Developmental Tissue Reconstruction

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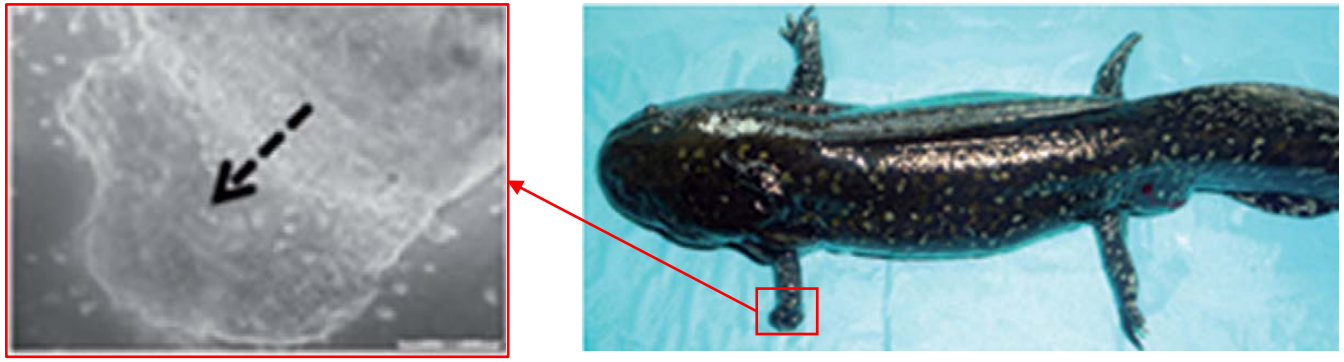
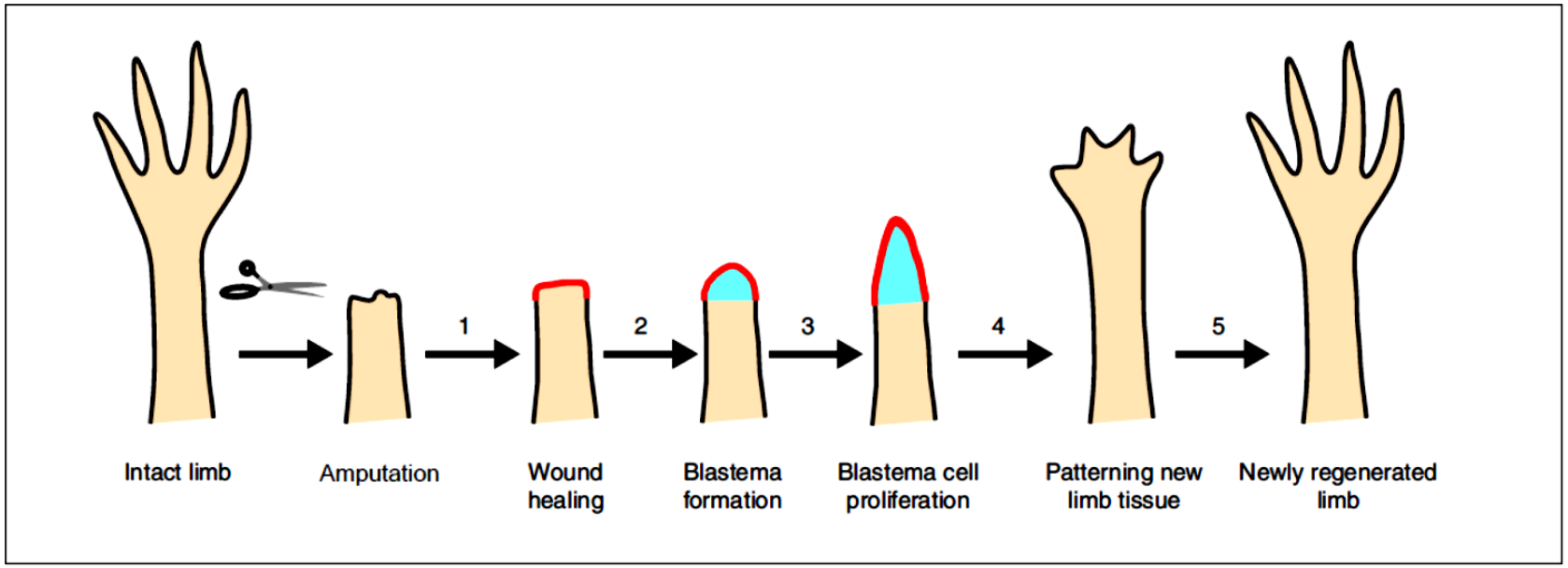
Current Life Science Topics

1. Fundamentals in Basic Biochemistry & Cell Biology
2. Introduction to Tissue Engineering & Regenerative Medicine
3. Developmental Tissue Reconstruction
4. Wound Healing & Regeneration
5. Natural Tissue Composition & Cell-ECM Interaction
6. Stem Cells & Cell-Based Therapy
7. Biomaterials
8. *Mid-Term Exam*
9. Mechano-transduction & Bioreactors
10. Discussions on Tissue Reconstruction
11. Regulation & Ethics
12. AI in Current Life Science
13. Machine Learning & Github
14. Deep Neural Network
15. Convolutional Neural Network
16. *Final Exam*

Study Materials

Principles of Regenerative Medicine by A. Atala *et al.*, Chapter 3




1
2
3
4
5
Intact limb
Amputation
Wound healing
Blastema formation
Blastema cell proliferation
Patterning new limb tissue
Newly regenerated limb

Adult Urodele Amphibians Regenerate the Limb after Amputation

Two major phases of limb regeneration

- Blastema formation
- Blastema redevelopment (Growth & differentiation)

Pattern formation spans both phases

Anuran amphibians (Xenopus), reptiles, birds, and mammals cannot form blastema after amputation

Chemical approaches to induce blastema formation in human appendages might be possible if we understand why some animals (such as urodele amphibians) are able to forma a regeneration-competent blastema after amputation while others are not

Blastema Formation

1. Hemostasis & Re-epithelialization
2. Histolysis & dedifferentiation
3. Blastema cell migration & accumulation

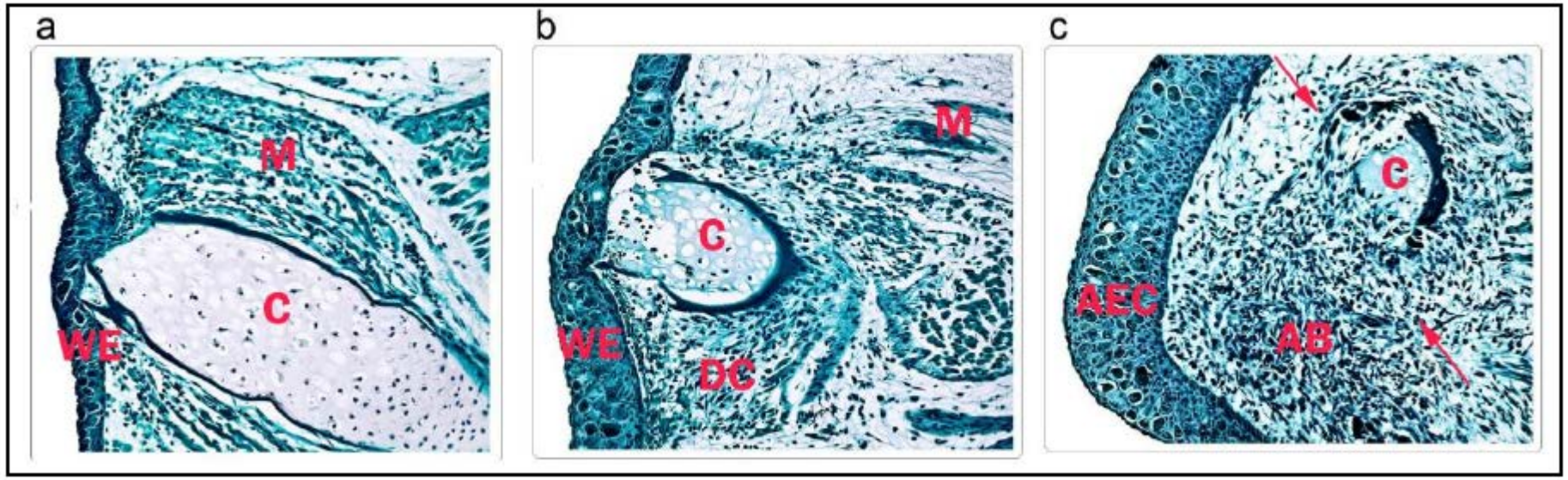
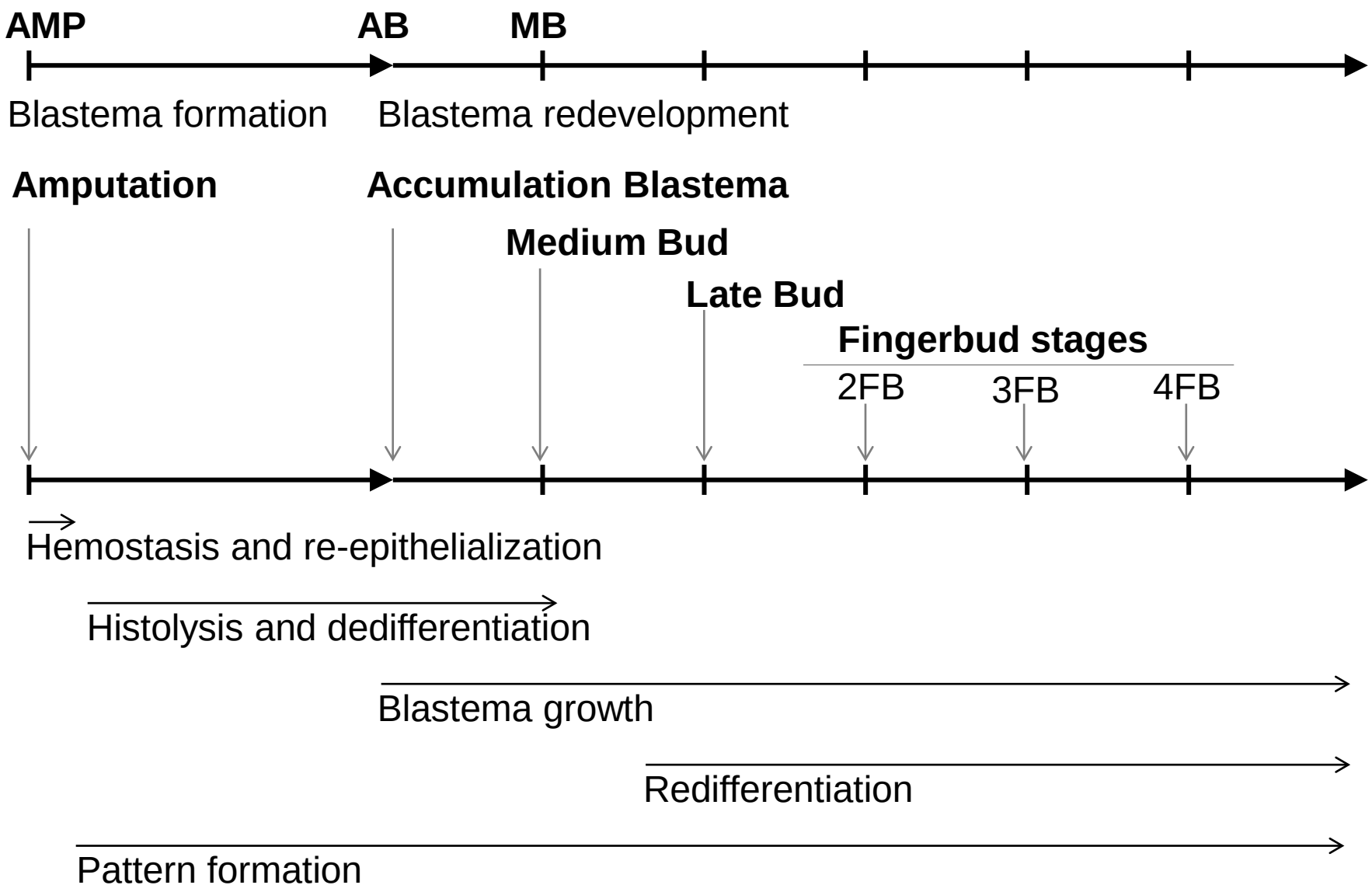


Diagram of Phases and Stages of Regeneration after Amputation of a Urodele Limb

EunAh Lee @ KHU



Skeletal Pattern

Stylopod – humerus, femur
Zeugopod – radius-ulna, tibia-fibula
Autopod – Carpal-fingers, Tarsal-toe

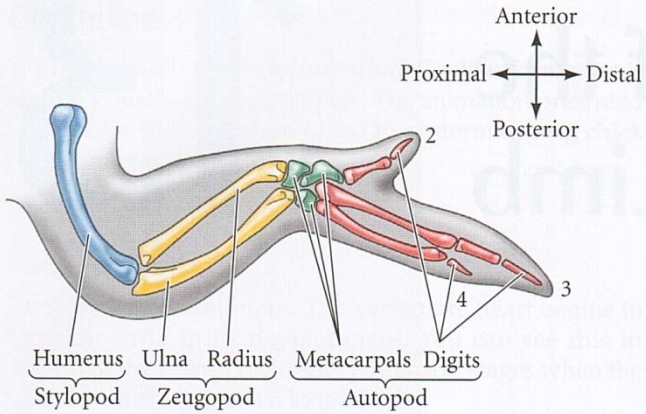


FIGURE 13.1 Skeletal pattern of the chick wing. According to convention, the digits are numbered 2, 3, and 4. The cartilage condensations forming the digits appear similar to those forming digits 2, 3, and 4 of mice and humans; however, new evidence (discussed later in the chapter) suggests that the correct designation may be 1, 2, and 3.

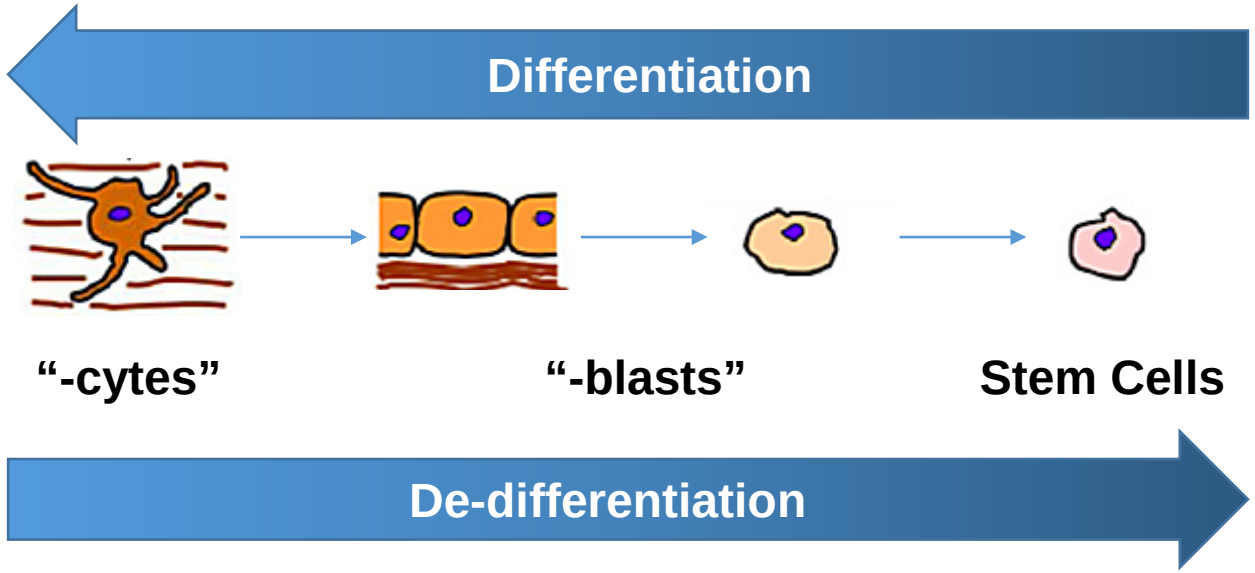
4 Dimension system

- 3 main axis
  - Proximo-distal axis (close-far/shoulder-finger): FGF family
  - Anterior-posterior axis (thumb-pinkie): SHH
    each hand develops as a mirror image of the other
  - Dorso-ventral axis (palms-knuckles): Wnt7a

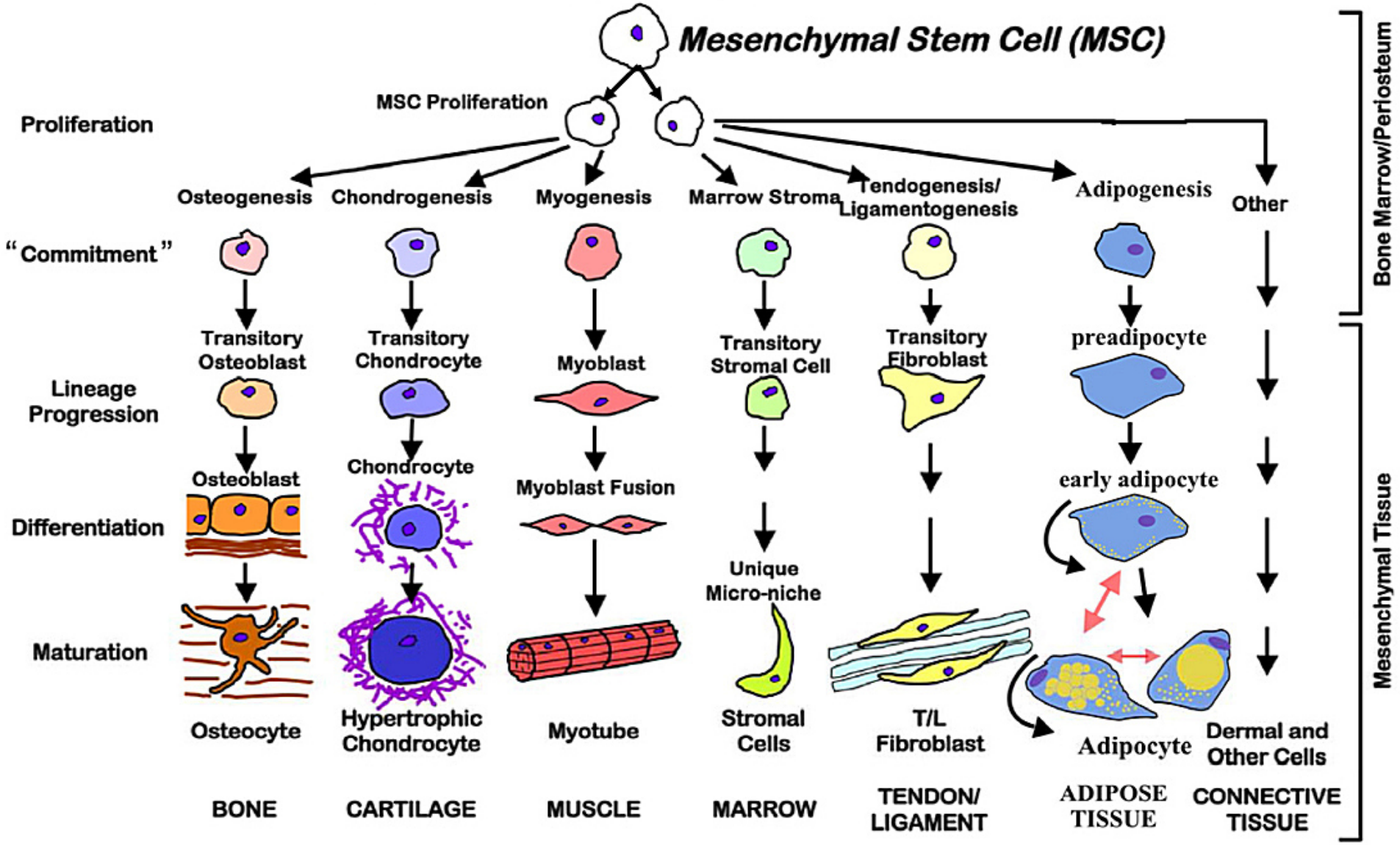
- Time

Blastema Formation

- Reverse developmental process
- Realized partly by cell dedifferentiation in local tissues of amputation plane
- Muscle stem cell partly contributes



THE MESENGENIC PROCESS



Growth & Redifferentiation of Blastema ≒ Embryonic Limb Bud Development

One major exception:

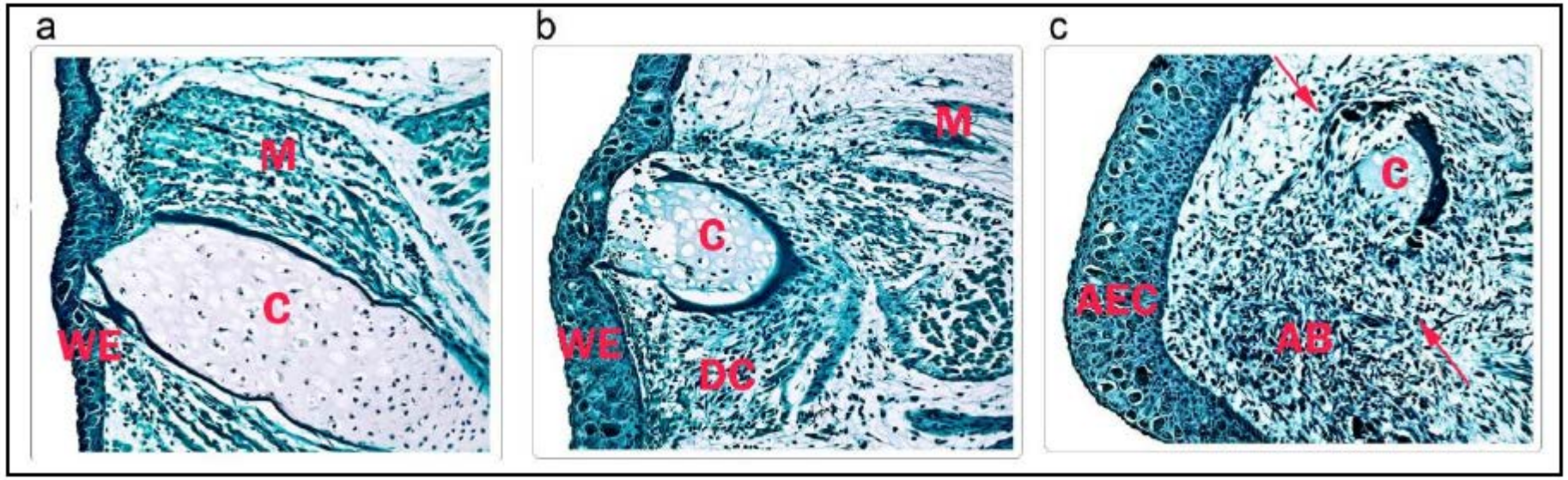
- Embryonic limb bud solely depends on signals from AER (apical ectodermal ridge)
- Blastema cell proliferation is dependent on signals from AEC (apical ectodermal cap: counterpart of AER) and the regenerating nerves

Musculoskeletal and dermal tissues from blastema redifferentiate in continuity w/ their parent tissues

Blood vessels and nerve regenerate by extension from the cut ends of the pre-existing blood vessels & axons.

Blastema Formation

1. Hemostasis & Re-epithelialization
2. Histolysis & dedifferentiation
3. Blastema cell migration & accumulation



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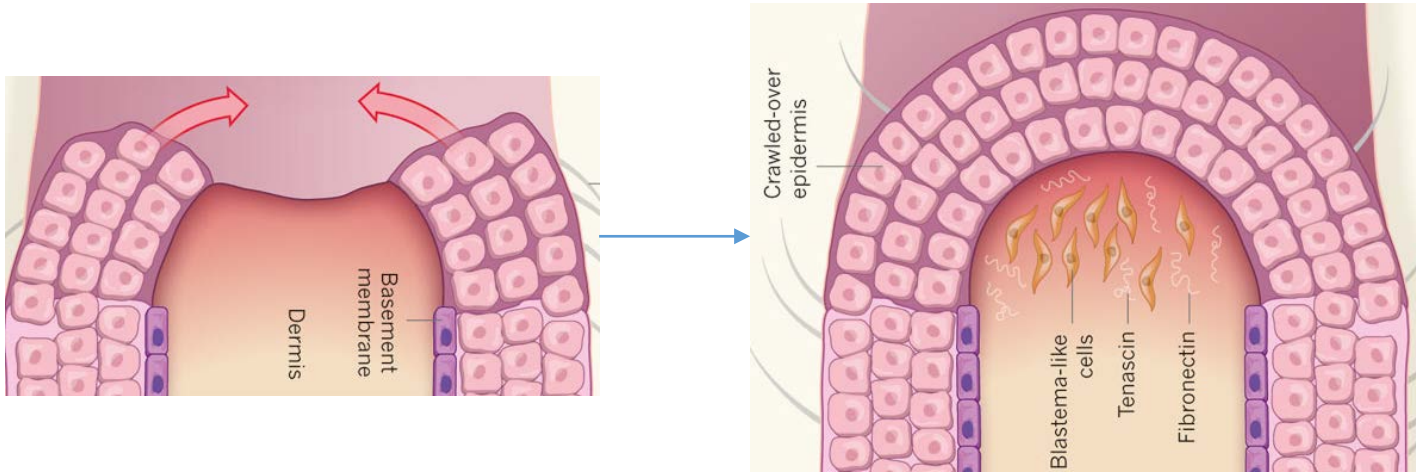


Hemostasis & re-epithelialization

Amputation

Vasoconstriction

Thrombin-catalyzed fibrin clot

An epithelium covers the wound surface (2~3 cells thick)

- **Basal epidermal cells at the cut edge migrate as a sheet**
- **Fibrin clot contains significant amounts of fibronectin, which the epithelial sheet uses as a substrate for migration**

Wound epidermis thickens to form the AEC

(Apical Epithelial Cap)

Basal cells & gland cells of wound epidermis/AEC have secretory function
-> essential for blastema formation
(evidenced by extensive ER & Golgi network)

Early Essential Signals for Limb Regeneration

Early wound epidermis has an important function in generating early signals for limb generation

Generate ionic current across the wound epidermis

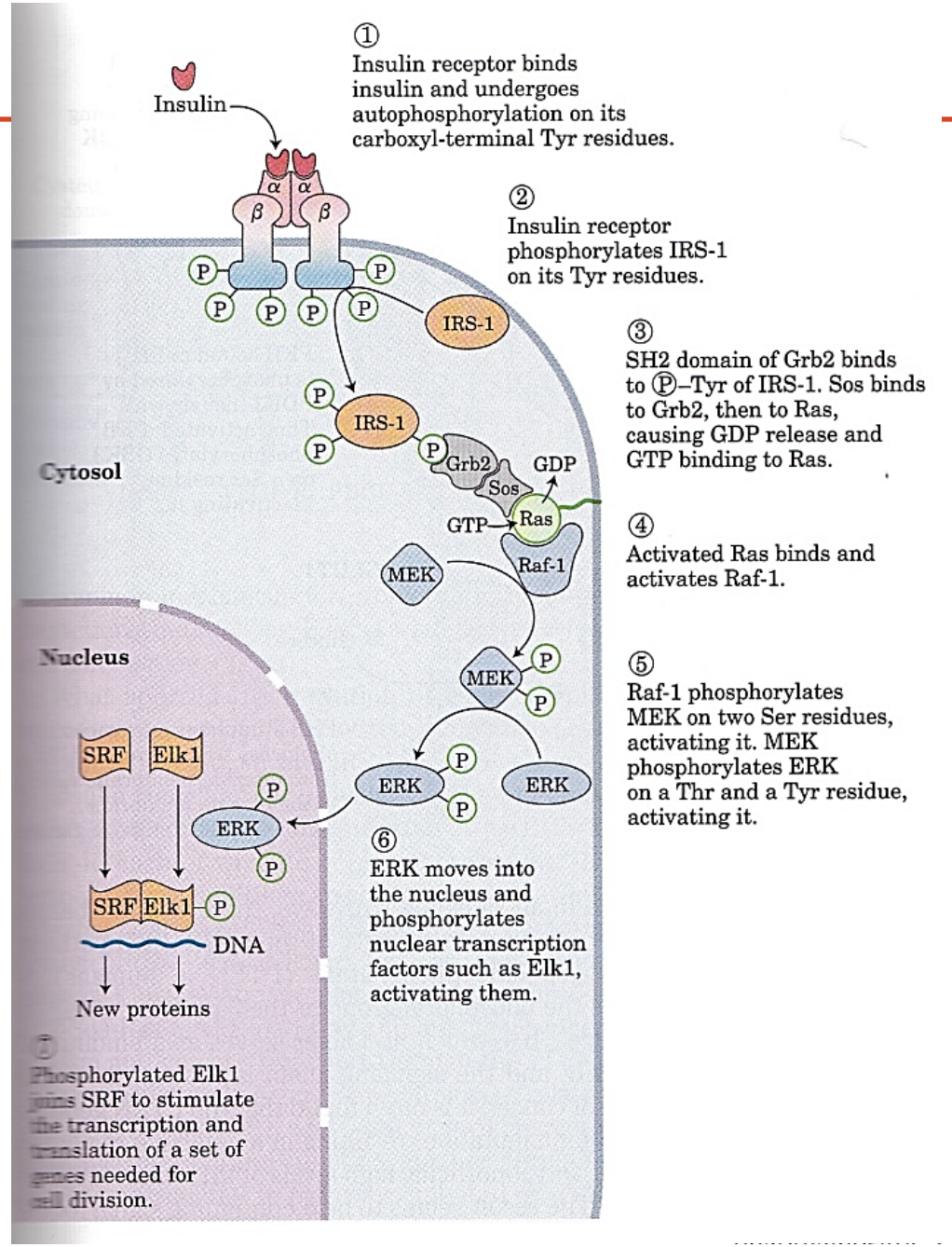
- Na2+ influx via sodium pump (amputated newt limb)
- H+ efflux via plasma membrane ATPase (amputated tail of Xenopus tadples)

- Drug-induced inhibition of either Na2+ or H+ movements during the first 24hr after amputation results in failure of blastema formation Jenkins et al., 1996; Adams et al, 2007)

Two other early regeneration signals

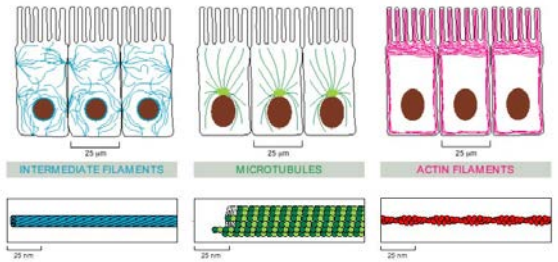
- NO & IP3 (Inositol-1,4,5-triphosphate)
- NO (synthesized by NOS1)
  - Strongly up-regulated in the wound epidermis of amputated limbs
  - Produced by macrophages and neutrophils (-> bacteriocidal effect)
  - NO -> activate proteases (important effectors of histolysis)
- IP3
  - PIP2 -> DAG + IP3
  - IP3 synthase: up-regulated during blastema formation
  - IP3 -> cytosolic Ca2+ ^^ -> PKC activation
  - Beryllium inhibition of IP3 formation prevents blastema formation (Tsonis et al., 1991)

Intracellular Signal Transduction



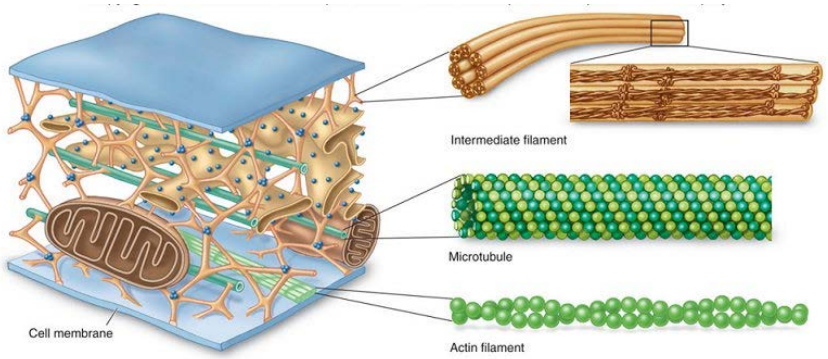
1. Ligand-receptor binding
2. Activation of receptor (phosphorylation)
3. Activation of adaptor proteins
4. Activation of MAPK
5. Activation of transcription factors
6. Induction of gene expression

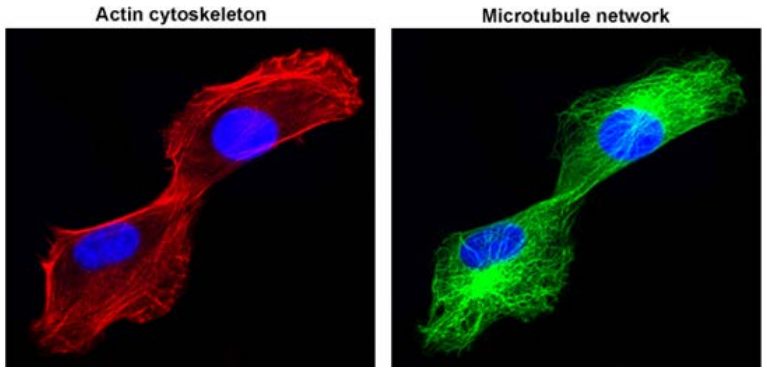
Cytoskeleton

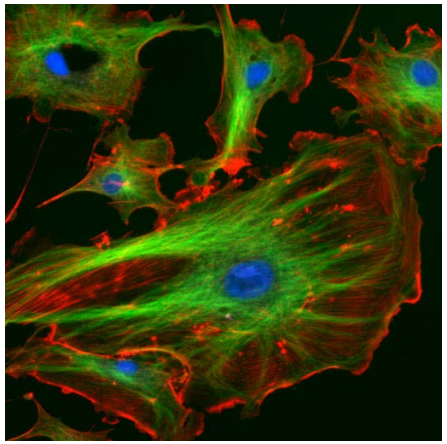


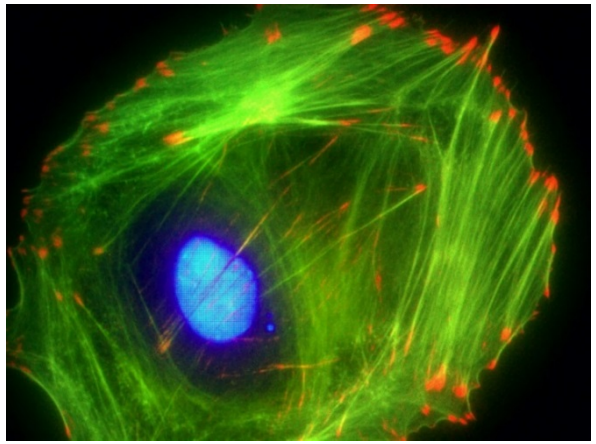
Cytoskeletal Functions

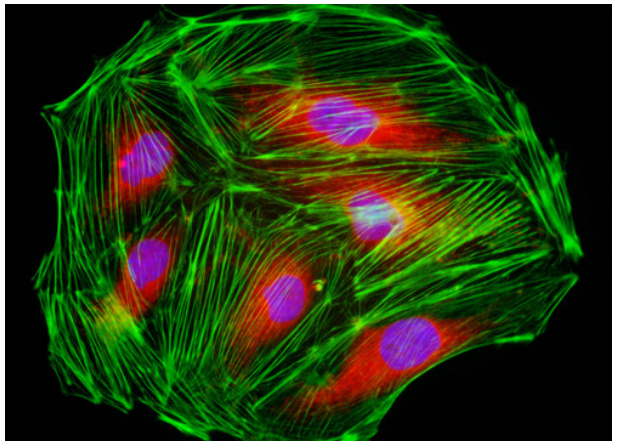
1. Structure and support
2. Intracellular transport
3. Contractility and motility
4. Spatial organization



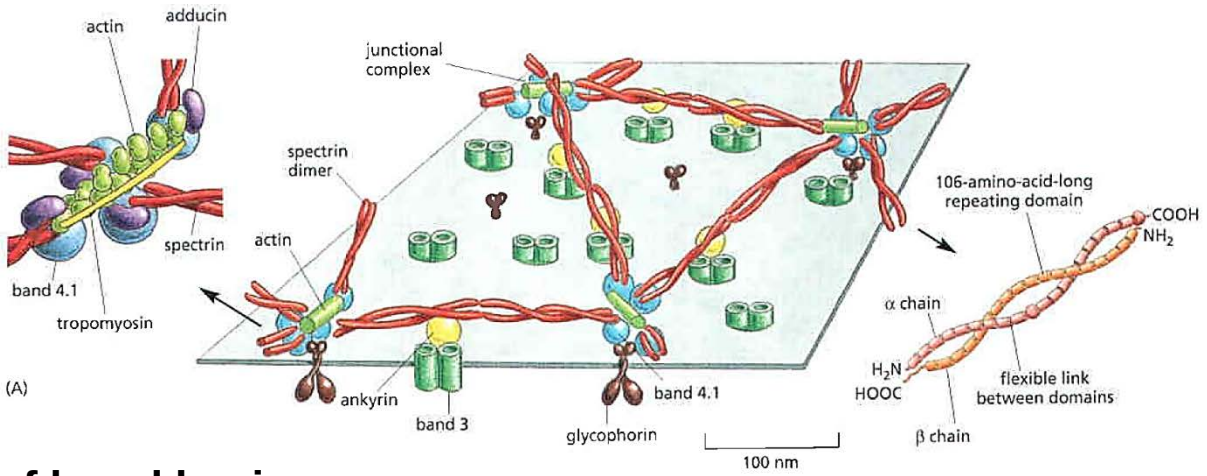




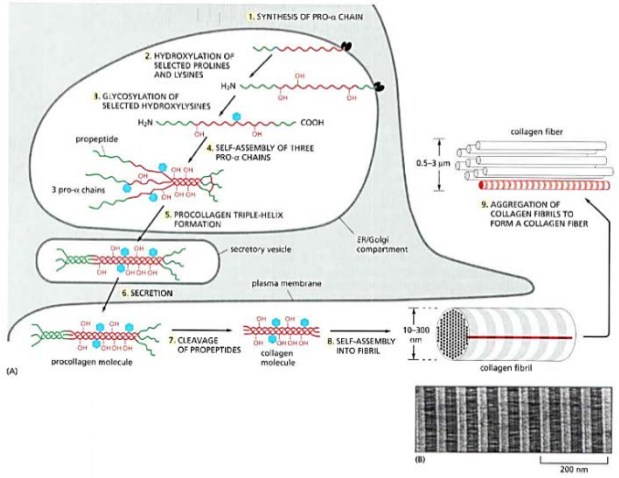


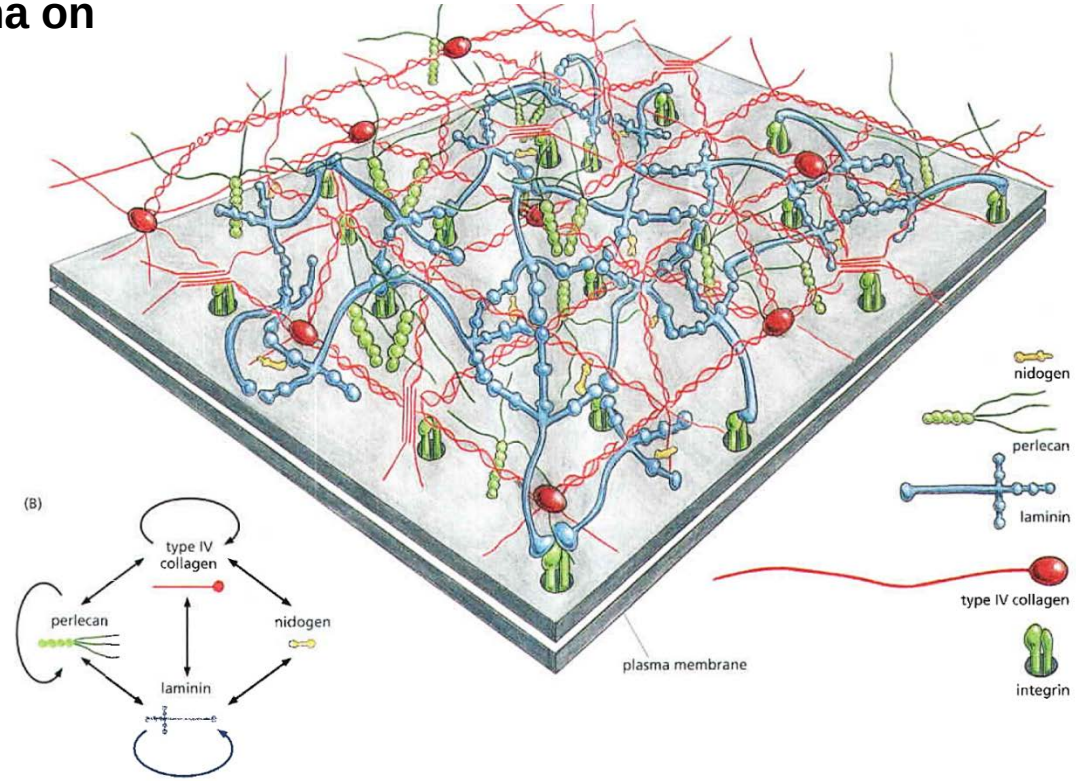


Cytoskeleton on the cytosolic side



Molecular structure of basal lamina on the extracellular side





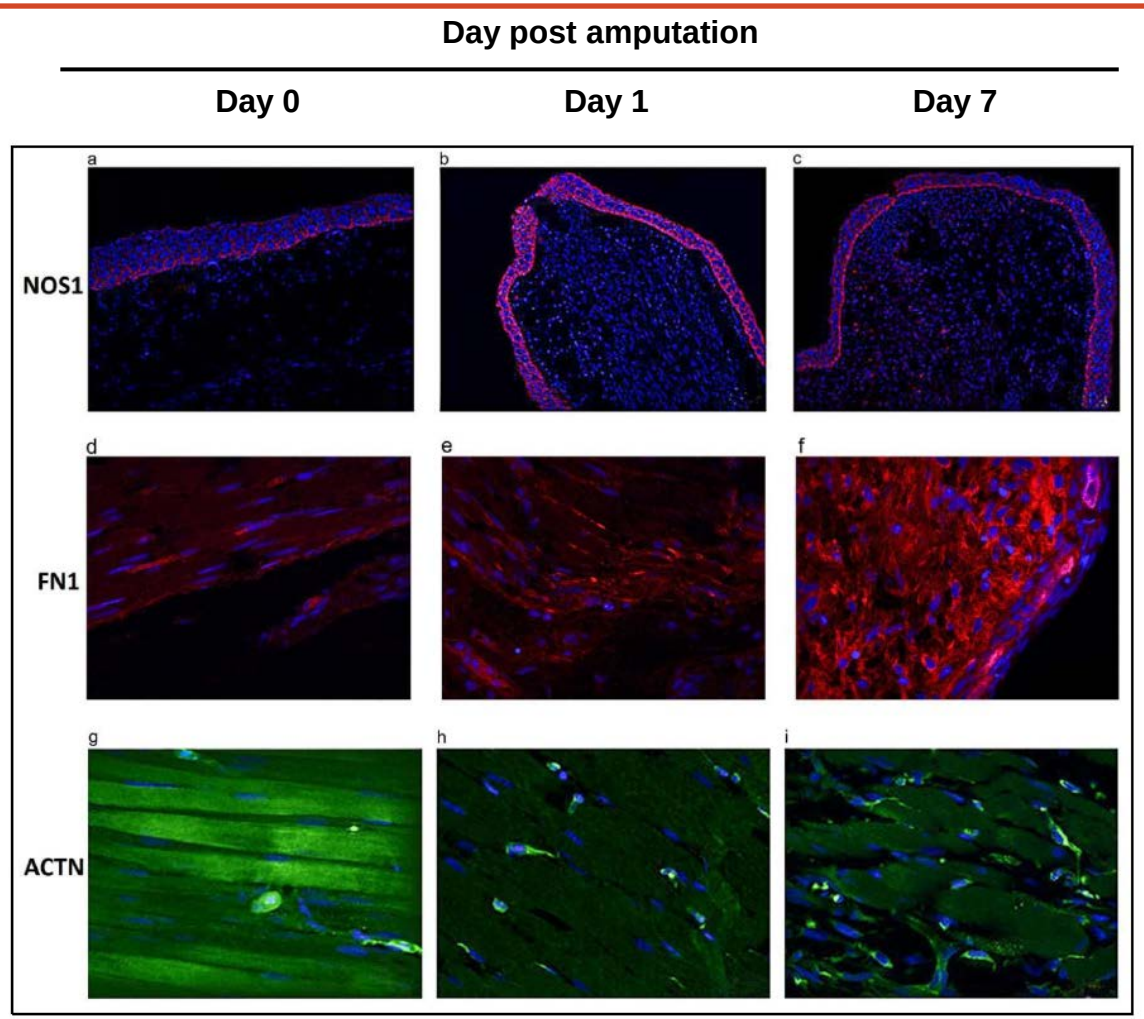
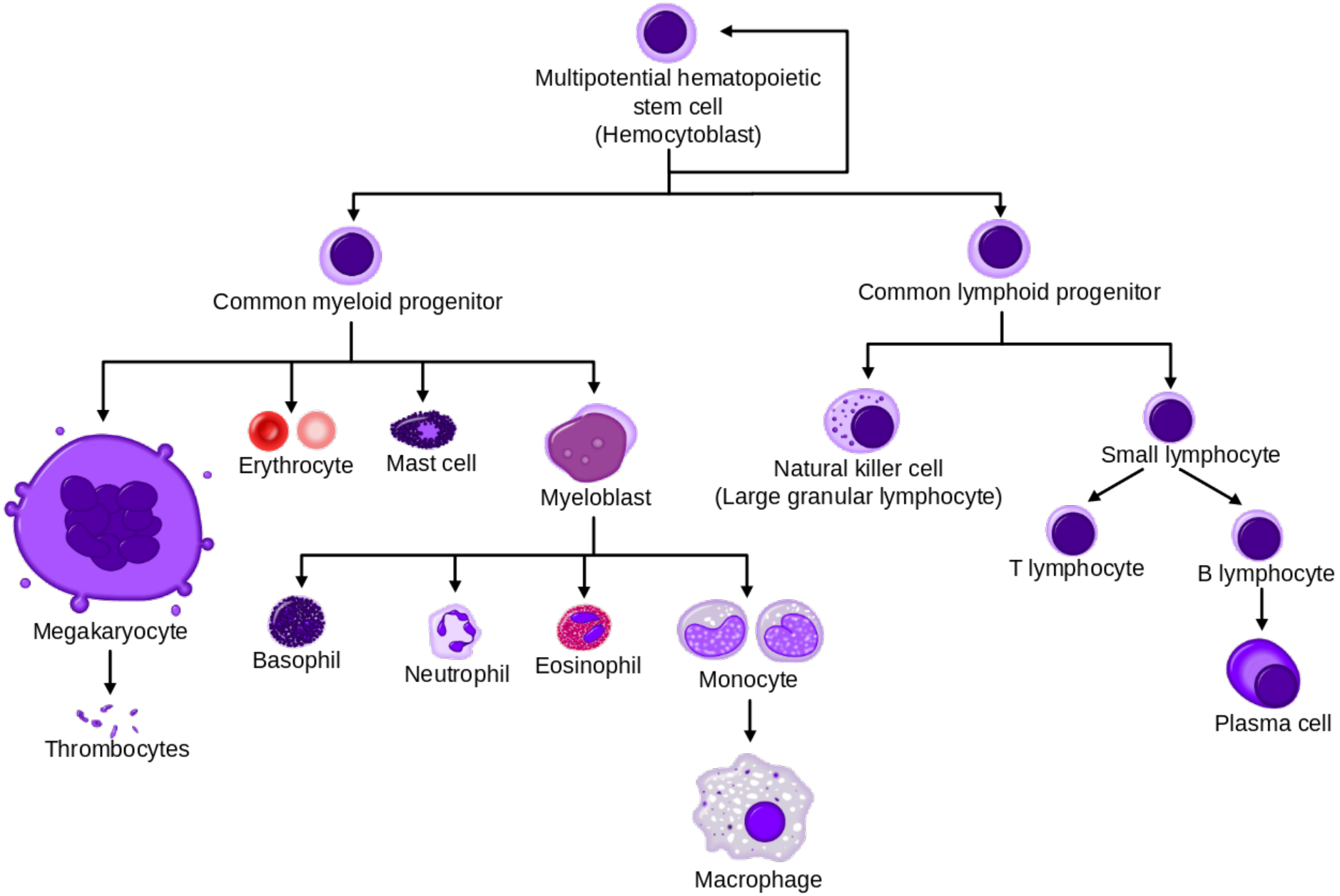


Figure 4
Immunostained sections of axolotl hindlimbs. Longitudinal sections of control **(a, d, g)** versus 1 day post amputation (dpa) **(b, e, h)** and 7 dpa **(c, f, i)** axolotl hindlimbs stained with primary antibodies to nitric oxide synthase 1 (NOS1) (a-c), fibronectin 1 (FN1) (d-f), α-actinin (ACTN) (g-i). Conjugated secondary antibodies were alexa-568 for fibronectin and NOS1, and alexa-488 for α-actinin. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). As expected from the proteomic data, fluorescence intensity of NOS1 showed a significant increase compared to control at 1 dpa, then decreased to a level slightly above control at 7 dpa. Fibronectin staining (red) at 1 and 7 dpa showed significant increases compared to controls, while α-actinin staining intensity (green) showed significant decreases.

Table 3: Liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) versus densitometry measurements

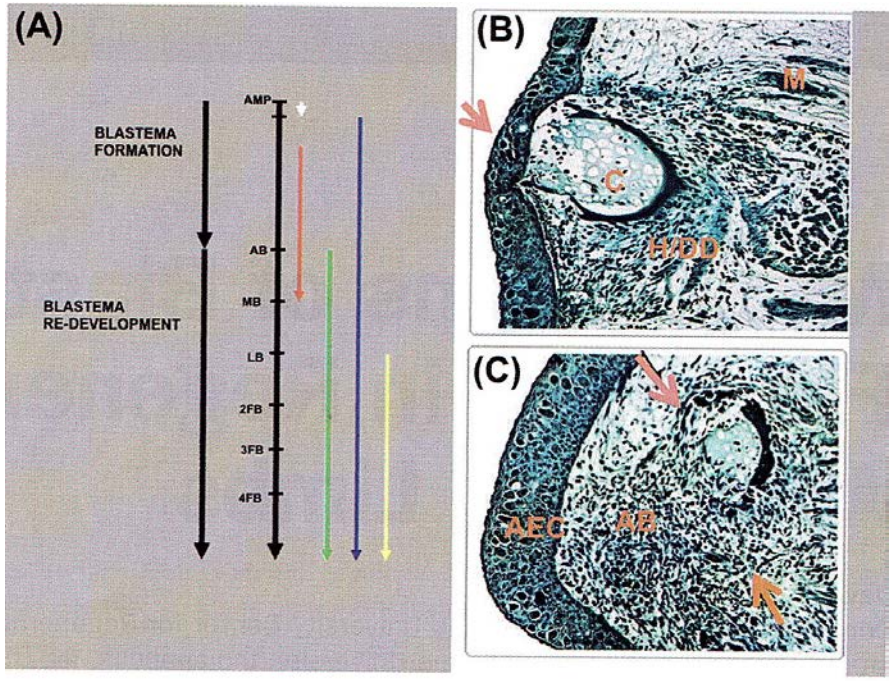
Protein	Comparison	Fold change	
		LC-MS/MS	Densitometry
NOS1	0 dpa vs 1 dpa	4.93	2.0*
	0 dpa vs 7 dpa	1.16	1.2***
Fibronectin	0 dpa vs 1 dpa	1.39	1.51**
	0 dpa vs 7 dpa	1.46	3.17*
α-Actinin	0 dpa vs 1 dpa	-1.16	-0.62***
	0 dpa vs 7 dpa	-1.58	-0.83

Rao, N 2009 BMC Biology


Multipotential hematopoietic stem cell (Hemocytoblast)
Common myeloid progenitor
Common lymphoid progenitor
Megakaryocyte
Thrombocytes
Erythrocyte
Mast cell
Myeloblast
Basophil
Neutrophil
Eosinophil
Monocyte
Macrophage
Natural killer cell (Large granular lymphocyte)
Small lymphocyte
T lymphocyte
B lymphocyte
Plasma cell

Wikipedia

1. Hemostasis & Re-epithelialization
2. Histolysis & dedifferentiation
3. Blastema cell migration & accumulation



Histolysis & Dedifferentiation

Histolysis: the loss of tissue organization resulting from the enzymatic degradation of ECM

Dedifferentiation: reversal of a given state of differentiation to an earlier state via nuclear reprogramming and loss of specialized structure and function

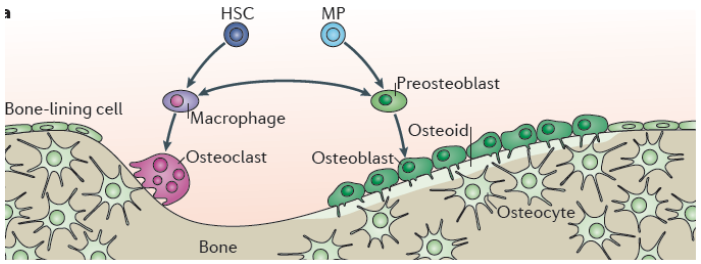
Tissue adjacent to wound epidermis

- **Intense histolysis for a distance of 1~2 mm**
- **Liberation of individual cells**
  (Dermal cells, Schwann cells, Skeletal cells, Satellite cells)

Mechanism of Histolysis - Acid hydrolases and MMPs

Osteoclasts are abundant in the region of histolysis -> degrade bone matrix via…

- Hydrochloric acid
- Acid hydrolases
- MMPs



Long, F 2012 Nature Rev Cell Biol

Acid hydrolases:

- Cathepsin D, acid phosphatase, beta-glucuronidase, carboxyester hydrolases, N-acetyl-glucosaminidase

MMPs (Matrix Metallo Proteinase):

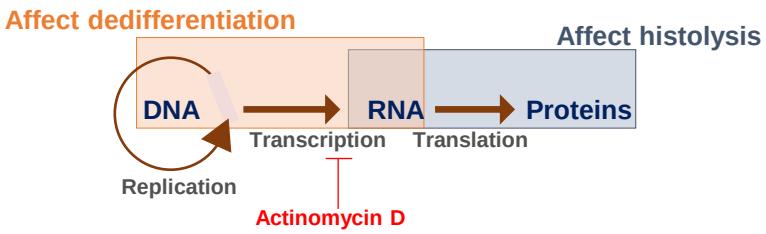
- MMP-2, MMP-9(gelatinases), MMP-3/10a and 10b (stromelysin)
- Inhibitor of MMP treatment -> failed blastema formation

Once the accumulation blastema begins to grow, histolysis gradually ceases due to the activity of TIMPs (Tissue inhibitors of metalloproteinases)

Mechanism of Dedifferentiation

Transcription

- Actinomycin D does not affect histolysis
- prevent or retard dedifferentiation



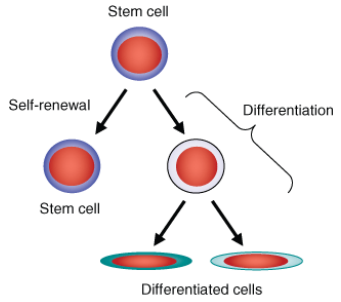
Dedifferentiation involves changes in transcriptional program to…

- Suppress differentiation genes
- Activating genes associated w/ stemness
- Reduction of cell stress
- Remodeling internal cell structure

Dedifferentiation ≒ Gaining Stemness

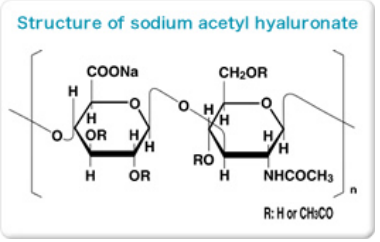
Stemness genes up-regulated during blastema formation: *msx1, nrad, rfrng, notch*

- *Msx1*: inhibiting myogenesis
- *Nrad*: correlated with muscle dedifferentiation
- *Notch*: a major mediator of stem cell self-renewal

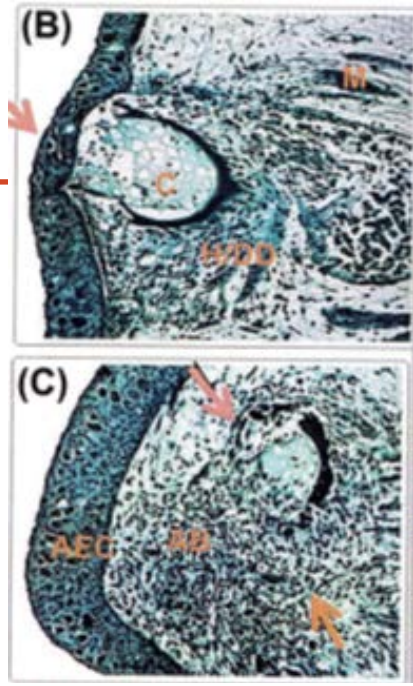


Dedifferentiated cells express a more limb bud-like ECM

- Decreased type I collagen synthesis & accumulation
- Increased fibronectin, tenascin, & hyaluronate



Characterization of Blastema Cells



Cellular changes tracked by several transplantation studies have shown that blastema cells are…

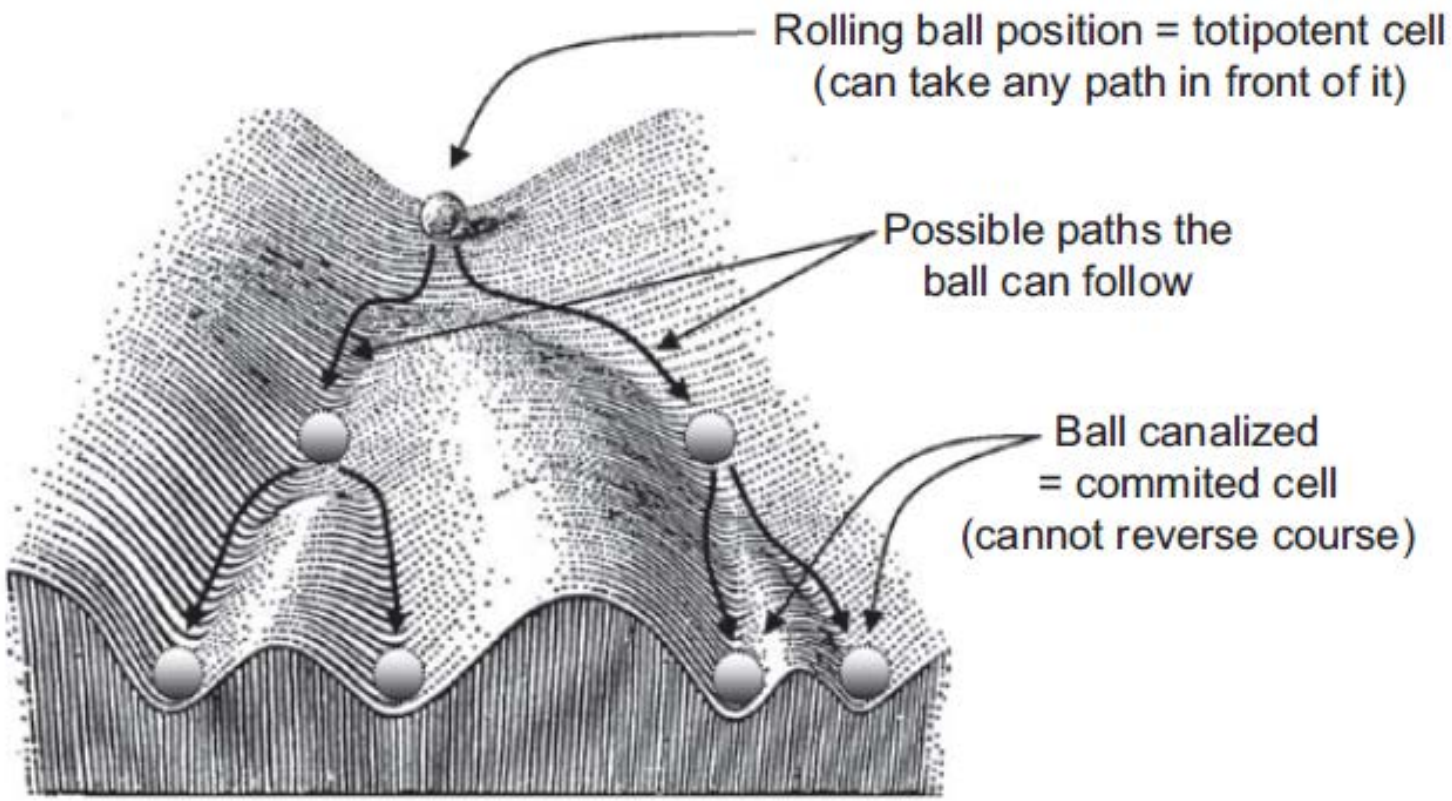
- **No pluri-potency or even multi-potency (No reprogramming)**
- **But are largely constrained to redifferentiate into their parent cell types (Terminally differentiated somatic cells -> progenitor cells)**

The exception is fibroblasts of the dermis: after dedifferentiation, they are able to transdifferentiate at high frequency into cartilage: similar to direct conversion

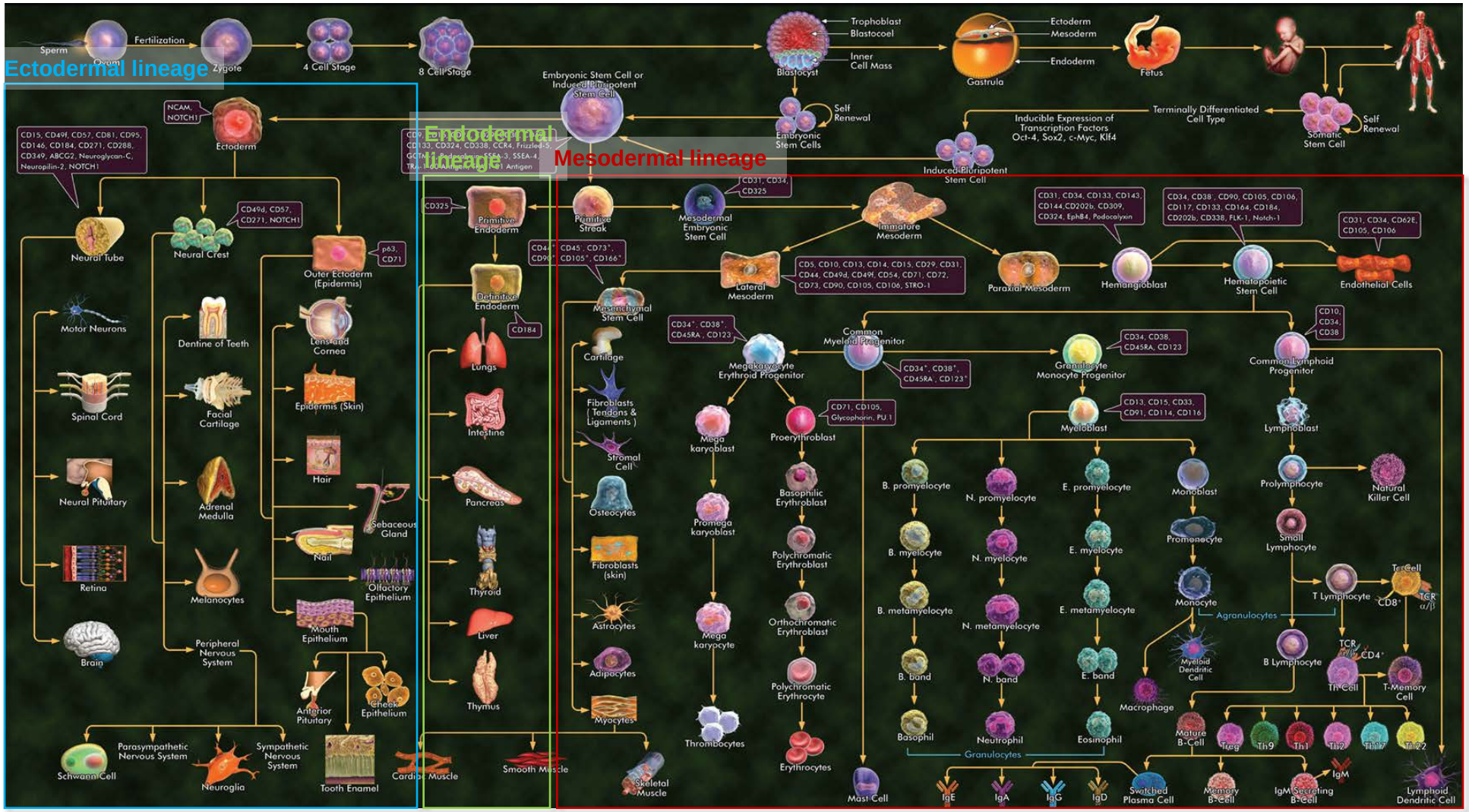
Regardless of this limited plasticity,

- **3 Transcriptional genes (klf-4, sox2, c-myc) are up-regulated during blastema formation**
- **Lin28 (the 4th Tc gene used to derive iPSCs) is up-regulated during blastema formation in axolotle limbs.**
- **This means that something other factors than transcriptional control are clearly in play. (Epigenetic change)**

Waddington's Epigenetic Landscape



Tissue Formation



Low Apoptosis in Regenerating Limbs

Apoptosis is known to be minimal in regenerating limbs

Pathways that protect cells from stress & apoptosis

- Reduced metabolic activity
- Upregulation of pathways that accelerate protein folding
- Upregulation of eliminating unfolded proteins
- Regulation on apoptotic pathway
  (to prevent apoptosis)

Molecular details of the remodeling process are largely uninvestigated…

Small molecules causing cellularization of C2C12 mouse myofibers: Dismantling of phenotypic structure and function (myofiber -> cellularization)

- Myoseverin
  - Disrupts microtubules
  - Up-regulate genes for GFs, immuno-modulatory molecules, ECM remodeling proteases, stress-response genes
- Reversine
  - C2C12 myofibers -> mononucleated cells that behave like MSCs (differentiate in vitro into osteoblast, adipocyte, muscle cells)

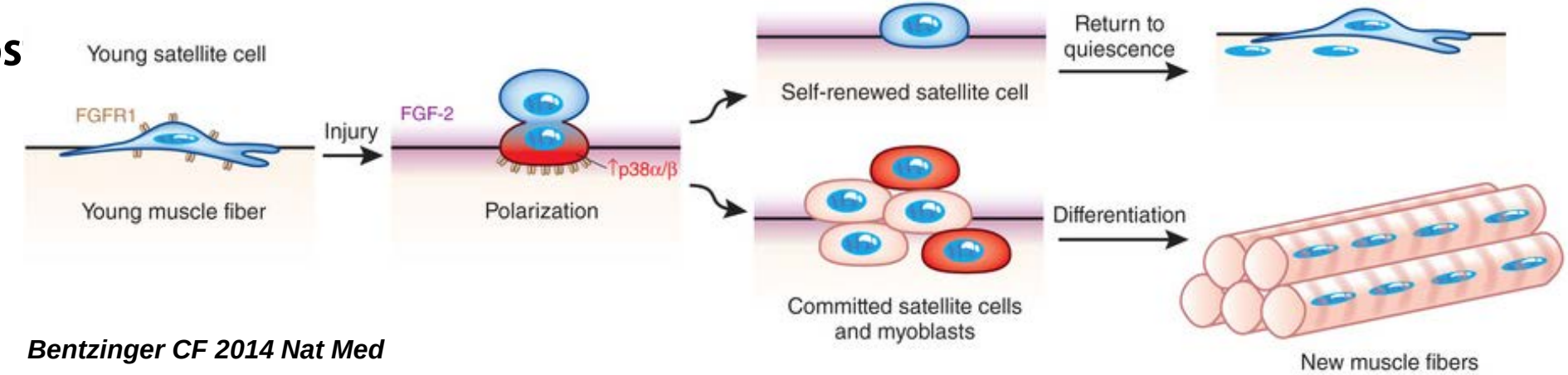
The signals that trigger the shift in transcription during dedifferentiation are largely unknown.

There are several possibility…

- Enzymatic degradation of ECM
- >cell morphology and integrin signal change
- >reorganization of cytoskeleton
- >activate signal transduction.

Differential tissue contributes to the blastema in the axolotle limb

- At amputation surface
  - Dermal cells – 19%,
  - Chondrocytes – 6%
- > contribution to blastema cells
  - 43% dermal cells
  - 2% chondrocytes
- Satellite cells of myofibers make a substantial contribution to the blastema
- Perios



Bentzinger CF 2014 Nat Med

Cell Cycle during Blastema Formation

Cells of amputated urodele limbs initiate cell cycle re-entry coincident with their histolysis and dedifferentiation

Both the labeling and mitotic indices rise as much as 10-fold when the accumulation blastema initiates growth

The low mitotic index prior to establishment of the accumulation blastema suggests that it forms primarily by the accumulation of dedifferentiated cells rather than their mitosis.

The fact that cells readily enter the cell cycle during histolysis and dedifferentiation but divide only infrequently suggests that a large proportion of dedifferentiating cells arrest in G2

Role of Wound Epidermis and Nerves

Blastema formation is inhibited w/o wound epidermis or nerves

Wound epidermis & nerve have differential effects on the cell cycle during blastema formation

- Role of WE:
  - Signal from wound epidermis -> histolysis
    -> accumulation of dedifferentiated cells -> (primarily responsible for establishment of blastema)

Role of nerve:

- Basal-level mitosis is nerve-dependent, not wound epidermis-dependent
- Denervation -> prevents blastema formation
  : not due to failure in dedifferentiation

- A major difference between the regenerates formed by **denervated** and **epidermis-free blastema**:
  - **Denervated blastemal** forms "**miniature limb**"
  - The pattern of skeletal elements formed by the **epidermis-free blastema** is more or less **distally truncated**, depending on the stage at which the epidermis is removed, suggesting that the AEC has a role in proximodistal patterning in addition to proliferation.

- **Nerve**
  - Stimulate mitosis
  - Involved in size control

- **AEC**
  - Affect differentiation & morphogenesis
  - Involved in pattern formation

1. Hemostasis & Re-epithelialization
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Blastema Cell Migration & Accumulation

The AEC apprears to direct the migration of blastema cells to form the accumulation blastema beneath it.

TGF-beta (strongly up-redulated during blastema formation)
-> induce fibronectin production
-> induce migration

Xenopus laevis -Blastema formation occurs, but no limb regeneration occurs

Similarity

- Hemostasis
- Re-epithelialization

Difference

- Little histolysis
- No dedifferentiation
- AEC is thinner
- No MMP production by WE
- Stress response exists

It has been well documented that young children, below the age of 10, regenerate amputated fingertips.

Pattern formation during Embryonic limb development

Skeletal Pattern

Stylopod – humerus, femur
Zeugopod – radius-ulna, tibia-fibula
Autopod – Carpal-fingers, Tarsal-toe

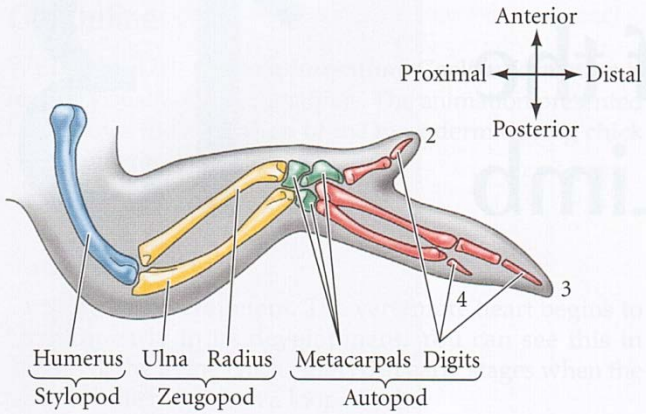


FIGURE 13.1 Skeletal pattern of the chick wing. According to convention, the digits are numbered 2, 3, and 4. The cartilage condensations forming the digits appear similar to those forming digits 2, 3, and 4 of mice and humans; however, new evidence (discussed later in the chapter) suggests that the correct designation may be 1, 2, and 3.

4 Dimension system

- 3 main axis
  - Proximo-distal axis (close-far/shoulder-finger): FGF family
  - Anterior-posterior axis (thumb-pinkie): SHH
    each hand develops as a mirror image of the other
  - Dorso-ventral axis (palms-knuckles): Wnt7a

- Time

Induction of the Early Limb Bud

Signal for limb bud formation:
FGF10 (lateral plate mesoderm cells)

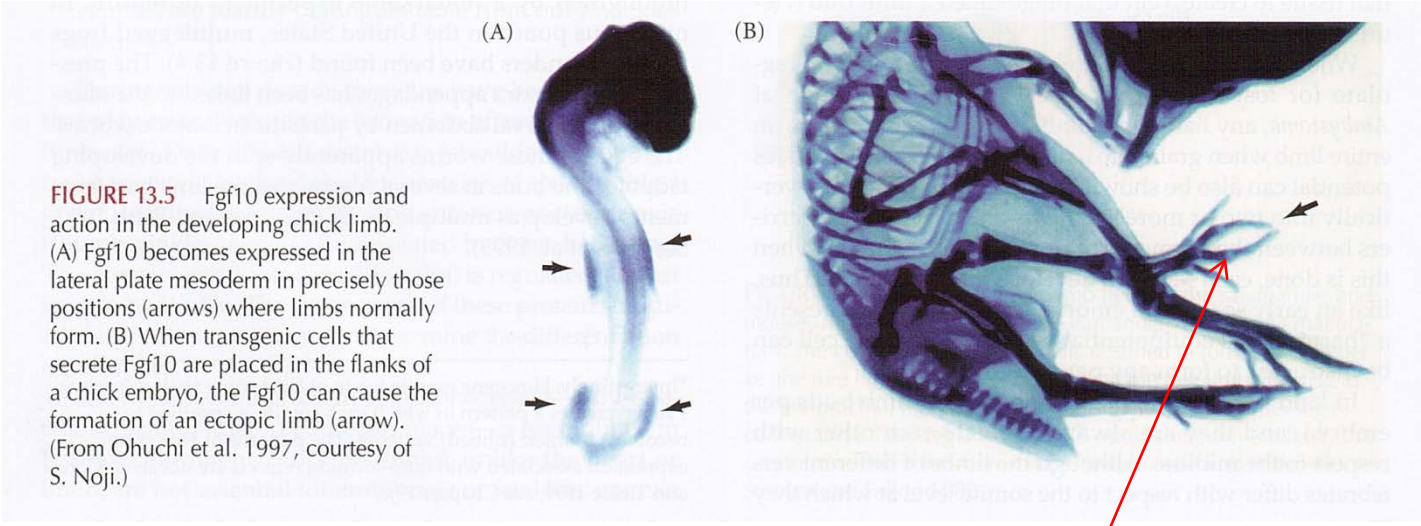
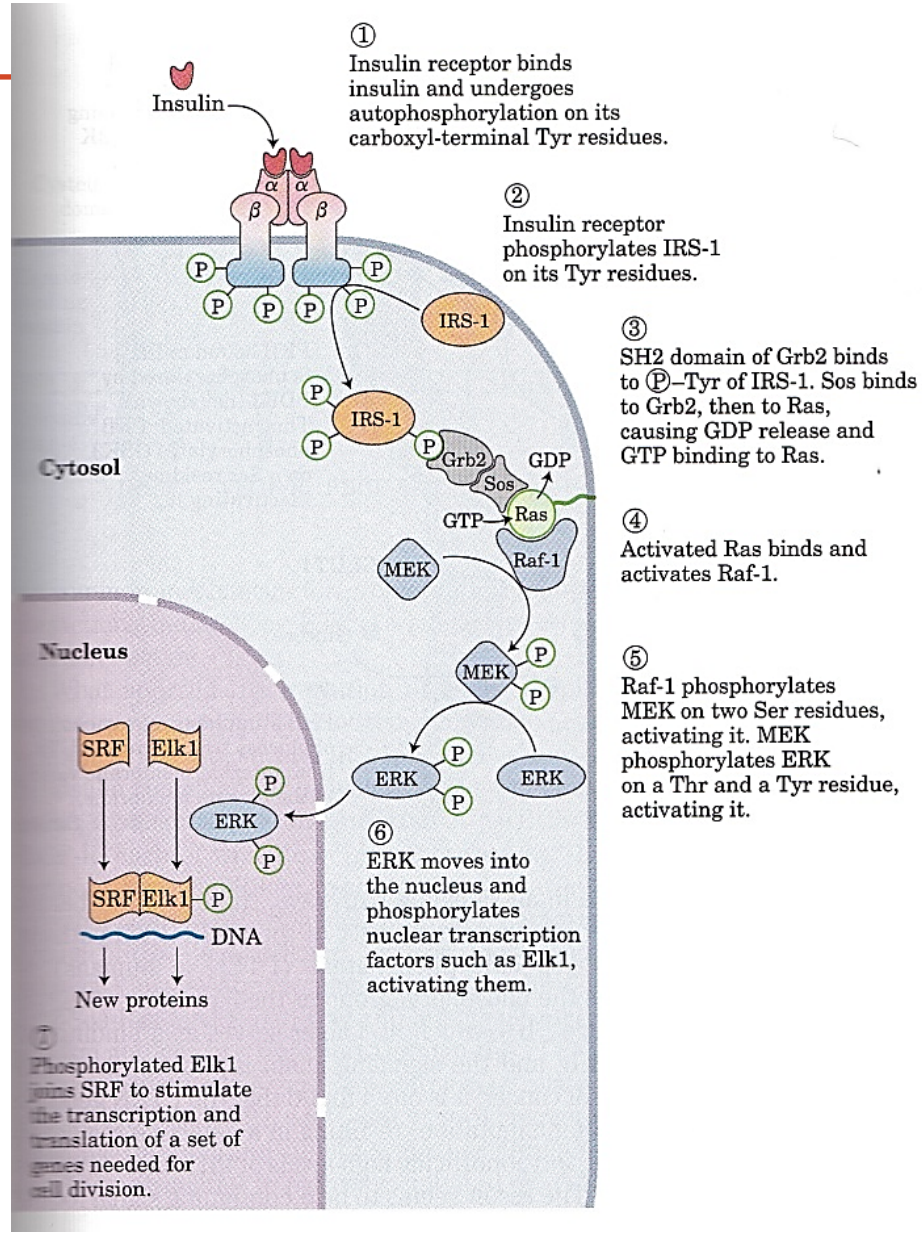


FIGURE 13.5 Fgf10 expression and action in the developing chick limb. (A) Fgf10 becomes expressed in the lateral plate mesoderm in precisely those positions (arrows) where limbs normally form. (B) When transgenic cells that secrete Fgf10 are placed in the flanks of a chick embryo, the Fgf10 can cause the formation of an ectopic limb (arrow). (From Ohuchi et al. 1997, courtesy of S. Noji.)

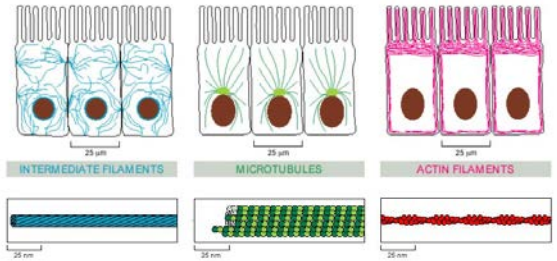
Ectopic limb formed by FGF10-releasing bead

Intracellular Signal Transduction



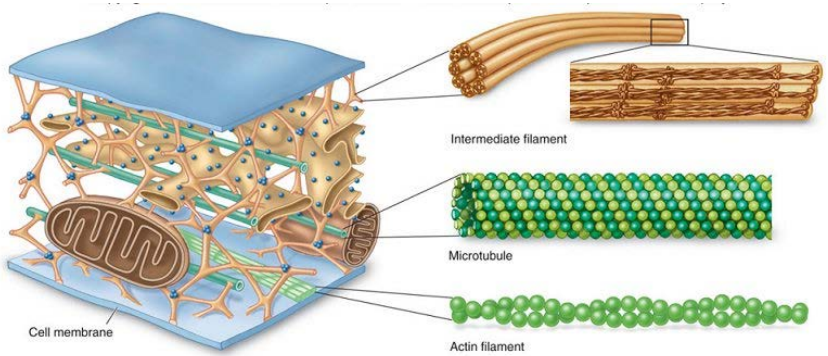
1. Ligand-receptor binding
2. Activation of receptor (phosphorylation)
3. Activation of adaptor proteins
4. Activation of MAPK
5. Activation of transcription factors
6. Induction of gene expression

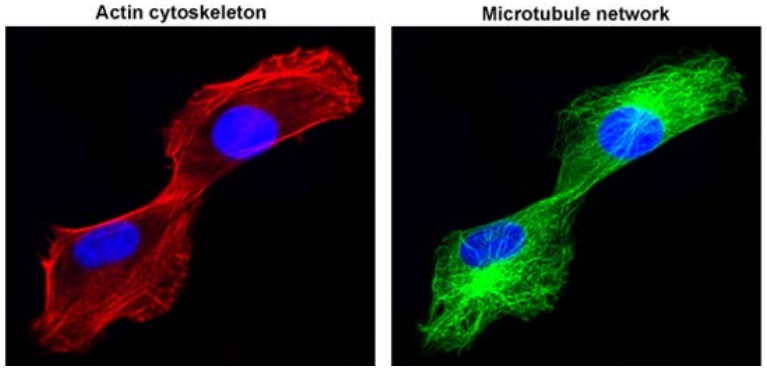
Cytoskeleton

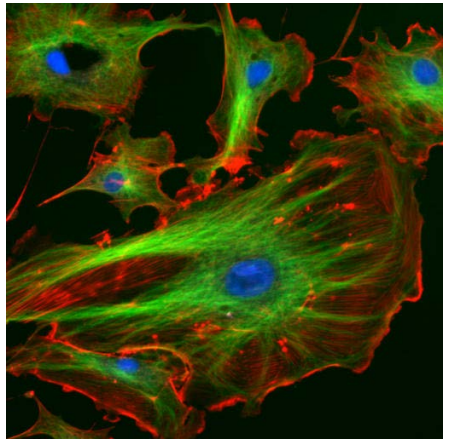


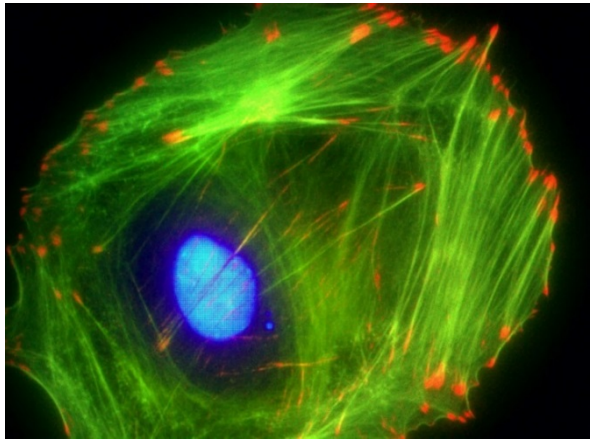
Cytoskeletal Functions

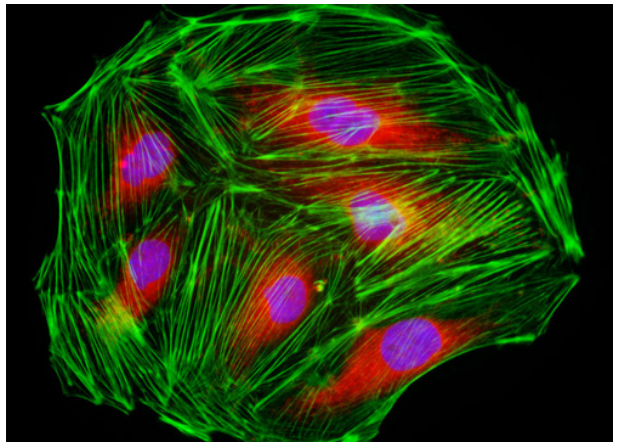
1. Structure and support
2. Intracellular transport
3. Contractility and motility
4. Spatial organization



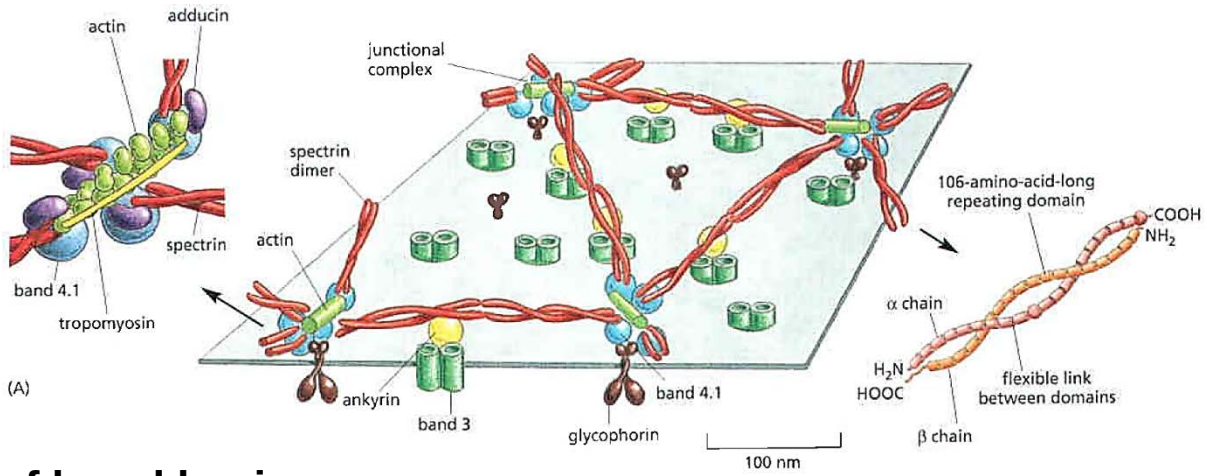




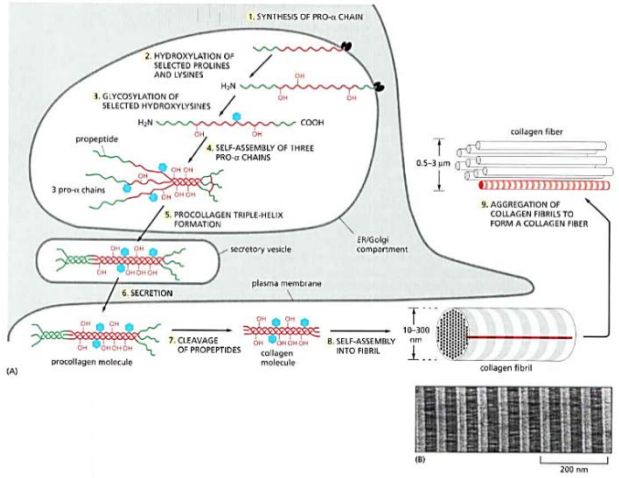


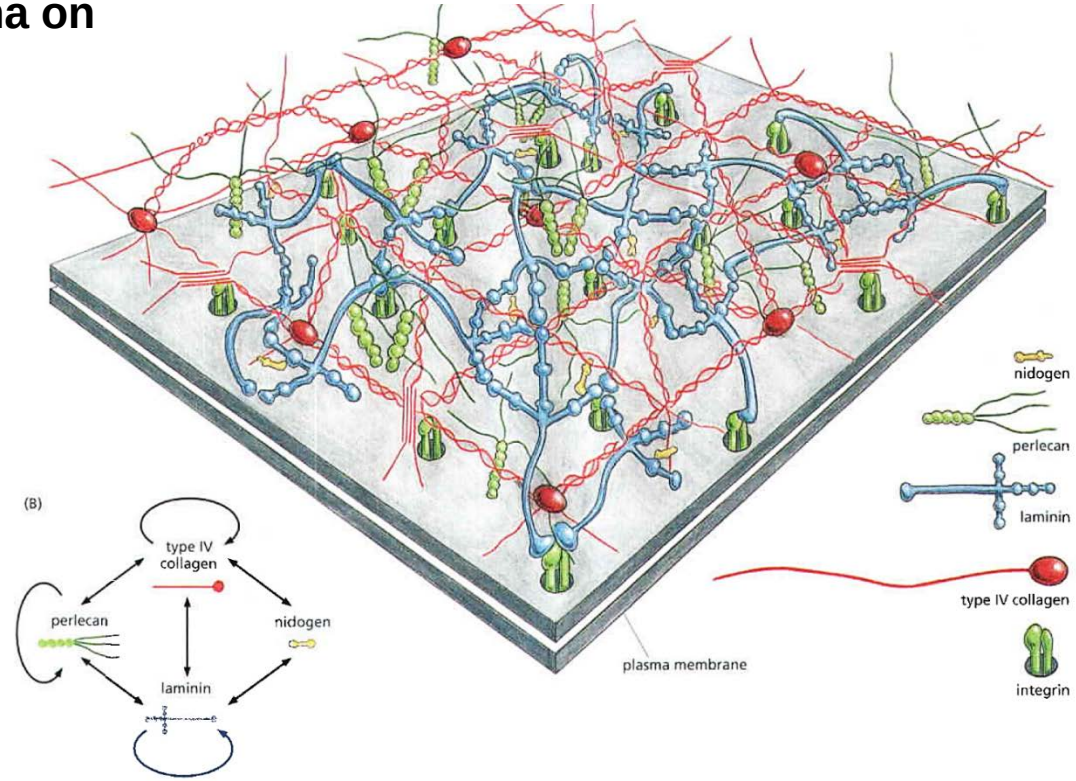


Cytoskeleton on the cytosolic side



Molecular structure of basal lamina on the extracellular side





Induction of the Early Limb Bud

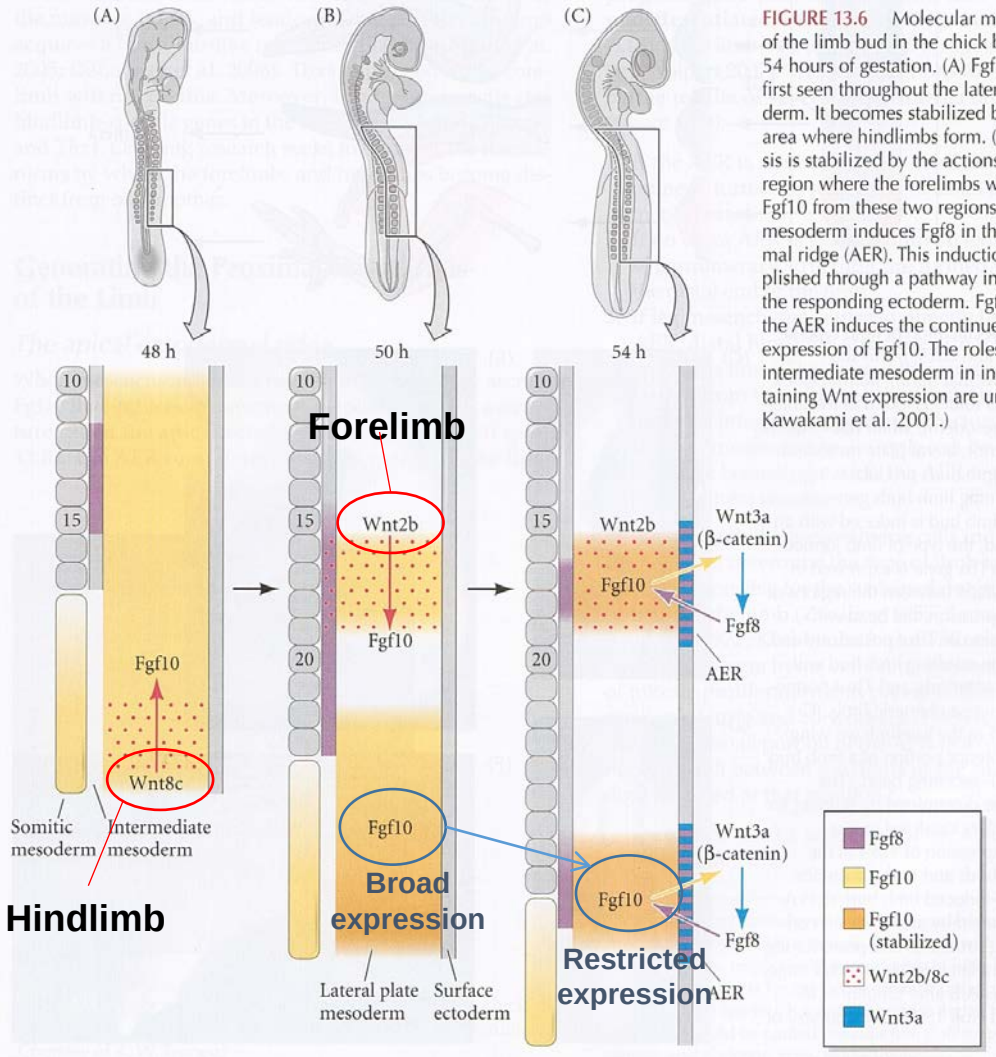
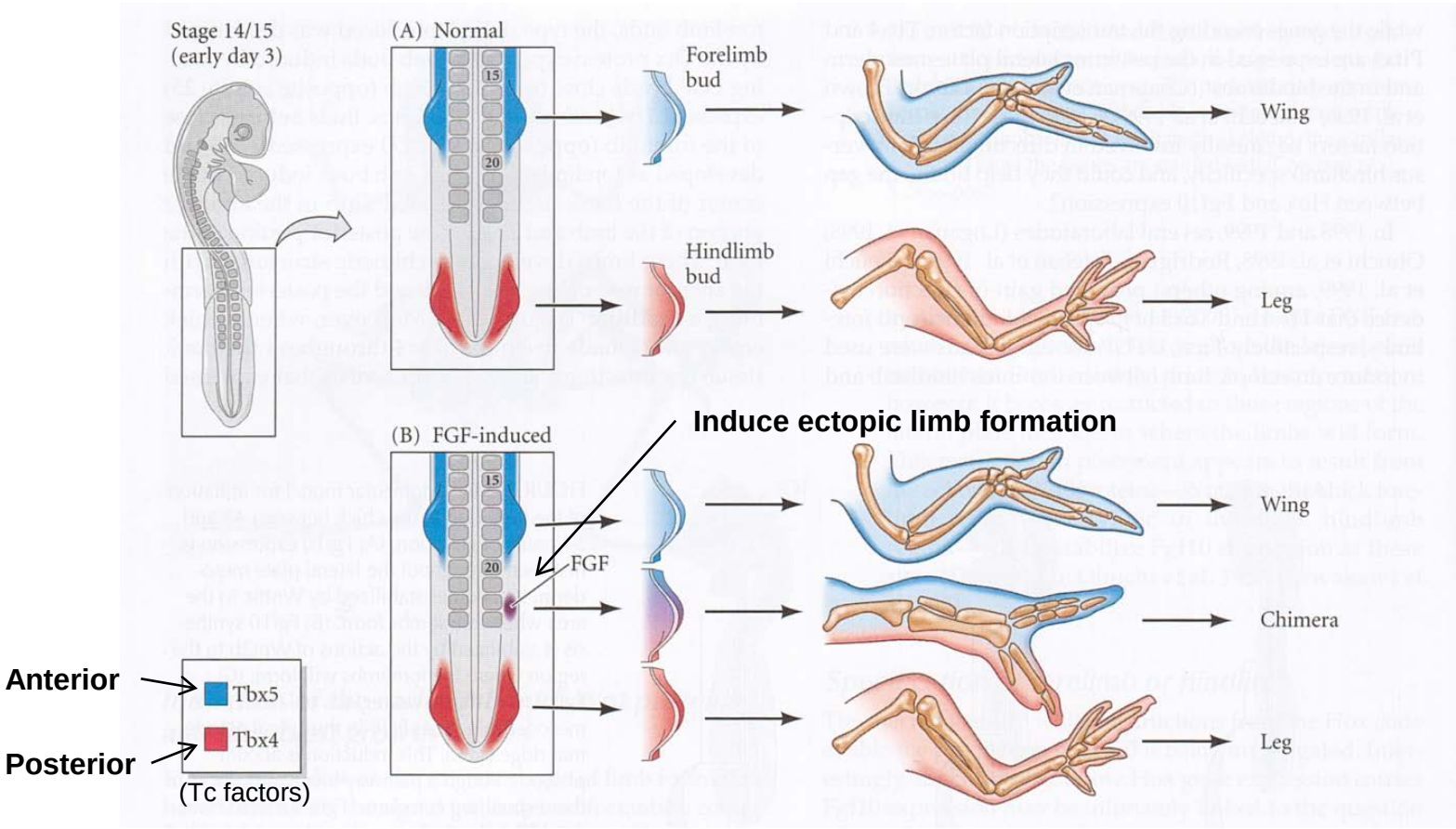


FIGURE 13.6 Molecular model for initiation of the limb bud in the chick between 48 and 54 hours of gestation. (A) Fgf10 expression is first seen throughout the lateral plate mesoderm. It becomes stabilized by Wnt8c to the area where hindlimbs form. (B) Fgf10 synthesis is stabilized by the actions of Wnt2b to the region where the forelimbs will form. (C) Fgf10 from these two regions of lateral plate mesoderm induces Fgf8 in the apical ectodermal ridge (AER). This induction is accomplished through a pathway involving Wnt3a in the responding ectoderm. Fgf8 secreted from the AER induces the continued mesodermal expression of Fgf10. The roles of Fgf8 in the intermediate mesoderm in inducing or maintaining Wnt expression are uncertain. (After Kawakami et al. 2001.)

Specification of Forelimb or Hindlimb



Generation of Proximo-distal Axis - AER

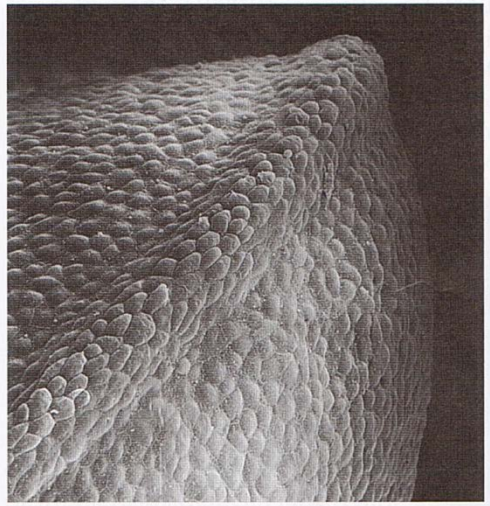


FIGURE 13.8 Scanning electron micrograph of an early chick forelimb bud, with the apical ectodermal ridge in the foreground. (Courtesy of K. W. Tosney.)

Mesenchymal cells enter limb field
-> secret Fgf10
-> induce *apical ectodermal ridge (**AER**)* formation

AER: Major signaling center for the developing limbs

1. Maintaining the mesenchyme beneath it in a plastic, proliferating state
   -> linear growth of the limb
2. Maintaining the expression of molecules (A-P axis)
3. Interact with proteins specifying A-P / D-V axis

Specification of the A-P Axis

- Shortly before a limb bud is recognizable
- Specified by a small block of mesodermal tissue.
  (Zone of polarizing activity: **ZPA**)

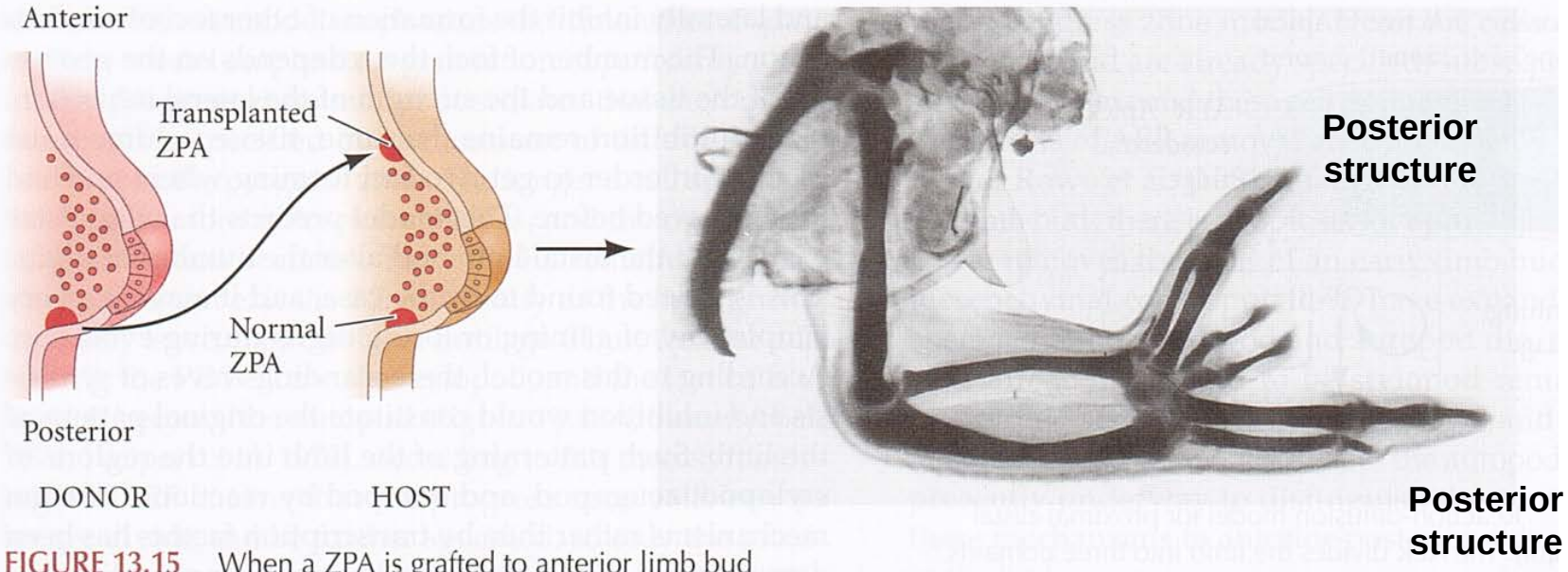
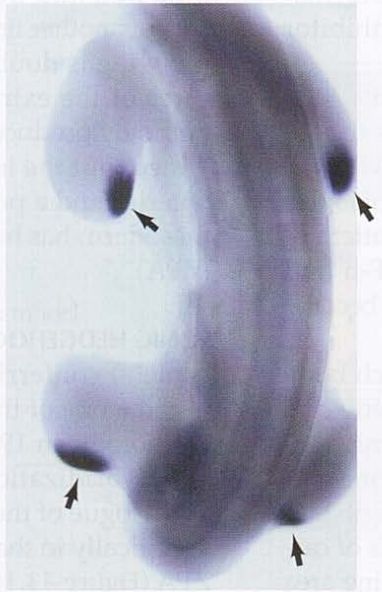


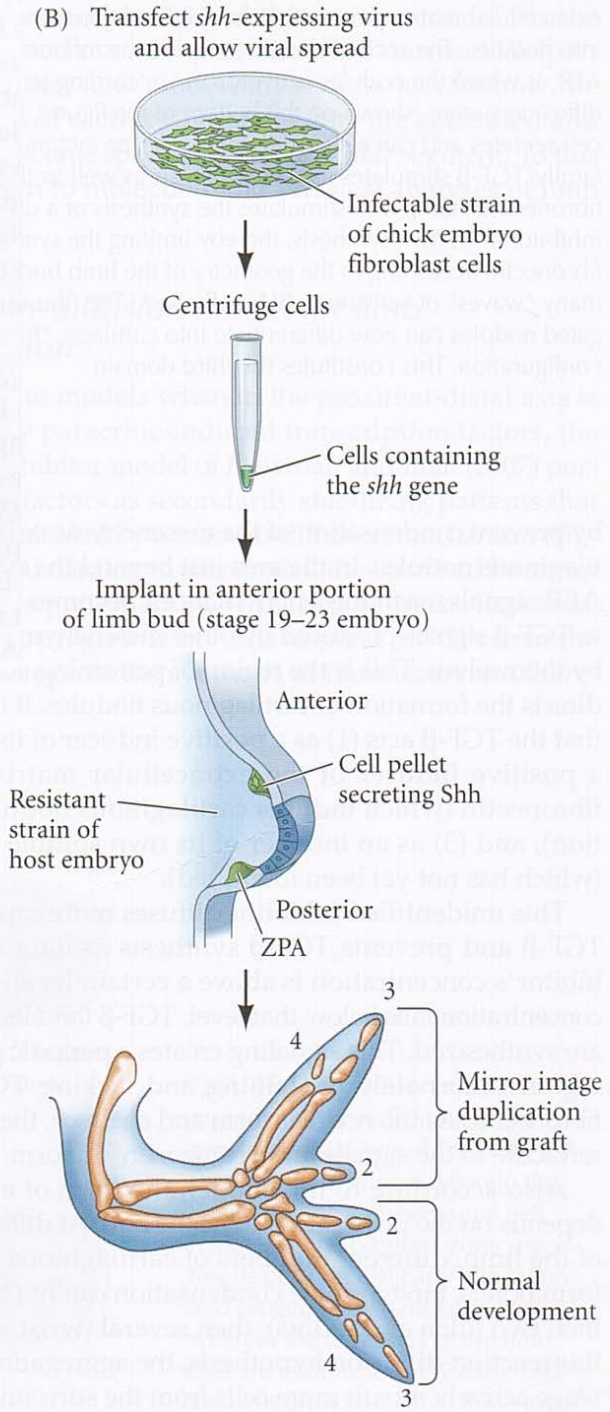
FIGURE 13.15 When a ZPA is grafted to anterior limb bud mesoderm, duplicated digits emerge as a mirror image of the normal digits. (From Honig and Summerbell 1985, photograph courtesy of D. Summerbell.)

Sonic Hedgehog Defines the ZPA

FIGURE 13.16 Sonic hedgehog protein is expressed in the ZPA. (A) In situ hybridization showing the sites of *sonic hedgehog* expression (arrows) in the posterior mesoderm of the chick limb buds. These are precisely the regions that transplantation experiments defined as the ZPA. (B) Assay for polarizing activity of Sonic hedgehog protein. The *shh* gene was inserted adjacent to an active promoter of a chicken virus, and the recombinant virus was placed into cultured chick embryo fibroblast cells. The virally transfected cells were pelleted and implanted in the anterior margin of a limb bud of a chick embryo. The resulting limbs produced mirror-image digits, showing that the secreted protein had polarizing activity. (A courtesy of R. D. Riddle; B after Riddle et al. 1993.)

(A) **SHH expression**





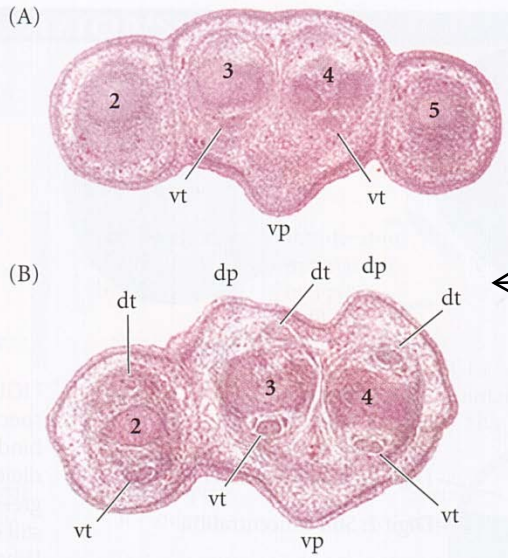
Generation of the Dorso-Ventral Axis

- Dorsal-ventral polarity of the limb bud is determined by the <u>ectoderm</u> encasing it.
- Ectoderm rotated 180 degree -> D-V axis partially reversed (distal elements are upside-down) – Late specification!
- Wnt7a: partially important (expressed in dorsal ectoderm)

Wnt7a

↓

Lim1 expression
(Tc factor essential for specifying dorsal cell fate in the limb.)

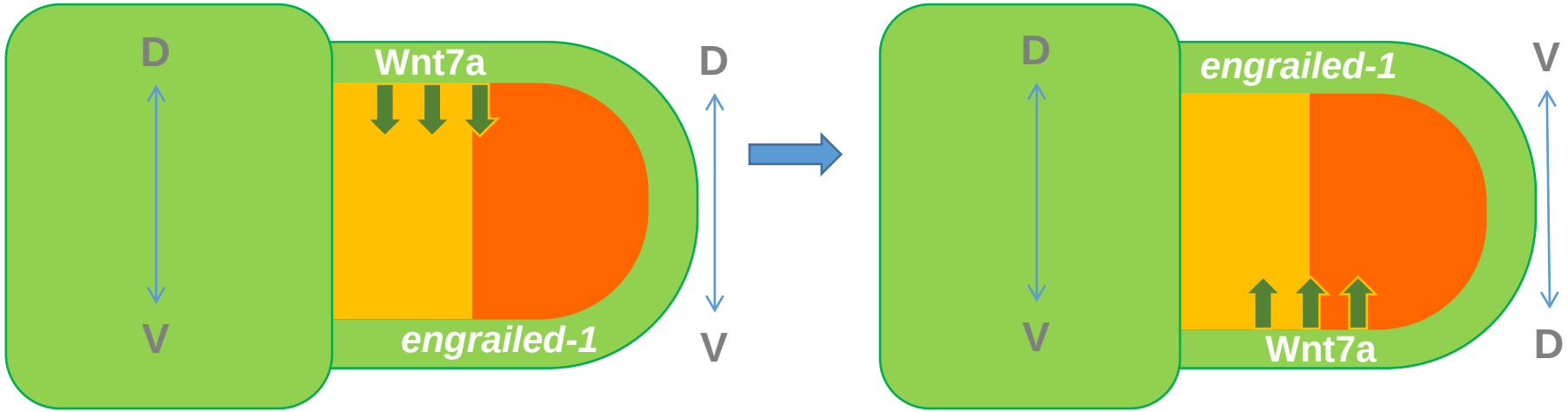


← Ventralized dortal tissue

FIGURE 13.23 Dorsal-to-ventral transformations of limb regions in mice deficient for both *Wnt7a* genes. (A) Histological section (stained with hemotoxylin and eosin) of wild-type 15.5-day embryonic mouse forelimb paw. The ventral tendons (vt) and ventral footpads (vp) are readily seen. Numbers indicate digit identity. (B) Same section through a mutant embryo deficient in *Wnt7a*. Ventral tendons and footpads are duplicated on what would normally be the dorsal surface of the paw—that is, the dorsal tendons (dt) and dorsal footpads (dp). (From Parr and McMahon 1995; photographs courtesy of the authors.)

Dorso-ventral patterning…

Can be inverted by inverting the epidermis at an early stage.



Coordinating the Three Axes

- 3 axis-specifying mechanisms- interrelated & coordinated

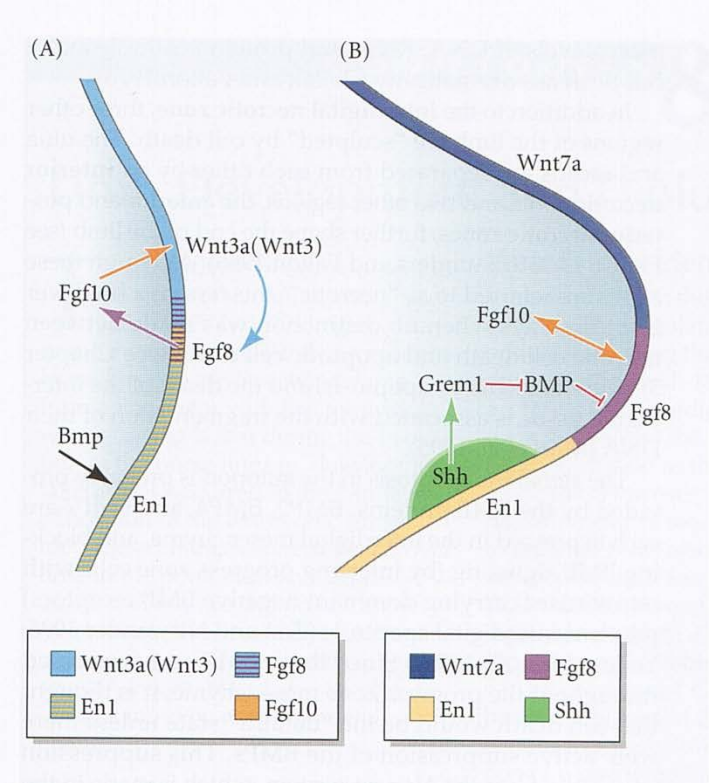


FIGURE 13.24 Early interactions between the AER and limb bud mesenchyme. (A) In the limb bud, Fgf10 from mesenchyme generated by the lateral plate mesoderm activates a Wnt (Wnt3a in chicks; Wnt3 in mice and humans) in the ectoderm. Wnt activates the β-catenin pathway, which induces synthesis of Fgf8 in the region near the AER. Fgf8 activates Fgf10, causing a positive feedback loop. (B) As the limb bud grows, Sonic hedgehog in the posterior mesenchyme creates a new signaling center that induces posterior-anterior polarity, and it also activates Gremlin (Grem1) to prevent BMPs from blocking FGF synthesis in the AER. (After Fernandez-Teran and Ros 2008.)

EOD