CARTILAGE TISSUE ENGINEERING & CELL CULTURE

Assoc. Prof. Murat ÖZMEN

Assoc. Prof. Emrah Şefik Abamor

Cartilage

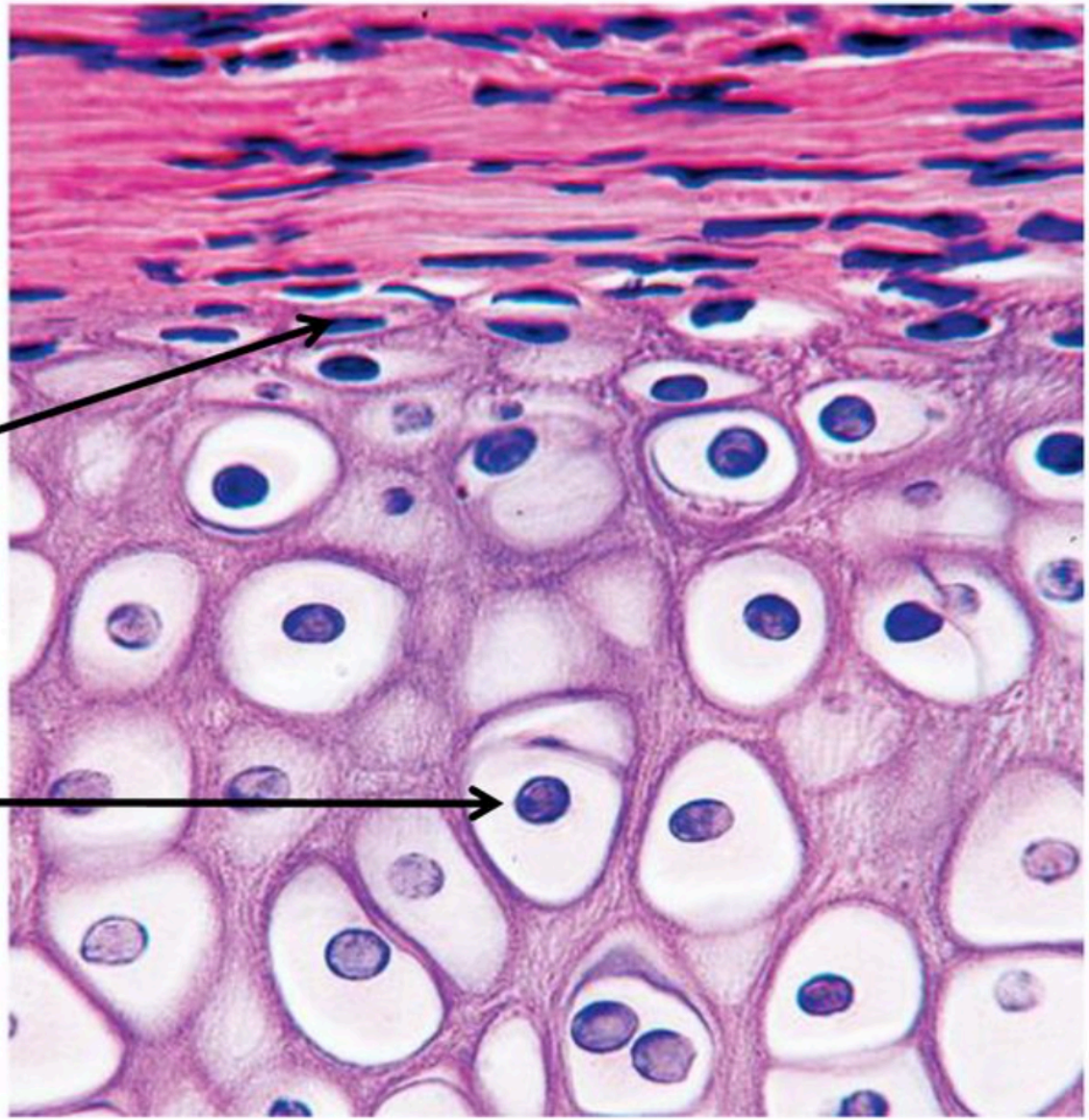
- Cartilage is a flexible type of connective tissue that is made up of cells called **chondrocytes** as well as from the materials that they secrete.
- The relatively stiff structure that cartilage boasts plays an important role in the development of the formation of bones during a fetus's early stages of progress.
- The skeleton is first laid out in its form as cartilage, thereafter being replaced by the more solid structure of bones.

Cartilage Cells

Cartilage is a specialized, supporting connective tissue composed of cells and extracellular matrix.

Chondroblasts
Precursor cells that differentiate into chondrocytes.

Chondrocytes
Mature cartilage cells that lie in little artifactual lacunae.

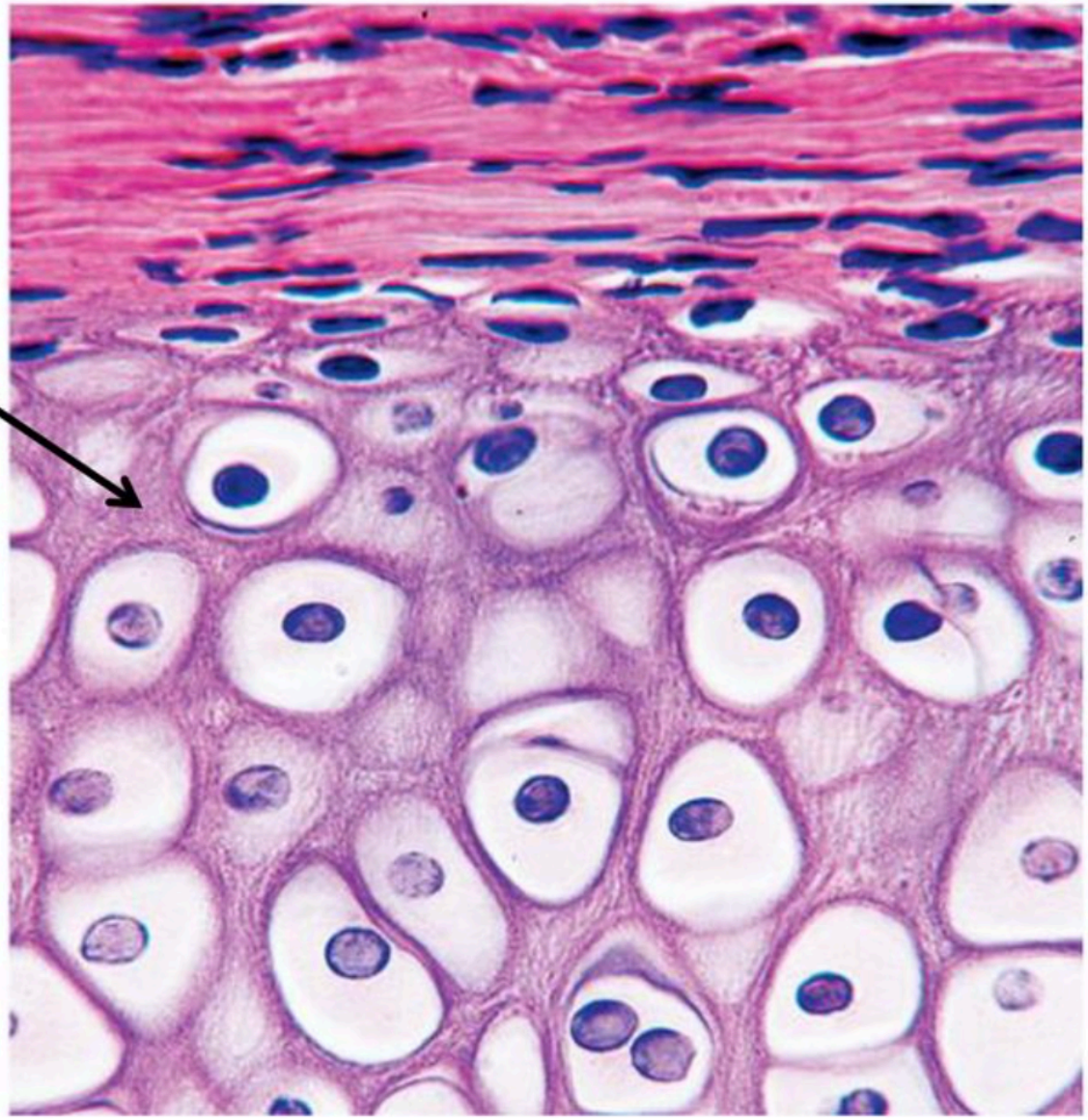


Cartilage Extracellular Matrix (ECM)

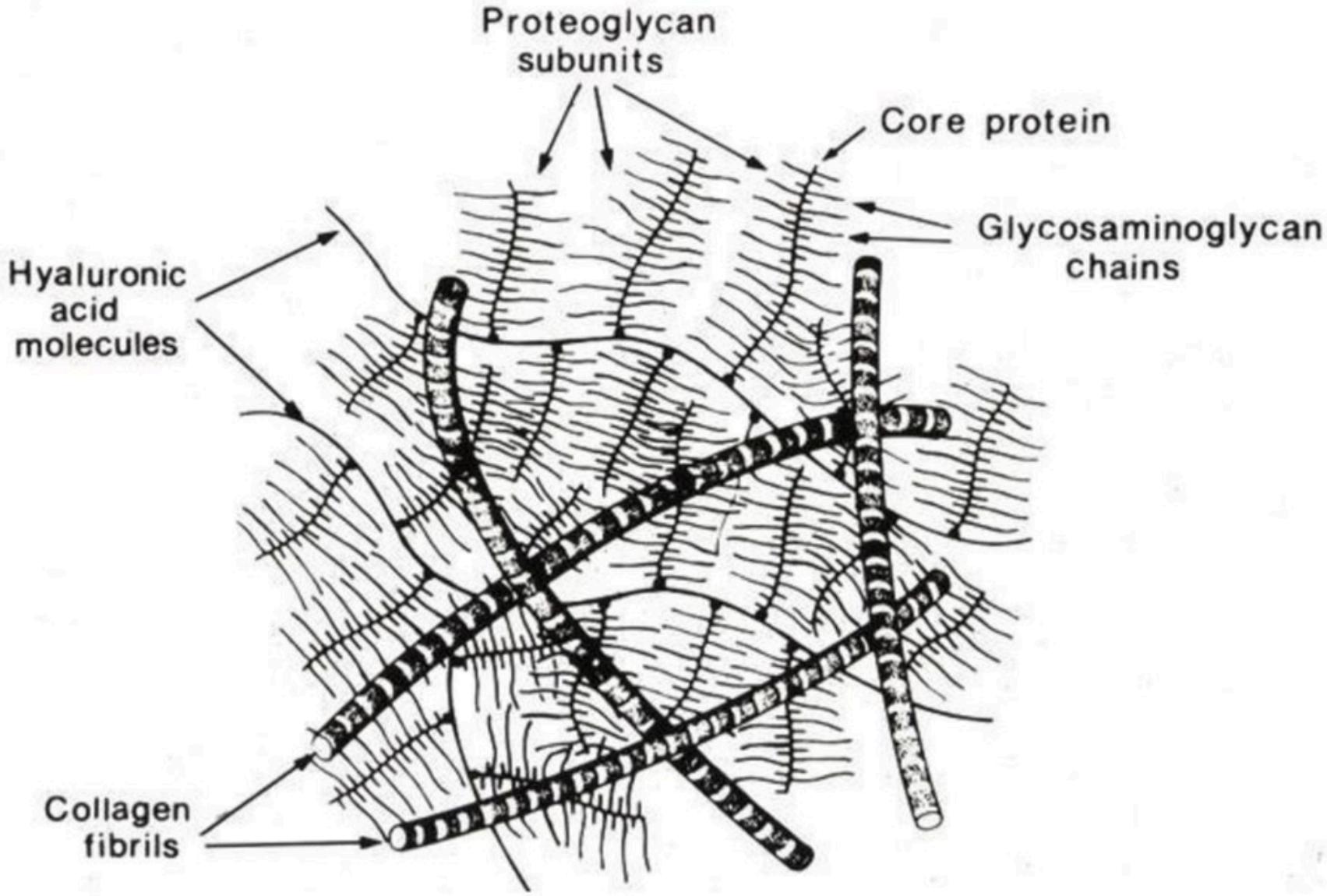
Cartilage ECM is composed of:

- Collagen and/or elastic fibers
- Lots of GAGs and proteoglycans

These substances make the ECM firm and resistant to mechanical forces.

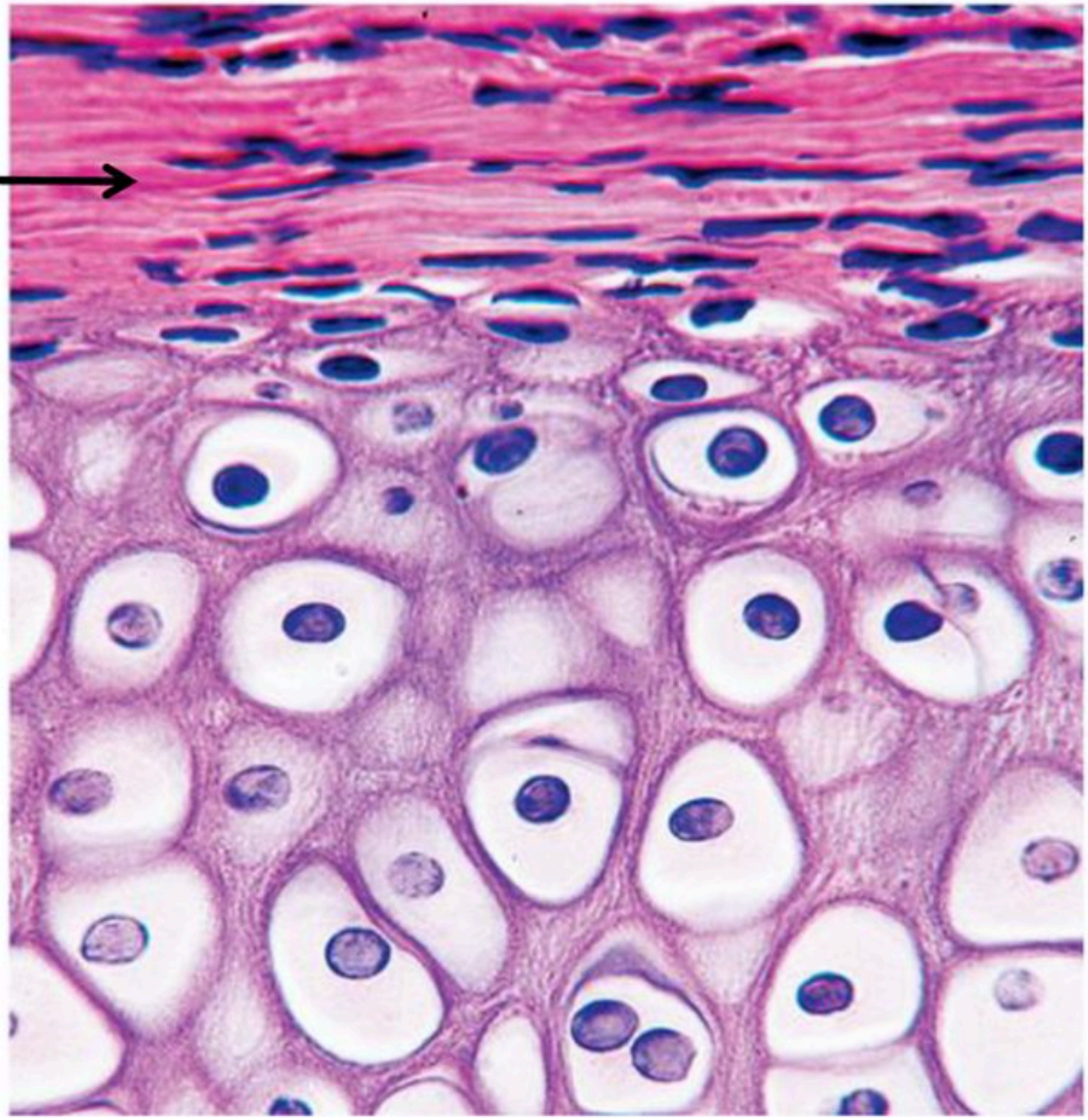


Composition of ECM in Cartilage

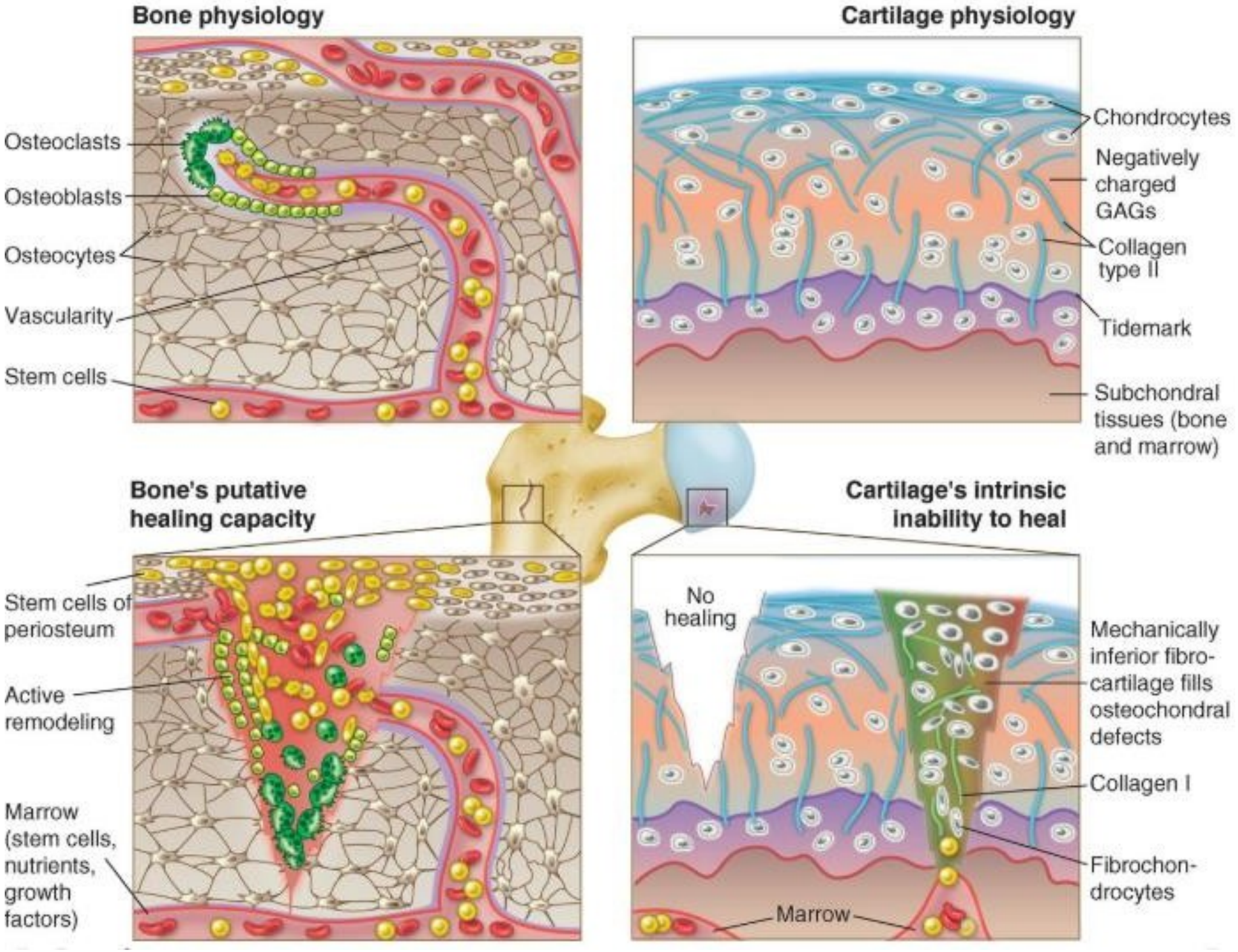


Perichondrium

- Perichondrium covers the surface of hyaline and elastic cartilage (but not fibrocartilage).
- Dense connective tissue composed of fibroblasts and type I collagen fibers.
- Contains blood vessels.



- The chondrocytes rely on a process of diffusion to obtain their needed nutrients.
- **Unlike bones, cartilage is avascular, meaning that is has no blood vessels for the transport of fresh blood to the cartilage.**
- Due to this lack of blood supply, cartilage **has a much longer healing process** and time when compared to the healing time of bones.
- The base structure of cartilage when observed under a microscope, is vastly less organized that the structure of a bone, further complicating the healing time of cartilage.


Bone physiology
Osteoclasts
Osteoblasts
Osteocytes
Vascularity
Stem cells
Cartilage physiology
Chondrocytes
Negatively charged GAGs
Collagen type II
Tidemark
Subchondral tissues (bone and marrow)
Bone's putative healing capacity
Stem cells of periosteum
Active remodeling
Marrow (stem cells, nutrients, growth factors)
Cartilage's intrinsic inability to heal
No healing
Mechanically inferior fibro-cartilage fills osteochondral defects
Collagen I
Fibrochon-drocytes
Marrow

1. Pre-chondrogenic stage

Main ECM components: hyaluronan, collagen type I and IIA

2. Aggregation and condensation into pre-cartilage nodules

Main ECM components: hyaluronan, collagen type I and IIA, fibronectin, tenascins and thrombospondins

3. Overt chondrocyte differentiation

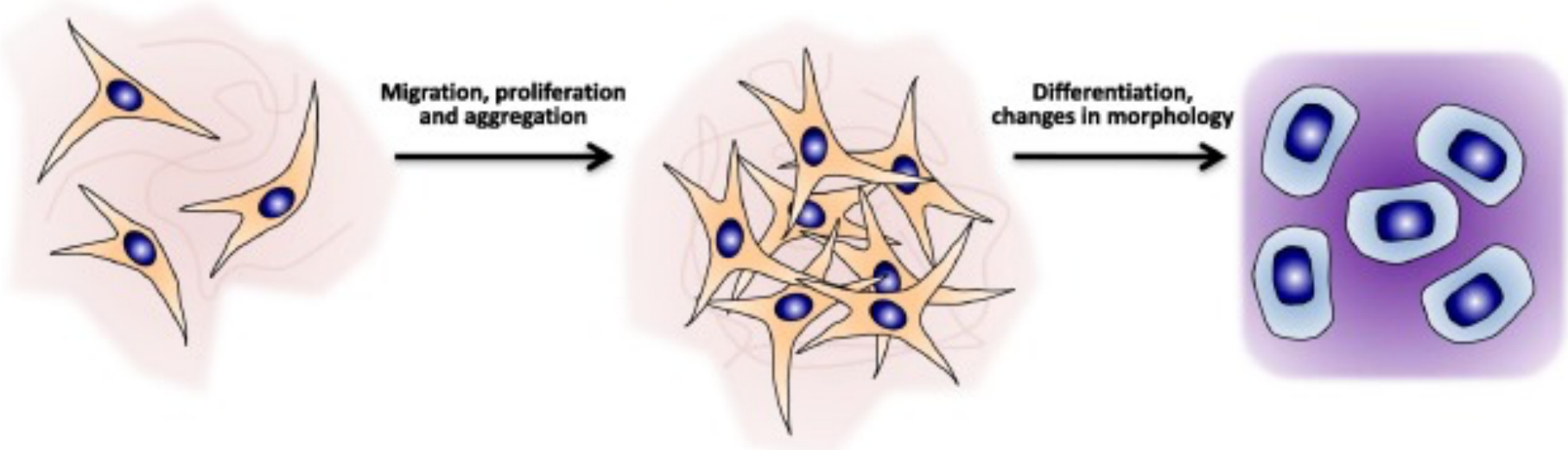
Main ECM components: hyaluronan, collagen type IIB, IX, and XI, and aggrecan

Transcription factors: Sox9, L-Sox5, Sox6, Runx2, HIF-2α, Barx2, Nkx3.2/Bapx1, Msx1 and 2, β-catenin, Smads, Lef1, C/EBPβ, AP-1 and AP-2, CREB

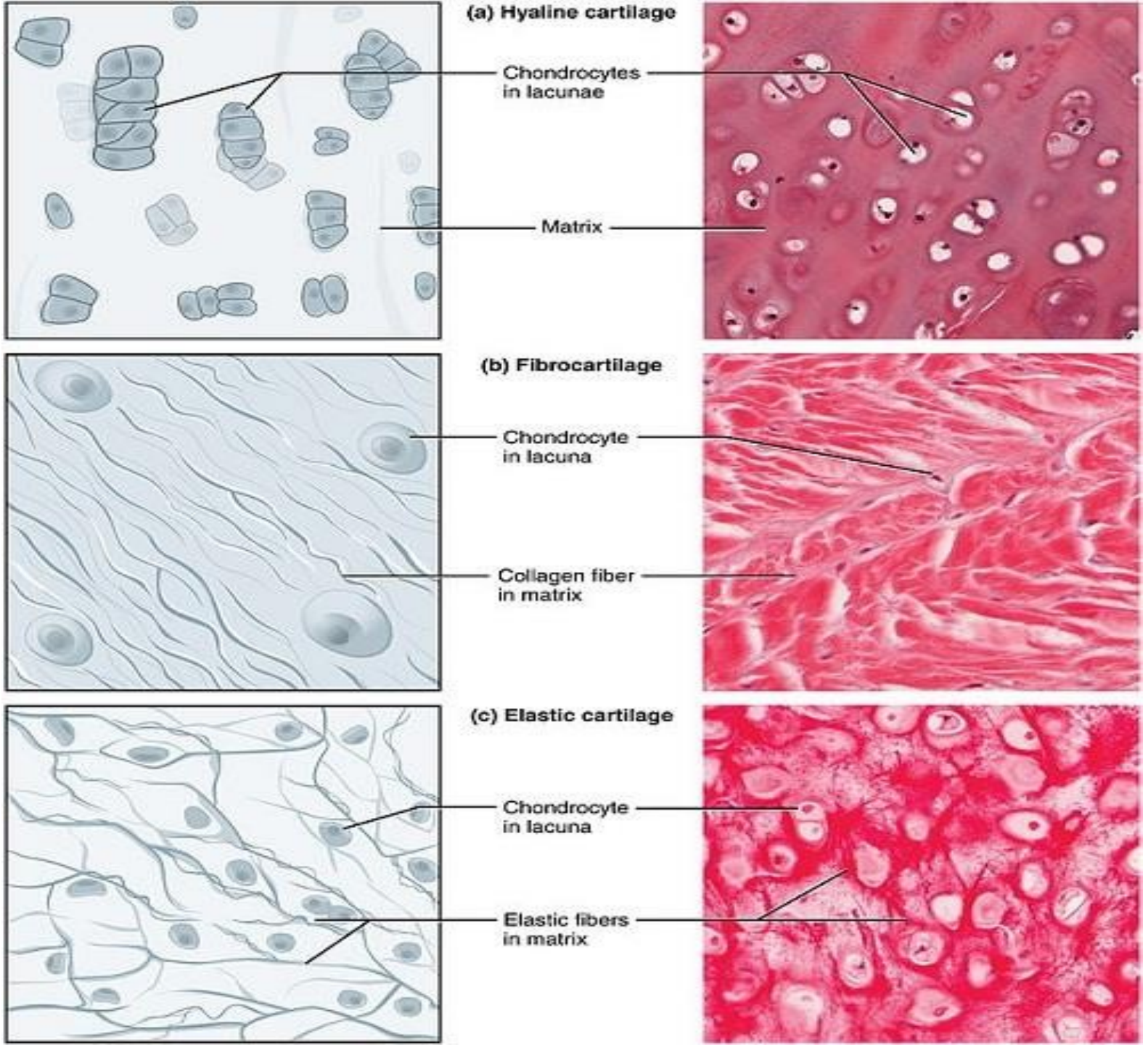
Extracellular signaling molecules: FGF, hedgehog, TGF-β1, 2 and 3, BMPs, PDGF, IGF, EGF, RA, Wnts, Jagged-1 and 2, DLL-1, 2 and 3, PTHrP

Protein kinases/phosphoprotein phosphatases: MAPKs (p38, ERK/2, JNK), PKA, PKC, ROCKI and II, PP1, PP2A, PP2B (calcineurin)

Cell adhesions and junctions: N-CAM, N-cadherin, gap junction



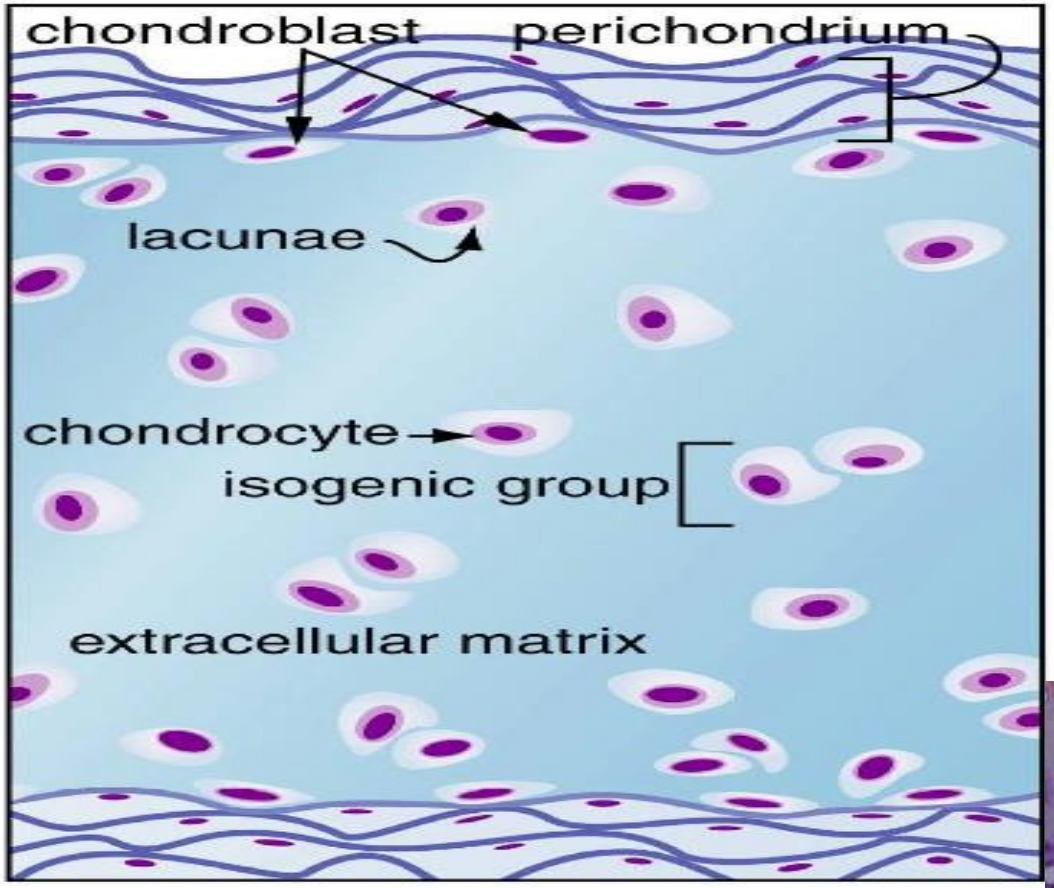
- There are 3 main types of cartilage that make up the human body:
- **Hyaline Cartilage**– the most common, and is found on the ribs, nose, larynx, trachea, and is the pre-cursor to a bone.
- **Fibrocartilage** – is the strongest cartilage, and is found in the in intervertebral discs, joint capsules and ligaments.
- **Elastic Cartilage** – provides strength and shape maintenance, and can be found in the external ear, epiglottis and the larynx.

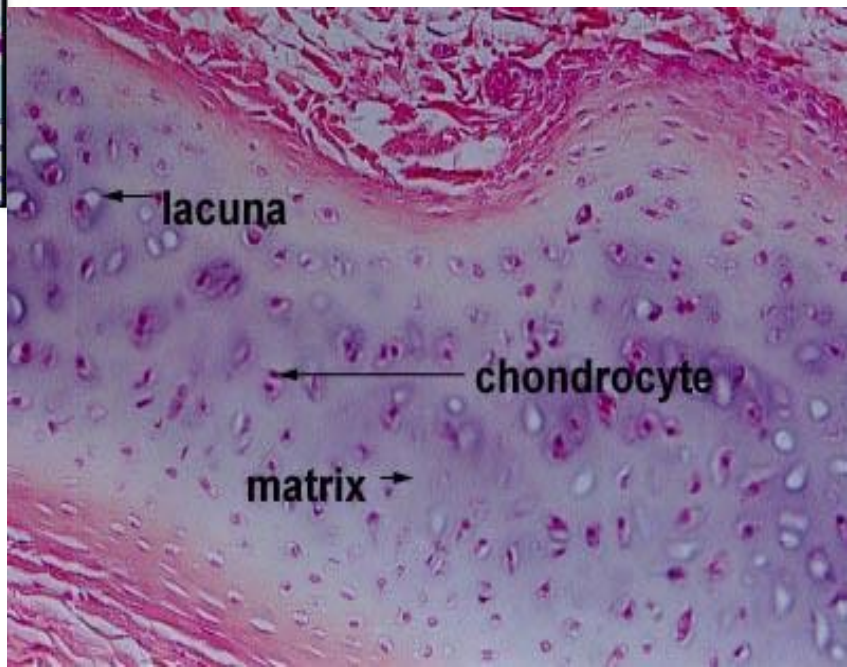

(a) Hyaline cartilage
Chondrocytes in lacunae
Matrix
(b) Fibrocartilage
Chondrocyte in lacuna
Collagen fiber in matrix
(c) Elastic cartilage
Chondrocyte in lacuna
Elastic fibers in matrix

Hyaline Cartilage

- Of the three main cartilages found in the body, in adults, hyaline cartilage is the most widespread.
- Forming the articular surfaces of long bones, between the ribs, the rings of the trachea in the throat and some parts of the skull.
- Hyaline cartilage has been given its name due to its glossy appearance, and is predominantly made up of collagen, although it displays few collagen fibers.
- In an embryo, hyaline cartilage is first formed before the bones solidify.

Hyaline Cartilage



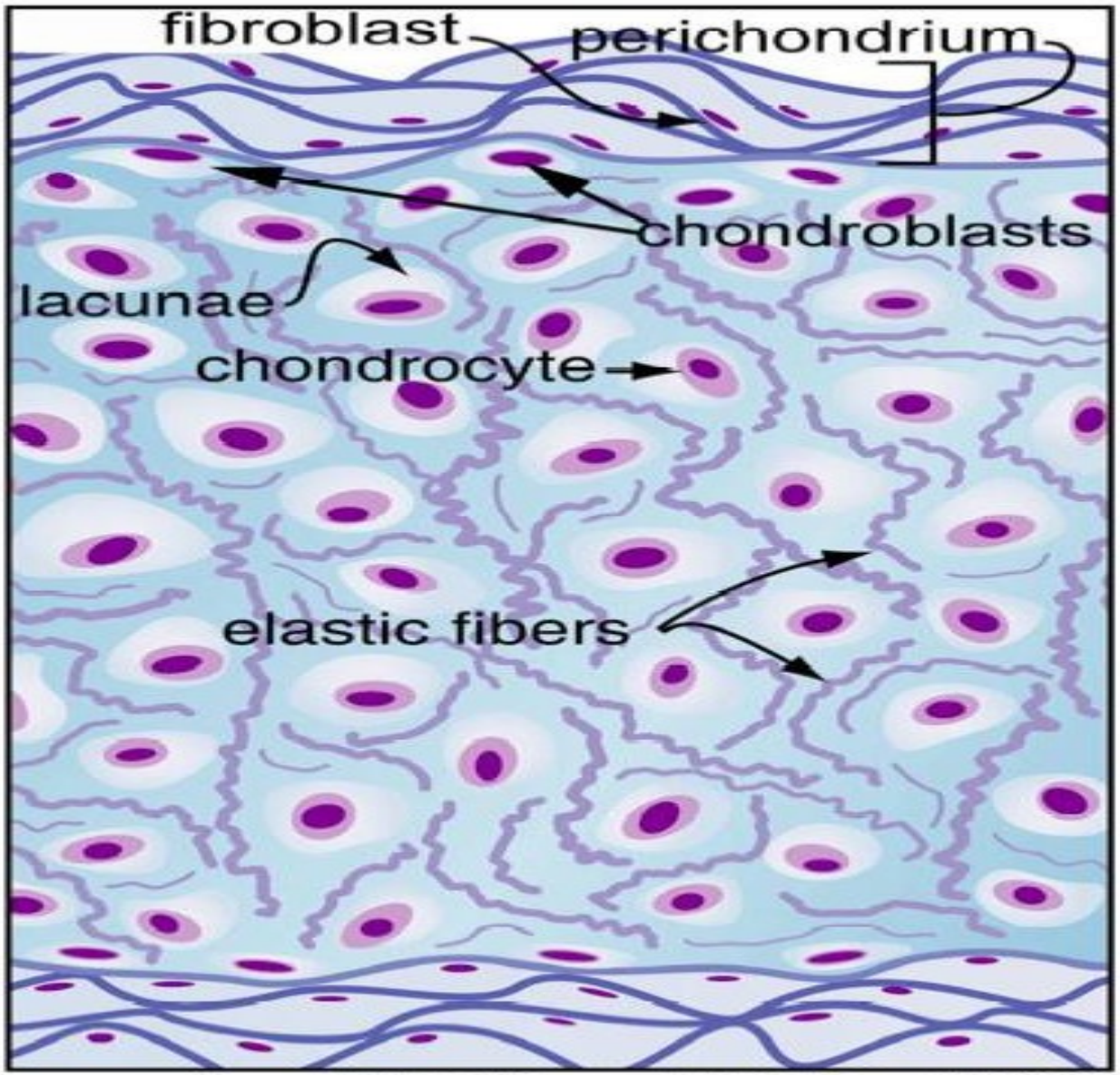


- The most common site where hyaline cartilage is found in adults include:
- The ***upper respiratory tract***: the nose, larynx, trachea and bronchi. Cartilage is used in these areas to prevent the airways from collapsing during inhalation.
- The ***articulating surfaces of bones:*** the cartilage here prevents bones from rubbing and creating friction against another bone as part of a synovial joint.
- The ***epiphyseal plates of bones:*** these are the growth plates attached to the ends of the body's long bones. They assist during adolescent growth and are replaced by solid bone once growth is complete.

Elastic Cartilage

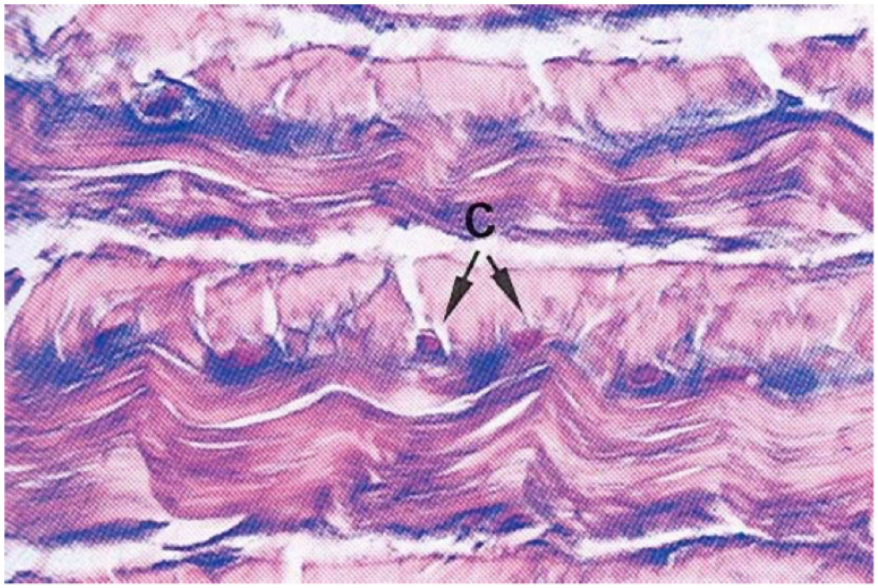
- Elastic cartilage, also known as yellow cartilage, is made up of networks of elastic and collagen fibers of which the principal proteins are elastin.
- Elastic cartilage has a high concentration of elastin fibers arranged in an extracellular matrix structure, and unlike hyaline cartilage, **it does not calcify for the formation of bones.**

Elastic Cartilage



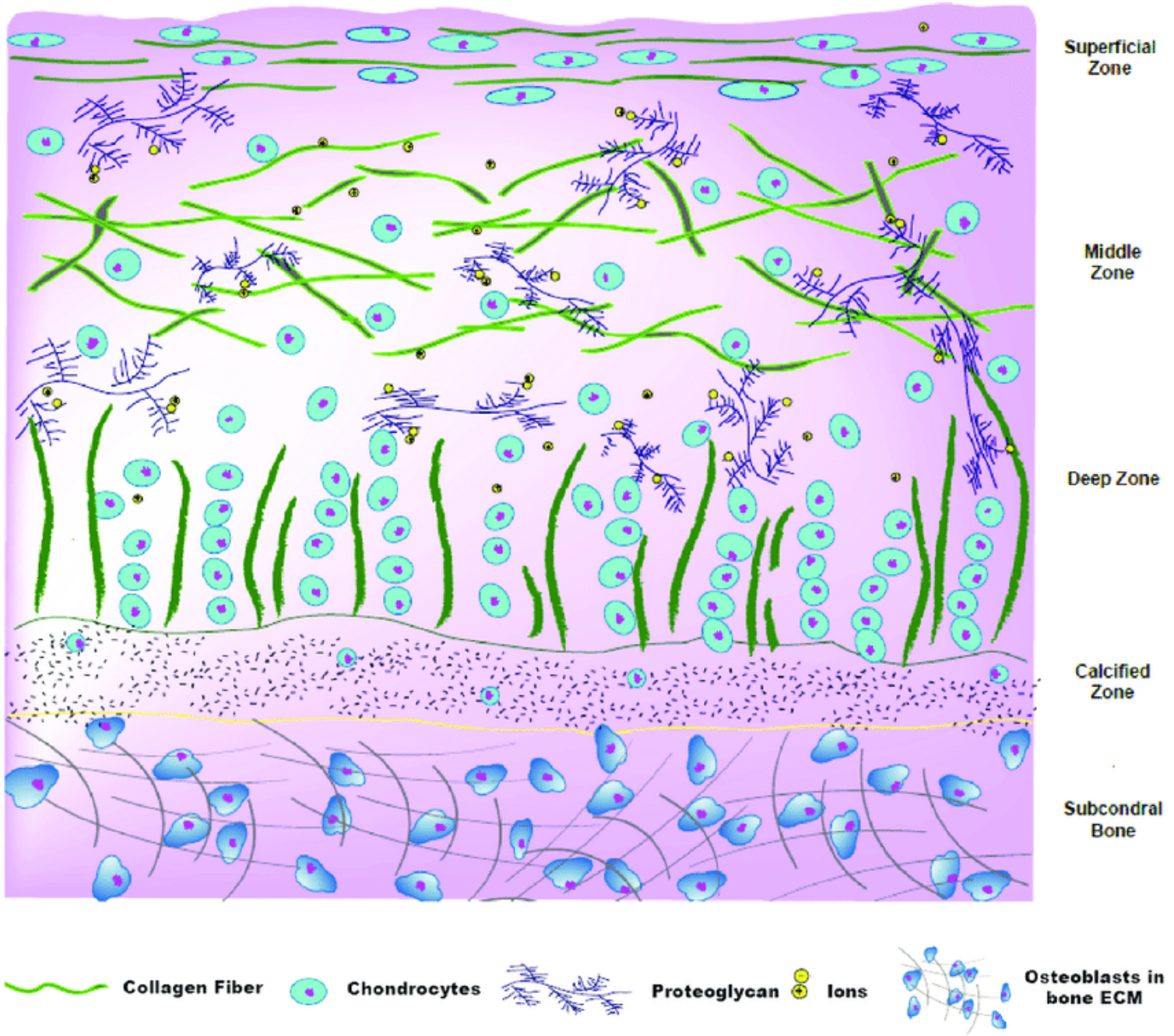
Fibrous cartilage

- Found in intervertebral disc, pubic symphysis, intrarticular disc of certain joints, menisci of knee joint & articular cartilage of temporomandibular cartilage
- Consists of bundles of collagen fibers embedded in minimal amount of matrix
- Cells are usually placed single in between the bundles of collagen fibers
- Not covered with perichondrium



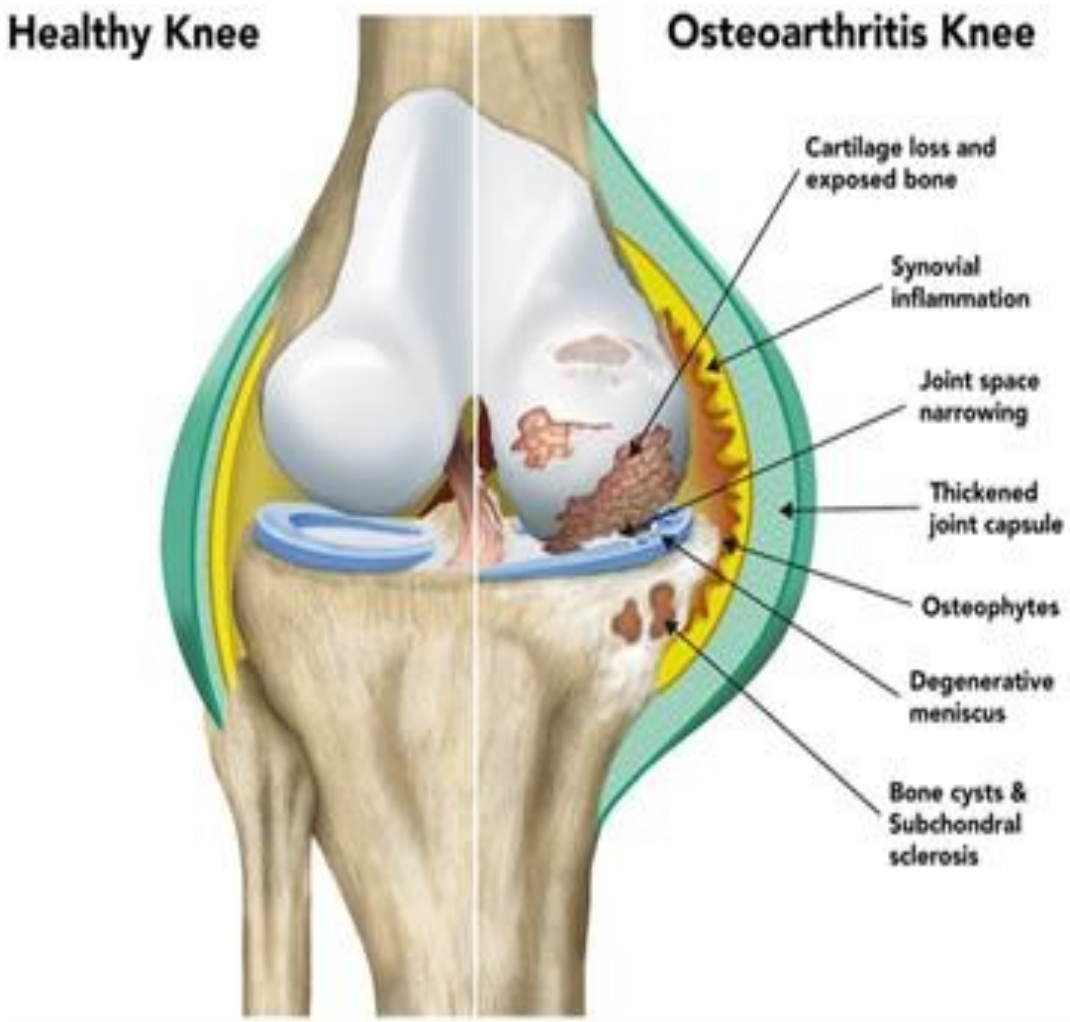
Articular Cartilage

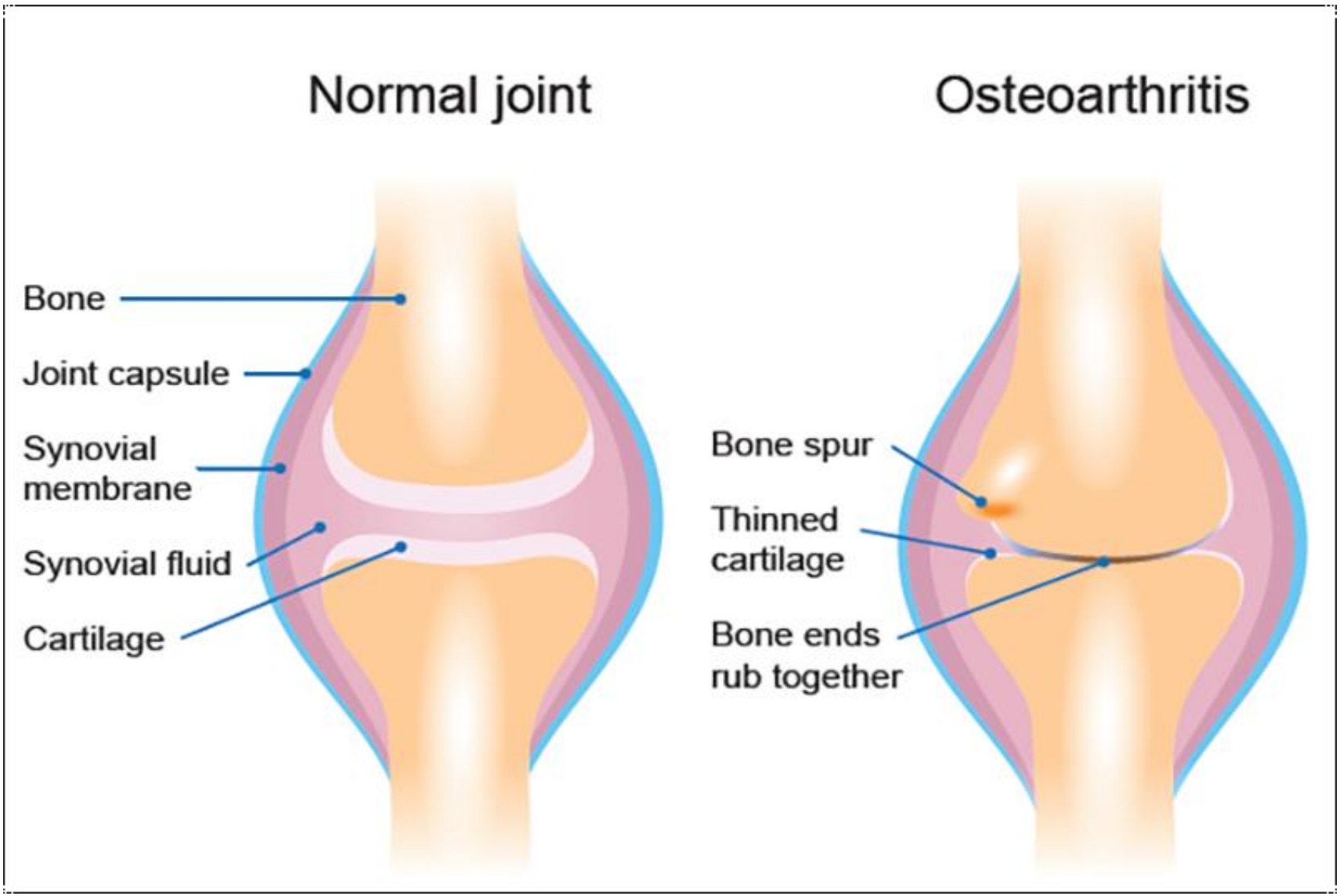
- Articular cartilage is a non-vascularized and poorly cellularized connective tissue that is frequently damaged as a result of trauma and degenerative joint diseases such as osteoarthritis.
- Because of the absence of vascularization, articular cartilage has low capacity for spontaneous repair.
- Today, and despite a large number of preclinical data, no therapy capable of restoring the healthy structure and function of damaged articular cartilage is clinically available.


Superficial Zone
Middle Zone
Deep Zone
Calcified Zone
Subcondral Bone
Collagen Fiber
Chondrocytes
Proteoglycan
Ions
Osteoblasts in bone ECM

Osteoarthritis

Osteoarthritis is accompanied by degradation of the articular cartilage, thickening of the subchondral bone, formation of osteophytes and variable degrees of synovium inflammation




Normal joint
Osteoarthritis
Bone
Joint capsule
Synovial membrane
Synovial fluid
Cartilage
Bone spur
Thinned cartilage
Bone ends rub together

Cartilage Tissue Engineering

- The ideal biomaterial should be biocompatible to prevent inflammatory and immunological reactions.
- It must provide a favorable environment for the 3D maintenance of chondrocyte phenotype and be adhesive to enable attachment of the cells within the lesion.
- It must be permeable to allow the diffusion of molecules, nutrients and growth factors.
- Finally, it should be biodegradable enough to be integrated in the physiological processes of tissue remodeling and ideally, **should be injectable**, thereby allowing for implantation by minimally invasive surgery.

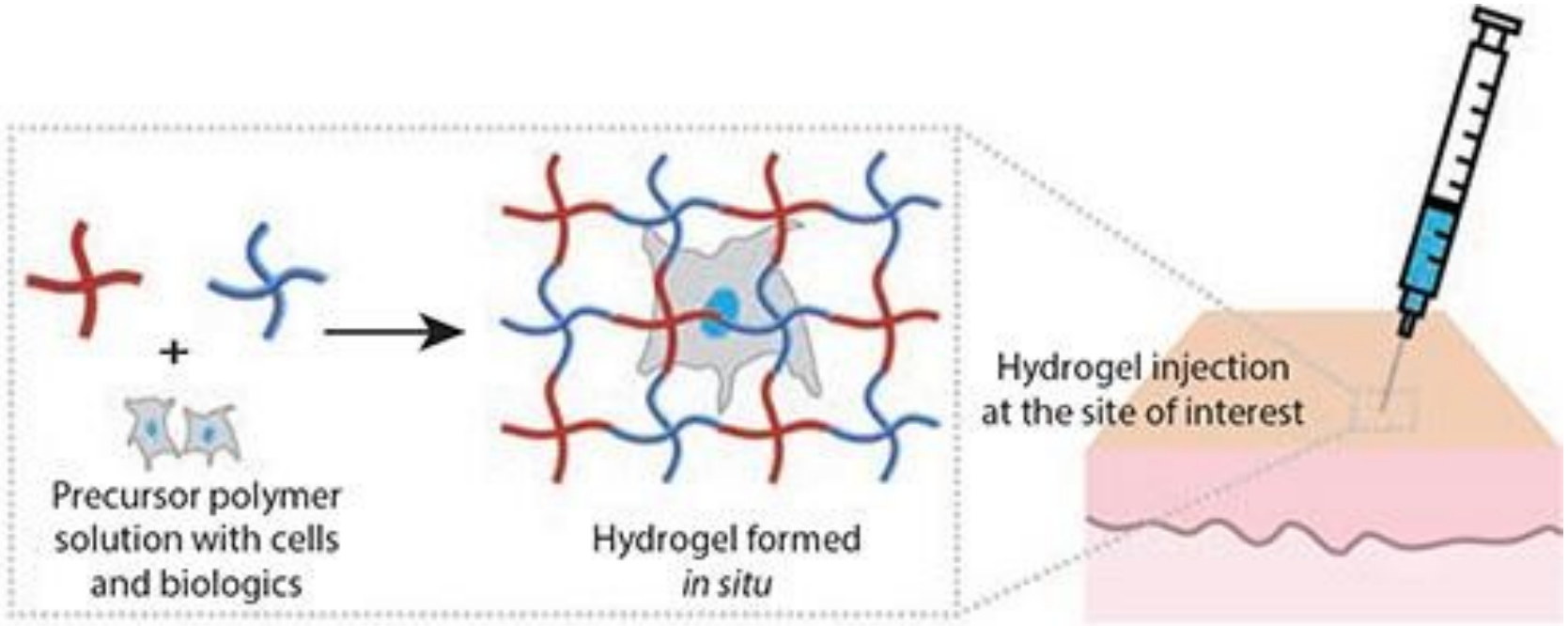
Table 1. Principal matrices used in cartilage engineering.

Matrices		
Type	**Component**	**Commercial product name**
Protein	Collagen	MACI®, Maix®, Atelocollagen®, MaioRegen®
	Fibrin	Tissucol kit®
	Silk	
Polysaccharides	Hyaluronic acid	HYAFF-11®
	Chitosan	BST-CarGel®
	Cellulose	
	Alginate	
Synthetic	Poly(lactic-co-glycolic acid)	Bio-Seed®-C
	Polylactic acid	
	Polyethylene glycol	

- Despite the large number of preclinical and clinical studies related to biomaterials for cartilage tissue engineering, **none of the solid matrices may be injected percutaneously.**
- In this context, recent studies are directed toward the development of **self-hardening and injectable materials that can be used in the percutaneous transplantation of chondrogenic cells.**

Hydrogels

- The properties and structures of hydrogels make them ideal candidates for implantation by minimally invasive surgery.
- Hydrogels are composed of chains of **synthetic or natural macromolecules capable of forming hydrogels after physical, ionic or covalent crosslinking.**
- They exhibit a high level of hydration close to that of the articular cartilage, which allows them to mimic the 3D environment of chondrocytes



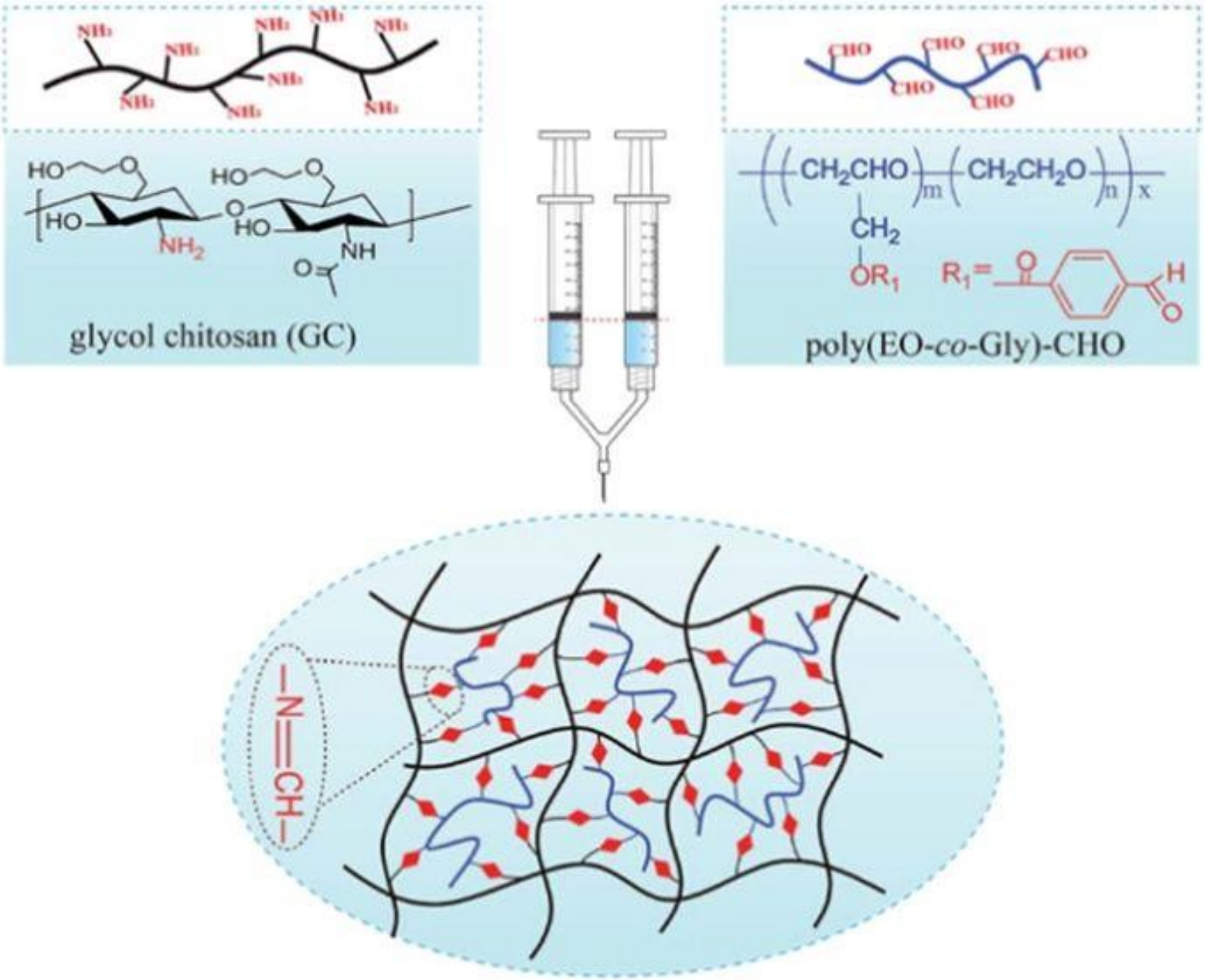
Building Blocks for Hydrogel Preparation
- Hyaluronic acid
- Chitasan
- Alginate
- Poly(ethylene glycol)
- Poly(*N*-isopropyl-acrylamide
- Proteins/peptides

Chemical Crosslinking
- Azide-alkyne
- Thiol-ene
- Diels-alder
- Oxime reactions

Physical Crosslinking
- Ionic interactions
- Thermoresponsive
- Hydrophobic interactions

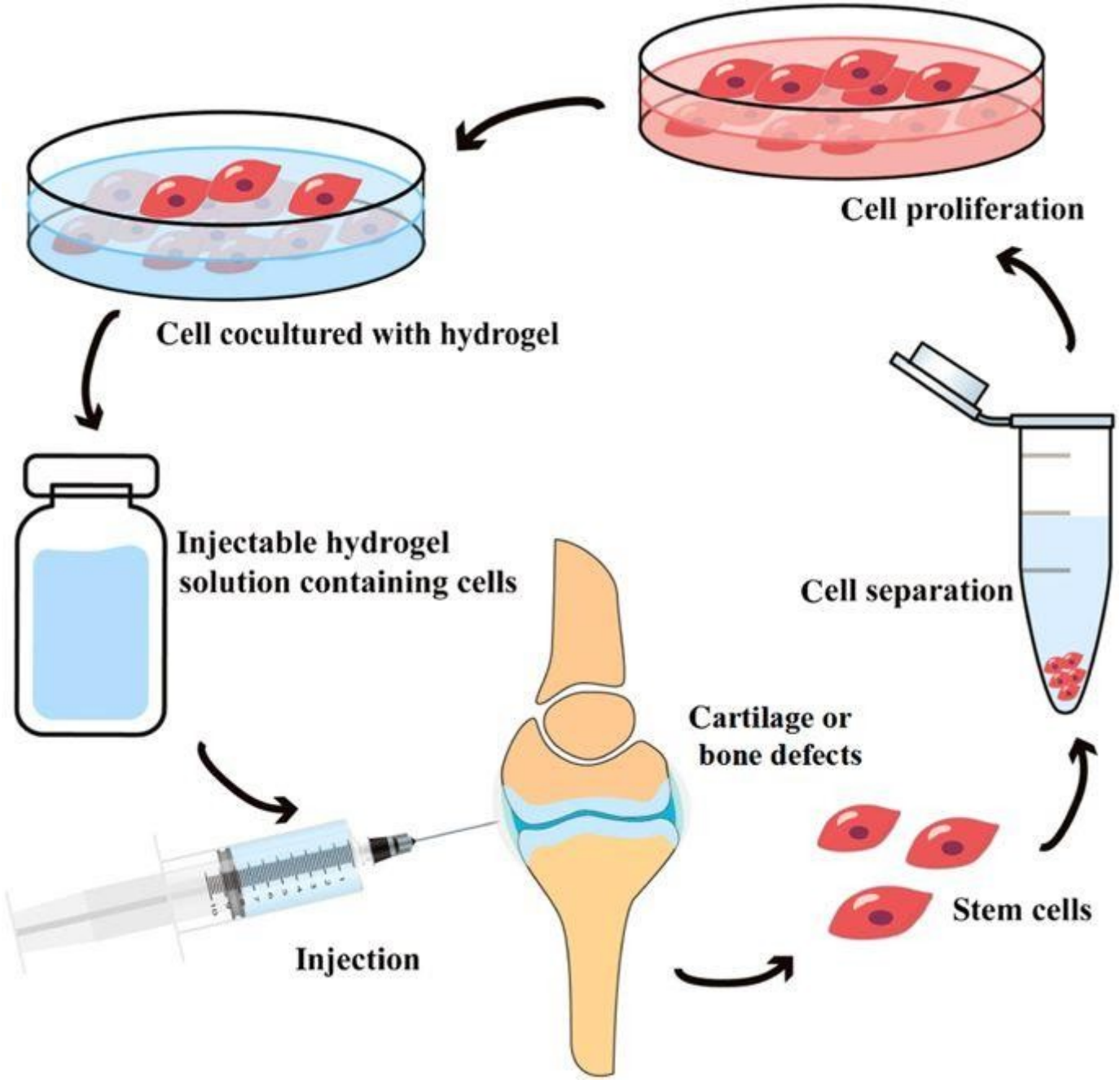
Cargo
- Therapeutic cells
- Growth factors
- Therapeutic peptides and proteins

Applications
- Bone regeneration
- Cartilage repair
- Cardiac regeneration
- Spinal cord repair


glycol chitosan (GC)
poly(EO-co-Gly)-CHO
—N=CH—

- As a scaffolding material for cartilage engineering, hydrogels have many advantages:

- They can exhibit similar mechanical, swelling, and lubricating behavior to articular cartilage
- their viscoelastic nature facilitates the transfer of mechanical loading ; and they allow their loaded cells to take on a spherical morphology, which is characteristic of the chondrogenic phenotype.
- Cell-laden hydrogels have also been extensively investigated for use as bioink for the 3D bioprinting of cartilage with biomimetic structure and function.
- Advanced hydrogels have been designed that permit the controlled release of chondroinductive factors or chondroprotective drugs.


Cell proliferation
Cell cocultured with hydrogel
Injectable hydrogel
solution containing cells
Cell separation
Cartilage or
bone defects
Stem cells
Injection

Cells

- Chondrocytes, fibroblasts, stem cells and genetically modified cells have all been tested in cartilage engineering.
- However, **chondrocytes and mesenchymal stem cells (MSCs)** remain the most largely investigated sources of chondrogenic cells for cartilage repair.
- Adult chondrocytes having the ability to form an ECM can be isolated from various sources such as articular cartilage, nasal septum, costal cartilage or auricular cartilage
- Nevertheless, auricular chondrocytes are derived from an elastic cartilaginous tissue, which does not have the mechanical properties of hyaline cartilage

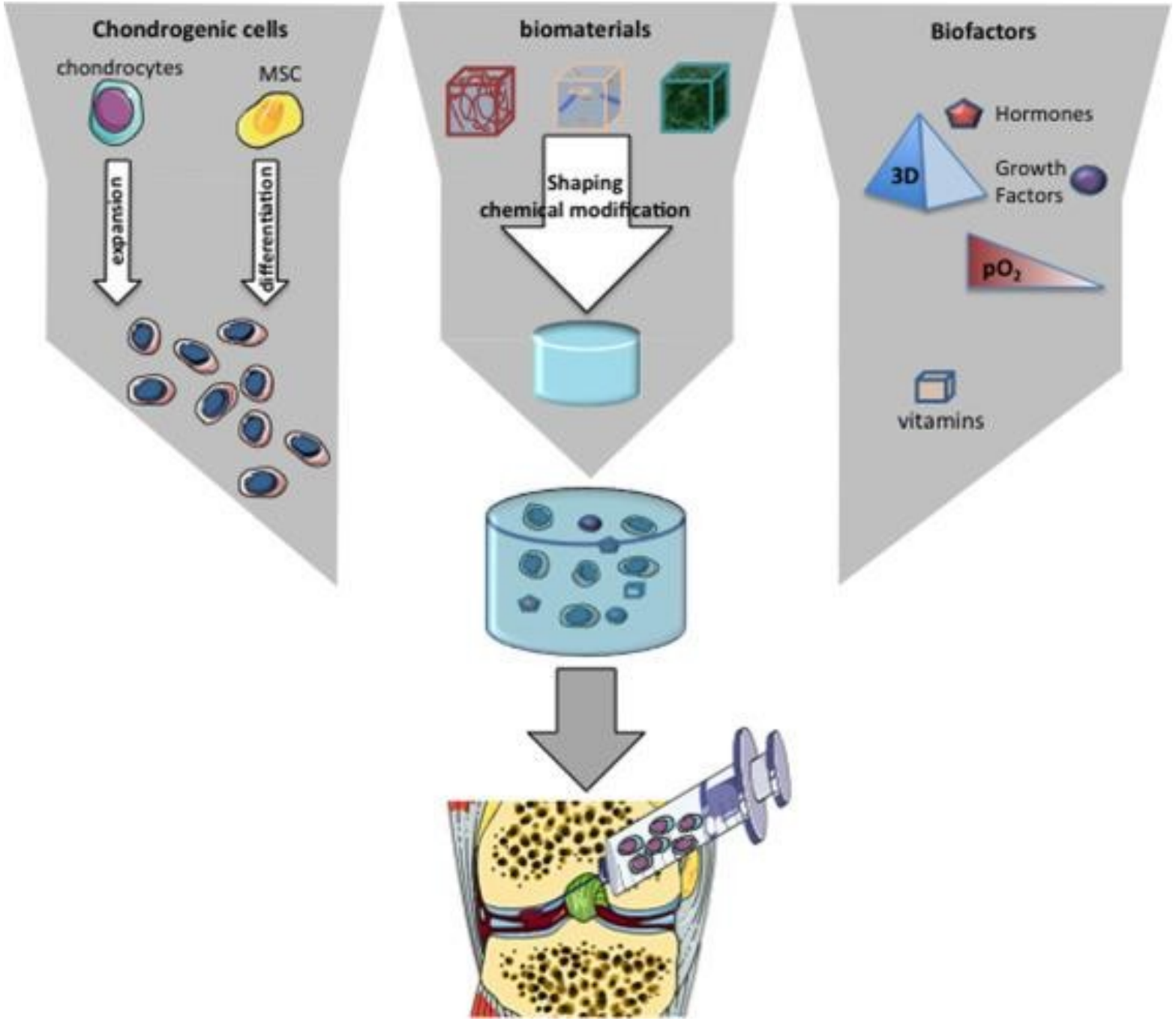
- One of the main drawbacks in the use of autologous chondrocytes is instability of their phenotype in monolayer culture.
- Indeed, chondrocytes cultured in monolayer rapidly lose their phenotype, as evidenced by the loss of expression of chondrocytic markers such as type II collagen, aggrecan and the superficial zone protein.
- This loss is accompanied by the orientation of cells toward a fibroblastic phenotype characterized by increased expression of type I collagen and adoption of the characteristic spindle shape of fibroblasts

Mesenchymal Stem Cells

- MSCs can give rise to several types of differentiated cells, including chondrocytes, adipocytes, osteoblasts, and myogenic and neuronal cells
- The most-studied source of MSCs is the bone marrow, but MSCs have also been isolated from muscles, adipose tissue, periosteum and perichondrium
- **In addition, MSCs isolated from bone marrow, adipose tissue, muscle and synovium showed chondrogenic differentiation ability.**
- Consequently, adult MSCs have been considered of major interest in tissue engineering

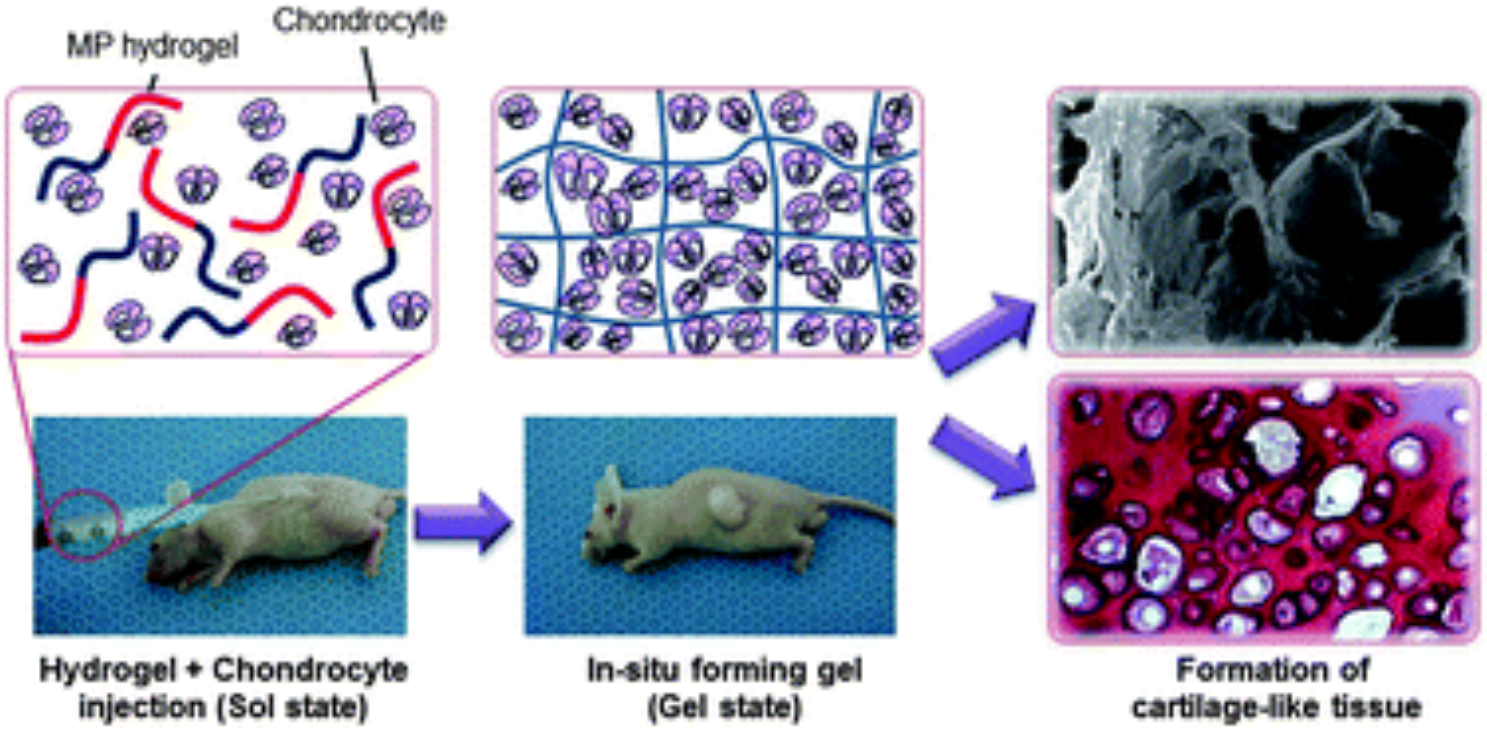
Growth Factors

- Several growth factors involved in the development of cartilage growth were identified as factors deeply affecting the chondrogenic differentiation of MSCs.
- Among these factors, the transforming growth factor β (TGF-β) superfamily members TGF-β1 and -β3 are the most commonly used to **induce the chondrogenesis of adult MSCs.**
- Alternatively, the use of bone morphogenetic protein with or without TGF-β has been extensively investigated


Chondrogenic cells
chondrocytes
MSC
expansion
differentiation
biomaterials
Shaping
chemical modification
Biofactors
Hormones
3D
Growth
Factors
pO2
vitamins

Growth Factors

- Articular cartilage is avascular and develops naturally in the presence of a low oxygen tension (hypoxia) ranging from 2% to 7%.
- Furthermore, hypoxia can upregulate the chondrocytic phenotype by hypoxia inducible factors (HIFs), which promote the expression of cartilage ECM genes. Therefore, placing MSCs in a hypoxic condition can promote the chondrogenesis of MSCs


MP hydrogel
Chondrocyte
Hydrogel + Chondrocyte injection (Sol state)
In-situ forming gel (Gel state)
Formation of cartilage-like tissue

What is Cell and Tissue Culture

- Tissue culture is the general name for the removal of cells, tissues or organs from an animal or plant and their subsequent placement into artificial environment conductive to growth
- This environment usually consists of a suitable glass or plastic culture vessel containing a liquid or semi-solid support medium that supplies the nutrients essential for survival and growth

What is Cell and TissueCulture

- **When the cells are removed from the organ fragments, thus disrupting their normal relationship with neighboring cells, it is called cell culture**

WHAT IS CELL CULTURE USED FOR?

Areas where cell culture technology is currently playing a major role:-

1) **Model systems :** for studying basic cell biology, interactions between disease causing agents and cells, effects of drugs on cells, process and triggering of aging & nutritional studies

2) **Toxicity testing :** Study the effects of new drugs

3) **Cancer research :** Study the function of various chemicals, virus & radiation to convert normal cultured cells to cancerous cells

4) **Virology :** Cultivation of virus for vaccine production, also used to study there infectious cycle.

5) **Genetic Engineering :** Production of commercial proteins, large scale production of viruses for use in vaccine production e.g. polio, rabies, chicken pox, hepatitis B & measles

6) **Gene therapy :** Cells having a functional gene can be replaced to cells which are having non-functional gene

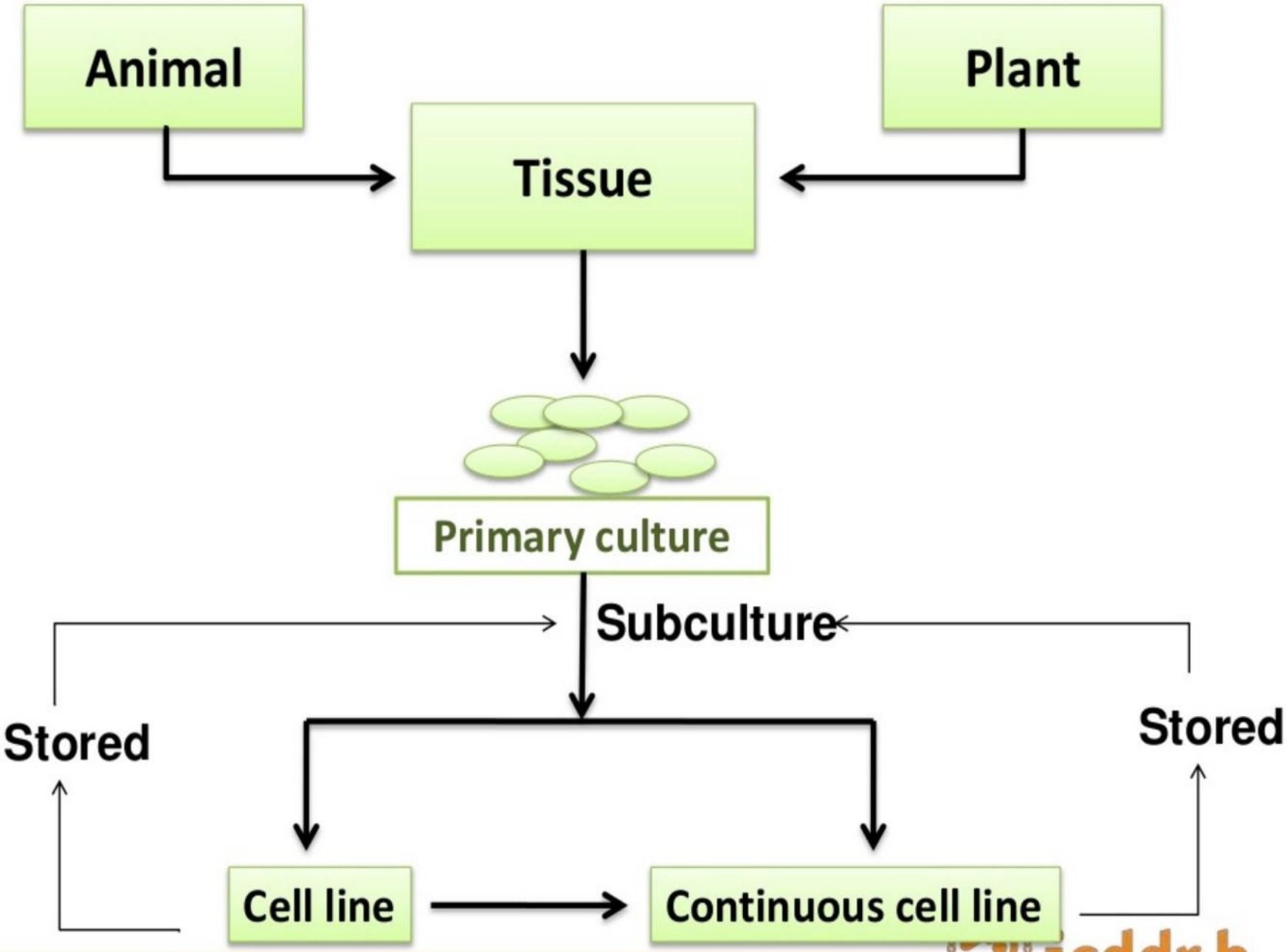
Classes of Culture Cells

- Cultures of animal cells are usually divided into 3 classes:

1. Primary cells
2. Cell lines
3. Continuous cell lines

1- Primary Culture

- When cells are surgically removed from an organism and placed into a suitable culture environment they will attach, divide and grow. This is called as primary culture
- Most of the primary culture cells have a finite lifespan of 5-10 divisions in vitro
- Due to their limited lifespan, one cannot do long-term experiments with these cells
- Primary cells are considered by many researchers to be more physiologically similar to in vivo cells

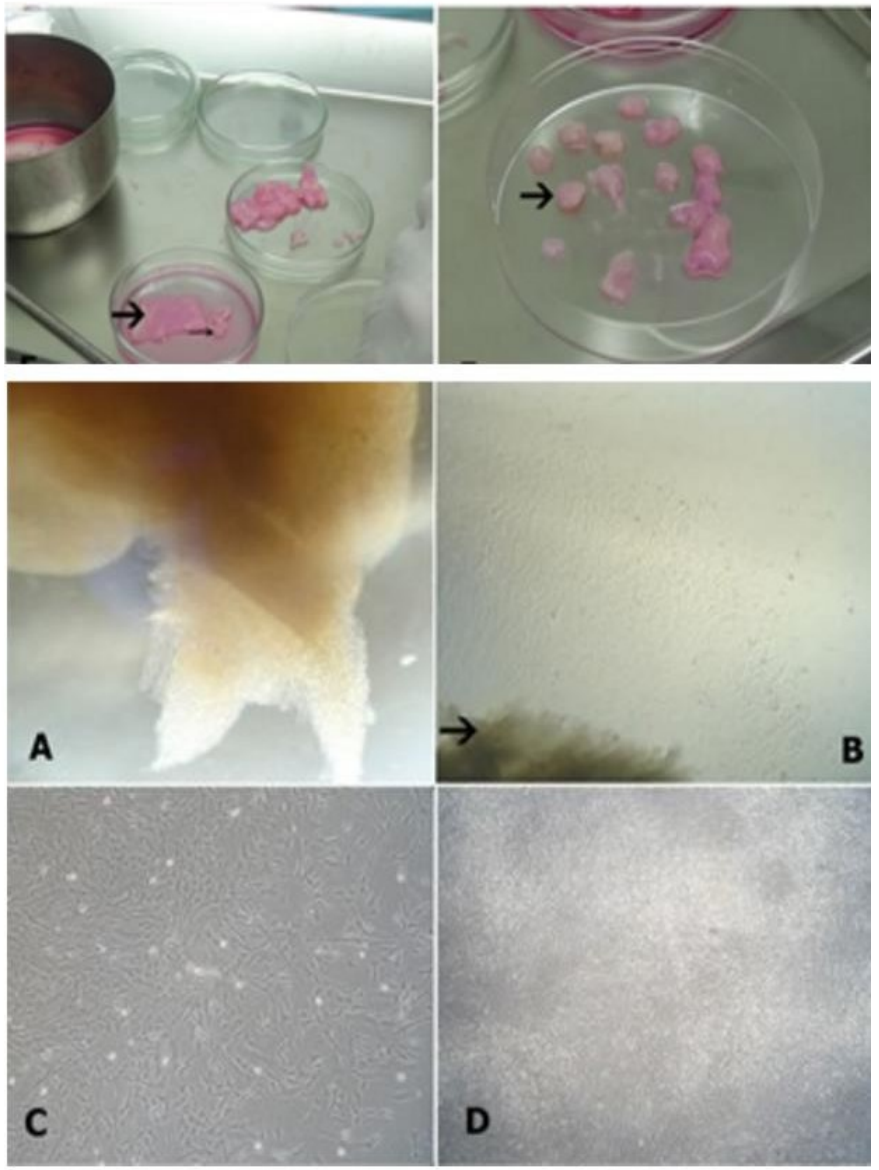

Animal
Plant
Tissue
Primary culture
Subculture
Stored
Stored
Cell line
Continuous cell line

Types of Cell culture

- Primary Cultures
  - Derived directly from excised tissue and cultured either as
    - Outgrowth of excised tissue in culture
    - Dissociation into single cells (by enzymatic digestion or mechanical dispersion)
  - Advantages:
    - **usually retain many of the differentiated characteristics of the cell in vivo**
  - Disadvantages:
    - initially heterogeneous but later become dominated by fibroblasts.
    - the preparation of primary cultures is labor intensive
    - **can be maintained in vitro only for a limited period of time**.

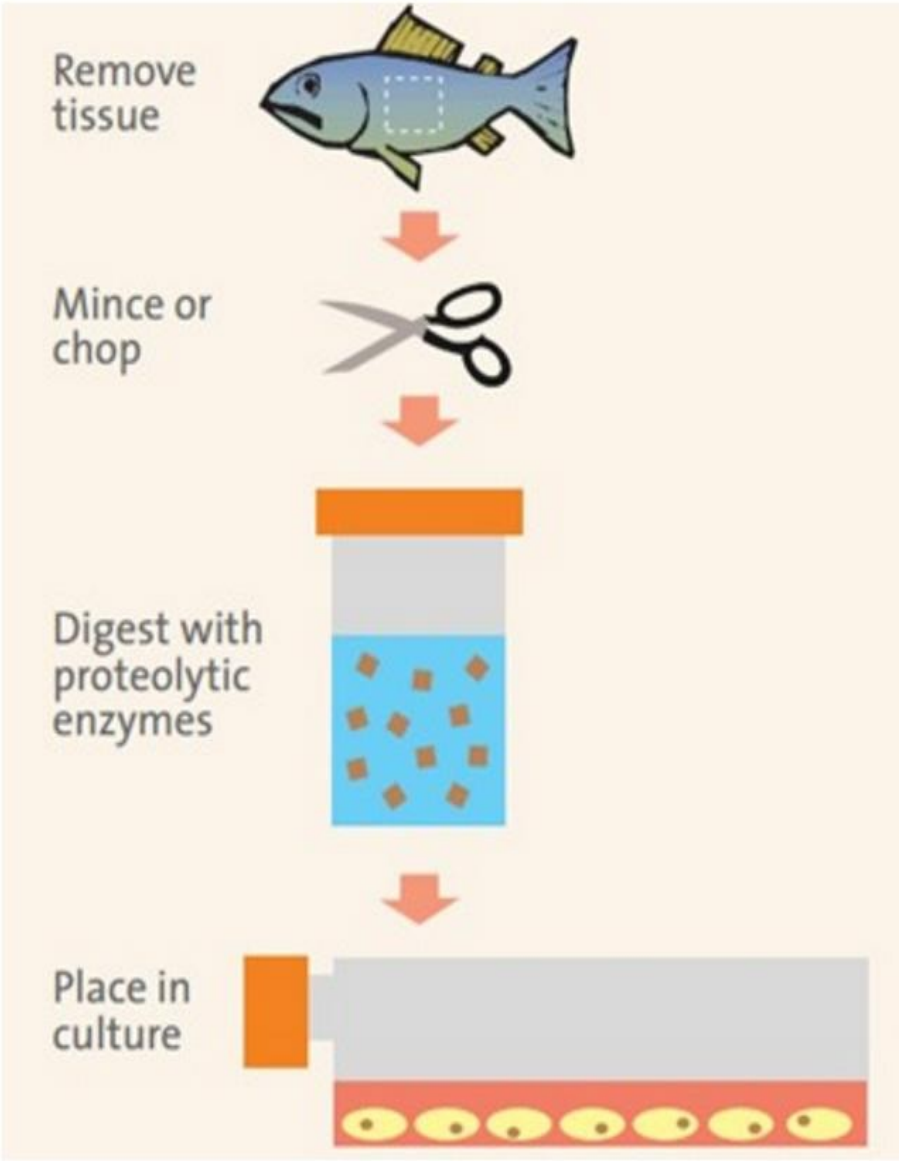
1- Primary Culture

- There are two basic methods for obtaining primary culture:
  1. **Explant cultures:**
     - Small pieces of tissue are attached (using plasma clots or fibrinogen) to a glass or treated plastic culture vessel and immersed in culture medium
     - After a few days individual cells will move from the tissue explant out onto the culture vessel surface or substrate where they will begin to divide and grow



-1 Primary Culture

2. **Enzymatic dissociation:**
   - More widely used
   - speeds up the process by adding digesting (proteolytic) enzymes such as trypsin or collagenase to the tissue fragments to breakdown bonds holding the cells together
   - This creates a suspension of single cells that are then placed into culture vessels containing culture medium and allowed to grow and divide

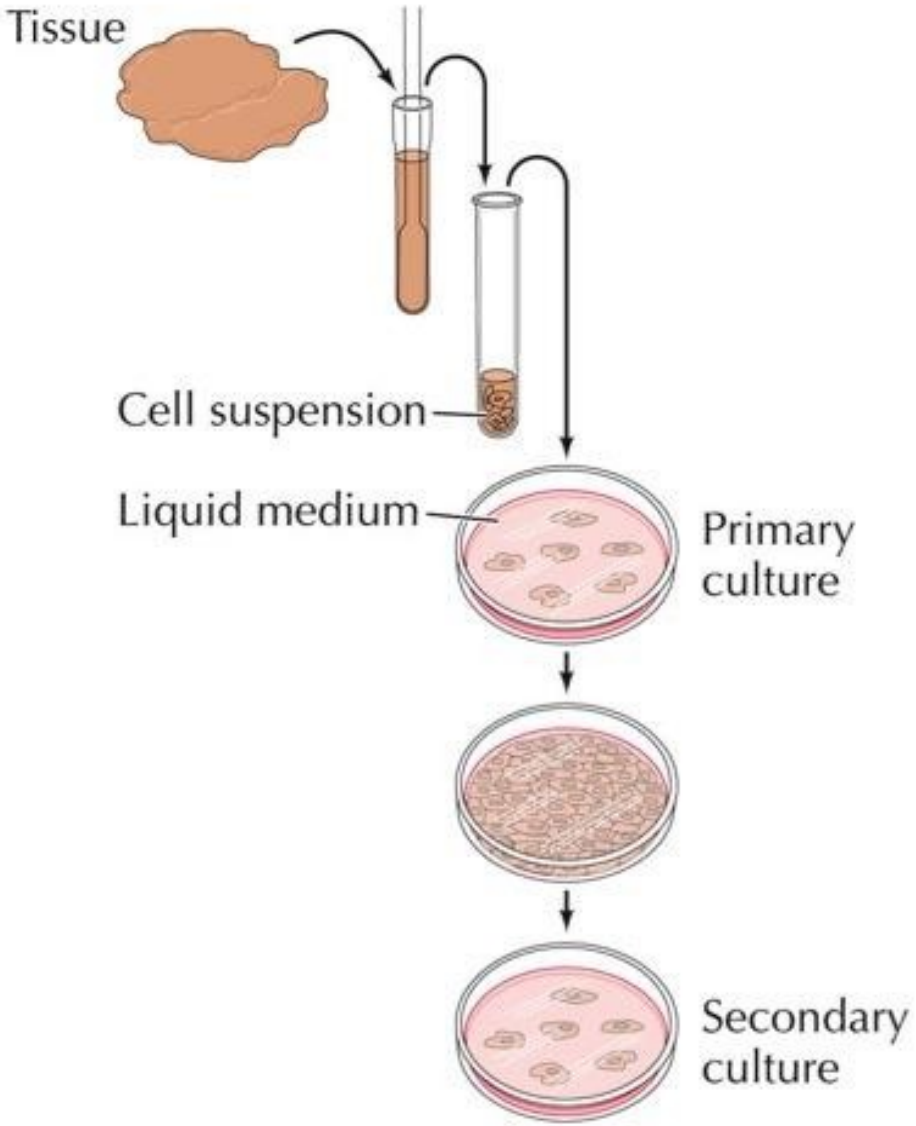


- In cell cultures, the next step after primary culture is subculture by passaging.
- Reproduction speeds decrease after cells in culture use the whole nutrients
- They consume a large proportion of the components in the breeding environment. Reproduction stops completely in time.
- In this period, the cul- ture division is the most appropriate process. This process is called subculture or passage.

Secondary Culture

- When a primary culture is sub-cultured, it becomes known as secondary culture or cell line.
- Subculture (or **passage**) refers to the transfer of cells from one culture vessel to another culture vessel.
- This is periodically required to provide fresh nutrients and growing space for continuously growing cell lines.
- The process involves removing the growth media and disassociating the adhered cells (usually enzymatically). Such cultures may be called secondary cultures.

1.41 Culture of animal cells



THE CELL, Fourth Edition, Figure 1.41 © 2006 ASM Press and Sinauer Associates, Inc.

Why sub culturing.?

- Once the available substrate surface is covered by cells (a confluent culture) growth slows & ceases.
- Cells to be kept in healthy & in growing state have to be sub-cultured or passaged
- It's the passage of cells when they reach to 80-90% confluency in flask/dishes/plates
- **Enzyme such as trypsin, dipase, collagenase in combination with EDTA breaks the cellular glue that attached the cells to the surface**

Continuous Cultures

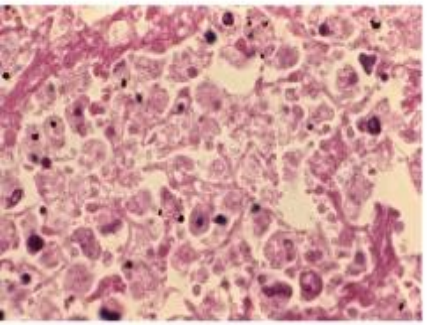
- derived from subculture (or passage, or transfer) of primary culture
  - Subculture = the process of dispersion and re-culture the cells after they have increased to occupy all of the available substrate in the culture
- usually comprised of a single cell type
- can be serially propagated in culture for several passages
- There are two types of continuous cultures
  - **Cell lines**
  - **Continuous cell lines**

Types of continuous culture

1) Cell lines
   - finite life, senesce after approximately thirty cycles of division (30 passages)
   - usually diploid and maintain some degree of differentiation.
   - it is essential to establish a system of Master and Working banks in order to maintain such lines for long periods

Types of continuous culture

2) Continuous cell lines
   - can be propagated indefinitely
   - generally have this ability because they have been transformed
     - tumor cells.
     - viral oncogenes
     - chemical treatments.
     - simultaneously
   - the disadvantage of having retained very little of the original *in vivo* characteristics



lung tissue

individual cells isolated from tissue

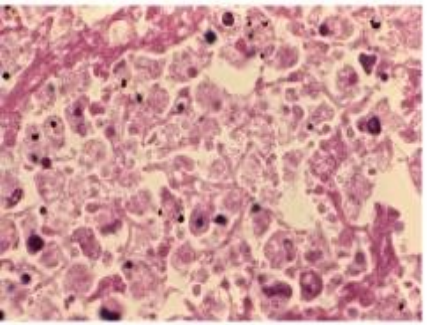
primary cell culture

contact inhibition

some cells transferred to new medium

secondary cell culture

(a)



lung tissue

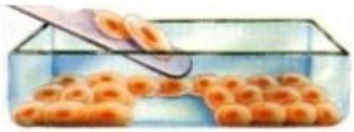
transformed cells or individual cells isolated from a tumor

continuous cell line

(b)

Demonstrating Contact Inhibition

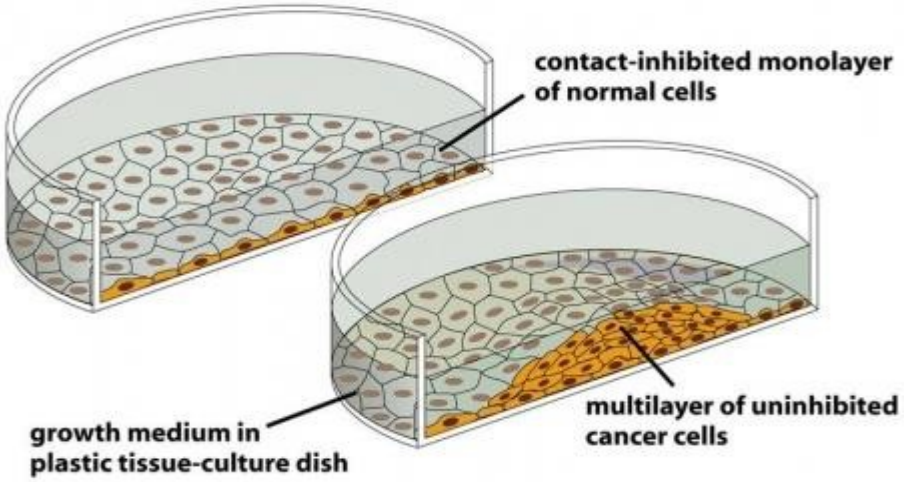
Normal cells in dish



When cells are removed, cells at the edge of gap begin to divide

Normal cells stop dividing when bottom of dish is covered.

Cancer cells continue to divide, piling on top of each other



Focus

COMMON CELL LINES

Human cell lines

- MCF-7 : breast cancer
- HeLa : Henrietta lacks cells

Mammalian cell lines

- Vero: African green monkey kidney (epithelial cells)
- BHK: Baby Hamster Kidney cells (fibroblast cells)
- MDCK: Mardin Darby Chicken Kidney cells

Insect cell lines

- C636: *Aedes albopictus* (mosquito cells line)

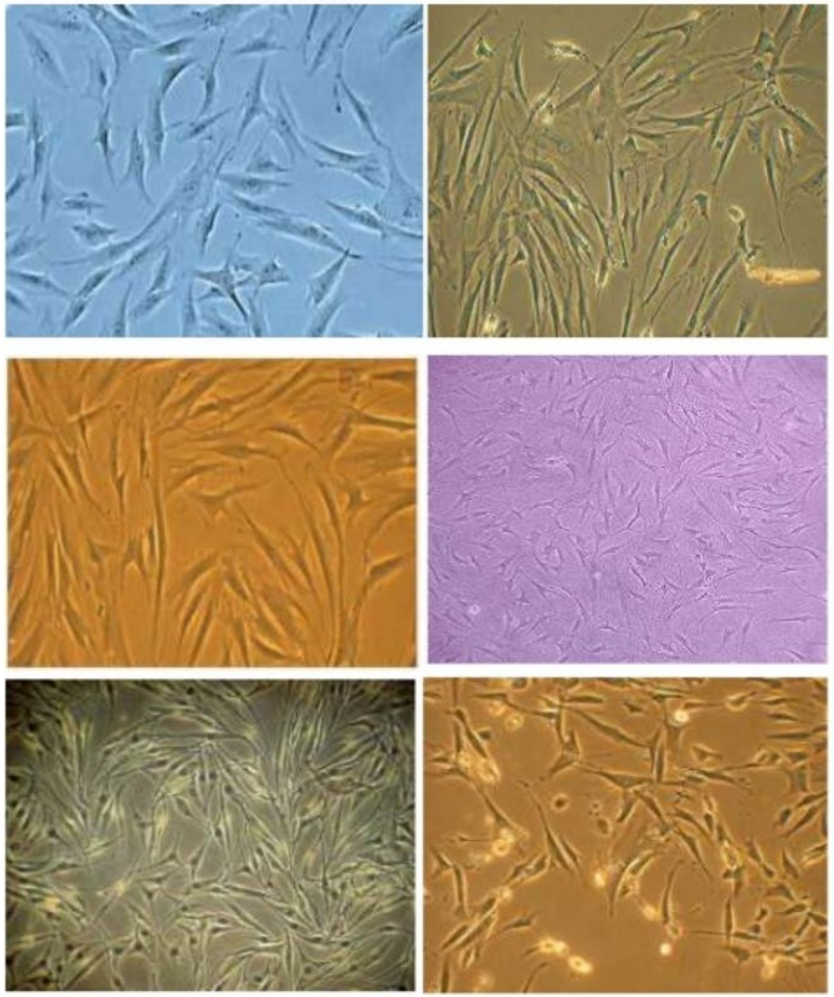
Morphology of Cells in Culture

❖ **Based on shape and appearance: 3 types**

- **Fibroblast-like**
- **Epithelial-like**
- **Lymphoblast-like**

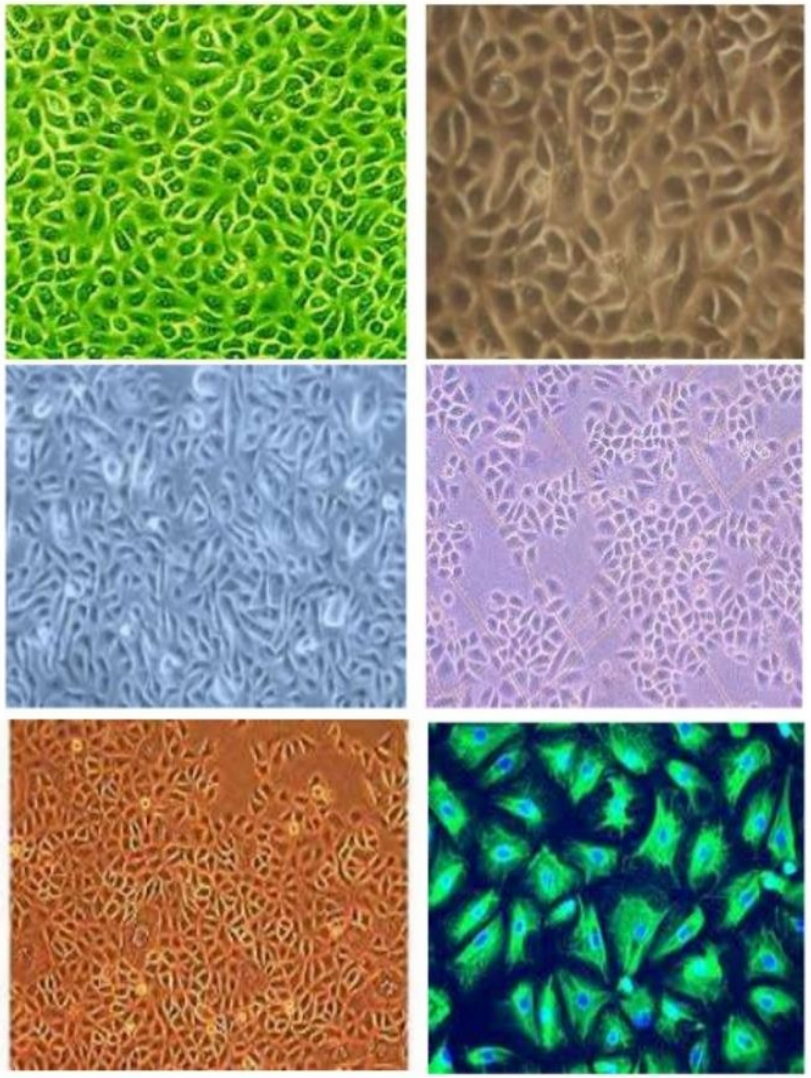
Morphology of Cells in Culture

- Fibroblast-like cells are bipolar or multipolar, have elongated shapes, and grow attached to a substrate



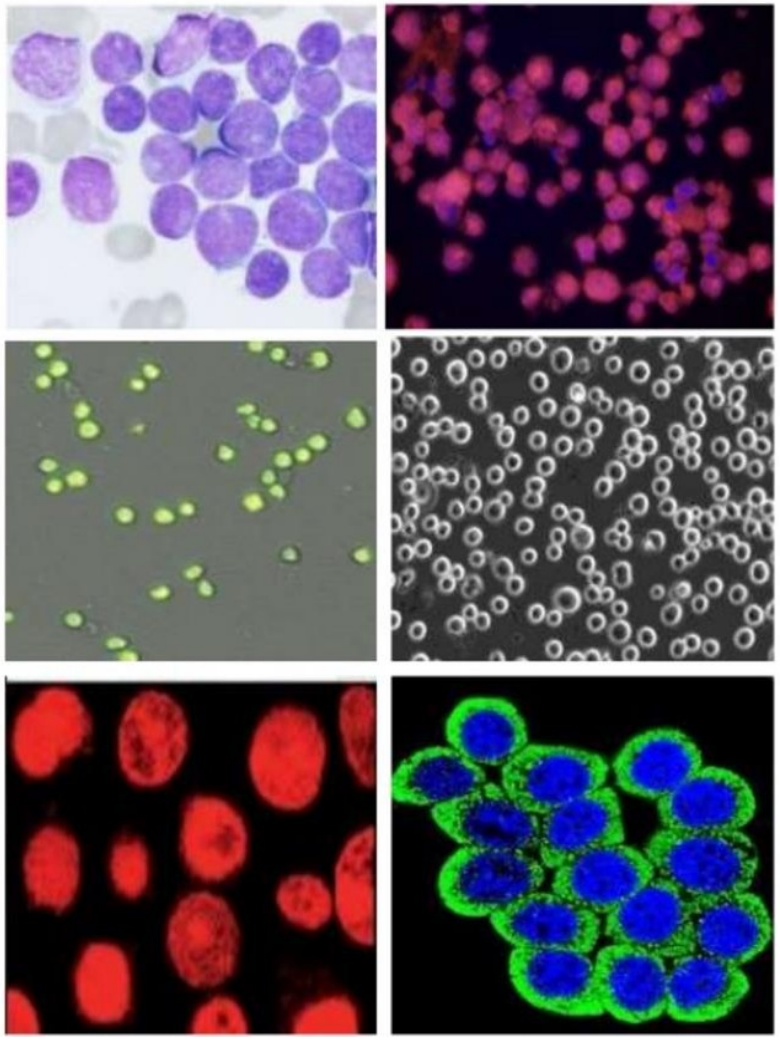
Morphology of Cells in Culture

- **Epithelial-like** cells are polygonal in shape with more regular dimensions, and grow attached to a substrate in discrete patches.



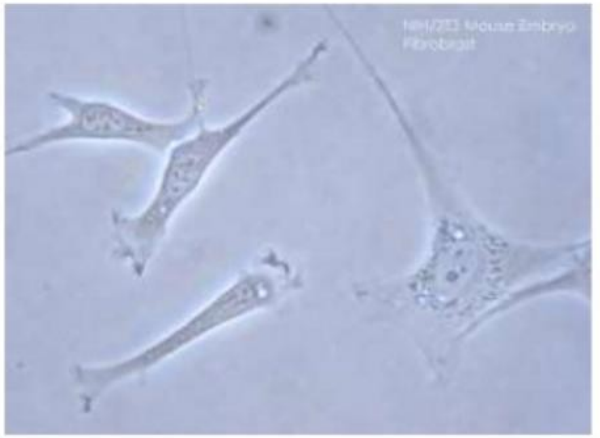
Morphology of Cells in Culture

- **Lymphoblast-like** cells are spherical in shape and usually grown in suspension without attaching to a surface.



Morphology of Cells in Culture

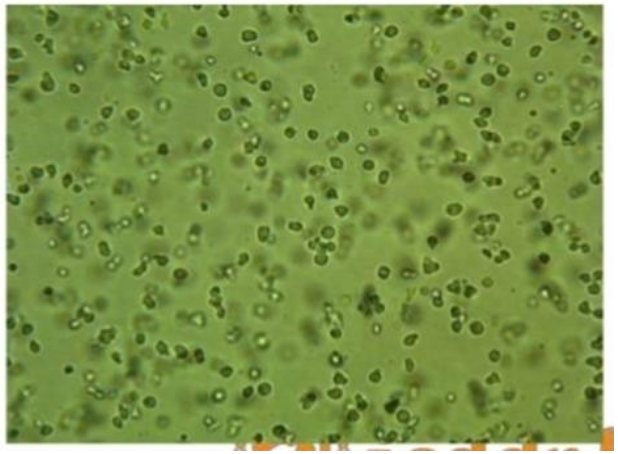
Depending on the adherence property : 2 types



Anchorage-dependent

Must be cultured while attached to a solid or semi-solid substrate (**adherent** or **monolayer culture**)

Example: MDCK, Vero



Anchorage-Independent (**suspension culture**)

Can be grown floating in the culture medium

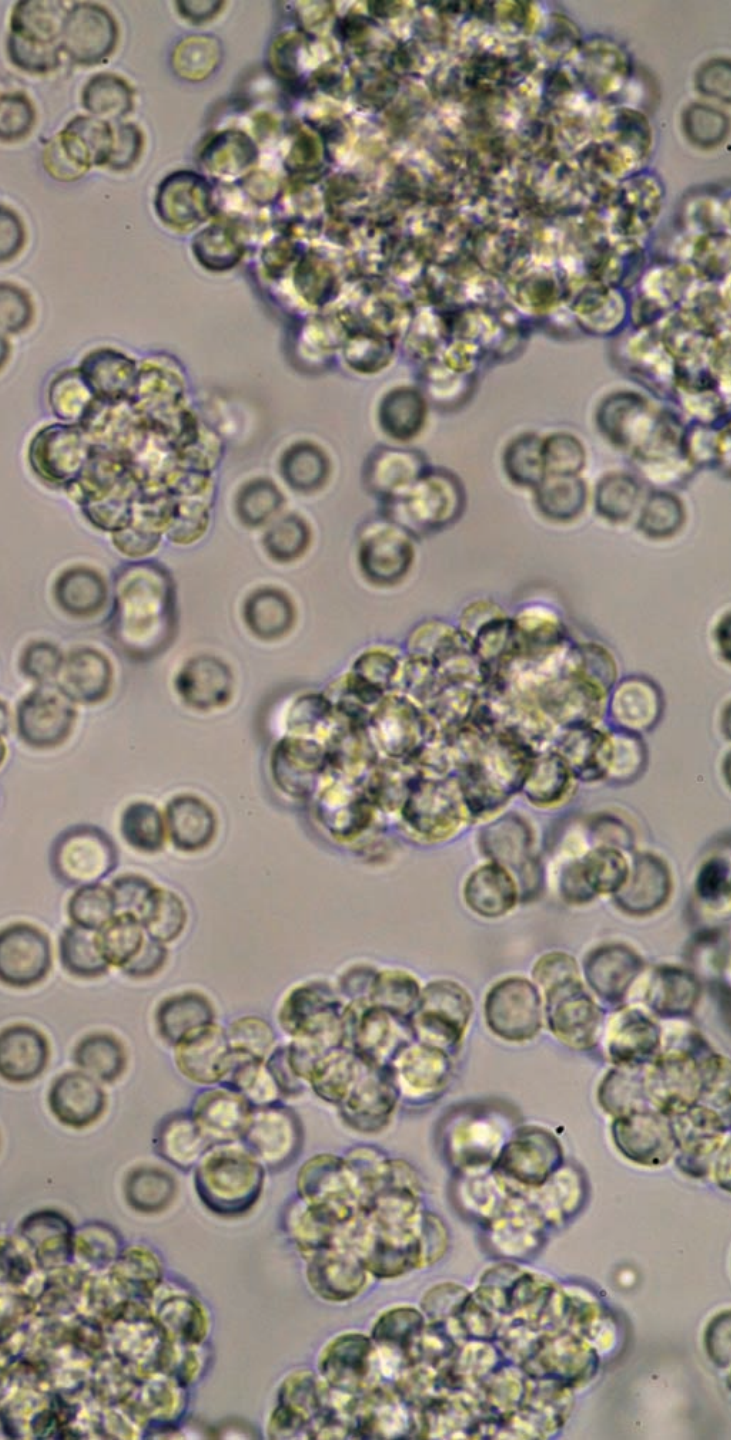
Example: MNFS-60

Culturing of cells

- Cells are cultured as anchorage dependent or independent
- Cell lines derived from normal tissues are considered as anchorage-dependent grows only on a suitable substrate e.g. tissue cells
- Suspension cells are anchorage-independent e.g. blood cells
- Transformed cell lines either grows as monolayer or as suspension

Adherent cells

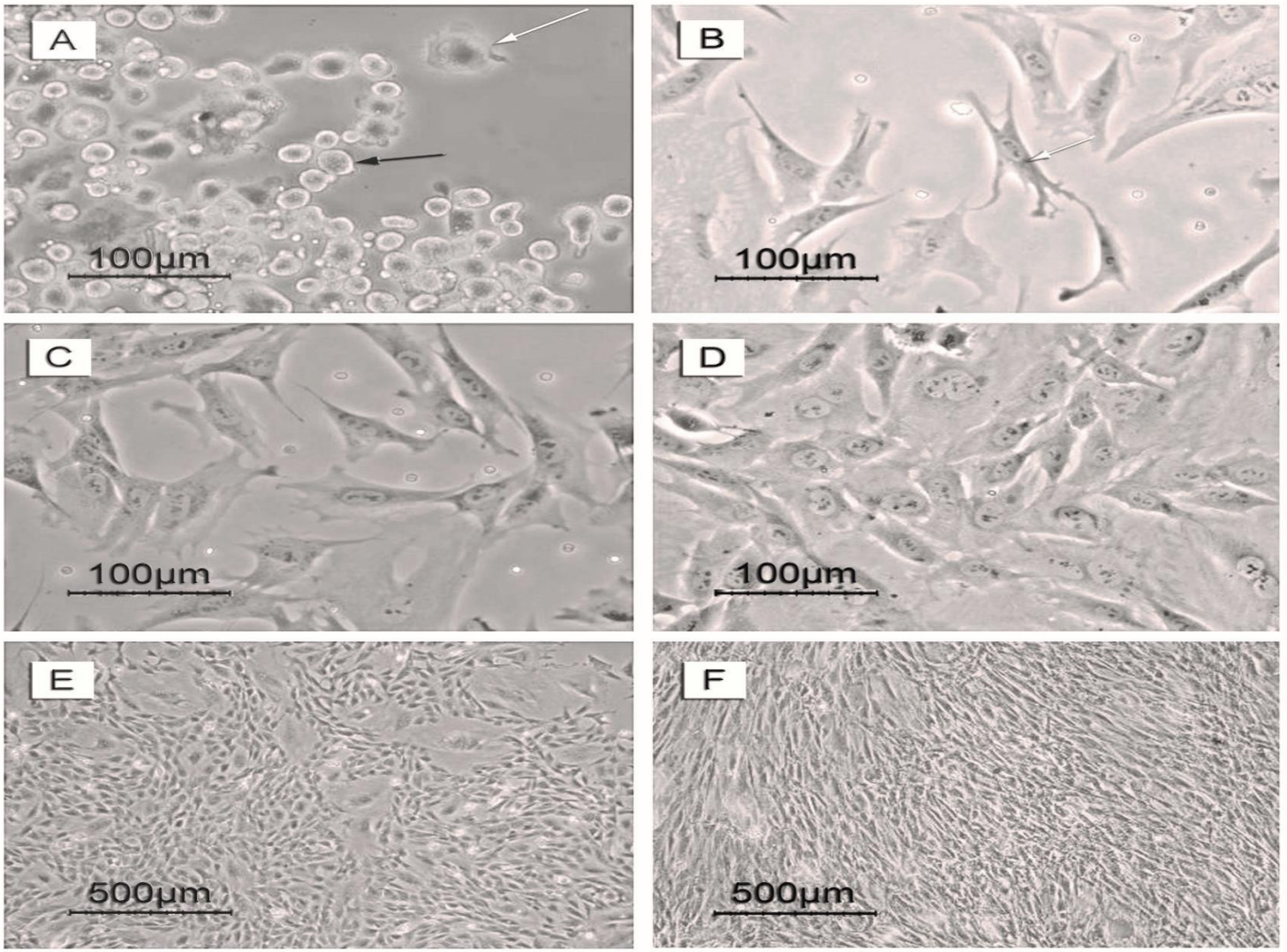
- Cells which are anchorage dependent
- Cells are washed with PBS (free of ca & mg) solution.
- Add enough trypsin/EDTA to cover the monolayer
- Incubate the plate at 37 C for 1-2 mts
- Tap the vessel from the sides to dislodge the cells
- Add complete medium to dissociate and dislodge the cells
- with the help of pipette which are remained to be adherent
- Add complete medium depends on the subculture
- requirement either to 75 cm or 175 cm flask



Suspension cells

- Easier to passage as no need to detach them
- As the suspension cells reach to confluency
- Asceptically remove 1/3rd of medium
- Replaced with the same amount of pre-warmed medium

Cell Confluency



Passaging Cells

Check confluency of cells

⇩

Remove spent medium

⇩

Wash with PBS

⇩

Incubate with trypsin/EDTA

⇩

Resuspend in serum containing media

⇩

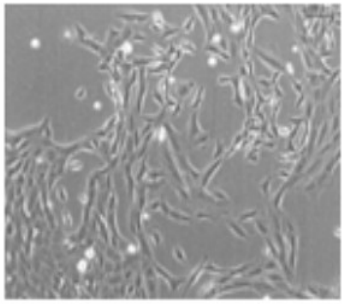
Transfer to culture flask

Why passage cells?

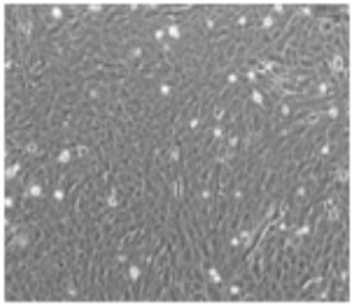
- To maintain cells in culture (i.e. don't overgrow)
- To increase cell number for experiments/storage

How?

- 70-80% confluency
- Wash in PBS to remove dead cells and serum
- Trypsin digests protein-surface interaction to release cells (collagenase also useful)
- EDTA enhances trypsin activity
- Resuspend in serum (inactivates trypsin)
- Transfer dilute cell suspension to new flask (fresh media)
- Most cell lines will adhere in approx. 3-4 hours



70-80% confluence



100% confluence