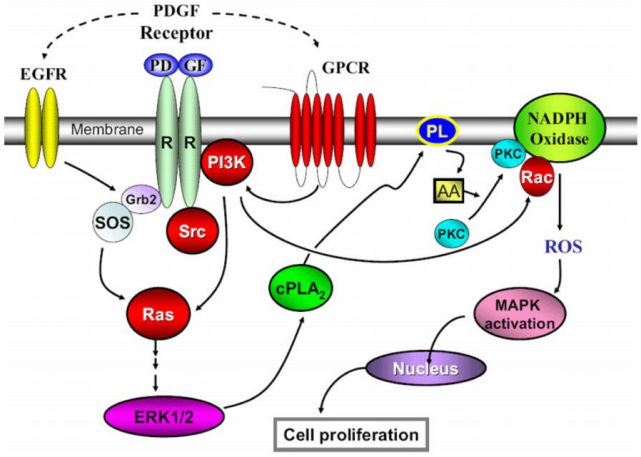

- PDGF receptors have huge number of phosphorylation sites
- Nearly every phosphorylationsite signals to a downstream effector
- Downstream effects:
  - **Mitogenicity** (via Src, Ras/MAPK, PLC-γ)
  - **Actin reorganisation and chemotaxis** (via PI3K/Rho)
  - **Survival** (via PI3K/Akt)

## REACTIVE OXYGEN SPECIES (ROS) AND THEIR DETOXIFICATION



## DISCOVERY OF $H_2O_2$ AS A SIGNALLING MOLECULE

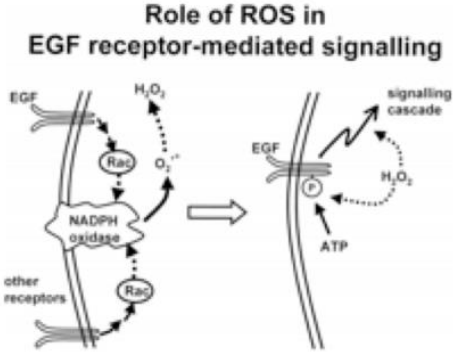
- Stimulation of cells with **PDGF** → intracellular concentration of **$H_2O_2$ increases**
- **$H_2O_2$ production** upon PDGF stimulation of cells is **PI3K dependent**: Activation of Rac by PI3K
  - Rac activates NADPH oxidase (superoxide production) → superoxide is converted to hydrogen peroxiase by superoxide dismutase
  - Hydrogen peroxide (or other ROS) is needed for MAPK activation
- **Depletion of $H_2O_2$** by catalase or N-acetylcysteine **inhibits PDGF receptor signalling** (including MAPK activation, DNA synthesis and cell migration)



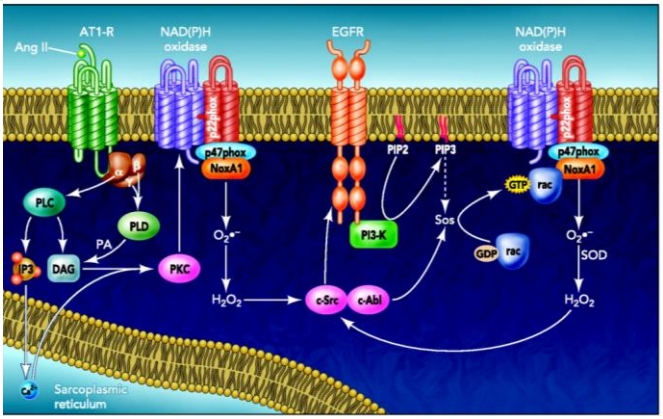

*Signalling by PDGF receptors: $H_2O_2$ as a signalling molecule*

## GENERATION OF SUPEROXIDE ANIONS AND $H_2O_2$

- **Rac** activates **NADPH oxidase**
- **NADPH oxidase** produces **superoxide ($O^{2-}$)**
- Dismutation of superoxide to hydrogen peroxide




- NADPH oxidase in the plasma membrane produces superoxide radicals



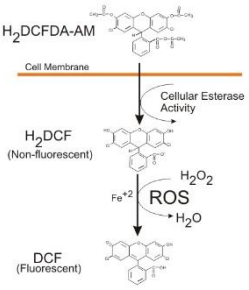

## DETERMINATION OF INTRACELLULAR ROS LEVELS:

### DCF

Reactions:

1. DCFDA-AM → $H_2$DCFDA (by cellular esterases)
2. $H_2$DCFDA → DCF (by any ROS)

- Only DCF is fluorescent
- Measures intracellular ROS only due to cellular presence of esterases
- Disadvantage: Method is not specific for 1 type of ROS

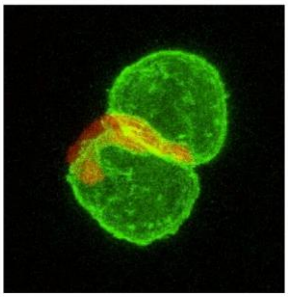



### HYPER

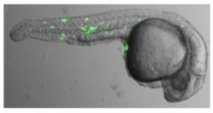
- **Genetically encoded fluorescent sensor** capable of detecting intracellular $H_2O_2$
- Yellow fluorescent protein (YFP) inserted into the regulatory domain of the E.coli protein OxyR
- Submicromolar affinity to $H_2O_2$, but not to other ROS (fairly specific)
- Suitable for Real-Time imaging by confocal microscopy
- In the presence of hydrogen peroxide, the reduced form of OxyR is converted into an oxidized DNA binding domain: Conformational change: Increased emission at 500 nm and decreased emission at 420 nm
- Monitoring of changes in green emission

## $H_2O_2$ IS A "CHEMOKINE"

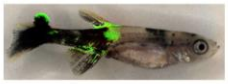
- Observation: Rapid attraction of immune cells upon tissue injury → *what is the chemoattractant?*
- **Hydrogen peroxide** is **produced at the wound site** and **attracts immune cells** *(shown by injection of HyPer RNA into zebrafish embryos and subsequent wounding)*
- Hydrogen peroxide **produced by tumor cells** attracts immune cells in zebrafish



Tumor cell (green) attracts a neutrophil (red) via release of hydrogen peroxide



Transformed melanoblast injected into zebrafish larvae



Development of melanomas in adult fish

# EPIDERMAL GROWTH FACTORS AND THEIR RECEPTORS

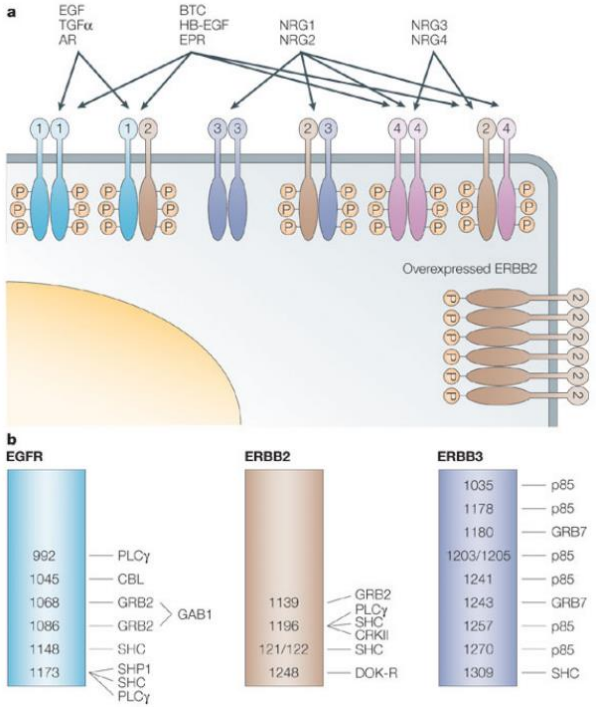
## EPIDERMAL GROWTH FACTOR **RECEPTOR** FAMILY

- **EGF receptor** = **HER1** = ErbB1
- **HER2** = ErbB2 = Neu
- **HER3** = ErbB3
- **HER4** = ErbB4

## EPIDERMAL GROWTH FACTOR RECEPTOR **LIGANDS**

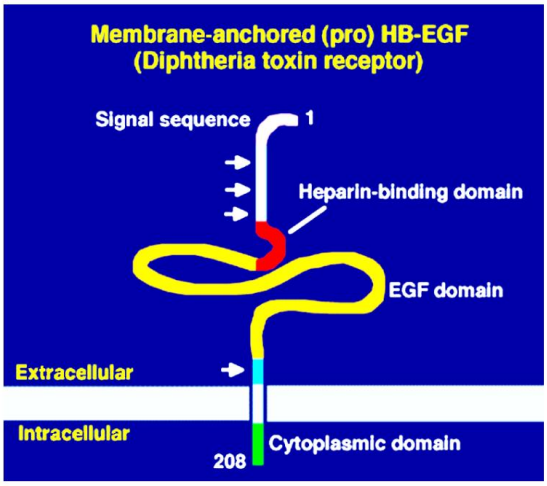
- **EGF receptor/ HER-1**: EGF, Transforming growth factor α, Betacellulin, Heparin-binding EGF, Amphiregulin, Epigen, Epiregulin
- **HER-2:** No ligands of HER-2 homodimers identified yet
- **HER-3**: Heregulins 1 and 2 (= Neu differentiation factor)
- **HER-4:** Heregulins 1, 2, 3, Betacellulin, Epiregulin, Heparin-binding EGF

## LIGAND BINDING SPECIFICITY OF THE EGFR FAMILY



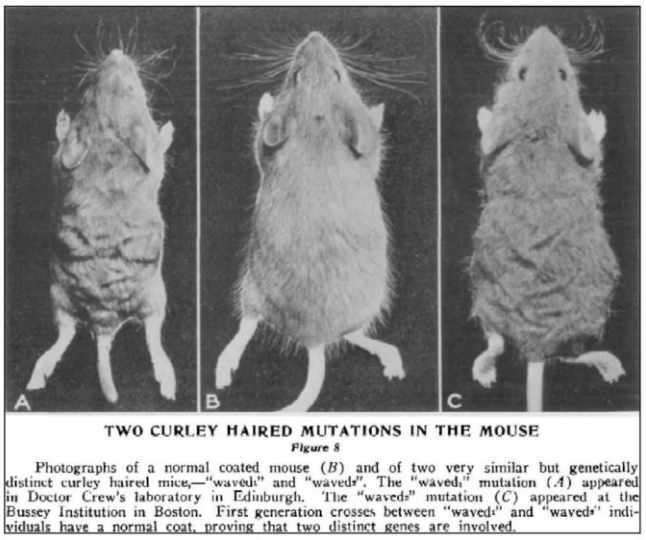

## CHARCATERISTICS OF EGF RECEPTOR LIGANDS

- Are **produced as membrane-anchored precursors** which can act in a juxtacrine manner or which are involved in cell-cell adhesion
- Soluble growth factor is produced by **proteolytic cleavage**: Autocrine or paracrine mechanisms of action

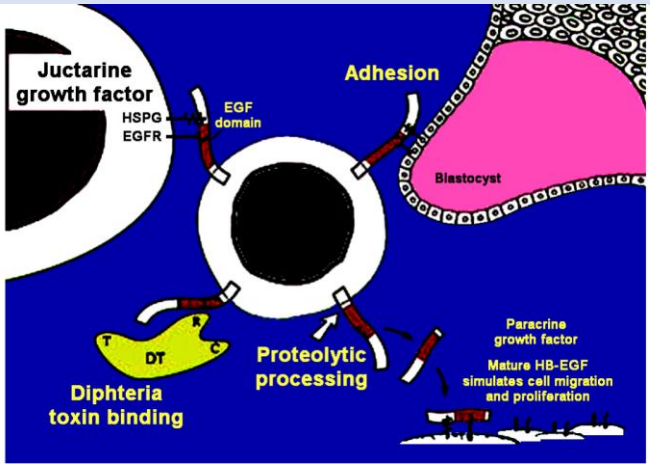



## EGF FAMILY: PHENOTYPES OF KNOCKOUT MICE

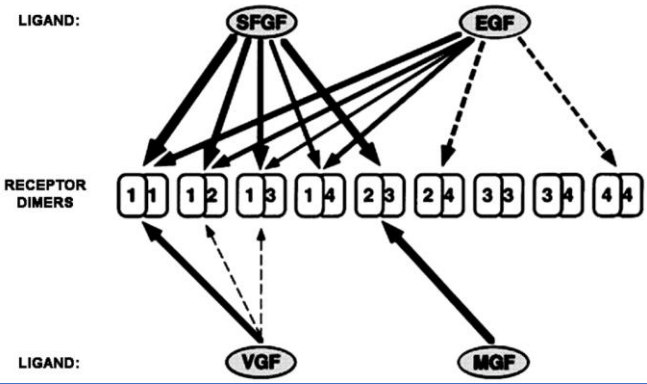
- **EGF receptor knockout**:
  - Phenotype is **strain-dependent** → Only mice of a certain genetic background survive until birth or even until postnatal day 20
  - Defects in skin, intestine, pancreas, tooth, eyelid, brain
- **HB-EGF knockout**: Develop severe heart failure
- **EGF knockout**, **Epigen knockout**: No phenotype detected
- **TGF-a knockout**: Curly hair
- **Amphiregulin knockout**: Underdeveloped mammary glands (minor phenotype)
- **EGF/TGF-a/amphiregulin triple knockout**: Survive to adulthood, but major mammary gland abnormalities (underdeveloped)




## DIFFERENT FUNCTIONS OF HEPARIN-BINDING EGF (HB-EGF)



## VIRUS-ENCODED EGF-LIKE GROWTH FACTORS AND THEIR RECEPTORS



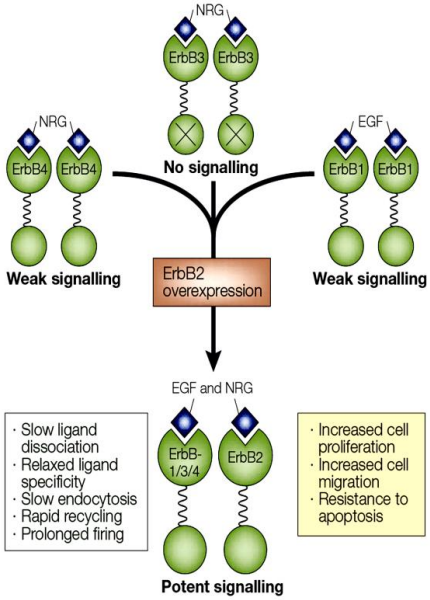

### POX VIRUSES

Pox viruses produce EGF-like growth factors

- *Shope fibroma virus growth factor (SFGF)*
- *Myxoma virus growth factor (MGF)*
- *Vaccinia virus growth factor (VGF)*
- **Function**: Not required for viral replication, but **enhance virulence and stimulate cell proliferation and migration** at the site of infection
- **Viral GFs use the host receptor for signaling**

## SIGNALLING VIA DIFFERENT ERBB DIMERS

- **HER2 = ErbB2** forms **heterodimers** with **ErbB1, ErbB3 and ErbB4** - Binding of ligands of ErbB2 dimerization partner
- Potent signalling via ErbB2 heterodimers: Stable signalling complexes with a long half-life




### ERBB2(HER2) HETERODIMERS

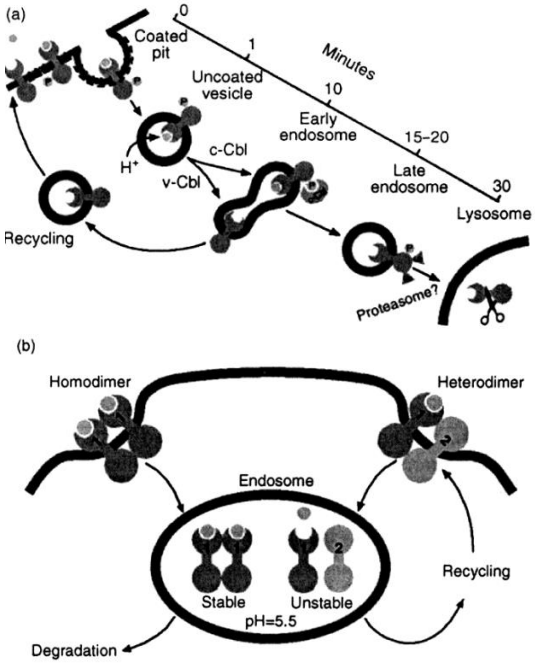
**ErbB2(HER2) heterodimers** elicit particularly **strong signals**

- More stable at the cell surface than complexes containing other EGFR family members
- HER2 doesn't bind EGF
- Although HER2 is not a receptor for EGF, it can decrease the rate of ligand dissociation from the EGFR
- HER2 heterodimers undergo endocytosis at a lower rate than do EGFR homodimers
- HER2-EGFR heterodimers are targeted for recycling, while EGFR homodimers are destined for degradation
-

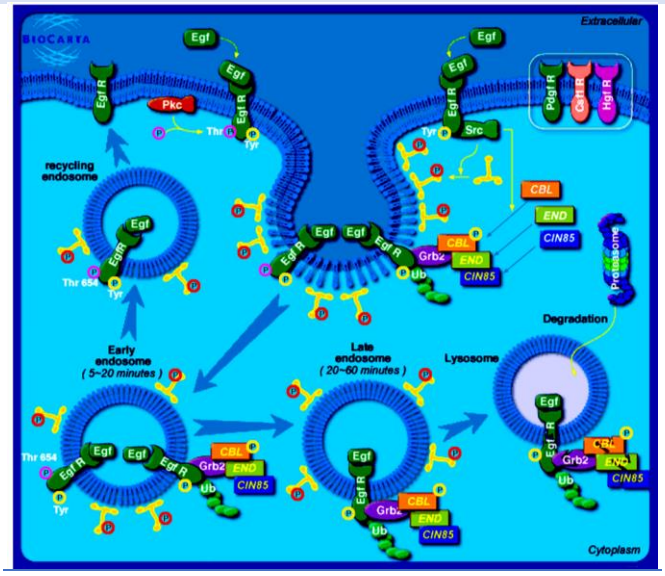
## ROLE OF C-CBL IN EGFR DEGRADATION

- c-Cbl binds to phosphorylated tyrosine 1045
- Ubiquitination of the receptor targets it for degradation in lysosomes
- In the absence of c-Cbl or when the c-Cbl binding site is mutated: receptor recycling, increased mitogenicity
- EGFR/HER2 heterodimers: unstable in the endosome, causing c-Cbl to dissociate from the receptor complex: recycling

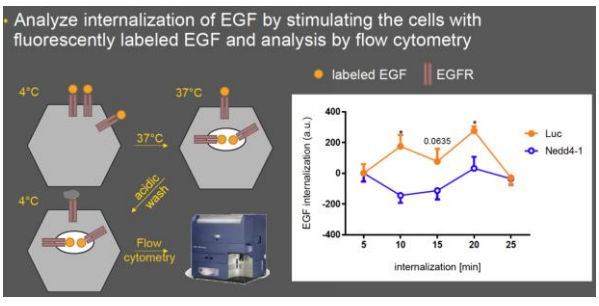
### C-CBL-MEDIATED RECEPTOR DEGRADATION OF EGF RECEPTOR HOMODIMERS, BUT NOT OF EGFR/HER2 HETERODIMERS



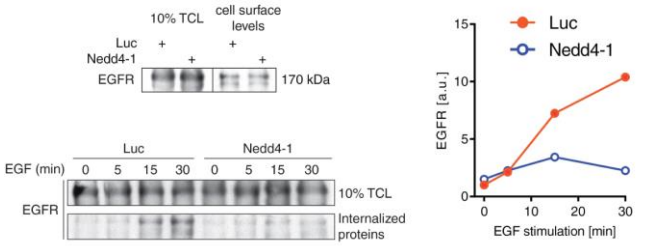

## EGF RECEPTOR INTERNALIZATION




### ANALYSIS OF EGF INTERNALIZATION



## ANALYSIS OF EGFR INTERNALIZATION




Culture of primary hepatocytes; biotinylation of surface proteins, incubation at 37°C and EGF treatment to allow internalization of the receptor; removal of surface biotin, pull-down with streptavidin beads and analysis by western blotting

## THE ROLE OF HER-2 IN CANCER

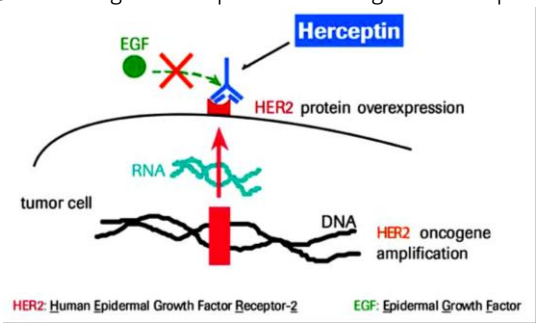
- Amplified and overexpressed in 30% of all mammary carcinomas *(Brustkrebs)* → Overexpression correlates with **poor clinical prognosis** (diagnostic marker)
- Overexpression in transgenic mice: Increased tumor malignancy, increased metastasis, increased resistance to chemotherapy, hormone-independent

## HER-2 AS A TARGET FOR CANCER THERAPY

- Inhibition by **antisense strategy**, **ribozymes**, **tyrosine kinase inhibitors** and recombinant, **humanized monoclonal antibody** (HERCEPTIN) → first approved recombinant protein for the treatment of cancer

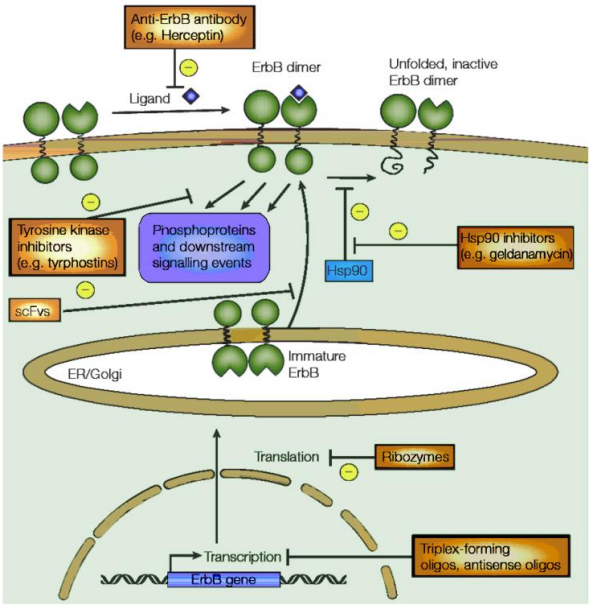
## USE OF HERCEPTIN FOR THE TREATMENT OF BREAST CANCER

- Herceptin is a recombinant, humanized monoclonal antibody
  - Blockage of receptor sites → signal interruption




- Used for the treatment of **advanced HER2-positive breast cancer** → But: Rapid development of resistance
- Side effects: Flu-like symptoms, nausea, rash, low white or red blood cell count. Most severe side effect: **cardiotoxicity** (toxicity of the heart muscle), in particular in combination with chemotherapy (doxorubicin)
- New strategy: Treatment of patients with **early stage HER2-positive breast cancer** as adjuvant therapy (not metastasized yet)
  - 50% reduction in relapse
  - Current issue: *Is this worthwhile considering the risk of developing heart disease?* (Yes)

## INHIBITION OF ERBB2 ACTION




## ERBITUX: MONOCLONAL ANTIBODY AGAINST EGFR

- **EGF receptor** is often **overexpressed in colon cancer** (Erbitux = Cetuximab)
- Erbitux = **monoclonal antibody against EGFR**
- Test whether they have a mutated Ras! If yes it won't work!
- Approved for the therapy of advanced colon and rectal carcinoma (with wild-type Ras), often together with chemotherapy, also approved for head and neck cancer
- Reasonable **success rate**, relatively **well tolerated**, but long-term studies still missing
- Side effect are not severe: Skin rash and inflammation - indicates that the patient responds to the drug

## IRESSA (= GEFITINIB) AND TARCEVA (ERLOTINIB)

- Inhibitors of the **EGFR tyrosine kinase**
- Used for the treatment of **locally advanced or metastatic non-small cell lung cancer** in patients who have previously received chemotherapy – in particular in patients with activating **EGFR mutations**
  - Clinical trials with Gefitinib showed efficacy in esophageal cancer
  - Erlotinib is also used for metastatic pancreatic cancer in combination with chemotherapy

# ANGIOGENESIS

## BLOOD VESSEL FORMATION

- Vasculogenesis: During **early development**
  - **De novo formation of vessels**
  - Can occur to a certain extent in the adult organism, e.g. during wound healing & carcinogenesis
- Angiogenesis: During **late development** & in the **adult organism**
  - Sprouting of new blood vessels **from pre-existing vessels**

## ANGIOGENESIS IN THE ADULT ORGANISM

- **Physiological angiogenesis**:
  - Menstrual cycle (uterus, ovary)
  - Hair cycling
  - Fat deposition
  - Muscle growth
- **Pathological angiogenesis**:
  - Wound healing
  - Cancer
  - Heart ischemia, Psoriasis, Diabetic retinopathy, Rheumatoid arthritis

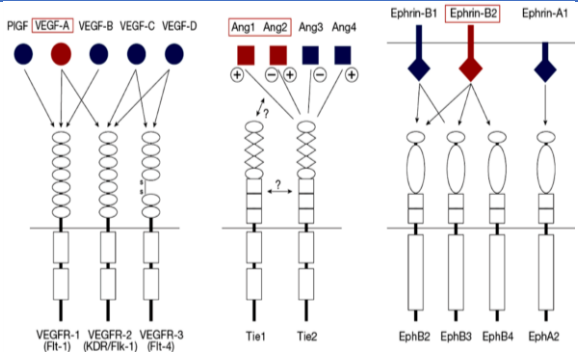
## EXCESSIVE AND INSUFFICIENT ANGIOGENESIS IN HUMAN DISEASE

In Disease often too much or too little angiogenesis
→ Treatment: Induce or inhibit angiogenesis


## DIFFERENT STEPS IN ANGIOGENESIS

1. **Angiogenic signal**: Inflammation, Hypoxia
2. **Degradation** of **extracellular matrix** (eg. by MMP)
3. **Migration** of **endothelial cells**
4. **Proliferation** of **endothelial cells**
5. **Contact** to the **extracellular matrix** (need to get a signal)
6. **Lumen** formation
7. **Stabilization** by association with **pericytes** and **smooth muscle cells**

# GROWTH FACTORS INVOLVED IN ANGIOGENESIS




*Which GF & GF receptors (GFR) are involved in angiogenesis?*

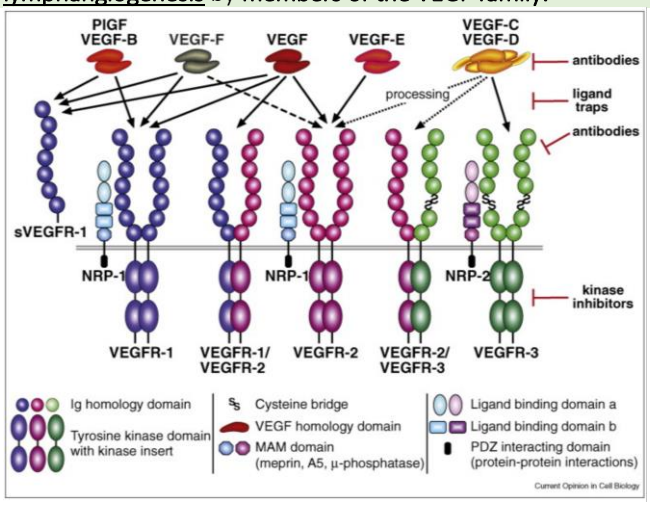
- VEGF-A → VEGFR-1, VEGFR-2
- Ang-1 and Ang-2 → Tie2
- Ephrin-B2 → EphB2, EphB3, EphB4

## VASCULAR ENDOTHELIAL GROWTH FACTORS

Big players that can **induce angiogenesis**

- **7 Ligands:**
  - VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E (produced by parapoxvirus Orf virus) and VEGF-F (in snake venom), Placenta growth factor (PLGF)
- **3 Receptors**
  - VEGFR1 and VEGFR2 are predominantly expressed on **blood vessel endothelial cells**
    VEGFR2 is also on lymphatic endothelial cells: activated by VEGF-A
  - VEGFR3 is predominantly expressed on **lymphatic endothelial cells**: Ligands VEGF-C and VEGF-D stimulate **lymphangiogenesis**

Regulation of vasculogenesis, angiogenesis and lymphangiogenesis by **members of the VEGF family:**




*(Are typical tyrosin kinases that form homo- and heterodimer depending on the ligand)*

- **VEGFR2 knockout**: Die in utero: No differentiation of endothelial cells, no blood vessel formation
- **VEGFR1 knockout**: Die between E8.5 and E9.5 due to an increase in the number of endothelial progenitors, leading to excessive proliferation of the vasculature *(negative regulator of VEGF-A effects?)*
- **VEGF-A heterozygous mice**: Die before birth from severe abnormalities in the cardiovascular system
- **VEGF-C knockout** mice show complete absence of lymphatic vessels
- **Overexpression of VEGF-C and VEGF-D** in transgenic mice **induces lymphangiogenesis**

## VEGF-A

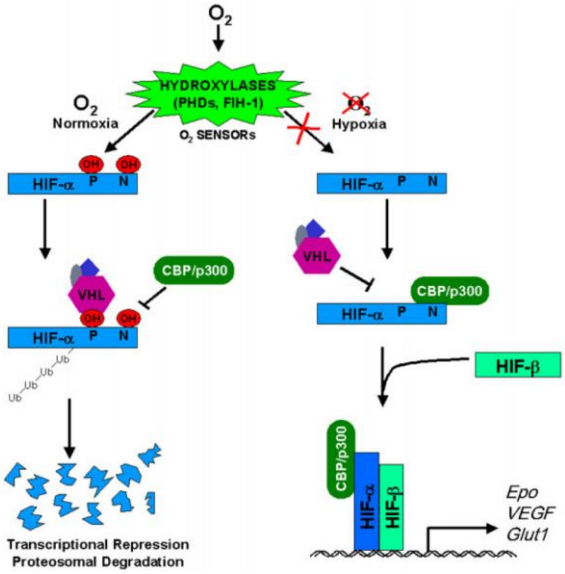
### BIOLOGICAL FUNCTIONS OF VEGF-A

- In vitro: Induces **migration** and **proliferation** of endothelial cells, **prevents apoptosis** of endothelial cells
- In vivo: Involved in various steps of **vasculogenesis** and **angiogenesis:**
  - **Induces sprouting** of capillaries
  - Increases **vascular permeability** (Blood can go in the interstitial fluid)
  - Induces **survival of blood vessels**
  - Can also **stimulate lymph-angiogenesis** via VEGFR2

### REGULATION OF VEGF-A PRODUCTION

- Most important stimulus: **Hypoxia**

→ **Hypoxia** activates **hypoxia-inducible transcription factor (HIF)**, which in turn activates VEGF gene expression

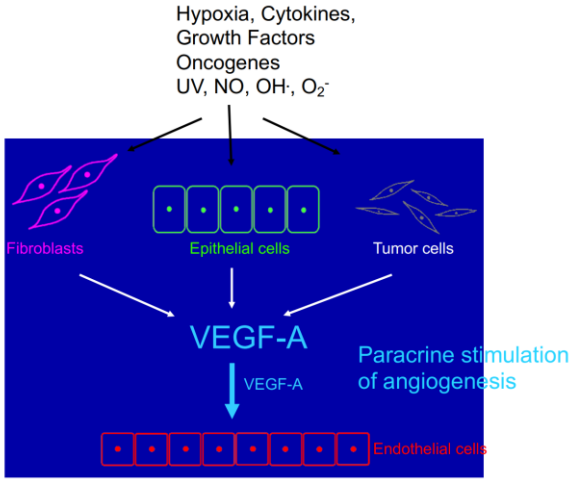



HIF-a is present in all of our cells

→ Under Normoxia, HIF-α is continuously degraded

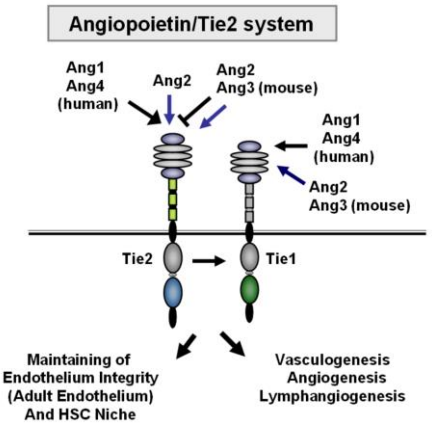
→ Under Hypoxia, HIF-α gets stabilized, associates with HIF-β and Dimer binds to promotor of target Genes *(VEGF, Glut1, Epo)*

- Other inducers:
  - Pro-inflammatory cytokines
  - Growth factors
  - Reactive oxygen species
  - UV
  - Oncogenes




## ANGIOPOIETINS

- Bind to **TIE-2 receptor** or to **TIE-1/TIE-2 heterodimers**
- **Angiopoietin-1** and -4 (human) are **agonists for TIE-2**
  - Ang-1 drives vasculogenic (immature → mature vessel)
- **Angiopoietin-2** and -3 (mouse) can be **antagonists or agonists** depending on the cell type and the growth conditions (possibly mediated via TIE-1)
  - Ang-2 destabilizes vessels (adult → unstable vessels)
- Angiopoietins are **NOT mitogenic for endothelial cells**, but **act in concert with VEGF**



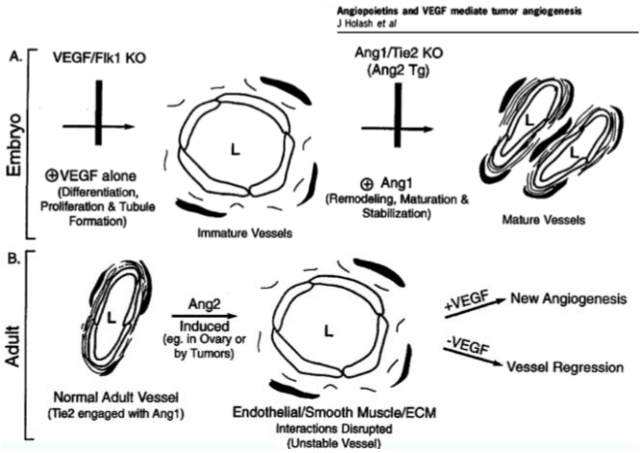

- TIE-2/Ang-1 knockout: Early stages of VEGF-dependent angiogenesis are normal, but **vessels are unstable**

  → Defect in the association of pericytes and smooth muscle cells (Ang-1/TIE-2 association is important for maintaining blood vessels)

### ANGIOPOIETIN-2

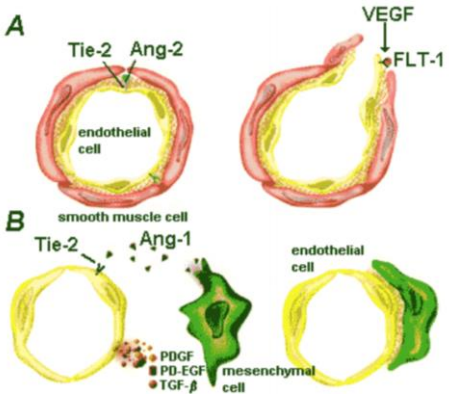
- Mostly antagonist of the Ang-1/TIE-2 interaction (Makes the vessels less stable)
- Ang-2 is expressed at sites of **vessel remodelling**:
  - Co-expression with VEGF induces **angiogenesis**
  - In the absence of VEGF it leads to **blood vessel regression** *(Rückbildung)*

### ROLE OF VEGF & ANGIOPOIETINS IN ANGIOGENESIS




Ang2: Cell detach from endothelial cells → vessels get more fragile (If one adds VEGF, angiogenesis gets induced)

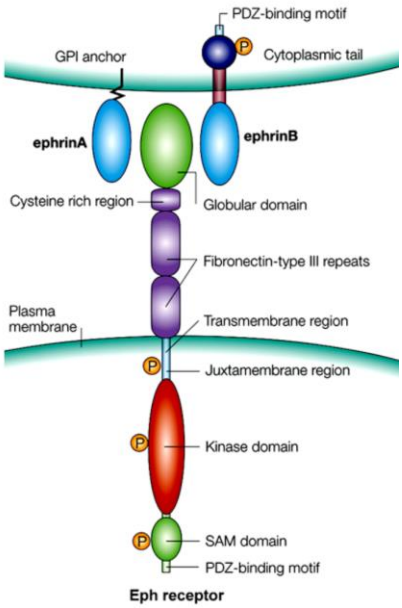
## INTERACTION BETWEEN ENDOTHELIAL CELLS AND PERI-ENDOTHELIAL CELLS VIA VEGF & ANGIOPOIETINS




**Paracrine loop**: Peri-endothelial cells (Pericytes) secrete Ang-1 which bind to the Tie-2 receptor of endothelial cells. Ang-1 leads to GF and chemokine secretion which leads to the recruitment of pericytes → vessel stabilisation

## EPHRINS AND EPH RECEPTORS

- **14 Eph receptors** and **8 ephrins** in mammals
- Ephrins are **transmembrane proteins**:
  - **Juxtacrine** mechanism of action
  - **Bidirectional signalling**
  - **Requires cell-cell contact**




- Eph receptors **dimerize** like other RTK
- Type B Ephrins: Have a **cytoplasmic domain** that becomes **tyrosine phosphorylated** upon receptor binding, no signalling molecules identified yet
- Type A Ephrins: Have **glycosyl-phosphatidyl inositol (GPI) anchor** - recruit adaptor proteins → able to signal

## BIOLOGICAL FUNCTIONS:

- Development of the nervous system, Vasculogenesis, Angiogenesis, and others

## EPHRINS REGULATE:

- **Migration, Cell-cell attachment, Cell-Matrix contacts**
- They **do NOT stimulate cell proliferation**
- Are expressed in the developing vasculature
- Knockout mice suggest a role of ephrins in vascular **assembly and differentiation of perivascular cells**

# TUMOR ANGIOGENESIS

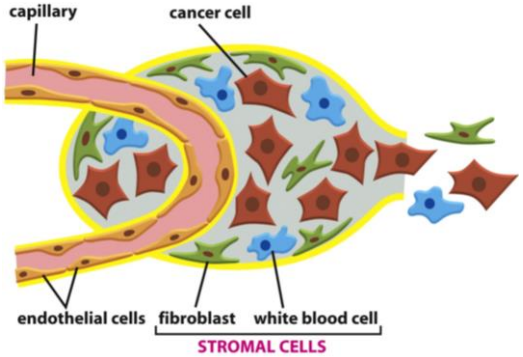
**1971**: *"All tumor growth is angiogenesis dependent"*

## ANGIOGENIC SWITCH

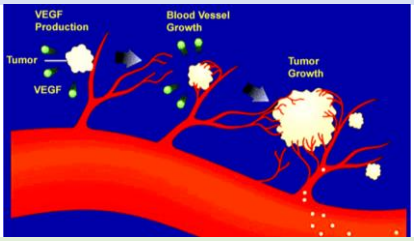
- Tumors can only grow up to a diameter of **0.2-0.4mm** when oxygen and nutrients are supplied via diffusion
- Further growth requires angiogenesis → angiogenic switch → Tumors induce angiogenesis which essential for tumor growth

## TUMOR-MICROENVIRONMENT

- Blood vessels are components of the tumor-microenvironment
- Tumor consists of **tumor cells** themselves and of **associated stromal cells** (required for tumor growth)
- Stromal cells:
  - Fibroblasts
  - Vascular and lymphatic endothelial cells (and associated pericytes and smooth muscle cells)
  - Different immune cells (macrophages, neutrophils, different T cells, B cells, mast cells etc.)

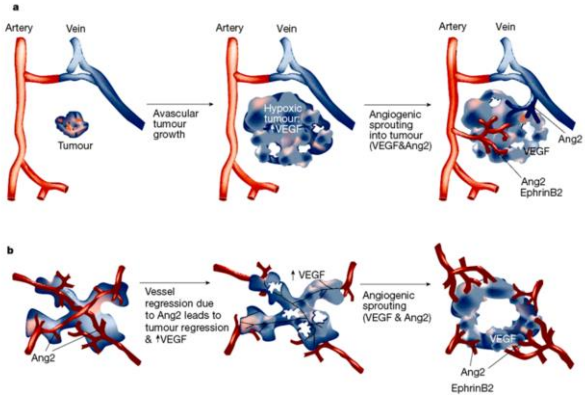



## THE ROLE OF VEGF IN TUMOR ANGIOGENESIS



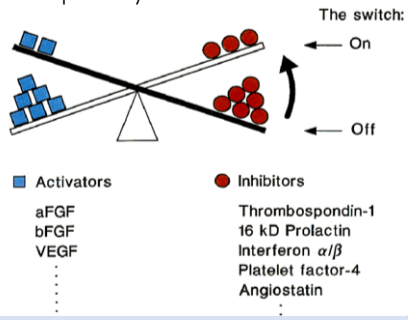

**Strongly upregulated** in almost all tumors by **hypoxia, inflammatory cytokines, oncogenes, ROS**

- Induces **survival, migration and proliferation of endothelial cells** and **sprouting of new vessels**
- **Recruits endothelial progenitor cells** from the bone marrow that contribute to the **formation of new vessels**
- Recruits **inflammatory cells** and also stimulates some tumor cells directly



## BALANCE HYPOTHESIS FOR ANGIOGENIC SWITCH

- Balance between activation/inhibition of angiogenesis
  - Essential for formation of more vessels
  - Inhibition due to regression of long vessels → more sprouting
- Balance regulation by angiogenesis activators (Ang2) and inhibitors (lack of VEGF)
- Some primary tumors secrete inhibitors themselves




## ANTI-ANGIOGENIC TUMOR THERAPY

- Observation: Frequent metastasis after removal of primary tumor
- Hypothesis: Primary tumor produces factors that **inhibit growth of metastasis** (insufficient for the inhibition of angiogenesis in the primary tumor, since the latter produces many pro-angiogenic factors)
- Evidence: Serum and urine of mice that suffer from lung cancer inhibit endothelial cell proliferation: Purification of **angiostatin**, a proteolytic product of plasminogen

## INHIBITORS OF ANGIOGENESIS

- **Synthetic inhibitors**: Metalloproteinase inhibitors, TNF-70 (homologue of antibiotic fumagillin), Thalidomide
- **Endogenous inhibitors**: Angiopoietin-2, Angiostatin, Endostatin, IL-12, Interferon-a
- **Biological antagonists**: VEGF antibodies (Avastin), VEGF antisense RNAs or Ribozymes, VEGF receptor tyrosine kinase inhibitors
- **Soluble VEGF receptors, soluble TIE-2**

### ENDOSTATIN:

- Naturally occurring 20 kD C-terminal **fragment from type XVIII collagen**
- **Inhibits angiogenesis** and tumor growth
- Advantage: Broad-spectrum inhibitor that blocks different angiogenic factors
- Disadvantage: Expensive production, dosing is critical and thus difficult (low concentrations are efficient, but high concentrations are not)
- Initial clinical trials failed, recent trials in China more promising: Now approved in China for the treatment of lung cancer in combination with chemotherapy

### AVASTIN (BEVACIZUMAB)

- Humanized monoclonal antibody against VEGF
- Approved in combination with chemotherapy (5- fluorouracil) for the treatment of metastatic colon and rectal cancer, non-small cell lung cancer and breast cancer
- Side effects: Bleeding, hypertension, holes in the colon, impaired wound healing, kidney damage
- Additional problem: Endothelial cells become resistant → they use other factors, e.g. FGF2 for angiogenesis

### VEGF antagonism **may increase tumor growth**

- Recruitment of bone marrow-derived pro-angiogenic cells (e.g. macrophages and vascular progenitor cells)
- Promotion of invasion and metastasis to provide access to normal tissue vasculature
- Other angiogenic factors (e.g. FGFs, ephrins or angiopoietins) are frequently upregulated
- New strategy: **Combined blockade of VEGF and FGF**

### SUTENT (SUNITINIB)

- Low molecular weight **tyrosine kinase inhibitor**
- Inhibits VEGFR1-3, PDGF receptors, KIT
- **Inhibits angiogenesis, reduces vessel stabilization** by pericytes and **inhibits (some) tumor cells directly**
- Side effects: gastrointestinal problems, cardiac problems, anorexia, skin discoloration, mucositis, hypertension, fatigue, bleedings
- Approved for the treatment of advanced gastrointestinal stromal cancer, for metastatic kidney cancer, and for pancreatic neuroendocrine cancer

### NEXAVAR (SORAFENIB)

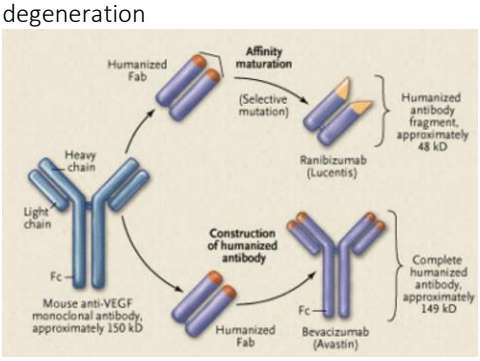
- Low molecular weight **tyrosine kinase inhibitor**
- Inhibits VEGFR2, PDGFRb and Raf kinase
- Inhibits angiogenesis and (some) tumor cells directly
- Approved for the treatment of advanced kidney cancer, non-resectable hepatocellular carcinoma and metastatic thyroid cancer

### MACUGEN

- Pegylated (polyethylene bound) **anti-VEGF aptamer**
- Direct injection into the eye
- Prevents excessive angiogenesis and vascular leakage in the eye
- Approved for the treatment of the wet form of macula degeneration

### RANIBIZUMAB (LUCENTIS)

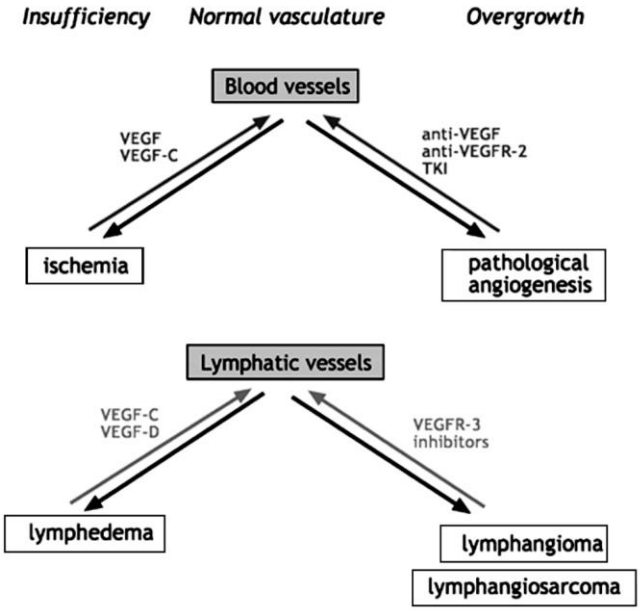
- Humanized antibody FAB fragment
- Affinity maturated (six amino acid changes compared to wild-type protein): Used for the treatment of macular degeneration



## THERAPEUTIC ANGIOGENESIS

- Use of **growth factors** or **gene transfer of growth factors** to **promote development of collateral blood vessels**
- New approach for the treatment of coronary artery and peripheral vascular disease
- Animal experiments and clinical trials with VEGF: Arterial VEGF gene transfer, intramuscular gene therapy (limb muscle, myocardium): First successful results
  - Arterial gene transfer of VEGF promotes angiogenesis in the ischemic limb
  - Healing of ischemic leg ulcers after VEGF gene therapy → Treatment of a non-healing wound with an angiogenesis-promoting growth factor → In-growth of granulation tissue in the lesion was observed

## PRO- AND ANTI-ANGIOGENIC THERAPY

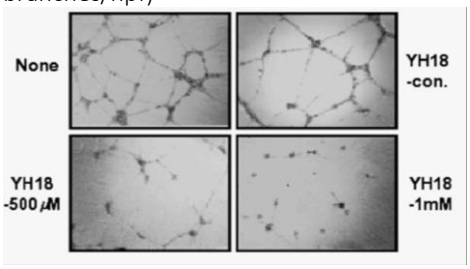



## ANGIOGENESIS ASSAYS

- In **vitro** tube formation assay on Matrigel
- Aortic ring assay
- In **vivo** Matrigel plug assay
- Chorioallantoic membrane assay
- Rabbit/mouse cornea assay

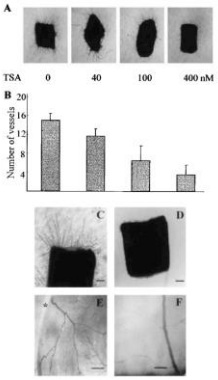
## IN VITRO TUBE FORMATION ON MATRIGEL

- Coat wells with **Matrigel** (mixture of laminin, collagen IV, nidogen and proteoglycans, resembling basement membrane)
- Seed **endothelial cells** on Matrigel surface
- Add **angiogenesis factor and/or inhibitor**
- Endothelial cells form capillary tubes within several hours
- Count number of capillary tubes (number of tube branches/hpf)




## AORTIC RING ASSAY

- Remove **thoracic aorta** of rats and cut into **1 mm rings**
- Embed rings in **collagen** polymerized in cylindrical **agarose wells**
- Add culture medium plus test substance
- Examine by phase contrast microscopy after 7-9 days




Increasing amounts of an angiogenesis inhibitor

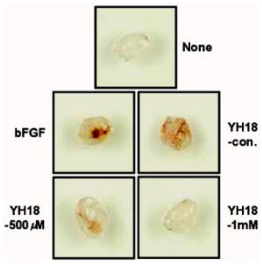



C, E: + VEGF

D, F: + VEGF, + inhibitor

## IN VIVO MATRIGEL PLUG ASSAY

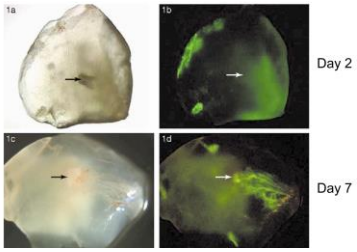
- Mix Matrigel and the potential angiogenic factor and/or inhibitor
- **Inject** cold liquid Matrigel mixture subcutaneously into **mice**
- Matrigel solidifies and **permits penetration** by host cells and the **formation of new blood vessels**
- **Count blood vessels** after several days




## SPONGE - MATRIGEL ASSAY

*In vivo Matrigel assay using a sponge implant with FGF2*

- Matrigel without test substance is first injected into a mouse
- Sponge or tissue fragment is then inserted into the plug
- Monitor new vessels, e.g. by injection of FITC-dextran




## CHORIOALLANTOIC MEMBRANE (CAM) ASSAY

- Incubate chicken eggs in a humidified chamber
- At day 7-9 of development: open a window in the egg shell
- Place tissue or organ grafts or membranes with test substance directly onto CAM
- Seal window and re-incubate egg
- Recover grafts and score for vascularization

## RABBIT/MOUSE CORNEA ASSAY

- Makes use of the fact that the **cornea is avascular**
- Make a pocket in the cornea and introduce test substance in a sponge or slow-release material
- Monitor vascularization with a stereo- microscope or by use of fluorochrome-labeled high molecular weight dextran

## LYMPHATIC VESSELS

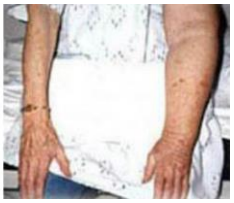
- **Collect the extravasated bloodless fluid** from tissues and **transfers it**, as lymph, via the collecting lymphatic vessels and thoracic duct back into venous circulation
- Serve an **immune function** by **transporting white blood cells** and **antigen-presenting cells to lymphoid organs**

**Characteristics of lymphatic vessels:**

- Lined by an **endothelium**
- Surrounded by **smooth muscle cells**
- **Discontinuous** or **fenestrated** basement membrane
- Lack of tight inter-endothelial junctions
- **Permeable to interstitial fluid and cells**
- Low flow and low pressure system
- Less complex network than blood vessels with fewer sprouts

### LYMPHEDEMA

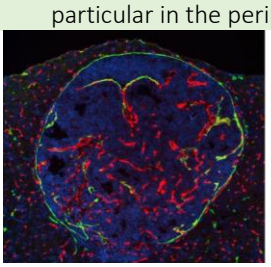
- Results from **impaired lymphatic drainage** (Cannot transport the interstitial fluid away)
- *Congenital lymphedema*: Caused by mutations in the VEGFR-3 gene, resulting in reduced tyrosine kinase activity

Right: Brestcancer patient that has removed lymphknotes → Lymphedema (lymphatic vessels are not functioning anymore)

## LYMPHANGIOGENESIS IN CANCER

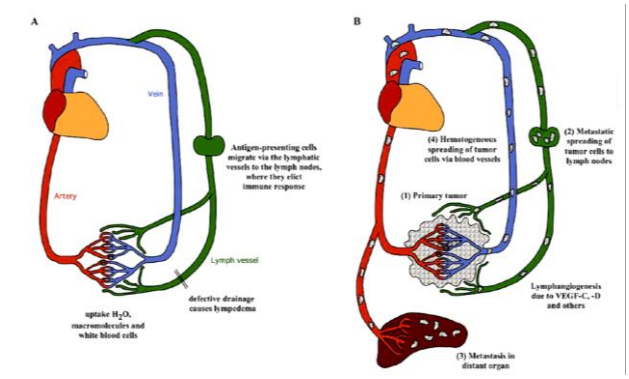
- Many tumors contain **functional lymphatic vessels**, in particular in the periphery of the tumors



Red: Blood vessels (CD31)
Green: lymphatic vessels (LYVE-1)

- Tumors **spread via lymphatic vessels**
- **VEGF-C overexpression** in mouse tumors **increases the rate of metastasis**
- **Treatment: Inhibition of VEGF-C, VEGF-D or VEGFR3** can be used to **suppress tumor formation and metastasis** in experimental mouse models

### RELATIONSHIP BETWEEN BLOOD VASCULAR AND LYMPHATIC SYSTEM IN NORMAL HEALTH AND IN CANCER



# FIBROBLAST GROWTH FACTORS (FGFS)

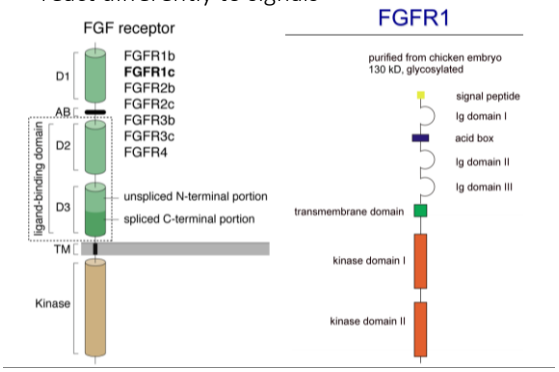
- Identified as **mitogens for fibroblasts**
- FGF Family has **22 FGFs** → Some have overlapping functions
- They regulate **proliferation, migration, differentiation and/or survival**
- FGFs are **defined through**
  - Their **sequence homology** and **conserved gene structure**
  - Most of them **bind to heparin**
  - Binding to:
    - FGFR1-4 (except FGF11-FGF14)
    - Heparan sulfate proteoglycans (except FGF 19, 21 and 23 and FGF11-FGF14)
    - FGF-BP (FGF binding protein: shown for some FGFs)
    - Other transmembrane / extracellular proteins
- FGFs are purified from **bovine pituitary gland**
  - aFGF = FGF1
  - bFGF = FGF2

## IDENTIFICATION OF FGFS:

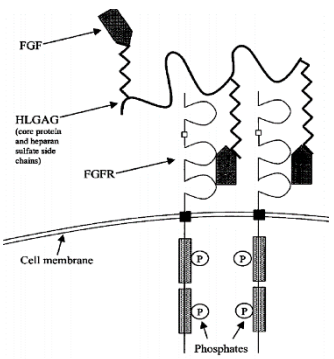
- Purification: 1,2,7,8,9
- Oncogenes: 3,4,5
- Target of oncogenes: 15
- PCR, in silico: 6,10,13,14,16-23

## FGFR

- Different types of FGFR result from **alternative splicing** and **polyadenylation**
  - **Differential splicing** in **extracellular domain** of FGFR1 generates **three receptor variants**
  - This results in **different ligand binding specificities** (e.g. FGFR1-IIIc and FGFR1-IIIb have different ligand binding specificities)
  - Splicing in a cell type-depending manner → different cells have different FGFRs and therefore react differently to signals




- **Heparan sulfate proteoglycans regulate binding** of FGFs to their receptors




→ **HLGAG** contains many heparan sulfate side chains
→ Side chains bind to **FGF**
→ FGF bind then to the Ig 3 domain of FGFR

FGFRs are monomers that bind FGF and come together through Heparin
→ Transphosphorylation

# FGF AND FGFR MUTATIONS/KO

## PHENOTYPES OF FGF KNOCKOUT MICE

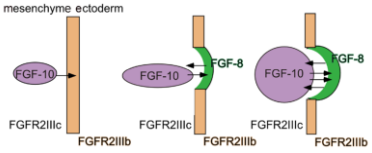
- There are **many phenotypes of FGF knockout mice**, some more severe than others → redundancy in subfamily

| FGF | Survival | phenotype |
|---|---|---|
| 1 | viable | no phenotype detected |
| 2 | viable | mild cardiovascular, wound repair, skeletal, neuronal |
| 3 | viable | mild inner ear, skeletal (tail) |
| 4 | lethal, E 4-5 | inner cell mass proliferation |
| 5 | viable | long hair, neuronal |
| 6 | viable | muscle regeneration |
| 7 | viable | hair follicle, ureteric bud growth |
| 8 | lethal, E 7 | gastrulation defect, CNS, limb development |
| 9 | lethal, P 0 | lung mesenchyme, XY sex reversal |
| 10 | lethal, P 0 | multiple organs including limb, lung, thymus, pituitary |
| 12 | viable | neuromuscular |
| 14 | viable | neurological |
| 15 | lethal, E 15.5 – P21 | heart abnormalities, impaired bile acid synthesis |
| 16 | viable | heart abnormalities |
| 18 | viable | Cerebellar development |

- *FGF 4,8,10, 15 KO are lethal*
- *FGF 3, 4 KO have strong phenotype*

FGF5 KO → mice have <u>long hair</u>
  - Negative regulator of hair growth

FGF10 KO → Mice have <u>no limbs</u> and <u>no FGF8</u>
  - Same result as without FGF2IIIb
  - Beads with FGF10 induce FGF9 expression and limb bud development → double paracrine mechanism




## PHENOTYPES OF FGFR KNOCKOUT MICE

FGFR1 → <u>die</u> during gastrulation
FGFR2 → <u>die</u> after implantation
FGFR3 → <u>bone deformation</u> and extension <u>inner ear defect</u>
FGFR4 → <u>liver</u> phenotype

<u>Consequences of FGFR1/2 loss in Keratinocytes:</u>

- Progressive **hair loss** → loss of hair follicle stem cells
- **Epidermal thickening** and **progressive inflammation** due to a defect in the epidermal barrier → resembles atopic dermatitis
  - Impaired barrier function
  - Severe skin dryness and infection
  - Similar inflammatory infiltrate; low numbers of neutrophils, High serum IgG and IgE levels
  - Epidermal thickening
  - Keratinocyte hyperproliferation
- Enhanced **transepidermal water loss**
  - Reduced expression of tight junction components which are under direct control of FGFR signaling

## EXCURSES: THE EPIDERMAL BARRIER

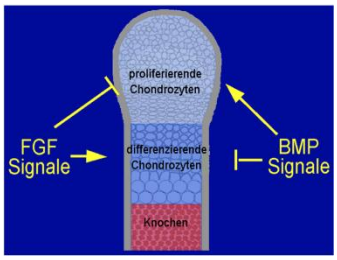
- Protects from **water loss, invasion of allergens, irritants and bacteria**
- Defects in barrier cause **inflammatory skin disease**
- Barrier mainly formed by cornified envelope → Cross-linked proteins and lipids in outermost epidermal layer
- Second component of barrier: Tight junctions
- 20-30% of patients with Atopic Dermatitis have a mutation in a filaggrin gene (component of cornified envelope)
- Is <u>not</u> **affected by loss of FGFR1/2**!

## MUTATIONS AFFECTING SKELETAL DEVELOPMENT

There are many possible mutations in FGFR1, 2 and 3, that are found in humans and lead to skeletal defects in embryos

### MUTATIONS IN FGFR3

- ***Achondroplasia*** → **Activating** mutations in **FGFR3** gene
  - FGFR3 is a **negative regulator of bone growth**
  - **Hyperactivation** leads to **less bone growth**
  - Mutations are generally transmitted by the father; Mutations stimulate proliferation of stem cells in the testis
- **FGFs & BMPs** have **antagonistc effects** on **bone growth**:




**FGF** inhibits proliferation of chondrocytes and activates their differentiation

**BMP** inhibits differentiation, but activates proliferation

### FGF23

- FGF23 is an **endocrine acting** growth factor
  - Is present in circulation & is produced by the bone
  - Regulates **vitamin D metabolism** and **phosphate homeostasis** in the kidney
  - Binding and receptor activation of FGF23 requires the transmembrane protein Klotho (co-receptor) → Klotho KO mice also show premature aging
- KO mice are **smaller** & show signs of **premature aging**
  - Show bone abnormalities as a consequence of high calcium and phosphate levels in the serum

## ENDOCRINE FGFS

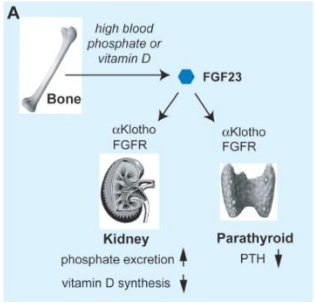
- FGF19 (FGF15 in mice), FGF21 and FGF23 **show reduced heparin binding** → act as **endocrine hormones**
- Receptor activation requires a **co-receptor protein (Klotho or Klotho β)**

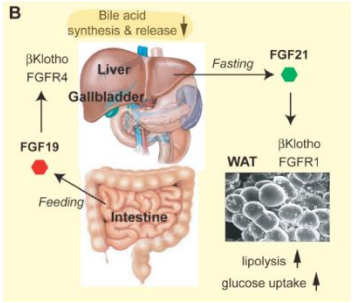
### FGF19

- Regulates **bile acid synthesis and gall bladder filling**
  - Produced in intestine
  - Stimulates hepatocytes of the liver
  - Is also involved in **energy and lipid homeostasis**

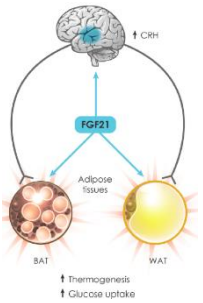
### FGF21

- Regulates the **response to fasting** by signaling to adipose tissue and brain
  - Produced in the liver
  - Involved in energy, lipid and glucose homeostasis
  - Important metabolism regulator

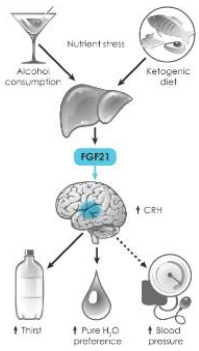



- Effects in the **adipose tissue:**
  - FGF21 induces corticotropin-releading hormone (CRH) in the hypothalamus, resulting in sympathetic outflow to the adipose tissue FGF21 also directly acts on adipose tissue
  - Net effect: Energy expenditure and weight loss in obese animals

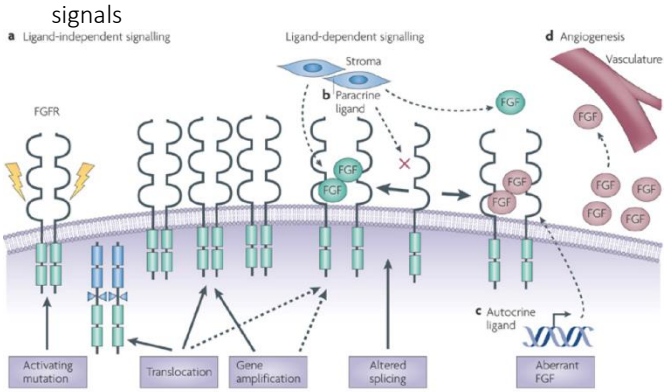



- FGF21 is induced in the liver by **dehydrating** metabolic stresses, including alcohol and ketogenic diet, and circulates to the nervous system, where it **stimulates thirst and drinking of pure water**
- FGF21 also stimulates **blood pressure**, possibly as a mechanism to prevent dehydration-associated hypotension




## PATHOGENIC FGF SIGNALLING IN CANCER

- **FGFRs** are **often mutated in cancer** → Mutations have different effects
  - Often: Proliferation and survival is enhanced
- **Altered splicing** is common in cancers → cells produce receptor that they are not supposed to → react to signals




Mechanisms of altering FGF signalling:

- Altered splicing
- Gene amplification
- Translocation
- Aberrant FGF
- Activating mutation in FGFR

## KGF (FGF7)

KGF (FGF7) is a **paracrine acting growth factor**

**Protective effect of FGF7:**

- On cells of bladder: Prevents ulcerative hemorrhagic cystitis after cyclophosphamide injection (→ chemotherapy)
- On alveolar cells: Prevents lung injury in various model systems (e.g. hyperoxia, acid)
- On cells of gastrointestinal tract: Pretreatment of mice with recombinant FGF7 reduces injury induced by radiation and/or chemotherapy → increased mucosal thickness, increased crypt cell survival

- Protective function for epithelial cells (less stress susceptibility)
- Enables tissue regeneration
- Often given before chemotherapy start

**Excursus: Chemo/radiotherapy-Induced *Oral Mucositis***

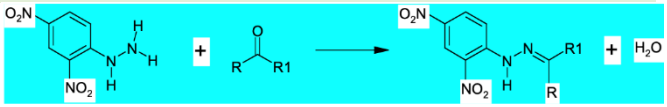
- Caused by toxic effects of chemo/radiotherapy on rapidly dividing cells of the normal mucosa
- The degree of injury is directly related to the type of chemo/radiotherapy regimen, schedule and dose
- Therapies with the highest incidence of mucosal toxicity (70%–90% rate of severe mucositis) include radio-/chemotherapy and bone marrow transplantation for the treatment of hematological malignancies, radio-/chemotherapy for the treatment of head and neck cancer
- It is associated with: pain (often requiring opioid analgesia), difficulty/inability to swallow (parenteral feeding), difficulty/inability to speak, gastrointestinal bleeding, NEGATIVE IMPACT on patients' quality of life

**FGF7** is used for treatment of chemo and radiotherapy-induced mucositis

FGF also **protects keratinocytes** from **toxicity of UV radiation** and **treatment with ROS** → Reduced oxidative protein damage → Reduced cell damage, reduced apoptosis

- <u>OxyBlot™ Protein Oxidation Detection</u>
  - Investigates protein oxidation through the detection of carbonyl groups on proteins that result from several types of oxidative damage
  - The carbonyl groups are chemically converted to their dinitrophenylhydrazone (DNP) derivatives
  - The protein samples are subjected to western blot analysis and the proteins bands on the membrane are detected by chemiluminescence

FGF7 **reduces protein oxidation** in response to ROS treatment



2,4-Dinitrophenylhydrazine — 2,4-Dinitrophenylhydrazone

FGF7 is **cytoprotective** for human hair follicle keratinocytes in organ culture:

- Chemotherapy → hairloss due to permanent oxidative damage → FGF could potentially be used for prevention

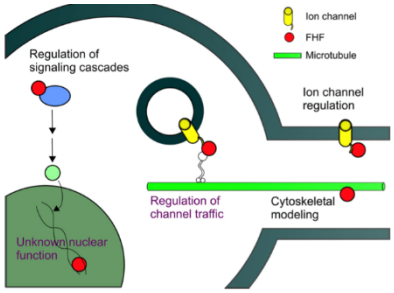
## FIBROBLAST GROWTH FACTOR HOMOLOGOUS FACTORS

- FGF11, FGF12, FGF13, and FGF14
  - Different variants produced by alternative splicing
  - No signal peptide and therefore not secreted
  - Cannot bind to FGF receptors due to structural changes

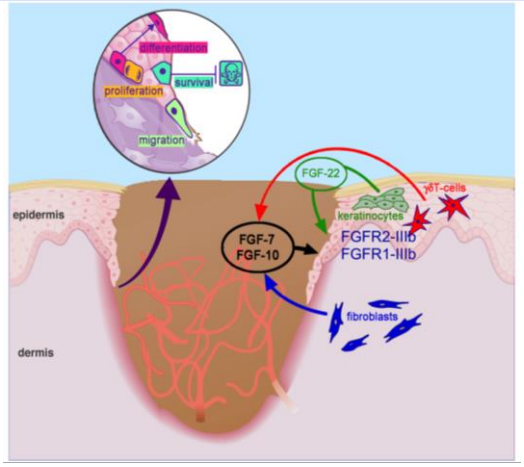
## FGHF BIND TO VOLTAGE-GATED ION CHANNELS

- High affinity binding to voltage-gaged $Na^+$- and $Ca^{2+}$-channels: Control of ion channels (in most cases delayed channel inactivation)
- Mutations in FGF11 associated with early onset epilepsy, ataxia, sensory deficits
- FGF14 SNPs (single nucleotide polymorphisms) are associated with major depression, schizophrenia, substance abuse and Alzheimer`s disease

Most likely also other binding partners and non-characterized nuclear functions

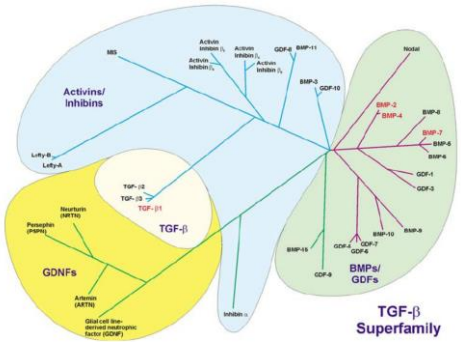



## ADDITIONAL: EXPRESSION OF FGF7, FGF10 AND FGF22 IN WOUNDS



# TRANSFORMING GROWTH FACTOR β (TGF-β)

1970: **Retrovirus-transformed fibroblasts** produce factors that induce malignant properties in normal fibroblasts: sarcoma growth factor, a mixture of TGF-α and TGF-β
  - They just have the same name because they were purified together → different functions!
- TGF-β is the prototype of a **large family of growth and differentiation factors**, which includes **TGF-β 1-3, activins, inhibins, bone morphogenetic proteins (BMPs), nodal, growth and differentiation factors (GDFs)** etc.



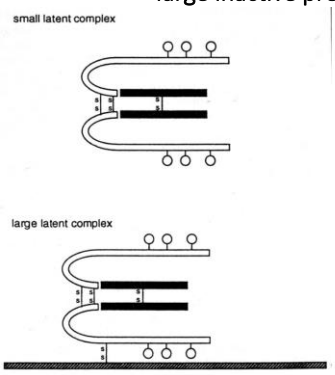

## TGF-B

### STRUCTURE

- **3 isoforms** in mammals (TGF-β 1-3)
- 25kD homodimers: Biologically active form
- 112 aminoacids, 9 **cystein residues**
  - 1 cysteine for **dimerization**
  - The other for the formation of **intramolecular disulfide bridges**

### PRECURSOR

- Produced as a **large inactive precursor**:



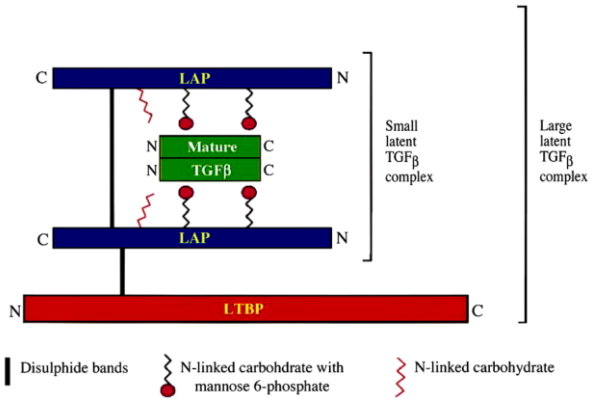

Black: Active TGF-β

White: LAP with sugar residues (LAP = latency associated peptide)

Grey: Latent TGF-β binding protein (LTBP)

Latent TGF-β has a longer half-life compared to the mature form: Storage form

  - The TGF-β homodimer is bound to **LAP** (= Latency associated peptide) → **small latent complex**
  - LAP can be bound to **LTBP** (= Latent TGF-β binding protein) → **large latent complex** → Latent TGF-β has a longer half-life compared to the mature form → **storage form**




- Homodimers are biologically active

### AKTIVATION OF TGF-B

*In vitro*:
- **Low pH**
- Proteinases
- Mechanical tension

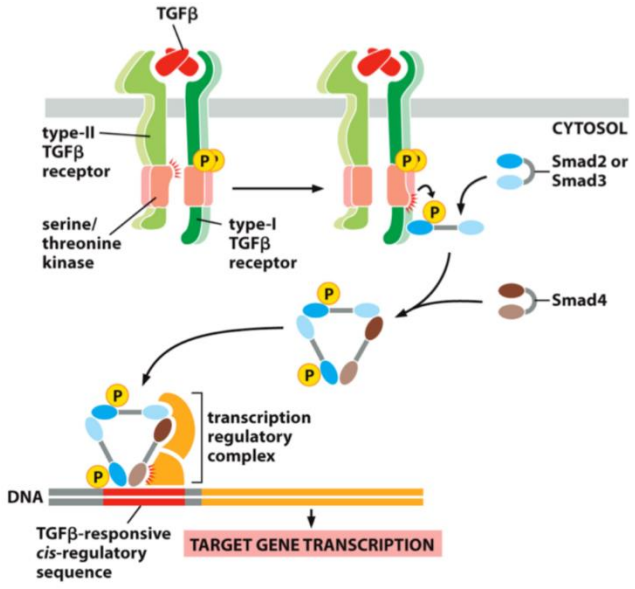
*In vivo*:
- Locally low pH
- Proteinases
- Thrombospondin (Induces conformational change)
- Reactive oxygen species (ROS)
- Binding of LAP to mannose-6-phosphate receptors or integrin avβ6 (Induces conformational change)
- Mechanical tension

## TGF-β RECEPTORS

**Type I and Type II receptors:**
- **Transmembranal protein** (One transmembrane domain)
  - Extracellular domain: Small, N-glycosylated
  - Intracellular domain: **Serin-threonine kinase** domain
- Dimeric ligands which combine type I with type II receptors → **Receptor dimerization**
  - Each type is present as a homodimer
  - Together they form heterotetramer upon activation
  - Type 2 R phosphorylates the type 1 receptor which then transduces the signal

**Type III receptor (β-glycan):**
- **Trans-membrane protein** with short cytoplasmic part **without enzymatic activity** and large extracellular domain with many carbohydrate residues
  - **Does not transduce a signal**, but **promotes binding** of the ligand to the signaling receptors

### TGF-β Receptor Signaling



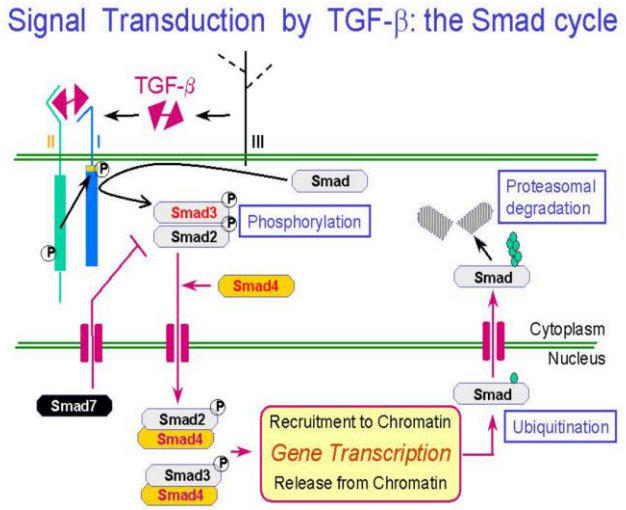

### STEPS OF ACTIVATION

1. Type 3 receptor binds dimeric ligand (extracellularly) and guides it to the type 2 receptor
2. Type 2 R binds ligand and forms tetramer with type 1 R
3. Type 2 R phosphorylates type 1 R
4. Type 1 R transduces the signal

## SMAD PROTEINS

- Smads bind **"smad binding element" (SBE)** on DNA with other TFs
- **Phosphorylated Type II receptor**: Docking site for Smads
- Receptor-Smads:
  - **Smad2** & **Smad3** bind to **TGF-β** and **Activin receptors**
  - **Smad1, Smad5, Smad8** bind to **BMP receptors**
- **Smad4** binds to receptor Smads → common nuclear binding partner/ DNA-binding site (transcriptional)
- **Smad6** and **Smad7** are **inhibitory** Smads
- Have positive feedback loop by promoting its own gene transcription
- Reverse Pathway by:
  - Reverse smads (see below)
  - Phosphatases (remove phosphorylation)
  - Ubiquitination of smads → degradation by proteasome

*You could test this pathway with immunofluorescence & WB (location of smad)*




## BIOLOGICAL FUNCTIONS OF TGF-Bs

**Inhibits proliferation** of most cell types, including epithelial cells

- Mutations in the TGF-β signaling pathway are frequently found in epithelial cancers
  - Smad4 Mutations in pancreatic carcinoma (DPC)
  - Smad2 mutations in colon carcinomas

**Exception:** Proliferation of fibroblasts is stimulated by TGF-B

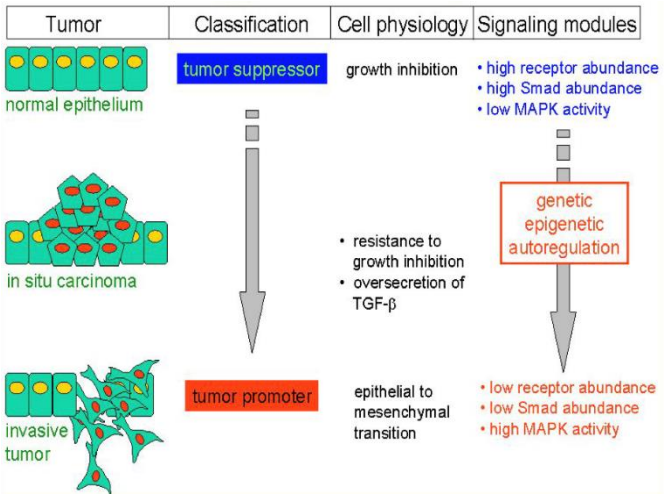
**Anti-inflammatory properties**:

- TGF-β1 knockout mice suffer from severe **inflammation**
- TGF-β1 stimulates **differentiation of regulatory T cells: Inhibits autoimmune processes**
- Possible use of TGF-β1 (viral delivery of TGF-β producing T cells) for the treatment of multiple sclerosis (good results in animal models)

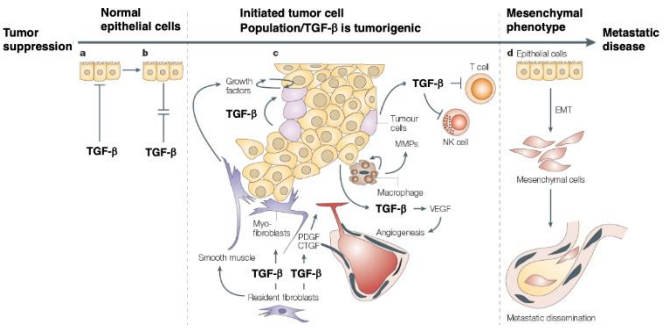
Induces **migration, proliferation of fibroblasts and matrix production** by these cells

- **Promotes differentiation of fibroblasts into myofibroblasts**, in particular in combination with mechanical tension: Cells acquire contractile properties
  - Often over-expressed in fibrotic disease where functional epithelial tissue is replaced by non-functional connective tissue, e.g. lung fibrosis, liver cirrhosis, hypertrophic scars and keloids etc.
  - Inhibition of TGF-β action for the **treatment of fibrotic disease**

## TGF-β IN CANCER




TGF-β has **complex roles in cancer**!




- Temporally restricted
- Main cancer drivers induced by TGF-β
  - GFs
  - Immune cell suppression
  - VEGF → angiogenesis