MOHAMMED V UNIVERSITY - RABAT
FACULTY OF MEDICINE AND PHARMACY - RABAT-

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Introduction to Cell Therapy and Tissue Engineering

THESIS

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BY

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FOR THE

DOCTOR OF MEDICINE DEGREE

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وَرَسُولُهُ وَالْمُؤْمِنُونَ وَسَتُرَدُّونَ إِلَى
عَالِمِ الْغَيْبِ وَالشَّهَادَةِ فَيُنَبِّئُكُمْ
بِمَا كُنْتُمْ تَعْمَلُونَ

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Dedications

To my defunct grandmother,

mmi Fatna bent m'hammed belhaj kaddor Eddahbi. She was my biggest cheerleader, she cried every time when I succeeded. She did not even know that I was always the best. She was the kindest human being ever existed. Strongest than men, she was the most sublime person.

To my grandfather, el haj Abdelkader ben Ahmed Lakhdar.

No words could describe the respect I have for him. I didn't spend much time with him but we shared all that he lived through genes and education of my father.

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I apologize if I misspoke or misbehave to you. You will never know how much I love you.

To the child Whose mother consulted me in hammam village, near khenifra, Morocco.

She cried for the hope that one day her son will live a normal life. She was so convinced that he will get another normal hand, that for a second we considered a psychiatric support for her.

To my friend Dr. Jihad lakssir, who doubted the existence of heart decellularized heart scaffold when I told him I read about it early 2012. And to all weak people of Morocco wherever they are.

To the stubborn teenager inside me who never stopped asking questions no matter what, who used and still use every string to reach wisdom and truth.

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Professor and director

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The president of the thesis judge's committee,

Pr. Eljoudi Rachid

Professor of Toxicology

It is a great honor for us see presiding over our thesis jury.

Please find in this work the expression of our deep gratitude, our thanks, and our moore sincere respect.

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My Professor and thesis judge,

Pr. Amale Hassani

Lieutenant Colonel and professor of pediatrics

I thank you very much for the honor you make me in accepting to judge this work.

I am very sensitive to your kindness and to your very kind welcome.

Please believe in myost distinguished regards.

Thank you.

My Professor and thesis judge,

Pr. Naima El Hafidi

Professor of pediatrics

I am very sensitive to the honor that you make me by agreeing to judge my job.

Please accept our thanks and the testimony of my respect and our gratitude.

Please believe in my most distinguished regards.

Thank you.

To myself in an other body,

Dr.Taha BAIZ

جزاك الله عني خيرا

To my sisters;

The kindest and most correct person I know; Soukaina

The most beautifull and best physician, Khaoula

The most like me, Salma.

To my dear family who loves me the way I am.

To my brothers Youssef and Adnane Baiz

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جزاكم الله عني خيرا

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Summary

Introduction

Maintaining life is not an acceptable end goal of human therapy anymore. Now, whole fields of reconstructive surgery emerged to improve the functional state, through rebuilding the body's original structures. In our current era, modern techniques of transplanting tissue and organs from one individual into another have been lifesaving. The immune phenomenon is clarified sufficiently to suppress the response in the clinical setting of transplantation and to produce prolonged graft survival and function in patients.

In a sense, transplantation can be viewed as the most extreme form of reconstructive surgery; transferring tissue from one individual into another.

Diverting urine into the colon can produce fatal colon cancers 20e30 years later. Making esophageal tubes from the skin can result in skin tumors 30 years later. Intestine for urinary tract replacement can result in severe scarring and obstruction over time.

Consequently; transplantation from one individual into another, although very successful, has severe constraints.

Moreover; accessing enough tissue and organs for all of the patients who need them is a major problem. Also, problems withthe immune system produce chronic rejection and destruction over time. Creating an imbalance of immune surveillance from immunosuppression can cause new tumor formation.

All these constraints produced a global need for new solutions to heal, treat or maybe replace the tissues and organs in question.

The field of regenerative medicine has emerged.

The main objective of this thesis is to introduce regenerative medicine as an important way to treat diseases and infirmity; treating two major parts: Cell therapy and tissue engineering. We did also summarize their current notable applications and discuss the challenges facing their development.

Methods

- The choice of English as language is based on the directives of the experts of the Moroccan association of Health Communication. The first recommendation of the latest conference (2014 FMPR) : «It is necessary to teach health sciences in Arabic to enable students to achieve the highest levels of achievement and absorption for their specializations, as well as to support the teaching of English language at all levels from primary to university level so that health professionals can open up to the world in this field.»
- Scientific online databases: PubMed; Science direct were consulted with the keywords: «cell therapy»; «tissue engineering».
- Other keywords used: «introduction to cell therapy»; « introduction to tissue engineering» «applications of tissue engineering»«regenerative medicine»«tissue engineering evaluation».
- Bibliographic manual research through our College Library http://biblio.medramo.ac.ma/bib/
- Bliographic research through the National Center for Biotechnology Information, U.S. National Library of Medicine.
- All the keywords were researched in both English and French using PubMesh.
- Finally, a manual search on the references of the selected articles was carried out for cross the information obtained.

Results

I. Part I: Cell therapy

1. History and Chronology

1.1. The history of stem cells

The few human survivors in immediate massive irradiation 1945 atomic bombings in Hiroshima and Nagasaki Evolved signs of anemia, serious infections and immune defect uncontrollable bleeding, related to the disappearance of the blood platelets essential for clotting. The study of their bone marrow, headquarters usual production of blood cells and platelets, the revealed deserted any hematopoietic cell: it was the suppression of the marrow of whom died secondarily all massively irradiated humans. (1)

It is from this observation sinister and comparison with biology normal individuals that further scientific work has been lead in human adults:

- the concept of "stem" cells, capable of dividing in case of decrease in the rate of circulating cells and give lines daughter cells. By an intercellular message, each cell division strain keeps constant the aggregate principal amount of cells and the cells strains. (1)
- the concept of cellular regenerative capacity of the skin and liver in particular. (1)
- the concept of transfer by transfusion of bone marrow stem cells (2)

In recent years, researchers have highlighted the presence of stem cells called adult or somatic several other tissues and body organs such as the gut, central nervous system, muscle ... This assumes that there are in all organs even in those where we have not yet localized them like kidney ... (1, 3)

While research on embryonic stem cells is to heart of biomedical science news due to their properties quite Exceptional it involves work on the embryo which is necessarily destructive and whose symbolic no shortage of raise ethical questions. (4)

1.2. Timeline and dates of important research.

Some important dates marked the dramatic advances in research Human stem cells.

- **1950-1960:** The concept of adult stem cells (ASC) is advanced to make account the renewal of blood and skin. (2)
- **1961:** Discovery of the first blood stem cells. (2)
- **1964:** Discovery of stem cells in embryonic carcinomas. (5)
- **1968:** First bone marrow transplant. (2)
- **Nineteen eighty one** Isolation and cultivation of embryonic stem (ES) mice.(6)
- **1994** Isolation of cells of the inner cell mass (ICM) of blastocysts humans and their maintenance in culture.(7)
- **1995:** Isolation of embryonic stem cells of primates.
- These Embryonic stem cells are diploid and have normal karyotypes. They are pluripotent and differentiate into cell types of all derivatives the three germ layers. It is found that the stem cells resemble primate embryonic carcinoma stem cells Human embryonic and suggest that it might be possible to produce and maintain human embryonic stem cells alive *in vitro*.(8)
- **1998-2000** Production of human embryonic stem cells from the inner cell mass of blastocysts transferred by pairs registered FLV. It was found that embryonic stem cells proliferate in vitro on prolonged periods while maintaining a normal karyotype. These cells differentiate spontaneously in somatic cell lines from three germ layers and form teratomas when injected at the immunodeficient mouse. (4, 9)
- **2000:** Production of human embryonic stem cells and their differentiation into neurons. (10)
- **2003:** Production of human embryonic stem cells from teeth Human milk naturally fallen. (11)
- **2005:** Obtaining human embryonic stem cells by transferring the nucleus of a patient in its somatic cell enucleated ovum .(12)

- **2006:** Obtaining human embryonic stem cells from human embryos considered dead of course.(13)
- **2009** : Hematopoietic stem cells in Morocco; simple, effective and well tolerated (14)

Current research focuses on the mechanisms intercellular communication, the mechanisms of differentiation and cell specialization. It also studies the harvesting conditions of these stem cells, culturing them, their proliferation and their administration local or systemic. This work aims the mastery of the regenerative potential these cells for many major therapeutic applications may emerge and allow for tissue and organ repair potentially vital and its various limitations and difficulties in the prospect of exceeding; it is the foundation of medicine regenerative.(3)

2. Recall biological development in humans.

Embryology is the study of embryonic development egg cell with an autonomous individual. Embryonic development includes various large steps that can be characterized by the differentiation and specialization most of the cells that make up the embryo. (15)

2.1. Fertilization:

12 hours after ovulation, the egg, when fertilized into the zygote; The process of fertilization occurs in the third distal fallopian tube, it takes about 24 hours. The date of fertilization is counted as the first day of human ontogeny or Embryonic development.

Fertilization means the fusion of two gametes of different sexes an egg cell at the origin of a new individual. The egg cell is **totipotent**.

Fertilization has three sets of fundamental eventsis found in all organisms:

1. Specific recognition of gametes which ensures the specificity of thefertilization;

2. activation of the ovum by the sperm which triggers a setof metabolic events scheduled;

3. the fusion of parental genomes as a prelude to the division of the egg anddevelopment of a new being diploid.(15, 16)

The egg cell will then be divided, the segmentation phenomenon.

2.2. Segmentation :

24 hours after fertilization, the zygote begins to undergo segmentation that is to say a series of mitosis leading to the formation of 2, daughter cells or blastomeres; (Figure 2)

The first division is vertical, is then obtained two blastomeres, the second division is also vertical but in a plane perpendicular to the first. At this stage, the embryo is composed of 4 cells, if isolated each of these cells and that reimplanted in the uterus can be obtained a stationary individual; these cells are totipotent. The third division is performed in a horizontal plane, thus obtaining 8 cells. This division between the animal pole that will give the embryo itself and vegetal pole which gives embryonic annexes. The cells from this division become pluripotent. The cell cycles will take place quickly and are synchronous to beginning and become asynchronous later: we obtain a morula.

In humans the morula stage is reached 4 days after fertilization. To stage, the cells are pluripotent, so they are able to differentiate in any cell type that makes up the body, but they can not give embryonic annexes. The embryo during this phase guard the same diameter and the same size, only the number of cells increases. At the blastula stage (16-64 cells), a cavity appears: the blastocoel. The cells of the inner mass of the blastocyst remain pluripotent. They have the capacity to differentiate into cells making up the three germ layers.(15, 17, 18)

The embryo then enters a step that aims to put in place three layers: this is the stage of gastrulation.

2.3. Gastrulation:

Gastrulation is a dynamic phase. During the second phase of embryonic development, a set of cell movements coordinated: morphogenetic movements, redoing available blastomeres of the blastula and divided them into three leaflets in humans: a outer layer: the ectoderm, a middle layer: the mesoderm and slips Internal: the endoderm, from which are built the embryo bodies then adult; (Figure 3)(15)

At this stage, the cells are multipotent, they are engaged ina leaflet. It was during gastrulation that we are witnessing the establishment the blastopore which is an opening in the blastula. It is from this blastopore, which is an organizing center, the morphogenetic movements will implement. The morphogens movements involve three types of mechanisms:

1. Intussusception superficial territories within the embryo.

2. The winding surface areas which are reflected on themselves by sliding over a network of molecules.

3. The extension of a surface area which spreads in a sheet on the surface of embryo involving cell proliferation, cell rearrangement pluristratified a layer which becomes singlestratum.(19)

During these phases, the cells lose their potentiality differentiation, this is done by the determining process.

2.4. Determination:

Sooner or later during development, a number of characteristics of the embryo and the adult are vested and will play an important role later in organogenesis. It is essentially spatial markers and cell fate. This determination step is not a clear step in the since embryogenesis begins after fertilization and continues throughout the embryonic development. On the cellular level, the determination results in no visible morphological changes that prepare their differentiation higher. They simply suffer a restriction of their potential differentiation become limited to one lane in which they are now engaged. (14, 20)

All of these mechanisms lead to the establishment of new cellular interactions reparing the embryo to the organogenesis phase.

2.5. Organogenesis:

The bodies of the training is done gradually over development of the embryo. It requires perfect coordination indifferentiation and scheduling of tissues involved in their construction. This coordination is provided by a series of interactions between Cell groups: a group of cells transmits a signal that causes the expression of certain genes in another group of cells and allows them differentiation in a particular way. Cell determination of presumptive territories will engender organ formation and cell differentiation. The majority of cells during this stage lose their potentiality; they become unipotent, . they can differentiate in a single cell type. However, some cells, as in adults, the cells remain Undifferentiated, in the case of embryonic germ cells that are pluripotent cells. During this stage, embryonic appendices are set up; they allow the development of the embryo, they will give the future placenta the umbilical cord. Stem cells are present in these two structures, they are pluripotent or multipotent.

Organogenesis is related to morphogenesis that is to say the body shaping to the embryo.(14, 21)

At six weeks all organs are formed, they must now grow to become truly functional, then we no longer talk but embryo fetus.

3. Stem cells

3.1. What is a cell?

The cell is the basic unit of living things. Its size is few hundredths of a millimeter. The cell comprises a core (except of prokaryotes and some special cells such as red blood cells), which is surrounded by cytoplasm. The nucleus contains most of the information genetic. In the cytoplasm, takes place most biochemical reactions necessary for the life of the cell (synthetic molecules and transformation energy).

A human being is made up of about a hundred thousand billion cells, owned about two hundred different types, most of which is divided Furthermore, while nearly twenty million of our body cells divide for maintaining a constant number of cells (replacement of cells disappearing by aging or lesion), these cells are called; stem cells. (22)

3.2. What is a stem cell?

A stem cell is a cell that is able to divide and multiply throughout life, ensuring the renewal of the cells of a individual. The division of a stem cell produces a new stem cell; "reserve" cell and a cell engaging in a process of differentiation that will lead to a specific function; (Figure 4)

Not all stem cells are equivalent. We distinguish 4 types of stem cells depending of what cell type they can give birth to. However we focalize on pluripotent and multipotent stem cells in cell therapy domain.(23)

·Totipotent Stem Cells: Present in the first 4 days of embryo; they are the only ones to have the ability to evolve to a complete organism.

· Pluripotent Stem Cells: or Stem Cells embryonic: present from the 5th to the 7th day after fertilization, they can give birth to more than 200 different types of tissues;

· Multipotent stem cells: these are the adult stem cells and fetal they can give rise to several types of cells differentiated.

· Unipotent stem cells: they only produce cells differentiated from a single tissue type and retain certain capabilities of self-renewal and proliferation.(24)

3.2.1. Cells embryonic or pluripotent stem:

a. Definition:

In humans, embryonic stem (ES) cells derived from theinner cell mass of the blastocyst preimplantation, also called button embryonic; (Figure 5, 6) (4, 25)

At Blastocyst stage (5th day of development); each cell of the interne blastocyst ass; (ES) is pluripotent. It can produce all the embryonic leaflets (mesoblast, endoblast, ectoblast) (Figure7) and germinal cells; derived tissus also.

b. Properties of ES cells.

Once separated blastocyst, ES cells that are extracted lose any opportunity to further develop into an embryo. However, they can be grown in the laboratory indefinitely while maintaining their pluripotency character and keeping an intact genome.

- ***Self-renewal.***

The main characteristic defining the ES cells is their ability to divide without differentiating for a pluripotent offspring identical. This is called self-renewal Permanent. this propertygives ES cells unlimited proliferative potential while maintaining a phenotypically normal and euploid karyotype.(26)

- ***Karyotype.***

The karyotype of ES cells is analyzed by the marking technique bands "G-banding". Many reports show that after culture long-term cells, normal karyotype is maintained.

Rosler and her colleagues cultivated for a hundred passages several lines of hESCs, they showed that they retained a karyotype euploïde over time; (Figure 8) However, other authors have reported the presence of abnormal karyotypes (Aneuploid) in ES cell lines, such as trisomy 12 and a Trisomy 17. Comparison of cytogenetic studies of long-term lineseuploid hES cells and other containing hES cells will be aneuploid therefore necessary to actually assess the cytogenetic stability of cells thus transformed. The researchers also questioned whether the frequencyaneuploidy or type of observed chromosomal abnormalities affect Ability y of ES cells to differentiate. (27)

c. Distinction between ES cells and stem cells of the embryo

ES cells are derived from stem cells of the embryo early. However, the stem cells of the embryo only exist sotransitional and differentiate during development, while cells ES can be cultivated indefinitely in vitro without differentiation. Cells ES are not identical to embryonic stem cells, even if they share many properties as pluripotency, and tumorigenicity the expression of specific markers.

ES cells should be considered as an adaptation Culture of existing stem cells in the early embryo.](25)

3.2.2. fetal stem cells

a. definition

Fetal stem cells are derived from fetal tissue at a stage much later (5-9 weeks) the stage of embryonic blastocyst umbilical cord or placenta. (28)

b. Classification

There are three classes of fetal stem cells(28)

- ***fetal somatic cells :***

These cells are multipotent that is to say capable of giving rise to several distinct tissues, without being able to regenerate a whole organism. Indeed, these cells have a character moredifferentiated the ES cells.

Two of the se cells are particularly important with a view therapy:

Stem cell germinal zones of the central nervous system, in the treatment of certain neurodegenerative diseases (disease Parkinson or Huntington's disease) .(29, 30)

Fetal hepatocytes which are the subject of active research to transplant .(31)

- ***Germ cells:***

These cells are derived from the draft germinal tissue of the fetus. Indeed, they are isolated from primordial germ cells of the crests gonadal fetus. These cells are pluripotent as ES cells. The culture of fetal stem cells is more difficult than that of cells Embryonic because they do not have the same properties and division proliferation of embryonic cells. Their genome is however less stable than ES, making them yet, unusable in a therapeutic perspective, as they offer important perspectives fondamental research.(32)

- ***Cord blood:***

The blood in the umbilical cord of the newborn is very precious medicine because it contains stem cells (Figure 10)

Stem cells from cord are characterized in part by a intermediate potential between those of the embryo and those in adults, can differentiate into hematopoietic precursor cells which, in turn, give blood cells. These cells are already used for transplantsBlood in children but also in adults. However these cells divide spontaneously and when they divide, they differentiate and die very quickly. Their culture is very difficult to succeed.

In cord blood, other stem cells such as liver, muscle, myocardium, vessels or cartilage were found. At present, many studies are conducted on these cells. He has been shown that they can proliferate in culture without losing their pluripotency and differentiate into homogeneous groups of adipocytes, hepatocytes, osteoblasts, chondroblasts, heart cells and neural cells. These results are encouraging, so this has prompted many countries to develop cord blood banks to replace those spinal cord as they offer advantages over uneven marrow registries because the grafts are collected safely and storable nearly 20 years. (33) And secondly, they express less HLA antigens their surface, thus avoiding rejection problems. (34)

3.2.3. Adult stem cells./

a. definition

Adult stem cells are probably present and dispersed in all organs of the adult human body; skin, intestine, bone marrow, brain, skeletal muscle, heart and liver.

More or less gathered in microenvironments improperly called "niches", they participate in the regeneration of the organ or tissues where they are located on the basis of biochemical signals (factors Specific growth) sent and received.

Adult stem cells are multipotent or unipotent but they seem to be with a certain plasticity.(35)

b. Function :

Somatic adult stem cell ensures homeostasis, that is to say physiological maintenance of an organ or tissue, replacing the cells dead, either naturally or after injury, thus ensuring the sustainability of organ function during the life of the individual. It fulfills this function, on the one hand by multiplying identical (which avoids the depletion of the reservoir of stem cells), on the other hand, by differentiating, acquiring and the characteristics of the tissue to be repaired. [4(35)

c. Location :

Stem cells that have been positively identified in humans are: neural stem cells, hematopoietic, epidermal, intestinal, bone, pancreas, hepatobiliary, and smooth muscle

- ***skeletal muscle; (Figure 11)***

Stem cells of three tissues (blood, s***k***in, intestine) operate times during life, to regularly renew all cells. Except those of the intestine, the other two are already used with success in therapy. As for those other tissues, they are not activated when the need for service arises.(35)

d. Features:

The numerous experimental studies performed in vitro, or after transplantation in animals, used to assign adult stem cells the following features that distinguish them from ES cells: [4(36)

- they do not multiply indefinitely in an undifferentiated state;
- they are very heterogeneous, given the variety of fabrics the organization to which they belong.
- Some are "multipotent": This is the case of stem cells hematopoietic and nervous.
- Others are rather "unipotent", ex: cells of the epidermis, produce only keratinocytes.
- Still others have an "intermediate potential" is the case of the cells mesenchymal stem, located in the bone marrow and produce bone cells, cartilage, and perhaps muscle; or still fetal hepatocytes, which produce hepatocytes and biliary cells. (37)

- ***The plasticity. (38-41)***

The discovery of the phenomenon of "plasticity" represents a disruption of some dogma And major therapeutic opening for virtually all areas of the medicine. Many studies in vivo and in vitro still necessary to determine whether these findings can drive celebration to cell therapy in humans.

The "Plasticity" is the ability of the stem cell to acquire different differentiation programs in certain conditions ofmicroenvironment.The more programs cell can acquire is high ; the more it is plastic.

- ***Plasticity by transdifferentiation***

The plasticityby "transdifferentiation" characterized the process by which a precursor or a differentiated cell is transformed into a cell differentiated from another cell type. ; (Figure 14) (42)

This phenomenon seems unlikely, despite the documentation of phenomena transdifferentiation essentially *in vitro*. (40)

In the literature, for lack of consensus definitions and about stem cells, the notions of "pluripotency" "transdifferentiation "" plasticity "are often amalgamated.

- ***Plasticity by dedifferentiation.***

Dedifferentiation characterizes the process by which a differentiated cell transforms into a differentiated cell from another cell type provided it is dedifferentiated in progenitor; (Figure 15)(43)

- ***The cell fusion mechanism.***

The cell fusion mechanism commonly observed in the body. By example, myoblasts fuse with each other to formmuscle fibers having a large number of nuclei. when a cell differentiated merges w ith an undifferentiated cell, it is like "Reprogrammed" by the differentiated cell.

The confusion can therefore move between the phenomenon of the suspected plasticity mergers and observed phenomena. Indeed, stem cells transplanted showed high frequency fusion with the cells of parenchyma while acquiring the phenotype of the past, giving the illusion a plasticity or a "trans-differentiation".

As such, it is the repopulation of the liver of a diseased mouse hematopoietic stem cells from a normal mouse. Nevertheless, Researchers estimate that the frequency of spontaneous fusion events cell appears very low, at least in normal tissues and does not appear able to explain all the results published. [53, 54](44, 45)

e. The different types of adult stem cells.

- ***Adult stem cells in the bone marrow.***
- ***Hematopoietic stem cells***

They are best known. They are capable of differentiating into White blood cells, red blood cells and platelets. Placed in an organ or tissue different, hematopoietic stem

cells can give muscle, liver or nerve cells These cells can be extracted from bone marrow or peripheral blood to beinjected as much as he needs. One can consider this same practice even from a related or unrelated donor, and more preferably from cord blood.

- ***The mesenchymal stem cells.***

These are cells of embryonic origin. They come from this structure particular embryo called the mesenchyme, they can differentiate into different tissues such as muscle, connective tissue or blood. We also know from an article published in 1999, they may cause the cartilage, bone, tendons and even adipose tissue [43, 55].(35, 46)

The results of these experiments performed in animals could open very promising if they were confirmed in humans.

- ***The endothelial progenitor cells.***

It also appears that the endothelial progenitor cells have been identified in the marrow. Nevertheless, a number of animal experiments confirm options open for their use. Thus, in animals, the injection of bone marrow stem cells containing so these endothelial progenitor cells, proved beneficial in promoting reperfusion or restoration f blood flow, in brain, retina and heart damage. [43, 56](35, 47)

- ***The stem cells that renew the skin and muscle***

The skin perfectly illustrates permanent regeneration of the activity of our organization. The superficial layer of the skin, the epidermis, is the subject of a rapid regeneration from a population of stem cells located in the deepest layer of the epidermis and hair follicles. These cells strains have a significant growth potential that is 1.7 x 1038. Their role is to renew not only the defective cell of the epidermis but also the sweat lands, sebaceous glands and appendages (hair, nails).

The muscle also provides an organ model with very high capacities regenerative. [43](35)

- ***The intestine, lung, liver and kidneys.***

Good knowledge of intestinal stem cells equaled the mischaracterization of liver stem cells as well as cells Stem lung and kidney. However, encouraging results have nevertheless obtained in animals. The presence of stem cells in the lining of the intestine allows probably the regeneration of enterocytes, mucous glands and other structures of the intestine. [43](35)

- ***Recent discoveries: the heart, eye and pancreas***

Research work had indicated that stem cells were discoveries in the heart. However, current clinical trials to find a therapeutic for cardiac diseases such as myocardial, use

Bone marrow cells of adult stem. The practical application of this method, making many needed further testing. [57](48) According to recent publications, precursor cells that can be transformed in Langerhans islet - cells responsible for insulin secretion in the pancréas- have been identified. However, if one were able to isolate precursor cells responsible for insulin secretion, extraordinary therapeutic approaches for treating diabetes could be considered. Current research on a fundamental point, do not allow to open Such rospects. [43](35)

Finally, researchers have shown that adult stem cells were localized in the ciliary area of the eye. [58](49)

f. Adult stem cells pluripotent potential

The pluripotency of certain adult stem cells appear to have been highlighted through. The multipotent adult progenitor Cells (MAPC) by a number of teams that are trying to demonstrate that stem cells with characteristics similar to Embryonic stem cells still exist in adults, localized in various tissues theywould, however, be very rare.

The most significant results are those obtained by the team of a Belgian biologist at the University of Minneapolis, Catherine Verfaillie. In one 2002 article, his team

shows that there is in the bone marrow, or in all organs, a type of precursor cells that can not really be distinguished from mesenchymal stem cells. These cells strains under the definitions given above are not only multipotent but pluripotent, such asEmbryonic stem cells. They were so named MAPC.

This discovery, although very difficult to reproduce, aroused interest in the scientific community, activists, supporters of research. Adult stem cells whose potential may be more important than what is expected. They therefore represent formidable prospects for regenerative medicine in the future. [59](50)

4. Cell therapy

4.1. Definition.

In practice, Cell therapy involves the injection of human cells in order to prevent, treat or mitigate disease. This is to repair damaged tissues with new cells that will rebuild: using cells to repair damaged tissue, or cells transformed to provide in tissues of missing molecules. Cell therapy for biological products for therapeutic, effects from preparation of human or animal living cells. [63, 64](51, 52)

4.2. Issues and prospects.

Cell therapy is an alternative to organ transplants and tissue which can treat some cases. In fact, many diseases cause cell destruction for which the only solution would be a graft. Compatibility issues, there is the low supply of grafts to the number of sick people. If you happen to produce fabrics from adult stem cells from the person himself (cells "Autologous"), we solve the problem of the donor and would remove the risk rejection! The purpose of this project is as simple as it is ambitious:

- grafted cells rather than replace organs or tissues
- maintain a functional capital
- avoid immunosuppressive treatments for life ...

The main difficulties are common to all teams working on this technique, whatever the field of application.

- Mastering the culture and the use of stem cells.
- The induction of good processing of stem cells, qualitatively and quantitatively.
- The development of an injection technique that will guarantee the survival implanted cells. The progress already made are numerous, those expected and hoped for are Moreover. One can expect a considerable development of therapy cell in the coming years, offering treatment options for even pathologies unsolved. [65, 66](53, 54)

4.3. Different therapeutic applications.

Conceptually, there is no area of "forbidden" to cell therapy. However, the extreme structural and functional complexity of cells, tissues and organs of our body shade prospects. (51) Nevertheless, the therapeutic areas in which prospects use of cell therapy seem realistic are numerous: hematology, dermatology, rheumatology, oncology, ophthalmology, neurology, cardiology, hepatology...

4.3.1. Hematologic diseases :

As every independent tissue; blood tissue is a combination of different cell types; cell products and sustaining net depending of his functions and sites. That make the blood tissue the main locomotive of stem cell therapies. Its accessibility and fast proliferation are very useful in the manufacturing of strategies targeting different pathological processes. We chose to repot the diseases with the latest updates and which have been subject of most scientific publications.

- ***Thalassemia:***

Thalassemia is an inherited autosomal recessive blood disorder, caused by mutations in globin genes or their regulatory regions. This results in a reduced rate of synthesis of one of the globin chains that make up haemoglobin. In ß-thalassaemia major there is an underproduction of ß-globin chains combined with excess of free α-globin chains. The excess free α-globin chains precipitate in red blood cells, leading to their destruction (haemolysis) and ineffective erythropoiesis.

The conventional approach to treatment is based on the correction of haemoglobin status through regular blood transfusions and iron chelation therapy for iron overload. Although conventional treatment has the capacity to improve the quality of life of people with ß-thalassaemia major, allogeneic hematopoietic stem cell transplantation is the only currently available procedure which has the curative potential.

We were unable to identify any randomised controlled trials or quasi-randomised controlled trials on the effectiveness and safety of different types of allogeneic stem cell transplantation in people with severe transfusion-dependant ß-thalassaemia major or ß0/+- thalassaemia variants requiring chronic blood transfusion. The absence of high-level evidence for the effectiveness of these interventions emphasises the need for well-designed, adequately-powered, randomised controlled clinical trials. (55)

- ***Multiple Myeloma:***

Multiple myeloma, also known as plasma cell myeloma, is a cancer of plasma cells, a type of white blood cell normally responsible for producing antibodies. Often, no symptoms are noticed initially.When advanced, bone pain, bleeding, frequent infections, and anemia may occur. Complications may include amyloidosis.

The cause is unknown. Risk factors include drinking alcohol, obesity, radiation exposure, family history, and certain chemicals. The underlying mechanism involves abnormal plasma cells producing abnormal antibodies which can cause kidney problems and overly thick blood. The plasma cells can also form a mass in the bone marrow or soft tissue.

When only one mass is present, it is known as a plasmacytoma while more than one is known as multiple myeloma. Multiple myeloma is diagnosed based on blood or urine tests finding abnormal antibodies, bone marrow biopsy finding cancerous plasma cells, and medical imaging finding bone lesions. Another common finding is high blood calcium levels. Multiple myeloma is considered treatable, but generally

incurable. Remissions may be brought about with steroids, chemotherapy, thalidomide or lenalidomide, and stem cell transplant. Bisphosphonates and radiation therapy are sometimes used to reduce pain from bone lesions. Autologous stem cell transplantation (ASCT) has been employed for patients with relapsed multiple myeloma (MM) after up-front ASCT.

Retrospective study among 446 patients showed the evidence for encouraging salvage ASCT for eligible patients.(56)

- ***Gaucher's disease***

Gaucher disease is a genetic disorder in which glucocerebroside a sphingolipid accumulates in cells and certain organs. The disorder is characterized by bruising, fatigue, anemia, low blood platelet count and enlargement of the liver and spleen, and is caused by a hereditary deficiency of the enzyme glucocerebrosidase (also known as glucosylceramidase. When the enzyme is defective, glucocerebroside accumulates, particularly in white blood cells and especially inmononuclear leukocytes. Glucocerebroside can collect in the spleen, liver, kidneys, lungs, brain, and bone marrow.

Manifestations may include enlarged spleen and liver, liver malfunction, skeletal disorders or bone lesions that may be painful, severe neurological complications, swelling of lymph nodes and (occasionally) adjacent joints, distended abdomen, a brownish tint to the skin, anemia, low blood platelet count, and yellow fatty deposits on the white of the eye (sclera). Persons seriously affected may also be more susceptible to infection. Some forms of Gaucher's disease may be treated with enzyme replacement therapy.

The disease is caused by a recessive mutation in the GBA gene located on chromosome 1 and affects both males and females. The carrier rate among Ashkenazi Jews is 8.9% while the birth incidence is one in 450.

Gaucher's disease is the most common of the lysosomal storage diseases.It is a form of sphingolipidosis (a subgroup of lysosomal storage diseases), as it involves dysfunctional metabolism of sphingolipids.

HSCT is a form of treatment that offers the potential of permanent cure. However, there are no clinical trials that have assessed the safety and efficacy of this treatment in comparison to other conservative measures (enzyme replacement therapy, substrate reduction therapy) now in use. (57)

4.3.2. Neuronal and psychiatric Diseases:

- ***Spinal muscular atrophy :***

Spinal muscular atrophy is one of the most common inherited forms of neurological disease leading to infant mortality. Patients have selective loss of lower motor neurons resulting in muscle weakness, paralysis and often death. Although patient fibroblasts have been used extensively to study spinal muscular atrophy, motor neurons have a unique anatomy and physiology which may underlie their vulnerability to the disease process. We report the generation of induced pluripotent stem cells from skin fibroblast samples taken from a child with spinal muscular atrophy. These cells expanded robustly in culture, maintained the disease genotype and generated motor neurons that showed selective deficits compared to those derived from the child's unaffected mother. This is the first study to show that human induced pluripotent stem cells can be used to model the specific pathology seen in a genetically inherited disease. As such, it represents a promising resource to study disease mechanisms, screen new drug compounds and develop new therapies. (58)

- ***Friedreich's ataxia***

The inherited neurodegenerative disease Friedreich's ataxia (FRDA) is caused by GAA·TTC triplet repeat hyperexpansions within the first intron of the FXN gene, encoding the mitochondrial protein frataxin. Long GAA·TTC repeats cause heterochromatin-mediated gene silencing and loss of frataxin in affected individuals. We report the derivation of induced pluripotent stem cells (iPSCs) from FRDA patient fibroblasts by transcription factor reprogramming. FXN gene repression is maintained in the iPSCs, as are the global gene expression signatures reflecting the human disease.

GAA·TTC repeats uniquely in FXN in the iPSCs exhibit repeat instability similar to patient families, where they expand and/or contract with discrete changes in length between generations. The mismatch repair enzyme MSH2, implicated in repeat instability in other triplet repeat diseases, is highly expressed in pluripotent cellsand occupies FXN intron 1, and shRNA silencing of MSH2 impedes repeat expansion, providing a possible molecular explanation for repeat expansion in FRDA.(59)

- ***Schizophrenia :***

Schizophrenia (SCZD) is a debilitating neurological disorder with a world-wide prevalence of 1%; there is a strong genetic component, with an estimated heritability of 80-85%. Although post-mortem studies have revealed reduced brain volume, cell size, spine density and abnormal neural distribution in the prefrontal cortex and hippocampus of SCZD brain tissue and neuropharmacological studies have implicated dopaminergic, glutamatergic and GABAergic activity in SCZD, the cell types affected in SCZD and the molecular mechanisms underlying the disease state remain unclear. To elucidate the cellular and molecular defects of SCZD, we directly reprogrammed fibroblasts from SCZD patients into human induced pluripotent stem cells (hiPSCs) and subsequently differentiated these disorder-specific hiPSCs into neurons. SCZD hiPSC neurons showed diminished neuronal connectivity in conjunction with decreased neurite number, PSD95-protein levels and glutamate receptor expression. Gene expression profiles of SCZD hiPSC neurons identified altered expression of many components of the cyclic AMP and WNT signalling pathways. Key cellular and molecular elements of the SCZD phenotype were ameliorated following treatment of SCZD hiPSC neurons with the antipsychotic loxapine. To date, hiPSC neuronal pathology has only been demonstrated in diseases characterized by both the loss of function of a single gene product and rapid disease progression in early childhood. hiPSC neuronal phenotypes and gene expression changes associated with SCZD, a complex genetic psychiatric disorder, are now reported. (60)

- ***Parkinson's disease :***

The recent generation of induced pluripotent stem cells (iPSCs) from a patient with Parkinson's disease (PD) resulting from triplication of the α-synuclein (SNCA) gene locus allows unprecedented opportunities to explore its contribution to the molecular pathogenesis of PD. We used the double-nicking CRISPR/Cas9 system to conduct site-specific mutagenesis of SNCA in these cells, generating an isogenic iPSC line with normalized SNCA gene dosage. Comparative gene expression analysis of neuronal derivatives from these iPSCs revealed an ER stress phenotype, marked by induction of the IRE1α/XBP1 axis of the unfolded protein response (UPR) and culminating in terminal UPR activation. Neuropathological analysis of post-mortem brain tissue demonstrated that pIRE1α is expressed in PD brains within neurons containing elevated levels of α-synuclein or Lewy bodies. Having used this pair of isogenic iPSCs to define this phenotype, these cells can be further applied in UPR-targeted drug discovery towards the development of disease-modifying therapeutics.(61)

- ***Familial Alzheimer's disease:***

By utilizing these cells as models to gain a greater understanding of the etiology of familial and sporadic AD, researchers are honing in on the intricate molecular mechanisms and therapeutic targets of these conditions. Naturally, as more is understood about the causes of both types of AD, there are more potential uses of iPSCs as interventional agents. This promising new frontier in iPSC research combines the benefits of pluripotent cells with the inherent advantages of using induced cells instead of embryonic cells. iPSCs eliminate the ethical concerns related to embryo destruction, and they can be obtained without depending upon the naturally limited harvest of ESCs. Perhaps the most powerful advantage of iPSCs-both for modeling and clinical intervention-is unique to the field of personalized, precision medicine.

Namely, iPSCs eliminate most of the immunological problems that may arise from other forms of stem cell therapies since they are autologous and genetically identical to the patient.

Nonetheless, challenges do remain in iPSCs' development, use in disease modeling, and therapeutic application. Animal research showing the efficacy of ESCs as potential clinical agents is promising for the future of iPSC-based medicine, but how well ESC-based experimentation will immediately translate into iPSC human therapy remains to be established. At the moment, ESCs show proof-of-concept for iPSC therapy, but they do not completely demonstrate identical properties between the two. Further, there is debate regarding the tumorigenic properties in stem cell therapy overall with specific concern to the use of viral vector iPSCs.

Fortunately, the gap in the differences between these two types of stem cell lines continues to narrow, and it is quite feasible that iPSCs will soon be as close to perfectly interchangeable with ESCs as is possible. Until we reach this point, ESCs will continue to be heralded as the gold standard for true pluripotency. As the scientific community moves closer to complete mimicry of ESCs with IPSCs, more researchers will find it prudent to pursue the ethically less controversial and therefore more clinically relevant iPSC-based experimentation.

Once these challenges are addressed, iPSCs have the potential to bring in a new era of AD treatment. From unlocking mysterious aspects of neurodegeneration to acting as a means of precisely individualized intervention, the inherent blank canvas characteristics of these cells will soon give the medical community an unprecedented level of control and creativity in disease therapy.(62)

4.3.3. Cardiac Diseases :

The generation of reprogrammed induced pluripotent stem cells (iPSCs) from patients with defined genetic disorders holds the promise of increased understanding of the aetiologies of complex diseases and may also facilitate the development of

novel therapeutic interventions. We have generated iPSCs from patients with LEOPARD syndrome (an acronym formed from its main features; that is, lentigines, electrocardiographic abnormalities, ocular hypertelorism, pulmonary valve stenosis, abnormal genitalia, retardation of growth and deafness), an autosomal-dominant developmental disorder belonging to a relatively prevalent class of inherited RAS-mitogen-activated protein kinase signalling diseases, which also includes Noonan syndrome, with pleomorphic effects on several tissues and organ systems. The patient-derived cells have a mutation in the PTPN11 gene, which encodes the SHP2 phosphatase. The iPSCs have been extensively characterized and produce multiple differentiated cell lineages. A major disease phenotype in patients with LEOPARD syndrome is hypertrophic cardiomyopathy. It shows that in vitro-derived cardiomyocytes from LEOPARD syndrome iPSCs are larger, have a higher degree of sarcomeric organization and preferential localization of NFATC4 in the nucleus when compared with cardiomyocytes derived from human embryonic stem cells or wild-type iPSCs derived from a healthy brother of one of the LEOPARD syndrome patients. These features correlate with a potential hypertrophic state. It also provides molecular insights into signalling pathways that may promote the disease phenotype.(63)

Acute myocardial infarction and chronic heart failure rank among the major causes of morbidity and mortality worldwide. Except for heart transplantation, current therapy options only treat the symptoms but do not cure the disease. Stem cell-based therapies represent a possible paradigm shift for cardiac repair. However, most of the first-generation approaches displayed heterogeneous clinical outcomes regarding efficacy. Stemming from the desire to closely match the target organ, second-generation cell types were introduced and rapidly moved from bench to bedside. Unfortunately, debates remain around the benefit of stem cell therapy, optimal trial design parameters, and the ideal cell type. It further emphasizes the importance of understanding the mechanisms of cardiac repair and the lessons learned from first-generation trials, in order to improve cell-based therapies and to potentially finally implement cell-free therapies. (64)

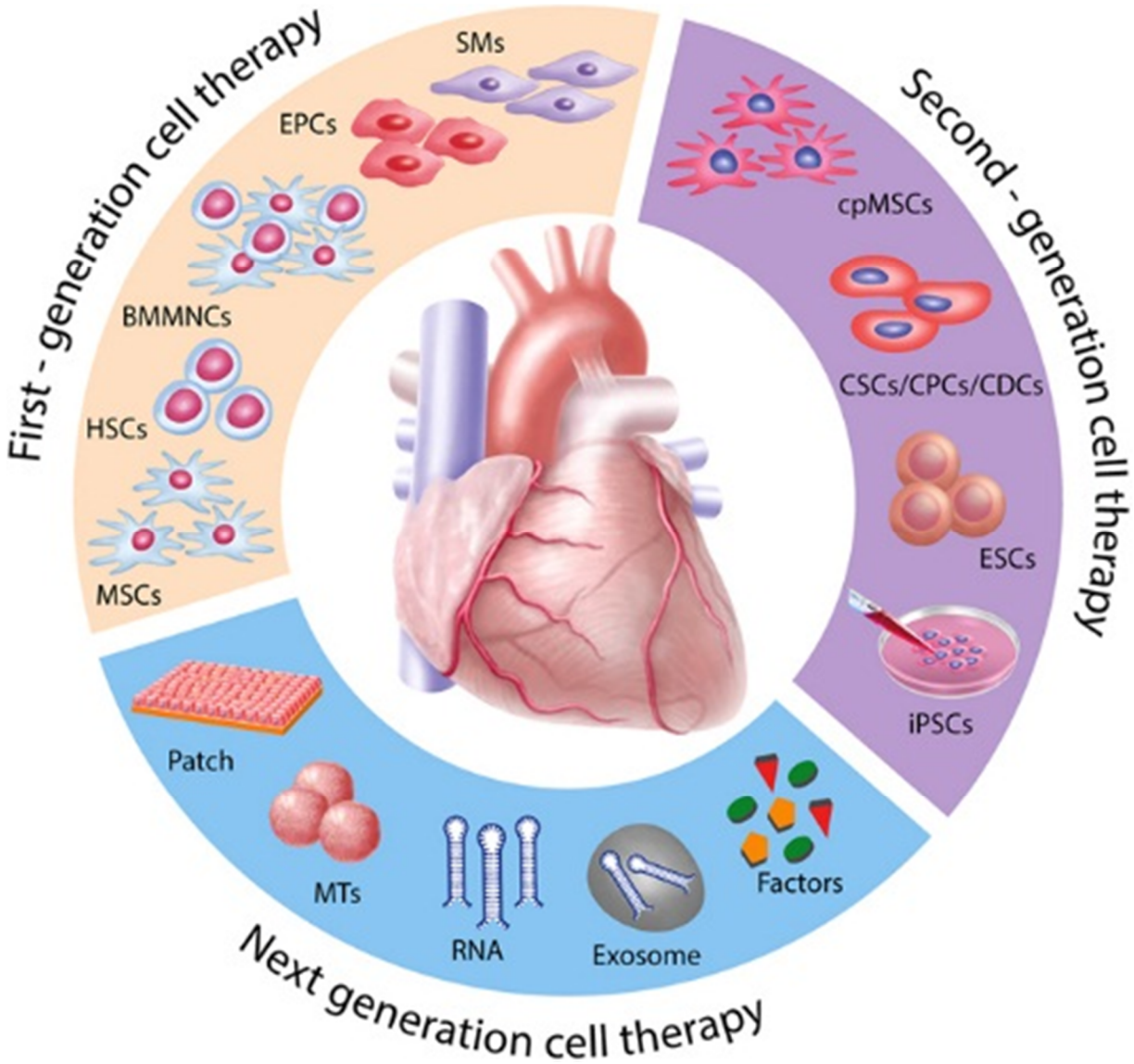

Figure 1: Evolution of translational cardiac regenerative therapies(64)

For the treatment of nonischemic CMP, SC therapy might improve LVEF, but not LVEDD. Further trials should aim to circumscribe the optimal SC regimen in this setting, and to assess long-term clinical outcomes as primary end points.(65) Subgroup analyses showed no evidence for differences in mortality between treatment groups when studies were grouped according to cell dose, baseline cardiac function, route of cell administration, cell type, participant diagnosis or use of co-interventions. Notably, cell therapy was associated with a lower risk of longterm mortality in patients irrespective of diagnosis: chronic IHD (RR 0.52, 95%CI 0.27 to 0.99), HF secondary to IHD (RR 0.33, 95%CI 0.19 to 0.58) and refractory angina (RR 0.11, 95%CI 0.01 to 0.91). Periprocedural adverse events associated with the mapping or cell/placebo injection procedure were infrequent; serious early postoperative adverse events were rare. Adverse events are described in detail in the full review.1 Cell therapy was also associated with a reduction in the incidence of non-fatal MI (RR 0.40, 95%CI 0.17 to 0.93, 9 studies) and incidence of arrhythmias (RR 0.46, 95%CI 0.22 to 0.97, 7 studies) over longterm follow-up, with both findings robust to selection bias. However, there was no evidence that cell therapy over long-term follow-up reduced the risk of rehospitalisation for HF or composite measures of MACE. No association was found between cell therapy and quality-of-life measures, although the number of studies reporting these outcomes was low. However, meta-analysis of nine studies suggested that cell therapy may be associated with a lower New York Heart Association class and improved exercise capacity over longterm follow-up. LVEF was measured as a surrogate measure for heart function using a range of different methods across studies (MRI, left ventricular angiography, singlephoton emission CT, echocardiography and radionuclide ventriculography), with several studies reporting LVEF as an outcome using more than one method of measurement. Separate analyses of LVEF were performed for each method of measurement, with conflicting results. In the analysis of LVEF measured by MRI, cell therapy was associated with an improvement in LVEF at short-term follow-up, but not at long-term follow-up. Cell therapy may reduce the risk of long-term mortality in IHD and CHD and that there are no major adverse events

associated with treatment. This is in agreement with other previous systematic reviews but is discordant with results obtained in systematic reviews and meta-analysis where cell therapies have been administered to people with acute MI. This suggests that people with chronic IHD, HF or both may benefit more from such treatments than patients with acute MI. These results may be clinically relevant, but the evidence for the reduction in the number of deaths with cell treatment relative to controls needs to be confirmed in larger clinical trials before cell-based treatment for these patients can be developed as clinical practice. It is important that all future clinical trials be prospectively registered and conducted appropriately to minimize the risk of bias. Future research should also focus on a better understanding of the cell therapies used (eg, mononuclear cells, circulating progenitor cells, mesenchymal stem cells or hematopoietic progenitor cells) and their mechanism of action, particularly in the presence of co-interventions. Additionally, patient-dependent outcomes need to be more thoroughly investigated to ascertain and distinguish between responders and non-responders, and to be able to tailor autologous, allogeneic or modified cell therapies to each patient group. (66)**:**

BMMNCs transplantation is associated with a moderate, but significant, improvement in LVEF in patients with non-ischemic DCM. This meta-analysis supports further RCT conductions using BMMNCs transplantation with larger patient's population and longer-term follow-up(67)

Meta-analysis demonstrated that stem cell therapy improves cardiac function and reduces mortality in dilated cardiomyopathy patients, which suggested that stem cell therapy may represent a new therapyoption for dilated cardiomyopathy.(68)

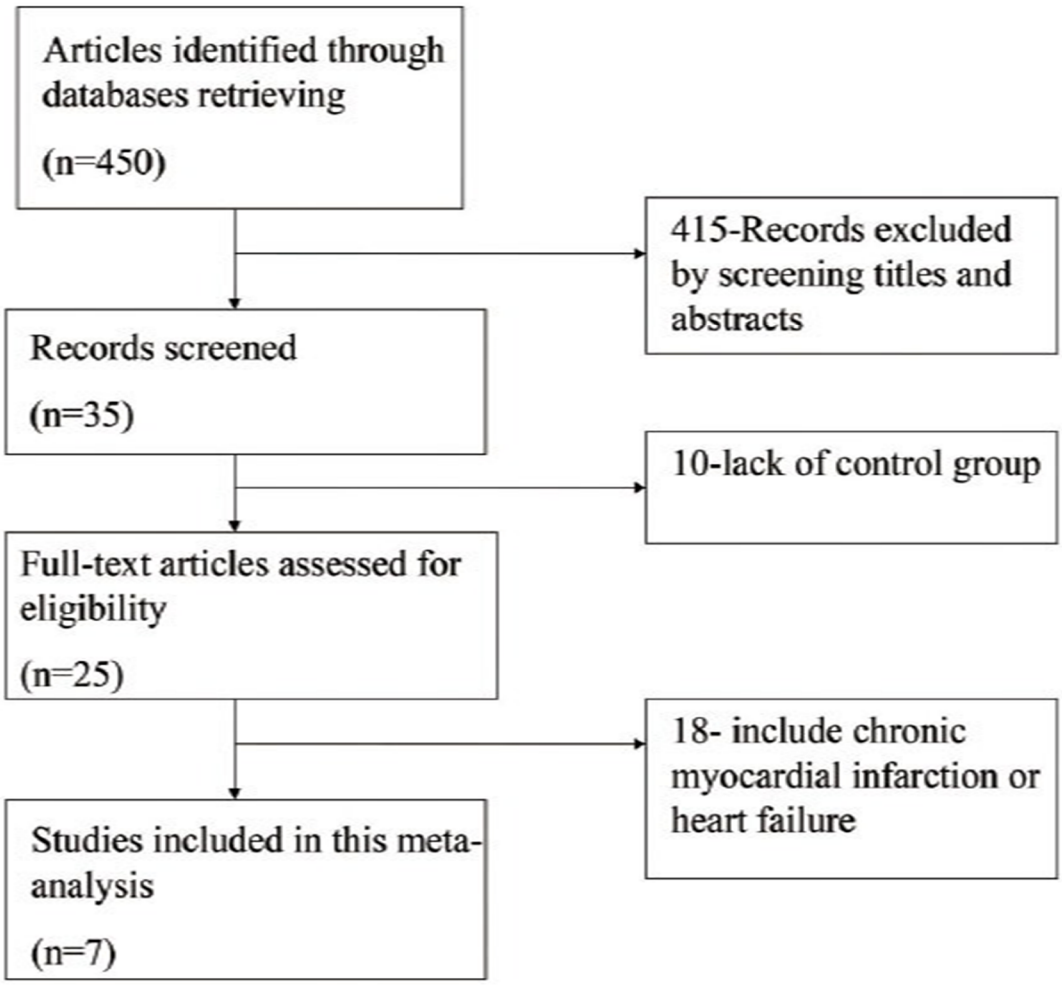

Figure 2: Flowchart of the study selection among 415 citations initially excluded at the title/abstract level. (68)

Inherited arrhythmia syndromes, including familial long QT syndrome, catecholaminergic polymorphic ventricular tachycardia, and Brugada syndrome, can cause life-threatening arrhythmias and are responsible for a significant proportion of sudden deaths in the young. Identification of genetic mutations and pathophysiological changes that underlie disease development can inform clinical practice and guide novel drug development. However, disease mechanisms in a large number of patients remain elusive and pharmacologic treatment is suboptimal, so many patients rely on implantable cardioverter-defibrillator therapy. Induced pluripotent stem cell models of

disease facilitate analysis of disease mechanisms in patient-specific cardiomyocytes, overcoming limitations of animal models and human tissue restrictions. Studies using induced pluripotent stem cell-derived cardiomyocytes are contributing to our understanding of the mechanisms that underpin disease pathogenesis and their potential to facilitate new pharmacologic therapies and personalized medicine.(69)

Long QT syndrome is a potentially life-threatening disease characterized by delayed repolarization of cardiomyocytes, QT interval prolongation in the electrocardiogram, and a high risk for sudden cardiac death caused by ventricular arrhythmia. The genetic type 3 of this syndrome (LQT3) is caused by gain-of-function mutations in the SCN5A cardiac sodium channel gene which mediates the fast Nav1.5 current during action potential initiation. Here, we report the analysis of LQT3 human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs). These were generated from a patient with a heterozygous p.R1644H mutation in SCN5A known to interfere with fast channel inactivation. LQT3 hiPSC-CMs recapitulated pathognomonic electrophysiological features of the disease, such as an accelerated recovery from inactivation of sodium currents as well as action potential prolongation, especially at low stimulation rates. In addition, unlike previously described LQT3 hiPSC models, we observed a high incidence of early after depolarizations (EADs) which is a trigger mechanism for arrhythmia in LQT3. Administration of specific sodium channel inhibitors was found to shorten action and field potential durations specifically in LQT3 hiPSC-CMs and antagonized EADs in a dose-dependent manner. These findings were in full agreement with the pharmacological response profile of the underlying patient and of other patients from the same family. Thus, data demonstrate the utility of patient-specific LQT3 hiPSCs for assessing pharmacological responses to putative drugs and for improving treatment efficacies(70)

4.3.4. Hepatic Diseases :

Liver diseases are commonand often require liver transplantation; however, donated organs are limited and thus alternative sources for liver cells are in high demand. Embryonic stem cells (ESC) can provide a continuous and readily available source of liver cells. ESC differentiation to liver cells is yet to be fully understood and comprehensive differentiation protocols are yet to be defined. Novel protocol for hESC differentiation into morphological and functional yet immature hepatocytes as an alternative method for hepatocyte generation was reported in 2011. The ability of hESC-derived hepatocytes to perform hepatocyte-specific functions was exploredand, hESC-derived hepatocytes produced high levels of albumin ,had elevated levels of functional alcohol dehydrogenase (ADH) ,produced alanin aminotransferase (ALT), expressed the LDL receptor and were capable of engulfing oxidized LDL . These data suggested that the hESC-derived cells produced using our differentiation protocol, gained functional activities of hepatocytes.(71)

Pluripotent stem cell derived hepatocyte-like cells can be generated from iPS cells and hESC in a reproducible and efficient manner. Review of several available methods reveals that there are multiple paths that lead from pluripotency to at least an immature hepatic phenotype that more closely resembles fetal rather than adult hepatocytes. This apparent incomplete differentiation state likely results from our poor understanding of the mechanisms underlying the developmental shift from fetal to adult liver. Moreover, the existing lack of standardization of morphologic, phenotypic, and functional characterization of iHLCs has made comparisons between published papers challenging, if not impossible. Reviews illustrated the importance of extensive phenotypic and functional characterization and have encouraged the community to apply various standards during hepatocyte-like cell characterization. In addition, the use of well-documented and functional hepatocyte reference controls is key to the future improvement of iHLC generation. This advance will lead to the rapid adoption of this key population and their use in a variety of applications including the study of

the mechanisms of human disease and development, and, perhaps in the longer term, as a platform for cell based therapeutics and to evaluate the efficacy and toxicity of pharmaceuticals.(72)Findings demonstrated that HGF mediated the enhancement of iPSC-Hep antioxidant/antiapoptotic capacities and hepatoprotection and that HGF-CHC is as an excellent vehicle for iPSC-Hep engraftment in iPSC-based therapy against AHF.Compared with PBS-delivered iPSC-Heps, the HGF-CHC-delivered iPSC-Heps exhibited higher antioxidant and antiapoptotic activities that reduced hepatic necrotic area. Importantly, these HGF-CHC-mediated responses could be abolished by administering anti-HGF neutralizing antibodies.(73)

Stem Cell Therapy is solution for several pathological processes. Curing; relieving symptoms; preventing diseases progress; it showed all abilities to explain different cell behaviors in pathological cases as we have seen.As cells regroup in tissues; phenotypical and functional unit of the organism; we can use these rules to to understand first the mechanisms of pathological cases; then figure out ways to correct them as much as possible.

One logical other way; is substitution of ill tissues by a new regenerated ones.

II. Part II :Tissue Engineering

1. Introduction:

Underpinned by the principles of rejuvenation, regeneration and replacement; Medicine is now moving from the era of curing symptoms to resolving etiologies. In the sixteenth century, Tagliacozzi of Bologna, Italy reported in his work 'Decusorum Chirurgia per Insitionem' a description of a nose replacement that he constructed from a forearm flap. In the nineteenth century, through the scientific understanding of the germ theory of disease and the introduction of sterile technique, modern surgery had its emergence.

The advent of anesthesia by the mid-nineteenth century enabled the rapid evolution of many surgical techniques. With anesthetized patients, innovative and courageous surgeons could save lives by examining and treating internal areas of the body: the thorax, the abdomen, the brain, and the heart. Initially, surgical techniques were primarily extirpative e for example, removal of tumors, bypass of the bowel in the case of intestinal obstruction, and repair of life threatening injuries. Maintenance of life without regard to the crippling effects of tissue loss or the psychosocial impact of disfigurement however was not an acceptable end goal. Techniques that resulted in the restoration of function through structural replacement became integral to the advancement of human therapy.

Reconstructive surgery have emerged to improve the quality of life by replacing missing function through rebuilding the body's structures. In our current era, modern techniques of transplanting tissue and organs from one individual into another have been revolutionary and lifesaving. In a sense, transplantation can be viewed as the most extreme form of reconstructive surgery; transferring tissue from one individual into another. As with any successful undertaking, new problems have emerged.

Techniques using implantable foreign body materials have produced dislodgment, infection at the foreign body/tissue interface, fracture, and migration over time. Techniques moving tissue from one position to another position have produced biologic changes because of the abnormal interaction of the tissue in its new location. Demand for regenerative medicine products has been driven by an increase in degenerative and chronic diseases which place cost pressures on healthcare providers, combined with advances in new technologies such as nanotechnology, bioengineering and stem cell therapy.

New and functional living tissue is fabricated using living cells, which are usually associated in one way oranother with a matrix or scaffolding to guide tissue development. New sources of cells, includingmany types of stem cells, have been identified in the past few years, igniting new interest in thefield. In fact, the emergence of stem cell biology has led to a new term; regenerative medicine.

Progress in this field has been so rapid that the 2012 Nobel Prize in Physiology or Medicine was awarded to Drs. Gurdon and Yamanaka for their insights into re-programming of cells and for the production of induced pluripotent stem cells (iPS cells).

Scaffolds can be natural, man-made, or a composite of both. A major advance in scaffolding hasoccurred with the description of gentle decellularization of vital organs with preservation of the architecture of the vascular supply by Harald Ott and colleagues. By perfusion through the skeleton of blood vessels in a bioreactor, cells can be reintroduced and therefore re-animate the organ before transplantation. Living cells can migrate into the implant after implantation, or can be associated with the matrix in cell culture before implantation. Such cells can be isolated as fully differentiated cells of the tissue they are hoped to recreate, or they can be manipulated to produce the desired function when isolated from other tissues or stem cell sources. Conceptually, the application of this new discipline to human health care can be thought of as a refinement of previously defined principles of medicine. The physician

has historically treated certain disease processes by supporting nutrition, minimizing hostile factors, and optimizing the environment so that the body can heal itself. In the field of tissue engineering, the same thing is accomplished on a cellular level. The harmful tissue is eliminated; the cells necessary for repair are then introduced in a configurationwhich optimizes the survival of the cells in an environment that will then permit thebody to heal itself. Tissue engineering offers an advantage over cell transplantation alone, inthat organized three-dimensional functional tissue is designed and developed. Tissue engineering can draw on theknowledge gained in the fields of cell and stem cell biology, biochemistry, and molecularbiology and apply it to the engineering of new tissues. Likewise, advances in materials science, chemical engineering, and bioengineering allow the rational application of engineering principles to living systems. Yet another branch of related knowledge is the area of human therapy as applied by surgeons and physicians. In addition, the fields of genetic engineering,cloning, and stem cell biology may ultimately develop hand in hand with the field of tissue Engineering in the treatment of human disease, each discipline depending on developmentsin the others.

We are in the midst of a biologic renaissance.

Interactions of the various scientific disciplines can elucidate not only the potential direction of each field of study, but also the right questions to address. The scientific challenge in tissue engineering lies both in understanding cells and their mass transfer requirements and the fabrication of materials to provide scaffolding and templates. Cells are necessary for normal development in morphogenesis and normal wound healing. In both of these circumstances, cells create or recreate functional structures using pre-programmed information and signaling.

Some approaches to tissue engineering rely on guided regeneration of tissue using materials that serve as templates for ingrowth of host cells and tissue. Other approaches rely on cells that have been implanted as part of an engineered device. As we understand normal developmental and wound healing gene programs and cell

behavior, we can use them to our advantage in the rational design of living tissues. Acquiring cells for creation of body structures is a major challenge, the solution of which continues to evolve. **The ultimate goal in this regard is the large-scale fabrication of structures and may be to create large cell banks composed of universal cells that would be immunologically transparent to an individual**. These universal cells could be differentiated cell types that could be accepted by an individual or could be stem cell reservoirs, which could respond to signals to differentiate into differing lineages for specific structural applications. Much is already known about stem cells and cell lineages in the bone marrow and blood. Studies suggest that progenitorcells for many differentiated tissues exist within the marrow and blood, and may very well be ubiquitous. Our knowledge of the existence and behavior of such cells in various mesenchymal tissues (muscle, bone and cartilage), endodermally derived tissues (intestine and liver), or ectodermally derived tissues (nerves, pancreas, and skin) expands on a daily basis.

The recent description of iPS cells is so promising that they may be the holy grail of therapeutic stem cells in unlimited numbers and with patient specificity to be immunologically neutral. These cells also avoid the ethical and regulatory pitfalls of embryonic stem cells. Much remains to be learned, but data continues to be positive.

As intermediate steps, tissue can be harvested as allograft, autograft, or xenograft. The tissues can then be dissociated and placed into cell culture, where proliferation of cells can be initiated. After expansion to the appropriate cell number, the cells can then be transferred to templates, where further remodeling can occur. Which of these strategies are practical and possibly applicable in humans remains to be explored. Large masses of cells for tissue engineering need to be kept alive, not only in vitro but also in vivo. The design of systems to accomplish this, including in vitro flow bioreactors and in vivo strategies for maintenance of cell mass, presents an enormous challenge towards which significant advances have been made. The fundamental biophysical constraint of mass transfer of living tissue needs to be understood and dealt with on an individual basis as we move toward human application.

The optimal chemical and physical configurations of new biomaterials as they interact with living cells to produce tissue-engineered constructs are under study by many research groups. These biomaterials can be permanent or biodegradable. They can be naturally occurring, synthetic or hybrid materials. They need to be developed to be compatible with living systems or with living cells in vitro and in vivo. Their interface with the cells and the implant site must be clearly understood so that the interface can be optimized. Their design characteristics are major challenges for the field, and should be considered at a molecular chemical level. Systems can be closed, semi-permeable, or open. Each design should factor in the specific replacement therapy considered. Design of biomaterials can also incorporate the biologic signaling that the materials may offer. Examples include release of growth and differentiation factors, design of specific receptors and anchorage sites, and three-dimensional site specificity using computer assisted design and manufacture techniques. New nanotechnologies have been incorporated to design systems of extreme precision. Combining computational modeling with nanofabrication can produce microfluidic circulations to nourish and oxygenate new tissues. *Tissue engineering* evolved from the field of biomaterials development and refers to the practice of combining scaffolds, cells, and biologically active molecules into functional tissues. The goal of tissue engineering is to assemble functional constructs that restore, maintain, or improve damaged tissues or whole organs.

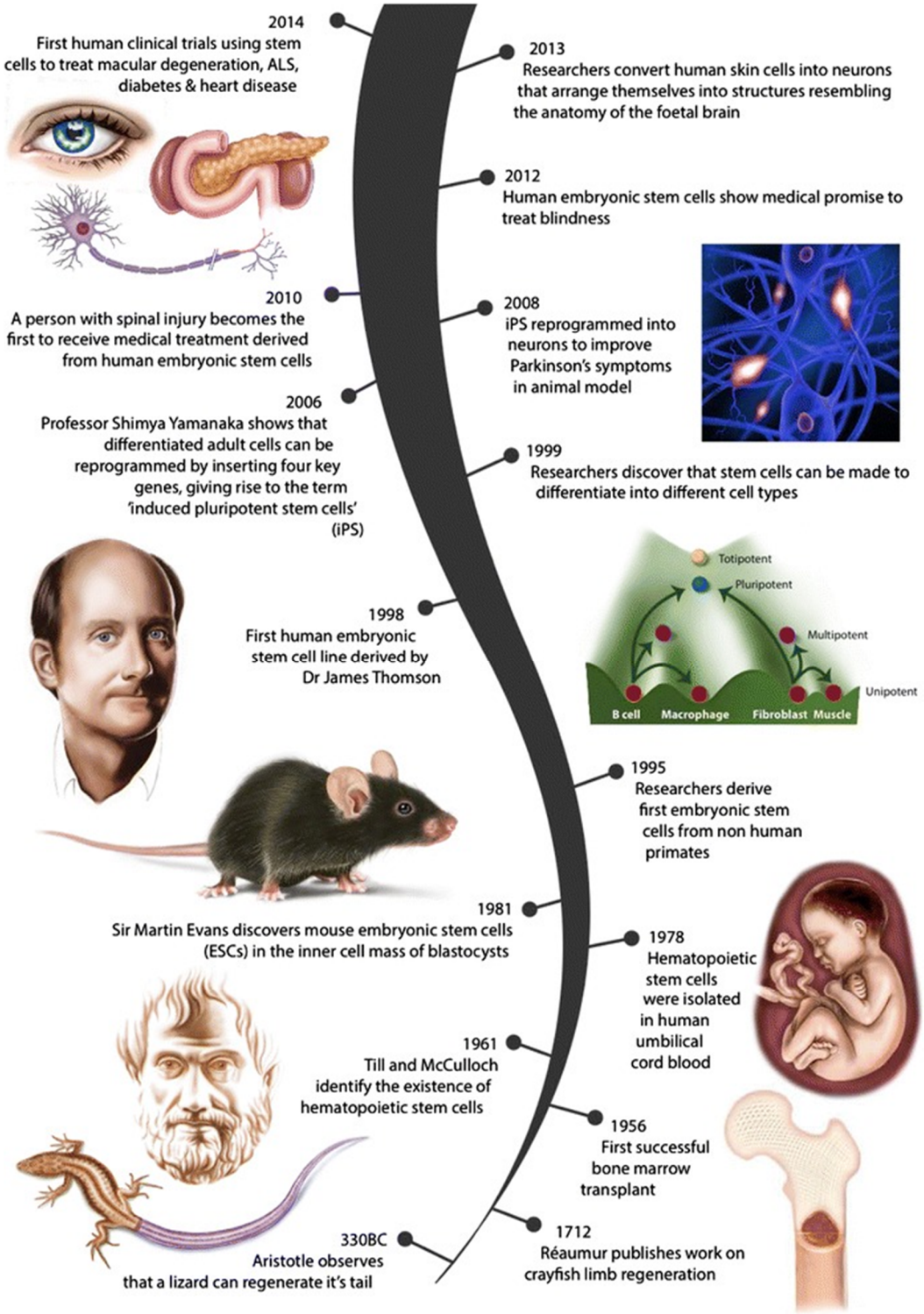

Figure 3: Regenrative medicine origins

- ***History of Tissue Engineering:***

Tissue engineering is a new field that uses living cells, biocompatible materials, and biochemical (e.g., growth factors) and physical (e.g., cyclic mechanical loading) factors to create tissue-like structures. Most frequently, the ultimate goal is implantation of these issue constructs into the body to repair an injury or replace the function f a failing organ.

The critical functions may be structural (e.g., bone, cartilage), barrier- and transport- related (e.g., skin, blood vessels), or biochemical and secretory (e.g., liver and pancreas).

Tissue engineering also applies to the development of specialized extracorporeal life support systems containing cells (e.g., bioartificial liver and kidney) as well as tissue units that may be used for diagnostic screening. In addition to clinical applications, other uses include drug testing forefficacy and toxicology as well as basic studies on tissue development and morphogenesis. The term regenerative medicine is often used synonymously with tissue engineering, although regenerative medicine often implies the use of stem cells as a cell source. Some historical highlights related to tissue engineering and regenerative medicine are shown below:

- 3000 BCE -Skin grafting described in Sanskrit texts of India
- 1794 -Autologous skin grafting in Europe by Bunger, Reverdin, and Baronio
- 1881 -Cadaveric skin allograft by Girdner
- 1944 -Refrigerated skin allografts by Webster
- 1949 -Cell cryopreservation at subzero temperatures developed by Polge
- 1952 -Skin cryopreservation developed by Billingham
- 1962 -Ivalon sponge developed as “synthetic substitute for skin” by Chardack
- 1975 -In vitro cultivation of keratinocytes by Rheinwald and Green
- 1979 -Cultured autologous epithelium, later commercialized as Epicel by Genzyme
- 1981 -Composite living skin equivalent by Bell, later commercialized as Apligraf by Organogenesis

- 1982 -Collagen-glycosaminoglycans (GAG)-based dermal matrix by Yannas, later commercialized as Dermal Regeneration Template by Integra Lifesciences
- 1987- “Tissue engineering” term coined
- 1988 -Cell transplantation in synthetic biodegradable polymers
- 1994 -Chondrocyte culture and transplantation by Brittberg, later commercialized as Carticel by Genzyme
- 2006 -Bioartificial bladder cultured in vitro and implanted in vivo
- 2008 -Engineered trachea from decellularized matrix seeded with human cells derived from stem cells

Skin grafting were the first tissue therapy based. Then came techniques to preserve cells and tissues that enabled allograft skin banking, making these skin grafts product reality. The first synthetic skin substitute reportedly used by more than one investigator was developed in 1962; however, the first successful tissue-engineered skin products were made in the late 1970s and early 1980s.

That is when modern tissue engineering really started, but tissue engineering” was apparently coined later, around 1987. Among the first tissue-engineered skin constructs was the product developed by Howard Greenand colleagues at Harvard Medical School, who described techniques to grow skin epidermis starting with a skin biopsy harvested from a patient.

Keratinocytes isolated from the biopsy couldbe proliferated by coculturing with a feeder layer of mouse mesenchymal cells, thus expanding the coverage area several thousand-fold within weeks. This technological breakthrough led to thefirst cell-based tissue-engineered product, Epicel, which was marketed by Genzyme (Cambridge,MA). Epicel consists of sheets of autologous (i.e., derived from the recipient) keratinocytes thatare used to cover patients suffering from catastrophic cutaneous burn injuries who do not haveenough viable skin remaining to be treated with traditional autografting techniques.

The productdoes not have a dermis and is only a few cells thick; therefore, it is extremely fragile and is notcommonly used (only approximately 60–70 patients per year on average). The U.S. Food andDrug Administration regulates Epicel as a xenogeneic (i.e., derived from another species, in thiscase nonhuman) product (because it uses a feeder layer of mouse cells), **the first of its kind**.

Mechanical engineer Ioannis Yannas at theMassachusetts Institute of Technology (MIT) in collaboration with burn surgeon John F. Burkeat the Boston ShrinersHospital for Children and their colleagues, developed another early product.

It consists of a bovine typeI collagen and shark chondroitin 6-sulfate mixture that is cross-linked and turned into a porousmatrix by controlled freeze-drying. A silicone sheet attached to one side functions as a temporaryepidermis-like barrier. Commercialized under the name **Dermal Regeneration Template** byIntegra Life Sciences (Plainsboro, NJ), this product is used to cover severe burn wounds wherethe damage extends deep into the dermis. Under these circumstances, the wound bed may notsupport a skin graft, or the absence of dermis may lead to extensive contraction and scarring of thehealed wound.

The matrix is biodegradable and presumably dissolves as the host's cells—primarilyfibroblasts, endothelial cells, and neural cells—migrate into it and deposit their own extracellularmatrix (ECM). Ultimately, the matrix disappears and is entirely replaced with a neodermis madeof the patient's own cells and matrix, thus promoting dermal regeneration while inhibiting woundcontraction and leading to better function and appearance of the healed wound.

At that point,the silicone film is removed, and the wound is covered with a skin graft. Interestingly, the productcontains no living cells, and its main purpose is to guide and stimulate the body's repair andregenerative processes.

Also early on, Eugene Bell at MIT and colleagues developed a composite skin product reconstituting both dermis and epidermis. The dermis is first made by seeding collagen gelwith dermal fibroblasts, which cause the gel to contract and form a neodermis.

The keratinocytesare grown on top of the neodermis, initially submerged in culture medium, and then at somepoint in the manufacturing procedure exposed to the air-liquid interface to induce differentiationand formation of a keratinized layer. The entire process takes approximately 3 weeks and usesallogeneic (i.e., derived from donors of the same species) cells isolated from neonatal humanforeskin, which provides the potential for off-the-shelf availability, but with the caveat that theallogeneic skin substitute can provide only temporary coverage, as the patient will eventuallyreject it. The current product based on this technology, Apligraf, marketed by Organogenesis (Canton, MA), is used to stimulate the host's wound healing response in recalcitrant venous legulcers and diabetic foot ulcers. Analogous skin constructs are also used for in vitro tests to measuretransdermal transport and chemical corrosive properties.

During the 1990s, several of these and other tissue-engineered skin and subsequently cartilageproducts were successfully commercialized. These early successes fueled much enthusiasm, andmany research laboratories embarked on applying tissue engineering to nearly every tissue inthe body. Several new companies were spun off with great fanfare and the hope that, as someprominent spokespeople predicted just 15 years ago, tissue engineers would be making complexbody parts by now.

The strategy of simply combining cells and matrix worked for skin andcartilage because these tissues do not require extensive vascularization and other significant tissueprocesses. Furthermore, technologies to grow and differentiate keratinocytes made it possible toadequately source the needed cells for these products. This was not the case for other tissues. As the same prominent spokespeople recently acknowledged; there remain significant hurdles toovercome, such as providing a functional vascular supply, controlling the complex arrangementof different cell types in a 3D tissue, and identifying qualitatively and quantitatively reliable cellsources to make those tissues.

In the early 2000s, the high-tech bubble burst, and weary investors stopped funding high-risk ventures including tissue-engineering companies, which led to a decline in the industry. A study conducted in 2004 found that activity in skin, cartilage, and other structural applications declined by more than 50% with a loss of 800 full-time employees.

The decrease was partially offset by an increase in stem cell firms, which added more than 300 employees. Except for this transient resurgence fueled by the promise of stem cells, financing of startup activity since 2008has been very limited. Although significant advances have occurred in some areas, such as bladder,cornea, and bronchial tubes, tissues such as blood vessels, heart, and liver—in spite of years ofresearch efforts—are still far from offering clinically acceptable solutions.

During the maturation of tissue engineering over the past three decades, several technologies have been developed based on advances in molecular and cellular biology and micro- and nano systems engineering. These technologies have been developed largely by basic scientists and engineers, who sometimes have a tendency to oversimplify the problem and do not always recognize ethe clinical issues. Nevertheless, some of these technologies have led to the development of molecular diagnostics, which as of 2002 comprised an industry market greater than $3 billion, growing at a rate of approximately 25% per .

That non therapeutic applications of tissue engineering are making strides may ultimately help support the development of new tissue engineered therapeutic products, which are much more difficult to produce than enthusiastic advocates originally thought. (74)

2. Basic Principles of Tissue Engineering:

2.1. Molecular Cell organization :

Multicellular tissues exist in one of two types of cellular arrangements; epithelial or mesenchymal. Epithelial cells adhere tightly to each other at their lateral surfaces and to an organized ECM (extracellular matrix) at their basal domain, thereby producing a sheet of cells resting on a basal lamina with an apical surface. Mesenchymal cells, in contrast, are individual cells with a bipolar morphology that are held together as a tissue within a three-dimensional ECM . The conversion of epithelial cells into mesenchymal cells, an 'epithelial-mesenchymal transition' (EMT), is central to many aspects of embryonic morphogenesis, adult tissue repair, as well as a number of disease states. The reverse process, whereby mesenchymal cells coalesce into an epithelium is a 'mesenchymal-epithelial transition' (MET). Understanding the molecules that regulate this transition between epithelial and mesenchymal states offers important insights into how cells and tissues are organized. The early embryo is structured as one or more epithelia. An EMT allows the rearrangements of cells to create additional morphological features. Well-studied examples of EMTs during embryonic development include gastrulation in Drosophila], the emigration of primary mesenchyme cells (PMCs) in sea urchin embryos , and gastrulation in amniotes (reptiles, birds, and mammals) at the primitive streak . EMTs also occur later in vertebrate development, such as the emigration of neural crest cells from the neural tube, the formation of the sclerotome from epithelial somites, and during palate fusion. The reverse process of MET is likewise crucial to development, and examples include the condensation of mesenchymal cells to form the notochord and somites, kidney tubule formation from nephrogenic mesenchyme, and the creation of heart valves from cardiac mesenchyme . In the adult organism, EMTs and METs occur during wound healing and tissue remodeling. The conversion of neoplastic epithelial cells into invasive cancer cells has long been considered an EMT process . However, there are also examples of tumor cells that have functional cell-cell adhesion junctions, yet are

still migratory and invasive as a group. This 'collective migration' also occurs during development . Hence, there is debate about whether an EMT model accurately describes all epithelial metastatic cancers. Similarly, the fibrosis of cardiac, kidney, lens, and liver epithelial tissue has also long been categorized as an EMT event .However, recent research into the kidney in vivo shows that the myofibroblasts induced following kidney injury are derived from mesenchymal pericytes, rather than the proximal epithelial cells . Therefore, the origin of the cells that contribute to fibrotic tissue scarring (epithelial or otherwise) may need to be carefully re-examined.

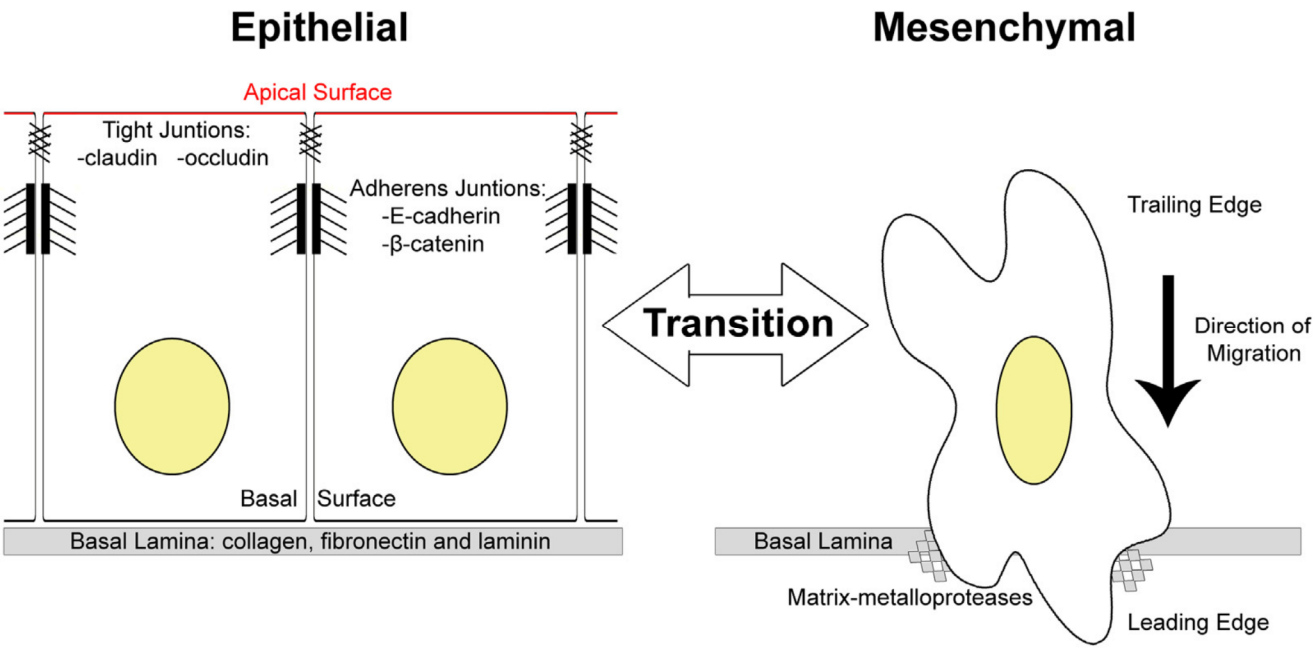

Figure 4: Epithelial vs. Mesenchymal.(75)

Epithelial cells adhere together by tight junctions and adherens junctions localized near the apical surface. Epithelial cells also have a basal surface that rests on a basal lamina (ECM). Mesenchymal cells in contrast do not have well- defined cell-cell adhesion complexes, have front-end/ back-end polarity instead of apical/basal polarity, and mesenchymal cells are characterized by their ability to invade the basal lamina.

2.2. Molecules that organize cells

The conversion of an epithelial sheet into individual migratory cells and back again requires the coordinated changes of many distinct families of molecules.

- ***Changes in cell-cell adhesion***

Epithelial cells are held together by specialized cell-cell junctions, including adherens junctions, desmosomes, and tight junctions. These junctions are localized in the lateral domain near the apical surface and establish the apical polarity of the epithelium. In order for an epithelial sheet to produce individual mesenchymal cells, cell-cell adhesions must be disrupted. The principle transmembrane proteins that mediate cell-cell adhesions are members of the cadherin superfamily. E-cadherin and N-cadherin are classical cadherins hat interact homotypically through their extracellular IgG domains with like-cadherins on adjacent cells. Cadherins are important mediators of cell-cell adhesion. For example, misexpression of E-cadherin is sufficient to promote cell-cell adhesion and assembly of adherens junctions in fibroblasts. In epithelial cancers (carcinomas), E-cadherin acts as a tumor suppressor. In a mouse model for b-cell pancreatic cancer, the loss of E-cadherin is the rate-limiting step for transformed epithelial cells to become invasive. Although the loss of cadherin-mediated cell-cell adhesion is necessary for an EMT, the loss of cadherins is not always sufficient to generate a complete EMT in vivo. For example, the neural tube epithelium in mice expresses N-cadherin, but in the N-cadherin knockout mouse an EMT is not induced in the neural tube. However, cadherins are essential for maintaining epithelial integrity, and the loss of cell-cell adhesion due to the reduction of cadherin function is an important step for an EMT. One characteristic of an EMT is ’cadherin switching’. Often, epithelia that express E-cadherin will downregulate E-cadherin expression at the time of the EMT, and express different cadherins such as N-cadherin. Cadherin switching may promote motility. For instance, in mammary epithelial cell lines, the misexpression of N-cadherin is sufficient for ncreased cell motility, and blocking N-cadherin expression results in less motility. However, the

misexpression of N-cadherin does not result in the complete loss of epithelial morphology Hence, cadherin switching may be necessary for cell motility, but cadherin switching alone isnot sufficient to bring about a complete EMT. There are several ways that cadherin expression and function are regulated. Transcription factors that are central to most EMTs such as Snail-1, Snail-2, Zeb1, Zeb2, Twist, and E2A, all bind to E-boxes on the E-cadherin promoter and repress the transcription of E-cadherin . Post-transcriptionally, the E-cadherin protein is ubiquitinated by the E3-ligase, Hakai, which targets E-cadherin to the proteasome. E-cadherin turnover at the membrane is regulated by either caveolae-dependent endocytosis or clathrin-dependent endocytosis, and p120- catenin prevents endocytosis of E-cadherin at the membrane. E-cadherin function can also be disrupted by matrix metalloproteases, which degrade the extracellular domain of E-cadherin. Some or all of these mechanisms may occur during an EMT to disrupt cell-cell adhesion.

In summary, cell-cell adhesion is maintained principally by cadherins, and changes in cadherin expression are typical of an EMT.

- ***Changes in cell-ECM adhesion***

Altering the way that a cell interacts with the ECM is also important in EMTs. For example, at the time that sea urchin PMCs ingress, the cells have increased adhesiveness for ECM. Cell- ECM adhesion is mediated principally by integrins. Integrins are transmembrane proteins composed of two non-covalently linked subunits, a and b, that bind to ECM components such as fibronectin, laminin, and collagen. The cytoplasmic domain of integrins links to the cytoskeleton and interacts with signaling molecules. Changes in integrin function are required for many EMTs, including neural crest emigration, mouse primitive streak formation , and cancer metastasis . However, the misexpression of integrin subunits is not sufficient to bring about a full EMT in vitro or in vivo.

The presence and function of integrins is modulated in several ways. For example, the promoter of the integrin b6 gene is activated by the transcription factor Ets-1 during colon carcinoma metastasis. Most integrins can also cycle between 'On' (high affinity) or 'Off' (low affinity) states. This 'inside-out' regulation of integrin adhesion occurs at the integrin cytoplasmic tail. In addition to integrin activation, the 'clustering' of integrins on the cell surface also affects the overall strength of integrin-ECM interactions. The increased adhesiveness of integrins due to clustering, known as avidity, can be activated by chemokines, and is dependent on RhoA and phosphatidylinositol 3' kinase (PI3K) activity .

In summary, **changes in ECM adhesion are required for an EMT. Cell-ECM adhesions are maintained by integrins, and integrins have varying degrees of adhesiveness dependent upon the presence, activity, or avidity of the integrin subunits.**

- ***Changes in cell polarity and stimulation of cell motility***

Cellular polarity is defined by the distinct arrangement of cytoskeletal elements and organelles in epithelial verses mesenchymal cells. Epithelial polarity is characterized by cell-cell junctions found near the apical-lateral domain (non-adhesive surface), and a basal lamina opposite of the apical surface (adhesive surface). Mesenchymal cells in contrast do not have apical/basal polarity, but rather front-end/back-end polarity, with actin-rich lamellipodia and Golgi localized at the leading edge.

Molecules that establish cell polarity include Cdc42, PAK1, PI3K, PTEN, Rac, Rho, and the PAR proteins. Changes in cell polarity help to promote anEMT. In mammary epithelial cells, the activated TGF-b receptor II causes Par6 to activate the E3 ubiquitin ligase Smurf1, and Smurf1 then targets RhoA to the proteasome. **The reduction of RhoA activity results in the loss of cell-cell adhesion and epithelial cell polarity**. In order for mesenchymal cells to leave the epithelium, they must become motile. Many of the same polarity (Crumbs, PAR, and Scribble complexes), structural (actin, microtubules), and regulatory molecules (Cdc42, Rac1, RhoA) that

govern epithelial polarity are also central to cell motility . Cell motility mechanisms also vary depending on whether the nvironment is 2D or 3D . Many mesenchymal cells express the intermediate filament vimentin, and vimentin may be responsible for several aspects of the EMT phenotype ..

In short, a wide variety of structural, polarity, and regulatory molecules must be re-assigned as cells transition between epithelial polarity and mesenchymal migration.

Invasion of the basal lamina In most EMTs the emerging mesenchymal cells must penetrate a basal lamina which consists of ECM components such as collagen type IV, fibronectin and laminin. The basal lamina functions to stabilize the epithelium and is a barrier to migratory cells. One mechanismthat mesenchymal cells use to breach the basal lamina is to produce enzymes that degrade it. Plasminogen activator is one protease associated with a number of EMTs, including neural crest emigrationand the formation of cardiac cushion cells during heart morphogenesisem. The type II serine protease, TMPRSS4, also promotes an EMT and metastasis when overexpressed in vitro and in vivo. Matrix metalloproteases (MMPs) are also important for many EMTs. When MMP-2 activity is blocked in the neural crest EMT, neural crest emigration is inhibited, but not neural crest motility.. In mouse mammary cells, MMP-3 overexpression is sufficient to induce an EMT in vitro and in vivo . Misexpressing MMP-3 in cultured cells induces an alternatively spliced form of Rac1 (Rac1b), which then causes an increase in reactive oxygen species (ROS) intracellularly, and Snail-1 expression. Either Rac1b activity or ROS are necessary and sufficient for an MMP-3-induced EMT. Hence, a number of extracellular proteases are important to bring about an EMT. While epithelial cells undergoing an EMT do eventually lose cell-cell adhesion, apical-basal polarity, and gain invasive motility, the EMT program is not necessarily ordered or linear. For example, in a study where neural crest cells were labeled with cell adhesion or polarity markers and individual live cells were observed undergoing the EMT in slice culture, neural crest cells changed epithelial polarity either before or after the complete loss of cell-cell adhesion, or lost cell-cell adhesions

either before or after cell migration commenced. Therefore, while an EMT does consist of several distinct phases, these steps may occur in different orders or combinations, some of which (e.g., the complete loss of cell-cell adhesion) may not always be necessary.

In summary, **changes in a wide range of molecules are needed** for an EMTas epithelial cells lose cell-cell adhesion, change cellular polarity, and gain invasive cell motility.

- ***The EMT transcriptional program***

At the foundation of every EMT or MET program are **the transcription factors that regulate the gene expression required for these cellular transitions**. While many of the transcriptionfactors that regulate EMTs have been identified, the complex regulatory networks are still incomplete. Here we review the transcription factors that are known to promote the various phases of an EMT. Then we examine how these EMT transcription factors themselves are regulated at the promoter and post-transcriptional levels.

- ***Transcription factors that regulate EMTs***

The Snail family of zinc finger transcription factors, including Snail-1 and Snail-2 (formerly Snail and Slug) are direct regulators of cell-cell adhesion and motility during EMTs. The knockout of Snail-1 in mice is lethal early in gestation, and the presumptive primitivestreak cells that normally undergo an EMT still retain apical/basal polarity, adherens junctions, and express E-cadherin mRNA. Snail-1 misexpression is sufficient for breast cancer recurrence in a mouse model in vivo, and high levels of Snail-1 predict the relapse of human breast cancer. Snail-2 is necessary for the chicken primitive streak and neural crest EMTs. One way that Snail-1 or Snail-2 causes a decrease in cell-cell adhesion is by repressing the E-cadherin promoter. This repression requires the mSin3A co-repressor complex, histone deacetylases, and components of the Polycomb 2 complex. Snail-1 is also a transcriptional repressor of

the tight junction genes Claudin and Occludinand the polarity gene Crumbs3 .The misexpression of Snail-1 and Snail-2 further leads to the transcription of proteins important for cell motility such as fibronectin, vimentin, and RhoB. Further, Snail-1 promotes invasion across the basal lamina. Themisexpression of Snail-1 represses laminin (basement membrane) production and indirectly upregulates mmp-9 transcription. Snail and Twist also make cancer cells more resistant to senescence, chemotherapy, apoptosis, and endow cancer cells with 'stem cell' properties. Hence, Snail- 1 or Snail-2 are necessary and sufficient for bringing about many of the steps of an EMT , including loss of cell-cell adhesion, changes in cell polarity, gain of cell motility, invasion of the basal lamina, and increased proliferation and survival. Other zinc finger transcription factors important for EMTs are zinc finger E-box-binding homeobox 1 (Zeb1, also known as dEF1), and Zeb2 (also known as Smad-interacting protein- 1, Sip1). Both Zeb1 and Zeb2 bind to the E-cadherin promoter and repress transcription. Zeb1 can also bind to and repress the transcription of the polarity proteins Crumbs3, Pals1-associated tight junction proteins (PATJ), and lethal giant larvae 2 (Lgl2). Zeb2 is structurallysimilar to Zeb1, and Zeb2 overexpression is sufficient to downregulate E-cadherin, dissociate adherens junctions, and increase motility in MDCK cells. The Lymphoid Enhancer-binding Factor/ T Cell Factor (LEF/TCF) transcription factors also play an important role in EMTs. For instance, the misexpression of Lef-1 in cultured colon cancer cells reversibly causes the loss of cell-cell adhesion. LEF/TCF transcription factors directly activate genes that regulate cell motility, such as the L1 adhesion molecule, and the fibronectin gene. LEF/TCF transcription factors also upregulate genes required for basal lamina invasion, including mmp-3 and mmp-7. Other transcription factors that have a role in promoting EMTs are the class I basic helix-loophelix factors E2-2A and E2-2B, the forkhead box transcription factor FOXC2, the homeobox protein Goosecoid, and the homeoprotein Six1.

To summarize, transcription factors that regulate an EMT often do so by directly epressing cell adhesion and epithelial polarity molecules, and by upregulating genes required for cell motility and basal lamina invasion.

- ***Regulation at the promoter level***

Given the importance of the Snail, Zeb and LEF/TCF transcription factors in orchestrating the various phases of an EMT, it is essential to understand the upstream events that regulate these EMT-promoting transcription factors.

The activation of Snail-1 transcription in Drosophila requires the transcription factors Dorsal (NF-kB) and Twist. The human Snail-1 promoter also has functional NF-kB sites and blocking NF-kB reduces Snail-1 transcription. Additionally, a region of the Snail-1 promoter is responsive to integrin-linked kinase (ILK), and ILK can activate Snail-1expression via poly-ADP-ribose polymerase (PARP). In mouse mammary epithelial cells, high mobility group protein A2 (HMGA2) and Smads activate Snail-1 expression, and subsequently Snail-2, Twist, and Id2 transcription. For Snail-2 expression, myocardin related transcription factors (MRTFs) interact with Smads to induce Snail-2 and MRTFs may play a role in metastasis and fibrosis. There are also several Snail-1 transcriptional repressors. In breast cancer cell lines, metastasis-associated protein 3 (MTA3) binds directly to and represses the transcription of Snail-1 in combination with the Mi-2/NuRD complex, as also does lysine-specific demethylase (LSD1). The Ajuba LIM proteins (Ajuba, LIMD1 and WTIP) are additional transcriptional corepressors of the Snail family.

The transcription of LEF/TCF genes such as Lef-1 is activated by Smads. The misexpression of Snail-1 results in the transcription of dEF-1 and Lef-1 through a yet unknown mechanism. Post-transcriptional regulation of EMT transcription factors

The activity of EMT transcription factors is also regulated post-transcriptionally, where alternative splicing, translational control, protein stability (targeting to the proteasome) and nuclear localization can all regulate an EMT.

One newly discovered layer of EMT regulation is the epithelial or mesenchymal specific expression of alternatively spliced transcripts. For example, epithelial splicing regulatory proteins 1 and 2 (ESRP1 and ESRP2) are two RNA-binding proteins that regulate the epithelialspecific expression of many molecules that are important to an EMT such as Rho regulators, integrins, and collagen. Blocking ESRP1 and 2 expression changes the pattern of alternatively spliced transcripts and causes cultured epithelial cells to upregulate vimentin and fibronectin, to reduce E-cadherin at cell-cell contacts, and to increase protease activity . Non-coding RNAs are also emerging as important regulators EMTs. In a breast cancer model, Myc activates the expression of microRNA-9 (miR-9), and miR-9 directly binds to and represses the E-cadherin promoter .Members of the miR-200 family repress the translation of Zeb1, and the expression of these miR-200 family members are repressed by Snail-1.

Additionally, Zeb2 transcription can be activated by naturally occurring RNA ntisense transcripts [1]. It is not yet known if there are non-coding RNAs that regulate Snail family members. However, the Y-box binding protein-1 (YB-1) is important for the selective activation of Snail-1 translation.

Protein stability is another layer of EMT control. Snail-1 is phosphorylated by GSK-3b and targeted for destruction. Therefore, the inhibition of GSK-3b activity by Wnt signaling may have multiple roles in an EMT, leading to the stabilization of both b-catenin and Snail-1. Some proteins that prevent GSK-3b-mediated phosphorylation (and thus promote Snail-1 activati on) are lysyl-oxidase-like proteins LOXL2, and ILK. A Snail-1 specific phosphatase (Snail-1 activator) is C-terminal domain phosphatase (SCP). Snail-2 is targeted for degradation by the direct action of p53 and the ubiquitin ligase Mdm2.

In addition to protein translation and stability, the function of Snail-1 also depends upon nuclear localization mediated by Snail-1's nuclear localization sequence. The phosphorylation of human Snail-1 by p21-activated kinase 1 (Pak1) promotes the nuclear localization of Snail-1 (and therefore Snail-1 activation) in breast cancer cells.

In zebrafish, LIV-1promotes the translocation of Snail-1 into the nucleus . Snail-1 also contains a nuclear export sequence (NES) that is dependent on the calreticulin (CalR) nuclear export pathway [46]. This NES sequence is activated by the phosphorylation of the same lysine residuestargeted by GSK-3b, which suggests a mechanism whereby phosphorylation of Sn ail-1 by GSK-3b results in the export of Snail-1 from the nucleus and subsequent degradation. LEF/TCF activity is also regulated by other proteins. b-catenin is required as a co-factor for LEF/ TCF-mediated activation of transcription, and Lef-1 can also associate with co-factor Smads to activate the transcription of additional EMT genes . In colon cancer cells, Thymosin b4stabilizes ILK activity.

In summary, EMT transcription factors such as Snail-1, Zeb1 and Lef-1 are regulated by a variety of mechanisms, both at the transcriptional level, and post-transcriptional level by alternative splicing, non-coding RNA translation control, protein degradation, nuclear localization, and co-factors such as b-catenin.

2.3. Molecular control of the EMT

The initiation of an EMT or MET is a tightly regulated event during development and tissue repair because deregulation of cellular organization is disastrous to the organism. A variet y of external and internal signaling mechanisms coordinate the complex events of the EMT, and these same signaling pathways are often disrupted or reactivated during disease. EMTs or METs can be induced by either diffusible signaling molecules or ECM components. Below we discuss the role of signaling molecules and ECM in triggering an EMT, and then present a summary model for EMT induction.

Ligand-receptor signaling During development, five main ligand-receptor signaling pathways are employed, namely TGF-b, Wnt, RTK, Notch, and Hedgehog. These pathways, among others, all have a role in triggering EMTs. While the activation of a single signaling pathway can be sufficient for an EMT, **in most cases an EMT or MET is initiated by multiple signaling pathways acting in concert.**

- ***TGF-b PATHWAY***

The transforming growth factor-beta (TGF-b) superfamily includes TGF-b, activin, and bone morphogenetic protein (BMP) families. These ligands operate through receptor serine/threonine kinases to activate a variety of signaling molecules including Smads, MAPK, PI3K and ILK. Most of the EMTs studied to date are induced in part, or solely, by TGF-b superfamily members. During embryonic heart development, TGF-b2 and TGF-b3 have sequential and necessary roles in activating the endocardium to invade the cardiac jelly and form the endocardial cushions. In the avian neural crest, BMP4 induces Snail-2 expression. In the EMT that transforms epithelial tissue into metastatic cancer cells, TGF-b acts as a tumor suppressor during early stages of tumor development, but as a tumor/EMT inducer at later stages. TGF-b-signaling may combine with other signaling pathways to induce an EMT.For example, in cultured breast cancer cells, activated Ras and TGF-b induce an irreversible.

EMT, and in pig thyroid epithelial cells, TGF-b and epidermal growth factor (EGF) synergistically stimulate the EMT. One outcome of TGF-b signaling is **to immediately change epithelial cell polarity**. In a TGF-binduced EMT of mammary epithelial cells, TGF-bR II directly phosphorylates the polarity protein, Par6, leading to the dissolution of tight junctions. TGF-b signaling also **regulates gene expression** through the phosphorylation and activation of Smads. Smads are important co-factors in the stimulation of an EMT. For example, Smad3 is necessary for a TGF-b-induced EMT in lens and kidney tissue in vivo. Smad3/4 also complex with Snail-1 and co-represses the promoters of cell-cell adhesion molecules. Further, TGF-bR I directly binds to and activates PI3K, which in turn activates ILK and downstream pathways. ILK is emerging as an important positive regulator of EMTs. ILK interacts directly with growth factor receptors (TGF-b, Wnt or RTK), integrins, the actin skeleton, PI3K, and focal adhesion complexes. ILK directly phosphorylates Akt and GSK-3b, and results in the subsequent activation of transcription factors such as AP-1, NF-kB,

and Lef-1. Overexpression of ILK in cultured cells causes the suppression of GSK-3b activity, translocation of b-catenin to the nucleus, activation of Lef-/b-catenin transcription factors, and the downregulation of E-cadherin. Inhibition of ILK in cultured colon cancer cells leads to the stabilization of GSK-3b activity, decreased nuclear b-catenin localization, the suppression of Lef-1 and Snail-1 transcription, and reduced invasive behavior of colon cancer cells. ILK activity also results in Lef-1-mediated transcriptional upregulation of MMPs. Hence, ILK (inducible by TGF-b signaling) is capable of orchestrating most of the major events in an EMT, including the loss of cell-cell adhesion and invasion across the basal lamina. WNT PATHWAY

Many EMTs or METs are also regulated by Wnt signaling. Wnts signal through seven- ass transmembrane proteins of the Frizzled family, which activates G-proteins, PI3K, inhibits GSK-3b and promotes nuclear b-catenin signaling. For example, during zebrafish gastrulation, Wnt11 activates the GTPase Rab5c, which results in the endocytosis of E-cadherin. Wnt6 signaling is sufficient for increased transcription of Snail-2 in the avian neural crest .Snail-1 expression increases Wnt signaling, which suggests a positive feedback loop.

One of the downstream signaling molecules activated by Wnt signaling is b-catenin. b-catenin is a structural component of adherens junctions. Nuclear b-catenin is also a limiting factor for the a tivation of LEF/TCF transcription factors. b-catenin is pivotal for regulating most EMTs. Interfering with nuclear b-catenin signaling blocks the ingression of sea urchin PMCs, and in b-catenin mouse knockouts, the primitive streak EMT does not occur, and no mesoderm is formed. b-catenin is also necessary for the EMT that occurs during cardiac cushion development . In breast cancer, b-catenin expression is highly correlated with metastasis and poor survival and blocking b-catenin function in tumor cells inhibits invasion in vitro. It is unclear if b-catenin overexpression alone is sufficient for all EMTs. If b-catenin is misexpressed in cultured cells, it causes apoptosis. However, the misexpression of a stabilized form of b-catenin in mouse epithelial cells in vivo results in metastatic skin tumors.

- ***Signaling by RTK ligands***

The receptor tyrosine kinase (RTK) family of receptors and the growth factors that activate them also regulate EMTs or METs.such as Ras/MAPK, PI3K/Akt, JAK/STAT, or ILK. Below we cite someexamples of RTK signaling in EMTs and METs. Hepatocyte growth factor (HGF, also known as scatter factor) acts through the RTK c-met. HGF is important for the MET in the developing kidney . HGF signaling is required for the EMTthat produces myoblasts (limb muscle precursors) from somite tissue in the mouse. In epithelial cells, HGF causes an EMT through MAPK and early growth response factor-1 (Egr-1) signaling.

Fibroblast growth factor (FGF) signaling regulates mouse primitive streak formation. FGF signaling also stimulates cell motility and activates MMPs. EGF promotes the endocytosis of E-cadherin. EGF can also increase Snail-1 activity via the inactivation of GSK3-band EGF promotes increased Twist expression through a JAK/STAT3 pathway. Insulin growth factor (IGF) signaling induces an EMT in breast cancer cell lines through the activation of Akt2 and suppression of Akt1. In prostate cancer cells, IGF-1 promotes Zeb-1 expression. In fibroblast cells, constitutively activated IGF-IR increases NF-kB activity and Snail-1 levels. In several cultured epithelial cell lines, IGFR1 is associated with the complex of E-cadherin and b-catenin, and the ligand IGF-II causes the redistribution of b-catenin from the membrane to the nucleus, activation of the transcription factor TCF-3, and a subsequent EMT.

Another **RTK known for its role in EMTs is the ErbB2/HER-2/Neu receptor**, whose ligand is heregulin/neuregulin. Overexpression of HER-2 occurs in 25% of human breast cancers, and the misexpression of HER-2 in mouse mammary tissue in vivo is sufficient to cause metastatic breast cancer . Herceptin_ (antibody against the HER-2 receptor) treatment is effective in reducing the recurrence of HER-2-positive metastatic breast cancers. HER-2 signaling activates Snail-1 expression in breast cancer through an unknown mechanism . **The RTK Axl is also required for breast**

cancer carcinoma invasiveness . Vascular endothelial growth factor (VEGF) signaling promotes Snail-1 activity by suppression of GSK3-b , and results in increased levels of Snail-1, Snail-2, and Twist . Snail-1 can also activate the expression of VEGF. In summary, RTK signaling is mportant for many EMTs.

- ***Notch Pathway***

The Notch signaling family also regulates EMTs. When the Notch receptor is activated by its ligand Delta, an intracellular portion of the Notch receptor ligand is cleaved and transported to the nucleus where it regulates target genes. Notch1 is required for cardiac endothelial cells to undergo an EMT to make cardiac cushions, and the role of Notch may be to make cells competent to respond to TGF-b2. In the avian neural crest EMT, Notch signaling is required for the induction and/or maintenance of BMP4 expression. Similarly, Notch signaling is required for the TGF-b-induced EMT of epithelial cell lines, and Notch promotes Snail-2 expression in cardiac cushion cellsand cultured cells.

- ***Hedgehog pathway***

The hedgehog pathway is also involved in EMTs. **Metastatic prostate cancer cells express high levels of hedgehog and Snail-1.** If prostate cancer cell lines are treated with the hedgehog pathway inhibitor, cyclopamine, levels of Snail-1 are decreased. If the hedgehog-activated transcription factor, Gli, is misexpressed, Snail-1 expression increases.

Additional signaling pathways Other signaling pathways that activate EMTs include **inflammatory signaling molecules, lipid hormones, ROS species, and hypoxia**. **Interleukin-6** (inflammatory and immune response) can promote Snail-1 expression in breast cancer cells , and Snail-1 in turn can activate Il-6 expression , providing a link between inflammation and EMTs . The lipid hormone prostaglandin E2 (PGE2) induces Zeb1 and Snail activity in lung cancer cells , and Snail-1 can also induce PGE2 expression . ROS species can also activate EMTs by PKC and MAPK

signaling. Hypoxia is important for initiating EMTs uring developmentand disease, often through hypoxia-inducible factor-1 (HIF-1), which directly activates Twist expression. Hypoxia also activates lysyl oxidases (LOX), which stabilize Snail-1 expressionby inhibiting GSK-3b activity.

In addition to diffusible signaling molecules, extracellular matrix molecules also regulate EMTs or METs. This was first dramatically demonstrated when lens or thyroid epithelium was embedded in collagen gels, and then promptly underwent an EMT. Integrin signaling appears to be important in this process, and involves ILK mediated activation of NF-kB, Snail-1, and Lef-1. Other ECM components that regulate EMTs include hyaluronan, the gamma-2 chain of laminin 5, periostin, and podoplanin. In summary, a variety of diffusible signals and ECM components can stimulate EMTs or METs. Fi

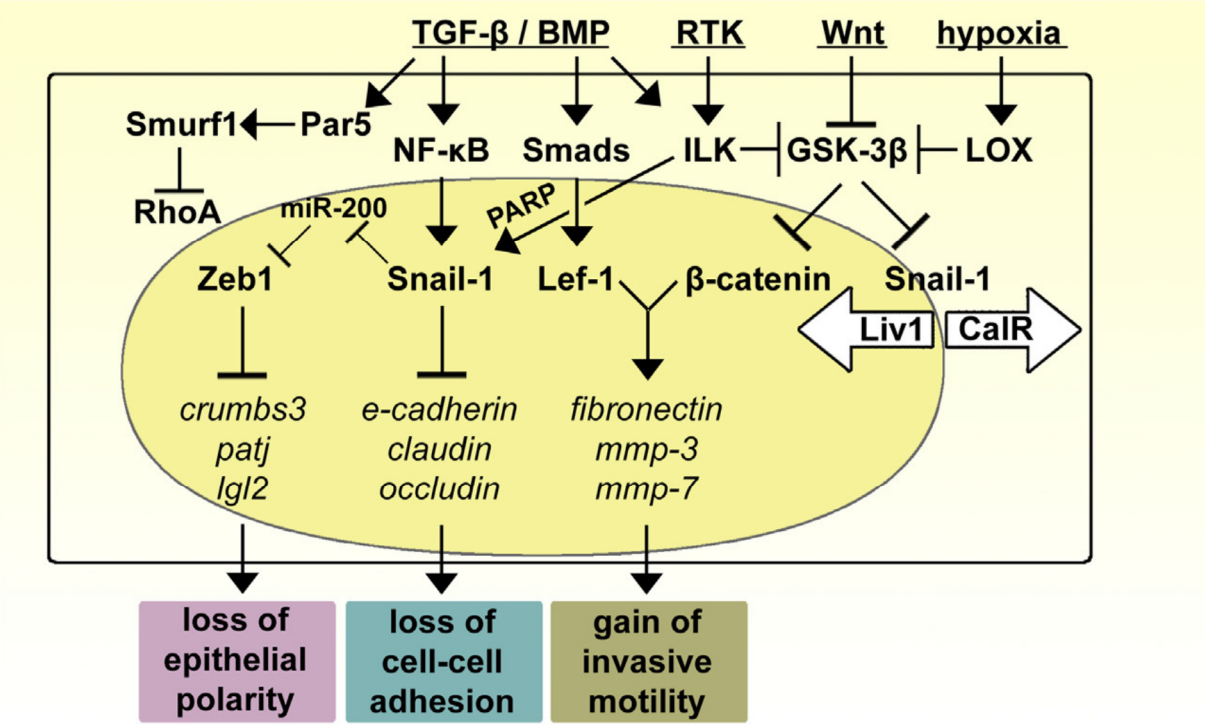

Figure 5: Induction of an EMT.(75)

This figure summarizes some of the important molecular pathways that bring about an EMT. Many of the signaling pathways converge on the activation of Snail-1 and nuclear b-catenin signaling to change gene expression, which results in the loss of epithelial cell polarity, the loss of cell-cell adhesion, and increased invasive cell motility.

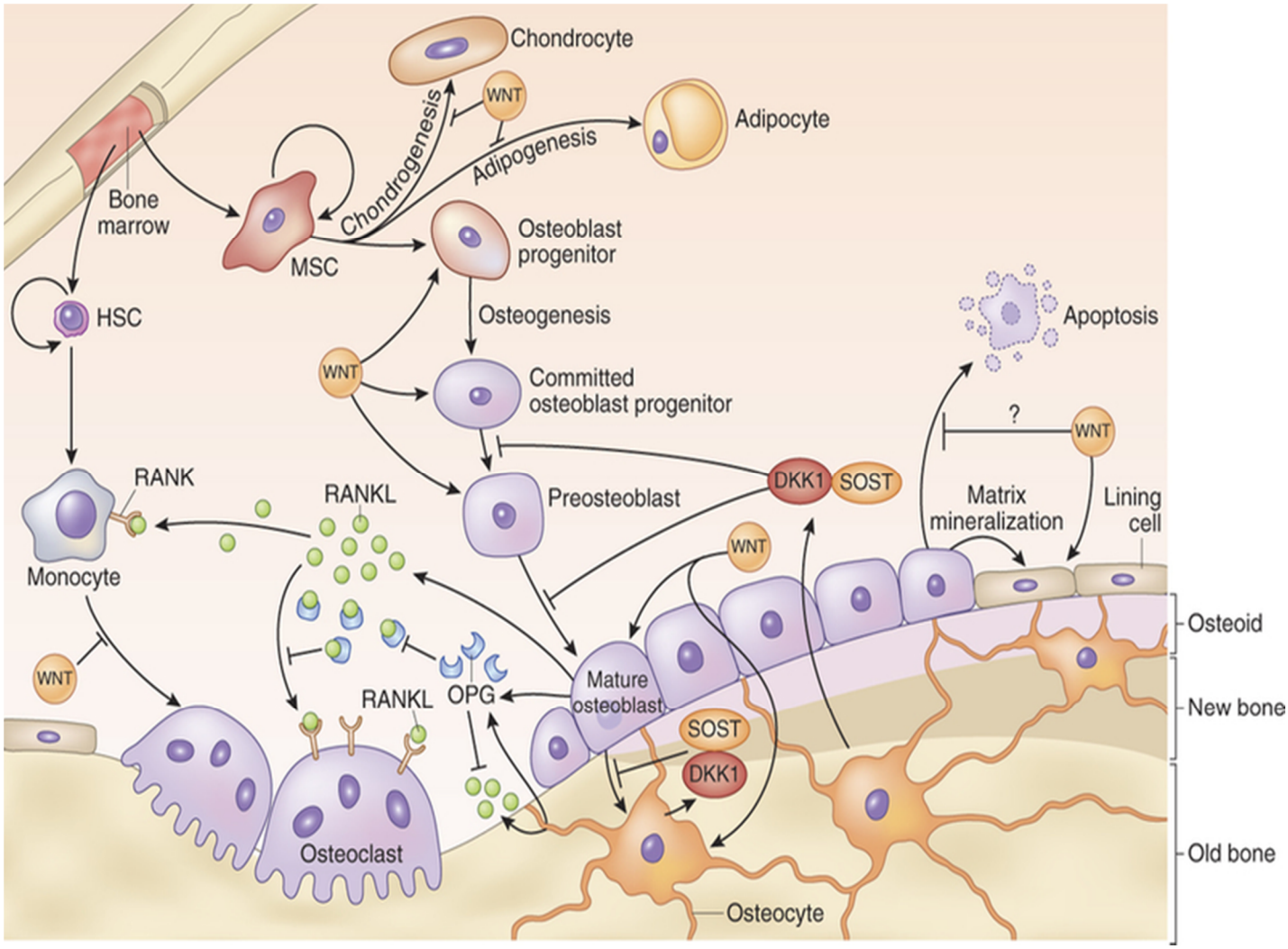

Figure 6: Impact of WNT/β-catenin signaling on bone cells.(76)

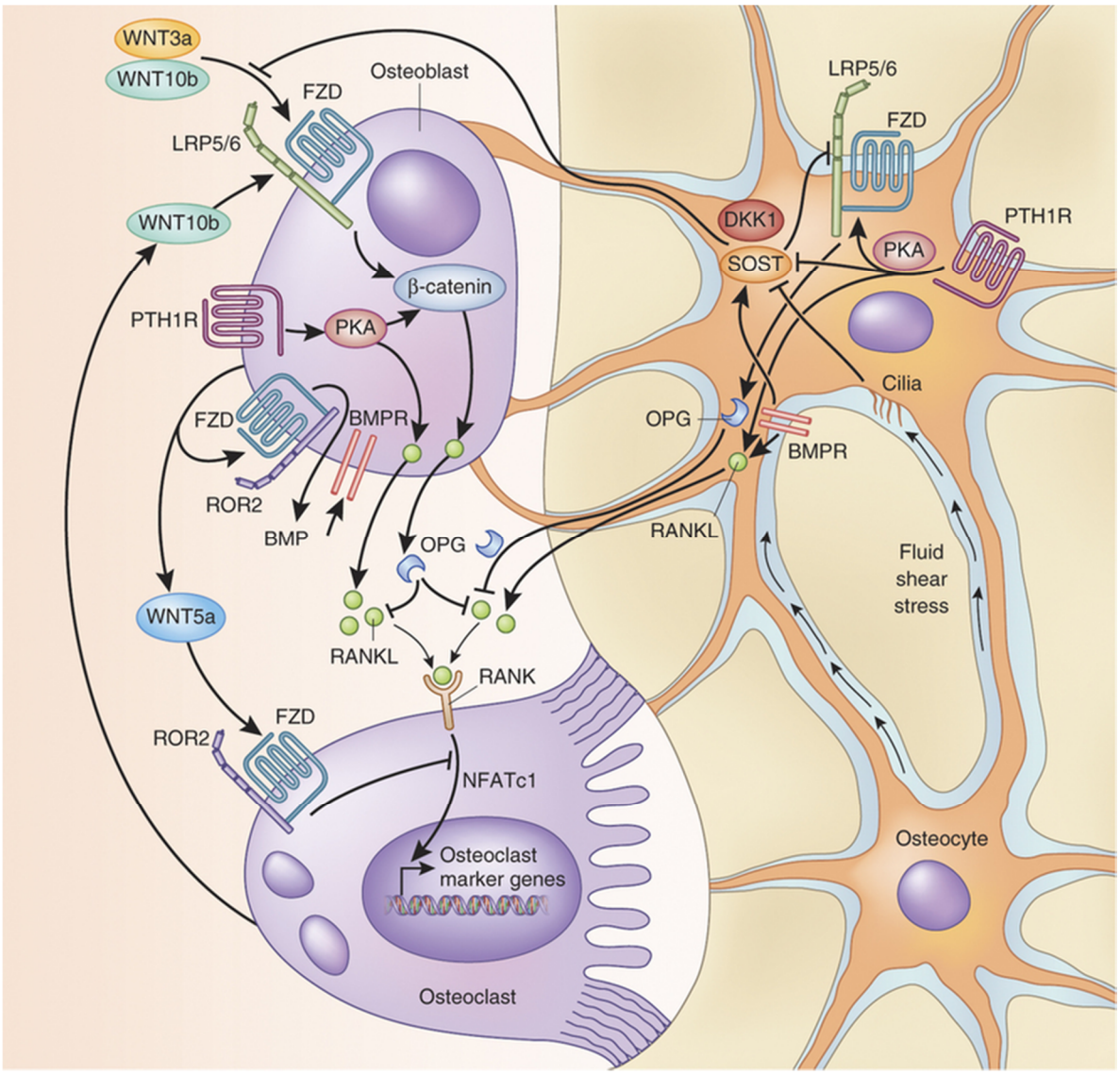

Figure 7: Crosstalk of WNT, PTH and BMP signaling between bone cells (76)

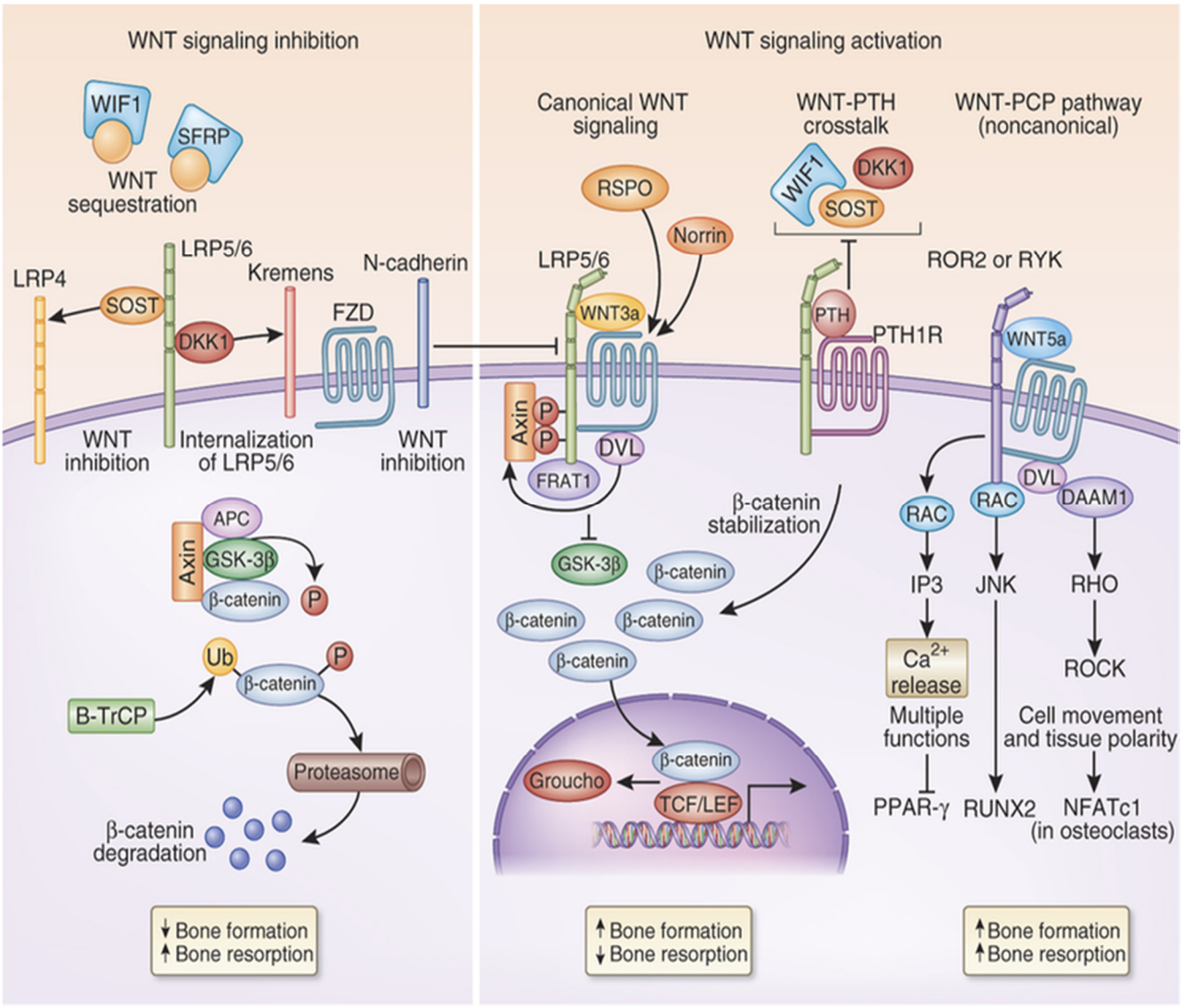

Figure 8: WNT signaling: a simplified view (76)

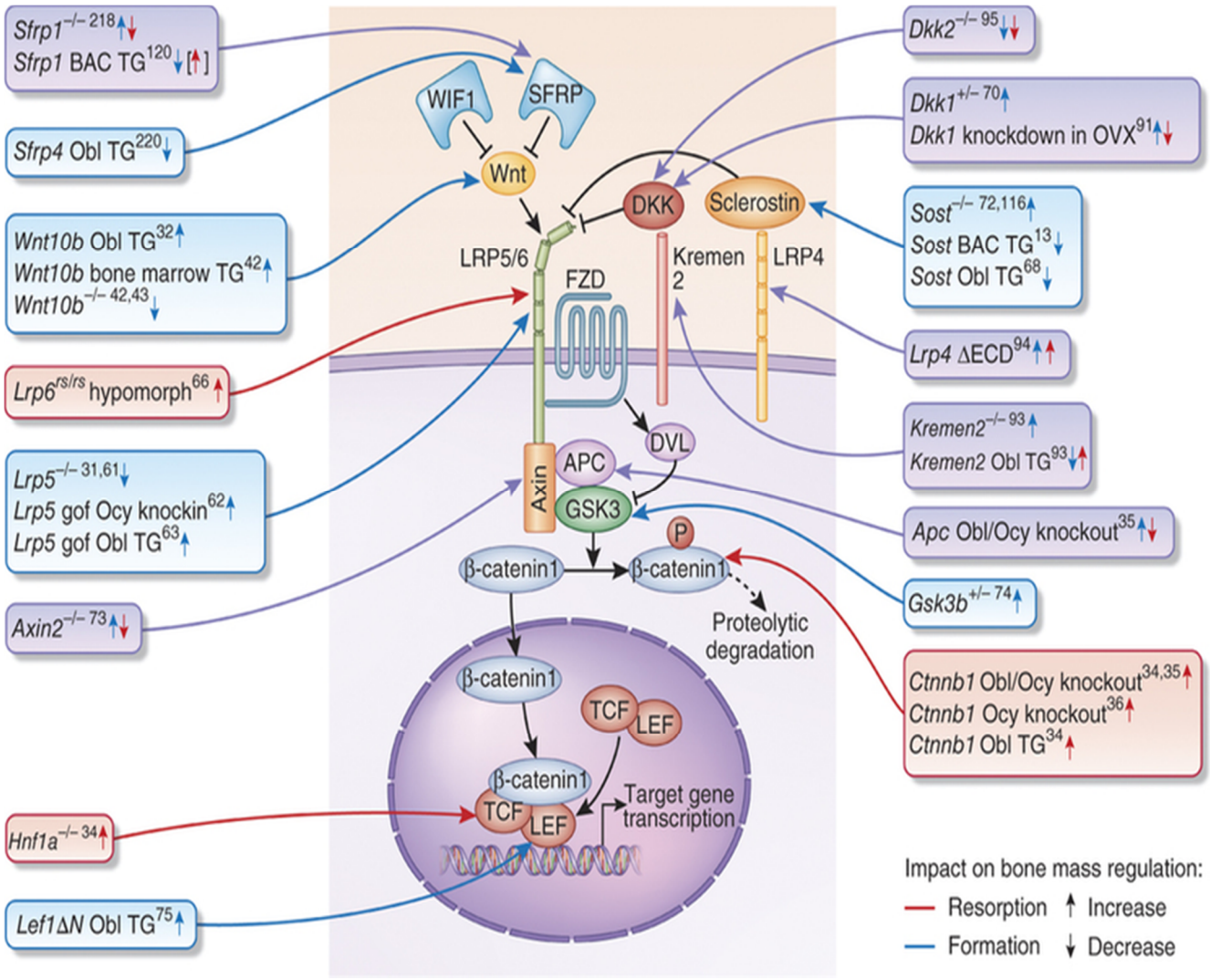

Figure 9: WNT signaling pathway members regulate bone mass: lessons from mouse genetics. (76)

- ***A model for EMT induction***

Many of the experimental studies on EMT mechanisms focus on one small part of the entire EMT process, and while great progress has been made in discovering EMT pathways, the entire EMT signaling network is still . From experimental evidence to date, it appears that many of the EMT signaling pathways converge on ILK, the inhibition of GSK-3b, and stimulation of nuclear b-catenin signaling to activate Snail and LEF/TCF transcription factors. Snail, eb and LEF/TCF transcription factors then act on a variety of targets to suppress cell-cell adhesion, induce changes in cell polarity, stimulate cell motility, and promote invasion of the basal lamina.

2.4. Cell-ECM Interactions:

- ***ECM composition***

The extracellular microenvironment contains both traditional structural matrix components, such as hyaluronic acid, proteoglycans, collagens, glycosaminoglycans, and elastins, and non-structural matricellular proteins, including secreted protein acidic and rich in cysteine (SPARC), tenascin, osteopontin, and thrombospondins. The distribution and organization of these molecules is not static, but varies from tissue to tissue and, during development, from stage to stage, which has significant implications for tissue function. For example, mesenchymal cells are immersed in an interstitial matrix that confers specific biomechanical andfunctional properties to connective tissue, whereas epithelial and endothelial cells contact a specialized matrix, the basement membrane, via their basal surfaces only, conferring mechanical strength and specific physiological properties to the epithelia. This diversity of composition, organization, and distribution of ECM results not only from differential gene expression of the various molecules in specific tissues, but also from the existence of differential splicing and post-translational modifications of those molecules. For example, alternative splicing may change the binding potential of proteins to other matrix molecules or to their receptors, variations in glycosylation can lead to changes in cell adhesion and migration, and proteolytic cleavage can generate fragments with diverse biological functions.

The ECM harbors many non-matrix proteins, including growth factors, cytokines, and matrix-degrading enzymes and their inhibitors. Tissue-specific and developmental-stage specific variation in matrix composition may determine the presence and localization of associated growth factors and cytokines. ECM molecules can influence the local concentration and biological activity of growth factors and cytokines by regulating their diffusion from theirsites of origin, by protecting them from degradation, by presenting them more efficiently to their receptors, and by altering levels of their receptors . Growth factor binding to intact ECM molecules or matrix fragments may also have inhibitory effects on growth factor signaling, by decreasing growth factor synthesis, impairing the interaction of these factors with their receptors, and/or decreasing levels of their receptors.

ECM/growth factor interactions can also involve the ability of specific domains of ECM molecules (e.g., laminin-332, tenascin-C, thrombospondin, and decorin) to activate growth factor receptors. The EGF-like repeats of laminin and tenascin-C bind and activate the EGFR. In the case of laminin, the EGF-like repeats interact with EGFR following their release by MMP-mediated proteolysis whereas tenascin-C repeats are thought to bind EGFR in the context of the full-length protein. Decorin also binds and activates EGFR, although this binding occurs via leucine-rich repeats rather than EGF-like repeats]. In contrast, the EGF-like repeats of thrombospondin 1 appear to activate the EGFR in an indirect, MMP9-dependent manner, likely via MMP9-mediated release of heparin-binding EGF (HB-EGF) . The ability of ECM molecules to activate growth factor receptors may facilitate a stable signaling environment for the associated cells due to the inability for the ligand to either diffuse or be internalized, thus serving as a long-term pro-migratory and/or pro-proliferative signal.

- ***Receptors for ECM molecules***

Integrins, a family of heterodimeric transmembrane proteins composed of a and b subunits, were the first ECM receptors to be identified. At least 18 a and 8 b subunits have been identified that pair in various combinations to yield 24 separate heterodimers, many of which recognize specific sequences on the ECM molecules . Some integrin heterodimers exhibit a high degree of ligand specificity, while others are able to interact with a variety of epitopes , facilitating plasticity and redundancy in specific systems. Although the a and b subunits of integrins are unrelated, there is 40e50% homology within each subunit with the highest divergence in the intracellular domain of the a subunit. Apart from integrin b4, the integrins have large extracellular domains and very small intracellular domains. Despite the small size of the cytoplasmic domains, integrins are able to bind to a variety of intracellular proteins, facilitating their interactions with the cytoskeleton and with signal transduction pathways.

Transembrane proteoglycans, including syndecans, a receptor for hyaluronan mediated motility (RHAMM), and CD44, can also serve as receptors for ECM molecules, including collagen, fibronectin, laminin, and hyaluronan. Syndecans, for example, mediate cell-ECM interactions via chondroitin- and heparan sulfate glycosaminoglycans, whose composition varies based upon the type of syndecan and the tissue in which it is expressed. These differential glycosaminoglycan modifications alter the binding capacity of particular ligands, such as fibronectin and tenascin. The short cytoplasmic domains of syndecans can interact with signaling proteins and the cytoskeleton, and thereby induce signal transduction upon binding to their ECM ligands, resulting in changes in cell adhesion and migration. In addition to syndecan's function as a matrix receptor, the protein core can bind nd activate some integrins directly, and activate other integrins indirectly, by facilitating their interactions with the matrix. Another proteoglycan receptor, CD44, undergoes tissue-specific splicing and glycosylation to yield multiple isoforms. CD44 has multiple ligands, including

collagen IV, collagen XIV, fibronectin, osteopontin, and laminin among others, in addition to its primary ligand, hyaluronan. CD44 can also interact with another hyaluronan receptor, RHAMM.

RHAMM, which is not an integral membrane protein, must bind transmembrane rotein(s), such as CD44, integrins, and/or receptor tyrosine kinases, to transmit the signal from hyaluronan to intracellular signaling proteins . Hyaluronan is present in most tissues in a high molecular weight, native form, but can be cleaved by various enzymes to renerate lower molecular weight fragments; this cleavage is frequently associated with tissue damage. The native and cleaved forms of hyaluronan elicit different cellular responses, potentially due to differential receptor selectivity. CD44 binds more stably to high molecular weight hyaluronan than low molecular weight hyaluronan fragments. In contrast, hyaluronan fragments, but not the native high molecular weight form, bind and activate toll-like receptors (TLRs), strongly suggesting that the fragments function as ’danger signals’ that sense tissue damage and induce inflammatory responses.

Additional extracellular matrix receptors have also been identified, including the elastin/ laminin receptor (ELR), CD36, annexin II, and receptor tyrosine kinases.

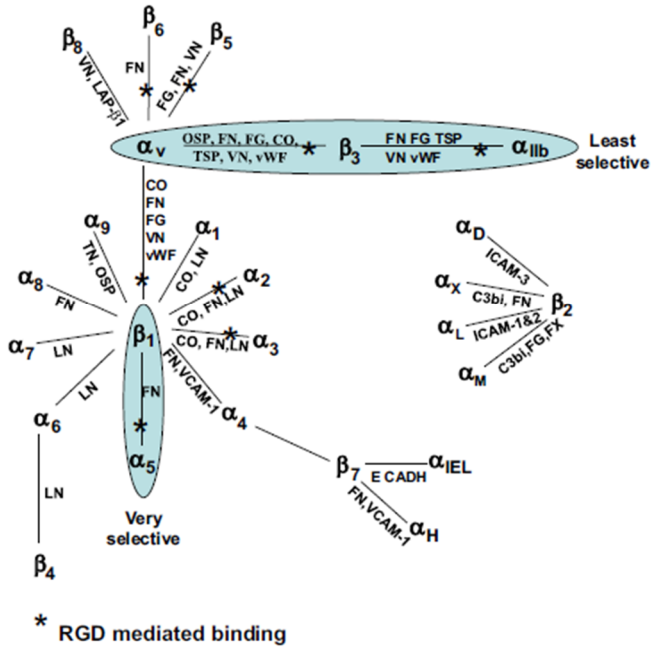

Figure 10 :Members of the integrin family of ECM receptors and their respective ligands.(75)

Members of the integrin family of ECM receptors and their respective ligands. These heterodimeric receptors are composed of one a and one b subunit, and are capable of binding a variety of ligands, including Ig superfamily cell adhesion molecules, complement factors, and clotting factors in addition to ECM molecules. Cell-cell adhesion is largely mediated through integrin heterodimers containing the b2 subunits, while cell-matrix adhesion is mediated primarily via integrin heterodimers containing the b1 and b3 subunits. In general, the b1 integrins interact with ligands found in the connective tissue matrix, including laminin, fibronectin, and collagen, whereas the b3 integrins interact with vascular ligands, including thrombospondin, vitronectin, fibrinogen, and von Willebrand factor. Abbreviations: CO, collagens; C3bi, complement component; FG, fibrinogen; FN, fibronectin; FX, Factor X; ICAM-1, intercellular adhesion molecule-1; ICAM-2, intercellular adhesion molecule-2; ICAM-3, intercellular adhesion molecule-3; LN, laminin; OSP, osteopontin; TN, tenascin; TSP, thrombospondin;VCAM-1, vascular cell adhesion molecule-1; VN, vitronectin; vWF, von Willebrand factor.

The ELR is a complex of three proteins, including neuraminidase 1, cathepsin A, and the elastin-binding protein (EBP). The EBP binds specifically to the GXXPG sequence found in elastin, fibrillin, laminin, and fragments of these matrix molecules, and is critical for elastin deposition The ELR can also bind to the YIGSR sequence in the b1 chain of laminin-111. Elastin-derived peptides generated by proteases activated in response to tissue injury can promote proliferation and/or migration of fibroblasts, epithelial/endothelial cells, and monocytes downstream of the ELR. These effects suggest that elastin-derived peptides are able to promote wound healing; indeed, these peptides enhance the healing of burn wounds when used in conjunction with more conventional treatments. CD36 can also function as a matrix receptor, despite its better-known role as a scavenger receptor, binding thrombospondin, collagen I, and collagen V. In endothelial cells, thrombospondin binding to CD36 induces apoptosis, and is thus anti-angiogenic in vivo. Another cell surface receptor, annexin II, is known to interact with alternative splice variants of tenascin-C, and mediates the cellular responses to these various forms of tenascin-C . Tyrosine kinase receptors, including the EGFR and the discoidin domain receptors DDR1 and DDR2, can also function as matrix receptors. The EGFR can be activated by EGF-like domains or by MMP-mediated release of HB-EGF (see above), while DDR1 and DDR2 serve as collagen receptors. Unlike most other receptor tyrosine kinases, which are activated by dimerization, DDR1 and DDR2 exist as constitutive homodimers, suggesting an alternative mechanism of activation. Upon ligand binding, the DDRs undergo autophosphorylationand induce multiple downstream signaling pathways that ultimately alter cell adhesion and migration. Several studies have suggested various roles for the DDR receptors in ECM remodeling. In smooth muscle cells, over-expression of DDR reduces the expression of several matrix molecules and receptors, including collagen, syndecan-1, and integrin a3, while increasing MMP activity, resulting in degradation of collagen and elastin. However, inhibition of DDR signaling in fibroblasts decreased collagen synthesis, suggesting that DDR may exert cell-type-specific effects on matrix deposition and remodeling.

Below, we will first discuss selected examples that illustrate the dynamics of cell-ECM interactions during development and wound healing, as well as the potential mechanisms involved in the signal transduction pathways initiated by these interactions. Finally, we will discuss the implications of cell-ECM interactions in tissue engineering.

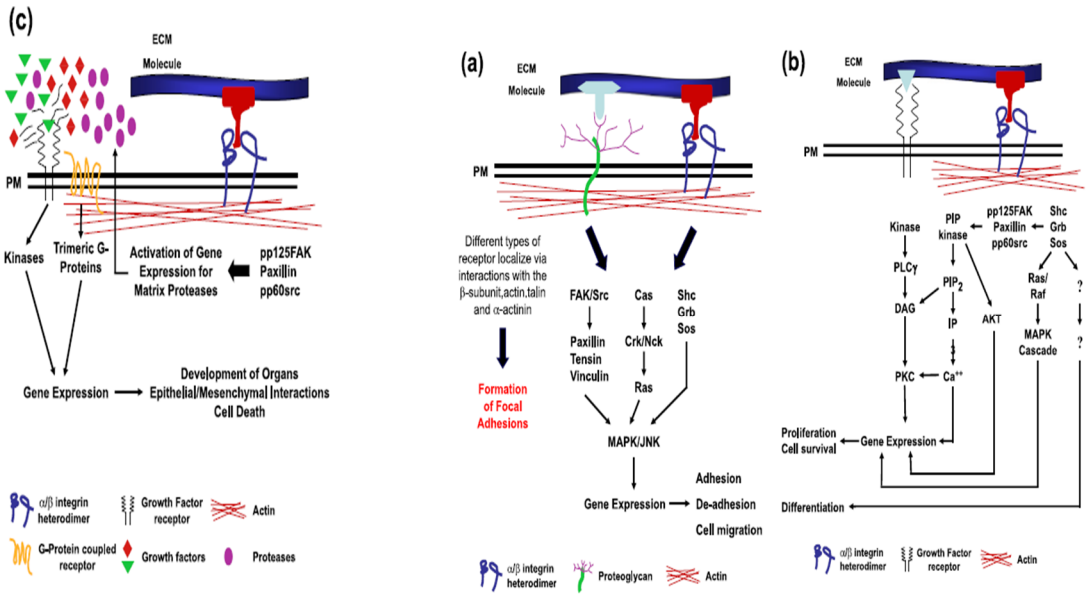

Figure 11: Schematic diagram of cell-ECM interactions present during healing and regenerative responses. Type I (a), type II (b) and type III (c) (75)

Such interactions between the ECM receptors and their respective ligands initiate signal transduction cascades culminating in a variety of cellular events important in repair andregeneration, including changes in cellular adhesion and migration and altered rates of proliferation and apoptosis. The presence and/or extent of such changesmay influence the balance of repair and regenerative responses to favor one outcome over another; thus, interventions that alter ECM signaling events may shiftthis balance to favor tissue regeneration and thus decrease scarring.

2.5. CELL-ECM Interactions

Multiple biological processes, including those relevant to development and wound healing, require both interactions between cells and their environment and modulation of such interactions. During development, the cellular crosstalk with the surrounding extracellular matrix promotes the formation of patterns, the development of form (morphogenesis), and the acquisition and maintenance of differentiated phenotypes during embryogenesis. Similarly, during wound healing these interactions contribute to the processes of clot formation, inflammation, granulation tissue development, and remodeling. As outlinedbelow, the current body of research in the fields of both embryogenesis and wound healing implicates multiple cellular behaviors, including cell adhesion/de-adhesion, migration, proliferation, differentiation and apoptosis, in these critical events.

- ***Development ADHESION AND MIGRATION***

Today, there is a vast body of experimental evidence that demonstrates the direct participation of ECM in cell adhesion and migration, but some of the most compelling experiments came from studies in gastrulation, migration of neural crest cells (NCC), angiogenesis, and epithelial organ formation. Cell interactions with fibronectin are important during gastrulation; inhibition of fibronectin-integrin interactions in amphibian embryos by the introduction of blocking antibodies or RGD-containing peptides into their blastocoel cavities, which compete with integrins for ECM binding and disrupt normal cell movement leading to abnormal development . Similarly, introduction of recombinant fibronectin lacking the RGD motif perturbs amphibian gastrulation. These effects are not unique to fibronectin, as they can also be introduced by manipulation of other molecules, such as hyaluronan and heparan sulfate proteoglycans. Inhibition of hyaluronan synthesis in zebrafish embryos interferes with cell movements in gastrulation, potentially due to a defect in Rac1 activation, as expression of constitutively active Rac rescued the observed migratory defects. In the case of HSPG, degradation of HSPG by injection of the blastocoel with

heparinitase interferes with gastrulation. More recent studies suggest that the interaction between HSPG and fibronectin is critical for gastrulation. Binding of fibronectin to HSPG causes a conformational change in fibronectin, exposing growth factor binding sites that then bind PDGF-AA, generating a stable PDGF gradient that promotes the directional cell migration that is critical for gastrulation. HSPGs are also necessary for FGF signaling during gastrulation, as inhibition of HSPG synthesis alters FGF localization, inhibits FGF signaling, and arrests embryonic development at gastrulation.

Cell-matrix interactions are also important for the migration of NCC, which develop in the dorsal portion of the neural tube just after closure of the tube, de-adhering from each other and then migrating extensively throughout the embryo in ECM-filled spaces, giving rise to a variety of phenotypes. The importance of cell-ECM interactions in NCC migration is supported by studies performed in the white mutant of Mexican axolotl embryos. The NCC that give rise to pigment cells fail to emigrate from the neural tube in these embryos, but when microcarriers containing subepidermal ECM from normal embryos are implanted into the appropriate area in these mutants, the NCC pigment cell precursors emigrate normally Laminin and fibronectin, the latter of which appears between chick NCC just prior to their emigration from the neural tube , play particularly critical roles in this process. Inhibition of fibronectin, laminin-111 (laminin-1), laminin-411 (laminin-8), or their integrin receptors using function-blocking antibodies, competing peptides, or antisense RNA prevents NCC migration, while exogenous laminin or fibronectin are sufficient to induce premature NCC migration . More recent studies have identified different subsets of NCC, which may exhibit different responses to specific matrix molecules. For example, cranial NCC do not migrate in response to fibronectin, but do migrate on laminin . Matrix remodeling may also contribute to NCC migration, as the matrix-remodeling protease MMP-9 is also required for NCC de-adhesion and migration, while exogenous MMP-9 induced premature de-adhesion and migration. The role of

MMP-9 in de-adhesion may be related to its ability to cleave the cell-cell adhesion molecule N-cadherin, while its role in migration may involve laminin degradation. Proteoglycans also participate in the NCC migration process. Aggrecan, a proteoglycan that predominates in the notochord and cartilage tissue, inhibits NCC migration and may thus delimit NCC migration pathways. The chondroitin sulfate proteoglycan versican may play a similar inhibitory role in NCC migration. In contrast, syndecan-4 is not required for cell migration, but is necessary for directional migration. In conjunction with a non-canonical Wnt pathway, syndecan-4 inhibits Rac at the rear of the cell, restricting Rac activity to the leading edge of the migrating cell and facilitating directional migration.

Endothelial cell interactions with ECM molecules and the type and conformation of the matrix are also crucial in cell adhesion and migration during angiogenesis, a process in which new blood vessels form from pre-existing vessels . Early indications of the role of ECM in angiogenesis were observed when human umbilical vein endothelial cells (HUVEC) were cultured on matrigel, a matrix synthesized by Engelbreth-Holm-Swarm (EHS) tumors. This specialized matrix has many of the properties of basement membrane; it consists of large amounts of laminin, as well as collagen IV, entactin/nidogen, and proteoglycans. When HUVEC are cultured on matrigel for 12 hours, they migrate and form tube-like structures. In contrast, when these cells are cultured with collagen I, they only form tube-like structures after they are maintained inside the gels for one week, at which time the cells have secreted heir own basement membrane molecules . The observation that tube formation occurs more rapidly on matrigel than within collagen gels strongly suggested an important role for one or more of the matrix molecules present within the basement membrane in the development of the capillary-like endothelial tubes. Indeed, **laminin-111, the predominant matrix molecule in matrigel, was later shown to participate in endothelial tube formation and angiogenesis**. Purified or recombinant laminin-111 induce cell adhesion, migration, and tube formation in vitro and angiogenesis in vivo, while a mutation in laminin a1 leads to defective vessel formation . Furthermore, studies in integrin a7-deficient mice suggest that

the laminin receptor integrin a7b1 is necessary for appropriate pericyte interactions with blood vessels, and is thus important in vessel maturation and stability. Recent studies have used knockout mice to implicate a number of ECM molecules in addition to laminin-111 in developmental blood vessel formation and maturation, including laminin a4, fibronectin, collagen IV, and the HSPG perlecan. Mice deficient in laminin a4, fibronectin, or collagen IV exhibit defects in basement membrane formation, a process known to be critical in vessel maturation and stability, while the basement membranes in mice lacking perlecan form normally but have decreased ability to withstand mechanical stress . Several integrin knockouts also exhibit defects in angiogenesis and vessel maturation. Like fibronectin, the fibronectin receptor integrin a5b1 is necessary for angiogenesis, as shown by substantial angiogenesis defects in integrin a5 and integrin b1 endothelial cell-specific knockouts . Abnormal pericyte-blood vessel interactions in integrin a4 edeficient animals suggest the importance of integrin a4b1 in this process, although in this case, the binding of this integrin to VCAM may be more important than its binding to fibronectin . Some of the ECM effects on angiogenesis involve matrix cooperation with growth factors, such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF). VEGF and PDGF interact with HSPG, which can sequester them until their release by proteases, limit their diffusion, and/or function as co-receptors to promote receptor binding and activation . Binding of VEGF to HSPG appears to be important in its localization during development. In mouse embryos, sole expression of a VEGF isoform that lacks the heparin-binding domain, VEGF121, increases diffusion of VEGF from the site of secretion and decreases blood vessel branching. This altered vascular patterning appears to result from impaired endothelial cellmigration, as shown by decreased filopodia formation in the migrating ’tip cell’ of nascentsprouts . In embryos solely expressing the HSPG-binding VEGF isoform, VEGF188, branching of the blood vessels and filopodia formation by endothelial tip cells were increasedwhen compared with wild type . HSPG are also important for vessel stability and maturation,as HSPG are required for PDGF-BB-induced signaling, which, in turn, is important forpericyte migration and interaction with nascent blood vessels .

In contrast to the pro-angiogenic activities of many matrix molecules, fragments generated by their proteolysis can exert anti-angiogenic effects. The YIGSR peptide derived from lamininb1, which binds the ELR rather than an integrin, prevents endothelial cell migration andangiogenesis. YIGSR binding to the ELR promotes laminin cleavage by cathepsin-B.

This cleavage could reveal cryptic binding sites on laminin, altering its ability to bind specific receptors, leading to changes in cell signaling that, in turn, inhibit angiogenesis.

Another possibility is that the YIGSR peptides exert anti-angiogenic properties due to competition for receptor binding with the intact laminin present in vivo. Indeed, if YIGSRpeptides can successfully compete with laminin, the displacement of the YIGSR sequence inthe intact laminin molecule by soluble YIGSR peptides would alter the presentation of theligand to its receptor, resulting in changes in mechanical resistance that alter signaling eventsdownstream of the receptor, yielding different cellular responses. A similar hypothesis hasrecently been proposed for the interactions of integrins with soluble versus intact ligands .

Many soluble integrin ligands generated through proteolytic activity are anti-angiogenic, suchas tumstatin and arresten (derived from collagen IV), endostatin (derived from collagen XVIII), and endorepellin (derived from perlecan), although their contribution to developmental angiogenesis remains unclear . Although the mechanisms generating different cellular outcomes are not completely understood, the fact that **soluble and intact ECM receptor ligands may, at times, lead to alternative outcomes is likely of importance in vivo following matrix degradation**. During angiogenesis, endothelial cell migration and invasioninto surrounding tissues is accompanied by the activation of matrix-degrading enzymes, whichthen cleave the matrix and release both matrix-bound growth factors as well as ECM fragments,providing additional angiogenic or anti-angiogenic cues to further influence the process .

As such, matrix molecules that initially facilitate angiogenesis may be proteolytically cleaved tocreate anti-angiogenic matrix fragments, preventing additional blood vessel formation and/orresulting in vessel maturation. Thus, the temporal and spatial production and cleavage ofmatrix molecules may have important consequences for tissue homeostasis.

- ***Proliferation***

Certain cell-ECM interactions modulate cell proliferation, with some matrix molecules inducing and others inhibiting proliferation. Tenascin-C stimulates cell proliferation in vitro via its EGF-like repeats, which bind and activate the EGFR. Similarly, the EGF-like repeats of laminin stimulate proliferation of a variety of different cell lines, likely via EGFR activation. Some of the ECM effects on cell proliferation involve matrix cooperation with growth factors. As mentioned above, under migration/adhesion, binding of growth factors to matrix molecules can affect their interactions with their receptors, limit their diffusion and/or sequester them until protease-mediated release. Several matrix-associated growth factors, including HB-EGF and TGF_b, can regulate cell proliferation during development.

Several studies have suggested that HSPG-associated molecules may regulate the proliferation of vascular smooth muscle cells (VSMC). Conditioned medium of aortic endothelial cells inhibits FGF-induced VSMC proliferation an effect that is abolished by pre-treatment with heparinase but not with proteases. One possible explanation for this result is that heparan-type molecules may directly inhibit aortic VSMC proliferation. However, it is also possible that heparinase treatment may release pro-proliferative molecules interacting with heparin or heparan sulfate, thus allowing these factors to interact with their receptors and either promote proliferation or block any anti-proliferative effects. One such mitogenic heparin-binding ECM molecule is HB-EGF, which is known to promote VSMC proliferation in vitro, in cultured cells. HB-EGF appears to regulate proliferation of additional mesenchymal cell types during development; a mouse embryo expressing of a mutant HB-EGF that could not be

cleaved and released by HSPG showed defects resembling the HB-EGF knockout, suggesting that HB-EGF release is necessary for its function. In contrast, embryos expressing a soluble version of HB-EGF that could not bind HSPG showed abnormal proliferation of multiple cell types, suggesting a role for soluble, and thus active, HB-EGF in regulating proliferation of these cell types during development . Another heparin-binding ECM molecule that could induce VSMC proliferation is thrombospondin, which is known to exert its mitogenic activities on VSMC via its amino terminal heparin-binding domain. Heparin blocks both thrombospondin binding to smooth muscle cells and its mitogenic effects. These results suggest that interactions between heparin and thrombospondin may interfere with thrombospondin-induced smooth muscle cell proliferation, and that the observed increases in VSMC proliferation following heparinase treatment mentioned previously may result, at least in part, from the removal of such inhibitory interactions.

The effects of heparin on VSMC may also result from its regulation of TGF-b, an inhibitor of VSMC proliferation; heparin increases TGF-b activation, and heparin-mediated antiproliferative effects are blocked by addition of a TGF-b antibody. As such, heparinase treatment may prevent TGF-b activation, abolishing the anti-proliferative effects. To complicate matters further, thrombospondin can promote TGF-b activation as well, although the importance of thrombospondin binding to HSPG in TGF-b activation is unclear. However, if heparin's effects are exclusively mediated by the inhibition of HB-EGF or thrombospondin and/or activation of TGF-b, one would expect that treatment of the endothelial cell conditioned medium with proteases should also eliminate the anti-proliferative effect. As theprotease treatment does not prevent these effects, it is likely that heparin-like moleculesalso have a direct anti-proliferative effect.

TGFb also cooperates with the ECM in the early developmental stages of the mammary gland during puberty. During this period, inductive events take place between the epithelium and the surrounding mesenchyme that are mediated by the basement

membrane (basal lamina and closely associated ECM molecules) and which play an important role in epithelial proliferation during branching of the gland. Endogenous TGFb produced by the ductal epithelium and surrounding mesenchyme forms complexes with mature periductal ECM . This TGF-b may participate in stabilizing the epithelium by stimulating expression of matrix molecules, by inhibiting cell proliferation, and/or by inhibiting matrix-degrading enzymes. In support of this possibility, the mammary gland epithelial cells in transgenic mice expressing a kinase-deficient TGF-b receptor showed excessive proliferation, whereas elevation of TGF-b decreased cell proliferation and increased matrix deposition . In the branching areas, TGFb is absent from newly synthesized ECM; thus, its inhibitory effects on epithelial cell proliferation and on production of matrix-degrading enzymes do not occur, allowing the basement membrane to undergo remodeling. **Along with the in vivo studies described above, in vitro studies using cultured cells suggest that various matrix molecules inhibit cell proliferation. For example, normal human breast cells do not growth arrest when cultured on plastic, but do so if grown in a basement membrane matrix**. More recent studies have shown that laminin-111 is particularly important in this inhibition of proliferation, as cells plated on this matrix molecule also stop dividing. Laminin appears to suppress breast epithelial cell proliferation via expression of Id-2, a transcriptional regulator that binds and inhibits basic helix-loop-helix (HLH) transcription factors, leading to growth arrest of this cell type.

- ***Differentiation***

Matrix molecules are critical in regulating the differentiation of keratinocytes, hepatocytes, and mammary gland epithelium. Keratinocytes form the stratified epidermal layers of the skin. The basal layer is highly proliferative, does not express the markers for terminal differentiation, and is the only cell layer in contact with the basement membrane. As these cells divide, the daughter cells lose contact with the basement membrane, move up to the suprabasal layers,and begin to express differentiation markers such as involucrin and keratins 1 and 10 . This suggests that physical interaction with the basement

membrane represses differentiation of the basal keratinocytes. Early studies implicated fibronectin and b1 integrins in preventing differentiation; however, the later studies showed that the keratinocytes of a conditional integrin b1 skin knockout mouse and those of mice deficient lacking various b1-associated a subunits do not undergo premature terminal differentiation. Additional studies investigating the importance of basement membrane components and basal keratinocyte integrins have found that these matrix-integrin interactions are critical in cell adhesion (and migration during wound healing), but do not appear to play an important role in differentiation. These results suggest that further studies are necessary to better understand the contribution of the basement membrane in differentiation.

In the mouse mammary gland, the basement membrane and its individual components, in conjunction with lactogenic hormones, are responsible for the induction of the differentiated phenotype of the epithelial cells. When mid-pregnant mammary epithelial cells are cultured on plastic, they do not express mammary-specific genes. However, when the same cells are plated and maintained on basement membrane components (EHS), they form alveolar-like structures and exhibit the fully differentiated phenotype with expression of the genes encoding milk proteins, s ch as b-casein . Function-blocking antibodies and conditional knockouts found that laminin-111 is the ECM molecule present in EHS ultimately responsible for the observed differentiation, and that integrins b1 and a6 are critical in maintaining the differentiated state . One role of laminin-111 in this process is in establishing mammary epithelial cell polarity, which re-distributes the prolactin receptor to the apical surface of the epithelium and facilitates ligand binding, receptor activation, and signaling necessary to induce expression of b-casein . ECM molecules also regulate expression of another milk protein, the whey acidic protein (WAP). EHS decreases the production of TGF-a by mammary gland epithelial cells; this increases WAP expression, which is otherwise inhibited by TGF-a . WAP may then participate in the maintenance of mammary epithelial differentiation by inhibiting the activity of laminin-degrading enzymes.

- ***Apoptosis***

Programmed cell death occurs during embryogenesis of higher vertebrates in areas undergoing remodeling, such as in the development of the digits, palate, nervous system, in the positive selection of thymocytes in the thymus, during mammary gland involution, and during angiogenesis. For example, **intact basement membrane molecules suppress apoptosis of mammary epithelial cells, whereas matrix fragments are thought to induce apoptosis during the involution of the mammary gland.** The numerous alveoli that produce milk during lactation regress and are resorbed during involution due to enzymatic degradation of alveolar basement membrane and programmed cell death. During this involution, apoptosis appears to proceed in two distinct phases, an early phase characterized by increased levels of pro-inflammatory and apoptosis-associated proteins, including several members of the tumor necrosis factor (TNF) and TNF receptor superfamilies and caspases, including caspase 1, a protein known to be important in promoting mammary epithelial cell apoptosis. This is then followed by a later apoptotic phase in which cell-ECM interactions are altered due to matrix degradation, preventing pro-survival integrin signaling and resulting in apoptosis. The importance of integrin signaling is underscored by experiments in which mammary epithelial cells undergo apoptosis when an antibody is used to disrupt interactions between a1 integrin and its ECM ligands. This later phase of matrix degradation and apoptosis may be regulated, at least in part, by decreased production of WAP, a milk protein that can inhibit the activity of proteases that cleave laminin. Decrease production of WAP by dying or de-differentiated cells could relieve WAP-mediated protease inhibition, promoting protease activation and matrix degradation. This, in turn, would decrease the interaction of intact matrix molecules with integrins, decreasing pro-survival signaling; it is also possible that integrins could then interact with soluble matrix fragments, which is known to induce apoptosis in endothelial cells (see below). In endothelial cells, avb3 integrin interactions with ECM play a crucial role in their survival during

embryonic angiogenesis. Disruption of these interactions with an antibody to avb3 inhibits the development of new blood vessels in the chorioallantoic membrane (CAM) by inducing endoth lial cell apoptosis. In contrast, avb3 binding to tumstatin, a proteolytic fragment of collagen IV, induces endothelial cell apoptosis, preventing angiogenesis and/or promoting vessel regression This interaction may promote apoptosis by interfering with normal integrin-ECM binding, thus removing a critical survival signal. Tumstatin may also promote apoptosis through a separate mechanism, such as via the recruitment and activation of caspase 8, as has been suggested previously for such soluble ligands. **Taken together, these findings suggest that disruption of cell-ECM interactions may lead to an increase in the expression or activation of pro-apoptotic molecules, and may also lead to the removal of pro-survival signals, which then directly or indirectly cause apoptosis.**

2.6. Wound healing

- ***Adhesion And Migration***

Early in the wound healing process, blood components and tissue factors are released into the wounded area in response to tissue damage, promoting both the coagulation cascade and platelet adhesion and activation, resulting in the formation of a clot consisting of platelets, cross-linked fibrin, fibronectin, and vitronectin, along with lesser amounts of SPARC, tenascin, and thrombospondin . Activated platelets, along with degranulating mast cells, release a number of cytokines and growth factors important in initiating the next phase of wound healing, the inflammatory response, and in regulating the wound healing process. The fibrinfibronectin clot has functions in addition to hemostasis, sequestering cytokines and growth factors while providing a temporary extracellular matrix that facilitates the adhesion and migration of multiple cell types, particularly leukocytes, into the wounded area . Leukocyte adhesion, migration, and secretion of inflammatory mediators are regulated by their interactions with various ECM molecules. After initial interactions between neutrophil and macrophage integrins with non-matrix molecules ICAM and VCAM, these leukocytes

interact with chemoattractants associated with HSPG, matrix molecules in the endothelial basement membrane, and the fibrin-based provisional matrix . HSPG binding to chemokines can create a stable gradient to promote leukocyte chemotaxis into the injured area. This HSPG chemokine binding is critical for appropriate leukocyte recruitment, as shown by their defective recruitment by mutant chemokines lacking the ability to bind glycosaminoglycans, in mice deficient in heparan sulfate biosynthesis, or in mice lacking syndecan-4 . Leukocyte adhesion to and migration through the basement membrane and provisional matrix are also mediated by matrix-receptor interactions. Neutrophils bind several matrix molecules, including fibronectin, vitronectin, laminin-511 (laminin 10), and the small leucine-rich proteoglycan (SLRP) lumican, and actively secrete laminin-411 (laminin 8) . Both laminin-411 and lumican are necessary for neutrophil xtravasation, the former via integrin a6b1 and the latter via b1 integrin(s). A proteolytic fragment of laminin-511 also promotes neutrophil migration, suggesting a potential role of proteases in this process as well .

Interaction with matrix molecules in the provisional matrix can also affect inflammatory cell adhesion, migration, and behavior. Integrin aMb2 expressed by various inflammatory cells interacts with fibrin, urokinase plasminogen activator (uPA), and thrombospondin 4, inducing inflammatory cell adhesion and migration . Binding of aMb2 to uPA also promotes plasmin activation and thus fibrin degradation and removal of the provisional matrix . Interactions between integrin aMb2 and its ligands also promote pro-inflammatory cytokine production; in monocytes, fibrin binding induces expression of pro-inflammatory cytokines and chemokines, including IL-1b, IL-6, TNF-a, MIP-1, MIP-2, and MCP-1, and thrombospondin-4 binding in neutrophils induces secretion of the chemokine IL-8 and the respiratory burst . Similarly, tissue macrophages release pro-inflammatory cytokines after TLR4 binding to low molecular weight hyaluronic acid. Due to the ability of matrix-leukocyte interactions to regulate the inflammatory process, the types of ECM molecules present in the injured area may greatly affect the inflammatory phase of wound healing.

During the re-epithelialization phase of cutaneous wound healing, keratinocytes migrate beneath the fibrin-rich provisional matrix, likely due, at least in part, to the fact that these cells do not express the fibrin-interacting integrin aVb3 . The keratinocytes do express multiple receptors for fibronectin, collagen, tenascin, and vitronectin, including the integrins a2b1, a3b1, a5b1, a6b1, a5b4, and av, and these receptor-matrix interactions, along with the activity of several proteases, promote keratinocyte migration and subsequent wound closure.

Keratinocyte migration requires the synthesis and deposition of laminin-332 and its interaction with integrin a3b1, initiating signaling leading to Rac activation and protrusion formation. Protease activity is also important in keratinocyte migration, likely through the release of HB-EGF from HSPG, which then induces keratinocyte migration through the EGFR.

Interactions between epithelial cells and ECM are also critical in the closure of other types of epithelial wounds. After wounding, retinal pigment epithelial cells exhibit a sequential pattern of ECM molecule deposition that is critical in the epithelial cell adhesion and migration associated with wound closure. Within 24 hours of wounding, these epithelial cells secrete fibronectin, followed shortly by laminin and collagen IV; if the cell adhesion to these ECM molecules is blocked with either cyclic peptides or specific antibodies, the epithelial cells fail to migrate and close the wound, underscoring the importance of such interactions in wound closure. During later stages of wound healing, macrophages and fibroblasts in the injured area deposit embryonic-type cellular fibronectin, which is important in the generation of the granulation tissue, a temporary connective tissue consisting of multiple types of ECM molecules and newly-formed blood vessels . The cellular fibronectin provides a substrate for the migration of endothelial cells into the granulation tissue, thus forming the wound vasculature, and also facilitates the recruitment of fibroblasts, myofibroblasts, and lymphocytes stimulated by a variety of chemotactic cytokines, or chemokines, that are produced by tissue fibroblasts and macrophages . Many chemokines have been

characterized in multiple species, including humans, other mammals, and even birds, and have been grouped into a large superfamily which is further subdivided based upon the position of the N-terminal cysteine residues . These chemokines, along with cell-ECM interactions, are critical in the adhesion and chemotaxis/migration of the cells that ultimately enter the wounded area and generate the granulation tissue.

One prototypical chemokine, IL-8, has several functions that are important in wound healing. Many of these functions have been elucidated in studies performed in the chick model system using chicken IL-8 (cIL-8/cCAF) . After wounding, fibroblasts in the injured area produce large quantities of cIL-8, most likely resulting from their stimulation by thrombin, a coagulation enzyme activated upon wounding that is known to induce fibroblasts to express and secrete cIL-8. The initial rapid increase in cIL-8 generates a gradient that chemoattracts neutrophils. These cells, in turn, produce monocyte chemoattractant protein, a potent chemoattractant for monocytes, which differentiate into macrophages in the wound environment. In addition, thrombin is able to induce IL-8 expression in cultured macrophages ; this, along with the additional IL-8 secreted from the endothelial cells of the wound vasculature and bound to various matrix components of the granulation tissue, further increases the presence of IL-8 in the granulation tissue. Indeed, IL-8 participates in granulation tissue formation by stimulating angiogenesis and matrix deposition; therefore IL-8 not only functions in the inflammatory phase of wound healing by serving as a leukocyte chemoattractant, but also plays an important role in granulation tissue formation. Angiogenesis relies heavily upon cell-ECM interactions, as described in detail earlier (under'Development'). The localization of the matrix molecules and the proteases that degrade them both have critical roles in this process. In some cases, interaction of an angiogenic factor or its receptor with a matrix molecule is important in ligand-receptor interactions and/or downstream signaling, whereas in others, matrix fragments signal differently from the parent matrix molecule. As mentioned earlier, many growth factors, including VEGF, bind HSPG, which can alter their diffusion and presentation

to their receptors. In addition to HSPG, VEGF-VEGFR signaling is heavily influenced by the presence of specific matrix molecules and integrins. Integrin avb3 is important in VEGFR2 activation and VEGF-induced angiogenesis, and a avb3 ligand, vitronectin, promotes the interaction of this integrin with VEGFR2 and enhances VEGF-induced VEGFR2 signaling. Fibronectin simultaneously binds VEGF and integrin a5b1, promoting VEGF-induced endothelial cell migration. VEGF can also bind integrin a9b1 directly, promoting VEGFR2-mediated signaling and endothelial cell migration. In contrast, collagen I binding to b1 integrins inhibits signaling downstream of VEGFR2. Taken together, these results underscore the importance of the microenvironment in regulation of VEGF-induced angiogenesis.

During angiogenesis, protease activity is needed for the endothelial cells to degrade and then migrate through the basement membrane and surrounding connective tissue. These proteases can regulate endothelial cell adhesion and migration during angiogenesis by releasing matrix-bound factors, as mentioned earlier, and also by generating functional matrix fragments and exposing previously concealed matricryptic sites. Some of these 'matricryptins' promote cell migration and angiogenesis. For example, a matricryptic site in collagen IV exposed by MMP-9-mediated proteolysis changes integrin binding from a1b1 to avb3, and blocking this site with an antibody or inhibiting integrin avb3 prevents endothelial cell adhesion and migration in vitro and angiogenesis in vivo. Many other matricryptins inhibit angiogenesis. For example, endostatin and tumstatin, matricryptins derived from collagen XVIII and collagen IV, respectively, inhibit VEGF-induced endothelial cell migration. These molecules may exert their inhibitory effects by binding VEGFR2 (endostatin) and/or integrin avb3 (endostatin and tumstatin), both of which are essential for VEGF-induced cell migration and angiogenesis, thereby blocking signaling downstream of the individual receptor(s) and the substantial crosstalk that occurs between them .

- ***Proliferation***

After wounding, the keratinocytes alter their proliferation and migration in order to close the wound, a process known as re-epithelialization. As this process occurs, the cells at the edge of the wound migrate, whereas the cells away from the wound proliferate in order to provide the additional cells needed to cover the wounded area. The proliferative state of these latter keratinocytes may be sustained by interactions with the ECM of the remaining basement membrane. Indeed, during the remodeling of normal skin, the proliferation of the basal layer of keratinocytes needed to replace the upper keratinocyte layers requires the presence of fibronectin in the epithelial basal lamina (see above). In addition, ECM derived from the basement membrane, when present in a dermal wound model, can maintain keratinocytes in a proliferative state for several days. It is likely that laminins-511 and -521 (laminins 10 and 11), in addition to fibronectin, participate in keratinocyte proliferation, as previous data indicates that these molecules can promote proliferation in vitro . Further studies have suggested important roles for integrins in regulating keratinocyte proliferation, although the functions of specific integrins remain unclear. For example, expression of integrin a9 is increased upon wounding, and keratinocytes in mice lacking epithelial integrin a9 or b1 exhibit reduced proliferation, suggesting a potential role for integrin a9b1 in keratinocyte proliferation; however, re-epithelialization is not substantially affected in these animals. In contrast, integrin avb8 is expressed specifically in non-proliferating suprabasal keratinocytes and not in the proliferating basal cells, suggesting a possible role in inhibiting keratinocyte proliferation.

While re-epithelialization progresses, the granulation tissue begins to form. This tissue is composed of ECM molecules, including embryonic fibronectin, type III collagen, type I collagen, and hyaluronic acid, along with multiple cell types, such as monocytes/macrophages, lymphocytes, fibroblasts, myofibroblasts, and the endothelial cells of the wound vasculature. Growth factors released by these cells and platelets cooperate with the aforementioned surrounding ECM molecules to provide pro-

proliferative signals to the granulation tissue fibroblasts and endothelial cells. Endothelial cells proliferate during angiogenesis, a process that is dependent upon growth factors, such as FGFs and VEGFs, and their interactions with matrix molecules. Signaling induced by both FGFs and VEGFs is enhanced by HSPG, as discussed earlier. In addition, VEGF binding to fibronectin or tenascin enhances its effect on endothelial cell proliferation . Furthermore, some growth factors appear to promote proliferation only when specific ECM molecules are present, as is seen in the fibronectin requirement for TGF-b1-mediated fibroblast proliferation . In contrast, SPARC and thrombospondin inhibit proliferation induced by VEGF and bFGF, indicating that interactions between growth factors and ECM can also be inhibitory. While ECM-growth factor interactions can significantly impact cell proliferation, specific ECM molecules, such as laminin, also affect proliferation directly . Previous studies suggest that the proliferative ability of laminin is mediated by its EGF-like domains, implicating EGFR activation in its proproliferative effects . In addition, certain ECM molecules and/or proteolytic fragments can inhibit proliferation; SPARC and decorin, as well as peptides derived from SPARC, decorin,collagen IV (tumstatin), and collagen XVIII (endostatin) are anti-angiogenic due to their inhibitory effects on endothelial cell proliferation.

- ***Differentiation***

As the granulation tissue forms, some of the fibroblasts within the wounded area differentiate into myofibroblasts, cells that express the protein a-smooth muscle actin (aSMA) and thus function similarly to smooth muscle cells; they also secrete a number of matrix molecules, including fibronectin and collagen I . Myofibroblast differentiation is **influenced by various matrix molecules, such as heparin, which decreases fibroblast proliferation while stimulating aSMA expression in vitro** . The effects of heparin on myofibroblast differentiation and aSMA expression likely result from the ability of heparin and heparan sulfate proteoglycans to interact with cytokines and/or growth factors like TGF-b1, which then modulate myofibroblast

differentiation. TGF-b1-induced differentiation also requires the ED-A-containing form of fibronectin and the binding of the ED-A domain to integrin a4b7 . Maintenance of myofibroblast differentiation is mediated by hyaluronan, as shown by reduced differe tiation when hyaluronan synthesis is inhibited . Interstitial collagens, in conjunction with mechanical tension, also participate in the differentiation process. Fibroblasts cultured on relaxed collagen gels fail to differentiate, whereas fibroblasts grown on stiffened collagen matrices exhibit myofibroblast characteristics . In addition, increased myofibroblast differentiation is observed when splints are used to increase mechanical tension in wounds, suggesting a role for mechanical tension in myofibroblast differentiation in vivo . Tensile stress may regulate cell signaling and cell-matrix interactions by revealing cryptic sites in intact matrix molecules; for example, the ED-A domain of fibronectin, which is necessary for TGF-b-induced myofibroblast differentiation, could be exposed by mechanical stress .Tensile stress exerted on the cell by the matrix may also induce the formation of stress fibers that exert intracellular tension on the integrin, changing its conformation and strengthening its adhesion to the matrix, promoting downstream signaling .

APOPTOSIS Late in the wound healing process, the granulation tissue undergoes remodeling to form scar tissue. This remodeling phase is characterized by decreased tissue cellularity due to the disappearance of multiple cell types, including fibroblasts, myofibroblasts, endothelial cells, and pericytes, and by the accumulation of ECM molecules, particularly interstitial collagens. The observed reduction in cell numbers during the remodeling phase occurs due to apoptosis; many of these apoptotic cells are endothelial cells and myofibroblasts, as shown bystudies using in situ DNA fragment end-labeling in conjunction with transmission electron microscopy . Myofibroblast apoptosis is regulated by mechanical tension; using a splint to maintain tension in a healing wound inhibits myofibroblast apoptosis, whereas the release of tension in this model promotes apoptosis . Apoptosis of fibroblasts and myofibroblasts may be important in preventing excessive scarring and facilitating the resolution of wound

healing. Indeed, there is a decreased apoptosis of these cells in keloids and hypertrophic scars, leading to increased matrix deposition and scarring . Inkeloids, reduced apoptosis may result from p53 mutations or growth factor receptor overexpression. In hypertrophic scars, however, the reduced apoptosis may result fromincreased expression of tissue transglutaminase, resulting in enhanced matrix degradation and diminished collagen contraction.

2.7. Signal transduction events during CELL-ECM

- ***Interactions***

As discussed above, ECM molecules are capable of interacting with a variety of receptors. Such interactions activate signal transduction pathways within the cell, altering levels of both gene expression and protein activation, thus ultimately changing outcomes in cell adhesion, migration, proliferation, differentiation, and death. The signaling pathways linked to these specific outcomes have been studied for many of the ligand-receptor interactions, particularly those involving integrins. In the case of integrins, it is important to remember that these receptors can participate in both ’outside-in’ and ’inside-out’ signaling. Outside-in signaling occurs when an extracellular ligand binds the receptor and initiates intracellular signaling, and in inside-out signaling, intracellular signaling increases the affinity of the receptor for its ligand. Binding of the receptor to the ligand, in turn, initiates outside-in signaling. Unless otherwise indicated, the signaling events discussed below refer to outside-in signaling. Based upon the many studies that investigate matrix-induced signaling, we postulate the **existence of three categories of cell-ECM interactions**, namely type I interactions that are involved in adhesion and migration, type II interactions involved in proliferation, differentiation, and survival, and type III interactions involved in apoptosis and epithelial mesenchymal transition.

- ***Type I interactions***

These are generally mediated **by integrin and proteoglycan receptors, and are important in the adhesion/de-adhesion processes that accompany cell migration**. These interactions are exemplified by fibronectin-mediated cell migration, which occurs when this matrix molecule simultaneously binds integrins and proteoglycan receptors, the latter via its heparin-binding domain . These receptors then co-localize and interact at cell adhesion sites, where the microfilaments interact with the cytoplasmic domain of integrin b1 through the structural proteins talin and a-actinin and the signaling molecule paxillin, which interacts with focal adhesion tyrosine kinase (FAK) . Binding of an integrin to its ligand triggers autophosphorylation of FAK on tyrosine 397; this then serves as the binding site for the SH2 domain of the c-Src tyrosine kinase. This kinase subsequently phosphorylates multiple proteins present in the focal adhesion plaques, including FAK itself at position 925, which increases its activity, and paxillin, tensin, vinculin, and p130cas . FAK PY925 binds the Growth factor receptor-bound protein 2 (Grb2)/Son of Sevenless (Sos) complex, thuspromoting the activation of Ras GTPase and the MAP kinase cascade, which may be involved in cell adhesion/de-adhesion and migration events . Paxillin may also participate in integrin-mediated signaling and motility, as evidenced by the reduced migration and decreased phosphorylation/activation of various signaling molecules observed in paxillindeficient fibroblasts, and the impaired migration seen in cells over-expressing a paxillin mutant unable to be phosphorylated by FAK/Src . Both phosphorylated paxillin and p130Cas bind Crk, an adaptor protein bound to DOCK180, a guanine nucleotide exchange factor (GEF) that activates Rac1, while paxillin is also able to recruit another Rac1/Cdc42 GEF, b-PIX, thereby inducing lamellipodia formation and cell migration . Src-mediated signaling also activates another Rac GEF, Vav2, along with a Rho GTPase activating protein (GAP) that inhibits RhoA. Interestingly, integrin-induced Src can phosphorylate and transactivate the EGFR, leading to additional pro-migratory signaling . The contribution of tensin to

cell adhesion and motility is poorly understood, although it is known to interact with the cytoskeleton and various phosphorylated signaling molecules via its SH2 domain; therefore, tensin may facilitate various signaling events downstream of integrin ligation . In cancer cells, tensin-1 interacts with a RhoGAP, and tensin mutations that blocked RhoGAP interaction decreased levels of Rho-GTP and reduced cell migration, suggesting a potential role in normal integrin-mediated signaling. **To this point, we have discussed the roles of FAK and Src in outside-in, traditional receptormediated signal transduction. However, these kinases can participate in inside-out signaling as well; FAK/Src signaling stimulates integrin activation, cell adhesion to matrix molecules, and focal adhesion strengthening**. Integrin activation and matrix binding then initiates outside-in signaling, promoting FAK/Src activity, suggesting a positive feedback loop of integrins and FAK/Src kinases that may facilitate directional migration. Matrix receptors other than integrins, such as proteoglycans and DDR1, are also important in cell adhesion and migration. Syndecans can function as co-receptors, associating with matrix molecules and integrins to promote cell adhesion and migration . Syndecan-4, for example, can bind the heparin-binding domain of fibronectin and the fibronectin receptor integrin a5b1, leading to activation of PKC and downstream activation of Rac and inactivation of RhoA, promoting migration. Following fibronectin binding, syndecan-4-induced PKC and RhoG promote integrin a5b1 internalization, thus inhibiting integrin-mediated signaling. Proteoglycan receptors other than syndecans induce cell migration following ligand binding. For example, hyaluronan binding to CD44 induces phosphorylation of its intracellular domain, resulting in fibroblast migration, as inhibition of CD44 with blocking antibodies or by expression of phosphorylation-deficient CD44 blocked hyaluronan-induced fibroblast migration. RHAMM, another hyaluronan receptor, facilitates fibroblast migration by regulating ERK1/2 activation downstream of CD44, and in smooth muscle cells, hyaluronan induces cell migration downstream of RHAMM via PI3K and Rac.

The roles of DDR1/2 in cell migration are less clear. DDR1 activation by collagen inhibits epithelial cell adhesion and migration induced by integrin ligation, likely through inhibition of Cdc42 and recruitment of the phosphatase SHP-2 . In contrast, collageninduced fibroblast migration depends upon DDR2 and downstream activation of MMP-2 and Erk1/2 .

- ***Type II interactions***

These involve processes in which the matrix-receptor interactions, in conjunction with growth factor or cytokine receptors, affect proliferation, survival, differentiation, and/or maintenance of the differentiated phenotype. These cooperative effects may occur in a direct manner, for example, by the direct interaction of EGF-like repeats present in certain ECM molecules with the EGF receptor, thereby promoting cell proliferation . Indirect cooperative effects are better understood at this time, particularly with regards to the anchorage dependence of cell growth. S-phase entry, even when growth factors are present, requires the interaction of cells with a substrate, while detachment of cells from matrix promotes anoikis, a type of apoptosis, underscoring the critical role of cell-ECM adhesion in cell survival and proliferation. The pro-survival function of matrix interactions is mediated by Fak signaling. In fibroblasts, Fak/Src-induced p130CAS activity leads to the activation of Rac, as mentioned above, which then promotes cell survival and proliferation via the JNK pathway . In epithelial cells, Fak/Src complexes promote cell survival through PI3K-induced Akt activation . Signaling downstream of Akt then increases levels of anti-apoptotic Bcl family members while decreasing levels of pro-apoptotic Bcl family members, resulting in cell survival . In addition to promoting cell survival, integrin signaling can promote cell proliferation, either alone or in conjunction with growth factor signaling. Integrin-induced activation of the Rac/JNK pathway can induce cell proliferation by stimulating expression of cyclin D and by promoting the degradation of p21, a cell cycle inhibitor . ERK1/2, also MAP kinases, can be activated by integrin ligation, which promotes the activation of Fyn and its binding to the Shc adaptor

protein, which then recruits Grb2/Sos and activates the Ras/ERK pathway, resulting in the phosphorylation of the transcription factor Elk-1 and the activation of genes important in cell cycle progression . Integrin signaling can also induce cell proliferation in conjunction with growth factor receptor signaling . Angiogenesis induced by the growth factors bFGF and VEGF requires the presence and activation of integrins avb3 and avb5, respectively]. In both cases, integrin signaling and FAK are necessary for activation of Erk1/2, but Erk1/2 activation by bFGF/avb3 is dependent upon Pak-1-induced Raf phosphorylation but not Ras, while Erk1/2 activation by VEGF/avb5 requires Ras . Furthermore, cell-ECM interactions are critical for the efficient and prolonged activation of MAPK by growth factors, likely participating in MAPK-induced proliferation . Cell-matrix interactions can also facilitate proliferation through transactivation of growth factor receptors, as in fibronectin-induced, Src-mediated EGFR activation . This EGFR transactivation is required for fibronectin-induced Erk activation, regulating genes involved in cell cycle progression, including Rb and cyclin D; however, fibronectin induced EGFR transactivation is not sufficient for cell division, but requires growth factorinduced signaling to induce expression of genes necessary for S phase. Cell-matrix interactions can also regulate growth factor receptor signaling through integrin or matrix binding to growth factors or their receptors and by modulating downstream signaling Multiple VEGF isoforms bind and activate integrin a9b1, which, along with VEGFR2, is necessary for VEGF-induced paxillin and Erk phosphorylation and angiogenesis. Similarly, integrin avb3 interacts with both IGF-1 and FGF-1 and is required for proliferation induced by both growth factors. Several growth factor receptors interact with integrins, including VEGFR2 and EGFR, and integrin-mediated adhesion can regulate growth factorinduced proliferation. Growth factor signaling, in turn, can activate associated integrins and promote matrix adhesion. For example, VEGFR2 signaling induces phosphorylation of integrin b3, leading to activation of Fak and p38 as well as VEGFR2-integrin binding and enhanced VEGFR2 signaling.

As mentioned earlier, matrix molecules themselves can interact with growth factors and/or their receptors. Fibronectin and collage interact with VEGF; binding to fibronectin increases VEGFR activation and endothelial cell proliferation, whereas binding to collagen activates a phosphatase and thus decreases VEGFR activity . HSPG can interact with many growth factors, including FGF and VEGF, limiting their diffusion, sequestering them until enzyme-mediated release, or promoting their receptor binding. FGF and VEGF binding to HSPG facilitates binding to their respective receptor and activates mitogenic signaling.

Matrix molecules can also bind growth factor receptors and activate them directly; for example, the EGF-like domains of laminin and tenascin-C bind and activate the EGFR, which may promote cell proliferation . In contrast, decorin binding to the EGFR promotes EGFR internalization, and thus inhibits mitogenic signaling downstream of this receptor.

Activation of several non-integrin matrix receptors, including CD44, RHAMM, and DDR2, are associated with cell survival and proliferation. Hyaluronan promotes Erk activation and fibroblast proliferation through RHAMM and endothelial cell proliferation through CD44 . Low molecular weight hyaluronan fragments also activate CD44, leading to Erk activation, cyclin expression, and proliferation in smooth muscle cells . In addition, the collagen receptor DDR2 promotes fibroblast and chondrocyte proliferation, although the signaling that underlies this mitogenic effect has not been elucidated . Similarly, cellular differentiation also relies upon cell interactions with ECM molecules, hormones, and growth factors. For example, the binding of laminin to integrin a2b1 in endothelial cells promotes the formation of capillary-like structures , whereas the binding of fibronectin to integrin a5b1 in these cells leads to cell proliferation . Additional signaling molecules are required to generate the capillary-like tubes; one such molecule is integrin-linked kinase (ILK), which, when over-expressed, rescues capillary-like tube formation in the absence of ECM molecules while expression of a dominant negative version of ILK blocks tube

formation even when ECM and VEGF are present. Once nascent vessels are formed, smooth muscle cells called pericytes are recruited to stabilize the endothelium and promote synthesis of the basement membrane. Pericyte differentiation relies upon cell-matrix interactions, as shown by impaired differentiation and vessel stabilization/ maturation in a mural cell-specific integrin b1 knockout . TGF-b-induced myofibroblast differentiation depends upon integrin a4b7 binding to the EDA domain of fibronectin and downstream activation of FAK, PI3K, and associated signaling pathways . Non-integrin receptors such as CD44 also regulate cellular differentiation. Hyaluronan induces keratinocyte differentiation in vitro in a CD44-dependent manner, and CD44 deficiency inhibits keratinocyte differentiation in vivo, suggesting a role for hyaluronan in this process . Similarly, TGF-b-induced myofibroblast differentiation requires hyaluronan.

- ***Type III interactions***

Type III interactions primarily involve processes leading to apoptosis and epithelial-tomesenchymal transitions. Normally adherent cells that fail to interact with matrix molecules undergo anoikis, a form of cell death. Anoikis results from a lack of pro-survival Fak/Src-induced PI3K/Akt or Erk signaling, coupled with pro-apoptotic signaling that inhibits anti-apoptotic Bcl family members, increases expression/activity of pro-apoptotic Bcl family members, and recruits and activates caspase 8. The mechanism involved in caspase 8 recruitment and activation by unligated integrins is unclear; one possibility is that caspase 8 is normally phosphorylated and inhibited by Src, and that the absence of integrin-activated Src signaling relieves caspase 8 inhibition . In many cases, integrins that do interact with ligands can still promote apoptosis. For example, CCN1 binding to syndecan-4 and integrin a6b1 induces expression of Bax, which promotes cytochrome C release from the mitochondria and subsequent caspase 9 activation, leading to fibroblast apoptosis . In endothelial cells, thrombospondin interaction with CD36 induces a pro-apoptotic pathway culminating in Fas ligand (FasL) expression; FasL then binds Fas, a death

domain receptor that induces apoptosis . Alterations in the ligand presentation by ECM can also regulate apoptosis. Studies have suggested that integrin ligation by soluble, rather than intact, ligands can function as integrin antagonists and promote apoptosis rather than survival or proliferation . Many of these soluble ligands can be created by matrix degradation during tissue remodeling. Endostatin, derived from collagen XVIII, and tumstatin, derived from collagen IV, promote endothelial cell apoptosis by interacting with a5b1 and avb3 integrins, respectively. The apoptosis stimulated by soluble ligands or other antagonists appears to occur via the recruitment and activation of caspase 8 by clustered integrins and decreased expression of pro-survival Bcl family members, without any requirement for death receptors . Although these three categories may not be exhaustive of the general types of cell-ECM interactions that occur during development and wound healing, they encapsulate the major interactions documented to date. Each category has its place in many developmental and repair events, and they may operate in sequence. A compelling example of the latter is the epithelial-to-mesenchymal transition and morphogenesis of the NCC system.These cells originate in the neural epithelium that occupies the crest of the neural folds. After the delamination event that separates the neural epithelium from the epidermal ectoderm , the folds fuse to form the tube. At this time, the NCC occupy the dorsal-most portion of the tube, they are not covered by basal lamina, and the subepidermal space above them contains large amounts of fibronectin . Just before the NCC emigrate from the neural tube, fibronectin appears between them, they separate from each other, and migrate away carrying fibronectin on their surfaces .During the period of emigration at any particular level of the neural tube, basal lamina is deposited progressively toward the crest from the sides of the tube . NCC emigration terminates as deposition reaches the crest of the tube. The NCC then follow specific migration pathways throughout the embryo, arriving at a wide variety of locations where they differentiate into many different phenotypes in response to external cues .

The appearance of fibronectin between the NCC just before emigration must be

the result of secretion by the adjacent cells or introduction from the epithelial cells after loss of cell-cell adhesions. In keeping with the cell-ECM interaction mechanism of type III, either alternative could initiate a positive feedback loop and release the NCC, leading to emigration: Enzymatic degradation of the stabilizing domain of fibronectin above the tube could cause enhanced secretion of specific enzymes by the NCC in response to the effect of the cell-binding domain acting alone, thus severing the cell adhesions and producing additional fibronectin fragments containing the cell-binding domain. These fragments, in turn, would bind to adjacent cells and stimulate further enzymatic secretion that would be self-perpetuating.

NCC emigrat on occurs in an anterior-to-posterior wave, thus, once enzymatic activity were initiated in the head of the embryo, it could propagate in a posterior direction, triggering NCC emigration in a wave from head to tail.

Clearly some controlling event(s) must terminate NCC emigration at each location along the neural tube. Such an event has already been identified. At the time of NCC emigration, the ventral and lateral surfaces of the neural tube are covered by an intact basal lamina, which stabilizes the epithelium and separates it from the fibronectin layer around the tube During the few hours of emigration at any one site, as the NCC are leaving from the dorsal most portion of the neural tube, basal lamina deposition progresses quickly up the sides of the tube and terminates local emigration when it becomes complete over the crest of the tube After they have emigrated from the neural tube, the NCC find themselves in an extracellular space filled with intact fibronectin and other ECM molecules that stimulate the focal adhesions of cell-ECM interactions of type I, thereby providing the substrate for migration. Upon arrival at their final destination, further interactions of type II stimulate differentiation into a wide range of phenotypes.

- ***Relevance for tissue engineering***

Designing tissue and organ replacements that closely simulate normal physiology is a challenging endeavor. One avenue to achieve this goal is to study how tissues and organs arise during embryogenesis and during normal processes of repair, and how those functions are maintained. When developing tissue replacements, one needs to consider the following:

➔ ***Avoiding an immune response that can cause inflammation and/or rejection***.

Onepossibility would involve using autologous cells; however, it is time-consuming and labor-intensive to collect and expand autologous cells for use in engineered tissues, decreasing the practicality of this approach. Alternatively, engineered tissues could incorporate progenitor cells that may suppress host immune responses directly or indirectly through decreased expression of MHC; these cells could be induced at a after time to differentiate into various cell types . One example of a progenitor cell that appears to decrease immune responses and also maintains a broad differentiation capacity is the mesenchymal stem cell, which is capable of differentiating into multiple cell types, and may thus prove to be an invaluable asset in tissue engineering.

➔ ***Creating the proper substrate for cell survival and differentiation.***

One of the strategies tofulfill this goal is the use of biocompatible implants composed of extracellular matrix molecules seeded with autologous cells or with heterologous cells in conjunction with immunosuppressant drugs. Addition of growth and differentiation factors to these matrices as well as agonists or antagonists that favor cell-ECM interactions can potentially increase the rate of successful tissue replacement. However, growth factor activity in the engineered tissue is dependent upon its stability and its ability to diffuse both within the engineered tissue and in peripheral host tissue. In normal tissue, VEGF has a short half-life (30 minutes), so it would be useful to increase the half-life, and thus activity, in an engineered tissue.

There are several methods that have been used to increase the duration and activity of growth factors: by adding components of the normal ECM that stabilize these factors and/or promote their activity, by covalently coupling the growth factors to a matrix molecule or biomaterial, and by removing proteolytic cleavage sites from growth factors to inhibit their degradation. In the case of VEGF, covalent linkage to a fibrin matrix or addition of heparin and/or fibronectin fragments to the matrix promotes angiogenesis to a greater extent than addition of VEGF to a fibrin matrix alone In addition to restricting VEGF diffusion, heparin and fibronectin fragments likely promote VEGF signaling . Preventing proteolytic cleavage of VEGF also increases its half-life and activity. Chronic wounds frequently exhibit excessive protease levels that would cleave wild type VEGF and limit its activity; in a mouse model of impaired healing, VEGF lacking proteolytic sites promoted VEGF duration and activity leading to improved angiogenesis and healing . It is possible that combinations of matrix molecules/fragments and inhibition of proteolytic activity could be even more effective in stimulating angiogenesis and wound healing in impaired wounds.

While the foregoing examples show that ECM molecules can be used successfully in tissue engineering, the use of natural ECM molecules in engineered tissue has several disadvantages, including the possibility of generating an immune response, possible contamination, and ease of degradation. Likewise, artificial biocompatible materials have drawbacks in that, unlike ECM, they are generally incapable of transmitting growth and differentiation cues to cells. Much attention is now devoted to the design of 'semisynthetic biomaterials' in which functional regions of ECM molecules, including thosethat interact with receptors or growth factors or those that are cleaved by proteases, are incorporated into artificial biomaterials to impart additional functionality .**The inclusion of ECM-like cell-binding sites that promote cell adhesion, growth, and/or differentiation into such biomaterials may be critical in developing and maintaining functional engineered tissues by providing the appropriate cellular microenvironment**. For example, because many

integrins interact with the RGD motif on matrix molecules, it can be used as an agonist to make synthetic implants more biocompatible and to allow the development of tissue structure, or as an antagonist to prevent or moderate unwanted cell-ECM interactions. Similarly, collagen, fibronectin, and gelatin have been used to coat synthetic biomaterials to increase their biocompatibility and promote successful biological interactions. However, the use of these biomaterials in engineered tissues requires additional knowledge regarding the types of cell-ECM interactions that result in the desired cellular effects.

➔ ***Providing the appropriate environmental conditions for tissue maintenance.***

To maintaintissue homeostasis, it is crucial to create a balanced environment with the appropriate cues for preservation of specific cell function(s). It is important to realize that such stasis on the level of a tissue is achieved via tissue remodeling e the dynamic equilibrium between cells and their environment. However, relatively little is known about the crosstalk between cells and ECM under such 'normal' conditions. As indicated above, the same ECM molecule may have multiple cellular effects; the ultimate cellular outcome likely depends upon the combination of variables, such as the domain of the molecule involved in the cellular interactions, the receptor used for these interactions, and the cellular microenvironment. These variables can, in turn, be influenced by matrix remodeling, as enzymatic degradation of the ECM can release functional fragments of ECM that then alter cell-ECM interactions by removing certain binding sites while exposing others. Another important consideration is the role of mechanical tension on matrix signaling, and, depending upon the desired effect, it may be necessary to control the stiffness and pore size of the biomaterial. One biomaterial that is 'tunable' in terms of stiffness and pore size is **starPEG,** which can use heparin as a functional crosslinker. Altering the concentration or molecular weight of the starPEG changes its stiffness and pore size, and can thus change the mechanical tension exerted on cells, while the use of heparin as a crosslinker yields additional functionality due to its ability to bind growth factors and enhance their signaling .

Combinations of this hybrid biomaterial with RGD and growth factors increased endothelial cell survival and tube-like organization, suggesting the potential of this approach in designing engineered tissues .(75)

3. Morphogenesis

Morphogenesis is the developmental cascade of pattern formation, the establishment of the body plan and architecture of mirror-image bilateral symmetry of musculoskeletal structures, culminating in the adult form. Tissue engineering is the emerging discipline of fabricating spare parts for the human body, including the skeleton, for the functional restoration and aging of lost parts due to cancer, disease or trauma. It is based on rational principles of molecular developmental biology and morphogenesis and is further governed by bioengineering. The three key ingredients for both morphogenesis and tissue engineering are inductive morphogenetic signals, responding stem cells, and extracellular matrix. Recent advances in the molecular cell biology of morphogenesis will aid in the design principles and architecture for tissue engineering and regeneration. The long-term goal of tissue engineering is to produce functional tissues in vitro for implantation in vivo to repair, enhance, and replace damaged tissue, and to preserve physiological function. Tissue engineering is based on the principles of developmental biology, evolution and self-assembly of supramolecular assemblies and higher hierarchal tissues and even whole embryos and organisms . Regeneration recapitulates embryonic developmentand morphogenesis. Among the many tissues in the human body, bone has considerable powers for regeneration and therefore is a prototype model for tissue engineering. On the other hand, articular cartilage, a tissue adjacent to bone, is recalcitrant to repair and regeneration. Implantation of demineralized bone matrix into subcutaneous sites results in local bone induction. The sequential cascade of bone morphogenesis mimics sequential skeletal morphogenesis in limbs and permits the isolation of bone morphogens. Although it is traditional to study morphogenetic signals in embryos, bone morphogenetic proteins (BMPs), the primordial inductive signals for bone were

isolated from demineralized bone matrix from adults. BMPs initiate, promote, and maintain chondrogenesis and osteogenesis and have actions beyond bone. The recently identified cartilage-derived morphogenetic proteins (CDMPs) are critical for cartilage and joint morphogenesis.

The symbiosis of bone's inductive and conductive strategies is critical for tissue engineering and is in turn governed by the context and biomechanics. **The context is the microenvironment, consisting of extracellular matrix scaffolding, which can be duplicated by biomimetic biomaterials, such as collagens, hydroxyapatite, proteoglycans, and cell adhesion proteins, including fibronectins and laminins. The rules of architecture for tissue engineering are**: an imitation and adoption of the laws and signals of developmental biology and morphogenesis, and thus they may be universal for all tissues, including bones and joints and associated musculoskeletal tissues in the limbs.

The traditional approach for identification and isolation of morphogens is first to **identify genes** in fly and frog embryos by means of genetic approaches, differential displays, substractive hybridization, and expression cloning. This information is subsequently extended to mice and men. An alternative approach is to **isolate morphogens from bone**, the premier tissue with the highest regenerative potential. Morphogenesis is the developmental cascade of pattern formation, the establishment of the body plan and architecture of mirror-image bilateral symmetry of musculoskeletal structures in the appendicular skeleton, culminating in the adult form. Our expanding knowledge of bone and cartilage morphogenesis is a prototypical paradigm for all of tissue engineering. The principles gleaned from bone morphogenesis and BMPs can be extended to tissue engineering of bone and cartilage and other tissues.

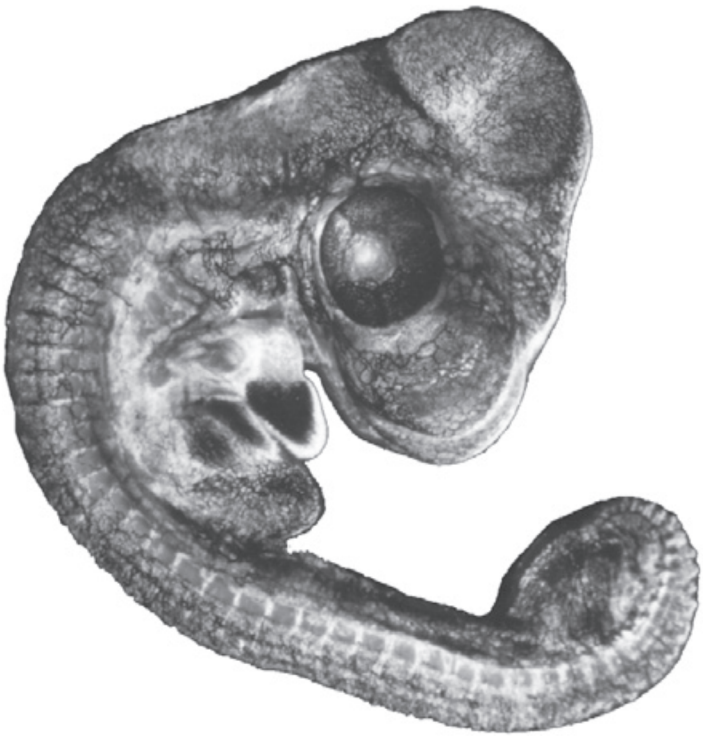

Figure 12: Developmental origins of skeleton in the chick embryo. (75)

The cranial neural crest gives rise to craniofacial skeleton. The lateral plate mesoderm gives rise to the limbs of the appendicular skeleton. The sclerotome of the somite gives rise to spine and the axial skeleton.

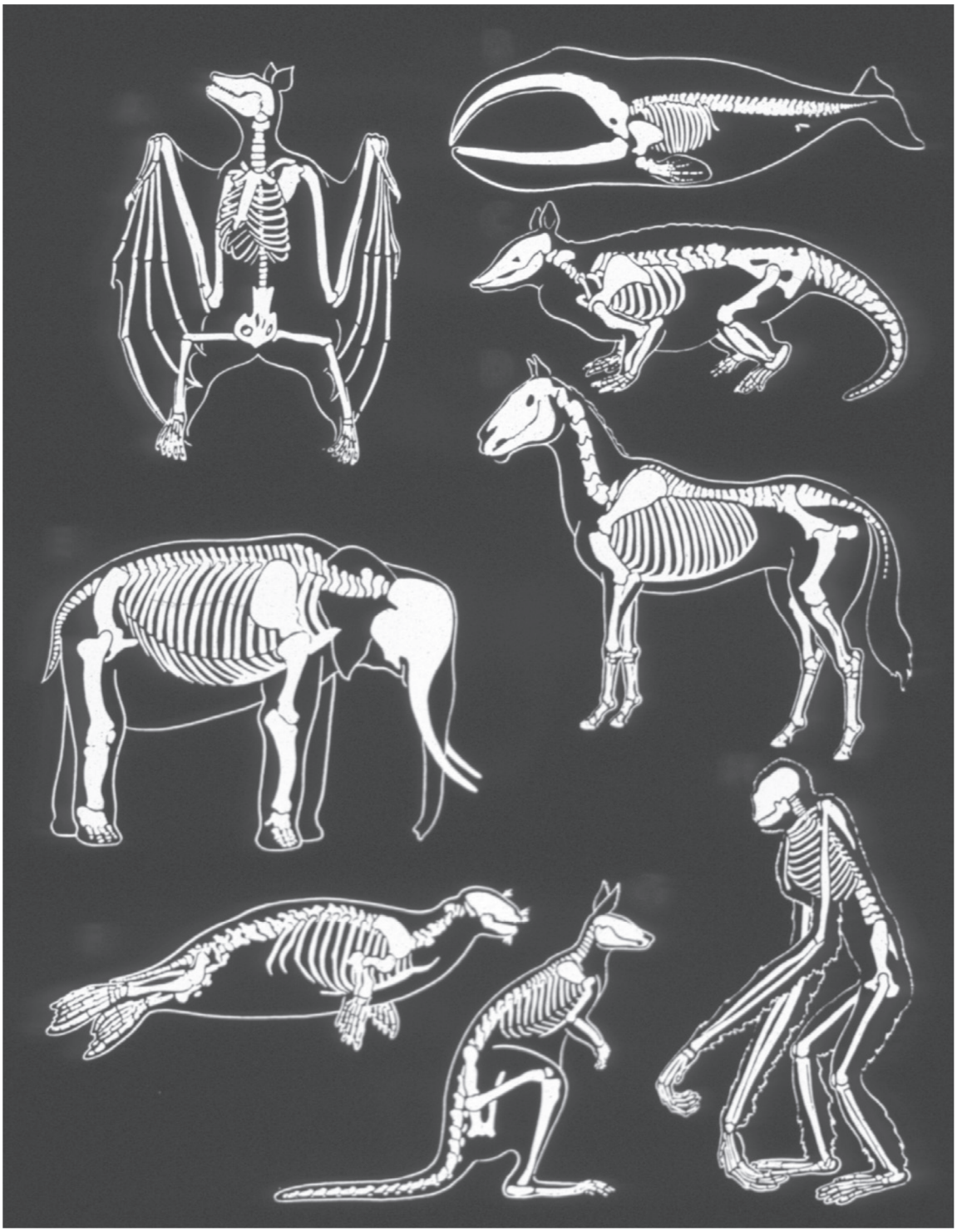

Figure 13: Evolution of skeletal structures in a variety of mammals adapted for flight (bat, a) and aquatic life (whale, b) and the use of hands in humans (h). (75)

3.1. Bone morphogenetic proteins (BMPS)

Bone grafts have been used by orthopedic surgeons for nearly a century to aid and abet recalcitrant bone repair. Decalcified bone implants have been used to treat patients with osteomyelitis. It was hypothesized that bone might contain a substance, osteogenin, which initiates bone growth. Urist made the key discovery that demineralized, lyophilized segments of rabbit bone, when implanted intramuscularly, induced new bone formation. The diaphysis (shafts) of long bones of rats were cleaned of marrow, pulverized and sieved. The demineralization of matrix was accomplished by 0.5 M HCl (. Bone induction, a sequential multistep cascade, is depicted in . The key steps in this cascade are chemotaxis, mitosis, and differentiation. Chemotaxis is the directed migration of cells in response to a chemical gradient of signals released from the insoluble demineralized bone matrix. The demineralized bone matrix is composed predominantly of type I insoluble collagen, and it binds plasma fibronectin. Fibronectin has domains for binding to collagen, fibrin, and heparin. The responding mesenchymal cells attached to the collagenous matrix and proliferated as indicated by [3H] thymidine autoradiography and incorporation into acid-precipitable DNA on day 3. Chondroblast differentiation was evident on day 5, chondrocytes on days 7e8, and cartilage hypertrophy on day 9. Vascular invasion was concomitant on day 9 with osteoblast differentiation. On days 10e12 alkaline phosphatase was maximal. Osteocalcin, bone g-carboxyglutamic acid containing gla protein (BGP), increased on day 28. Hematopoietic marrow differentiated in the ossicle and was maximal by day 21. This entire sequential bone development cascade is reminiscent of bone and cartilage morphogenesis in the limb bud. Hence, it has immense implications for isolation of inductive signals initiating cartilage and bone morphogenesis. In fact, a systematic investigation of the chemical components responsible for bone induction was undertaken.

The foregoing account of the demineralized bone matrix-induced bone morphogenesis in extra-skeletal sites demonstrated the potential role of morphogens tightly associated with the extracellular matrix. Next, we embarked on a systematic study of the isolation of putative morphogenetic proteins. A prerequisite for any quest for novel morphogens is the establishment of a battery of bioassays for new bone formation. A panel of in vitro assays was established for chemotaxis, mitogenesis, and chondrogenesis, and an in vivo bioassay was established for bone formation. Although the in vitro assays are expedient, we monitored routinely a labor-intensive in vivo bioassay, for it was the only bona fide bone induction assay. A major stumbling block in the approach was that **the demineralized bone matrix is insoluble and exists in the solid state**. In view of this, dissociative extractants such as 4 M guanidine HCl or 8Murea as 1% sodium dodecyl sulfate (SDS) at pH 7.4 were usedto solubilize the proteins. Approximately 3% of the proteins were solubilized from demineralized bone matrix, and the remaining residue was mainly insoluble type I bone collagen. The extract alone or the residue alone was incapable of new bone induction. However, addition of the extract to the residue (insoluble collagen) and then implantation in a subcutaneous site resulted in bone induction. Thus, it would appear that in **optimal osteogenic activity collaboration takes place between the soluble signal in the extract and insoluble substratum or scaffolding Thus, an operational concept of tissue engineering was established, in which soluble signals bound to extracellular matrix scaffold act on responding stem/progenitor cells to induce tissue digestion.** This bioassay was a useful advance in the final purification of bone morphogenetic proteins and led to the determination of limited tryptic peptide sequences leading to the eventual cloning of BMPs. In order to scale up the procedure, a switch was made to bovine bone. Demineralized bovine bone was not osteoinductive in rats, and the results were variable. However, when the guanidine extracts of demineralized bovine bone were fractionated on an S-200 molecular sieve column, fractions less than 50 kD were consistently osteogenic when bioassayed after reconstitution with allogeneic insoluble. Thus, **protein fractions inducing bone were not species specific, and**

appeared to be homologous in several mammals. It is likely that larger molecular mass fractions and/or the insoluble xenogeneic (bovine and human) collagens were inhibitory or immunogenic. Initial estimates revealed 1 mg of active osteogenic fraction in a kilogram of bone. Hence, over a ton of bovine bone was processed to yield optimal amounts for amino acid sequence determination. The amino acid sequences revealed homology to TGF-b1. The important work of Wozney and colleaguesinvolved cloning BMP-2, BMP-2B (now called BMP-4), and BMP-3 (also called osteogenin). Osteogenicprotein-1 and -2 (OP-1 and OP-2) were cloned by Ozkaynak and colleagues. **There arenearly 10 members of the BMP family**. The other members of the extended TGF-b/ BMP superfamily include inhibins and activins (implicated in follicle-stimulating hormone release from pituitary), Mullerian duct inhibitory substance (MIS), growth/differentiation factors (GDFs), nodal, and lefty, a gene implicated in establishing right/left asymmetry . **BMPs are also involved in embryonic induction.** BMPs are dimeric molecules, and their conformation is critical for biological action. Reduction of the single interchain disulfide bond resulted in the loss of biological activity. The mature monomer molecule consists of about 120 amino acids, with seven canonical cysteine residues. There are three intrachain disulfides per monomer and one interchain disulfide bond in the dimer. In the critical core of the BMP monomer is the cysteine knot. The crystal structure of BMP-7 has been determined. It is highly possible that in the near future the crystal structure of BMP receptor and receptor contact domains will be determined.

3.2. Cartilage-derived morphogenetic proteins (CDMPS)

Morphogenesis of the cartilage is the key rate-limiting step in the dynamics of bone development. Cartilage is the initial model for the architecture of bones. Bone can form either directly from mesenchyme, as in intramembranous bone formation, or with an intervening cartilage stage, as in endochondral bone development. All BMPs first induce the cascade of chondrogenesis, and therefore in this sense they are cartilage morphogenetic proteins. The hypertrophic chondrocytes in the epiphyseal

growth plate mineralize and serve as a template for appositional bone morphogenesis. Cartilage morphogenesis is critical for both bone and joint morphogenesis. The two lineages of cartilage are clear-cut. The first, at the ends of bone, forms articulating articular cartilage. The second is the growth plate chondrocytes, which, through hypertrophy, synthesize cartilage matrix destined to calcify prior to replacement by bone, and are the 'organizer' centers of longitudinal and circumferental growth of cartilage, setting into motion the orderly program of endochondral bone formation. The phenotypic stability of the articular (permanent) cartilage is at the crux of the osteoarthritis problem. **The maintenance factors for articular chondrocytes include TGF-b isoforms and the BMP isoforms**.

An in vivo chondrogenic bioassay with soluble purified proteins and insoluble ollagen scored for chondrogenesis. A concurrent reverse transcriptionepolymerase chain reaction (RT-PCR) approach was taken with degenerate oligonucleotide primers. Two novel genes for CDMPs 1 and 2 were identified and cloned. **CDMPs 1 and 2 are also called GDF-5 and GDF-6. CDMPs are related to BMPs. CDMPs are critical for cartilage and joint morphogenesis. CDMPs stimulate proteoglycan synthesis in cartilage. CDMP 3 (also known as GDF-7) initiates tendon and ligament morphogenesis**.

3.3. Pleiotropy and thresholds

Morphogenesis is a sequential multistep cascade**. BMPs regulate each of the key steps: chemotaxis, mitosis, and differentiation of cartilage and bone**. BMPs initiate chondrogenesis in the limb. The apical ectodermal ridge is the source of BMPs in the developing limb bud. The intricate dynamic, reciprocal interactions between the ectodermally derived epithelium and mesodermally derived mesenchyme sets into motion the train of events culminating in the pattern of phalanges, radius, ulna, and the humerus. The chemotaxis of human monocytes is optimal at femtomolar concentrations. The apparent affinity was 100e200 pM. The mitogenic response was optimal at the 100 pM range. The initiation of differentiation was in the nanomolar

range in solution. However, caution should be exercised, because BMPs may be sequestered by extracellular matrix components, and the local concentration may be higher when BMPs are bound on the extracellular matrix. A single recombinant BMP human 4 can govern chemotaxis and mitosis differentiation of cartilage and bone, maintain phenotype, stimulate extracellular matrix, and promote survival of some cells but cause the death of others. **Thus BMPs are pleiotropic regulators that act in concentration-dependent thresholds.**

3.4. BMPS bind to extracellular matrix

It is well known that extracellular matrix components play a critical role in morphogenesis. These structural macromolecules and their supramolecular assembly in the matrix do not explain their role in epithelialemesenchymal interaction and morphogenesis. **This riddle can now be explained by the binding of BMPs to heparan sulfate heparin and type IV collagen of the basement membranes**. In fact, this binding might explain in part the necessity for angiogenesis prior to osteogenesis during development. In addition, the actions of activin in development of the frog, in terms of dorsal mesoderm induction, are modified to neuralization by follistatin. Similarly, Chordin and Noggin from the Spemann organizer induce neuralization via binding and inactivation of BMP-4. Thus neural induction is likely to be a default pathway when BMP-4 is non-functional. Thus, an emerging principle in development and morphogenesis is that binding proteins can terminate a dominant morphogen's action and initiate a default pathway. Finally, the binding of a soluble morphogen to extracellular matrix converts it into an insoluble matrix-bound morphogen toact locally in the solid state.

Although BMPs were isolated and cloned from bone, work with gene knockouts has revealed aplethora of actions beyond bone. Targeted disruption of BMP-2 in mice caused embryonic lethality. The development of the heart is abnormal, indicating a need for BMP-2 in heart development. BMP-4 knockouts exhibit no mesoderm induction, and gastrulation is impaired. Transgenic overexpression of BMPs under the

control of keratin 10 promoter leads to psoriasis. The targeted deletion of BMP-7 revealed the critical role of this molecule in kidney and eye development. Thus the BMPs really are true morphogens for such disparate tissues as skin, heart, kidney and eye. In view of this, BMPs may also be alled body morphogenetic proteins.

3.5. BMP Receptors

Recombinant humanBMP-4 and BMP-7 bind to BMP receptor IA (BMPR-IA) and BMP receptor IB (BMPR-IB) . CDMP-1 also binds to both type I BMP receptors. There is collaboration between type I and type II BMP receptors. The type I receptor serine/threonine kinase phosphorylates a signal-transducing protein substrate called Smad 1 or 525. Smad is a term derived from the fusion of the Drosophila Mad gene and the Caenorhabtitis elegans (nematode) Sma gene. Smads 1 and 5 signal in partnership with acommon co-Smad, Smad 4. The transcription of BMP-response genes are initiated by Smad 1/Smad 4 heterodimers. Smads are trimeric molecules, as discovered by X-ray crystallography. The phosphorylation of Smads 1 and 5 by type I BMP receptor kinase is inhibited by inhibitory Smads 6 and 7. Smadinteracting protein (SIP) may interact with Smad 1 and modulate BMP-response gene expression. The downstream targets of BMP signaling are likely to be homeobox genes, the cardinal genes for morphogenesis and transcription. BMPs in turn may be regulated by members of the hedgehog family of genes, such as Sonic and Indian hedgehog, including receptors patched and smoothened and transcription factors such as Gli 1, 2, and 3. The actions of BMPs can be terminated by specific binding proteins, such as noggin.

3.6. Responding STEM CELLS

It is well known that the embryonic mesoderm-derived mesenchymal cells are rogenitors for bone, cartilage, tendons, ligaments, and muscle. However, certain stem cells in adult bone marrow, muscle and fascia can form bone and cartilage. The identification of stem cells readily sourced from bone marrow may lead to banks of stem cells for cell therapy and perhaps gene therapy with appropriate 'homing' characteristics to bone marrow and hence to the skeleton. The pioneering work of Friedenstein et al. and Owen and Friedenstein identified bone marrow stromal stem cells. These stromal cells are distinct from the hematopoietic stem cell lineage. **The bone marrow stromal stem cells consist of inducible and cells have the propensity to form bone cells, without any external cues or signals**. **On the other hand, inducible osteogenic precursors require an inductive signal, such as BMP or demineralized bone matrix**. It is noteworthy that operational distinctions between stromal stem cells and hematopoietic stem cells are getting more and more blurry! **The stromal stem cells of Friedenstein and Owen are also called mesenchymal stem cells**, with potential to form bone, cartilage, adipocytes, and myoblasts in response to cues from the environment and/or intrinsic factors. There is considerable hope and anticipation that these bone marrow stromal cells may be excellent vehicles for cell and gene therapy. From a practical standpoint, these stromal stem cells can be obtained by bone marrow biopsies and expanded rapidly for use in cell therapy after pretreatment with BMPs. The potential uses in both cell and gene therapy is very promising. There are continuous improvements in the viral vectors and efficiency of gene therapy. For example, it is possible to use BMP genes transfected in stromal stem cells to target the bone marrow.

4. Morphogens and gene therapy

The recent advances in morphogens are ripe for techniques of regional gene therapy for orthopedic tissue engineering. The availability of cloned genes for BMPs and CDMPs and the requisite platform technology of gene therapy may have immediate applications. Whereas protein therapy provides an immediate bolus of morphogen, gene therapy achieves asustained, prolonged secretion of gene products. Furthermore, recent improvements in regulated gene expression allow the turning on and off of gene expression. The progress in vectors for delivering genes also bodes well. The use of adenoviruses, adeno-associated viruses, and tetroviruses is poised for applications in bone and joint repair. Although gene therapy has some advantages for orthopedic tissue engineering, an optimal delivery system for protein and gene therapy is needed, especially in the replacement of large segmented defects and in fibrous non-unions and mal-unions.

5. Biomimetic biomaterials

Inductive signals (BMPs) and responding stem cells (stromal cells) lead us to the scaffolding (the microenvironment/extracellular matrix) for optimal tissue engineering. The natural biomaterials in the composite tissue of bones and joints are collagens, proteoglycans, and glycoproteins of cell adhesion, such as fibronectin and the mineral phase. The mineral phase in bone is predominantly hydroxyapatite. In its native state, the associated citrate, fluoride, carbonate, and trace elements constitute the physiological hydroxyapatite. Its high protein-binding capacity makes hydroxyapatite a natural delivery system. Comparison of insoluble collagen, hydroxyapatite, tricalcium phosphate, glass beads, and polymethylmethacrylate as carriers **revealed collagen to be an optimal delivery system for BMPs** It is well known that collagen is an ideal delivery system for growth factors in soft and hard tissue wound repair. Hydrogels may be of great utility in cartilage tissue engineering.

During the course of systematic work on hydroxyapatite of two pore sizes (200 or 500 mm) in two geometrical forms (beads or disks), an unexpected observation was made. **The geometry of the delivery system is critical for optimal bone induction. The disks were consistently osteoinductive with BMPs in rats, but the beads were inactive** . The chemical compositions of the two hydroxyapatite configurations were identical. In certain species, the hydroxyapatite alone appears to be 'osteoinductive'. **In subhuman primates, hydroxyapatite induces bone, albeit at a much slower rate. One interpretation is that osteoinductive endogenous BMPs in circulation progressively bind to an implanted disk of hydroxyapatite. When an optimal threshold concentration of native BMPs is achieved, the hydroxyapatite becomes osteoinductive**. Strictly speaking, most hydroxyapatite substrata are ideal osteoconductive materials. **This example in certain species also serves to illustrate how an osteoconductive biomimetic biomaterial can progressively function as an osteoinductive substance by binding to endogenous BMPs**. Thus, there is a physiological-physicochemical continuum between the hydroxyapatite alone and progressive composites with endogenous BMPs. Recognition of this experimental nuance will save unnecessary arguments among biomaterials scientists about the osteoinductive action of a conductive substratum such as hydroxyapatite. Complete regeneration of baboon craniotomy defect was accomplished via recombinant human osteogenic protein (rhOP-1; human BMP-7). Recombinant BMP-2 was delivered by a poly(-hydroxy acid) carrier for calvarial regeneration . Copolymers of polylactic acid and polyglycolic acid with recombinant BMP-2 were used in a non-union model in rabbit ulna, and complete unions were achieved in the bone . An important problem in the clinical application of biomimetic biomaterials with BMPs and/ or other morphogens is its **sterilization**. Although gas (ethylene oxide) can be used, one should always be concerned about reactive free radicals. Using allogeneic demineralized bone matrix with endogenous native BMPs, as long as a low temperature (4_C or less) is maintained, the samples tolerated up to 5e7 M rads of irradiation. The standard dose acceptable to the Food and Drug Administration is 2.5 M rads. The ambient sample temperature during irradiation that is absolutely critical.

6. Tissue engineering of bones and joints

Unlike bone, with its considerable prowess for repair and even regeneration, cartilage is recalcitrant. But why? In part this may be due to the relative avascularity of hyaline cartilage and the high concentration of protease inhibitors and perhaps even of growth inhibitors. The wound debridement phase is not optimal for preparing the cartilage wound bed for the optimal milieu interieur for repair. Although cartilage has been successfully engineered to predetermined shapes, true repair of the tissue continues to be a real challenge, in part due to hierarchical organization and geometry . However, considerable excitement in the field has been generated by a group of Swedish workers in Gothenburg, using autologous culture-expanded human chondrocytes. **A continuous challenge in chondrocyte cell therapy is progressive dedifferentiation and loss of characteristic cartilage phenotype. The redifferentiation and maintenance of the chondrocytes for cell therapy can be aided by BMPs, CDMPs, TGF-b isoforms, and IGFs**. It is also possible to repair cartilage using muscle-derived mesenchymal stem cells. The possibility of problems posed by cartilage proteoglycans in preventing cell immigration for repair was investigated by means of chondroitinase ABC and trypsin pretreatment in partial thickness defects, with and without TGF. Pretreatment with chondroitinase ABC followed by TGF revealed a contiguous layer of cells from the synovial membrane, hinting at the potential source of repair cells from synovium. Multiple avenues of cartilage morphogens, cell therapy with chondrocytes and stem cells from marrow and muscle, and a biomaterial scaffolding may lead to an optimal tissue-engineered articular cartilage. Recombinant human and BMP-2 and BMP-7 were approved in 2002 by the Food and Drug Administration (FDA) for tibial non-unions and single-level spine fusion. BMPs have been used in healing segmental defects. The proof of the principle of tissue engineering based on

BMPs as signals, molding via a scaffold, and responding cells can be demonstrated.

- ***Future challenges***

It is inevitable that as humans age that they will be confronted by impaired locomotion due to wear and tear in bones and joints. Therefore, the repair and possibly complete regeneration of the musculoskeletal system and other vital organs, such as skin, liver, and kidney, may potentially need optimal repair or a spare part for replacement. Can we create spare parts for the human body? There is much reason to be optimistic that tissue engineering can help patients. We are living in an extraordinary time with regard to biology, medicine, surgery, bioengineering, computer modeling of predictive tissue engineering and technology. The confluence of advances in molecular developmental biology and attendant advances in inductive signals for morphogenesis, stem cells, biomimetic biomaterials, and extracellular matrix biology augers well for imminent breakthroughs.

The symbiosis of biotechnology and biomaterials has set the stage for systematic advances in tissue engineering. **The recent advances in enabling platform technology include molecular imprinting. In principle, specific recognition and catalytic sites are imprinted and receptor recognition sites**. For example, the cell-binding RGD site in fibronectinor the YIGSR domain in laminin can be imprinted in complementary sites.

The rapidly advancing frontiers in morphogenesis with BMPs, hedgehogs, homeobox genes, and a veritable cornucopia of general and specific transcription factors, coactivators, and repressors will lead to co-crystallization of liganderecептor complexes, protein DNA complexes, and other macromolecular interactions. This will lead to peptidomimetic agonists for large proteins, as exemplified by erythroprotein. To such advances one can add new developments in the self-assembly of millimeter scale structures floating at the interface of perfluorodecalin and water and interacting by means of capillary forces controlled by the pattern of wettablity. The final self-assembly is due to minimization of free energy in the interface. These are advances that will lead to man-made materials that mimic extracellular matrix in tissues. It

faithfully reproduces the structural features and may be imprinted with morphogens, inductive signals, and cell adhesion sites. This assembly can be loaded with stem cells and BMPs and other inductive signals, with a nutrient medium optimized for the number of cell cycles, and then it predictably exits into the differentiation phase to reproduce a totally new bone femoral head. In fact, such a biological approach with vascularized muscle flap and BMPs yielded new bone with a defined shape and has demonstrated proof of the principle for further development and validation.

We indeed are entering a brave new world of prefabricated biological spare parts for the human body, based on sound architectural rules of inductive signals for morphogenesis, responding stem cells with lineage control, and with growth factors immobilized on a template of biomimetic biomaterial based on extracellular matrix. Like life itself, such technologies evolve with continuous refinements to benefit humankind by reducing the agony of human pain and suffering. In conclusion, based on principles of evolution, development, and self-assembly, the fields of tissue engineering and regenerative medicine are poised to make explosive advances with immense applications in the clinic.

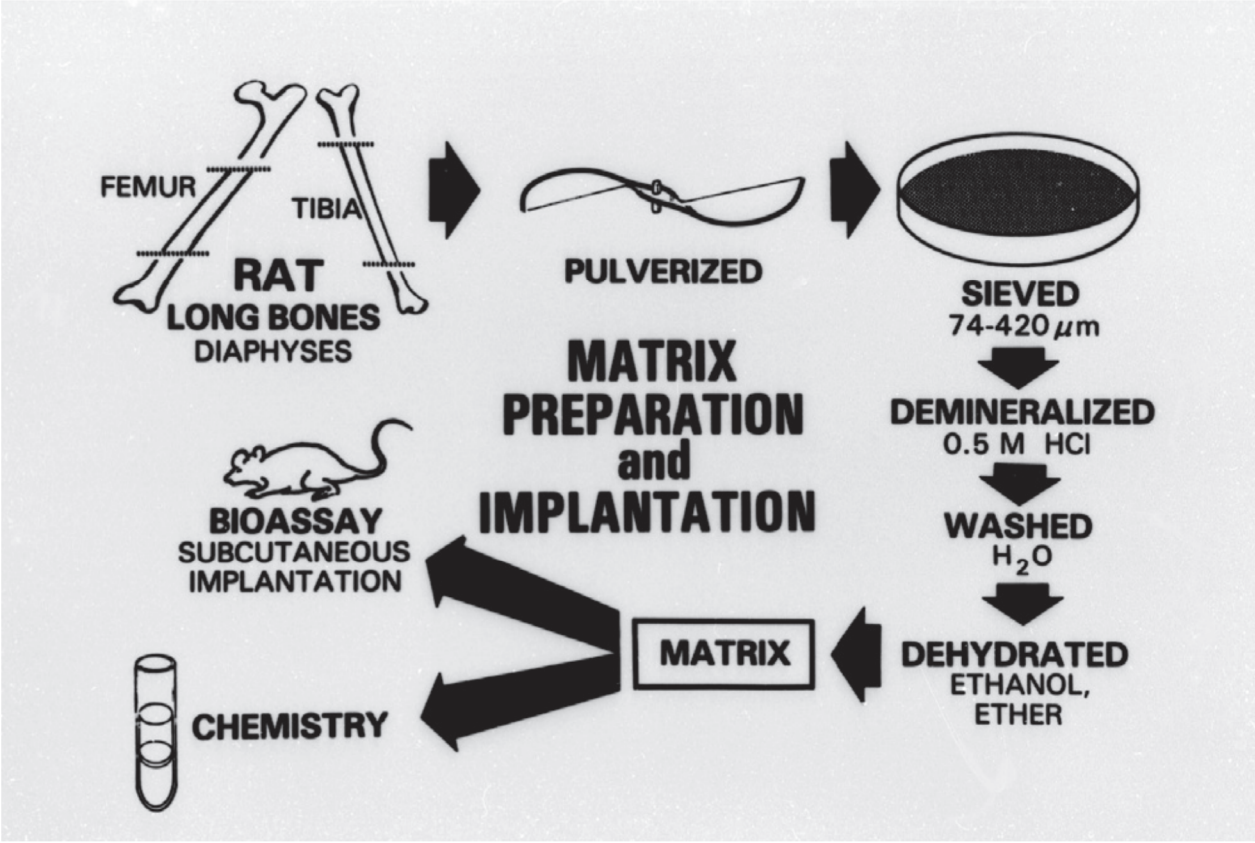

Figure 14: Preparation of the demineralized bone matrix (DBM). (75)

The diaphysis (shafts) of femur and tibia are cleansed of marrow and dried prior to pulverization. The pulverized bone matrix is sieved to a particle size of 74e420 mm and demineralized by 0.5 M HCl, dehydrated in ethanol and diethyl ether. The resulting matrix is a potent inducer of cartilage and bone, and we isolate, by means of chemical techniques, the active osteoinductive agent BMP.

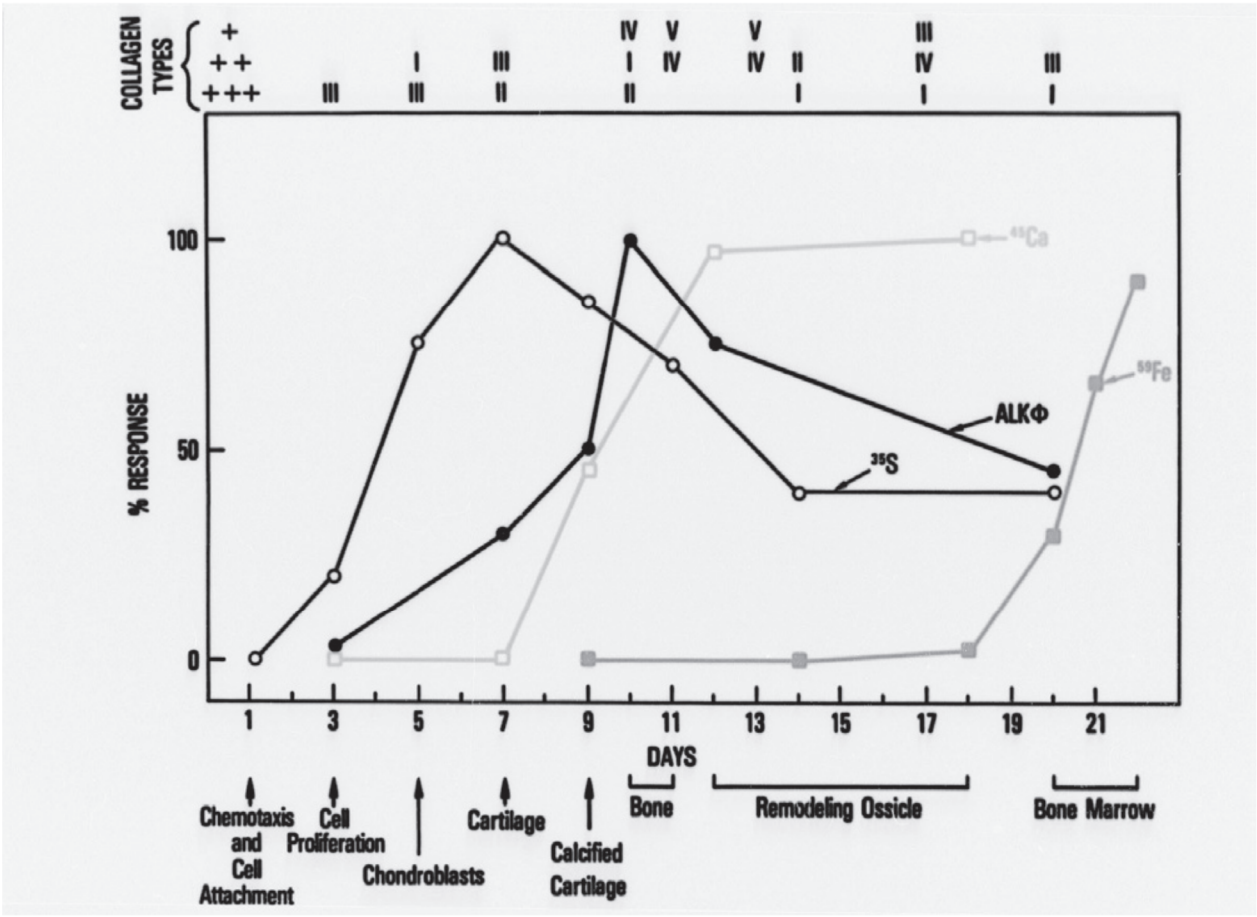

Figure 15: Developmental sequence of extracellular matrix-induced cartilage, bone, and marrow formation. (75)

Changes in 35SO4 incorporation into proteoglycans and 45Ca incorporation into the mineral phase indicate peaks of cartilage and bone formation, respectively. The 59Fe incorporation into heme is an index of erythropoiesis, as plotted from the data of Reddi and Anderson (1976) [5]. The values for alkaline phosphatase indicate early stages of bone formation [4]. The transitions incollagen types I to IV, summarized on top of the figure, are based on immunofluorescent localization ¼ polymorphonuclear leukocytes. (Source: Ref. [6], with permission.)

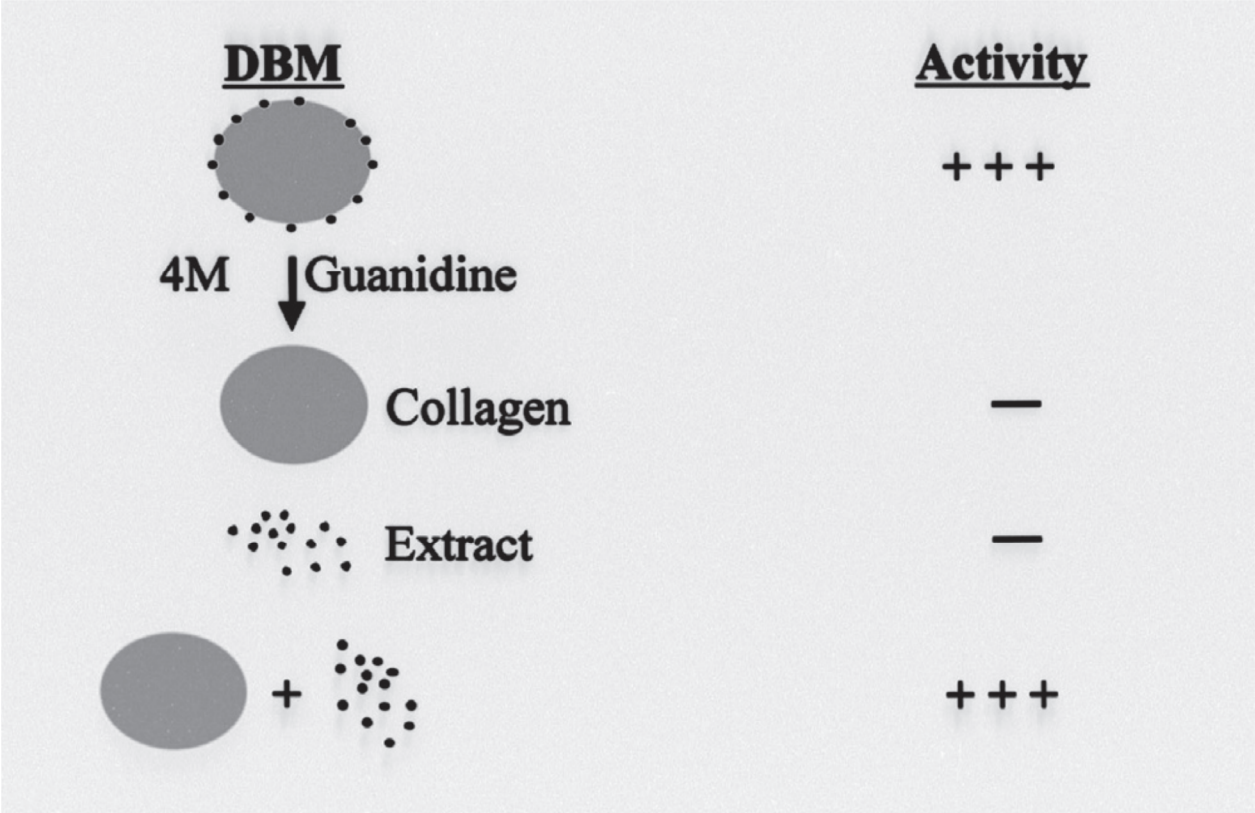

Figure 16: Dissociative extraction by chaotropic reagents such as 4M guanidine and reconstitution of osteoinductive activity with insoluble collagenous matrix. (75)

The results demonstrate a collaboration between a soluble signal and insoluble extracellular matrix. This experiment further established the basic tenets of tissue engineering in 1981 as signals, scaffolds, and responding stem cells.

Bone morphogenetic proteins

BMP Other names Function

BMP-2 BMP-2A Bone and cartilage morphogenesis

BMP-3 Osteogenin Bone morphogenesis

BMP-3B GDF-10 Intramembranous bone formation

BMP-4 BMP-2B Bone morphogenesis

BMP-5 Bone morphogenesis

BMP-6 Cartilage hypertrophy

BMP-7 Osteogenic protein-1 Bone formation

BMP-8 Osteogenic protein-2 Bone formation

BMP-8B Spermatogenesis

BMP-9 Liver differentiation

BMP-10 ?

Tooth differentiation

BMP-11 GDF-11 Odontoblast regulation

BMP-15 ?

Cartilage-derived morphogenetic proteins

CDMP Other names Function

CDMP 1 GDF-5 Cartilage condensation

CDMP 2 GDF-6 Cartilage formation, hypertrophy

CDMP 3 GDF-7 Tendon/ligament morphogenesis

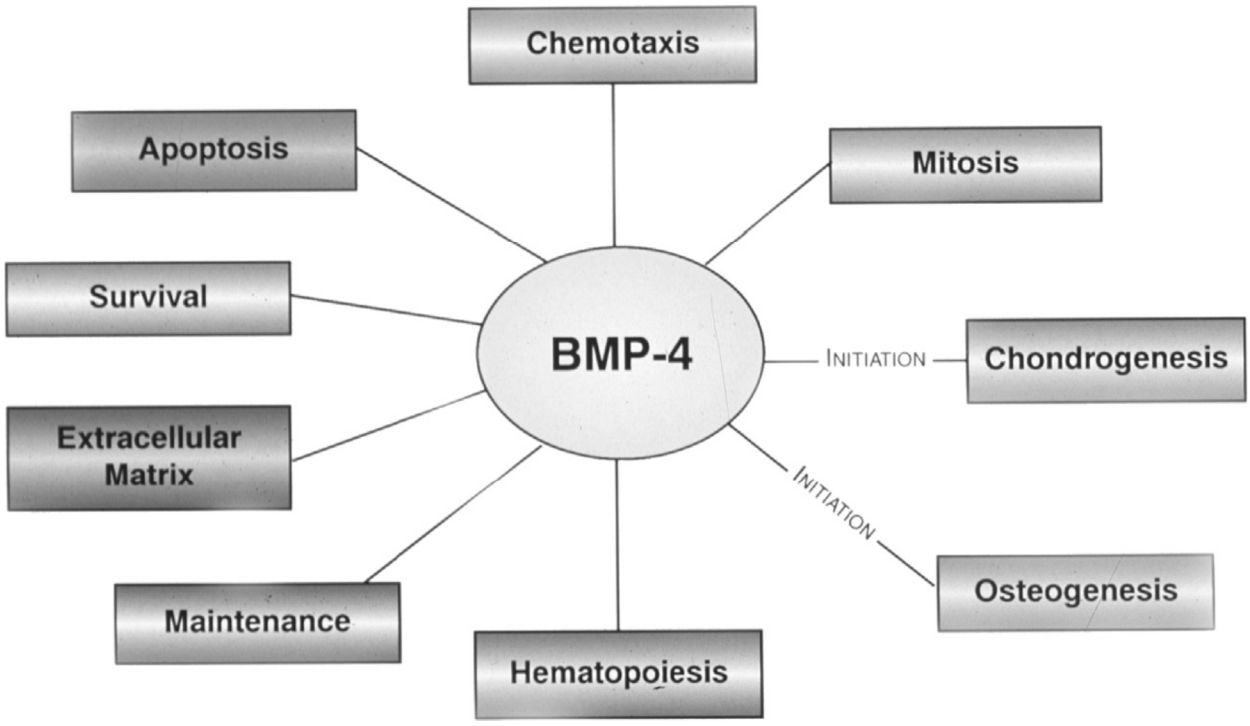

Figure 17: BMPs are pleiotropic molecules. Pleiotropy is the property of a single gene or protein to act on a multiplicity of cellular phenomena and targets. (75)

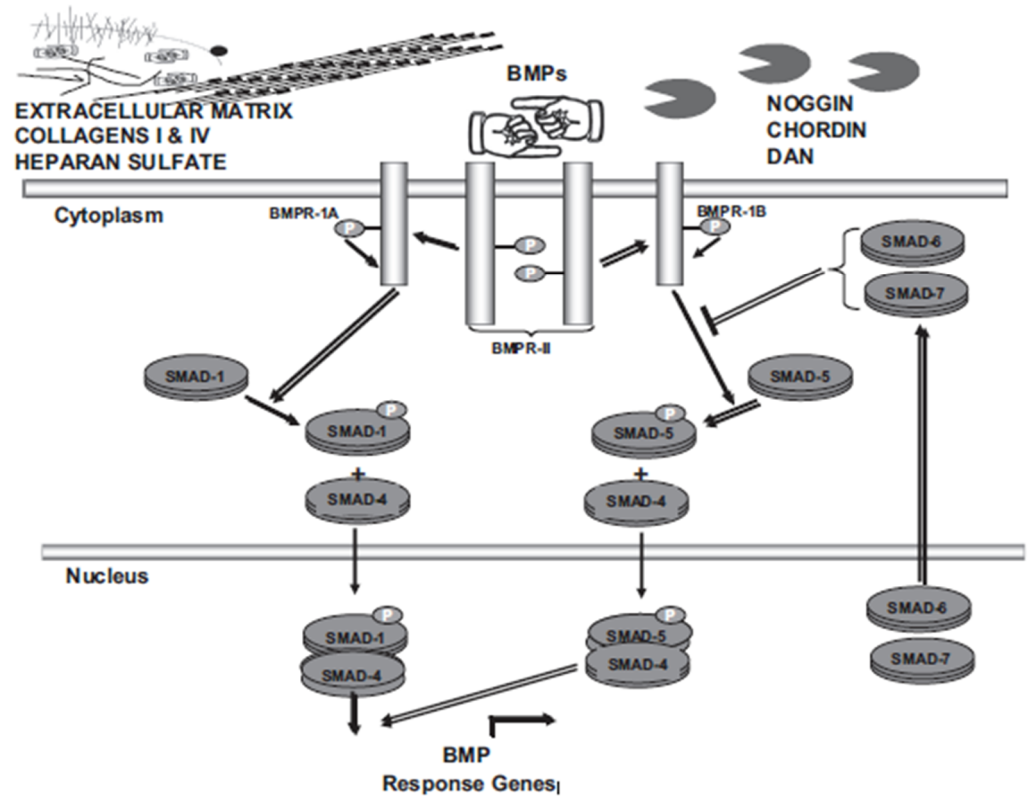

Figure 18: BMP receptors and signaling cascades (75)

BMPs are dimeric ligands with cysteine knot in each monomer fold. Each monomer has two b-sheets, represented as two pointed fingers. In the functional dimer, the fingers are oriented in opposite directions. BMPs interact with both type I and II BMP receptors. The exact stoichiometry of the receptor complex is currently being elucidated. BMPR-II phosphorylates the GS domain of BMPR-I. The collaboration between type I and II receptors forms the signal-transducing complex. BMP type I receptor kinase complex phosphorylates the trimeric signaling substrates Smad 1 and Smad 5.

This phosphorylation is inhibited and modulated by inhibitory interacts with Smad 4 (functional partner) and enters the nucleus to activate the transcriptional machinery for early BMP-response genes. A novel Smad-interacting protein (SIP) may interact and modulate the binding of heteromeric Smad 1/Smad 4 complexes to the DNA.

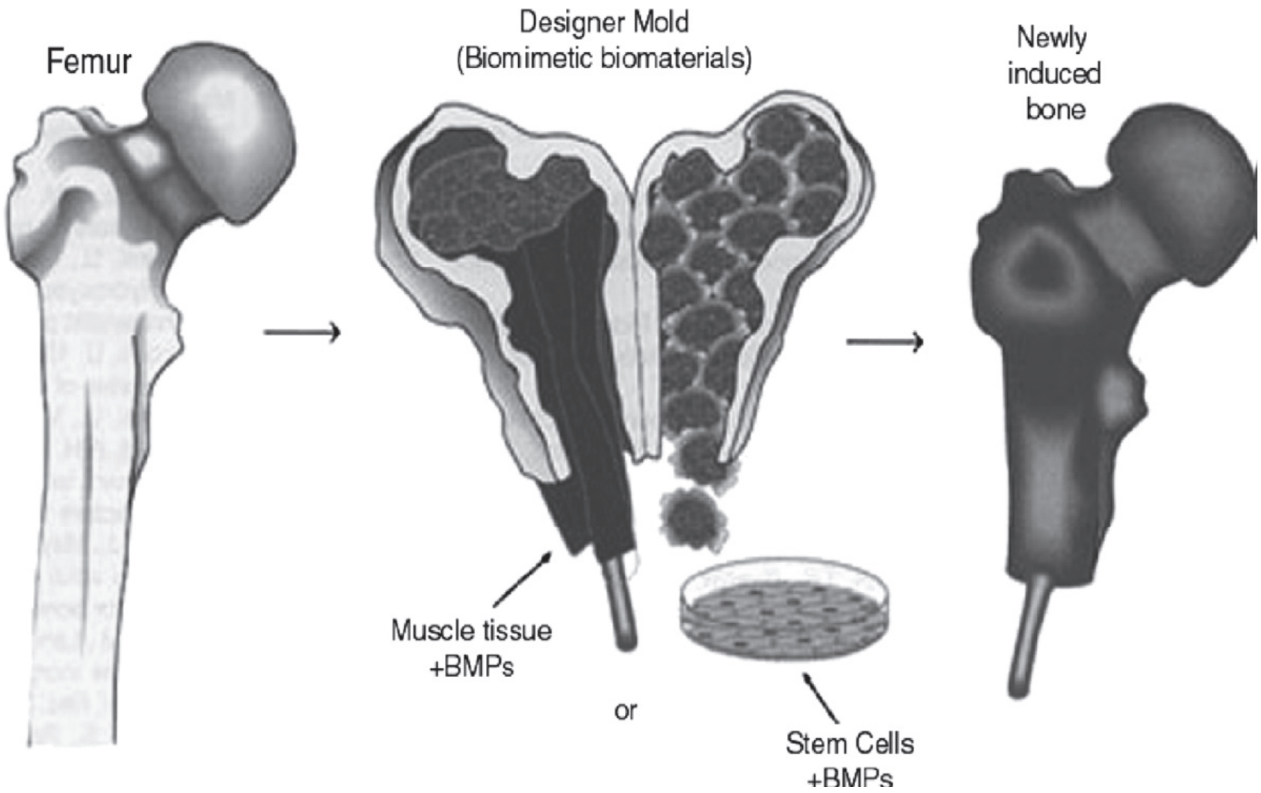

Figure 19: Proof of the principles of tissue engineering was established

A mold was used to contain the vascularized muscle flap and treated with purified BMPs and collagen scaffold. The newly formed bone faithfully reproduced the shape of the mold. In the future, one can use stem cells directed by recombinant BMPs to induce bone.

Gene Expression, Cell Determination, and Differentiation :

Studies of skeletal muscle development were the first to provide the principles for understanding the genetic and molecular bases of determination and differentiation. Molecular signals from adjacent embryonic structures activate specific genetic pathways within target cells. Important families of transcriptional regulators are expressed in response to these cues to initiate important developmental processes in skeletal muscle as well as in other tissues and organs. Both activators and repressors are essential to control the time and location at which development occurs, and self-regulating, positive feedback loops ensure that, once begun, development can proceed normally. An understanding of the mechanistic basis of the embryonic commitment to a unique developmental pathway, and the subsequent realization of the adult phenotype, are essential for understanding stem cell behavior and how this might be manipulated for therapeutic goals. It has been through the study of muscle development that the genetic basis of these processes was first revealed, laying out a mechanistic basis for understanding determination and differentiation. Following success in studies of skeletal muscle (reviewed in references), genetic pathways involved in the determination of other systems, have alsobeen uncovered, largely because of the underlying conservation of structure among the various effector molecules and mechanisms.

6.1. Determination and differentiation

Determination describes the process whereby a cell becomes **committed to a unique developmental pathway**, which, under conditions of normal development, appears to be a stable state. In many cases, cells become committed early in development yet remain highly proliferative, expanding exponentially for long periods of time before differentiation occurs. Until recently, determination could only be defined post hoc. Prior to the discovery of transcriptional regulators there were **few markers to indicate whether or not a cell wascommitted** to a unique phenotype. Thus, determination was operationally defined as that state that existed immediately

prior to differentiation, that is, before expression of a cell-type-specific phenotype. The identification of transcription factors that control the differential expression of large families of genes changed the concept.

Determination and differentiation are processes that are coupled during embryogenesis, where a small number of pluripotent cells (stem cells), expand and enter pathways through which they form the diverse cell types of the adult. The process of differentiation describes the acquisition of the phenotype of a cell, most often identified by the expression of specific proteins achieved as a result of differential gene expression. The differentiated state is easily determined by simple observation in most instances, because most differentiated cells display a unique phenotype as a result of the expression of specific structural proteins. Skeletal muscle cells are an extreme example of this, having a cytoplasmic matrix filled with highly ordered myosin, actin, and other contractile proteins within sarcomeres e the functional units of contraction e giving the fibers their cross-striated pattern. As development proceeds, there is a gradual narrowing of the possible final cell phenotypes that individual cells can adopt, with the final cell fate (set of genes expressed) determined by factors both extrinsic and intrinsic to the cell .

Changes in gene expression responsible for directing cells to differentiate along particular developmental pathways result from the **response to stimuli received from surrounding cells and the specific cellular phenotype of the cell itself at the time of interaction**. For example, cells of recently formed somites have the potential to form either skeletal muscle or cartilage in response to adjacent tissues, and the fate adopted is a result of their location with respect to adjacent structures e the notochord, neural tube, and overlying ectoderm e that produce signaling molecules that determine phenotype . In addition to the activation of musclespecific structural and enzyme-encoding genes, the differentiated state is maintained by the continued expression of specific regulatory transcription factors, which can now be identified using modern tools of cellular and molecular biology, including monoclonal antibodies, antisense

nucleic acid probes, and gene chip analyses. Commitment and differentiation to a skeletal muscle fate begins in the somites of the early vertebrate embryo. Within the embryonic somites, two distinct anatomical regions contain muscle progenitors. Specified by signals from the adjacent structures, the dorsal portion of each somite forms an epithelial structure, the dermomyotome, which contains the precursor cells of all skeletal muscles that will form in the vertebrate body (with the exception of those found in the head). The medial portion of dermomyotome contains cells that form the axial musculature surrounding the vertebral column, while cells of the ventral-lateral portion of the dermomyotome undergo a process of delamination and migrate into the forming appendages to produce the appendicular musculature of the limbs and body wall . While the muscle fibers that form from the different regions of the somite are nearly indistinguishable, myogenesis in the axial and appendicular muscles is regulated by different effectors, demonstrating the complexity of determination and differentiation in the early embryo.

6.2. MyoD and the bhlh family of developmetal regulatory factors

It was not until late in the twentieth century that experiments first demonstrated that cellular commitment to specific developmental fates could be determined by the expression of a single gene or a very small number of genes . With the improvements in tissue culture methods and rapid advances in molecular biology that permitted the introduction of foreign genes into mammalian cells, the first factor capable of specifying a cell to a particular cellular phenotype, MyoD, was isolated and characterized in the laboratory of Dr. Harold Weintraub . MyoD expression is specific to skeletal muscle, and introduction of MyoD cDNA into fibroblasts of the 10T1/2 cell line converts them at a high frequency into stable myoblasts, which in turn express skeletal muscle proteins. MyoD was only the first of a family of yogenic regulatory factors (MRFs) to be discovered; others include myf-5 , myogenin , and MRF4 ; reviewed by Berkes and Tapscott . The importance of MyoD and myf-5 to the determination of skeletal muscle was demonstrated when double knockout of these two genes in transgenic mice resulted in a nearly complete absence of skeletal muscle .

MRF members share a common structure, a stretch of basic amino acids followed by a stretch of amino acids that form two amphipathic helices separated by an intervening loop (the helixloop- helix [HLH] motif), and they are nuclear-located DNA-binding proteins that act as transcriptional regulators . Experiments have demonstrated that the basic amino acids are required for DNA binding and are essential for the myogenic conversion of fibroblasts to muscle, while the HLH motif plays an essential role in the formation of heterodimers with other ubiquitously expressed HLH proteins (products of the E2a gene) as well as in DNA binding .

Nature being conservative, it is not surprising that the bHLH motif was found in transcriptional factors regulating the determination of cell types other than muscle. Based on homology with MyoD, the transcription factor NeuroD was isolated by the Weintraub laboratory and shown to act as a neuronal determination factor . Expression of NeuroD in presumptive epidermal cells of Xenopus embryos converted many into fully differentiated neurons. Interestingly, NeuroD also plays an important role in the differentiation of pancreatic endocrine cells and the retina. While NeuroD is involved primarily in neuronal differentiation and survival, neurogenin, whose expression precedes that of NeuroD in the embryo, functions more like a determination factor . Overexpression of Xenopus neurogenin Ind uces ectopic neurogenesis as well as ectopic expression of NeuroD. Additional bHLH family members, including HES, Math-5, and Mash-1, have been isolated and participate in the determination of neural cells as well.(75)

Differences in the expression of various members of the neurogenic bHLH family help to explain the diversity of neuronal cell types. For example, genetic deletion of the Mash-1 gene eliminates sympathetic and parasympathetic neurons and enteric neurons of the foregut, while knockout of NeuroD leads to a loss of pancreatic endocrine cells as well as cells of the central and peripheral nervous system. In addition to the various bHLH activators, other homeodomain-containing transcription factors are required for specification of neuronal subtypes.

Because cardiac muscle has so much in common with skeletal muscle, including

a large number of contractile proteins, an exhaustive search was made for MyoD family members in the heart. Surprisingly, MyoD family members were not found in the developing heart, and thus they play no part in the differentiation of cardiac muscle cells. However, a different family of bHLH-containing factors, including dHAND and eHAND, were found in the developing heart, autonomic nervous system, neural crest, and deciduum. In the heart these factors are important for cardiac morphogenesis and the specification of cardiac chambers. Unlike their MyoD family cousins, neither of the HAND proteins plays a role in the differentiation of cardiac muscle cells.

Acting as dominant negative regulators of the bHLH family of transcriptional regulators is a ubiquitously expressed family of proteins that contain the helix-loop-helix structure but lack the upstream run of basic amino acids essential for specific DNA binding by MyoD family members. Termed inhibitors of differentiation (Id), these proteins can associate specifically with MyoD or products of the E2A gene, and attenuate their ability to bind DNA by forming non-functional heterodimeric complexes. In proliferating myoblasts, Id inhibit the terminal differentiation program by complexing with the E12/E47 protein until the cell receives an appropriate stimulus. Id levels decrease on terminal differentiation.

Neuronal development is also regulated in part by repressors of the neurogenic family of bHLH activators. The HES family of HLH-containing proteins is expressed in neural stem cells, where they maintain proliferation of neuronal precursors and prevent premature differentiation in cells expressing NeuroD and neurogenin. The interaction of unique sets of positively acting bHLH activators and negatively acting members of the HES family elps explain how different subsets of neurons undergo differentiation at different times during development, so that the complex structure of the brain can be produced.

6.3. Mefs AND microRNAs e coregulators of development

MEF2 proteins are transcriptional activators that bind to AþT-rich DNA sequences found in many muscle-specific genes, including those encoding contractile proteins, muscle fiber enzymes, and the muscle differentiation factor myogenin. While some members of this family of MADS-box regulatory factors show a nearly ubiquitous distribution among tissue types, a few show expression that is more restricted to striated muscle. Because they do not act alone, MEF2 family members must physically interact with MyoD family members at their DNA-binding domains to positively regulate transcription of downstream muscle-specific differentiation genes . Additionally, the transcriptional activation of muscle-specific genes requires that either the MyoD or MEF2 protein provide a transcriptional activation domain. Interestingly, although the wide tissue distribution of some MEF2 family members suggested that they may act in combination with bHLH family members found in other cell types (such as neurogenin in neural precursors) to activate downstream genes (Molkentin and Olson, 1996), no evidence has been found to that effect .

A common method of regulation in most differentiating cells which results in pecification, maintenance, and regulation of all complex regulatory circuits, are non-coding RNAs which includes microRNAs . Differentiation requires not only the acquisition of new functions through gene activation and transcription, but also the suppression of gene transcription at the expression level. MicroRNAs (miRNAs), which regulate gene expression at the transcriptional and post-transcriptional levels, play key roles in differentiation. They can suppress gene expression by binding to mRNAs through complementarity in sequence between the two and altered expression by alternative splicing. In particular, they have been shown to play key roles in the post-transcription regulation in skeletal muscle cell differentiation. Specific microRNAs interact with MyoD and other bHLH transcription factors, such as Twist, in cooperation with MEF2 by binding of MyoD and myogenin. Some of the key functions of MEF2 that are central to muscle cell gene transcription are controlled by

microRNAs. Particular miRNAs are absent from undifferentiated myoblasts and are strongly upregulated upon differentiation. Experiments suggest that miRNAs also act as modulators of myogenic differentiation, affecting expression of genes not involving Mef2. An example is that an increase of miR1 expression in muscle cell culture accelerates myoblast differentiation by downregulating histone deacetylase 4 (HDAC4), a repressor of muscle differentiation. The interplay between transcription factors and microRNAs provides the fine tuned modulation of protein expression characteristic of differentiating cells.

6.4. Pax IN Development

Much of what has been learned about the role of MyoD and myf-5 in the determination of skeletal muscle has come from studies on transgenic mice in which various genetic loci have been deleted. This work suggested that MyoD and myf-5 acted as redundantactivators of myogenesis, albeit with some slight distinctions. Subsequently, Pax3, a DNAbinding protein with both a paired box and a paired-type homeodomain, was identified as a key regulator of myogenesis. Double knockout of Pax3 and myf-5 leads to a complete absence of skeletal muscle and places Pax3 genetically upstreamof MyoD. Using knock-in experiments where the lacZ marker gene replaced Pax3, Buckingham's group demonstrated that Pax3 and myf-5 are activated independently of one another. The implication is that axial muscle (myf-5-dependent) and appendicular muscle (MyoD-dependent) are specified separately in the embryonic somites by two different pathways, and that a Pax gene(or more than one) is required for this specification (determination).

A second member of the paired box transcription factor family, Pax7, has also been implicated in myogenesis. Pax7 was isolated from satellite cells, a population of muscle-committed stem cells found in intimate association with mature muscle fibers and involved in muscle growth and repair in the adult. Pax7 is specifically expressed in proliferative myogenic precursors, both embryonic myoblasts as well as satellite cells, and is down regulated at differentiation. Transgenic mice lacking the Pax7 gene

have normal musculature, albeit with reduced muscle mass, but a complete absence or markedly reduced numbers of satellite cells . These investigators found that in these Pax7 mutants, satellite cells, which are cells responsible for postnatal growth of skeletal muscle, are progressively lost by cell death. These results suggest that specification of skeletal muscle satellite cells requires Pax7 expression, or that Pax7 expression is responsible for survival of satellite cells. The interplay of the many factors that control the initiation and maintenance of myogenesis are shown in Figures.

6.5. Satellite and stem cells in skeletal muscle

- ***Differentiation and repair***

The reason that a discussion of skeletal muscle differentiation is so central to a basic understanding of cell differentiation is that this tissue unites concepts of tissue formation and tissue repair. The unifying concept is the stem cell. Among the first to be considered as stem cells were the satellite cells of skeletal muscle (reviewed by Wang and Rudnicki). These cells lie just beneath the basal lamina that surrounds each muscle fiber within an anatomic muscle. These cells meet the definition of stem cellsbecause they are self-renewing. They are cells set aside in early muscle development, during which they contribute to fiber lengthening as muscles grow, as well as to the formation of additional fibers as the neonate matures. On the other hand, muscle hypertrophy, as in weight lifters, is primarily produced by expansion is size of the fibers rather than fiber number. However, because of injury of small numbers of fibers, satellite cells can fuse with existing fibers during this process. They are located within special niches formed by the basal lamina. These niches keep satellite cells in a quiescent state until there is injury to the fibers of a muscle on which they lie. Injury results in the release of cytokines that initiate the proliferation and migration of the satellite cells on the surface of the injured fibers. The cell divisions of satellite cells are asymmetric e one daughter forms another satellite cell (stem cell) and is retained in the niche and the other goes forward as a myogenic cell, which most often fuses with an injured fiber to reconstitute its multinuclear state, or occasionally, depending on the

nature of the injury, to form a new fiber . Thus, while muscle regeneration in higher vertebrates is very limited, new fibers can form and old fibers can be repaired while still retaining a supply of stem cells for future tissue repair. The challenge to investigators of tissue engineering is to develop strategies to facilitate extensive muscle stem cell proliferation, cell survival, and efficient cell differentiation of transplanted cells. Equally challenging is gaining an understanding of the cells that form the connective tissue scaffolding of an anatomic muscle, if such satellite cells or stem cells are to successfully be used to treat neuromuscular disease or to reconstitute muscles following trauma .

Among the most promising new approaches to repair of skeletal muscles damaged by disease or trauma are embryonic stem cells (ES cells) and induced pluripotent stems (iPS cells). In recent times there has been a flurry of experimental activity in model systems for regenerating and primary muscular diseases, which exploit these two types of cells.

iPS cells can be obtained through direct reprogramming of different human somatic cells to a pluripotent state by a limited number of genes, or by transfection with retroviruses, lentiviruses or plasmids . There is also a variety of other cells types that have been specifically linked to skeletal muscle differentiation , including bone marrow-derived side-population cells , mesenchymal stem cells , pericytes, CD133þ progenitor cells, and mesoangioblastsbecause they show a high myogenic propensity in vitro and in vivo. But only ES and iPS cells overcome the disadvantage of the latter cell types because they can be produced in sufficient quantities for study and transplantation.

The use of ES cells from humans is complicated by ethical considerations, whereas iPS cells are not, because they do not require human embryos. iPS cells also have the advantage that each individual could have their own cells (autologous patient-specific stem cells) used to form iPS cells, thus obviating the immunological rejection that accompanies the use of ES cells. The hurdles to overcome in tissue engineering of

muscle are the size of muscles in humans (human anatomic muscles are relatively large), which require large numbers of cells for adequate repair. This means that transplanted cells must proliferate and endure in vivo, and that they become co-opted into the satellite cell lineage for subsequent hypertrophy of the muscle or its repair. In any situation where cells for transplantation are encouraged to proliferate and differentiate in vitro following viral transfection, an important challenge is the formation of teratomasor insertional mutagenesis.

The advantage of iPS cells for skeletal muscle transplantation has been exploited in murine model systems. Darabi and colleaguesand Mizuno and colleagueshave used iPS cells from the mouse transfected with a gene (Pax 7) fostering myogenic differentiation showing that these engineered cells are effective in engraftment, improving muscle function in disease models. The studies demonstrate in principle that the use of stem cell transplantation can be effective.

However, for human transplantation it will be important that human iPS cells be directed into the myogenic lineage without the use of transfection or other approaches that alter the structure of the genome . The use of non-transfection strategies is desirable, as the integration of transfected genes can be problematic for the functioning of the cells. Recently, the hurdle of making human iPS cells initially obtained from retrovirally transformed cells that can undergo myogenesis in vivo may have been overcome when tested in an immunoincompetent model mouse systems . Under specific culture conditions, myogenic iPS cells were obtained which formed human muscle when transplanted, supplying satellite cells for subsequent regeneration in response to injury, and reportedly do not form tumors.While these approaches with human iPS cells are exciting and hold great promise, additional work is needed before induced pluripotent stem cell transplantation can be applied in humans.

Determination and differentiation are in large part controlled by the expression of transcriptional regulators. The processes begin early in development and involve the formation of stem cells that become committed to specific pathways of regulated gene

expression. The regulators responsible were first characterized in studies examining commitment to, and differentiation of, skeletal muscle, and muscle development serves as a model for the mechanisms involved. Some other developing organs, such as the central and peripheral nervous systems and the pancreas, employ very similar mechanisms and closely related members of the bHLH family of proteins to drive development. However, for reasons that are not clear, other organs, such as the heart, in which cardiac cells express many of the same contractile protein genes as their skeletal muscle cousins, use other mechanisms. As our understanding of the mechanisms and effectors of determination and differentiation during embryonic development increase, we will be better able to conceive and apply strategies to engineer stem cells, embryonic or adult-derived, to address medical problems through

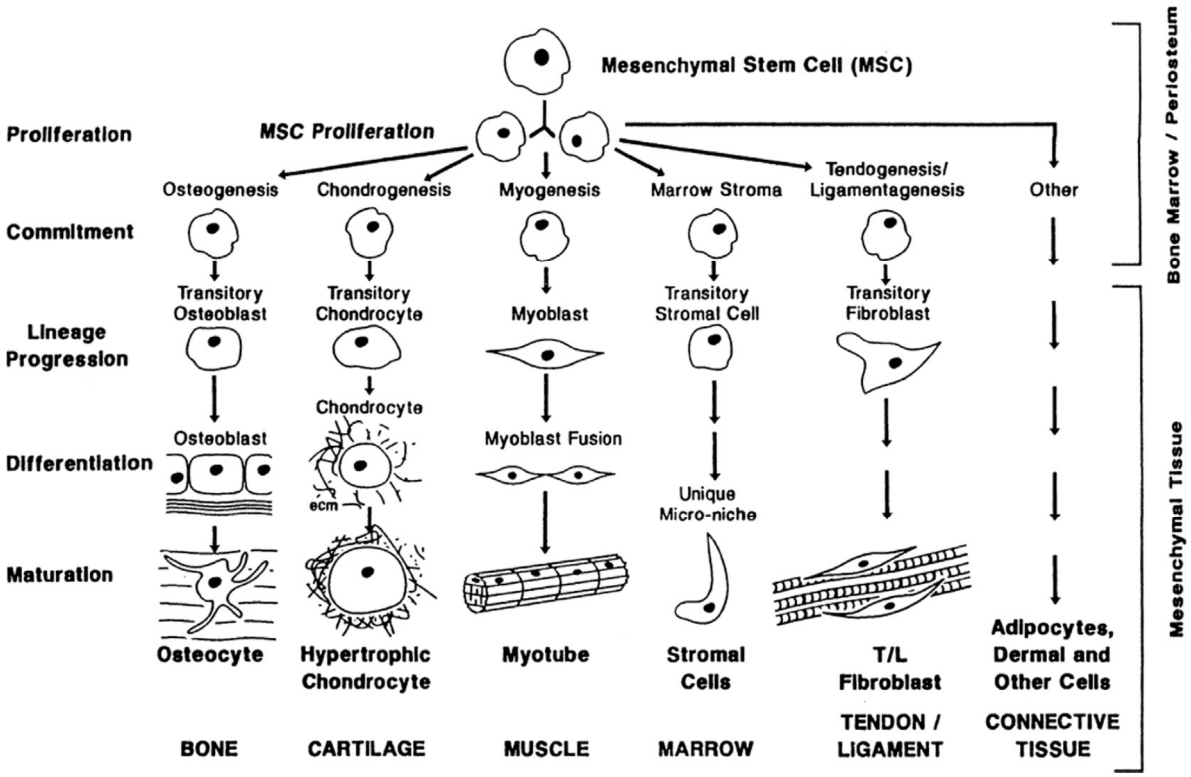

Figure 20: The process of commitment and differentiation. (75)

Cells arise during gastrulation in the vertebrate embryo that subsequently produce all the different cell type of the body. Cells that can be designated as mesenchymal stem cells (MSC) proliferate and, in response to cues from the cellular environment, enter lineages that undergo differentiation and subsequent maturation into the mature cell types.

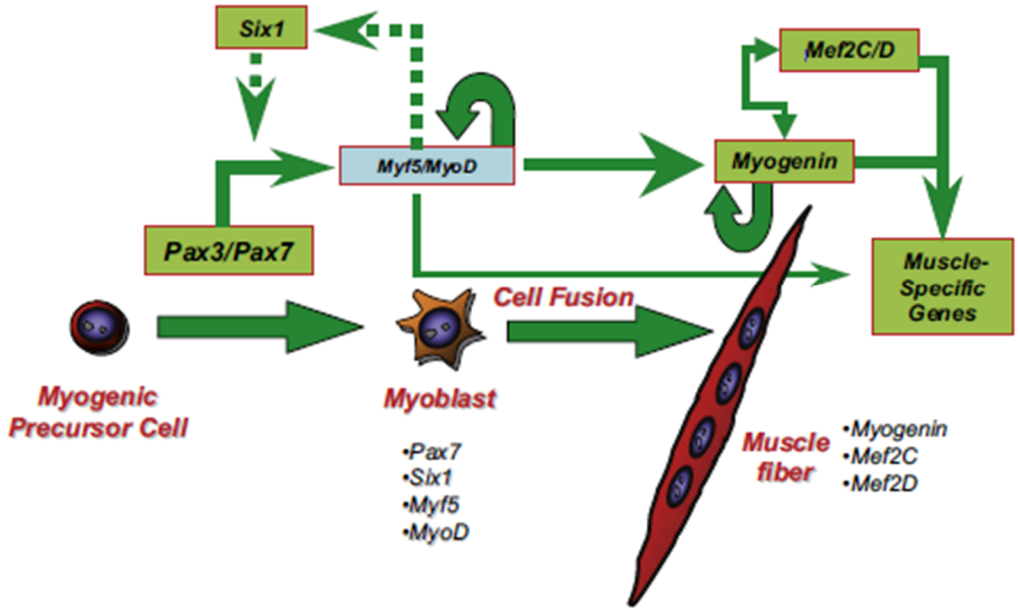

Figure 21: A regulatory network controls muscle cell differentiation.(75)

7. Engineering FunctionalTissues:

7.1. Introduction

Clinical disorders typically associated with musculoskeletal and cardiovascular tissues (i.e., osteoarthritis and myocardial infarction, respectively) often result in the loss of native tissue structural organization and mechanical function. Tissue engineering is a rapidly growing field that seeks to restore the structure and function of tissues damaged due to injury, aging or disease through the use of cells, biomaterials, and biologically active molecules . Despite many early successes, there are few engineered tissue products available for clinical use, and significant challenges remain with regard to translating tissueengineering technologies to the clinic for successful long-term repair of dysfunctional tissues. The precise reasons for graft failure are not fully understood, but they include a combination of factors that lead to the breakdown of repair tissues under conditions of physiologic loading.

Two critical aspects of tissue-function in many tissue and organ systems are the transmission or generation of mechanical forces and the maintenance of blood circulation. In particular, articular cartilage and myocardium have highly specialized tissue composition and structure to allow for the specific mechanical and transport properties of these tissues. Although different in many respects, cartilage and cardiac tissues both perform critical biomechanical functions in vivo and lack intrinsic capacity for self repair. Therefore, cartilage and cardiac tissues are ideal targets for functional tissue engineering.

At the time of implantation, tissue-engineered constructs rarely possess mechanical properties that can withstand the high magnitudes of mechanical stresses experienced in vivo. For many native tissues, however, the potential range of in vivo stresses and strains are not well characterized, thus making it difficult to incorporate a true ’safety factor’ into the design criteria for engineered tissues. In addition, matching a single mechanical parameter, such as modulus or strength, is rarely sufficient, as most tissues possess complex viscoelastic, non-linear, and anisotropic mechanical

properties that may vary with age, site, and other factors. A number of complex interactions must also be considered, as the graft and surrounding host tissues are expected to integrate and remodel in response to their changing environments post-implantation. For example, convective transport of oxygen, nutrients, and waste products may become a limiting factor. Currently, most tissue-engineered constructs do not contain a functioning vasculature at the time of implantation, instead relying on either anastomosis of implanted capillary networks to the host vasculature or de novo angiogenesis to provide transport capability. Because oxygen has low solubility in aqueous media and diffuses over distances of only 100 to 200 mm, oxygen transport is often a limitation in cell survival within large, anatomically relevant grafts.

An evolving discipline referred to as ’functional tissue-engineering’ has sought to address the aforementioned challenges by developing guidelines for rationally investigating the role of biological and mechanical factors in tissue engineering. A series of formal goals and principles for functional tissue engineering have been proposed in a generalized format. In brief, these guidelines include development of:

1) Improved definitions of functional success for tissue-engineering applications;
2) Improved understanding of the in vivo mechanical requirements and intrinsic properties ofnative tissues;
3) Improved understanding of the biophysical environment of cells within engineered constructs;
4) Scaffold design criteria that aim to enhance cell survival, differentiation, and tissue mechanical function;
5) Bioreactor design criteria that aim to enhance cell survival and the regeneration of functional tissue-engineered constructs;
6) Construct design criteria that aim to meet the metabolic and mechanical demands of specific tissue-engineering applications; and

7) Improved understanding of biological and mechanical responses of an engineered tissue construct following implantation. Since these guidelines were proposed, significant progress has been made toward developing in vitro culture systems and techniques to enhance graft mechanical properties and engineer functional tissues.

One of the key challenges in functional tissue engineering is optimizing the in vitro culture environment in order to produce three-dimensional (3D) implants that can meet the requirements of the in vivo milieu. In particular, the ability to precisely define and control in vitro culture conditions can be exploited to improve and ultimately control the structure, composition and mechanical properties of engineered tissues.

7.2. Key concepts for engineering functional tissues

Fundamental parameters for engineering functional tissues Many tissue-engineering approaches involve the in vitro culture of cells on biomaterial scaffolds to generate functional engineered constructs. The working hypothesis is that in vitro culture conditions have a significant influence on the structural and mechanical properties of engineered tissues, and therefore can be exploited to manipulate the growth and functionality of engineered tissues. In vitro culture conditions will refer to tissue-engineering scaffold systems, bioreactors, growth factors, and mechanical conditioning regimens that mediate cell behavior and functional tissue assembly . Scaffolds will be defined as 3D material structures designed to perform some or all of the following functions:

1) Promote cell-biomaterial interactions, cell adhesion, and extracellular matrix (ECM) deposition;
2) Permit sufficient transport of gases, nutrients and regulatory factors to allow cell survival, proliferation and differentiation;
3) Biodegrade at a controllable rate that approximates the rate of tissue regeneration under the culture conditions of interest; and
4) Provoke a minimal degree of inflammation or toxicity in vivo .
5) Bioreactors will be defined as laboratory devices designed to perform some or all of thefollowing functions:

6) Provide control over the initial cell distribution on 3D scaffolds;
7) Provide efficient mass transfer of gases, nutrients, and growth factors to tissue-engineered constructs during their in vitro cultivation; and
8) Expose the developing constructs to convective mixing, perfusion, and/or mechanical, electrical, or other biophysical factors in a controlled manner .

Mechanical conditioning will be defined as the in vitro application of dynamic mechanical loads (i.e., compression, tension, pressure, and/or shear) to cells, tissues, and/or three-dimensional (3D) engineered tissue using custom designed systems. These fundamental in vitro culture parameters can be controlled independently or in combination to strategically meet the requirements of the specific tissue to be engineered.(75)

7.3. Fundamental criteria for engineering functional tissues

The biological and mechanical requirements of an engineered tissue depend on the specific application. For example, engineered cartilage should provide a low-friction, articulating surface and be able to withstand and transmit load in compression, tension, and shear; whereas engineered cardiac tissue should propagate electrical signals, contract in a coordinated manner, and withstand dynamic changes in pressure, tension, and shear. In addition to tissuespecific requirements that serve as design principles for functional tissue engineering, there are fundamental criteria that all engineered tissues should meet:

1) At the time of implantation, an engineered tissue should possess sufficient size and mechanical integrity to allow for handling and permit survival under physiological conditions;
2) Immediately following implantation, an engineered tissue should provide some minimal level of biomechanical function that should improve progressively until normal tissue function has been restored; and
3) After implantation, an engineered tissue should mature and integrate with surrounding host tissues.

7.4. Importance of in vitro studies for engineering functional tissues

Cells cultured in vitro tend to retain their differentiated phenotype and function under conditions that resemble their natural in vivo environment; these conditions may be generated using a combination of scaffolds, bioreactors, growth factors, and mechanical conditioning. In vitro-grown tissue-engineered constructs can potentially be transplanted in vivo for tissue engineering and regenerative medicine applications or utilized as platforms for in vitro testing of cell and tissue-level responses to molecular, mechanical, or genetic manipulations. In vitro studies relevant to tissue engineering and regenerative medicine

In vivo models are essential for clinical translation of engineered tissues as they provide insight into the functional performance of engineered tissues. However, these models are complicated by high variability and biological and mechanical environments that differ from those existing in the human condition. To overcome these limitations, in vitro studies can be designed to understand the performance of the engineered tissue in vivo by: (i) addressing the challenges of in vivo complexity in more controllable in vitro systems, and (ii) exploring how an in vitro-grown construct may behave when implanted in vivo. For example, tissueengineered cartilage constructs pose a significant challenge with regard to variable and incomplete integration upon implantation in vivo . To address the challenge of tissue integration, researchers have taken the approach of evaluating the integration of engineered tissues with native tissues using not only in vivo models , but also in vitro models . In one study, cells were combined with three types of scaffolds [fibrin, agarose, and poly(glycolic acid) (PGA)], incorporated with explants of native articular cartilage, and cultured as composites for 20 or 40 days . The presence of native cartilage significantly altered cell proliferation and matrix accumulation in the composites. Additionally, although engineered constructs based on all three scaffold materials adhered to the native cartilage, there were significant differences in the adhesive strength between the groups, suggesting that the type of scaffold may influence construct integration in vivo.

In another in vitro study, cells were combined with hyaluronan benzyl ester scaffolds, incorporated with three types of explants (native articular cartilage, vital bone, or devitalized bone), and cultured in rotating bioreactors for 4 or 8 weeks. Engineered cartilage constructs interfaced with the solid matrix of adjacent cartilage without any gaps or intervening capsules. Additionally, focal intermingling between construct collagen fibers and native cartilage collagen fibers provided evidence of structural integration. Interestingly, adhesive strength was higher for constructs cultured adjacent to bone than cartilage and highest for constructs cultured adjacent to devitalized bone . These findings could be explained by the differences in adjacent tissue architecture (histological features) and transport properties (diffusivity) . Perfused bioreactors consistently yielded significantly higher amounts of glycosaminoglycans (GAG) and total collagen in engineered cartilage co-cultured adjacent to engineered bone than either engineered cartilage, native cartilage, or native bone . These results suggest that the type of native tissue with which the engineered tissue is combined may influence construct integration in vivo. Collectively, information can be gleaned from in vitro studies with respect to the integration potential of a tissue-engineered construct prior to in vivo implantation.

In vitro platforms relevant for high throughput screening of drugs and other agents

In vitro models can also be utilized to generate physiologically responsive tissue for creening pharmaceutical and therapeutic drugs (reviewed in Vandenburgh et al.). This type of model can be designed for high throughput screening, thereby reducing the need for human tissue and organ harvest. Moreover, if based on human rather than animal cells (e.g., Schaaf et al.), in vitro models can provide a more relevant system than in vivo animal models, which may differ significantly from the human case in their physiologic responses.

For example, a tissue-engineered drug screening platform referred to as ‘engineered heart tissue’ was developed by combining neonatal rat heart cells with

ECM components such as Matrigel_ and fibrin. The cell-ECM-fibrin mixture was cast between two flexible posts such that maturation of the neonatal cells and condensation of the maturing tissue between the flexible posts yielded strips of cardiac-like tissue which contracted regularly. Pharmaceutical agents with known cardiac effects induced the expected changes within the engineered heart tissue in a repeatable fashion, suggesting that engineered tissues of this type could be used for relatively high throughput drug screening. Moreover, human embryonic stem cells (ESCs) were recently cultured to form engineered heart tissue that was successfully used to screen a panel of drugs with known pro-arrhythmic effects . Engineered heart tissue may also be used as a surrogate cardiac tissue to explore in vitro the integration into native heart tissue of various cell populations, such as murine cardiomyocytes, cardiac fibroblasts, and murine ESC-derived cardiac cells . These studies together suggest that engineered heart tissue can provide a valuable platform for screening cardiac pharmaceutical and cell therapeutics. In vitro experiments, such as those described above, are designed to elucidate cell- and tissuelevel responses to molecular and mechanical stimuli, and thus improve the understanding of complex in vivo phenomena and promote clinical translation of tissue-engineering technologies. In this context, the in vitro culture environment plays a key role by allowing for controlled and reproducible test conditions that limit the variability associated with the in vivo environment.(75)

7.5. Influence of selected in vitro culture parameters on the development and performance of engineered tissues

It is well-understood that traditional two-dimensional (2D) in vitro culture techniques cannot sufficiently recapitulate the microenvironment experienced by cells and tissues in vivo. Furthermore, physical forces are critical for the development of tissues and organs during embryogenesis and post-natal growth and remodeling. Therefore, functional tissue engineering strategies aim to recreate a 3D microenvironment in vitro that mimics the in vivo milieu through the use of scaffold systems, bioreactors, growth factors, and mechanical conditioning.

7.6. Cartilage tissue engineering

With increasing in vitro culture duration, chondrocytes assemble a mechanically functional ECM (e.g., Buschmann et al.), and cardiomyocytes develop contractile responsiveness to electrical impulses (e.g., Radisic et al.). For example, over a 40 day period of in vitro culture, constructs based on bovine calf chondrocytes and PGA scaffolds contained increasing amounts of GAG and type II collagen, and decreasing amounts of the PGA scaffold. In this system, the relatively high rates of ECM synthesis and deposition by the calf chondrocytes approximately matched the relatively high degradation rate of the PGA scaffold, a finding that did not hold true when the same scaffold was studied with other cell types (e.g., bone-marrow-derived mesenchymal stem cells [MSCs]). The structural and functional properties of engineered cartilage constructs can be improved to some degree by further extending the culture duration. For example, 7 month long cultures carried out in rotating bioreactors operated on Earth yielded engineered cartilage constructs with very high GAG fractions (w8% of wet weight) and compressive moduli (w0.9 MPa) that were comparable to normal articular cartilage, although the collagen fraction and dynamic stiffness of the 7 month constructs remained sub-normal .

Optimal maturation of tissue-engineered cartilage in vitro may improve cartilage repair with respect to ECM quality and integration after implantation in vivo. A recent study correlated engineered cartilage maturity with in vivo repair. Engineered cartilage was prepared by culturing chondrocytes on ECM scaffolds for 2 days, 2 weeks, or 4 weeks. Constructs were then implanted into full thickness cartilage defects in rabbit knee joints. In this study, the use of more mature engineered cartilage improved osteochondral defect repair; however, another study showed that prolonged in vitro culture of engineered constructs prior to in vivo implantation may lead to ECMdegradation, resulting in sub-optimal performance in vivo . Collectively, these findings suggest that while in vitro tissue maturation may enhance the in vivo performance of the engineered tissue, the optimal tissue maturation conditions are likely to be cell type-, scaffold-, culture condition-, and/or animal model-dependent.

7.7. Cardiac tissue engineering

Engineered cardiac tissue slowly forms contractile units over time in culture, beginning with spontaneous beating of single cells or groups of cells. As neonatal rat heart cells mature, elongate, and form networks, coordinated, coherent contractions develop throughout the construct. For example, engineered cardiac tissue cultured for up to 8 days exhibited a temporal increase in contractile amplitude. In another study, engineered heart tissue exhibited coherent contraction at 7e9 days in culture, increased in force of contraction between 9 and 15 days in culture, and then stabilized. Cells within these constructs showed an elongated phenotype consistent with more mature cardiomyocytes. For cardiac tissue engineering, however, increasing culture duration alone is not sufficient to produce functional tissue for implantation. At present the maximal contractile force generation reported for an engineered cardiac construct (w4 mN/mm2) remains more than an order of magnitude below that of normal heart muscle.

7.8. Cartilage Tissue-Engineering Scaffolds

Novel 3D scaffold designs have attempted to mimic aspects of native ECM using composite scaffold structures. As biomimetic physical and mechanical properties are difficult to achieve with a single, homogeneous material, several approaches for tissue engineering have employed composite scaffold systems, which are often designed with fiber reinforcement and layered structures.

Fiber-reinforced constructs for cartilage repair This type of design typically utilizes a fibrous phase comprised of a non-woven, knitted, or 3D woven fabric within a cell-supporting phase comprised of a hydrogel or sponge-like material. The cell-supporting phase generally provides a favorable environment for proliferation, differentiation, and ECM synthesis, while the fiber phase functions as a mechanical reinforcement and stabilizer for the construct. For example, hydrogels known to support chondrogenesis (e.g., agarose, alginate, and fibrin) have been combined with degradable non-woven or woven 3D scaffolds to engineer functional cartilage. To test

the influence of a hydrogel cell-carrier system for chondrogenesis, non-woven poly (lactic-co-glycolic acid) (PLGA) meshes were seeded with either dissociated bovine chondrocytes or with chondrocytes suspended in alginateand then implanted subcutaneously in nude mice. The alginate cell-carrier increased seeding efficiency by assisting in the retention and uniform distribution of cells throughout the pores of the non-woven mesh. The fiber-reinforced hydrogel also yielded a physically robust construct that maintained its initial geometry over time, without a negative effect on ECM synthesis. Similar studies showed success when non-woven PGA was either combined with a chondrocyte-laden fibrin gel or with MSCs in a type I collagen and alginate gel. Woven 3D fabrics embedded with hydrogels have also been utilized and replicate the complex biomechanical behavior of native articular cartilage. A microscale 3D weaving technique was employed to fabricate multiple layers of continuous fibers in three orthogonal directions. Composite scaffolds comprised of 3D woven fiber bundles of PGAor poly(e-caprolactone) (PCL)were used in combination with fibrin gel to mimic the physical properties of native articular cartilage, specifically, its inhomogeneous, anisotropic, non-linear, and viscoelastic mechanical properties. Construct compressive mechanical properties and equilibrium coefficient of friction were found to be similar to those of native articular cartilage throughout the defined culture period. Further, constructs seeded with human adipose-derived stem cells (ASCs) supported the elaboration of ECM which stained positive for the presence of chondroitin 4-sulfate and type II collagen (Fig. 13.4),.

In other studies, the infiltration of this 3D woven PCL scaffold with a slurry of cartilage-derived ECM enhanced the chondrogenesis of ASCs, while providing a mechanically functional construct that resisted cell-mediated contraction. A unique advantage of this composite structure is that scaffolds can be designed and fabricated with predetermined control of site-dependent variations in mechanical properties and porosity within a biocompatible matrix.

Fiber reinforcement can also improve the mechanical properties of sponge-like caffolds that otherwise have insufficient mechanical properties to support mechanical loading. For example, a scaffold comprising a web-like collagen micro-sponge and knitted PLGA fabric was fabricated for engineering cartilage tissue. The knitted fabric provided the mechanical integrity lacking in the collagen micro-sponge, and the collagen micro-sponge filled in the large pores of the fabric to facilitate uniform cell distribution and cartilage-like tissue formation. Similarly, reinforced PLGA foam scaffolds were produced by embedding short PGA fibers into the bulk polymer prior to foaming. The mechanical properties of these scaffolds could be tailored for potential use in articular cartilage repair by adjusting the material composition. Collectively, this work demonstrates that fiber reinforcement is a controllable design variable that can be manipulated in order to engineer scaffolds to suit the loadbearing requirements of an engineered tissue.

Stratified constructs for cartilage repair In an attempt to recapitulate the structural, compositional and mechanical inhomogeneity of articular cartilage, which varies with depth from the tissue surface, several groups have designed stratified constructs (reviewed in Klein et al.). In one study, a two-layered stratified structure based on agarose was developed in an effort to mimic the depth-dependent mechanical properties of articular cartilage. Constructs with a top layer composed of 2% agarose and a bottom layer composed of 3% agarose exhibited zonal differences in the compressive elastic modulus immediately after fabrication. Another study reported on the use of a photopolymerizing poly (ethylene glycol) diacrylate (PEGDA) hydrogel system that supported chondrocyte-mediated matrix synthesis which histologically resembled the depthdependent composition of native articular cartilage. Furthermore, in a recent study the layered organization of PEG-based hydrogels comprised of chondroitin sulfate (CS), matrix metalloproteinase-sensitive peptides (MMP-pep), and/or hyaluronic acid (HA), promoted the differentiation of bone-marrow derived MSCs into chondrogenic phenotypes that resembled those found in the zones of

articular cartilage. The three-layered, PEG-based construct incorporated CS and MMP-pep into the top layer (superficial zone), CS into the middle layer (transitional zone) and HA into the bottom layer (deep zone), thus resulting in a construct with spatially-varying mechanical and biochemical properties. Overall, these stratified structures resulted in depth-dependent variations in construct properties, thereby mimicking some of the inhomogeneous aspects of native articular cartilage.

- ***Osteochondral constructs***

Since bone-to-bone interfaces are known to integrate more effectively than cartilage-to-bone interfaces, osteochondral constructs are considered a promising technique for repairing full thickness articular cartilage defects. Progress in the development of osteochondral constructs using multi-material strategies has been reviewed by several groups. In one study, an anatomically-shaped osteochondral construct was developed by casting a layer of chondrocyte- seeded agarose gel on top of devitalized trabecular bone. Other approaches have included: engineered cartilage grown on non-woven PGA sutured to a subchondral support made of Collagraft_ , a hyaluronan benzyl ester sponge for cartilage regeneration attached with fibrin glue to a calcium phosphate ceramic sponge for bone regeneration , differentially applied chondrogenic and osteogenic growth factors on MSCs seeded in stacked silk scaffolds, a 3D printed scaffold of mixed PLGA/PLA for the upper cartilage region with a PLGA/tricalcium phosphate mix for the lower bone region , a collagen gel for chondrogenesis cultured upon a bone-inducing hydroxyapatite base , a cartilaginous tissue layer overlying a porous biodegradable calcium polyphosphate substrateand chondrocyteladen agarose hydrogels layered on constructs composed of mineral-containing microspheres or directly containing a mineral phase .

7.9. Cardiac tissue-engineering scaffolds

Scaffolds designed for cardiac tissue engineering must meet very different requirements to those developed for cartilage tissue engineering. Cardiac tissue-engineering scaffolds should provide the necessary microvascular and mechanical properties to meet the demands of a continuously contracting tissue, considering the cellular, geometric, mass transport, and oxygen supply concerns of native cardiac tissue. Scaffold approaches for cardiac tissue engineering have been reviewed by several groupsand are discussed briefly here. Many natural and synthetic materials have been examined for use in cardiac tissue engineering. As described previously, Matrigel_, combined with fibrin and/or thrombinor with collagen spongeshas been used to generate contractile engineered cardiac tissue in vitro. However, when considering clinical translation of these scaffolds, material limitations become apparent. Specifically considering the mechanical requirements of cardiac tissue, collagen gels and sponges may lack the mechanical strength required to withstand suturing and/or repeated cycles of stretch and relaxation. On the other hand, synthetic polymers popular in other tissue-engineering applications (e.g., PCL, PLGA) may be too stiff to form engineered cardiac tissue that possesses appropriate contractile properties. Therefore, development of other scaffolds that more closely mimic the native structural, mechanical, and transport properties of cardiac tissue is an area of active research.

In pioneering work, Ott et al. developed a biomimetic scaffold for cardiac tissue engineering through de-cellularization of adult rat hearts. These de-cellularized hearts were repopulated by intramural injection and coronary artery perfusion with neonatal heart cells and aortic endothelial cells and cultured with pulsatile flow and electrical stimulation to regenerate the nascent pump function of the heart. Although this system for cardiac tissue engineering has proven successful in many respects, the efficiency of re-cellularization remains low and the mechanical stiffness of the de-cellularized and re-cellularized tissues remains considerably higher than that of native myocardium.

In order to design a scaffold to address some of the specific structural and mechanical requirements of cardiac tissue, an elastomeric scaffold with accordion-like honeycomb pores was microfabricated from poly(glycerol sebacate) (PGS) . The accordion-like honeycomb pores were chosen as a first step toward mimicking the native structure of collagen within the myocardial ECM . These scaffolds, when combined with neonatal rat heart cells, yielded constructs with anisotropic mechanical properties that closely matched to the right ventricular myocardium, coordinated contractions in response to electrical stimulation, and allowed for some degree of elongation and alignment of the neonatal heart cells . In addition, PGS scaffolds designed based on finite element simulations provided a platform for cell delivery while simultaneously recapitulating the mechanical properties of the left ventricular myocardium . Many other scaffold designs have also utilized PGS as an elastomer for cardiac tissue engineering. One study used salt leaching to produce a porous scaffold, combined with laser cutting to produce microvascular channels for transport and oxygen diffusion , and another utilized semi-automated layer-by-layer assembly of planar polymer sheets with through-pores to create 3D structural patterns that directed the orientation of meso-scale cardiac muscle-like fibers . Scaffold modifications that sought to provide greater oxygen, nutrient, and waste transport (i.e., microvascular channels), or to condition the construct with mechanical stresses (i.e., cyclic stretch), can potentially increase the viability of the cells within the scaffold over time.

7.10. Bioreactors and growth factors

Bioreactors, which are capable of initiating, maintaining, and directing cell growth and tissue development in a well-defined and tightly controlled culture environment, have proven to be crucial tools for 3D tissue culture. Tissue culture bioreactors represent a controllable model system for:

1) Studying the effects of biophysical stimuli and therapeutic agents on cells and developing tissues;
2) Simulating responses of an in vitro-grown construct to in vivo implantation and thereby helping to define its potential for survival and functional integration; and
3) Developing and testing physical therapy regimens for patients who have received engineered tissue implants. As described in the studies highlighted below, bioreactors have been utilized to:
4) Improve cell infiltration and distribution by dynamically seed cells within 3D scaffolds;
5) Overcome the limitations associated with oxygen and nutrient transport that are often observed in tissues cultured in static environments; and
6) Enhance matrix synthesis and mechanical properties by biophysical stimulation of the developing constructs.

7.11. Cell seeding

Applying an appropriate cell type to a biomaterial scaffold is the first step in the tissue-engineering process, and the seeding technique may have a critical role in directing subsequent tissue formation. Scaffold cell seeding has traditionally been done statically and performed manually using pipettes, but these static seeding techniques often lead to inefficient and spatially non-uniform cell distribution within the scaffold, which may result in disparate ECM deposition throughout the construct . In order to overcomethe challenges associated with static seeding, bioreactor-based dynamic cell seeding techniques have been developed.Numerous types of bioreactors have been explored, including spinner flasks , wavy-walled reactors , and perfused vessels .

For some combinations of cells and scaffolds, dynamic cell seeding resulted in sxaffolds with high and spatially uniform initial cell densities, which increased ECM deposition and compressive modulus in the resulting engineered cartilage. For other

combinations, perfusion of a cell suspension directly through a scaffold enabled spatially uniform seeding and enhanced tissue regeneration. Engineered cartilage seeded in perfused bioreactors with alternating medium flow reportedly exhibited higher cell viability and uniformity than controls seeded statically and in spinner flasks. Likewise, engineered cardiac tissue seeded in perfused bioreactors with alternating medium flow exhibited higher cell viability and spatial uniformity than controls seeded in mixed petri dishes.

7.12. Construct cultivation

Cellular apoptosis and the formation of necrotic regions within 3D engineered constructs cultured under static conditions suggest that diffusion alone does not provide sufficient mass transport of oxygen, nutrients, and wastes for cell survival within a construct. Bioreactors help to mitigate these mass transfer limitations and provide a controlled microenvironment for 3D construct development. Several groups have demonstrated that mass transport limitations could be minimized, cell viability, differentiation, and function enhanced, and matrix synthesis improved within engineered constructs with the use of bioreactors that induce convective mixing (spinner flasksand rotating bioreactors), and perfusion .

Convective mixing, flow, and mass transport are required to supply the oxygen, nutrients, and regulatory factors that are in turn required for the in vitro cultivation of large tissue constructs. Oxygen is the factor that generally limits cell survival and tissue growth, due to its relatively low solubility, slow diffusion rate and high consumption rate. Different tissue types have different oxygen requirements, depending on cell type(s), concentrations and metabolic activities. For example, articular cartilage, an avascular tissue, has a lower requirement for oxygen than myocardium, a highly vascularized tissue. Experimental and modeling studies have correlated oxygen gradients within engineered tissues with morphology and composition.

7.13. Cartilage tissue-engineering bioreactors

A variety of bioreactors have been used to engineer cartilage constructs. Rotating bioreactors supported the growth of engineered cartilage constructs 5 to 8 mm thick based on bovine calf chondrocytes and PGS scaffolds. More recently, human chondrocytes were expanded in 2D and then cultured on hyaluronan benzyl ester scaffolds in rotating bioreactors for up to 4 weeks. While constructs cultured statically and in bioreactors contained similar amounts of GAG and collagen, the bioreactor-grown constructs exhibited a bi-zonal structure, consisting of a collagenous surface capsule deficient in GAG and an inner region that stained more positively for GAG. As compared to bovine calf chondrocytes, expanded human chondrocytes deposited relatively lower amounts of matrix. In another study, a wavy-walled bioreactor was used to culture bovine calf chondrocytes on PGA scaffolds, and increased construct growth, defined by weight, cell proliferation, and ECM deposition, was observed in bioreactors as compared to spinner flasks.

In an effort to explore the influences of bioreactors and exogenous growth factors, an oscillatory perfused bioreactor providing slow, bi-directional perfusion was used to study cartilage constructs made by culturing adult human MSCs on 3D woven PCL scaffolds for 3 weeks. Constructs cultured in bioreactors had higher aggregate moduli, higher total collagen contents, and similar GAG contents compared to constructs cultured statically. Constructs cultured statically in medium containing chondrogenic growth factors but not serum exhibited better chondrogenesis and more homogeneously positive matrix staining for GAG and collagen type II than otherwise identical medium containing serum. Constructs cultured in medium without chondrogenic growth factors and with serum did not exhibit chondrogenic differentiation. Together, these studies show that bioreactors and growth factors can influence cell morphology, proliferation, and ECM deposition in engineered cartilage.

7.14. Cardiac tissue-engineering bioreactors

In the case of engineered cardiac tissue, convective mixing in rotating bioreactors and spinner flasks supported the growth of a tissue-like surface layer w100 to 200 mm thick.

Moreover, perfusion of culture medium directly through an engineered cardiac construct can significantly improve construct thickness and spatial homogeneity. Specific design of perfusion bioreactors for cardiac tissue engineering is described in, and bioreactors designed specifically for electrical stimulation of cardiac constructs are described in. In one example, perfused bioreactors enhanced the survival of heart cells cultured on porous collagen sponges by increasing the transport of oxygen and insulin-like growth factor-I .

Neonatal rat heart cells were seeded on scaffolds at high density by hydrogel-entrapment, and then slow, bi-directional perfusion culture was carried out in an oscillatory perfused bioreactor for 8 days. Bioreactor-grown constructs grown exhibited improvements over static controls with respect to several benchmarks, including reduced apoptosis, increased contractile amplitude, and increased expression of the contractile protein cardiac troponin-I . In the static control group, heart cells remained rounded and did not exhibit cross-striations, whereas in the bioreactor group, some cells were elongated and striated, albeit to a lesser degree than native adult rat ventricular myocardium. Moreover, perfusion of medium supplemented with IGF-I yielded further improvements in construct properties, presumably due to enhanced transport of growth factor to the heart cells within the 3D construct. In another study done in the oscillating perfused bioreactor, heart cells cultured on twolayered, 500 mm thick PGS scaffolds with fully interconnected accordion-like honeycomb pores exhibited increases in the gap junctional protein connexin-43 and MMP-2, an enzyme associated with tissue remodeling . In other studies, sustained release and delivery of IGF or sequential delivery of IGF-I and hepatocyte growth factor (HGF)enhanced survival and maturation of heart cells in a 3D model and protected against oxidative stress.

Together, these studies suggest that bioreactors and growth factors may work in tandem to enhance heart cell viability, contractility and differentiation in 3D myocardial grafts.(75)

Bioreactors and mechanical forces

Bioreactors can be utilized to apply biomechanical signals (shear, compression, tension, pressure, or a combination thereof) to growing tissues. Chondrocytes are quite responsive to mechanical signals and remodel the matrix according to the loads applied; thus the choice of loading regime can directly influence the development of the structure, composition, and mechanical properties of cartilaginous constructs (reviewed in Grad et al.). Likewise, skeletal, smooth, and cardiacmuscle cells are quite responsive to mechanical signals.

7.15. Effects of hydrodynamic forces

Hydrodynamic forces associated with convective mixing can have a significant effect on the composition, structure, and properties of engineered tissues. For example, engineered cartilage cultured in rotating bioreactors had thinner surface capsules and higher fractional amounts of GAG than constructs grown in spinner flasks . The flow field in the spinner flask was unsteady, turbulent (Reynolds number of 1758), and characterized by large spatial variations in the velocity field and maximum shear stresses . In contrast, the flow field in the rotating bioreactor (slow turning lateral vessel, STLV) was predominately laminar with shear stresses ofw1 dyn/cm2and a well-mixed interior due to secondary flow patterns induced by the freely-settling constructs . A model of tissue growth in the rotating bioreactor that accounted for the intensity of convection over 6 weeks of in vitro culture was used to predict the morphological evolution of an engineered cartilage construct . In particular, the model predicted that high shear and mass transfer at the lower corners of a settling, discoid construct would preferentially induce tissue growth in these regions, and that temporal changes in construct size and shape would further enhance local variations in the flow field in a manner that accentuated localized tissue growth. The computed velocity

fields and shear stress data corresponded well with the morphological evolution of engineered cartilage, as shown by superimposing a calculated flow field on a histological cross-section of an actual construct. The above examples suggest that combining experimental studies and computational modeling of hydrodynamic shear stresses and concentration gradients in bioreactors may help to explain underlying mechanisms that control the growth of engineered tissue constructs.

7.16. Effects of mechanical tension, compression, and shear loading

It is well known that mechanical forces are critical for determining the architecture of native tissues such as bone , and there is growing evidence that mechanical factors are important factors in determining stem cell fate . In particular, the role of in vitro mechanical stress in maintaining and promoting the chondrogenic phenotype has been the topic of several investigations, but the specific influences of different physical stimuli and their interactions with the biochemical environment are not fully understood. For example, dynamic compression caused a w2-fold increase in cartilage nodule density and a 2.5-fold increase in GAG synthesis in stage 23/24 chick limb-bud cells cultured in agarose gel . Likewise, cyclic hydrostatic pressure significantly increased the amounts of proteoglycan and collagen in aggregates of human bone-marrow-derived MSCs . In another study, compression enhanced chondrogenic differentiation in mouse embryonic E10 stage cells embedded in collagen type I compared to unloaded controls, as shown by upregulation of SOX-9 and downregulation of IL-1b expression .

A variety of devices have been custom designed and built to study the effects of mechanical conditioning (i.e., compression, tension, pressure or shear) on cells and tissues in vitro (reviewed in Brown et al. , Darling et al. , andWaldman et al.). For engineering cartilage, devices typically apply dynamic compression (e.g., Buschman et al. , Mauck et al.), hydrostatic pressure (e.g., Mizuno et al. , Toyoda et al.) or mechanical shear (e.g., Waldman et al.). For engineering skeletal, smooth, and cardiac muscle tissues, devices typically apply dynamic tensile strainor pulsatile hydrostatic pressure.

7.17. Mechanical effects on engineered cartilage tissue

In the case of engineered cartilage, a number of studies have shown that mechanical conditioning can enhance chondrogenesis. Dynamic loading has been shown to increase AG accumulation and ECM assembly, and therefore, the mechanical properties of constructs based on bovine calf articular chondrocytes and a variety of 3D scaffolds including agarose gel , PGA non-woven mesh , and self-assembling peptide gel . Similar results were also observed for adult canine chondrocytes in an agrose gel under dynamic loading conditions . Application of dynamic loading was also investigated in a layered agarose construct, with encapsulated bovine articular chondrocytes, with varying mechanical properties (2% agarose vs. 3% agarose). These results indicated preferential matrix formation in the 2% agarose layer and an increased elastic modulus in only the initially softer, more permeable layer (2% agarose). Although the aforementioned studies focused on cells encapsulated in hydrogels, similar results (i.e., increased ECM production and compressive modulus of construct) were found when dynamic loading was applied to calf chondrocytes cultured within a porous calcium polyphosphate scaffold. Overall, the response of chondrocytes to dynamic loading depended on the amount and composition of ECM in the developing construct , and in some studies loading increased both synthesis of new GAG and its loss into the culture media.

The influence of dynamic loading on engineering cartilage has also been investigated n MSCladen hydrogel systems. In a hyaluronic acid-based hydrogel seeded with human MSCs, dynamic compressive loading enhanced cartilage-specific matrix synthesis and more uniform distribution, increased construct mechanical properties, and suppressed the expression of hypertrophic markers . In another study , mechanical loading of MSC-laden agarose constructs prior to chondrogenesis decreased functional maturation and ncreased chondrogenic gene expression. In contrast, loading initiated after chondrogenesis and atrix elaboration further improved the mechanical properties of engineered constructs, but only when TGF-b3 levels were

maintained and under specific loading parameters. Overall, these results demonstrated that the combination of dynamic compressive loading initiated after chondrogenesis and sustained TGF-b exposure may enhance the mechanical properties and matrix distribution of engineered cartilage constructs. The effects of mechanical stimuli on engineered cartilage may vary among different scaffold systems. In one study, fibrin hydrogels seeded with chondrocytes were cultured under unconfined compression (static and oscillatory). Compared to the free-swelling control condition, static loading had minimal influence on matrix synthesis or construct stiffness. When comparing the constructs exposed to static versus oscillatory loading, the constructs cultured under dynamic conditions were found to be softer with less matrix accumulation. Although dynamic compressive loading often results in favorable outcomes in terms of engineering functional cartilage tissue, the scaffold material in which the cells are cultured may influence the effects of mechanical conditioning. In the aforementioned studies, the cell-laden constructs were loaded in dynamic compression; however, the influence of shear, hydrostatic pressure, and tensile forces has also been explored for cartilage regeneration. For example, the application of shear stress yielded constructs with higher amounts of ECM and higher compressive moduli than those exposed to compressive stress . Likewise, application of dynamic hydrostatic pressure affected chondrogenesis in 3D cultures of bovine and humanchondrocytes. In another study, the influence of intermittent loading induced with hydrostatic pressure was investigated during cartilage formation. The application of hydrostatic pressure increased the GAG content of the engineered cartilage construct. Finally, the influence of oscillatory tensile loading on chondrocytes derived from distinct tissue zones of articular cartilage (superficial, middle, and deep) was investigated. Tensile loading stimulated proteoglycan synthesis only in superficial zone chondrocyte populations. The results of these studies collectively suggest that loading conditions other than compression may enhance the properties of engineered cartilage constructs, and a combination of loading regimens may be necessary to engineer the different zones of articular cartilage (reviewed in Klein et al.).

7.18. Mechanical effects on engineered muscle tissue

In the case of engineered muscle, dynamic tensile and pulsatile loading can affect construct composition, contractility, and pharmacological responsiveness. Cyclic stretch affects not only the structure (e.g., orientation of cells and collagen)but also the mechanical function (i.e., contractility) of engineered cardiac tissue. Recently, scaffolds have been specifically designed for mechanical stimulation in cardiac tissue engineering. In this study, chitosan-collagen scaffolds with an array of parallel channels were seeded with rat neonatal heart cells and subjected to dynamic tensile stretch for 6 days using a custom designed bioreactor. Mechanical conditioning promoted cardiomyocyte alignment and elongation, and increased cell-to-cell connections as evidenced by increased connexin-43 expression, although these results were dependent on high local stress conditions and were not achieved in areas of the scaffold with lower stress.

Instead of utilizing cyclic stretch to provide mechanical stimulation, some studies utilize pulsatile hydrostatic pressure. One such example used a bioreactor designed to provide physiologically relevant shear stresses and flow rates via pulsatile perfusion . Culture under these pulsatile perfusion conditions enhanced the contractility of the constructs by increasing the contractile amplitude and lowering the excitation threshold.

Together, these mechanical conditioning studies demonstrate that specific loading protocols may be exploited to stimulate specific responses in engineered tissue constructs, and emphasize the potential utility of bioreactors which provide mechanical conditioning in the form of stretch or pulsatile flow in studying and promoting in vitro construct formation.

Conclusions

As the field of functional tissue engineering progresses, the development of engineered tissue replacements may require additional exogenous influences to

achieve many of the important requirements for long-term success. Scaffolds can provide structural, biochemical and mechanical cues, and in combination with bioreactors, growth factors, and mechanical conditioning, may enhance in vitro generation of functional tissue-engineered constructs. In vitro models can be used to assess construct physiologic and pharmacologic responses, including responses to environments mimicking those into which the constructs will eventually be implanted in vivo. Nonetheless, it is important to note that other rapidly evolving technologies also may have a significant impact on tissue engineering, and it is important to consider in vitro culture parameters in light of the role of novel molecular and gene therapies, and other changing technologies.

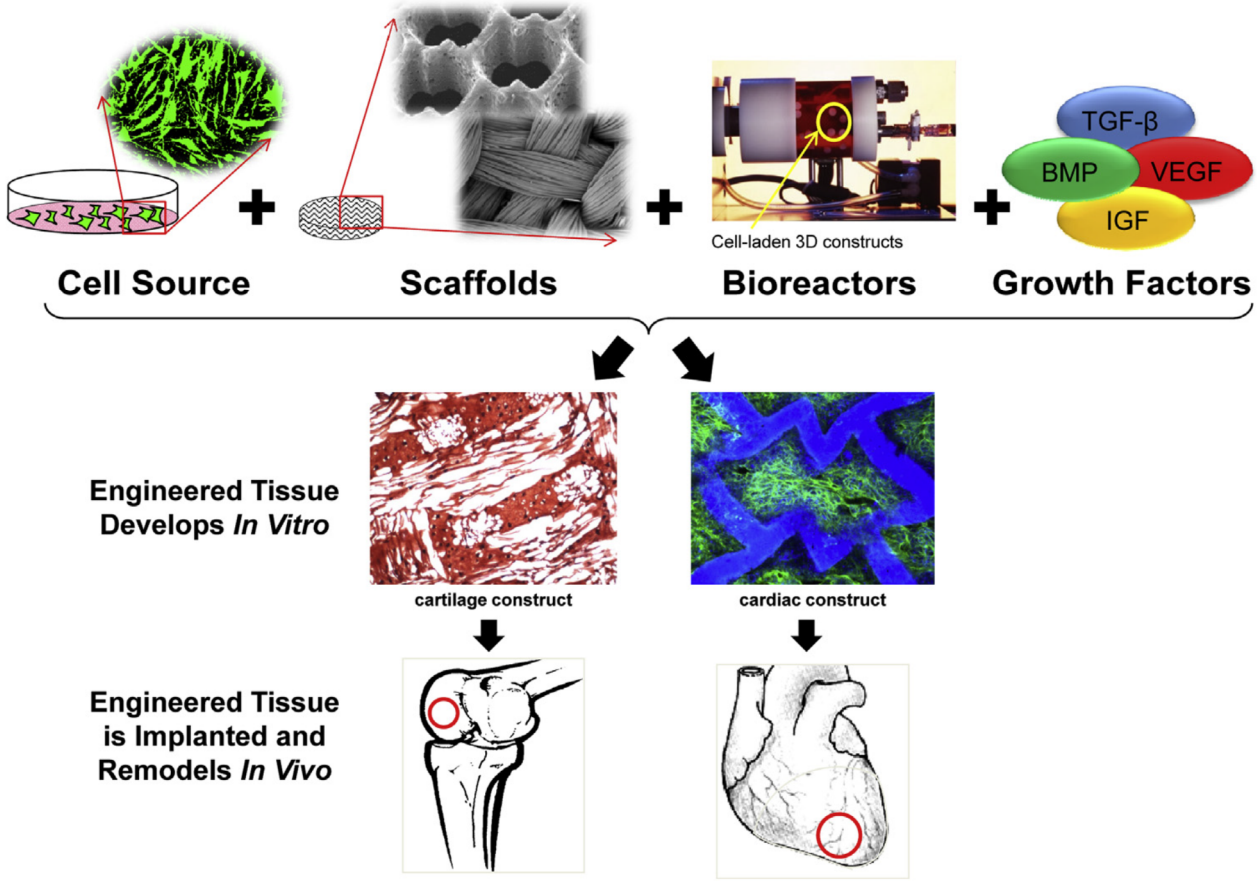

Figure 22: Strategy for engineering functional tissues in vitro. (75)

Cells, scaffolds, bioreactors, and growth factors are used as tools to create functional engineered tissues.

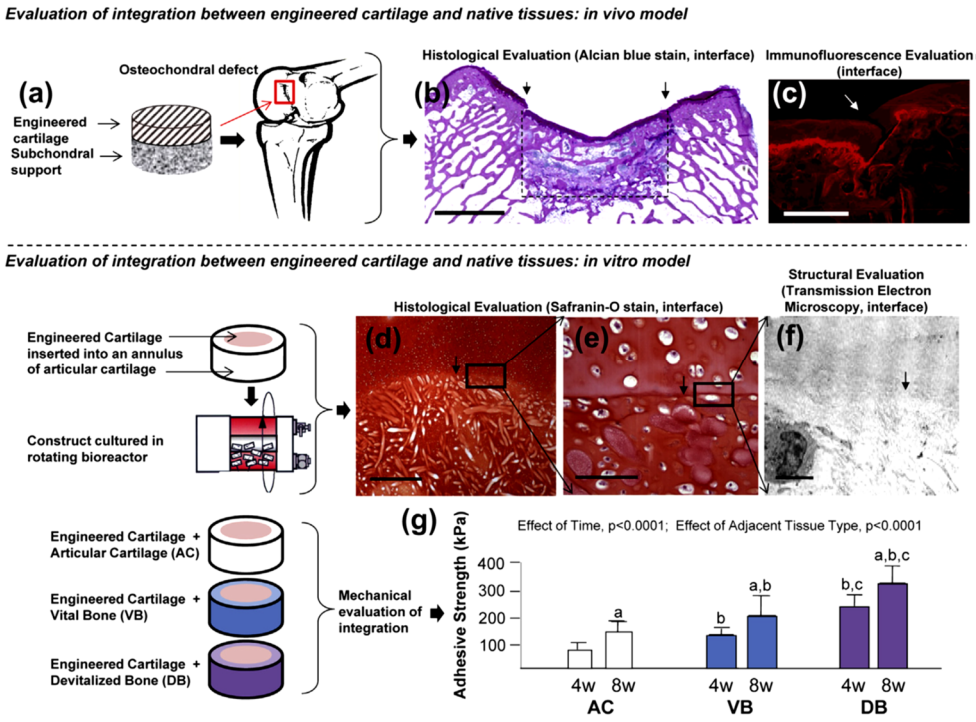

Figure 23: Studies of the clinically relevant problem of engineered cartilage integration. (75)

a) Osteochondral implant integration was studied using adult rabbits with surgically created osteochondral defects. b, c) Histological appearances of explants harvested after 6 months. b) Alcian blue stain, scale bar: 2.5 mm, dashed line shows borders of original defect; c) Immunofluorescence stain, scale bar: 400 mm. Arrows indicate areas of incomplete integration between engineered and host cartilages. Bottom Panel: Engineered cartilage integration was studied using rotating bioreactors to culture engineered cartilage construct disks within rings of articular cartilage (AC), vital bone (VB), and devitalized bone (DB). d, e) Histology of the construct-AC interface (Safranin-O stain, scale bars: 500 mm and 50 mm for D and E, respectively). f) Transmission electron micrograph of the construct-AC interface (scale bar: 5 mm). Arrows at interfaces point toward the construct and arrowheads indicate the scaffold. g) Adhesive strength for construct disks cultured in rings of AC (white), VB (blue) or DB (purple) for 4 or 8 weeks (4w, 8w). Data represented are mean _ SD. aSignificant difference due to time; bSignificantly different from the corresponding AC composite; cSignificantly different from the corresponding VB composite. Top panel: adapted from Schaefer et al., Arthritis & Rheumatism, 46(9):2524, 2002 [19]. Middle and Bottom panels: adapted from Tognana et al., Osteoarthritis Cartilage 13(2):129, 2005 [21].

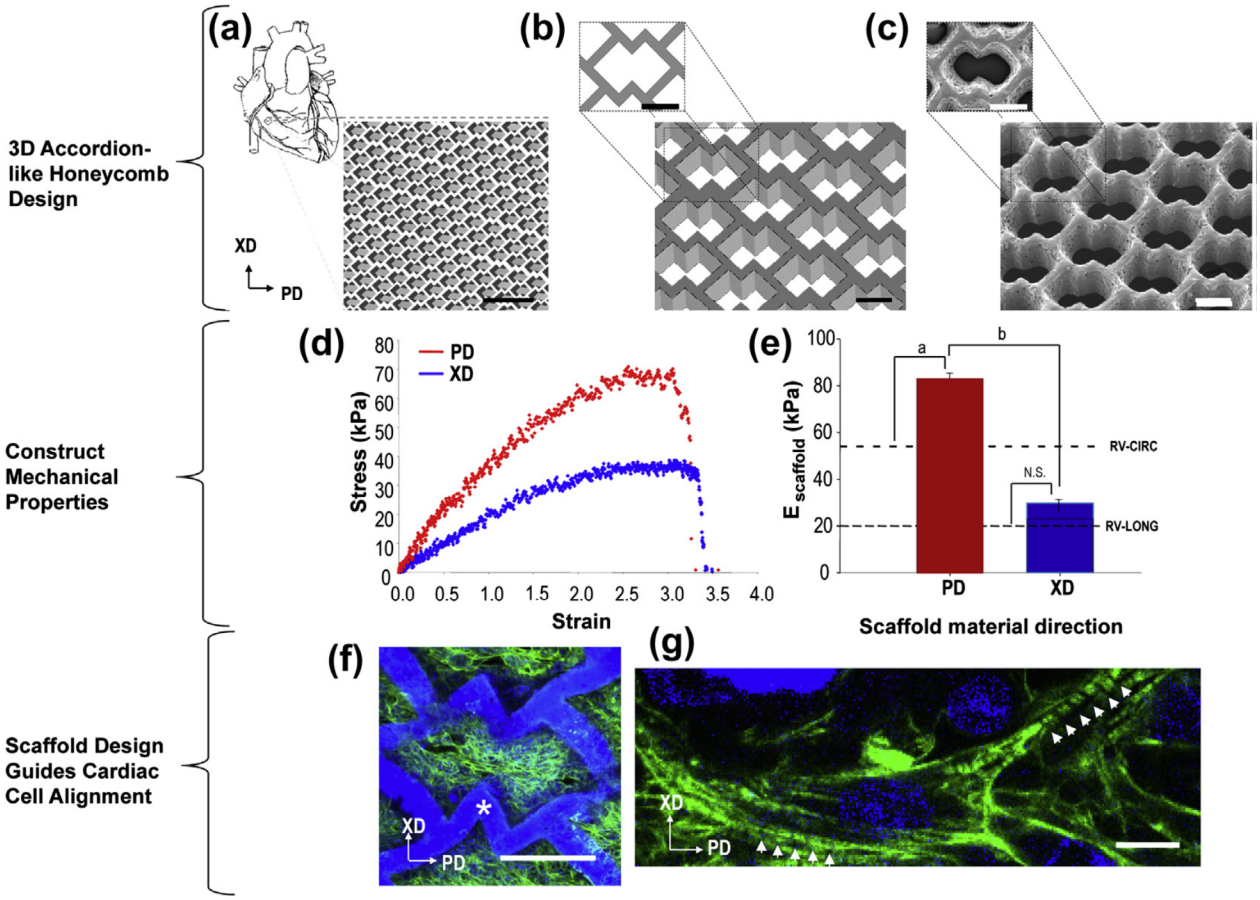

Figure 24: Scaffold design for engineering cardiac tissue. (75)

Top Panel: a, b) Schematics of accordion-like honeycomb scaffold design (a e scale bar: 1 mm, b e scale bar: 200 mm). c) Scanning electron micrograph of the scaffold architecture fabricated by laser microablation of a biodegradable elastomer, poly(glycerol sebacate) (PGS) (scale bar: 200 mm). Preferred direction (PD) and orthogonal cross-preferred direction (XD) material directions are indicated. Middle

Representative uniaxial stress strain plot of PGS scaffold with cultured neonatal rat heart cells (scaffolds were fabricated from PGS membranes cured for 7.5h at 160_C; cells were cultured for 1 week). e) Scaffold anisotropic effective stiffnesses (EPD and EXD) are compared to specimens of native adult rat right ventricular (RV) myocardium harvested in two orthogonal directions (RVCIRC and RVLONG, respectively). Data are represented as mean _ SD. aSignificant difference between scaffold and RV; bSignificant difference due to scaffold test direction; N.S. indicates not significant. Bottom Panel: f, g) Confocal micrographs of neonatal rat heart cells cultured on accordion-like honeycomb scaffolds for 1 week. (Stain: filamentous F-actin (green), counterstain: DAPI (blue), scale bars: 200 mm and 10 mm, for f and g, respectively). Scaffold indicated by white asterisks; cross-striations indicated by white arrows. Adapted from Engelmayr, Jr., G. C. et al., Nature Materials 7: 1003, 2008 [67].

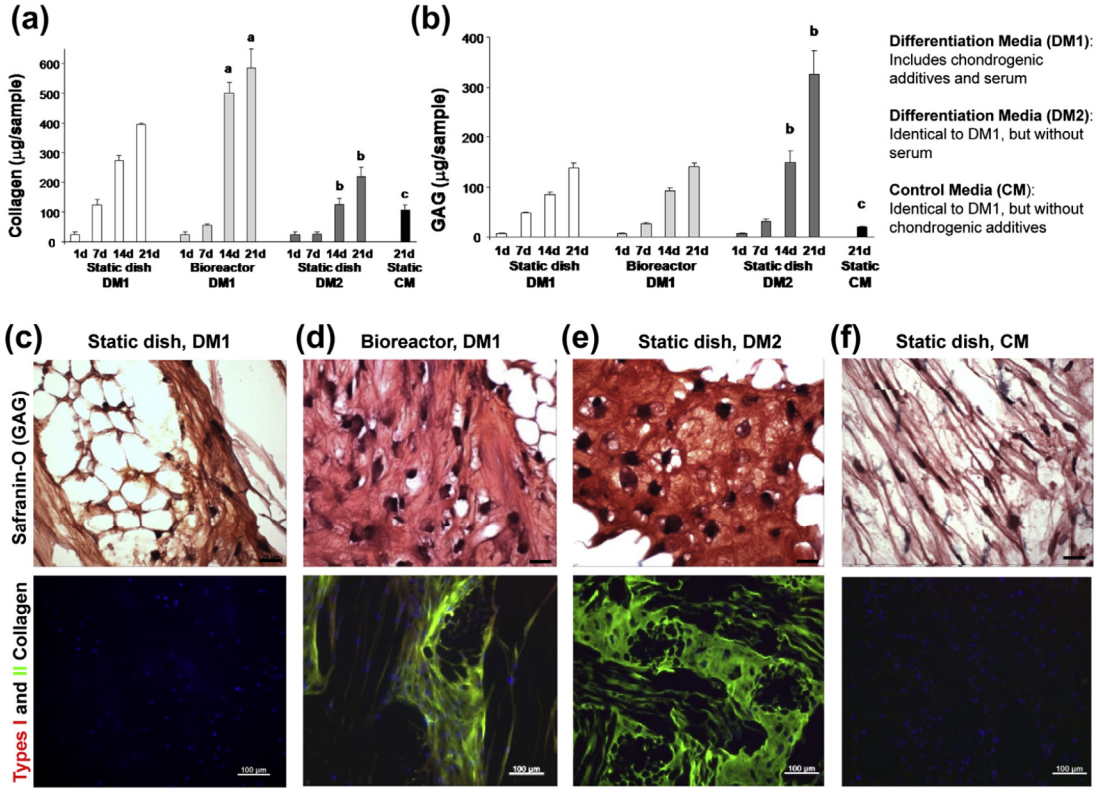

Figure 25: Bioreactors and growth factors influence the structure and composition of engineered cartilage. (75)

Human MSCs were cultured on 3D woven PCL scaffolds in static dishes or bioreactors and in three different culture media (differentiation medium 1 (DM1), differentiation medium 2 (DM2), and control medium (CM)) for up to 21 days. Top Panel: Time evolutions of construct amounts of a) total collagen and b) GAG. Data are represented as mean _ SEM. aSignificant difference due to type of culture vessel, bSignificant difference due to presence of serum, cSignificant difference due to chondrogenic additives, which included TGF-b-3, insulin-transferrin-selenium (ITS), dexamethasone, and ascorbic acid. Bottom Panel: Histological sections of 21-day constructs cultured c) statically in DM1, d) in bioreactors in DM1, e) statically in DM2, and f) statically in CM and stained with safranin-O for GAG (top, scale bars: 20 mm) or immunostained for collagen type II (green) and type I (red, not seen) with DAPI (blue) counterstain (bottom, scale bars:100 mm). Adapted from Valonen, P.K., et al., Biomaterials, 31(8): p. 2193, 2010 [93]. Mechanobiology, Tissue

8. Development and Organ Engineering :

8.1. Introduction

Tissue engineering has as its main goal the fabrication of artificial tissues for use as replacements for damaged organs and complex body structures. Great advances have been made in terms of developing prosthetic devices that can repair structural defects (e.g., vascular grafts) and even replace complex mechanical behaviors (e.g., artificial joints). However, the challenge for the future is to develop tissue substitutes that restore the normal biochemical functions of living tissues and organs, in addition to their structural features. To accomplish this feat, we must first establish precise design criteria for tissue engineering and develop new fabrication approaches to create complex organomimetics that reconstitute the tissue-tissue interfaces and physical microenvironments of living organs. These design features should be based on a thorough understanding of the molecular, cellular and biophysical basis of tissue regulation. In particular, they must take into account the important role that insoluble extracellular matrix (ECM) scaffolds and mechanical stresses play in control of cell phenotypes or ’fates’ (e.g., growth, differentiation, motility, apoptosis, different stem cell lineages) during tissue formation, maintenance, and repair. This latter point is

critical, since the spatial organization of cells and the mechanical constraints imposed on them as they grow actively regulate tissue development, as well as tissue and organ function throughout adult life. Our analysis of this regulatory mechanism should be of particular interest to the tissue engineer, because it has led to the identification of critical chemical and mechanical features of ECMthat are responsible for control of cell fate switching in developing tissues. In addition, the reader will be introduced to some unanswered puzzles in developmental biology which, if deciphered, could provide powerful new approaches to tissue regeneration and repair. Finally, recent advances will be described in terms of microengineering human 'organs-on-chips' that recapitulate complex structures and functions of living human organs, which have opened new avenues of investigation for drug screening as well as modeling complex disease processes in vitro.

8.2. Extracellular matrix structure and function

8.2.1. Composition and organization

One of the most critical elements of tissue engineering is the ability to mimic the ECM scaffolds that normally serve to organize cells into tissues. ECMs are composed of different collagen types, large glycoproteins (e.g., fibronectin, laminin, entactin, osteopontin), and proteoglycans that contain large glycosaminoglycan side chains (e.g, heparan sulfate, chondroitin sulfate, dermatan suflate, keratan sulfate, hyaluronic acid. While all ECMs share these components, the organization, form, and mechanical properties of ECMs can vary widely in different tissues depending on the chemical composition and three-dimensional (3D) organization of the specific ECM components that are present. For example, interstitial collagens (e.g., types I, III) self assemble into 3D lattices which, in turn, bind fibronectin and proteoglycans. This type of native ECM hydrogel forms the backbone of loose connective tissues, such as dermis. In contrast, basement membrane collagens (types IV, V) assemble into planar arrays; when these collagenous sheets interact with fibronectin, laminin, and heparan sulfate proteoglycan, a planar ECM results (i.e., the 'basement membrane'). The

ability of tendons to resist tension and of cartilage and bone to resist compression, similarly result from local differences in the organization and composition of the ECM.

In vivo foundation for cell anchorage The first and foremost function of ECM in tissue development is its role as a physiological substratum for cell attachment. This feature is easily visualized by treating whole tissueswith ECM-degrading enzymes (collagenase, proteases); cell detachment and loss of cell and tissue morphology rapidly result. Cells that are dissociated in this manner can reattach to an artificial culture substrate (e.g., plastic, glass). However, adhesion is again mediated by cell binding to ECM components that are either experimentally immobilized on the culture surface, deposited de novo by the adhering cells, or spontaneously adsorbed from serum (e.g., fibronectin, vitronectin). In fact, standard tissue culture plates are actually bacteriological (non-adhesive) plastic dishes that have been chemically treated using proprietary methods to enhance adsorption of serum- and cell-derived ECM proteins. To summarize, cells do not attach directly to the culture substrate (i.e., plastic or glass), rather they bind to intervening ECM components that are adsorbed (or derivatized) to these substrates. For this reason, cell adhesion can be prevented by coating normally adhesive culture surfaces with polymers that prevent protein adsorption, such as poly (hydroxyethylmethacrylate).

Spatial organizer of polarized epithelium Living cells exhibit polarized form as well as function (e.g., basal nuclei, supranuclear Golgi complex, apical secretory granules in secretory epithelia). Dissociated cells fail to orient in a consistent manner when cultured on standard tissue culture substrata or on interstitial connective tissue. In the case of epithelial cells, the normal polarized form is often restored if the cells synthesize and accumulate their own ECM, or if they are cultured on exogenous basement membrane (i.e., the specialized epithelial ECM) . These types of studies suggest that basement membrane normally serves to integrate and maintain individual cells within a polarized epithelium. Clearly, there are many intracellular and

intercellular determinants of polarized cell form and function (e.g., cytoskeletal organization, organelle movement, junctional complex formation). However, anchorage to ECM appears to provide an initial point of orientation and stability on which additional steps in the epithelial organization cascade can build. ECM may regulate the orientation of other cell types (e.g., chondrocytes, osteoclasts) as well.

Scaffolding for orderly tissue renewal All tissues are dynamic structures that exhibit continual turnover of all molecular and cellular components. Thus, it is the maintenance of tissue pattern integrity that is most critical to the survival of the organism, and regeneration of normal tissue architecture is one of the main goals of tissue engineering. Maintenance of specialized tissue form requires that cells that are lost due to injury or aging must be replaced in an organized fashion. Importantly, orderly tissue renewal depends on the continued presence of insoluble ECM scaffoldings which act as templates that maintain the original architectural form and assure for accurate regeneration of pre-existing structuresFor example, when cells within a tissue are killed by freezing or treatment with toxic chemicals, all of the cellular components die and are removed; however, the basement membranes often remain intact. These residual ECM scaffoldings assure the correct repositioning of cells (e.g., cell polarity) and restore different cell types to their correct locations (e.g., muscle cells within muscle basement membrane, nerve cells in nerve sheaths, endothelium within vessels, etc.), in addition to promoting the cell migration and growth which are required for the repair of all the component tissues. Conversely, loss of ECM integrity during wound healing results in disorganization of tissue pattern, and thus, scar formation. Uncoupling between basement membrane extension and cell doubling also leads to disorganization of tissue morphology during neoplastic transformation.

8.2.2. Establishment of tissue microenvironments

ECMs often establish a physical boundary between neighboring tissues. For example, the basement membrane normally restricts mixing between the epithelium

and underlying connective tissue, and compromise of basement membrane integrity is indicative of the onset of malignant invasion when seen in the context of tumor formation. The ECM boundary also may regulate macromolecular transport between adjacent tissues given that the basement membrane forms the semi-permeable filtration barrier in the kidney glomerulus. However, little is known about this potential function of the ECM in other local tissue microenvironments.

Sequestration, storage, and presentation of soluble regulatory molecules ECMs may modulate tissue growth and morphogenesis through their ability to bind, store, and eventually release soluble regulators of morphogenesis. For example, the soluble mitogen, basic fibroblast growth factor (FGF) exists in an immobilized form in ECMs deposited by cells cultured in vitro , as well as in normal tissues (e.g. corneal basement membranes) in vivo . In fact, many cytokines (e.g., FGF, VEGF, HGF, insulin-like growth factor, hematopoietic growth factors, etc.) are normally stored bound to heparin, heparan sulfate or other specific binding proteins within natural ECMs . The low growth rate observed in most normal tissues may result from sequestration of mitogens by ECM, whereas release of these stored factors ('stormones') due to injury or hormonally-induced changes in ECM turnover may help to switch growth on locally. Binding of other types of regulatory molecules to the endothelial basement membrane (e.g., plasminogen activator inhibitor) also may play a role in tissue physiology (e.g., blood coagulation, cell migration). Regulator of cell growth, differentiation, and apoptosis Most normal (non-transformed) cells only grow when attached and spread on a solid substrate.

Cells attach and spread in vitro either by depositing new ECM components or by binding to exogenous ECM. In fact, cell spreading and growth can be suppressed by inhibiting ECM deposition in vitro using drugs . Cell growth stimulated by soluble mitogens has also been shown to vary depending on the type of ECM component used for cell attachment (e.g., collagen versus fibronectin) as well as on the mechanical properties of the ECM (e.g., malleable gel versus rigid ECM-coated dish).

Interestingly, ECM substrates that promote growth tend to suppress differentiation and vice versa. For examples, many cells proliferate and lose differentiated features when cultured on attached type I collagen gels that can resist cell tension and promote cell spreading. In contrast, the same cells cease growing and increase expression of tissue-specific functions (e.g. albumin secretion in hepatocytes, milk secretion and acinus formation by mammary cells, capillary tube formation by endothelial cells) if cultured on the same gels that are made flexible by floating them free in medium or on attached ECM gels that exhibit high malleability (e.g., basement membrane gels, such as Matrigel). Under these conditions, the cells exert tension across their adhesions, resulting in contraction of the ECM gel and cell rounding which, in turn, shuts off growth and turns on differentiation-specific gene functions. The differentiationinducing effects of these malleable ECM substrates can also be suppressed by making the gels rigid through chemical fixation , thus confirming the critical role of cell-generated mechanical forces in this response.

While local changes in ECM turnover may promote tissue remodeling, large-scale breakdown of the ECM may force the same growing tissues to undergo involution. Many cultured cells rapidly lose viability and undergo programmed cell death (i.e., apoptosis) when detached from ECM and maintained in a round form . Loss of basement membrane integrity is also observed in regions of tissues that are actively regressing and growing tissues (e.g., capillaries, mammary gland) can be induced to involute using pharmacological agents (e.g., proline analogues) that inhibit ECM deposition and lead to basement membrane dissolution in vivo . Recent transgenic mice studies confirm that growing tissues can be made to involute by shifting the endogenous proteolytic balance such that total ECM breakdown results . These findings suggest that local changes in ECM composition and flexibility may regulate cell sensitivity to soluble mitogens, and thereby control cell growth, viability, and function in the local tissue microenvironment.

8.3. Pattern formation through ECM remodeling

Mesenchymal control of epithelial pattern Probably the greatest insight into the role of ECM in tissue development comes from analysis of embryogenesis. In the embryo, genesis of a tissue's characteristic form (e.g., tubular versus acinar) and deposition of ECM are both controlled by complex interactions between adjacent epithelial and mesenchymal cell societies. The epithelial cell is genetically programmed to express tissue-specific (differentiated) functions and to deposit the insoluble basement membrane which functions as a common attachment foundation that both separates adjacent tissues and stabilizes tissue form . However, while production of tissue-specific cell products (cytodifferentiation) is determined by the epithelium, tissue pattern (histodifferentiation) is usually directed by the surrounding mesenchyme. For example, when embryonic mammary epithelium is isolated and combined with salivary mesenchyme, the mammary epithelial cells take on the form of the salivary gland although they still produce milk proteins. However, the specificity of these epithelial-mesenchymal interactions can vary widely from organ to organ. For instance, embryonic salivary epithelia specifically require salivary mesenchyme for successful development, while pancreatic epithelia will undergo normal cytodifferentiation and histodifferentiation in response to mesenchyme isolated from a variety of embryonic tissues.

- ***Tissue patterning through localized ECM remodeling***

The complex tissue patterns that are generated through epithelial-mesenchymal interactions result from the establishment of local differentials in tissue growth and expansion in a microenvironment that is likely saturated with soluble mitogens. The classic work on salivary gland development by Bernfield and coworkers revealed that the epithelium imposes morphological stability through production of its basement membrane, whereas the mesenchyme produces local changes in tissue form, specifically by degrading basement membrane at selective sites. An increased rate of cell division is observed in the tips of growing lobules where the highest rate of ECM

breakdown and re-synthesis (i.e., highest turnover rate) is also observed. At the same time, the mesenchyme slows basement membrane turnover and suppresses epithelial cell growth in the clefts of the glands. This is accomplished by secretion of fibrillar collagens which slow ECM degradation locally, and thereby promote basement membrane accumulation in these regions. Similar local coupling between ECM turnover, cell growth rates and tissue expansion is observed in many other developing tissues, including growing capillary blood vessels.

It is important to note that increased ECM turnover involves enhanced rates of matrix synthesis as well as degradation. In fact, net basement membrane accumulation (i.e., increased area available for cell attachment) must result for epithelial tissues to grow and expand laterally, and thus the local rate of ECM synthesis must be greater than that of degradation in these high turnover regions. If the rate of ECM degradation is significantly greater than synthesis, then net basement membrane dissolution along with cell retraction and rounding results. As described above, this would lead to cell death and tissue regression rather than expansion. Role of mechanical stresses

Before ending discussion of the role of ECM in pattern formation, it is critical to emphasize that while chemical regulators mediate tissue morphogenesis, the signals that are actually responsible for dictating tissue pattern are often mechanical in nature. The patterngenerating effects of compression on bone, shear on blood vessels, and tension on muscle are just a few examples. Mechanical stresses are also important for embryological development; however, internal cell-generated forces appear to play a more critical role . For example, mechanical tension that is generated via actomyosin filament sliding within the cytoskeleton of the cells that compose the embryo plays a key role during gastrulationas well as during tissue morphogenesis. In fact, the pattern of development can be experimentally altered by applying external stresses to the embryo using micropipettes, or by altering the level of cytoskeletal tension. Mechanical compression of mesenchyme associated with the ’condensation’ of mesenchyme that commonly precedes formation of new organ rudiments has been

recently shown to be sufficient to induce whole tooth organ formation during embryogenesis. The pattern-generating capabilities of mesenchyme isolated from different developing tissues also correlates with differences in their ability to exert mechanical tension on external substrates. Local changes in ECM turnover may drive morphogenetic patterning of tissues, in part, by altering the mechanical compliance of the ECM and thereby, changing cell shape or cytoskeletal tension, as will be described below.

8.4. Mechanochemical switching between Cell fates

Given the pivotal role that ECM plays in tissue development, many studies have been carried out to analyze how changes in cell-ECM interactions act locally to regulate cell sensitivity to soluble mitogens, and thereby establish the differentials of growth, differentiation, apoptosis and motility that are required for tissue morphogenesis. To accomplish this, simplified in vitro model systems have been developed which retain the minimal determinants necessary for maintenance of the physiological functions of interest (i.e., cell growth and differentiation) . For example, to determine the effects of varying cell-ECM contacts directly, we precoated bacteriological petri dishes that were otherwise non-adhesive with different densities of purified ECM molecules, such as fibronectin, laminin, or different collagen types. Quiescent, serum-deprived cells were plated on these dishes in a chemically-defined medium thatcontained a constant and saturating amount of soluble growth factor.When capillary endothelial cells were studied, DNA synthesis and cell doubling rates increasedin an exponential fashion as the density of immobilized ECM ligand was raised and cellspreading was promoted. When higher cell plating numbers were used to promote cell-cellinteractions as well as cell-ECM contact formation, the capillary cells could be switched between growth and differentiation (capillary tube formation) in the presence of saturating amounts of soluble mitogen (FGF) simply by varying the ECM coating density.

Specifically, when plated on a high ECM density (e.g., >500 ng/cm2 fibronectin), the cells attached, spread extensively, formed many cell-cell contacts, and organized into a planar cell monolayer. When the same cells were plated on a low ECM density (<100 ng/cm2, the cells attached but they could not spread, and thus only cell clumps or aggregates were observed. When the same capillary cells were plated on a moderate density, cells first attached, spread, and formed cell-cell contacts as they did on the higher ECM densities. However, the tensile forces generated by the cells appeared to overcome the resistance provided by their relatively weak ECM adhesions and thus, the cell aggregates began to retract over a period of hours until a mechanical equilibrium was attained. Under these conditions, formation of an extensive network comprised of interconnected capillary tubes resulted. Many of these capillary tubes became elevated above the culture surface, although the network remained adherent to the culture dish at discrete points through interconnected multicellular aggregates. The importance of mechanical forces for this switching between growth and differentiation was confirmed by demonstrating that similar capillary tube formation could be induced on the high ECMdensity that normally promoted spreading and growth, simply by increasing the cell plating numbers and thereby amplifying the level of cell tension. The same system was used to demonstrate similar shape (stretch)-dependent switching between growth and differentiation in other cell types. For example, the growth and differentiation of primary rat hepatocytes could be controlled independently of cell-cell contact formation by varying cell-ECM contacts and cell spreading using the method described above.

Additional studies confirmed that ECM exerts its regulatory effects at the level of gene expression and that these effects are mediated at least in part through modulation of the cytoskeleton. These results are consistent with those from other laboratories, which demonstrate that malleable ECM gels (e.g., Matrigel, native collagen gels) that promote cell rounding also induce differentiation and suppress growth e whereas the opposite effects are produced when these gels are fixed and

made rigid. Altering cell-ECM contacts by varying ECM coating densities influences cell function via two distinct but integrated mechanisms. First, increasing the local density of immobilized ECM ligand promotes clustering of transmembrane ECM receptors on the cell surface which are known as ’integrins’ . Integrin clustering, in turn, activates a number of different chemical signaling pathways (e.g., tyrosine phosphorylation, inositol lipid turnover, Naþ/Hþ exchange, MAP kinase) that are also utilized by growth factor receptors to alter cellular biochemistry and gene expression . Activation of these signaling pathways likely plays an important role in control of cell differentiation and survival; however, integrin-dependent chemical signaling alone is not sufficient to explain how cells are induced to enter S phase and proliferate . A second mechanism that involves tension-dependent changes in cell shape and cytoskeletal organization, or changes in the level prestress (isometric tension) within the cell and linked ECM, also comes into play. The importance of tension-dependent changes of cell shape and cytoskeletal organization was demonstrated directly by developing a model system in which cell distortion was varied independently of the local density of immobilized ECM molecule by controlling the spatial distribution of ECM anchors that can resist cell-generated traction forces. This was made possible by adapting a technique for forming spontaneously assembled monolayers (SAMs) of alkanethiolsto create micropatterned surfaces containing adhesive ECM islands with defined surface chemistry, shape and position on the cell (micrometer) scale, separated by non-adhesive regions. The method involves the fabrication of a flexible elastomeric stamp that exhibits the particular surface features of interest using photolithographic techniques. The topographic high points on the stamp (e.g., 40 x 40 mm square plateaus raised above recessed intervening regions) are coated with an alkanethiol ink and the stamp is then apposed to a gold-coated surface. The alkanethiol forms SAMs covering only the regions where the stamp contacts the surface (i.e., 40 x 40 mm squares). Then the surrounding uncoated regions are filled with a SAM composed of similar alkanethiols that are conjugated topoly(ethylene glycol) (PEG) that prevents protein adsorption. The result is a chemicallydefinedculture surface that is completely

covered with a continuous SAM of alkanethiols,however, the local adhesive islands of defined geometry support protein adsorption, whereasthe surrounding boundary regions coated with PEG do not. Thus, when these substrates arecoated with a high density of purified ECM protein, such as laminin or fibronectin, adhesive islands of defined shape and position, coated with a saturating density of matrix molecule result. Using this technique, cell position and shape can be precisely controlled because the cells only attach to the ECM-coated adhesive islands. In fact, even square and rectangular shaped cells exhibiting 90_ corners can be engineered using this approach .

This micropatterning method was first used to ask the question: if cells are restricted to a small size similar to that produced on a low ECM coating concentration, but the local density of immobilized integrin ligand is increased 1000-fold, which is the critical determinant of cell function e the ECM density or cell shape? The answer was that it was cell shape. Primary hepatocytes remained quiescent on small adhesive islands coated with a high ECM density, even though the cells were stimulated with high concentrations of soluble growth factors, and cell growth (DNA synthesis) increased in parallel as the size of the adhesive island was increased. Similar results also were obtained with capillary endothelial cells. Moreover, inhibition of hepatocyte growth on the small islands also was accompanied by a concomitant increase in albumin secretion. In the case of endothelial cells, the cells were similarly induced to differentiate into capillary tubes when cultured on linear patterns that supported cell-cell contact as well as a moderate degree of cell distension whereas the cells were induced to undergo apoptosis (cellular suicide) when cultured on the smallest islands that fully prevented cell extension. Thus, cells shape and function can be engineered simply by altering the geometry of the cell's adhesive substrate.

Intracellular signals elicited by integrin receptor clustering due to ECM binding have been shown to be critical for control of cell growth and function. Thus, one could argue that cell shape and mechanical distortion of the cytoskeleton (CSK) are not important. Instead, it might be the increase in the total area of cell-ECM contacts and

the associated enhancement of integrin binding that dictates whether cells will grow, differentiate or die on large versus small adhesive islands. To explore this further, substrates were designed in which a single small adhesive island (which would not support spreading or growth) was effectively broken up into many smaller islands (3e5 mm in diameter) that were separated by nonadhesive barrier regions. When capillary cells were plated on these substrates, their processes stretched from island to island and the cells exhibited an overall extended form similar to cells on large islands. However, the total area of cell-ECM contact was almost identical to that exhibited by non-growing cells on the smaller islands. These studies revealed that in the presence of optimal growth factors and high ECM binding, DNA synthesis was high in the cells that spread over multiple small islands whereas apoptosis was completely shut off , thus confirming that cell shape distortion is the critical governor of this response. Importantly, cell distortion also impacts cell migration as well as stem cell lineage switching. For instance, when cells on square islands are stimulated with motility factors, the cells preferentially extend new motile processes (e.g., lamellipodia, filopodia, microspikes) from their corners, whereas they extend in all directions along the edge of round cells on circular islands. Cells on polygonal ECM islands also prefer to form new lamellipodia from corners with acute angles rather than obtuse ones . In contrast, when mesenchymal stem cells are cultured on different sized ECM islands, those on small islands switch on the fat cell lineage, whereas the spread cells on larger ECM islands become bone cells. Thus, taken together, these results suggest that microfabrication methods might lead to new approaches to tissue engineering using microfabricated substrates. With this approach, it may be possible to direct cell migration, growth and differentiation of stem cells in specific locations by modifying the surface chemistry and topography of artificial materials, instead of creating gradients of soluble chemokines.

The effects of shape distortion on cell fate switching may, in part, be mediated by changes in the level of isometric tension or 'prestress' within the cytoskeleton. For example, both flexible and poorly adhesive culture dishes that inhibit growth and induce differentiation dissipate prestress, whereas substrates that stimulate cell spreading and growth (e.g., rigid dishes) support high levels of cytoskeletal tension. Small ECM islands that inhibit cell spreading also prevent pulmonary vascular smooth muscle cells from responding to vasconstrictors, such as endothelin-1 (as measured by increases of myosin light chain phosphorylation), and similar effects are produced by culturing cells on flexible ECM substrates that dissipate tensional prestress in the ECMand in the interconnect cytoskeleton (i.e., linked through transmembrane integrins) . In addition, cells preferentially differentiate on ECM substrates that are compliance-matched with their cytoskeleton and that have a mechanical stiffness similar to that of their natural tissues. Cells also migrate up gradients of ECM stiffness, a process known as durotaxis. These observations suggest that tissue formation may be controlled by changing the physical properties of either the ECM or of the cell, or by altering tension generation in the cytoskeleton.

Regional variations of cell shape distortion or of cytoskeletal prestress similarly drive tissue patterning in the embryo. For example, during tissue morphogenesis, only a subset of cells must respond to soluble growth factors by proliferating locally and sprouting or budding outward relative to neighboring non-growing cells. This process is repeated along the sides of the newly formed sprouts and buds, and the whole process reiterates over time; this is how the fractal-like patterns of tissues develop. This process is mediated by regional changes in ECM structure: the ECM thins in regions where new buds or sprouts will form due to local enzymatic degradation. Because tissues are prestressed (due to the action of cytoskeletal contractile forces), a local region of the tensed ECM may thin more than the rest like a 'run' in a woman's stocking. Cells anchored to this region will also stretch or spread, whereas the shape of neighboring cells on intact ECM would remain unchanged. If cell spreading promotes growth, then local cell growth differentials would result.

This possibility that tissue morphogenesis may be controlled through changes in the mechanical force balance between the cytoskeleton and the ECM is supported by recent experimental studies in embryonic and adult systems. Analysis of the effects of cell shape in cell cycle progression in vitro have revealed that these effects are mediated by altering signal transduction through the small GTPase Rho and its downstream target, Rho-associated kinase (ROCK) that controls cytoskeletal tension generation. Importantly, when cytoskeletal contractility was suppressed in whole embryonic lung rudiments by inhibiting Rho or ROCK, both epithelial branching morphogenesis and angiogenesis were inhibited, and this was accompanied by decreased basement thinning (as if there was no tension in the stocking) . In contrast, stimulating the Rho pathway had the opposite effect (i.e., it increased morphogenetic branching) at moderate levels of activation and caused complete organ contraction and inhibition of tissue growth at very high levels of stimulation . Altering the cellular force balance by modulating ECM rigidity also can alter expression of the angiogenic factor receptor VEGFR2 (via control of nuclear transport of opposing transcription factors) and thereby, regulate angiogenesis both in vitro and in vivo . Thus, local changes in ECM structure and cell shape caused by altering cytoskeletal prestress may govern how individual cells respond to chemical signals in their microenvironment in vivo, just as experimental studies using microfabricated ECM islands have demonstrated in vitro. This mechanism for establishing local growth differentials may play a critical role in morphogenesis in all developing systems .

8.5. Mechanobiology summary

In summary, it is shown that the development of functional tissues, such asbranching capillary networks, requires both soluble growth factors and insoluble ECM molecules. The ECM appears to be the dominant regulator, however, since it dictates whether individual cells will proliferate, differentiate, undergo directional motility or die locally in response to soluble stimuli. This local control mechanism is likely critical for the establishment of the local differentials in cell growth, motility, differentiation, apoptosis and stem cell commitment that mediate pattern formation in all developing tissues.

Analysis of the molecular basis of these effects has revealed that ECM molecules alter cell growth via both biochemical and biomechanical signaling mechanisms. ECM molecules cluster specific integrin receptors on the cell surface and thereby activate intracellular chemical signaling pathways, stimulate expression of early growth response genes (e.g., c-fos, jun-B) , and induce quiescent cells to pass through the G0/G1 transition. However, in addition, the immobilized ECM components must physically resist cell tension and promote changes of cell shapeand cytoskeletal tensionin order to promote full progression through G1 and entry into S phase. In fact, studies with living and membrane-permeabilized cells confirm that changes in cell shape result from the action of mechanical tension which is generated within microfilaments and balanced by resistance sites within the underlying ECM. Local changes in ECM structure or mechanics will therefore alter the ECM's ability to resist cell tractional forces exerted on integrins, and thereby produce changes in cytoskeletal organization. This will, in turn, alter the level of isometric tension (prestress) in the cytoskeleton, as well as modulate the activity of various signal transduction pathways signal inside the cell. Taken together, this work suggests that the pattern-regulating information that the ECM conveys to cells is both chemical and mechanical in nature. Thus, design of future artificial ECMs for tissue and organ engineering applications must take into account the critical role that mechanical forces and these mechanotransduction mechanisms play in biology.

Early tissue-engineering efforts by reconstructive surgeons and material scientists started with knowledge of the clinical need and of the mechanical behavior of connective tissues on the macroscopic scale, and worked backwards. The long-term goal for the field is to design and fabricate tissue and organ substitutes starting from first principles. This includes incorporating biologically inspired mechanical and architectural principles, as well as an in depth understanding of the molecular and biophysical basis of tissue regulation. Clearly, given the potent and varied functions of the ECM, fabrication of artificial ECMs will play a central role in all of these future

efforts. We and others have already begun to explore the utility of synthetic bioerodible polymers as cell attachment substratesand the potential usefulness of immobilized synthetic ECM peptides for controlling cell growth and function . These materials offer a major advantage in terms of biocompatibility since the artificial substrates completely disappear over time, and thus the implanted donor cells become fully incorporated into the host. They also provide great chemical versatility, as well as the potential for large-scale production at relatively low cost. In addition, use of synthetic chemistry reduces the likelihood of batch to batch variation during large-scale production, a problem which can potentially complicate use of purified ECM components. For these reasons, polymer chemistry and novel fabrication techniques will likely lead to development of more effective tissue substitutes.

However, if we understood the fundamental principles that guide ECM remodeling and pattern formation in tissues, perhaps tissue engineering might take a different approach in the future. For example, one could envision entirely new methods of clinical intervention if we understood how tissue-specific mesenchyme generates tissue pattern; how ECM turnover is controlled locally; how cell-generated contractile forces contribute to tissue repair and remodeling; or how compressing or pulling tissues alter their growth and form. This knowledge could lead to methods for identifying and isolating relevant 'pattern-generating' cells; for developing pharmacological modifiers of ECM remodeling that may be incorporated in local regions of implants to promote or suppress tissue expansion locally; and for fabricating 'biomimetic' scaffolds that mimic the mechanical and architectural features of natural ECMs necessary to switch on or off the function of interest (e.g., growth vs. differentiation or apoptosis) at a particular time or place. These are the just a few of the challenges for the future. Finally, one glimpse of the future comes fromour recent work that focuses on the development of human 'organs-on-chips' in which microscale-engineering technologies are used to create microfluidic devices lined by cultured living human cells that recapitulate the physiological microenvironments of

whole living organs. The goal is to develop organomimetic microdevices that enable study of complex human physiology in an organ-specific context. Our first success in this area was the development of a human breathing 'lung-on-a-chip' that reproduces key structural, functional, and mechanical properties of the fundamental functional unit of the living human lung e the alveolar-capillary interface. We did this by microfabricating a microfluidic system containing two closely apposed microchannels separated by a thin (10 mm) porous flexible membrane made of poly (dimethylsiloxane) (PDMS), which was coated with ECM. Human alveolar epithelial cells were cultured on one side of the membrane with air flowing above their apical survace, and human pulmonary microvascular endothelial cells were cultured on the other with flowing medium containing human white blood cells to mimic blood flow. Breathing movements were reproduced in this engineered microsystem by applying cyclic suction to neighboring hollow microchambers; this rhythmically deforms the central porous membrane and attached cell layers much as occurs at the living alveolar-capillary interfaces during breathing. The lung-on-a-chip was shown to replicate the complex organ-level responses of living human lung, including the inflammatory response triggered by bacteria or cytokines introduced into the air space . The endothelial cells rapidly become activated in response to these cues, and increase their expression of ICAM-1, which induces adhesion of primary human neutrophils flowing in the capillary channel; this is followed by their transmigration across the capillary-alveolar interface and into the alveolar space where the white blood cells engulf the bacteria. Because the PDMS has high optical clarity, the entire human inflammatory response can be visualized in real-time within this biochip.

Importantly, we found that the breathing motions and rhythmic distortion of the cells are absolutely critical for them to mimic organ-level physiology and pathophysiology. For example, we found that physiological breathing movements greatly accentuate toxic and inflammatory responses of the lung to silica nanoparticles (12 nm) that were introduced into the air channel to mimic airborne particulates.

Mechanical strain also enhanced epithelial and endothelial uptake of nanoparticulates and stimulated their transport into the underlying microvascular channel. Importantly, similar effects of physiological breathing on nanoparticle absorption are observed in whole mouse lung. Moreover, we have recently extended this work by developing a microengineered human disease model of pulmonary edema, which closely mimics this life-threatening side effect that is induced by the cancer chemotherapic interleukin-2 (IL-2) in human patients, and is characterized by fluid accumulation and fibrin deposition in the alveolar airspace. Again, we found that physiological breathing motions are responsible for the majority of the effects of IL-2 on pulmonary vascular leakage, and we confirmed this in an animal model.

Finally, when we adapted the same microengineering approach to produce a human 'gut-on-a-chip' lined by human intestinal epithelial cells, we also found that by mimicking peristaltic-like mechanical motions and the trickling fluid flows of the living gut, we obtained levels of differentiation and functionality never seen before in vitro. These included spontaneous formation of intestinal villi, restoration of epithelial barriers, and gaining the ability to co-culture intestinal bacterial (microbiome) on the human epithelium without compromising tissue viability or function.

Human organ-on-chips models could provide a potential new approach to enhance our fundamental understanding of complex disease processes and to enable more rapid, accurate, cost-effective and clinically relevant testing of drugs, as well as cosmetics, chemicals and environmental toxins. On-chip human disease models also could help to facilitate the translation of basic discoveries into effective new treatment strategies.

But most importantly, these results demonstrate the power of combining insights into mechanobiology with advanced engineering approaches as we seek to confront future challenges in tissue and organ engineering.

9. In Vivo Synthesis of Tissues and Organs :

The goal of achieving in vivo induced regeneration for a variety of tissue and organs following severe injury remains at the forefront of current tissue-engineering investigations. Typically, an analog of the extracellular matrix is utilized as a template that, when properly formulated, induces regeneration of lost or damaged tissue. Currently, successful regeneration has been induced in the skin and peripheral nerves , while progress has been made in developing appropriate extracellular matrix analogs to alter the typical organismic response to injury in a variety of tissues, including kidney, cartilage, bone, central nervous system, and brain dura. These investigations, active for the previous three decades, have primarily focused on identifying the optimal extracellular matrix analog components to block organized wound contraction and scar tissue formation, while inducing regeneration of physiological tissue. Althoughmany tissue engineering investigations currently focus on developing techniques appropriate for synthesis of tissues and organs in vitro, such products must eventually be implanted in the appropriate anatomical site of the host. Since implantation of an organ construct is almost always preceded by a surgical procedure that generates a severe wound, it is essential to master the evolving methodology of in vivo wound healing in order to synthesize appropriate neo-organ constructs in vitro for eventual implantation.

9.1. Mammalian response to injury

Defect scale Treatment options for organ injury depend significantly on the scale of the defect. Microscopic defects can be treated using a wide variety of soluble factors (i.e., herbs, potions, pharmaceuticals, vitamins, hormones and antibiotics). However, organ-scale defects present a significantly larger wound site, require considerably different treatment practices, and constitute the focus of this article. These defects, primarily created by disease or by an acute or chronic insult that result in millimeter or centimeter scale wounds, cannot be treated with drugs because the problem is the failure of a mass of tissue including cells, soluble proteins and

cytokines, and insoluble extracellular matrix (ECM). Significant loss of function in the affected tissue or organ, termed the ’missing organ’, leads to consequences such as lack of social acceptance in cases of severe burns and facial scars, loss of mobility and sensory function in the case of neuroma, and life-threatening symptoms in cases such as cirrhotic liver, large-scale severe burns, and ischemic heart muscle.

9.2. Regeneration versus repair

Certain organisms have the ability to regenerate significant portions of damaged tissue. An example is the amphibian newt, which regenerates functional limbs following amputation. The mammalian fetus has displayed the ability to regenerate damaged organs and tissue spontaneously up to the third trimester of gestation; however, adult mammals do not typically exhibit spontaneous regeneration following severe organ injuries . Instead, the adult mammal response to severe injury is closure of the wound by contraction and scar tissue formation, a process termed repair. Cell-mediated contraction of the wound site is observed in many different species in varying degrees, at many organ sites . Distinct from repair, regeneration is characterized by synthesis of physiological (normal, functional) replacement tissue in the wound site that is structurally and functionally similar to the original tissue. Based on these observations, for the remainder of this text the term ’early fetal’ refers to the fetal response to injury that leads to regeneration, while ’adult’ refers to all mammals (adult as well as late fetal) that respond to injury via repair.

The contractile fibroblast phenotype, termed the myofibroblast, plays a critical role in determining the nature (repair or regeneration) of mammalian wound healing. During adult repair, myofibroblast-mediated organized wound contraction and scar tissue synthesis is observed. During early fetal healing, differentiation of myofibroblasts has not yet occurred and regeneration of damaged tissue and organs occurs in the absence of contraction. The data suggest that induced organ regeneration in the adult may be encouraged by developing techniques to stimulate partial reversion to early fetal healing. Additionally, the ransforming growth factor-b (TGF-b) family of

molecules has been implicated in this ontogenetic transition between fetal regeneration and adult repair response to injury. TGF-bs are multifunctional cytokines with widespread effects on cell growth and differentiation, embryogenesis, immune regulation, inflammation, and wound healing . In terms of their relationship with repair processes, TGF-b1 and TGF-b2 are known to promote scarring, while TGF-b3 may reduce scarring . As such, deficient levels of TGF-b1 and b2 and increased levels of TGF-b3 are observed in early gestational (’fetal’ regenerative healing response) compared to late gestational (’adult’ repair healing response) mice. These results implicate increased TGF-b1, b2, and decreased TGF-b3 expression along with myofibroblast activity in late gestation and post-partum fetal scar formation. The available evidence suggests future experiments utilizing procedures for control of TGF-b in conjunction with other tissue-engineering constructs that modify myofibroblast behavior, such as bioactive scaffolds, to induce regeneration of tissues that are known to be non-regenerative.

- ***Tissue triad***

There are three distinct tissue types, termed the tissue triad, which together define the structureof most organs: the epithelial layer, the basement membrane layer, and the stroma . Developmental and functional similarities between this triad in a variety of tissues and organs such as skin and peripheral nerves have been observed, suggesting that it can be used as an illustrative device to understand injury response in other organs as well . A layer of epithelial cells (the epithelial layer) covers all surfaces, tubes, and cavities of the body; this layer is cell-continuous and avascular; unlike the basement membrane and stroma, the epithelial layer does not comprise a significant amount of ECM. The basement membrane is an acellular, avascular, continuous layer of ECM components separating the epithelial layer and the stroma. The stroma is cellular, contains ECM and connective tissue components, is heavily vascularized, and provides a reservoir for nutrient uptake to and waste removal from the basement membrane and epithelia.

Following injury to a variety of tissues, such as the skin, peripheral nerves, blood vessels, lung, kidney, and pancreas, the epithelial and basement membrane layers regenerate spontaneously when the stroma remains intact, while the damaged stoma heals through repair-mediated contraction and scar formation processes. Understanding the injury response of the tissue triad has suggested a paradigm for inducing regeneration in non-regenerative tissues: the repair mechanisms appear to be activated by disruption of the stromal architecture, and proper replacement of the stromal layer is critical for any regeneration to occur. As such, development of materials to act as analogs of the ECM to replace the lost stromal architecture has been a primary focus of studies of regenerative medicine over the past two decades.

9.3. Methods to treat loss of organ function

Six approaches have been used to restore some level of functionality to a damaged tissue or organ: transplantation, autografting, implantation of a permanent prosthetic device, use of stem cells, in vitro synthesis of organs, and induced regeneration. The last three techniques are often collectively referred to as ‘tissue engineering’. In vivo induced regeneration is the only methodology to date which has modified the adult mammalian wound healing response to induce regeneration. All six techniques will be briefly discussed in the following sections; a more detailed description has been previously published.

9.3.1. Transplantation

Transplantation is widely used to replace complex tissues and organs, but is limited by two significant factors. While transplantation of a select few tissues such as the eye and testis occur without rejection, a significant challenge facing transplantation is the immunological barrier between the donor and host. After transplantation, the donor organ is attacked and rejected by the host's immune system. The primary clinical method for preventing such rejection is the use of immunosuppressive drugs for the remainder of the host's life to suppress their immune system. However, immunosuppression also makes the host vulnerable to infections . A second major obstacle is the difficulty in finding immunocompatible donors and the shortness of supply of suitable organs.

9.3.2. Autografting

With autografting, the donor and the recipient are the same individual; a fraction of the tissue or organ is harvested from an uninjured site and grafted at the non-functional site.

Autografting removes issues related to immune response, but necessitates the creation of a second wound site (donor site), subjecting the patient to a second severe trauma and additional loss of functionality. Therefore, autografting is utilized only when sufficient autograft tissue is available and when the loss of functionality or morbidity at the primary wound site outweighs that at the harvest site, giving it limited applicability. Typical applications of autografting follow severe burns and peripheral nerve injuries in the hand.

9.3.3. Permanent prosthetic device

Permanent, prosthetic devices are typically fabricated from biologically inert materials such as metals, ceramics, and synthetic polymers that do not provoke the immune response problems inherent to many transplanted tissues. Even though these devices are fabricated from bio-inert materials, interactions between the prosthesis and

the surrounding biological environment still lead to a number of unfavorable physical and biological manifestations. Specific examples are the formation of a thick, fibrous scar tissue capsule around the implant, stressshielding of the surrounding tissue, platelet aggregation to implanted surfaces, and accumulation of wear particles both at the site of implantation and in the lymphatic system

The spontaneous remodeling process of the tissues surrounding the implant can also be significantly altered, leading to further tissue degradation. These often-serious side effects illustrate the difficulty of replacing bioactive tissues with bio-inert implants fabricated from materials possessing drastically different material and mechanical properties.

9.3.4. Stem cells

The pluripotent nature of stem cells offers a multitude of therapeutic possibilities.

Current efforts in stem cell research have focused on understanding stem cell plasticity, aging, and ways of controlling differentiation. Mesenchymal, epithelial, and neural stem cellshave been grown in vitro and studied. More recently, experimental investigations of stem cells have also focused on utilizing hematopoietic and embryonic stem cells. In particular, techniques for harvesting and identifying them , expanding and differentiating them in culture , and re-implanting them at an injury site have been at the forefront of stem cell research. Importantly, many of these studies have made the transition to exploring biomaterial-mediated effects on basic cell bioactivityand regenerative potential. An improved understanding of stem cell behavior and the development of stem cell-based technologies also raise a number of important ethical questions, consideration of which will play a significant role in the development of stem cellbased tissue-engineering solutions.

9.3.5. In vitro synthesis

In vitro synthesis requires the growth of a functional volume of tissue in vitro. It allows total control over the culture environment, including soluble regulator content (i.e., growth factors, cytokines), insoluble regulator content (i.e., ECM proteins), and a variety of cell culture media and loading conditions. In order to develop large (critical dimension >1 cm), bioactive scaffolds, it is important to metabolically support the cells within these constructs. There are two mechanisms available for the transport of metabolites to and waste products from cells in a scaffold: diffusion, and with in vivo applications, transport through capillary networks formed in the scaffold via angiogenesis. While angiogenesis becomes the limiting factor in vivo, significant angiogenesis is not observed for the first few days after implantation, and is not present at all in vitro. As a result, current tissue engineering constructs are size limited (<1 cm) due to diffusion constraints. Improving metabolite influx is critical for larger, more complex scaffolds. Additionally, the complexity of biological systems, specifically cytokine, growth factor, and intercellular signaling needs throughout the volume of developing tissue have to date precluded, with a few exceptions such as in vitro culture of replacement heart valvesand epithelial sheets for severe burn patients , the formation of complex tissues in vitro.

9.3.6. Induced in vivo organ synthesis (induced regeneration)

Induced organ synthesis in vivo relies on the processes inherently active in the wound site to regenerate lost or damaged tissue.

A highly porous analog of the ECM, also termed a scaffold, is utilized to induce regeneration at a wound site where the organism would normally respond via repair processes. Induced organ synthesis was made possible by the development of fabrication techniques to produce ECM analogs with well-defined pore microstructure, specific surface area, chemical composition, and degradation rate. Its first application was the use of a collagen-glycosaminoglycan (CG) scaffold (termed dermal regeneration template e DRT) that induced skin regeneration following severe injury.

The DRT displayed high biological activity when implanted into a full-thickness skin wound and was capable of inducing regeneration of the underlying dermal layer of skin as well as the epidermal and basement membrane layers . Efforts using decellularized matrices have presented a new avenue for generating material platforms for tissue regeneration such as in the area of urogenital and intestinal repair.

For the remainder of this review, we will focus on studies on the structure and function of ECM analogs used in tissue-engineering applications as well as an in-depth discussion of the tissueengineering approaches utilized to induce regeneration of a number of tissues, namely skin, peripheral nerves, and orthopedic tissues.

9.4. Active extracellular matrix analogs

9.4.1. Fundamental design principles for tissue regeneration scaffolds

Porous, three-dimensional scaffolds have been used extensively as biomaterials in the field of tissue engineering for in vitro study of cell-scaffold interactions and tissue synthesis and in vivo induced tissue regeneration studies. These scaffolds, analogs of the ECM, act as a physical support and, more importantly, as an insoluble regulator of biological activity that affects cell processes such as migration, contraction, and division. For the remainder of this review, the term active or bioactive ECM analog will refer to scaffolds that induce regeneration of normally non-regenerative tissues following severe injury. It has been hypothesized that these active ECM analogs induce regeneration by establishing an environment that selectively inhibits wound contraction by **preventing the organized contractile response and blocking scar synthesis**, the two processes normally responsible for closing a wound following severe injury. Recent efforts suggest that porous collagen scaffolds enhance regeneration both in injured adult skin and peripheral nerves by **disrupting the formation of a contractile cell capsule at the edges of the wound**. Notably, capsules or clusters of contractile cells were hypothesized to impose a universal mechanical barrier during wound healingwhich, if disrupted appropriately, enhances the quality of induced regeneration in a wider range of organs. Four physical and structural

properties must be controlled to critical levels for the fabrication of an active ECM analog: the degradation rate which defines the template residence time, the chemical composition, the pore microstructure (mean pore size, shape, and orientation), and the scale of the scaffold that is defined by the critical cell path length. These characteristics and any governing models that help to describe cell behavior in active ECM analogs will be discussed briefly in the following section.

9.4.2. Template residence time

The length of time that the scaffold remains insoluble in the wound site (residence time) is critical in defining its bioactivity. For physiologic tissue to be synthesized at a wound site, the scaffold must initially support cell migration, proliferation, and organization, but the scaffold must then degrade in such a manner that it does not interfere with the native tissue synthesis and remodeling processes. These considerations require a scaffold residence time with an upper and lower bound**, a concept that has been formalized as the isomorphous tissue replacement model: the scaffold residence time must be approximately equal to the time required to synthesize a mature tissue via regeneration at the specific tissue site under study** . In the case of a full-thickness skin wound, for example, this healing time is approximately 25 days, and the degradation kinetics of the active ECM analog that induces skin regeneration was optimized for that time period . Alternatively, the healing time of a peripheral nerve injury is dependent on the gap length: peripheral nerve trunks grow unidirectionally, fromthe proximal toward the distal stump, at a rate of approximately 1 mm/day. Accordingly, during induced regeneration of peripheral nerves across a gap, a scaffold in contact with the nerve stumps (either the tube into which the stumps are inserted or the scaffold structure in the tube lumen) must remain in an insoluble state over a period approximately equal to the time, of order 10e20 days, during which axon elongation proceeds from the proximal to the distal stump.

An intact scaffold cannot diffuse away from the wound bed; therefore the simplest method for achieving isomorphous tissue replacement requires the insoluble

scaffold structure to be degraded by enzymes in the wound bed into low molecular weight fragments. The lifetime of the scaffold is defined by the degradation time constant (td) and can be compared to the normal healing process time constant for a wound at the anatomic site of interest (th) . When the scaffold remained in the wound bed as a non-degradable implant (td/th > 1), dense fibrous tissue similar to scar was synthesized underneath the scaffold. When the scaffold degraded rapidly (td/th < 1), wound healing was marked by wounds contraction and scar synthesis similar to the reparative healing process observed in an ungrafted wound and no regeneration was observed . Because different wound sites and even the same wound site in different species may have different time constants for healing (th), it is necessary to adjust the degradation rate of the ECM analog for each wound site and species in order to satisfy the isomorphous tissue replacement requirement and induce regeneration.

Scaffold resistance to degradation can be increased by increasing the relative density (solid content) of the scaffold. However, a more elegant option that does not involve changing the structural characteristics of the scaffold is also available: **scaffold degradation resistance increases with increasing crosslink density between the fibers that make up the scaffold structure** In the case of collagen-based scaffolds, degradation in the wound bed is accomplished primarily by native collagenases. Decreased degradation rates for collagen-based scaffolds has been achieved by introducing glycosaminoglycans (GAGs) into the collagen mixture, which results in the formation of additional crosslinks, and by further crosslinking the resultant scaffold using a multitude of physical and chemical crosslinking processes. Dehydrothermal (DHT) crosslinking is a physical technique where the scaffold is exposed to a high temperature under vacuum, leading to the removal of water from the scaffold. Drastic dehydration (<1% water content) of the scaffold leads to the formation of inter-chain amide bonds through condensation . DHTcrosslinking is adjustable; exposure to higher temperatures or longer lengths of time produces a higher crosslink density and slower degradation rate . UV treatment is a second

physical crosslinking technique that can create crosslinks between collagen fibers due to the effects of radiation . Chemical crosslinking treatments, such as glutaraldehydeor carbodiimideexposure can also be utilized to induce covalent bonds between collagen fibers. These crosslinking techniques are considerably more powerful, resulting in significantly higher crosslink densities and slower degradation rates. However, chemically crosslinked scaffolds must be extensively washed to remove all traces of the typically cytotoxic chemicals. Additionally, some chemical crosslinkers integrate a portion of the chemical compound into the crosslink; degradation of these scaffolds releases cytotoxic agents into the wound site so these techniques must be used with care . **While described in detail for collagen-based scaffolds, these crosslinking tools and techniques can be applied to a multitude of scaffolds, fabricated from both natural and synthetic materials**.

9.4.3. Chemical composition

The chemical composition defines the ligands displayed on the scaffold surface. Cell behaviors such as attachment, migration, proliferation, and contraction are all mediated by interactions between the focal adhesions and integrins expressed on the cell surface and the ligands available on the scaffold surface. An active ECM analog must be fabricated in a manner, and from specific materials, that leads to expression of a chemical environment conducive to cellscaffold interactions that prevent organized wound contraction and scar synthesis and that instead induce regeneration.

A multitude of different materials have been used to fabricate scaffolds for many tissueengineering applications including studies of induced in vivo regeneration. Several synthetic, non-degradable polymers such as (poly)dimethyl siloxane have been occasionally utilized; these polymers, which parenthetically violate the principle of isomorphous tissue replacement, do not express ligands on their surface and have not induced regeneration. Degradable synthetic polymers such as poly(L-lactide) and poly(lactide-co-glycolide) variants have been fabricated to satisfy isomorphous tissue replacement and with surfaces that have been modified chemically to display appropriate ligands; however, these materials have not been observed to prevent

contraction and scar formation nor to induce regeneration of stromal tissue. A chemical composition that has been used successfully to induce regeneration is a graft copolymer of type I collagen and a sulfated glycosaminoglycan (GAG) . Collagen is a significant constituent of the natural ECM, and collagen-based scaffolds have been used in a variety of applications due to many useful properties: low antigenic response and a high density of ligands that interact specifically with integrin receptors in fibroblasts (the cell type predominantly responsible for cell-mediated contractions processes during repair). Particular collagen scaffolds have been observed to promote cell and tissue attachment and growth as well as to induce regeneration of tissue following injury . However, in order to induce regeneration, the periodic banding (~64 nm) of the collagen fiber structure must be selectively abolished to prevent platelet aggregation that leads to repair. A number of other natural protein-based scaffolds (i.e., hyaluronate-based, fibrin-based, and chitosan-based scaffolds) have also shown great promise in the field of tissue engineering. These natural polymers are also capable of facilitating cell binding similar to that observed with collagen scaffolds in part due to their expression of natural ligand binding sites, but these materials have not been developed to the point of inducing regeneration at this point.

9.4.4. Template pore microstructure

The biological activity of any ECM analog also depends significantly on its three-dimensional pore microstructure. Having migrated into the scaffold, the cell interacts with the surrounding scaffold environment, making use of its cell surface receptors to bind to specific ligands on the scaffold surface. The first critical component of ECM analog microstructure to consider is the open- or closed-cell nature of the scaffold. An open-cell pore microstructure exhibits pore interconnectivity which a closed-cell microstructure exhibits membrane-like faces between adjacent pores, effectively sealing the environment of one pore from its neighbors. Pore interconnectivity is critical for scaffold bioactivity because cells to be able to migrate through the construct and to interact with other cells in a manner similar to that observed in vivo.

A second critical structural feature is the relative density (Rd) or porosity of the ECM analog.

The relative density of the scaffold is calculated as the ratio of the scaffold density to the density of the solid from which the scaffold is made. Porosity, a measure of how porous the scaffold is, is the pore volume fraction of the scaffold. Additionally, the relative density and porosity are inversely related: scaffold porosity can also be defined as (1 _ Rd). These variables both define the amount of solid material in the scaffold; when the pores are closed, too small, or when the relative density is too large, cells are not able to migrate through the scaffold, a significant impediment for a tissue-engineering scaffold. An active ECM analog must possess an open-cell pore structure with a relative density below a critical value that is characteristic of each application, but is typically significantly <10% (bioactive porosity typically >90%). This structural criteria, determined from the results of a number of experiments studying cell-scaffold interactions, suggest that a critical number of cells are required within a bioactive scaffold.

The mean pore size of the ECM analog significantly influences its bioactivity; hence the effect of mean pore size will now be discussed in conjunction with the application of cellular solids modeling and a discussion of scaffold specific surface area. To describe even a simple cellscaffold interaction, a highly detailed model describing the number of receptors utilized per bound cell and the nature of the binding and receptor sites needs to be developed. However, a more generic explanation can be used to describe the complexity of the cell-scaffold interaction and the significant influence the pore size has on its bioactivity. At first pass, it is apparent that there is a minimum pore size requirement for a bioactive ECM analog: the pores must be large enough to allow cells to fit through the pore structure and populate the analog. There is also an observed upper bound to pore size ; it has been hypothesized that this upper bound in mean pore size if due to the effects of scaffold specific surface area. To further test this hypothesis, cellular solids modeling techniques have been

integrated to better describe scaffold microstructure using a quantitative framework. The complex geometry of foams (and of scaffolds) is difficult to model exactly; instead, dimensional arguments that rely on modeling the mechanisms of deformation and failure in the foam, but not the exact cell geometry, will be used . Scaffold relative density (Rd) and mean pore size (d) together define the scaffold specific surface area (SA/V), the total surface area of pore walls available for cell attachment. A cellular solids model has been developed and experimentally validated to accurately predict SA/V of collagen-based scaffolds:

Increasing mean pore size in a series of constructs and keeping Rd constant decreases the overall SA/V, while increasing Rd and keeping the mean pore size constant increases SA/V. The primary feature of this structural analysis to consider is that a change in the construct SA/V indicates a change in the available area to which cells bind. This calculation and experimental result suggests the significance of the scaffold SA/V in defining its bioactivity. If the SA/V is too small (i.e., due to a relatively large mean pore size), an insufficient number of cells will be able to bind to the scaffold and the cells that remain free will contribute to the spontaneous repair mechanism. As previously discussed, there is also a minimum mean pore size, defined by the characteristic dimension of the cell: approximately 10e50 mmfor most cells.When the scaffold pore size is smaller than this critical dimension, cells will be unable to migrate through the scaffold whereas when the pore size is too large an insufficient specific surface area will be available. These upper and lower bounds of the scaffold mean pore size, mediated by cell size and specific surface requirements, have been determined experimentally for each cell type for tissues where regenerative templates have been used.

The shape of the pores that make up the porous scaffold must also be considered. Cells have been observed to be exquisitely sensitive to the mechanical properties of the underlying substrate, and slight changes in the mean shape of the pores can result in significant variations in the extracellular mechanical propertiesand overall construct bioactivity

Changes in mean pore shape may also play a role in defining the areas of the scaffold available or unavailable for binding and the predominant direction of cell migration as well as in the geometrical organization of cells within the scaffold.

9.4.5. Critical cell path length

Successful migration of cells into an active ECM analog and their initial survival is critically important for successful regeneration. While the effect of the structural characteristics (i.e., pore size, shape, relative density) on the bioactivity of an ECM analog has been discussed, there is another important characteristic to consider: an adequate source of metabolites (i.e., oxygen, nutrients). There are two mechanisms available for transport of metabolites to and waste products from the cells: diffusion to and from the surrounding wound bed or transport along capillaries that have sprouted into the scaffold as a result of angiogenesis. While angiogenesis becomes the limiting factor for long-term cell survival and growth, significant angiogenesis is not observed for the first few days after implantation. Therefore, early cell survival inside the scaffold is defined solely by diffusion. The critical scaffold thickness, the maximum scaffold thickness than can be supported by metabolite diffusion, has been observed empirically in to be on the order of a few millimeters.

A quantitative model of cell metabolic requirements and nutrient diffusion characteristics that defines the critical cell path length for cell migration into a scaffold has been developed to describe the salient features of this process . Here, the complexity of the nutritional requirements of the cell is simplified by generically considering a critical nutrient that is required for normal cell function; such a nutrient is assumed to be metabolized by the cell at a rate R in moles/mm3/sec. The nutrient is pictured being transported from the wound bed, where the concentration of nutrient is assumed to be a constant, Co, over a distance L via the exudate until it reaches the cell within the scaffold. Immediately following implantation of the scaffold, nutrient transport is performed exclusively via diffusion that can be modeled using the scaffold diffusivity D in mm2/sec. Dimensional analysis readily yields the cell lifeline number (S): S ¼ RL2=DCo (2)

The cell lifeline number characterizes the relative ratio of the rate of nutrient supply to nutrient consumption by the cell. If the rate of consumption of the critical nutrient exceeds greatly the rate of supply, S >> 1, the cell will soon die. At steady state (S ¼ 1) the rate of consumption of nutrient by the cell equals the rate of transport via diffusion over a distance L; at steady state, the value of L is the critical cell path length, Lc, the longest distance away from the wound bed that the cell can migrate without requiring nutrient in excess of that supplied by diffusion. For many cell nutrients of low molecular weight, Lc is of order a few hundred micrometers to a few millimeters, a distance short enough to suggest the need for very close proximity between wound bed and implant and to indicate that in vivo regeneration of large tissue or organs requires incorporation of special promoters to angiogenesis .

As a result, recent experimental work has focused on understanding the relationship between scaffold pore microstructure and permeability; scaffold permeability controls diffusion-based metabolite and waste transport to and from the scaffold and influences the final hydrostatic pressure distribution in the scaffold. Both of these parameters can significantly influence cell behavior and overall scaffold bioactivity. Cellular solid modeling tools that quantitatively describe scaffold permeability in terms of salient microstructural features have been utilized in this analysis. Both scaffold pore size and compressive strain can vary significantly with different applications, making them the primary features to characterize in terms of scaffold permeability. An open-cell foam, cellular solids model has been developed to accurately model scaffold permeability (K) in terms of scaffold mean pore size (d), percent compression (3), a system constant (A), and scaffold relative density (r*/rs) was developed:

The cellular solids model (Eq. 3) of scaffold permeability suggests that scaffold permeability increases with increasing pore size and decreases with increasing compressive strain, a result that has also been observed experimentally for a series of collage-based scaffolds. The excellent comparison between experimentally measured

permeability and that which is predicted from the cellular solids model suggests that such predictive modeling tools can be used to describe scaffold permeability for many different scaffold architectures under a variety of physiological loading conditions.

9.4.6. Active collagen-glycosaminoglycan scaffolds

Collagen-glycosaminoglycan scaffolds have been observed to have a high degree of bioactivity and be able to induce regeneration of non-regenerative tissues at a variety of anatomical sites.

Three specific wounds and the appropriate ECM analogs have been studied in our laboratory: skin regenerated via the DRT , peripheral nerves regenerated by the nerve regeneration template (NRT) , and the conjunctiva regenerated by a modified DRT.

As predicted, the bioactivity of the DRT and NRT has been found to be closely related to specific physical parameters of the scaffold. Scaffold mean pore size , degradation rate , chemical composition, pore orientation , and the pore volume fraction have all been shown to have a significant effect on the quality of regeneration and it appears that only a narrow range of structural features satisfies the criteria for bioactivity.

9.5. Basic parameters for in vivo regeneration studies: Reproducible, non-regenerative wounds

In vivo regeneration of injured or excised tissue can be modeled as a process taking place within a bioreactor that is surrounded by a reservoir with constant properties; the bioreactor symbolizes the entire organism with its complex homeostatic mechanisms. From an engineering standpoint, the bioreactor must have a defined and consistent anatomical and physicochemical environment for quantitative study of any biological process. In order to study induced in vivo regeneration, it is therefore critical to standardize the wound site where studies are performed. Without a standardized, reproducible wound site, it is impossible to accurately and statistically

assess differences between ECM analogs within a single laboratory or to compare results from different, independent laboratories. Billingham and Medawar introduced the concept of an anatomically constant wound for the study of massive skin injuries. This concept has been amplified and standardized; it is referred to as the anatomically well-defined woundand has been applied to the study of injuries in skin, peripheral nerves and a variety of other wound sites. The anatomically well-defined wound for studies of skin regeneration is the full-thickness skin wound where all tissue (epithelia, basement membrane, stoma) is removed down to the underlying fascia . For peripheral nerve regeneration studies, the standardized wound is complete transection (total or full axotomy) of the nerve trunk, typically at the midpoint of the sciatic nerveFor studies of cartilage repair following injury, the standardized wound model is complete removal of the cartilage down to the underlying subchondral bone . These models standardize the experimental wound environment, making it possible to obtain statistically significant results that can be compared meaningfully. However, in clinical cases, the nature of the wounds typically varies on a case-bycase basis, and it is important to understand the applicability of each treatment methodology to the range of injuries that are encountered. For example, while a tubular scaffold can be used to study peripheral nerve regeneration using the full axotomy model, a tubular implant would be difficult to implement clinically in the case of severe crush injuries that do not result in complete nerve trunk transection.

9.6. Examples of in vivo organ regeneration

9.6.1. Skin regeneration

Patients exhibiting skin wounds with loss of a substantial fraction of total body surface area (TBSA) face an immediate threat to their survival, originating primarily from the loss of their epidermis. One result of this loss is an increase by an order of magnitude of the moisture evaporation rate, which if left uncorrected, leads to excessive dehydration and shock.

Another result is a sharp increase in risk of massive bacterial infection, which if allowed to progress, frequently resists treatment and leads to sepsis. Even when patients manage to survive these immediate threats, there is a residual serious problem of quality of life, originating from the occurrence of crippling contractures and disfiguring scars due to the physiological repair process. Conventional treatment is based on use of autografting, which yields excellent results at the treatment site but which is burdened by the trauma caused at the donor site as well as by the unavailability of donor sites when the TBSA exceeds about 40-50%.

Of the three major tissue types that together comprise skin, the epidermis and basement membrane layers regenerate spontaneously following injury provided there is a dermal substrate (stromal layer) underneath. Complete regeneration of the damaged epithelia and basement membrane is typically observed following first and second degree burns, small cuts and scrapes, and blisters. However, complete skin regeneration is not observed in the case where substantial damage to the underlying stroma occurs; such cases include third-degree burns, deep cuts, and scrapes. In these cases, similar to the full-thickness, anatomically welldefined wounds created for studies of skin regeneration, organized wound contraction and scar synthesis is observed. A more in-depth description of the skin tissue triad, salient anatomical features, and its regenerative capacity has been previously published.

In addition to the clinical success of the collagen-based DRT, there are four other technologies developed to induce skin regeneration that have achieved variable levels of success that will be briefly discussed here to motivate further thought about scaffold-based options for treating severe injuries. Since its launch, this device (Integra Dermis Regeneration TemplateR) has been used with over 200,000 patients suffering skin loss around the world (Trasca T., Integra

Life Sciences, Plainsboro, NJ). In the following section, the DRT, Cultured Epithelial Autograft (CEA), Living Dermal Replacement (LDR), Living Skin Equivalent (LSE), and the Naturally Derived Collagen Matrix (NDCM, Alloderm) will be discussed.

The DRT is a collagen-glycosaminoglycan scaffold whose microstructural and materials properties have been optimized to produce a bioactive ECM analog that, when implanted, induces sequential regeneration of the underlying dermis and resultant regeneration of the basement membrane and epithelial layers. The effectiveness of the DRT has been demonstrated clinically with a population of massively burned patients and in animal experiments utilizing a full-thickness (anatomically well-defined) skin wound.

The DRT is typically used as an acellular implant that induces regeneration of the dermis, thereby providing the essential substrate for spontaneous regeneration of the epidermis and basement membrane layers. Following dermal regeneration by the unseeded DRT, epidermal cells fromthe wound edges migrate into the center of the wound and form a mature epidermis and basement membrane in a process termed sequential regeneration. The resultant regeneration of appropriate tissue layers (tissue triad) along with associated structures (i.e., rete ridges) has indicated that the DRT is capable of inducing regeneration of mature skin in a full-thickness skin wound model.

Clinically, the DRT is used as an acellular, bilayer device consisting of an inner layer of the active ECM analog and an outer layer of elastomeric poly (dimethyl siloxane). The layer of silicone is removed about two weeks after grafting the device, having served the important temporary role of controlling moisture flux and bacterial invasion at the site of organ synthesis. The DRT is fabricated from collagen-glycosaminoglycan copolymer with a 98:2 ratio of microfibrillar, type I collagen to chondroitin 6-sulfate. The microstructure of the DRT has been optimized with both a lower and upper pore size bound of 20 _ 4 mm and 125 _ 35 mm, respectively. Additionally, the biodegradation rate of the DRT has been optimized with lower and upper bounds of residence time in the wound bed of 5 and 15 days, respectively.

Clinical use of the DRT has emphasized the treatment of patients with massive burns, as well as those who require resurfacing of large or small scars from burns. The DRT is responsible for regenerating the underlying dermal architecture, providing the

appropriate stromal layer upon which native regenerative processes of the basement membrane and epithelial layer can take place. For wounds of relatively small characteristic dimension, e.g., 1 cm, epithelial cells migrating at speeds of order 0.5 mm/day from the wound edges can provide a confluent epidermis within 10 days. In such cases, the unseeded DRT fulfills all the design specifications set for successful in vivo skin regeneration. However, the wounds incurred by a massively burned patient are typically of characteristic dimension of several centimeters, often more than 20e30 cm. These wounds are large enough to preclude formation of a new epidermis by cell migration alone within a clinically acceptable time frame. Current clinical protocol favors harvesting and use of a very thin autoepidermal graft from another part of the patient's body, thin enough not to leave behind a scarred donor site, to cover the neodermis , or application of a CEA (described below) on top of the DRT following removal of the poly(dimethyl siloxane) membrane.

A keratinocyte-seeded DRT has been used in guinea pig and swine animal models; the keratinocyte-seeded DRT induces simultaneous formation of a dermis as well as the epidermal and basement membrane layers and removes the requirement for epithelial cell-mediated migration from the wound edges for successful skin regeneration. Although the keratinocyteseeded DRT induces simultaneous regeneration of skin in the guinea pig model, an animal model characterized by extensive organized wound contraction that is significantly more severe than that observed with humans, thesum total of the results of studies using theDRTsuggest that in vivo synthesis of skin in the clinical setting does not require anything more than the DRT .

CEAs are an epidermal graft formed from a sheet of keratinocytes grown in vitro and then implanted into the wound site. A small epidermal sample is removed from the patient, dissociated, and the resultant cells are then cultured in vitro until they form an epithelial membrane. However, in the case of full-thickness skin wounds where there is no underlying dermis to support the epidermal sheet, rapid CEA degradation is

typically observed and long-term clinical applicability has not been observed; in particular, one persistent problem is the formation of blisters under large areas of the graft (avulsion). However, in the case of partial-thickness wounds where a significant portion of the stroma remains, the 'take' of the CEA graft has been very good. As such, the CEA has been used to cover significant areas, as large as 50% of the TBSA, for both partial-thickness and as a temporary solution for fullthickness wounds to prevent immediate wound dehydration and infection. Additionally, regardless of whether the graft was placed on a partial- or full-thickness skin wound, the resulting CEA graft exhibited mechanical fragility due to a lack of the 7-S domain of type IV collagen, anchoring fibrils, and rete ridges. These structures, required for the formation of a physiological dermo-epidermal junction, as well as the formation of a physiological collagen and elastin fiber architecture observed in the normal, adult dermis are not formed after grafting of the wound site with the CEA. Without these structures, the CEA cannot be used as a permanent skin replacement; instead, the CEA is often used as a temporary coverage as part of a more substantial treatment regimen such as autografting or DRT implantation.

The LDR is a polyglactin-910 nylon surgical mesh scaffold fabricated from a copolymer of 90 wt% glycolic acid and 10 wt%lactic acid termed PGL that was developed to be an analog of the ECM and induce skin regeneration following injury. The PGL fibers, approximately 100 mm in thickness, were knitted into a mesh that exhibits a pore microstructure with a characteristic dimension of 280e400 mm. This mesh structure presented a large-weave structure, relative to the characteristic cell dimension of approximately 10 mm that allowed rapid cell incorporation and ample nutrient diffusion. Prior to implantation, the acellular PGL mesh was cultured in vitro with fibroblasts until the cells were observed to synthesize several important ECM components such as collagen and elastin in vitro. Immediately prior to implantation, the upper surface of the PGL scaffold was seeded with keratinocytes to produce a bilayer graft. Once the keratinocytes reached confluence on the surface of the PGL

mesh, the entire structure was grafted into the wound site. A thin, fragile epidermal layer typically developed by 10 days post-grafting and became cornified as early as 20 days post-grafting. Additionally, by 20 days post-grafting the LDR scaffold had degraded completely with minimal inflammatory response. While the interface between the graft and the wound bed stained positive for laminin, consistent with the synthesis of a lamina lucida layer, no other component of the basement membrane was synthesized. In addition, rete ridges were not synthesized and a thick layer of fibrotic tissue with a large fibroblast population and vascular in-growth, characteristic of scar tissue formation, was observed below the newly synthesized epidermal layer. In summary, while the LDR showed the ability to induce regeneration of a neo-epidermis, it did not exhibit the ability to induce regeneration of a complete basement membrane or dermal layer.

The LSE was formed by populating a collagen lattice with dermal fibroblasts that in vitro contracted the lattice and synthesized additional ECM proteins, forming a neo-dermal layer. Similar to the LDR, after the initial culture period, the upper surface of the neo-dermal layer was seeded with a suspension of keratinocytes. Once seeded, the keratinocytes attached to the collagen construct, proliferated, and differentiated to form a multilayered, epidermal structure within one to two weeks, all in vitro. Although short segments of the lamina densa were observed along the dermo-epidermal convergence in vitro, the cultured LSE did not exhibit a complete basement membrane layer, Rete ridges, or any other skin appendages at the end of the in vitro culture period. At this point, the construct was then implanted into the wound site. Continued structural and biological changes were observed in the LSE following grafting, indicating that remodeling was taking place. A functional, fully differentiated epidermis was observed as early as seven days following grafting, and by fourteen days a vascularized subepidermal layer with many of the structural characteristics of normal dermis, such as a ’basketweave’ collagen fiber pattern, was present. However, continued maturation into a formalized dermal region was not observed. Experimental

results across multiple animal models and experimental trials were consistent in indicating that the LSE was able to induce regeneration of a mature epidermis and basement membrane, but a mature dermal layer was not observed and the dermo-epidermal junction remained flat. Instead of relying on technologies to fabricate a three-dimensional scaffold structure from either synthetic or natural materials (i.e., LSE, LDR, DRT), the NDCM technology uses decellularized dermal tissue as a scaffold to induce skin regeneration. Decellularized matrices were explored as platforms that possess favorable mechanical properties for surgical handling, and which contained a surface chemistry in many ways similar to that of the native

ECM. The main advantage of the NDCM over a homograft (cellularized dermal tissue from a human donor) and xenograft (cellularized dermal tissue from an animal donor) is that owing to decellularization, the antigenicity of the scaffold is significantly reduced, thereby eliminating the incidence of rejection. The NDCM has been used primarily to treat fullthickness burns and burns to areas of the skin where contraction and scar formation would inhibit functionality (i.e., feet and hands). The NDCM is typically implanted into full-thickness skin wounds and is often covered by a thin autograft of the patient's own epidermis to speed the healing process. This treatment, similar to that observed with the use of the DRT, results in a high percentage of graft take; additionally, the thin autograft of epidermal tissue significantly decreases the time for complete graft re-epithelialization. Patients showed normal range of motion, grip strength, motor control, and functionality along with formation of appropriate anatomical structures (i.e., rete ridges) following treatment, demonstrating the utility of a naturally derived material in tissue-engineering applications.

9.6.2. Peripheral nerve regeneration

The mammalian peripheral nervous system also consists of a distinct tissue triad. Thousands of axons make up a nerve trunk where each axon is surrounded sequentially by an epithelial, basement membrane, and stromal layer. Schwann cells wrap around the individual axon, forming the myelin sheath that constitutes the cell-continuous, epithelia. Surrounding the epithelia is the acellular ECM layer (basement membrane) connecting the myelin sheath and the stromal layer . The endoneurium is the stromal layer of cells, blood vessels, and ECM that surrounds, insulates, and protects all nerve fibers. Similar to that observed with skin, the myelin sheath (epithelia) and basement membrane regenerate spontaneously following injurywhile the endoneurium (stromal layer) does not.

9.7. General findings

While there have been a large number of studies investigating many different device designs, few have been shown to perform as well as the peripheral nerve autograft (typically the sural nerve from the leg), and none appear to have been able to improve over the autograft for gaps larger than 10mm. For studies of peripheral nerve regeneration, complete axotomy (total severance of the nerve trunk) was utilized as the standardized wound model. However, the large number of assays and experimental arrangements employed by independent investigators has made it difficult to effectively compare experimental outcomes. A new methodology has recently been formalized to standardize this comparison, allowing for effective assessment of the quality of peripheral nerve regeneration. This technique compares the frequency of reinnervation (%N) across the experimental gap. The frequency of reinnervation across a gap is reported as percent of nerve trunks fitted with an experimental chamber where axons are observed to bridge the gap between the transected stumps; %N has been used by a large number of investigators as a dimensionless measure of nerve regeneration and, when not reported directly, can often be calculated from data presented by individual investigators.

The investigator reported %N can be normalized to correct for the effect of changes in gap length, animal species, and experimental chamber; the normalization process calculates the critical axon elongation length (Lc) , and is based on analysis of data from an early study which showed that a relatively small increase in the gap length bridged by a silicone tube is followed by a sharp drop in %N . Data from this and several other reports have been used to construct a sigmoidal curve, termed a 'characteristic curve', for %N versus gap length (L) for the silicone tube configuration. The critical length (Lc) is a single quantitative parameter that describes the effectiveness of a given nerve repair device in promoting nerve regeneration, and is calculated as the length where the particular device results in 50% reinnervation (and 50% formation of neuroma). Reinnervation at the midpoint is used in this calculation rather than reinnervation at the distal stump because elongating axons reach the gap midpoint faster than the distal stump so midpoint data reach a time-independent state earlier than distal stump data. Given the widespread use of the unfilled silicone tube in studies of rat sciatic nerve defects, its characteristic curve (Lc ¼ 9.7 _ 1.8 mm) is often used to define a laboratory standard for all comparisons, allowing comparison of other experimental devices to the silicone standard by comparing their respective characteristic curves and Lc.

Using the silicone tube or any other well-defined construct as a control, it is possible to estimate the length shift, 6L, the difference between values of Lc for the experimental chamber and that of the control. 6L can be used as a simple measurement of relative regenerative activity exhibited by any device. Data obtained from different animal models can also be compared by normalizing each species result to the silicone tube standard tested in most animal models.

9.8. Effect of tubulation

Peripheral nerve regeneration is not observed in the absence of a tubular device connecting the two ends of a transected nerve stump if the gap between the two transected stumps is greater than a few millimeters. It has also been observed that

insertion of the distal stump into the tube is required for successful regeneration; this result indicates that even though axon elongation takes place from the proximal to the distal stump, the distal stump appears to provide a critical cytokine field responsible for guiding axon elongation (neurotrophic effect).

However, while it has been observed that a tube is all that is required to induce regeneration across a gap of modest length following complete transection, the physical parameters of the tube and any material in the tube lumen significantly affect the kinetics and quality of regeneration; these effects will be discussed in the remaining sections of this review.

9.9. Effect of tube chemical composition

A variety of natural and synthetic polymers have been used in peripheral nerve regeneration conduits, and the choice of tube (conduit) composition has been found to significantly affect the quality of regeneration; for a complete review of the literature, please consult previous publications by the authors. Early conduits were produced from naturally occurring materials that were easily harvested and implanted such as bone, dura, perineurium, and blood vessels. More recent devices have utilized non-degradable, synthetic materials such as stainless steel, rayon, and silicone as well as degradable, synthetic polymers such as polyester, polyglactin, and polylactate, and natural polymers such as collagen, laminin, and fibronectin.

Non-degradable tubes have typically resulted in a poor quality of regeneration marked by significant formation of a neural scar (neuroma). Conversely, conduits fabricated from

ECM components, specifically collagen, fibronectin, and laminin, have been generally shown to enhance the quality of peripheral nerve regeneration. Collagen tubes in particular have been observed to induce the highest quality of regeneration, as measured by both morphological and electrophysiological methods. It has been hypothesized that this optimal chemical composition was observed because collagen

tubes contain inherently bioactive binding sites (ligands) for attachment and migration of various cell types, and can be manufactured in such a way that the tube wall pore structure can display a range of cell and protein permeabilities. Additionally, the degradation rate of the collagen tubes can be tailored to degrade with half-lives that vary over a very wide range to meet the requirements of the isomorphous tissue replacement model.

9.10. Effect of tube permeability

Conduit permeability significantly affects the mechanism and quality of peripheral nerve regeneration. Tube permeability can be defined by its initial structural features (i.e., scaffold porosity, pore size) or its degradation characteristics (i.e., rapid degradation quickly permeabilizes the tube). Additionally, the conduits may be protein permeable but cell impermeableor cell and protein permeable. No appreciable increase in the quality of peripheral nerve regeneration was observed for the protein permeable and cell impermeable conduit compared to the impermeable conduit. However, cell (and therefore protein) permeable tubes exhibited significantly superior regenerative capacity compared to impermeable as well as protein permeable and cell impermeable conduits . Device permeability also significantly influences the contractile response following peripheral nerve injury; permeable collagen tubes display a significantly thinner contractile capsule surrounding the regenerating nerve trunk than biodurable silicone tubes. It is hypothesized that device permeability reduces myofibroblast-mediated contraction of the wound site by permitting migration of the contractile cells away fromthe wound site through the tube walland by allowing connective tissue cells from the surrounding environment access to the tube lumen.

9.11. Effect of tube degradation rate

A biodurable conduit, such as a silicone tube, initially induces partial reinnervation between proximal and distal stumps following implantation. However, as the initially regenerated nerve trunk remodels and matures, the silicone tube constricts this process, resulting in pain and eventual degeneration of the regenerated

nerve; prevention of the ultimate degeneration of the initially regenerated nerve trunk requires a difficult second surgical procedure that can further harm the nerve trunk. For this reason, one of the historical goals in studies of peripheral nerve regeneration studies is identification of a suitable degradable conduit. Superior performance of biodegradable tubes compared to tubes made from materials that were either biodurable or which had a very low degradation rate has since been reported in studies by several investigators. Examples include comparison of tubes made of two biodurable polymers, silicone and poly (tetrafluorethylene), and two degradable polymers, a copolymer of poly (lactic acid) and 3-caprolactone (PLA/PCL) as well as collagen This study indicated that the degradable tubes induced a higher quality of regeneration compared to the biodurable tubes over a 6 mm gap in the mouse sciatic nerve. Additionally, a number of degradable collagen tubes have been shown to perform as well as the autograft, considered to be clearly superior to regeneration via a silicone tube and commonly thought to fall short functionally only of physiological (pre-injury) nerve .

While the regenerative advantage of a degradable tube versus a biodurable tube is supported by the evidence, there have not been many extensive studies on the effect of the magnitude of the degradation rate and whether an optimal degradation rate exists as has been found in the study of ECM analogs (the DRT) to induce skin regeneration. Recently, one of the first comprehensive studies of the effect of the degradation rate of collagen tubes on the quality of peripheral nerve regeneration has been published by these authors; this study utilized a 15mm gap in the rat sciatic nerve and evaluated the regenerative capacity of a homologous series of porous, collagen tubes, showing a significant effect of degradation rate on the quality of peripheral nerve regeneration. The chemical composition, pore structure, and permeability of the conduits in this study were kept constant while the crosslink density was steadily increased to create a series of five devices with in vivo degradation half-lives varying between <1 week to >100 weeks, The quality of peripheral nerve regeneration was

observed to vary significantly with tube degradation rate; the highest quality of peripheral nerve regeneration was observed for tubes with a degradation half-life of two to three weeks . This data suggests that the positive effects of tubulation in treating peripheral nerve injuries are due to the presence of the tube immediately following injury; tubulation appears to significantly affect the early mechanisms of peripheral nerve regeneration. A speculative view of the maximum regenerative activity observed in the present study can be based on the putative existence of a lower and higher limit in tube degradation rate similar to that observed for the DRT. When the degradation rate of the collagen tubes is excessively slow, the tubes behave as if they were biodurable, remaining intact long enough to interfere with tissue remodeling. In contrast, tubes that degrade very rapidly fail to maintain a protected environment for regeneration that is the basis for the use of tubulation to promote nerve regeneration.

9.12. Effect of tube filling

A wide variety of solutions, ECM analogs, and cell suspensions have been introduced into the tube lumen in an effort to improve the quality of peripheral nerve regeneration. Use of ECM macromolecules, such as collagen, laminin and fibronectin, in both solution and gel form, has been observed to have no significant effect on peripheral nerve regeneration; furthermore, when gel concentrations exceeded certain critical levels, a negative effect on the quality of regeneration is observed. However, a laminin-coated collagen-based scaffold has been observed to improve the quality of regeneration, implying the requirement of an insoluble structure within the tube lumen in addition to any soluble regulators to improve the quality of regeneration.

Several insoluble substrates, ECM analogs inserted into the empty lumen of the tube prior to implantation, have shown significant regenerative activity. Examples include highly oriented fibrin fibers and axially oriented polyamide filaments that significantly improved the quality of regeneration. Specific ECM analogs have been observed to significantly increase the maximal gap length that can be bridged by axonal tissue, the speed of axonal bridging, and the quality of regeneration.

The ECM analog that has been found to induce the highest quality of regeneration is a collagen-based scaffold termed the NRT. The NRT has induced regeneration of a functional peripheral nerve across gaps varying from 10 to 15 mm in the rat sciatic nerve

Like the DRT, the microstructural and material properties of the NRT have been optimized. The highly bioactive NRT is characterized by axially (extending between the proximal and distal stumps) elongated pore tracks defined by axially oriented ellipsoidal pores with a mean pore size of approximately 35 mm. This pore structure is hypothesized to improve the quality of peripheral nerve regeneration by providing directional guidance to the formation of linear Schwann cell columns which act as tracks for axon elongation (microtube hypothesis). The positive effect of an axially oriented fiber structure has also been observed using a fibrin fiber-based ECM analog in the tube lumen42]. The degradation rate of the NRT has also been found to significantly affect the quality of regeneration, with an in vivo degradation half-life on the order of six weeks found to be optimal; NRT variants that degraded too rapidly or too slowly led to significantly poorer functional recovery . The long-term morphological structure and electrophysiological function of nerves regenerated using the NRT has been observed to be at the level of an autografted nerve, the current gold-standard for peripheral nerve injury treatment.

Soluble regulators and cell suspensions have also been introduced into the tube lumen in an effort to improve the quality of regeneration. Suspensions of Schwann cells showed very significant regenerative activity, further supporting the microtube hypothesis which describes nerve regeneration as dependent on early formation of linear columns of Schwann cells extending from the proximal towards the distal nerve trunk. In addition to the use of a cell suspension, solutions of acidic (aFGF) and basic fibroblast growth factor (bFGF) were also observed to improve the quality of regeneration. However, the use of nerve growth factor (NGF) was not found to improve the quality of regeneration. A variety of conclusions have been drawn about

the relative efficacy of the various biomaterials and devices employed in the study of peripheral nerve regeneration. Nerve chamber configurations that had the highest regenerative activity were those in which the tube wall comprised certain synthetic biodegradable polymers such as collagen, was cell-permeable rather than protein permeable or impermeable, and had an in vivo degradative half-life on the order of 2 to 3 weeks. Introduction of an insoluble regulator (ECM analog in the form of a scaffold) into the tube lumen, but not of ECM components in a gel or solution form, also significantly improves the quality of regeneration. The optimal ECM structures were found to be highly porous with controlled degradation rates and an axially aligned microstructure. In addition, suspensions of Schwann cells as well as solutions of either acidic or basic fibroblast growth factor placed within the tube lumen with or without an insoluble ECM analog have been shown to improve the quality of regeneration.

Cartilage and fibrocartilage disk tissue-engineering applications Articular cartilage contains an avascular, non-neural ECM composed primarily of type II collagen and glycosaminoglycans. Compared to other tissues, cartilage possesses a very low cell density and a very high ECM density; the chondrocytes populating the cartilaginous ECM display low proliferative activity and, due to the high ECM protein density, are unable to migrate through the tissue. Due to the low proliferative activity, avascularity, and high ECM protein density, injuries to cartilage display an injury response distinct from traditional injury responses characterized by inflammatory processes, cell-mediated contraction, and scar synthesis. No repair or regeneration processes are observed in vivo following cartilage injuries; instead, the scope of injury increases as the wound edges become increasingly degraded, eventually compromising the joint . Severe cartilage injuries are extremely prevalent in today's active society, resulting in pain, decreased patient activity, and eventually disability, profoundly impacting quality of life. The current methods for treating such focal cartilage defects include microfracture, autologous chondrocyte implantation, and osteochondral

autografting. In the case of microfracture and autologous chondrocyte implantation, a flap of periosteum is sewn over the cartilage defect area and is sealed with fibrin glue. With microfracture, immediately prior to application of the periosteal flap, microfractures in the subchondral bone are created to allow bone marrow cells access to the then-sealed cartilage defect in an attempt to utilize the stem cell population in the bone marrow to regenerate the damaged cartilage. With autologous chondrocytes implantation, a biopsy of cartilage tissue is removed from the patient prior to surgery and cultured in vitro to obtain a large chondrocyte population that is then injected back into the periosteal flap sealed cartilage defect. In the case of osteochondral autografting, a series of osteochondral plugs consisting of cartilage, the underlying subchondral bone, and the tidemark region separating them are removed from a non-loading region at the edge of the damage joint. These plugs are then implanted into the primary cartilage defect using an approach termed mosaicplasty, named after the mosaic pattern of the implanted osteochondral plugs. These procedures, however, exhibit limited long-term success in treating the cartilage injury. Preclinical studies implementing tissue-engineering approaches have yielded results that represent improvements over the currently employed cartilage repair procedures.

Various synthetic and natural materials have been employed to fabricate porous, bioresorbable scaffolds for articular cartilage tissue engineering. Among the list of bioresorbable or partially resorbable materials used for cartilage repair are collagen, hyaluronan, fibrin, polylacticacid (PLA) and polyglycolic acid (PGA), and chitosan scaffolds and gels, devitalized cartilage, hydroxyapatite, demineralized bone matrix, and bioactive glass . Natural polymers such as collagen provide a more native surface to cells, and have been the primary focus of tissue-engineering studies due to previous successes in peripheral nerve and skin regeneration.

Studies have confirmed that the addition of cells seeded within these 3D scaffolds enhance matrix synthesis and increase type II collagen production in vivo and in vitro

There are various cell types that may be used to enhance cartilage synthesis when seeded into matrices, including articular chondrocytes and chondroprogenitor cells derived from bone marrow, periosteum, or perichondrium.

Preliminary investigations have been focused on the bioactivity of chondrocytes in a series of ECM analogs. Promising results have been observed for adult articular chondrocytes cultured in vitro in type II CG scaffolds, where the chondrocytes have retained high biosynthetic capacity for producing type II collagen, the predominant ECM component of cartilage.

Type II CG scaffolds seeded with autologous, articular chondrocytes have also been evaluated for their in vivo regenerative capacity in a full-thickness cartilage injury model; the full-thickness cartilage injury, with cartilage removed down to the subchondral bone, is the standardized, anatomically well-defined wound utilized for in vivo studies of cartilage regeneration.

The greatest total amount of reparative tissue was found in the cell-seeded type II CG scaffolds as opposed to unseeded type II CG scaffolds and seeded or unseeded type I CG scaffolds. Moreover, examination of the reparative tissue formed in the subchondral region of defects treated with the chondrocyte-seeded type II collagen scaffolds indicated that the majority of the tissue was positive for type II collagen and that good integration was observed between the implant and the surrounding cartilage. These results indicate an influence of the exogenous chondrocytes on the process of chondrogenesis . Such studies of the healing of chondral defects in animal models have revealed that there is some potential for regeneration of this connective tissue. The introduction of certain biomaterial scaffolds along with selected surgical procedures and cell therapies has been demonstrated in animal studies to facilitate the cartilage reparative process and now offers the promise of extending the longevity of clinical treatments of cartilage defects. Recently, CG scaffolds populated with.

TGF-b1 transfected meniscus cells were used to successfully fill avascular zone meniscus lesions with repair tissue. Other work has compared type I and type II CG scaffolds for intervertebral disk tissue-engineering applications, finding that type II CG scaffolds were preferential to type I on the basis of cell number as well as protein and GAG synthesis .

In a separate series of studies, the regenerative potential of chondrocytes encapsulated in photopolymerized poly(ethylene oxide) hydrogels and self-assembling peptide hydrogels gels have been tested using an in vitro culture model followed by in vivo implantation. Preliminary results suggest that photocrosslinkedand self-assembling hydrogels are promising scaffolds for tissue-engineering cartilage as cell viability was maintained, uniform cell seeding was achieved, the biochemical content of the ECM proteins synthesized within the construct were similar to those found in native cartilage . Additionally, the importance of the biomechanical environment during in vitro culture of chondrocytes within a threedimensional construct has been observed; improved biosynthesis of ECM components, notably native proteoglycans, is observed when a cyclic loading environment is applied to in vitro chondrocytes cultures within a series of hydrogel and scaffold constructs.

Collectively these findings provide the basis for the rational development of approaches for the more complete regeneration of articular cartilage, and demonstrate that meaningful clinical outcomes can be achieved even if complete regeneration is not achieved.

- ***Bone, osteochondral regeneration applications***

In addition to solidification-induced structural modifications, the CG construct can be chemically modified to create collagen composites. Mineralized CG scaffolds are of particular interest for orthopedic applications due to their potential to mimic the native biochemistry of bone: interpenetrating collagenous matrix (organic) and CaP (mineral) content.

The addition of a mineral phase to the classic CG scaffold archetype allows for the development of materials with the requisite biochemical and biomechanical properties for bone and osteochondral tissue engineering. Mineralized CG scaffolds have been created via two distinct mechanisms: coating a fully formed CG scaffold, or synthesizing mineralized CG chemistries prior to scaffold fabrication. Mineralized collagen-GAG have been fabricated via a surface coating process . Here, CG scaffolds were initially synthesized using conventional freezedrying and then sequentially soaked in phosphate (NaNH4HPO4) and calcium (CaCl2) solutions. This treatment improved construct compressive modulus nearly 70-fold, from 0.3 kPa to 31 kPa, while maintaining high porosity (95%) . Alternatively, CGCaP scaffolds have been fabricated from a triple co-precipitate suspension of collagen, GAG, and calcium phosphate (CaP).

The CGCaP scaffold technology has been used as the basis for creating multiphase collagen scaffolds for the repair of interfacial tissues, notably osteochondral defects. The multicompartment scaffold was designed to be a single biomaterial construct containing multiple regions (’compartments’), each with distinct microstructural, chemical, and mechanical properties that are connected via a continuous interface between regions. The initial multiphase scaffold developed via this approach contains an osseous compartment for subchondral bone regeneration (type I collagen, chondroitin 6-sulfate, and CaP) and a cartilaginous compartment for cartilage regeneration (type II collagen and chondroitin 6-sulfate). The continuous interface can be created by layering the cartilaginous compartment and the osseous compartment suspensions in a conventional freeze-drying mold, but then incorporating a processing step to enable partial diffusive mixing between the two suspensions near their interface. After forming an interdiffusion zone, freeze-drying was used to form the final multicompartment scaffold microstructure. This approach can reduce some complications observed in layered scaffolds with abrupt interfaces including delamination, foreign body contamination (from glue or other adhesive), and

inefficient cellular transport between scaffold phases. The differential chemistry, microstructure, and mechanics of the osseous and cartilaginous compartments enable these layered scaffolds to exhibit compressive deformation behavior that mimics behavior observed in natural articular joints

These layered scaffolds are currently the subject of numerous in vitro and in vivo experiments for various orthopedic tissue-engineering applications. This scaffold system has shown improved healing of osteochondral defects in a caprine modeland is currently undergoing Phase I clinical trials for primary and secondary (backfill of traditional mosaicplasty harvest sites) osteochondral defects in the knee. The developed technologies and techniques may hold promise for the regeneration of not only osteochondral defects, but also other orthopedic interfaces such as the osteotendinous insertion.(75)

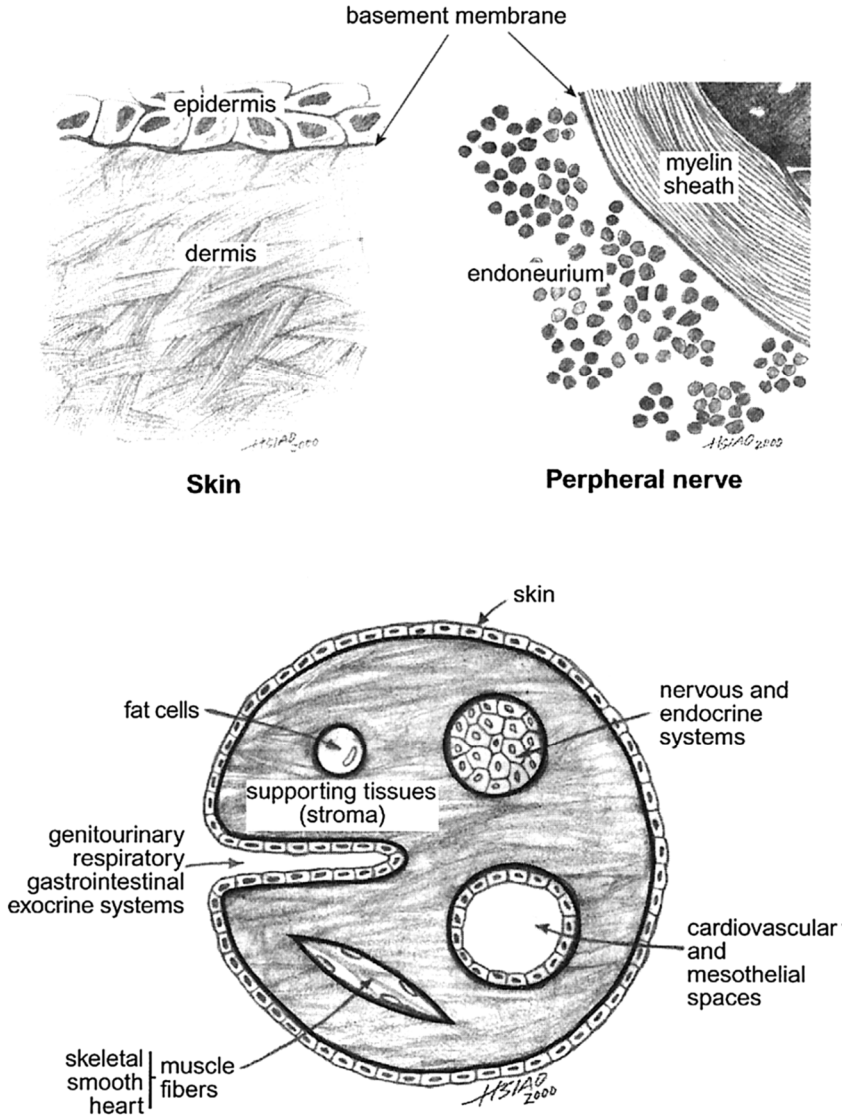

Figure 26: Schematics of the tissue triad structure observed in mammalian tissue.

Top: Tissue triad of skin and peripheral nerves. The basement membrane is a thin extracellular matrix tissue located between the cellular and non-vascular epithelia (epidermis, myelin sheath) and the cellular, vascularized stroma (dermis, endoneurium). Bottom: Diagram of the distribution of epithelial, basement membrane, and stromal tissues in the mammalian system. Examples of stromal tissues are bone, cartilage, and their associated cell types as well as elastin and collagen. Examples of epithelial tissues are those covering the surface of the genitourinary, respiratory, and gastrointestinal tracts as well as surfaces of the mesothelial cells in body cavities, muscle fibers, fat cells, and endothelial cells in the cardiovascular system [1].

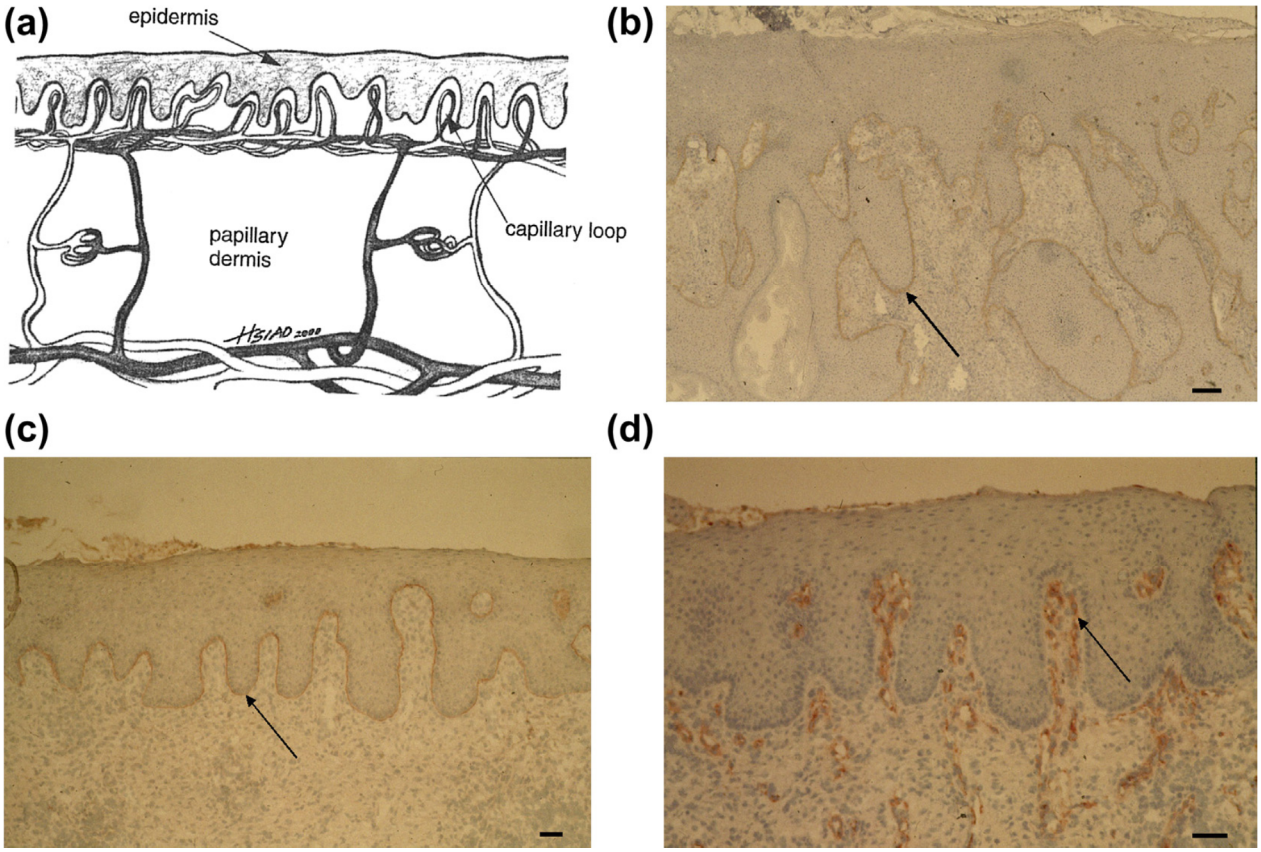

Figure 27: Diagram of normal skin showing the characteristic rete ridges at the dermo-epidermal junction and the vascular network (capillary loops) that populates the subepidermal region (75).

As early as 12 days after grafting a full-thickness skin wound with a keratinocyte-seeded DRT, anchoring fibrils were observed in the regenerating basement membrane (arrow). The basal surface epithelium and the periphery of the epithelial cords are labeled with type VII collagen immunostaining, identifying the anchorage structures at the dermo-epidermal interface. Bar: 150 mm (b). As early as 35 days after grafting a full-thickness skin wound with a keratinocyte-seeded DRT, a confluent hemidesmosomal staining pattern is observed at the dermo-epidermal junction (arrow) by immunostaining for the a6b4 integrin. The pattern observed in the regenerating skin is identical to that observed in physiologic skin. Bar: 100 mm (c). A full-thickness skin wound grafted with a keratinocyte-seeded DRT was observed to regenerate many of the structure observed in normal skin. Immunostaining for Factor VIII 35 days after grafting revealed that capillary loops had formed in the rete ridges of the regenerated dermis (arrow) similar to those observed in physiologic skin. Bar: 75 mm. (d) [100].

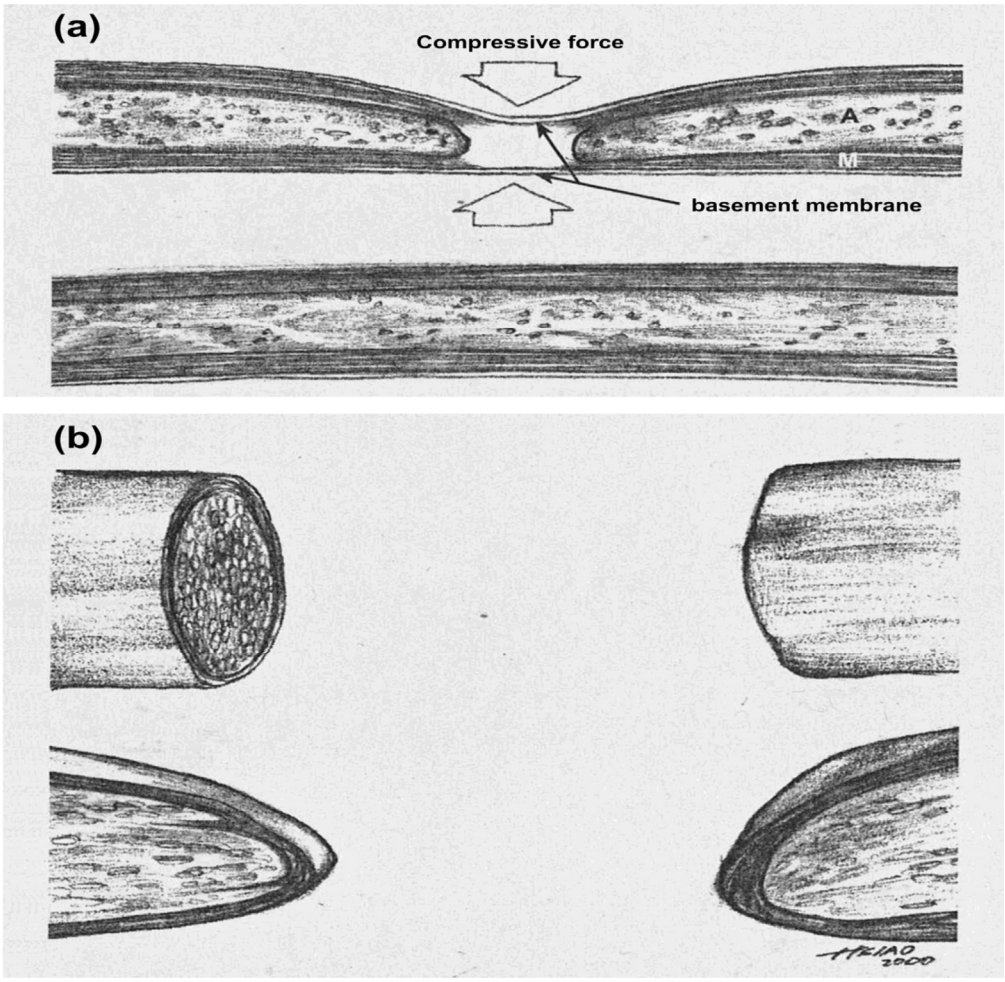

Figure 28: Axons and myelin sheath inside a nerve fiber are regenerative.

Following mild crush injuries, the axoplasm (A) and myelin sheath (M) degenerates along the length of the crushed nerve fiber, but the basement membrane remains intact throughout. Spontaneous regeneration of the nerve fiber occurs within a few weeks of injury (a). Most of the supporting tissues (stroma) surrounding nerve fibers are not regenerative. Although axons are regenerative following transection, the remainder of the nerve trunk is not. Following transection, each stump is closed via cellmediated contraction and neuroma synthesis (b) [1].

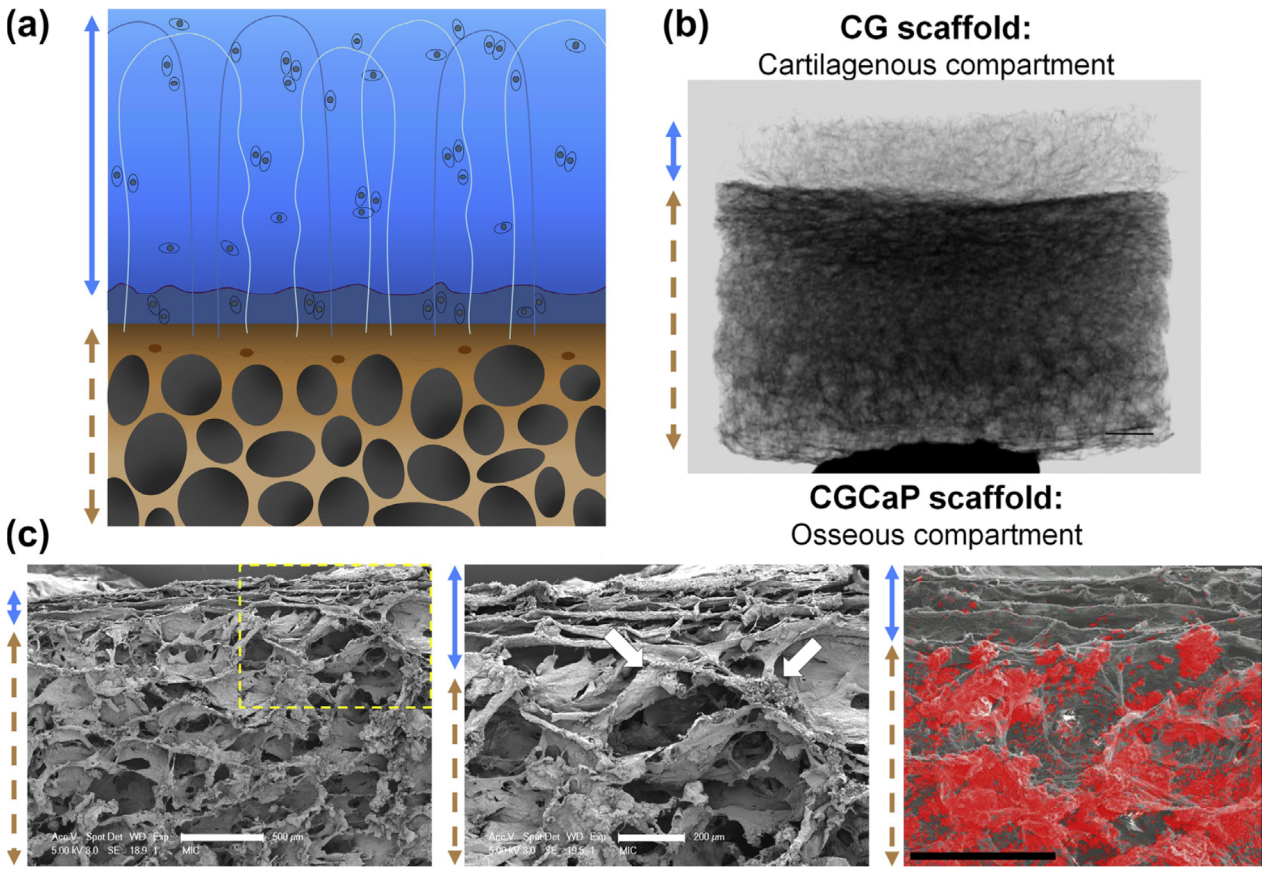

Figure 29: Structure of the natural articular joint

a) Structure of the natural articular joint showing articular cartilage and subchondral bone joined by a continuous interfacial region. b) X-ray mCT image of the layered osteochondral scaffold showing distinct cartilaginous and osseous compartments (scale bar 1 mm). c) Scanning electron microscope (SEM) images of the osteochondral scaffold showing the complete scaffold microstructure (left; scale bar 500 mm), and the interfacial region (middle; scale bar 200 mm) showing continuity between the osseous (tan dashed arrow) and cartilaginous (blue solid arrow) compartments including collagen struts extending across the transition (white arrows). No regional areas of delamination or debonding are observed between the compartments. Distribution of Ca mineral (P similar but not shown) content (red shading) superimposed over an SEM image of the osteochondral scaffold showed distinct mineralized (high CaP content, tan dashed arrow) and non-mineralized (low/zero CaP content, blue solid arrow) layers (right; black scale bar 400 mm). Reprinted with permission from.

Control of the cellular environment is crucial for understanding the behavior of cells and for engineering cellular function. We summarize examples where these tools have helped to control the microenvironment of cells, and have been useful in solving problems in fundamental cell biology. The methods described here are experimentally simple, inexpensive, and well suited for patterning biological materials. How do tissues assemble in vivo? How do cells interact with each other in tissues? How do cells respond to stimuli? How do abnormal stimuli give rise to pathological conditions? Answering these fundamental biological questions, and using the information thus obtained for medical applications, requires understanding the behavior of cells in well-controlled microenvironments.

Many of the challenges in trying to control the environment experienced by individual cells lie in the relevant scales of size, as well as the character of the stimuli. These scales of size range from angstroms (for molecular detail), through micrometers (for an individual cell), to millimeters and centimeters (for groups of cells); the types of stimuli that must be addressed include the molecular composition of the liquid in which the cell is immersed, the topographical and chemical composition of the surface to which the cells attach, the nature of neighboring cells, and the temperature. Researchers have used a number of techniques to pattern cells and their environment.

Before the 1990s, the most commonly used technique was photolithography, used in biological studies with varying degrees of success. Examples include topographical features that confine the growth of snail neurons to silicon chips, as first demonstrations of interfacing natural computation with artificial ones. However, photolithography is not a technique best suited for biological studies. It is an expensive and time-consuming technology; it is poorly suited for patterning non-planar surfaces; it provides too little control over surface chemistry to pattern sufficiently diverse types of biomolecules on surfaces; it is poorly suited for patterning materials such as hydrogels; the equipment required to use it is rarely routinely accessible to biologists; and it is directly applicable to patterning only a limited set of photosensitive materials (e.g., photoresists).

Microfabrication and micropatterning using stamps or molds fabricated from elastomeric polymers (’soft lithography’) provide versatile methods for generating patterns of proteins and ligands on surfaces, micro-scale chambers for culturing cells, and laminar flows of media in capillaries, all in the 0.1e100 micrometer size range . Soft lithographic methods are relatively simple and inexpensive. The elastomeric polymer most often used in these procedures e polydimethylsiloxane (PDMS) e has several characteristics (optical transparency, ease in manipulation, and low cost) that make it attractive for biological applications

. As a new technology, soft lithography is being increasingly used in cell biology, due to its biocompatibility, simplicity, and adaptability to biological and biochemical problems. Different types of cells make up tissues and organs hierarchically, and communicate within a complex, three-dimensional environment. It has become evident that 3D cell culture provides more physiological cellular environments compared to conventional 2D cell cultures.

Culturing cells in hydrogel has been a widely used method, since the architecture and chemical composition of hydrogels can be easily engineered. However, previous hydrogel cultures lack precise cell positioning and reversibility, which are important characteristics in engineering cellular microenvironments.We have reported a simple reversible hydrogel patterning method for 3D cell culture. In a pre-gelled alginate solution, calcium is chelated by DM-nitrophen (DM-n) to prevent crosslinking. After sufficient UV exposure, the caged calcium is released from DM-n, causing the alginate to cross-link. By exposing a pre-gelled solution to UV selectively, we formed an alginate gel in specific regions of a microfluidic device through the light-triggered release of caged calcium . Since the amount of crosslinking is based on the calcium concentration, the cross-linked alginate can easily be dissolved by EDTA, thus releasing the patterned cells for further analysis.

The density of cells in real tissues is about 108e109 cells cm_3 which is difficult to recapitulate in most present in vitro models, due to the difficulty of replicating the

spatial distributions of oxygen, metabolites, and signaling molecules in tissues. To tackle this problem, we developed a device for the 3D culture of mammalian cells in microchannels by combining microfluidicsand soft lithographic molding of gels containing mammalian cells. Collagen or Matrigel_made up the matrix of each module of cell-containing gel. Each module had at least onedimension below ~300 mm. The flux of oxygen, nutrients, and metabolic products into andout of the modules was sufficient to allow cells to proliferate to densities comparable tothose of native tissue. Multiple cell types with high survival ratios (99%) could be spatiallyorganized in the microfluidic channels. We also stacked and destacked layers of paperimpregnated with suspensions of cells in ECM hydrogel ('cells-in-gels-in-paper' or CiGiP) to make it possible to control oxygen and nutrient gradients in 3D and to analyze molecular and genetic responses. Stacking assembles the 'tissue', whereas destacking disassembles it, and allows its analysis. The study offers a uniquely flexible approach to study cell responses to 3D molecular gradients and to mimic tissue- and organ-level functions. In vivo, cells in different locations of 3D tissues are physiologically different, because they are exposed to different concentrations of oxygen, nutrients, and signaling molecules, and to other environmental factors (temperature, mechanical stress, etc). The majority of high throughput assays based on 3D cultures can only detect the average behavior of cells in the whole 3D construct. Isolation of cells from specific regions of 3D cultures is possible, but relies on low-throughput techniques such as tissue sectioning and micromanipulation. To tackle this problem, we developed a simple method for culturing arrays of thin planar sections of tissues, either alone or stacked to create more complex 3D tissue structures based on the 'cells-in-gelsin-paper' approach. The procedure starts with sheets of paper patterned with hydrophobic regions that form 96 hydrophilic zones. Serial spotting of cells suspended in ECM gel onto the patterned paper creates an array of 200 micron-thick slabs of ECM gel containing cells. Stacking the sheets with zones aligned on top of one another assembles 96 3D multilayer constructs. Destacking the layers of the 3D culture, by peeling apart the sheets of paper, 'sections' all 96 cultures at once. Because the 3D

cultures are assembled from multiple layers, the number of cells plated initially in each layer determines the spatial distribution of cells in the stacked 3D cultures. This capability made it possible to compare the growth of 3D tumor models of different spatial composition, and to examine the migration of cells in these structures .

At the core of tissue engineering is the construction of 3D scaffolds out of biomaterials to provide mechanical support and guide cell growth into forming new tissues or organs. Recently, we reported a new fabrication strategy that results in stable tubular tissue with a high structural similarity to many biological tubular tissues. Using a stress-induced rolling membrane (SIRM) technique, we used two fabrication steps for their tubular structures. First, different types of cells were delivered and patterned on a two dimensional SIRM using microfluidic channels. Then the SIRM was released to roll up into a 3D tube. The tubes have different types of cells at specific locations, i.e., different parts of the tube wall are made up of different cells . Mimicking structural and functional features is a prerequisite for fabricating functional tubular tissues in vitro, and the realization of structural-tissue mimicry may have wide applications in simulation of many tubular tissues and enriches the toolbox for 3D micro/nanofabrication by initially patterning in 2D and transforming it into 3D.

Discussion

Concerning the form, we chose to treat the subject in two clauses to give each part the attention, organization and sufficient search amount. Cell therapy is a domain of tissue engineering. We gave it the first part, going from particular to general to reach a level of brightness and ease in explanation. Adding to the fact the historical chronology put the cell therapies in the locomotive of tissue engineering.

The analysis of searching consists the results of selected studies and publications targeting clinical and biological aspects. The biotechnological view of the subject could be more patent.

The purpose of this thesis was to introduce these two concepts which are very vast and richly multidisciplinary. That, had pushed us to limit ourselves to the most recent achievements and applications instead of making an exhaustive listing. The clinical studies presented are all retrospective. In th next steps, We will need more prospective studies to prove the security of each method of regenerative medicine. The religious aspect was not relevant to the purpose of this thesis and could be discussed in another work in other circumstances.

1. Challenges of Cells Therapies:

1.1. Embryonic Stem Cells ESCs (pluripotent):

Given the thousands of surplus embryos currently available in laboratories and, the possibility to derive lines of cells extracted these embryos donated for research, embryonic stem cells seem accessible and unlimited, allowing multiple research to take place.

The proliferation ability of embryonic stem cells is large, allowing to obtain lines more easily than the cells of Adult stem. Thus, ES cells would offer the prospect of a product allogeneic therapy immediately available with a high performance both in culture and conservation, which is a definite advantage for pathologies affecting a large number of patients.

Embryonic stem cells are much more spontaneously able to survive and multiply in vitro stem cells than harvested in adults.

Moreover, ES cells differentiate into a particular tissue due to the addition of regulatory molecules and appropriate growth factors:

- neurons, astrocytes, and oligodendrocytes
- hematopoietic cells
- muscle cells
- cardiomyocyte
- keratinocyte
- cells producing insulin
- hepatocyte
- ...

In the other hand, these cells are isolated from surplus embryos or derived from abortions, therefore their use raises ethical issues. In particular, for some, religious or not, who consider the extraction of stem cells Embryonic as an attack on the integrity of the embryo.

The diversity of regulation in Europe alone shows how this is a difficult question for public authorities.

Adding to that, Embryonic stem cells implanted in a body other than that they are from can induce an immune response because they are recognized as a non-self. We can see appearing rejection phenomena. Studies on mice have shown that the use of these ES cells resulted in the appearance of tumors. Indeed, these cells have a high proliferative capacity that can eventually be uncontrollable.

The risk of carcinogenesis after administration of ES cells could be proportional to their proliferative capacity. This risk would come from increased opportunities for change when the cell is undifferentiated it undergoes a high number of divisions.

1.2. Fetal stem cells:

These cells have many advantages; it is where research multiply in order to use them in protocols of cell therapy.

Their plasticity is closer to embryonic stem cells and they do not induce tumor development after transplantation. In the case of cell transplants from cord blood and even position allogeneic rejection risks are significantly lower due to the low expression of HLA antigens on the membrane surface of the like cells. Their regulation that is much more flexible and the use of these cells raises no ethical problem.

But very few stem cells are present in the umbilical cord blood. It is therefore important to learn about these cells and their needs so to improve performance and availability for therapeutic purposes.

1.3. Adult stem cells

Adult stem cells could prove to be the best choice for cell therapy considering their multiple advantages:

All transplant process allogeneic adult stem cells have the risk of rejection since the antigens of the major histocompatibility system (HLA) are clearly expressed in their membrane surfaces. this difficulty arises obviously, not the case of an autograft.

Then, Embryonic stem cells have a high regenerative potential but to tolerate their chromosomal instability poses a problem for efficiency therapeutic. Adult stem cells do not face the same difficulties because of their greater differentiation. It is technically more easy to control their evolution.

And, harvesting techniques such as places of obtaining the most adult stem cells are not a problem: the procedures application is coded, the collection of such cells legally falls the gift of an informed and consenting adults.

In addition, it may be possible to ultimately benefit from their plasticity and reprogram them to abandon their specialization and again become stem cells, or coerce specialized cells other tissue types.

There is, facing a series of difficulties not negligible due to insufficient knowledge about their properties as their biological mechanisms of differentiation, specialization and which will, therefore, be the major research challenges in the future.

The first difficulty with the harvesting of adult stem cells regarding their location and rarity. The lack of specific markers. This type of cells complicates their identification. Even if one assumes their presence in all human organs, searches are yet unable to demonstrate it...

It appears very difficult, to date, to produce good environments, conducive to the proliferation of adult stem cells. These cells, cultured ex vivo, lose some of their potential for self-renewal but also their multipotent character. They are, moreover, very difficult to keep.

They do not cover all potential disease accessible to cell therapy. They appear effective in treating some extensive lesions, they reveal disappointing for serious neurodegenerative diseases. They have a low capacity for homing.

The decrease in number as and extent of the subject aging.

The risk of developing cancer after autologous cells strains is often linked to their rarity in withdrawals that grows duty to cultivate in vitro, to proliferate: the probability of appearing cultured cancer cells is likely proportional to the number of successive divisions to which we must submit these cells to collect sufficient numbers for therapeutic action considered. Carcinogenesis depends may be of uncontrolled runaway this multiplication. This risk appears to be lower with stem cells differentiated adult, with embryonic stem cells.

In allogeneic transplant adult situations, there is a carcinogenic risk specifically relating to the transmission to the recipient of diseased cells possibly present in the donor before.

2. Challenges of Tissue engineering

2.1. Practical considerations:

Timing is an inherent limitation of many tissue-engineering applications, not the least open systems. Although autologous approaches obviate the risk of immunologic rejection, they typically involve weeks, if not months, of processing in order to culture a sufficient number of cells for a given composite construct. Yet time is often an unaffordable luxury for many patients, particularly those with life-threatening conditions and/or for whom limited treatment alternatives currently exist.

Because elaborate and expensive infrastructures are often necessary for the development and manufacture of engineered tissues, products designed to address relatively rare disease processes are often difficult to remain sustainable in the long term. Indeed, many firms with considerable interest in tissue engineering and regenerative medicine therapies have exited the market despite early studies suggesting efficacy. Production within Good Manufacturing

Practice (GMP) facilities are a prerequisite for Food and Drug Administration (FDA) approval of cell-based therapies, which, in turn, cannot be pursued without a critical mass of highly trained personnel. Furthermore, certain tissues require preconditioning in complex bioreactors, which may not be readily amenable to scaled-up manufacturing and shipping.

All of these issues translate into a chronic difficulty in establishing multicentric clinical trials, which are in turn essential for the widespread application of new therapeutic strategies.

2.2. Scientific limitations

The rapidly changing technologies within the broad, multidisciplinary field of tissue engineering have sometimes made clinical evaluations fairly difficult. For example, the ideal cell type or even cell source for many clinical applications remain undetermined.In many cases, while differentiated autologous cells would be ideal,

their use simply may not be a viable option in humans, either because of current isolation and expansion limitations (e.g., hepatocytes, neurons, cardiomyocytes, pancreatic islet cells) or because they tend to dedifferentiate over time.

At this time, infectious risks cannot be completely eliminated with these xenogeneic techniques. Another scientific limitation is the lack of an optimal biomaterial for many clinical tissue engineering applications. Many of the currently available synthetic scaffolds are still metabolized by the body leaving a significant foreign-body reaction behind. These conditions can lead to a reduction in the diffusion of nutrients and waste products, fibrosis, and other complications. Additionally, the cytotoxic effects of macrophage-generated nitric oxide can reach and destroy the transplanted cells. Thus, it is not surprising that most of the scaffolds that have been implanted in humans to date are derived from natural scores (e.g., bone, dermis, intestinal submucosa).

Unfortunately, at the same time, natural scaffolds have been associated with unfavorable mechanical properties (e.g., rapid or inconsistent degradation, low or erratic tensile strength). Further, some chemicals used in the decellularization process are known to negatively affect the properties of the scaffold.

For these reasons, there remains a continued interest in the development of novel incompatible synthetic biomaterials amongst them scientists and others. For example, electrospinning is somewhat novel, an alternative approach for creating scaffolds that can be made with finely tuned biomechanical specifications. The advantages of this technique include the ability to make scaffolds with high porosity as well as a high surface area to volume ratio while mimicking the dimensions and structure of native collagen and elastin fibrils. Finally, scaffolds impregnated with growth factors or specific peptide sequences may also allow for a better control of the surrounding microenvironment. Indeed, these newer synthetic materials, discussed in depth in other chapters, will be instrumental in helping to broaden the types of engineered tissues that can be rendered viable for human use.

2.2. Vascularization hurdles

Typically, any tissue greater than 1 cm in thickness cannot rely solely on vascular ingrowth from the host's vascular bed in order to remain viable in vivo. It is therefore not surprising that most successful clinical applications involving tissue engineering have dealt with the implantation of either surface lining (e.g., skin); simple, hollow organs (e.g., bladder); or relatively small, vessel-like conduits (e.g., blood vessels, trachea). A major challenge in the clinical implementation of more sizable and complex engineered structures and organs such as liver, heart, kidney, and lungs continues to revolve around how to best optimize the blood supply to the graft after implantation.

One interesting, already preclinical approach to address this problem has been to create a preformed microcirculation within the engineered scaffold itself (77). This strategy has been employed by Vacanti and his colleagues using microelectromechanical systems (MEMS) technology in a large animal model (78).

Preliminary work has enabled this group to develop a robust computational model of the vascular circulation, which includes the fractal nature of network topology, the rheology of blood flow through this computational system, and the mass transfer of oxygen and nutrients across the vascular bed.

Vascular channels can be etched onto silicon wafers, which can then be transferred to biodegradable polymer systems. Multiple monolayers of this architecture can then be stacked to form three-dimensional structures. Another described approach to organ revascularization has involved seeding of the vasculature of a decellularized organ with endothelial cells in a bioreactor prior to anastomosis of the engineered graft, albeit so far only in rodent models (79). Conceivably, some of these approaches might possibly become instrumental in facilitating the formation of other complex accessory networks (e.g., neural, lymphatic, biliary) between the host and engineered tissue itself. Two other approaches to enhance construct vascularization, namely gene therapy and scaffold-based encapsulation of angiogenic growth factors (e.g., vascular endothelial growth factor), have also been pursued in preclinical models (80).

3. Regulation

Unfortunately, we do not have any legal texts tackling regenerative medicine.

Our approach has highlighted many points that should be framed in official texts. For successful integration of cell therapy products or drugs, several critical points need to be considered. First, allow altruistic donations of tissues or organs to people in their lifetime (for therapeutic purposes or for medical research) under regulatory control. It should also allow ex-vivo manipulations of stem cells and the production of stem cell lines, cell products or medical devices (eg heart valves, keratinocytes ...) for cell therapy.

The integration of cell therapy also involves the institutionalization of the institution involved in the manipulation of stem cells (or other cell types) and their conservation, as well as their placing on the market via the creation of a national bank of stem cells or cell therapy products. Finally, it would be wise to promote a private or public-private partnership, the only model of operation and success of a stem cell project, to allow biomedical research with cell therapy products through the conduct of tests to validate new treatment protocols and to authorize the use of these products in public and private institutions previously approved by the Ministry of Health. However, it is necessary to establish the legal status of cell therapy products without giving them the status of pharmaceutical drugs that would delay their integration in Morocco, to allow the export of these products to international transplant centers because they could provide compatible donations to our Moroccans living abroad (MRE) and, create a biovigilance service that would be responsible for taking care of patients with side effects related to the use of biotherapy products. (81)

Conclusion and recommandations

Because a lot of diseases are idiopathic; and because organ transplantation is one of the least cost-effective therapies and not always available, regenerative medicine offers exploration of pathology, further application of knowledge and hope for more consistent and rapid treatment of those in need of body part replacement, and therefore has greater potential to improve patient quality of life.

The selected examples presented above illustrate that further advances in tissue engineering require additional knowledge of the basic mechanisms of cell function and the ways they interact with the environment. The recent surge in research on ECM molecules themselves and their interactions with particular cells and cell-surface receptors has led to the realization that these interactions are various and complex, allowing the modulation of fundamental events during development and wound repair, and are crucial for the maintenance of the differentiated phenotype and tissue homeostasis. As such, the manipulation of specific cell-ECM interactions has the potential to modulate particular cellular functions and processes in order to maximize the effectiveness of engineered tissues.

Finally, we recommend to start by:

1. Adding regenerative medicine as a strong suggestion in all the therapeutic chapters in medicine courses where its studies presented proof of effectiveness.
2. Making these suggestions clear; practical, comprehensible and closer to medical academics by presenting examples from real registred cases.
3. Creating a Moroccan bank of cells, tissues and organs which it role will be to control, collect, and to condition the prelevements in order to see latter on, a first transplanted Moroccan engineered organ.

Abstract

Abstract :

Title: Introduction to Cell Therapy and Tissue Engineering

Realized by: Lakhdar Mehdi to get his doctorate in medicine

Keywords: Cell therapy, Tissue engineering, Regenerative medicine.

The main objective of medicine was always to protect the integrity of the human body and mind and to treat what is altered to the limit of scientific accurate knowledge. A large specter of lethal pathology had only to be removed or replaced for treatment. The cell therapies and tissue engineering as parts of regenerative medicine opened the gate to new perspectives for curing these kinds of tissues alteration.

This thesis has two major purposes: To introduce cell therapies and tissue engineering as concepts. To demonstrate and raise awareness of their current clinical applications.The literature study started October the 25th, 2017 and it did end the 19th February 2018. Databases such as PubMed, National Center for Biotechnology Information, U.S. National Library of Medicine, and the FMPR College Library websites were all used to collect the articles, based on the latest date of appearance, relevance, and pertinence of the content.

Stem Cell Therapy is showed all abilities to explain different cell behaviors in pathological cases. In a wider view, it is the emerging discipline of fabricating spare parts for the human body. Imitation and adoption of the laws, signals of developmental biology and morphogenesis are the rules of architecture for tissue engineering. In vitro synthesis allows control over the culture environment by using bioactive Scaffolds metabolically supported. Induced organ synthesis in vivo relies on the processes inherently active in the wound site to regenerate lost or damaged tissue. Efforts using decellularized matrices have presented a new avenue for generating material platforms for tissue regeneration. Regenerative medicine is the door to new levels of appropriate health care.

نبذة مختصرة

ملخص الاطروحة العنوان: مقدمة في العلاج الخلوي وهندسة الأنسجة

أنجزت من قبل: لخضر المهدي للحصول على الدكتوراه في الطب

الكلمات الرئيسية: العلاج الخلوي ، هندسة الأنسجة ، الطب التجديدي

كان الهدف الرئيسي للطب هو حماية سلامة جسم الإنسان ونفسه ومعالجة ما يتم تعديله في حدود المعارف العلمية الدقيقة الحالية. كان لا بد لطيف كبير من الأمراض الفتاكة من الزرع أو الاستئصال للعلاج. ففتحت العلاجات الخلوية وهندسة الأنسجة كأجزاء من الطب التجديدي, البوابة على منظورات جديدة لعلاج هذه الأنواع من الأمراض.

لهذه الاطروحة هدفان رئيسيان: إدخال العلاج الخلوي وهندسة الأنسجة كمفاهيم جديدة ثم شرح ورفع مستوى الوعي حول التطبيقات السريرية الحالية. بدأت ابحاث هذه الدراسة في 25 أكتوبر 2017 وانتهت في 19 فبراير 2018. تم استخدام قواعد البيانات مثل PubMed والمركز الوطني لمعلومات التقنية الحيوية والمكتبة الوطنية الأمريكية للطب ومواقع مكتبة كلية الطب والصيدلة في الرباط لجمع المقالات، استنادًا إلى أحدثها وأكثرها ملائمة للمحتوى.

أظهر استعمال الخلايا كل القدرات لشرح مختلف السلوكيات الخلوية المرضية، من الفشل الكبدي، فشل القلب، الورم النقوي المتعدد، التلاسيميا، حرق الجلد، مرض جوشر إلى الباركنسون والفصام. من منظور أوسع، تمثل هندسة الأنسجة العلم الناشئ لتصنيع قطع الغيار لجسم الإنسان. قليد واعتماد القوانين ،عوامل النمو البيولوجي والتشكيل هي قواعد المعمارية لهندسة الأنسجة. التركيب المختبري يسمح بالتحكم الكامل في بيئة الزراعة باستخدام السقالات النشطة بيولوجيًا المدعمة استقلابيا. فتعقيد النظم البيولوجية، وتحديدًا الستوقين، وعامل النمو، واحتياجات التشوير بين الخلايا عبر حجم الأنسجة النامية قد حالت حتى الآن دون تشكيل أنسجة معقدة في المختبر. يعتمد تخليق العضو المستحث، في الجسم الحي، على العمليات النشطة بطبيعتها في موقع الجرح لتجديد الأنسجة المفقودة أو التالفة. فتستخدم مناظرة شديدة التناسق للمصفوفة الزائدة الخلوية، التي يطلق عليها أيضًا سقالة ، للحث على التجدد في موقع الجرح. قدمت الجهود باستخدام مصفوفات لا خلويه لتجديد الأنسجة كوسيلة جديدة لتوليد منصات المواد لتجديد الأنسجة. في الختام ، إن الطب باب لمستويات جديدة من الرعاية الصحية المناسبة.

Resumé :

Titre: Introduction à la therapie cellulaire et ingenieurie tissulaire

Realisée par: LAKHDAR EL MEHDI

Mots clés: Thérapie cellulaire, Ingénierie tissulaire, Médecine régénérative

L'objectif principal de la médecine a toujours été de protéger l'intégrité du corps et de l'esprit humainet de traiter toute altération à la limite de la connaissance scientifique précise et actuelle. Un grand spectre de pathologie létale avait pour traitement seulement la greffe ou l'exérèse. Les thérapies cellulaires et l'ingénierie tissulaire en tant que parties de la médecine régénérative, ont ouvert la porte à de nouvelles perspectives pour traiter ces types d'altérations tissulaires.

Cette thèse a deux objectifs majeurs: Introduire les thérapies cellulaires et l'ingénierie tissulaire en tant que concepts. Démontrer et sensibiliser sur leurs applications cliniques actuelles. L'étude bibliographique a débuté le 25 octobre 2017 et s'est terminée le 19 février 2018. Des bases de données : PubMed, le Centre national d'information sur la biotechnologie, la National Library of Medicine des États-Unis et les sites Web de la FMPR ont été consultés selon dernière date d'apparition, la pertinence du contenu des articles.

La thérapie par cellules souches a montré ses capacités à expliquer différents comportements cellulaires pathologiques. Dans une perspective plus large, l'ingénierie tissulaire représente la discipline émergente de la fabrication de pièces de rechange pour le corps humain. La synthèse in vitro, permet un contrôle total sur l'environnement de culture en utilisant des échafaudages bioactifs métaboliquement supportés. La synthèse d'organes induite in vivo, repose sur les processus intrinsèquement actifs dans le site de la plaie pour régénérer les tissus endommagés. Les efforts utilisant des matrices décellularisées présente une nouvelle avenue pour générer des plates-formes matérielles pour la régénération tissulaire. Pour conclure, la médecine regenerative est la porte vers de niveaux de soins appropriés.

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Serment d'Hippocrate

Au moment d'être admis à devenir membre de la profession médicale, je m'engage solennellement à consacrer ma vie au service de l'humanité.

- Je traiterai mes maîtres avec le respect et la reconnaissance qui leur sont dus.
- Je pratiquerai ma profession avec conscience et dignité. La santé de mes malades sera mon premier but.
- Je ne trahirai pas les secrets qui me seront confiés.
- Je maintiendrai par tous les moyens en mon pouvoir l'honneur et les nobles traditions de la profession médicale.
- Les médecins seront mes frères.
- Aucune considération de religion, de nationalité, de race, aucune considération politique et sociale ne s'interposera entre mon devoir et mon patient.
- Je maintiendrai le respect de la vie humaine dés la conception.
- Même sous la menace, je n'userai pas de mes connaissances médicales d'une façon contraire aux lois de l'humanité.
- Je m'y engage librement et sur mon honneur.

قسم أبقراط

بسم الله الرحمان الرحيم

أقسم بالله العظيم

في هذه اللحظة التي يتم فيها قبولي عضوا في المهنة الطبية أتعهد علانية:

- بأن أكرس حياتي لخدمة الإنسانية.
- وأن أحترم أساتذتي وأعترف لهم بالجميل الذي يستحقونه.
- وأن أمارس مهنتي بوازع من ضميري وشرفي جاعلا صحة مريضي هدفي الأول.
- وأن لا أفشي الأسرار المعهودة إلي.
- وأن أحافظ بكل ما لدي من وسايل على الشرف والتقاليد النبيلة لمهنة الطب.
- وأن أعتبر ساير الأطباء إخوة لي.
- وأن أقوم بواجبي نحو مرضاي بدون أي اعتبار ديني أو وطني أو عرقي أو سياسي أو اجتماعي.
- وأن أحافظ بكل حزم على احترام الحياة الإنسانية منذ نشأتها.
- وأن لا أستعمل معلوماتي الطبية بطريق يضر بحقوق الإنسان مهما لاقيت من تهديد.
- بكل هذا أتعهد عن كامل اختيار ومقسما بشرفي.

والله على ما أقول شهيد.

جامعة محمد الخامس – الرباط
كلية الطب والصيدلة بالرباط

سنة : 2018 أطروحة رقم: 142

مقدمة في العلاج الخلوي وهندسة الأنسجة

أطروحة

قدمت ونوقشت علانية يوم:.....

من طرف

السيد: المهدي لخضر

المزداد في: 17 أكتوبر 1991 بالدار البيضاء

لنيل شهادة الدكتوراه في الطب

الكلمات الأساسية: العلاج الخلوي – هندسة الأنسجة – الطب التجديدي.

تحت إشراف اللجنة المكونة من الأساتذة

السيد: رشيد الجودي أستاذ في علم السموم	**رئيس**
السيد: عز الدين إبراهيمي أستاذ في التكنولوجيا الحيوية	**مشرف**
السيدة: أمال حسني أستاذة في طب الأطفال	**أعضاء**
السيدة: نعيمة الحفيظي أستاذة في طب الأطفال	