Asok Mukhopadhyay *Editor*

Regenerative Medicine: Laboratory to Clinic

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Foreword

Stem cell biology and its applications have received a large boost in India over the last 20 years. Yet, it has been practiced in select centres across the country even earlier. One example is the work on bone marrow transplantation in the clinic for patients suffering from anaemia. The group led by Dr. Mammen Chandy of the Christian Medical College, Vellore, started work in this area well over 20 years ago. The other is the work on the basic biology of limb development in frogs and amphibians, and the work by Professor Priyamvada Mohanty Hejmadi in Orissa.

But a focused push and support for research in stem cells—basic biology, and its applications in regenerative medicine was initiated by the Department of Biotechnology (DBT) of the Ministry of Science and Technology, India, which set up a Task Force on Stem Cells and Regenerative Medicine (SCRM) in the year 2001. Its mandate was to formulate and implement strategy, support research and develop programmes in the area, create a platform for clinical research and schemes for setting up infrastructure and equipment, institutional development, and to put together a framework for regulation. In collaboration with the Indian Council for Medical Research (ICMR) of the Ministry of Health, DBT put together a set of national guidelines for stem cell research and its applications. Together, they have set up the National Apex Committee for Stem Cell Research and Therapy. Two comprehensive reviews of the current status in the area of SCRM in India have been published [1, 2].

Dedicated funding for this area of SCRM has been provided over these 15 years by the Task Force of DBT, which has support for research programmes, included workshops for training manpower, visits by external experts (e.g., Profs. John Gurdon, Martin Evans, Irwing Weissman and others) to centres across the country, clean rooms, cGLP and cGMP facilities, high-end equipment and support for scientists to travel abroad for conferences and short-term training. DBT has also supported scientist-industry collaboration through joint funding mechanisms.

As a result of such promotion by DBT (and ICMR), today India has over 30 centres across the country—involved in basic research, production of stem cells from various sources, clinical applications, production facilities and other areas. Dr. Asok Mukhopadhyay has wisely chosen to edit this book, which is a state-of-the-art update on *Regenerative Medicine: Laboratory to the Clinic*. The chapters here capture the work on the lab bench on one side and the bedside on the other.

Some representative examples of the basic research are captured in Part I on Basic Stem Cells and Disease Biology. We note contributions on the sources and production, and differentiation of stem cells of relevance to chosen diseases.

Part II focuses not only on specific applications in the liver, pancreas and the lacrimal gland but also on scaffolding, bio-printing and strategies for tissue engineering. A particularly relevant chapter by Nagarajan discusses issues such as sites of delivery, number of cells to be delivered, animal studies and their extension to humans.

Part III describes some exciting and successful applications being practiced at some centres in India. It is particularly satisfying that India has declared haematopoietic stem cell based treatment for anaemia as 'proven therapy', and we must appreciate the efforts of haematologists in having brought this forth. In a country where anaemia of various types is rampant, such a stem cell treatment is of special value.

A second example of regenerative medicine, done at a few centres in India, is the repair and successful regeneration of the corneal outer surface and vision improvement in patients whose corneas have been damaged by chemical or thermal burns. Called Cultivated Limbal Epithelial Transplantation (CLET for short), and its simpler in situ in vivo version SLET, this treatment is being considered by the national regulatory body as 'proven therapy'. Application in cardiology (particularly in cases of myocardial infarction, already shown successful in multi-centre trials across India) is the third exciting application of SCRM. And the work on physeal regeneration, discussed by Vrisha Madhuri and colleagues, is an example of the productive bringing together of scaffolding and bioreactor-based expansion on one hand, and transplantation on the subject on the other hand—thus illustrating the oft-quoted phrase 'bench to bedside'.

For Asok, who is well known not only for his noteworthy research in mesenchymal stem cell biology and liver regeneration, but has brought together 28 groups of researchers to contribute to this timely and diverse examples of the practice and applications of SCRM, this has been a labour of love. We are deeply appreciative of this effort on this part.

Congratulations, Asok!

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Prof S. Balasubramanian

References

1. Balasubramanian D, Sharma A. Stem cell research: beyond the headlines. In: Biotechnology: an agent for sustainable socio-economic transformation. Nature India. 2016; p. 18–23. https://www.natureasia.com>nindia>pdf.
2. Sharma A. Stem cell research and policy in India: current scenario and future perspective. J Stem Cells. 2009;4:133–40.

Preface

Regenerative medicine, a broad subject, deals with the process of creating living, functional tissues to repair or replace tissue or organ function lost due to congenital defects, disease, damage, or age. Tissue regeneration is a concept that has roots dating back to 1000 BC, to the earliest known records of medical interventions by a renowned surgeon of ancient India, "Susruta," which are later recognized in modern medical sciences as "plastic surgery." The basic operative principles allow a plastic surgeon to reconstruct primarily external defects like cleft lip and microtia, perform breast augmentation/implant surgery, treat burn injury, etc. On the other hand, regenerative medicine aims to develop new approaches to restore lost functions of damaged internal and external body parts by replacement with tissues from autologous/allogenic sources or inducing the body's own tissue regeneration potential by providing a suitable microenvironment. Thus, it covers a wide range of unmet medical needs to improve the quality of life and in many cases protect patients from untimely demise. In molecular level, the vastly different clinical scenarios can be amalgamated with basic understanding of developmental biology, immunological tolerance, wound healing process, and cell-cell and cell-matrix interaction. Therefore, regenerative medicine has been considered a multidisciplinary field involving biology, chemistry, engineering, medicine, and surgery.

The present book is divided into three parts: disease biology and basic stem cells, potential clinical studies, and bedside applications. The most vital issue in any functional tissue regeneration process is understanding the disease biology and the single unit of a tissue, "cell," and its modification. The first part of the book represents some of these aspects like the dynamics of wound healing in diabetic condition and the treatment of osteoarthritis in the perspective of developmental biology. Interestingly, this part also consists of a few chapters that address fundamental questions on the use of pluripotent stem cells in tissue regeneration, functions of long noncoding RNAs in neuronal commitment, etc. The second part deals with upcoming prospects in the regeneration of the pancreas, liver, and lacrimal gland and tissue engineering in general. The banking of cord blood in India and the potential applications of cord blood stem cells in different clinical indications have been described in this part. The pillar of success of translational regenerative medicine is to perform well-designed preclinical studies in a suitable animal model. These are conducted as a proof of concept to understand the survival and proliferation of the cells, cell migration, bio-distribution, the epigenetic memory of the differentiated cells, safety,

tissue integration, immune reactions, and the manufacturing challenges of cell-based products. The remaining chapter of this part covering some of the above aspects of the translational research that are normally overlooked. The last part describes the experiences of the clinicians and scientists in the bedside translation of regenerative medicine in different clinical indications starting from the induction of transplant tolerance during organ transplantation to the treatment of aging, from construction of the cornea to physeal regeneration.

It is an impossible task to cover each and every aspect of regenerative medicine in a new publication like this; I hope that the readers will liberally consider the constraint of the first edition of the book. I hope that sharing the Indian experience of the bedside applications of regenerative medicine alone will not confound readers, as it is known that clinical practice has no geographical boundaries. It is expected that this edited book will be an immense support to the research and clinical practice of regenerative medicine in all corners of the globe.

New Delhi, India Asok Mukhopadhyay

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About the Editor

Asok Mukhopadhyay has just retired from the very prestigious National Institute of Immunology, New Delhi, after more than 25 years of service as a Scientist and Principal Investigator. He received Ph.D. from the Indian Institute of Technology, New Delhi, and worked as a Post-Doctoral Research Fellow at MD Anderson Cancer Center, Houston, TX, USA. His main area of research is stem cells in regenerative medicine. He has published 75 research papers, contributed to seven book chapters and has also written the textbook *Animal Cell Technology* [Published by IK International, New Delhi; ISBN: 978-81-89866-96-9 (2009)]. Further, he holds two Indian patents.

Dr. Mukhopadhyay is a member of the Scientific Advisory Committee of several leading Indian institutions, as well as the Task Force on 'Stem Cell Research and Regenerative Medicine'—Department of Biotechnology, Government of India. He also serves as a reviewer for various reputed journals such as *Tissue Engineering*, *World Journal of Stem Cell Research*, *Biotechnology and Bioengineering*, and *Stem Cells and Development*.

Part I

Disease Biology and Basic Stem Cells

Impact of the Diabetic State on Wound Healing Dynamics and Expression of Soluble Cellular Mediators

1

Stephanie E. Grant and William J. Lindblad

Abstract

Diabetes mellitus impacts virtually every organ system of the body due to the influence that altered glucose metabolism imparts on cellular physiology and because of the effect chronic hyperglycemia can have on protein glycosylation states. As a physiological process involving multiple cell types, biomolecules, and a requirement for cell activation and activity, wound healing processes from formation of a transitional extracellular matrix after tissue destruction to altered neutrophil activation to a reduction in effective mesenchymal cell function have all been shown to be impacted by diabetes. In this chapter, we will review numerous studies that have documented changes in different components of classic dermal wound healing due to chronic hyperglycemia producing an overall diminished capacity to heal tissues and to even lead to the formation of ulcerations. Lastly, we will briefly discuss recent findings from our own studies that suggest that the diabetic state may alter the ability of fibroblasts to respond to activation stimuli with the appropriate expression of pro-inflammatory mediators. This aberrant expression could ultimately lead to an over-recruitment of neutrophils and/or monocyte/macrophages leading to a failure to heal a wound.

Keywords

Cell migration • Cytokines • Fibroblasts • Hyperglycemia • Inflammation

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Abbreviations

bFGF	Basic fibroblast growth factor
CCR	CC chemokine receptor
CXCR	CXC chemokine receptor
ECM	Extracellular matrix
HIF-1α	Hypoxia-inducible factor-1α
IL	Interleukin
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattractant protein-1
MMP	Matrix metalloproteinase
NO	Nitric oxide
PDGF	Platelet-derived growth factor
TGFβ	Transforming growth factor-β
TIMP	Tissue inhibitor of matrix metalloproteinase
TLR	Toll-like receptor
TNFα	Tumor necrosis factor-α

1.1 Introduction

Diabetes mellitus represents one of the most common endocrine diseases worldwide. Representing a variety of causative mechanisms, diabetes is characterized by chronic hyperglycemia with alteration in the ability of target cells to utilize insulin or an inability of the pancreas to secrete insulin. While impacting many cellular processes and physiological systems, diabetes has a significant detrimental effect on wound healing. Wound healing has traditionally been described as occurring in four distinct phases of hemostasis, inflammation, proliferation, and remodeling; however, a more fluid and overlapping series of processes is a more accurate view of the overall biological response. As the coagulation cascade is beginning, inflammatory cells have already begun to invade the tissue. Proliferation and migration begin as the cells at the wound margin respond to the free-edge effect and disrupted oxygen supply immediately after injury. Remodeling begins as proliferation and migration lead to the deposition of cells to fill in the defect. The progressive nature of the healing mechanism highlights the importance of the proper function of the cellular machinery (Table 1.1). It now appears that many, if not all, of these cellular processes are impacted by the hyperglycemic state characteristic of diabetes, although the influence of the hypoinsulinemic state may also be a significant factor on these events. We will describe these different cellular stages and provide an overview of how the diabetic state may alter the normal response of the tissues to injury.

Table 1.1 Summary of diabetes-induced changes in major dermal wound healing cellular processes

Cell/process	Impact	Healing effect
Coagulation	Nonenzymatic glycosylation of fibrinogen	Denser clots resistant to fibrinolysis
Neutrophils	Poor chemotaxis, reduced phagocytic activity, lower respiratory burst, failure to be terminated	Inadequate acute inflammatory response, prolonged intense chronic inflammation
Monocyte/macrophages	Poor chemotaxis, reduced phagocytic activity	Reduced production of growth factors, particularly VEGF, and delayed granulation tissue formation and neovascularization
Keratinocytes	Reduced migration, reduced proliferation	Delayed reepithelialization
Fibroblasts	Alteration in type I collagen synthesis and α-smooth muscle actin expression	Disruption in deposition of ECM and wound contraction
Endothelial cells	Reduced VEGF and stromal cell-derived factor-1 expression, enhanced vascular permeability	Delayed and poorly organized granulation tissue

1.2 Blood Coagulation, Platelet Activation, and Immediate Vascular Effects

Beginning immediately following injury with the exposure of subendothelial collagen to the blood, circulating von Willebrand factor binds to exposed collagen, which allows platelet binding via GP1b [1]. The bound platelets now begin the activation process which is accompanied by the release of preformed α-granules containing histamine, serotonin, platelet-derived growth factor (PDGF), and transforming growth factor-β (TGFβ). Histamine induces vascular permeability and vasodilation, which helps to reduce blood flow in the area and set the stage for increased fluid flow to the damaged tissues, and serotonin induces a short-lived vasoconstriction to minimize blood loss if the smooth muscle layer is exposed. PDGF has many functions including the stimulation of proliferation of mesenchymal cells and chemoattraction of neutrophils, monocytes, and mesenchymal cells, while TGFβ, at low levels, functions as a chemoattractant of neutrophils and monocytes. Next, the platelets begin to synthesize thromboxane A2 (TxA2), which enhances platelet aggregation, and leukotriene B4, a strong neutrophil chemoattractant. The rearrangement of the platelet cell membrane allows for the dimerization of GPIIb and GPIIIa which function as the fibrinogen receptor. Factor V is shuttled to the platelet surface to facilitate the conversion of circulating prothrombin to

thrombin. Thrombin can then convert fibrinogen to fibrin which allows fibrin cross-linking and the formation of the clot.

Numerous studies have shown that clot formation in diabetic individuals results in an altered fibrin matrix [2]. One mechanism for this alteration in matrix is through nonenzymatic glycation of fibrinogen, resulting in denser clots that are resistant to fibrinolysis [3, 4]. This denser matrix may lead to changes in the ability of the fibrin matrix to be modified, converting into a transitional extracellular matrix (ECM) and supporting the organized migration of cells into the wound area.

1.3 Recruitment of Neutrophils

As hemostatic mechanisms are put in place, the inflammatory cascade begins. The key step in the initiation of this phase is the activation of endothelial cells to allow immune cell entry into the wound bed. Platelet-activating factor (PAF) secreted by platelets and macrophages and interleukin-1 (IL-1) secreted by macrophages induce a conformational change in the endothelial cells that loosen formerly tight cellular junctions, allowing for movement of plasma components into the subendothelial space [5]. Endothelial cells begin synthesizing PAF within minutes of stimulation, which serves to activate neutrophils that are moving into the area [5].

Movement of neutrophils into damaged tissue involves a multistep process involving adhesion molecules and active cellular events in both the neutrophil and endothelial cell. The first phase of what is termed “the adhesion cascade” begins with the slow trafficking or “rolling” of neutrophils through areas of inflammation. This occurs within minutes of tissue injury and continues for at least 2 hours [6]. The rolling process is believed to be mediated by an interaction between L-selectin expressed by neutrophils and E-selectin expressed by activated endothelial cells [5]. Soon after the rolling process begins, L-selectin is shed from the neutrophil surface, which then activates CD11/CD18 integrins. Activated endothelial cells constitutively express ICAM-1 and ICAM-2, which are the ligands for the neutrophil integrins. This facilitates a strong adhesive interaction between the neutrophil and the activated endothelial cell that induces a morphological change in the neutrophil from a spherical shape to a flattened shape. At this point, a chemotactic gradient of IL-1, IL-8, or tumor necrosis factor-α (TNFα) must be present for the neutrophil to undergo diapedesis. The neutrophil must also secrete proteases such as matrix metalloproteinase-9 (MMP-9) and lysozyme to degrade the basement membrane [7]. Once in the extravascular space, the neutrophil begins to clear the cellular debris and bacterial threats through phagocytosis and the release of antimicrobial peptides and reactive oxygen species.

Alterations in neutrophil function are expected to contribute to the development of chronic wounds in diabetic patients. Although there seems to be a very broad and non-consistent range of defects in these patients, poor metabolic control is frequently associated with more severe neutrophil dysfunction [8]. Weak chemotaxis

to the wound bed, decreased phagocytic rate, lowered oxidative burst activity, decreased toll-like receptor (TLR) and TNFα expression, and chronic neutrophil presence occur in the later phases of wound repair [9]. The clinical result of these dysfunctions is an inadequate early inflammatory response followed by a prolonged, intense inflammatory phase that interferes with the normal proliferative and remodeling phases of wound repair.

1.4 Monocyte/Macrophage Recruitment and Activation

Macrophages are the second major inflammatory cell population to arrive at the wound bed [10]. At 24–48 h post-injury, the neutrophil population in the wound bed is expected to decrease through apoptotic and phagocytic mechanisms which allow for monocyte/macrophage domination of the repair process [11]. The major chemoattractant of monocytes in the early wound is PDGF. IL-6 production by neutrophils and fibroblasts may also stimulate activated endothelial cells to produce monocyte chemoattractant protein (MCP-1) which would help to drive the shift from neutrophil to monocyte/macrophage wound bed domination [10]. Monocyte homing to the wound bed occurs in a similar fashion to neutrophil migration. The rolling phase of monocyte homing is mediated by monocyte-expressed L-selectin and endothelial cell VCAM-1. Tight adherence and flattening of the monocyte to achieve diapedesis require β1 and β2 integrin interactions [12]. The release of granular proteins by neutrophils as they enter the wound bed is believed to facilitate the homing and extravasation of monocytes through direct monocyte activation, β2 integrin activation, and enhanced CAM expression [7].

Once monocytes enter the wound bed, differentiation to macrophages occurs [13]. Depending on the cytokine environment, two distinct macrophage phenotypes can be elicited. M1 macrophages are primarily inflammatory cells, while M2 macrophages main function is repair oriented through the promotion of angiogenesis and tissue remodeling/repair. M2 macrophages exist in several subtypes, M2a, M2b, M2c, and M2d [14]. In the early phases of normally healing wounds when the removal of damaged tissue is paramount, primarily M1 macrophages are present, whereas in the later phases when tissue generation is the prime directive, M2 macrophages predominate [15]. M1 macrophages are believed to be generated in the presence of IFNγ or lipopolysaccharide (LPS) through the upregulation of interferon regulatory factor 5 [14]. However, bacterial wound invasion or T-cell involvement is not necessary for the activation and differentiation of M1 macrophages as the presence of TNFα has also been shown to promote this process [16]. M2a macrophages are generated in the presence of IL-4 and IL-13 through the action of interferon regulatory factor (IRF) 4; however, they have been found in the absence of these cytokines [14]. Although the M2 subtypes are generally associated with repair functionality and not inflammation, they have been shown to markedly upregulate their production of pro-inflammatory cytokines in response to LPS exposure. This is a classic example of the extreme plasticity of the activated macrophage. The M2b subtype comes about in the presence of IL-1β and/or LPS and the M2c subtype

in the presence of IL-10 and/or TGFβ. The M2d subtype has a novel pathway of differentiation. This macrophage is first differentiated to the M1 phenotype and in the presence of both TLR agonists and adenosine 2A receptor agonists is then further differentiated into the M2d phenotype. This phenotype shows increased angiogenic function.

Monocyte/macrophage dysfunction in the diabetic leads to a variety of deficits in proper wound healing. Much of the research is inconsistent with regard to the specific mechanism of the deficit. This may be attributed to the large spectrum of differences in the degree of metabolic dysfunction, glucose control, and age. Poor chemotaxis to the wound bed combined with reduced phagocytic activity leads to a prolonged inflammatory state due to the inability to remove neutrophils, as well as reduced ability to remove pathogenic material [17]. This initially poor chemotaxis also prolongs the production of granulation tissue and lymphatic vessels as the lower numbers of macrophages cannot produce sufficient amounts of VEGF [18]. The ability of the macrophage in the diabetic to produce adequate amounts of cytokines and growth factors including VEGF, IL-1β, and TNFα also appears to be impaired [19]. However, some studies in genetically diabetic mice have shown an early and persistent elevation in inflammatory cytokine production by the macrophage, as well as the prolonged presence of these cells in the later phases of repair [20]. This appears to be related to the downregulation of phagocytic ability of some dysfunctional macrophages as the ingestion of apoptotic bodies is believed to be a prerequisite for the downregulation of inflammatory cytokine production.

1.5 Fibrocyte Contribution to Wound Healing

The classic assumption regarding fibroblast and myofibroblast proliferation and migration was that these cells primarily originated from the healthy tissue surrounding the wound. However, Bucala et al. [21] reported a population of spindle-shaped circulating cells similar in appearance to fibroblasts that would enter the wound bed alongside inflammatory cells within the first 48 h post-injury. The cell surface phenotype shared some characteristics with fibroblasts, namely, vimentin, fibronectin, collagen I, and collagen III. However, they also expressed CD45 (leukocyte common antigen) and CD34 (hematopoietic stem cell marker), suggesting that fibrocytes have similarities to leukocytes and may have a bone marrow origin [21]. Scanning electron microscopy indicated prominent cell surface projections on fibrocytes, which distinguished them morphologically from leukocytes. A study which introduced whole male bone marrow into female mice who had received lethal radiation showed that fibrocytes are of hematopoietic origin [22].

Circulating fibrocytes express multiple chemokine receptors on their surface [23]. In vivo injection of secondary lymphoid tissue chemokine promoted fibrocyte chemotaxis, which suggests that vascular endothelium-derived SLC could promote fibrocyte chemotaxis to the early wound bed through interaction with CCR (CC

chemokine receptor)-7. In vitro exposure of peripheral blood fibrocytes to MCP-1 increased cell migration and induced proliferation and differentiation to myofibroblasts and production of alpha smooth muscle actin which suggests that MCP-1 production in the early wound bed could also promote fibrocyte chemotaxis through interaction with CCR-2 [24]. Other chemokine receptors that are expressed by peripheral blood fibrocytes may have a role in fibrocyte chemotaxis which include CCR-3, CCR-5, and CXCR (CXC chemokine receptor)-4 [23].

To date, examination for chronic hyperglycemia-related impairment of fibrocyte influx and/or function is lacking and represents an area for potential investigation.

1.6 Migration of Resident Cell Populations

1.6.1 Keratinocytes

Reepithelialization of the wound is critical to successful healing as it will protect the wound from further environmental insult. The first signs of reepithelialization are visible within hours after injury with keratinocytes migrating outward in a "tongue-like projection" from the epithelial root sheath of the hair follicles at the wound edge [25]. The epidermal cells are capable of migrating over the newly formed transitional ECM, and therefore reepithelialization is not rate limited by the formation of granulation tissue [26]. The keratinocytes in the epidermis proximal to the wound initially become larger which appears as a thickening of the wound margin [27]. Cell-cell junctions are dissolved, allowing migration, with multiple integrins displayed on the cell surface to provide directionality toward the fibrin and fibronectin that have leaked from the damaged vasculature. Keratinocyte proliferation becomes apparent at the basal layer 1–2 days post-injury and appears to reach a peak just as ECM production is well underway [28]. Critical to the ability of keratinocytes to migrate is the balanced expression of matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) [29]. As the basement membrane is reestablished, both hemidesmosomes and desmosomes once again become visible at the wound margin [28].

Human keratinocytes cultured in high-glucose conditions exhibit significantly altered phenotype compared to keratinocytes cultured in normoglycemic conditions [29]. Migratory ability over type I collagen was significantly reduced, as was proliferative capacity. The expression of MMP-2 and MMP-9 was greatly reduced; however, the expression of TIMP-1 was upregulated. These results suggest that in diabetic patients with poor metabolic control, reepithelialization of acute wounds may be impaired, prolonging the wound healing process and increasing the risk of infection due to the lack of barrier from pathogens. Keratinocytes treated with high-glucose peripheral blood monocyte-conditioned media showed that a significantly lower expression of IL-22 by these monocytes led to poor keratinocyte migration and reduced MMP-3 expression, leading to delayed wound closure in a diabetic rat model [30].

1.6.2 Mesenchymal Cells

As the inflammatory phase of wound healing begins to subside at day 3–4 post-injury, the repopulation of the wound bed with mesenchymal cells becomes apparent [31]. The presence of PDGF-AB, PDGF-BB, and TGFβ1 leads to the activation and chemotaxis of resident fibroblasts and smooth muscle cells [28]. Migration is accomplished by the extension of the leading edge of the cell plasma membrane through the polymerization of actin filament. As this extension grows farther ahead of the cells original position, adhesions at the rear of the cell let go, effectively moving the cell forward [32]. Once they have reached the wound bed, fibroblasts begin to proliferate rapidly, and production of matrix proteins begins. The formation of granulation tissue occurs as the fibrin clot is lysed and replaced with hyaluronan and fibronectin, as well as the formation of new vascular structures. In the early wound, type III collagen is most abundant; however, later in the wound healing process, the stronger but more slowly manufactured type I collagen dominates [33].

At approximately 1 week post-injury, fibroblasts under the influence of TGFβ will begin differentiating into contractile myofibroblasts which facilitate closure of the wound. Despite the need for myofibroblasts for wound contraction, these cells also produce elevated levels of type I collagen and are felt to contribute too many fibrotic conditions. Cardiac fibroblasts from type II diabetic individuals show an enhanced myofibroblastic phenotype characterized by increased ability to contract 3D collagen matrices, elevated production of type I collagen, and high levels of α-smooth muscle actin [34]. These effects may well lead to the increased prevalence of cardiac fibrosis in diabetic patients; however, how this finding relates to dermal wound healing is unclear.

1.7 Neovascularization Process

Early granulation tissue is formed first at the periphery of the wound and proceeds centrally [35]. Within blood vessels immediately adjacent to the wound bed, endothelial cells begin to proliferate in response to the angiogenic stimulation of basic fibroblast growth factor (bFGF) secreted by activated platelets as well as from damaged connective tissue cells [36]. Around day 3 post-injury, these endothelial cells begin to express fibronectin and the integrins αvβ3, α1β1, and α2β1 which allow them to adhere to and migrate through the early granulation tissue. Concurrently with the arrival of migrating fibroblasts to the wound bed, new capillary buds begin to form. Angiogenesis at this stage is believed to be mainly driven by VEGF produced by keratinocytes, fibroblasts, endothelial cells, and macrophages [37]. VEGF induces the differentiation and migration of peripheral blood-derived endothelial progenitor cells which contribute to the formation of vasculature [38]. It also causes increased vascular permeability and the subsequent leakage of fibrinogen and fibrin into the wound bed, thus allowing for the adherence of endothelial cells, leukocytes,

and fibroblasts [39]. In the presence of high concentrations of VEGF, endothelial cells produce nitric oxide (NO) which then further upregulates the production of VEGF and microvascular permeability. Degradation of the basement membrane and ECM mediated by MMPs leads to further release of angiogenic growth factors from the damaged tissue. The chemotaxing endothelial cells begin to form cell-cell junctions. As the vascular lumen takes shape, smooth muscle cells and pericytes are incorporated into the design. Although vascular remodeling will continue for quite some time, the interaction between endothelial cells and smooth muscle cells and pericytes leads to the production of TGFβ which downregulates their migration and proliferation [40].

Microvascular complications are a common issue in diabetic patients. Alteration of endothelial and inflammatory cell function due to transcriptional changes induced by the formation of advanced glycation end products leads to increased vascular permeability, vascular occlusion, and eventually cell loss [41]. In the setting of the acute wound, lack of oxygenation due to damaged vasculature upregulates the production of hypoxia-inducible factor-1α (HIF-1α). HIF-1α then signals the upregulation of VEGF, leading to angiogenesis. However, in the setting of chronically elevated blood glucose, both the expression of HIF-1α and VEGF are reduced, as well as the endothelial progenitor cell mobilizing chemokine stromal cell-derived factor 1 [42]. In addition, endothelial cell populations are markedly decreased in diabetic patients, particularly those with peripheral vascular disease [43]. The clinical result is a slow and poorly organized granulation tissue formation which prolongs the inflammatory phase, impacts wound closure, and leads to an increased risk of infection.

1.8 Process for Elimination of Cell Populations

1.8.1 Induced Apoptosis

The prolongation of neutrophil activity in a healing wound has been associated with numerous detrimental outcomes. In a normally healing wound, neutrophil populations are expected to begin to decline by days 2–3 post-injury. It appears that the β2 integrin-mediated process of neutrophil trans-endothelial migration may initiate the apoptosis cascade following exposure to TNFα [44]. As the wound healing process proceeds from the inflammatory stage to the proliferative phase, monocyte populations begin to wane. This is believed to be influenced by the presence of VEGF [45].

As new tissue is formed in the wound, granulation tissue must be broken down. In the case of an inefficient breakdown process, pathological scarring can result. As granulation tissue is broken down, fibroblasts differentiate into contractile myofibroblasts which facilitate wound closure. This process mechanically loads the cells until stable tissue is formed [46]. As the wound stabilizes, this mechanical tension is gradually released on the myofibroblasts, triggering apoptosis within 3-6 hours.

1.8.2 Pro-resolving Lipid Mediators

A number of lipid-based mediators, including lipoxins, resolvins, protectins, and maresins, have been identified which function as downregulators of inflammation. The lipoxins are generated from arachidonic acid that is produced in the inflammatory response [47]. They have several immune-modulatory effects in the wound healing process. The key step in the resolution of the inflammatory phase is the downregulation of neutrophil-mediated tissue destruction [48]. As neutrophil populations begin to decline as a result of induced apoptosis and/or necrosis, macrophages are stimulated by lipoxin A4 to upregulate phagocytosis of neutrophil apoptotic bodies [49]. Limited studies to date suggest that the production of pro-resolving lipid mediators may be altered in diabetes. In a murine model of type II diabetes, neutrophils showed an impaired responsiveness to resolving E1 in stimulating the phagocytosis of *Porphyromonas gingivalis* [50].

1.9 Influence of the Hyperglycemic State on Epigenetic and microRNA Dynamics

Plasma microRNAs (miRs) contained within microvesicles that provide protection from degradation have been found to regulate aspects of the inflammatory and angiogenic response [51, 52]. These noncoding RNAs bind to the 3′ untranslated region of mRNA, leading to a decrease in translation. A "plasma microRNA signature" has been described for diabetic patients [52]. Significant differences between diabetic and nondiabetic patients have been elucidated in the expression of 41 plasma miRs. Of particular interest are miR-126, miR-200b, and miR-191. Decreased plasma levels of endothelial cell expressed miR-126 characteristic of diabetic patients have been associated with the development of peripheral vascular disease, presumably due to a negative angiogenic effect. In diabetic patients with chronic wounds, the normal hypoxia-downregulated expression of miR-200b by endothelial cells and platelets is significantly higher, leading to the downregulation of VEGF expression by endothelial cells. miR-191 expression by endothelial cells was also found to be dysregulated in diabetic patients with chronic wounds. Increased plasma miR-191 levels resulted in delayed wound healing due to the suppression of ZO-1-mediated angiogenesis and migration in endothelial cells and fibroblasts.

The role of histone methylation status in diabetic wound healing is a new area of investigation that has shown interesting discoveries. Macrophages isolated from punch biopsy samples taken from diabetic lower-extremity chronic wounds following amputation were found to express significantly less M2 phenotypical markers compared to macrophages isolated from nondiabetic wounds [53]. The diabetic macrophages also produced significantly higher levels of the pro-inflammatory cytokine IL-12, resulting in increased expression of IL-1β, IL-6, and TNFα. M1 domination was duplicated in a murine model of the diabetic wound and was

believed to be the result of a significantly decreased repression of the IL-12 gene by histone lysine trimethylation (H3K27me) as a result of increased demethylation by the Jumonji C domain-containing protein (Jmjd3).

1.10 Expression of Inflammatory Mediators by Fibroblasts

Given the large number of cell types involved with the healing of damaged tissue, it is apparent that overall coordinated regulation must occur through the selective expression, secretion, and receptor binding of a large number of soluble mediators. As already mentioned in this chapter, multiple growth factors (PDGF, TGFβ, VEGF) along with interleukins (IL-1β, IL-6, IL-8, IL-17) and other factors (lipid-based resolvins) are able to modulate the function of circulating and fixed cell types. While it is beyond the scope of this review, inflammatory cells have been the primary focus of much research on these soluble mediators. However, another source of these factors may contribute significantly to the outcome of the healing response, namely, fibroblasts. Studies by Tredgett and colleagues showed that hypertrophic scar fibroblasts expressed functional TRL4 which responded to LPS administration with the induction of a number of pro-inflammatory genes including IL-6, IL-8, and MCP-1 [54]. They also showed that hypertrophic scar fibroblasts showed a greater induced expression of these inflammatory mediators upon LPS stimulation compared to control fibroblasts.

Reports on fibroblasts from other tissue sources have also shown the expression of IL-6, IL-8, and MMP-1 following exposure to LPS, implicating TLR4 signal transduction in this gene expression [55]. Recently, in studies in our laboratory, we have data to support the expression of IL-1β, IL-6, IL-8, TNFα, and IL-17 by human dermal fibroblasts after treatment with LPS. Induction of these interleukins was time dependent with significant increases in mRNA at 6 h after exposure to LPS. Of note the expression of these mediators was dependent on whether they were obtained from individuals with type I diabetes, individuals with maturity-onset diabetes of the young, or non-affected controls. Expression of mRNA for these mediators was significantly higher in the fibroblasts obtained from type I diabetic individuals compared to non-affected control cells. These data suggest that fibroblasts may contribute to the inflammatory environment of wounds by expressing pro-inflammatory mediators in response to wound stimuli and that this expression is upregulated in the diabetic.

Conclusions

It has become apparent that the negative impact of diabetes on tissue wound healing is multifactorial involving virtually every process involved with the repair of damaged tissue. As summarized in Table 1.1, cells of the innate immune system, mesenchymal cells, and endothelial cells all show phenotypic changes that can reduce a person's ability to heal dermal damage. Along with these characterized changes, our preliminary data suggests that diabetes can shift the inflammatory environment within a wound by also altering the ability

of fibroblasts to secrete pro-inflammatory mediators that control many of the cellular activities in the wound. Continued efforts to characterize these many interacting effects will be needed to fully understand the impact of diabetes on wound healing.

References

1. Hoffman M, Monroe DM. A cell-based model of hemostasis. Thromb Haemost. 2001;85:958–65.
2. Undas A, Ariës RAS. Fibrin clot structure and function: a role in the pathophysiology of arterial and venous thromboembolic diseases. Int J Biochem Cell Biol. 2012;44:1800–12.
3. Jaleel A, Halvatsiotis P, Williamson B, et al. Identification of Amadori-modified plasma proteins in type 2 diabetes and the effect of short-term intensive insulin treatment. Diabetes Care. 2005;28:645–52.
4. Dunn EJ, Philippou H, Ariës RA, et al. Molecular mechanisms involved in the resistance of fibrin clot lysis by plasmin in subjects with type 2 diabetes mellitus. Diabetologia. 2006;49:1071–80.
5. Albelda SM, Smith CW, Ward PA. Adhesion molecules and inflammatory injury. FASEB J. 1994;8:504–12.
6. Tedder TF, Steeber DA, Chen A, et al. The selectins: vascular adhesion molecules. FASEB J. 1995;9:866–73.
7. Soehnlein O, Zernecke A, Weber C. Neutrophils launch monocyte extravasation by release of granule proteins. Thromb Haemost. 2009;102:198–205.
8. Geerlings SE, Hoepelman AI. Immune dysfunction in patients with diabetes mellitus (DM). FEMS Immunol Med Microbiol. 1999;26:259–65.
9. Nguyen KT, Seth AK, Hong SJ, et al. Deficient cytokine expression and neutrophil oxidative burst contribute to impaired cutaneous wound healing in diabetic, biofilm-containing chronic wounds. Wound Repair Regen. 2013;21:833–41.
10. Kaplanski G, Marin V, Montero-Julian F, et al. IL-6: a regulator of the transition from neutrophil to monocyte recruitment during inflammation. Trends Immunol. 2003;24:25–9.
11. Ross R, Odland G. Human wound repair. II. Inflammatory cells, epithelial-mesenchymal interrelations, and fibrogenesis. J Cell Biol. 1968;39:152–68.
12. Luscinskas FW, Kansas GS, Ding H, et al. Monocyte rolling, arrest and spreading on IL-4-activated vascular endothelium under flow is mediated via sequential action of L-selectin, beta 1-integrins, and beta 2-integrins. J Cell Biol. 1994;125:1417–27.
13. Deonarine K, Panelli MC, Stashower ME, et al. Gene expression profiling of cutaneous wound healing. J Transl Med. 2007;5:11. doi:10.1186/1479-5876-5-11.
14. Ferrante CJ, Leibovich SJ. Regulation of macrophage polarization and wound healing. Adv Wound Care. 2012;1:10–6.
15. Nagorsen D, Deola S, Smith K, et al. Polarized monocyte response to cytokine stimulation. Genome Biol. 2005;6:R15.
16. Italiani P, Boraschi D. From monocytes to M1/M2 macrophages: phenotypical vs. functional differentiation. Front Immunol. 2014;5:1–22.
17. Katz S, Klein B, Elian I, et al. Phagocytotic activity of monocytes from diabetic patients. Diabetes Care. 1983;6:479–82.
18. Maruyama K, Asai J, Ii M, et al. Decreased macrophage number and activation lead to reduced lymphatic vessel formation and contribute to impaired diabetic wound healing. Am J Pathol. 2007;170:1178–91.
19. Zykova SN, Jenssen TG, Berdal M, et al. Altered cytokine and nitric oxide secretion in vitro by macrophages from diabetic type II-like db/db mice. Diabetes. 2000;49:1451–8.
20. Wetzler C, Kampfer H, Stallmeyer B, et al. Large and sustained induction of chemokines during impaired wound healing in the genetically diabetic mouse: prolonged persistence of neutrophils and macrophages during the late phase of repair. J Invest Dermatol. 2000;115:245–53.

21. Bucala R, Spiegel LA, Chesney J, et al. Circulating fibrocytes define a new leukocyte subpopulation that mediates tissue repair. Mol Med. 1994;1:71–81.
22. Mori L, Bellini A, Stacey MA, et al. Fibrocytes contribute to the myofibroblast population in wounded skin and originate from the bone marrow. Exp Cell Res. 2005;304:81–90.
23. Abe R, Donnelly SC, Peng T, et al. Peripheral blood fibrocytes: differentiation pathway and migration to wound sites. J Immunol. 2001;166:7556–62.
24. Ekert JE, Murray LA, Das AM, et al. Chemokine (C-C motif) ligand 2 mediates direct and indirect fibrotic responses in human and murine cultured fibrocytes. Fibrogenesis Tissue Repair. 2011;4:23.
25. Krawczyk WS. A pattern of epidermal cell migration during wound healing. J Cell Biol. 1971;49:247–63.
26. Braiman-Wiksman L, Solomonik I, Spira R, et al. Novel insights into wound healing sequence of events. Toxicol Pathol. 2007;35:767–79.
27. Coulombe PA. Wound epithelialization: accelerationg the pace of discovery. J Invest Dermatol. 2003;121:219–30.
28. Singer AJ, Clark RA. Cutaneous wound healing. N Engl J Med. 1999;341:738–46.
29. Lan CCE, Liu IH, Fang AH, et al. Hyperglycaemic conditions decrease cultured keratinocyte mobility: implications for impaired wound healing in patients with diabetes. Br J Dermatol. 2008;159:1103–15.
30. Huang SM, Wu CS, Chao D, et al. High-glucose-cultivated peripheral blood mononuclear cells impaired keratinocyte function via reduced IL-22 expression: implications on impaired diabetic wound healing. Exp Dermatol. 2015;24:639–41.
31. Welch MP, Odland GF, Clark RAF. Temporal relationships of F-actin bundle formation, collagen and fibronectin matrix assembly, and fibronectin receptor expression to wound contraction. J Cell Biol. 1990;110:133–45.
32. Darby IA, Hewitson TD. Fibroblast differentiation in wound healing and fibrosis. Int Rev Cytol. 2007;257:143–79.
33. Ramasastry SS. Acute wounds. Clin Plast Surg. 2005;32:195–208.
34. Fowlkes V, Clark J, Fix C, et al. Type II diabetes promotes a myofibroblast phenotype in cardiac fibroblasts. Life Sci. 2013;92:669–76.
35. Tonnesen MG, Feng X, Clark RAF. Angiogenesis in wound healing. J Invest Dermatol Symp Proc. 2000;5:40–6.
36. McClain SA, Simon M, Jones E, et al. Mesenchymal cell activation is the rate-limiting step of granulation tissue induction. Am J Pathol. 1996;149:1257–70.
37. Lingen MW. Role of leukocytes and endothelial cells in the development of angiogenesis in inflammation and wound healing. Arch Pathol Lab Med. 2001;125:67–71.
38. Asahara T, Takahashi T, Masuda H, et al. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. EMBO. 1999;18:3964–72.
39. Lingen MW. Role of leukocytes and endothelial cells in the development of angiogenesis in inflammation and wound healing. Arch Pathol Lab Med. 2001;125:67–71.
40. Goddard LM, Luisa Iruela-Arispe M. Cellular and molecular regulation of vascular permeability. Thromb Haemost. 2013;109:407–15.
41. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. Nature. 2001;414:813–20.
42. Thangarajah H, Yao D, Chang EI, et al. The molecular basis for impaired hypoxia-induced VEGF expression in diabetic tissues. Proc Natl Acad Sci U S A. 2009;106:13505–10.
43. Fadini GP, Miorin M, Facco M, et al. Circulating endothelial progenitor cells are reduced in peripheral vascular complications of type 2 diabetes mellitus. J Am Coll Cardiol. 2005;45:1449–57.
44. Walzog B, Jeblonski F, Zakrzewicz A, et al. Promote apoptosis of human. FASEB J. 1997;11:1177–86.
45. Petreaca ML, Yao M, Ware C, et al. Vascular endothelial growth factor promotes macrophage apoptosis through stimulation of tumor necrosis factor superfamily member 14 (TNFSF14/LIGHT). Wound Repair Regen. 2008;16:602–14.
46. Grinnell F, Zhu M, Carlson MA, et al. Release of mechanical tension triggers apoptosis of human fibroblasts in a model of regressing granulation tissue. Exp Cell Res. 1999;248:608–19.

47. Serhan CN. Pro-resolving lipid mediators are leads for resolution physiology. Nature. 2014;510:92–101.
48. Mitchell S, Thomas G, Harvey K, et al. Lipoxins, aspirin-triggered epi-lipoxins, lipoxin stable analogues, and the resolution of inflammation: stimulation of macrophage phagocytosis of apoptotic neutrophils in vivo. J Am Soc Nephrol. 2002;13:2497–507.
49. Godson C, Mitchell S, Harvey K, et al. Lipoxins rapidly stimulate nonphlogistic phagocytosis of apoptotic neutrophils by monocyte-derived macrophages. J Immunol. 2000;164:1663–7.
50. Herrera BS, Hasturk H, Kantarci A, et al. Impact of resolvin E1 on murine neutrophil phagocytosis in type 2 diabetes. Infect Immun. 2015;83:792–801.
51. Dangwal S, Stratmann B, Bang C, et al. Impairment of wound healing in patients with type 2 diabetes mellitus influences circulating microRNA patterns via inflammatory cytokines. Arterioscler Thromb Vasc Biol. 2015;35:1480–8.
52. Zampetaki A, Kiechl S, Drozdov I, et al. Plasma MicroRNA profiling reveals loss of endothelial MiR-126 and other MicroRNAs in type 2 diabetes. Circ Res. 2010;107:810–7.
53. Gallagher KA, Joshi A, Carson WF, et al. Epigenetic changes in bone marrow progenitor cells influence the inflammatory phenotype and alter wound healing in type 2 diabetes. Diabetes. 2015;64(4):1420–30.
54. Wang J, Hori K, Ding J, et al. Toll-like receptors expressed by dermal fibroblasts contribute to hypertrophic scarring. J Cell Physiol. 2011;226:1265–73.
55. Cho J-S, Kang J-H, Um J-Y, et al. Lipopolysaccharide induces pro-inflammatory cytokines and MMP production via TLR4 in nasal polyp-derived fibroblast and organ culture. PLoS One. 2014;9(11):e90683. doi:10.1371/journal.pone.0090683.

Etiology and Treatment of Osteoarthritis: A Developmental Biology Perspective

2

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Abstract

Osteoarthritis is a debilitating disorder of the joints during which cartilage lining the articular surface of the bones undergoes progressive, irreversible damage, ultimately resulting in disability in locomotion. The current understanding about the pathogenesis of osteoarthritis is far from complete, and no effective therapy is available to tackle osteoarthritis. Analyzing the pathogenesis of osteoarthritis from the vantage point of a developmental biologist indicates that the molecular and histological changes observed during osteoarthritis closely recapitulate embryonic cartilage differentiation, thereby offering a new paradigm to understand this disease. In order to come up with new strategies for halting disease progression or initiating regeneration, it is important to understand the etiology of osteoarthritis from a molecular perspective afforded by developmental biological studies.

Keywords

Articular cartilage • BMP • Development • Osteoarthritis • Transient cartilage

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Abbreviations

ACI	Autologous chondrocyte implantation
ChM-I	Chondromodulin-I
CSPCs	Cartilage stem/progenitor cells
MACI	Matrix-assisted chondrocyte implantation
NSAIDs	Nonsteroidal anti-inflammatory drugs
PCL	Polycaprolactone
PTHrP	Parathyroid hormone-related peptide

2.1 Introduction to Osteoarthritis

Osteoarthritis is a painful and chronic disorder of the joints which affects a large number of people across the world. It affects all joints in the body but the most commonly affected are hands, hips, and knee joints. The tissue that is principally affected in osteoarthritis is articular cartilage, which is a thin tissue that lines the ends of long bones in adult vertebrate skeleton and makes locomotion possible at joints. The incidence rates of osteoarthritis have witnessed a steep rise in the last century or so [1]. It was not however without mention in the older medical literature as it was described by Hippocrates, Galen, and Avicenna.

It was scientifically described for the first time in *De Humanis Corporis Fabrica* by Vesalius in 1541 where articular cartilage and synovial fluids were discussed. The modern scientific description dates to 1829 when it was described by Benjamin Brodie. In 1890, Archibald Garrod coined the term osteoarthritis to describe a spontaneous inflammation or degradation of the articular cartilage. Osteoarthritis comes from the Greek words *osteo,* bone; *arthr,* joint; and *itis,* inflammation. During the last century, it has been recognized as a major musculoskeletal disorder that affects senior people with high incidence.

In the Global Burden of Disease report by WHO in 2010, osteoarthritis had a worldwide age-standardized prevalence of 3.8% of the global population. The incidence in females was 4.8%, while in males, it was 2.8%, and the peak prevalence was at the age of 50. Osteoarthritis was the 15th major cause of years lived in disability (YLD) in 2000, while in the 2010 report, it was the 11th leading cause of YLD, i.e., a 64% change between the two studies conducted 10 years apart [2]. The economic burden associated with osteoarthritis has also witnessed a steady rise with sustained medical costs, increased workplace absences, and reduced efficiency, resulting from osteoarthritis-induced disability [3]. The rise in the prevalence of osteoarthritis is attributed to multiple factors which include lifestyle changes, obesity, etc.

The chronic nature of osteoarthritis and its widespread prevalence has led to intensive efforts by groups to develop effective therapeutic strategies to tackle this disease. Till date there is no effective therapy for the treatment of osteoarthritis. The

principal approaches to manage osteoarthritis involve nonsurgical and surgical means primarily aimed at reducing the pain and distress. At present there is no disease modifying therapy either for osteoarthritis. A major limitation of the current therapies is that they do not take into account the molecular changes or the actual cause of degradation of articular cartilage which is the principal tissue that is affected during osteoarthritis. Pathogenesis of osteoarthritis is intricately linked to the biology of articular cartilage, and thus it warrants specific attention because degenerative changes that are hallmarks of osteoarthritis can be understood better from the perspective of developmental biology of articular cartilage during endochondral ossification.

2.2 The Development of Articular Cartilage

2.2.1 Endochondral Ossification

In order to understand articular cartilage development, it is important to understand the process of endochondral ossification. The long bones in the appendicular (limbs) skeleton and elements of axial (vertebrae and ribcage) skeleton of vertebrates develop by the process of endochondral ossification, while the bones in the craniofacial skeleton and clavicle develop by the process of intramembranous ossification. The principal feature of endochondral ossification is that the bone develops from within an initial cartilage template, while in intramembranous ossification, ossification proceeds without any cartilage intermediate (Gr. *endo,* within; *chondro*, cartilage).

Endochondral ossification starts with condensation of a bunch of mesenchymal cells in the developing limb bud which turn on the expression of a transcription factor *Sox9*, a member of the high mobility group (HMG) of transcription factors, and is essential for the formation of cartilage [4]. These *Sox9*-expressing cells then turn on the expression of cartilage-specific transcripts such as *Col 2a1*, *aggrecan*, *and Col 11a1*. The cells at this stage have turned on the cartilage differentiation program which is followed by proliferation and an increase in the size of the limb bud [5].

The next principal event is the start of hypertrophic differentiation in the cells at the center of the cartilage primordium. During condensation of limb mesenchymal cells, a layer of cells is excluded which surround the developing cartilage primordium. This layer is referred to as the perichondrium. The initially contiguous cartilage primordium is segmented to give rise to the distinct skeletal elements of the developed limb. The tissue at the site of the future joint is referred to as the interzone. The distal and proximal ends of the perichondrium as well as cells of the interzone secrete parathyroid hormone-related peptide (PtHrP, also referred to as PtHlh). The cells of the developing cartilage express the receptor for PtHrP, PtH1R. As long as the PtH1R-expressing cartilage cells are within the range of diffusion of PtHrP, the cells remain proliferative. However, due to proliferation and growth, once the cells are beyond the range of PtHrP diffusion, they turn on the

expression of Indian hedgehog (Ihh), a key driver of hypertrophic differentiation. Hypertrophic differentiation thus starts in the middle of the cartilage anlagen and spreads toward the ends of the primordia. Ihh and Pthrp, which regulate the early phases of hypertrophic differentiation, also regulate the number of cells that undergo hypertrophy and the formation of the growth plate which is the center of subsequent skeletal development [6]. Hypertrophic cells express *Col 10a1* and *Runx2*. The cells undergo a massive increase in cell size during hypertrophy, and this process is principally responsible for the growth and final proportion of different skeletal elements [7]. The growth plate acts as a source of hypertrophic cells during the continued phase of longitudinal growth. Apart from Ihh and Pthrp, BMP and FGF signaling pathways play major roles in regulating hypertrophic differentiation.

Following hypertrophy, and expression of vascular endothelial growth factor (VEGF), vascularization takes place in most of the elements, and matrix remodeling follows which is mediated by matrix-degrading enzymes such as MMP13. The degradation of matrix paves way for calcification and subsequent bone formation. The source of bone cells has been the center of a century-long debate with one school arguing the incoming vasculature as the source of osteoblasts or bone progenitor cells, while the other sect arguing that hypertrophic cells get trans-differentiated into osteoblasts, yet other groups think that the inner layer cells of the perichondrium/periosteum are induced by the hypertrophic cells to differentiate as osteoblasts [8]. In adults nestin-expressing hematopoietic stem cells have been demonstrated to contribute to bone lineage [9], while during embryonic bone development, both periosteum [10] and hypertrophic cells [11] have been shown to differentiate as osteoblasts. The osteoblasts secrete a matrix rich in Col 1a1 which is the principal component of the bone along with hydroxyapatite. Wnt signaling is known to promote osteoblast proliferation and differentiation [12, 13].

During postnatal stages, the secondary ossification center is set up toward the ends of the developing skeletal elements where further ossification occurs culminating in the formation of epiphysis. The epiphyseal growth plate acts as a source of chondrocytes during most of the processes of endochondral ossification by forming an arrayed structure which consists of proliferating, resting, and hypertrophic cells arranged in columns (Fig. 2.1). After a certain stage of postnatal development, the growth plate ceases to be a seat of chondrocyte maturation and proliferation and is said to be closed by estrogen receptor-mediated signaling though the actual mechanisms underlying this process remain to be uncovered.

After completion of endochondral ossification, most of the initial cartilage template is replaced by the bone except for the cartilage at the ends of the bones which is not invaded by vasculature, does not undergo hypertrophy, and retains the expression of Col 2a1 throughout adult life. From a developmental biology perspective, the cartilage which gets converted into or replaced by the bone is referred to as "transient cartilage," while the cartilage that remains as cartilage at the ends of the bones is known as "permanent or articular cartilage." The process of endochondral ossification is regulated by multiple signaling pathways and transcription factors which orchestrate this process in a fine-tuned manner so as to ensure proportionate development of various skeletal elements [5].

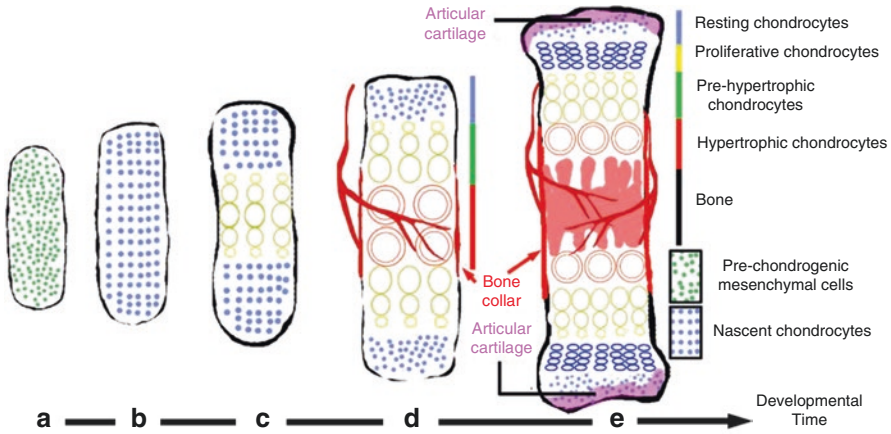

Fig. 2.1 Schematic of endochondral ossification. During endochondral ossification, bone formation takes place within an intermediate transient cartilage template (*A*–*E*). (*A*) It starts with successive differentiation steps of a homogenous proliferative population of pre-chondrogenic mesenchymal cells lined by the perichondrium. (*B*) Following this step, the condensed population of mesenchymal cells differentiates as chondrocytes and forms a cartilage template. (*C*) A small proportion of chondrocytes at the center of the maturing element undergo a terminal hypertrophic differentiation during which they become nonproliferative. (*D*) As development progresses, distinct zones of differentiated chondrocytes appear. At the distal ends, chondrocytes still continue to proliferate and is known to contribute to elongation of bones. Hypertrophic chondrocytes secrete VEGF at the center of the element that enable blood vessels to invade this domain. (*E*) Finally, the hypertrophic zone is replaced by the bone. During early stages of osteogenesis, multiple zones at distinct stages of endochondral ossification may be appreciated. The zones can be broadly categorized as the bone, hypertrophic chondrocytes, pre-hypertrophic chondrocytes, and proliferative chondrocytes which differ morphologically as well as molecularly. Most of the cartilage primordium is eventually replaced by the bone. However, a small population of cells at the end of the skeletal elements will maintain their chondrogenic nature throughout adult life and is referred to as articular cartilage

2.2.2 Articular Cartilage Development

Articular cartilage is a tissue that is a few layers thick and lines the endings of the bones in a mature vertebrate skeleton. It is primarily found at the sites where two bones articulate, i.e., the joints. The principal function of articular cartilage is to provide a smooth, lubricated frictionless surface so that locomotion can occur at joints. It is a highly specialized tissue which has a very organized arrangement of cells and extracellular matrix. The principal component of articular cartilage is the extracellular matrix, which is composed typically of water, Col 2a1, and charged macromolecular aggregates called proteoglycans, e.g., aggrecan, decorin, etc. The articular cartilage is avascular, aneural, and alymphatic and is practically devoid of cells which make up less than 2% of the tissue volume [14]. The development of articular cartilage occurs during the process of endochondral ossification concurrently with synovial joint formation. Thus, any discussion about the development of articular cartilage is incomplete without referring to the development of synovial joints.

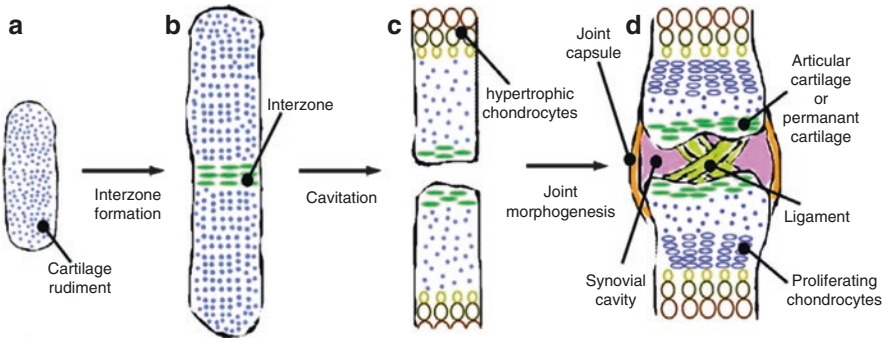

Fig. 2.2 Schematic of synovial joint formation in the appendicular skeleton. (*A*) Formation of synovial joint in vertebrates starts within a contiguous rudiment of homogenous cartilage cells. At this early phase, the skeletal rudiment does not exhibit any overt sign of joint morphogenesis. (*B*) Morphological changes appear at the future joint sites as chondrocytes lose their round shape to acquire a flattened morphology perpendicular to the longitudinal axis. During this step the cells undergo compaction. This result in the formation of a distinct tissue called the interzone. (*C*) Interzone becomes the site of segmentation and eventually splits the contiguous rudiment into two separate elements by the process of cavitation. Bulk of the chondrocytes in each of these elements mature as transient cartilage by endochondral ossification. However, a small portion of the original rudiment adjoining the plane of segmentation resists this change and instead differentiates as articular cartilage. (*D*) After cavitation, joint morphogenesis takes place that leads to formation of synovial joint components such as the joint capsule, ligaments, synovial cavity and synovium, and articular cartilage. During this phase, at the joint site, the ends of the skeletal elements form interlocking shapes that enable frictionless movement across the surface. The articular cartilage is a distinct cartilaginous tissue that maintains chondrogenic nature throughout adult life and is also referred to as the permanent cartilage

The development of synovial joints starts soon after the initiation of cartilage differentiation following the condensation of limb mesenchymal cells at the center of a developing limb bud. As the cartilage primordium proliferates and expands at some previously specified sites, this uninterrupted cartilage template starts to get segmented (Fig. 2.2). The cells at the prospective joint sites become flattened and further compacted to give rise to a specialized region referred to as the interzone. This is followed by actual segmentation of the cartilage primordium, along the middle of the interzone, by a process known as cavitation. Interzone formation and cavitation are associated with downregulation of the expression of Col 2a1 and aggrecan which is crucial for this process to occur [15]. The interzone then undergoes morphogenetic changes which culminate in the formation of articular cartilage and other components of the synovial joint such as the synovial cavity, meniscus, etc.

It was generally believed that transient (i.e., the cartilage that undergoes hypertrophic differentiation) and permanent (or articular) cartilage cells have distinct tissue origins and that the interzone cells give rise to the articular cartilage [16]. However, in a recent study by Ray et al., it was shown that a population of highly proliferative cells, referred to as the distal proliferative zone (DPZ), gives rise to both transient cartilage cells and articular cartilage cells. The cells of the DPZ express Col2a1. These cells as they proliferate and expand come either under the

influence of BMP signaling emanating from the hypertrophic zone and become transient cartilage or come under the influence of Wnt signaling emanating from the interzone and become articular cartilage [17].

A critical point in this context is that interzone is a source of Wnt ligands. The cells of the interzone however are not responsive to Wnt signaling. Wnt signaling is known to be comparatively a short-range signaling pathway and hence affects the cells in the sub-articular zone which is populated by cells from the distal proliferative zone. The cells under the influence of Wnt signaling are protected from BMP signaling, which is a relatively long-range signal, by a tight domain of Noggin expression, which is an inhibitor of BMP signaling. Abrogation of Noggin in developing cartilage leads to transient cartilage differentiation throughout and abolition of articular cartilage differentiation [17, 18]. Moreover, ectopic expression of BMP in developing articular cartilage leads to transient cartilage differentiation at the expense of articular cartilage differentiation. Similarly, misexpression of Wnt ligands in developing chicken limb also leads to ectopic expression of articular cartilage markers. Moreover, exposure of BMP or Wnt to differentiated articular or transient cartilage cells promotes ectopic transient or articular cartilage, respectively [17].

Taking these observations together, Ray et al. proposed a model for simultaneous differentiation of articular cartilage and transient cartilage from a common population of cells (Fig. 2.3). In this context, it may be noted that till date Wnt signaling is the only signaling pathway identified which promotes articular cartilage differentiation, while BMP and Ihh signaling pathways promote transient cartilage differentiation. Components of TGFβ signaling pathway are expressed in the perichondrium as well as in the interzone. Existing literature suggests that TGFβ signaling pathway prevents hypertrophic differentiation. Smad3 is a major transcriptional mediator of TGFβ signaling pathway. A Smad3 mutant mouse expresses ectopic type X collagen in the articular cartilage cells [19]. It needs to be stressed that while critical cells that will undergo articular cartilage differentiation are protected from hypertrophic differentiation but mere prevention of hypertrophic differentiation is not sufficient for articular cartilage development, it needs a pro-articular cartilage signal, i.e., Wnt to proceed in that direction.

2.2.3 Adult Articular Cartilage

Adult articular cartilage is a highly functionally specialized tissue. The articular cartilage is found in diarthrodial or synovial joints and acts as a lubricating surface capable of redistributing mechanical and compressive loads experienced at synovial joints. The composition of extracellular matrix of articular cartilage is responsible for its unique functional capabilities, and it is highly organized, with respect to cells as well as collagen fibers. Extracellular matrix of adult articular cartilage is composed primarily of water, collagen II, collagen IX, collagen XI, collagen VI, and macromolecular protein aggregates known as proteoglycans, principal among which is aggrecan, which is discussed previously. Adult articular cartilage is avascular, aneural, and alymphatic and is hypocellular.

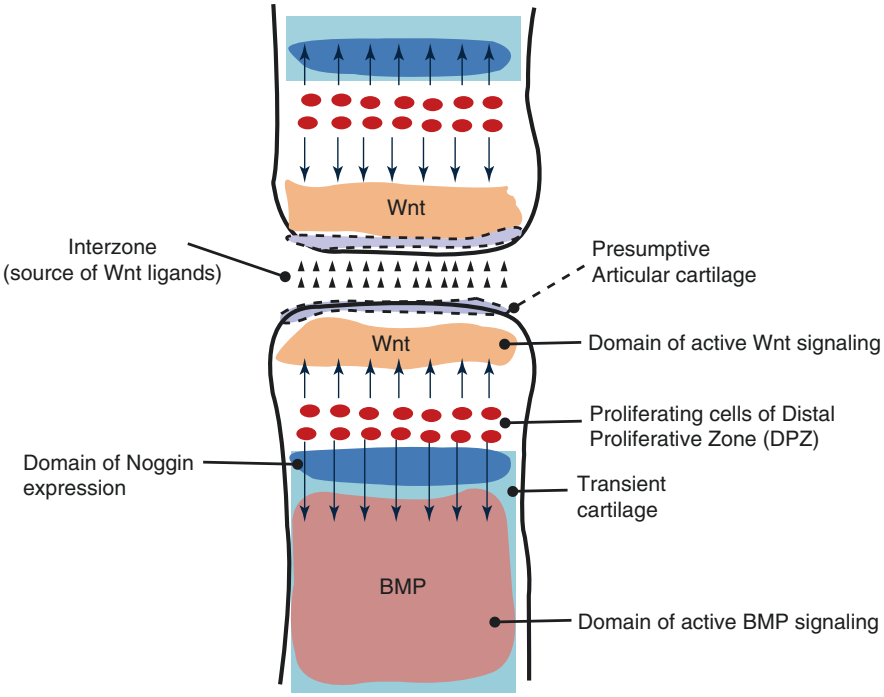

Fig. 2.3 Model for differentiation of articular cartilage and transient cartilage simultaneously from a common pool of progenitor cells. During embryonic development, soon after segmentation has proceeded, cells on either side of the plane of segmentation are specified as interzone cells which are flattened and nonproliferative. The region immediately adjacent to the interzone will develop into articular cartilage, while the rest of the element will undergo transient cartilage differentiation. However, there exists a band of proliferative, bipotential cells referred to as the distal proliferative zone (DPZ). These bipotential cells express Col 2a1, divide, and expand toward transient cartilage and articular cartilage domains. The transient cartilage is a field of BMP signaling, whereas the interzone is a source of secreted Wnt ligands. Cells of the DPZ expanding toward BMP signaling domain will undergo transient cartilage differentiation, while cells expanding toward interzone will be exposed to Wnt ligands and differentiate into articular cartilage. These two opposing signaling domains are separated by a thin band of Noggin expression (an inhibitor of BMP signaling). This expression domain of Noggin insulates the cells expanding toward articular cartilage domain from BMP signaling and differentiating as transient cartilage, ensuring the simultaneous differentiation of articular cartilage and transient cartilage

The principal cell type found is articular chondrocytes which make up to 2% of the tissue volume and are arranged in functionally and structurally varied zones. The superficial layer is composed of flattened cells which are specialized to secrete a proteoglycan known as lubricin or Prg4, which creates a lubricated surface for frictionless articulation at joint surface, and the collagen fibers in this layer are aligned in parallel direction to that of the surface. These superficial fibers possess high tensile strength and superior tolerance to mechanical strains and stresses that the articular cartilage is subjected to. The superficial zone is also known to possess a population of progenitor cells that can contribute to cartilage repair [20]. As these

cells possess stem cell markers, they are also referred to as cartilage stem/progenitor cells (CSPCs) [21].

The intermediate or middle zone is composed of round chondrocytes where the collagen fibers are arranged oblique to the surface. This layer helps to protect the cartilage from high compressive loads. This layer is fairly devoid of cells while rich in matrix, thus making up the major volume of the tissue. The deeper layer of articular cartilage possesses the highest resistance to compressive forces, given that collagen fibers are arranged perpendicularly to the surface and the cells are arranged in columns. This zone possesses the highest proteoglycan content and largest collagen fibrils and is largely devoid of water and replete with chondrocytes.

The deep zone is followed by the tidemark which is a transition between the cartilage and the subchondral bone. The tidemark marks the beginning of calcified zone, wherein the collagen fibers of articular cartilage attach to the bone and provide anchorage to the cartilage. The calcified zone is populated with hypertrophic cells which express collagen X. The subchondral bone following the calcified bone is also believed to be a source of bone marrow mesenchymal cells as it is the seat of secondary ossification [14, 22].

Articular chondrocytes, the major cell type of adult cartilage, are unique cells which are highly specialized to secrete the cartilage matrix and do not possess any mitotic potential in normal conditions, a property thought to be responsible for the low regenerative capacity of articular cartilage. These cells are critical for the functioning of the normal cartilage and are the epicenters of articular cartilage pathologies as well.

2.3 Molecular and Histological Changes in Osteoarthritis

2.3.1 Overview of Principal Changes

Osteoarthritis is a disease that is primarily characterized by a progressive, irreversibly degradation of the articular cartilage which is attributed to a multifactorial etiology. Since the earliest mention of osteoarthritis in the Greek medical literature and the scientific description in the mid-nineteenth century, there has been much progress in our understanding of this disease on the whole, but a lucid understanding of the molecular processes responsible for the development of this disease still eludes us. The stages in pathogenesis can be characterized molecularly and histologically into multiple phases depending on the severity of the disease. These changes mirror the increasing degradation of articular cartilage.

The principal changes during the development of osteoarthritis can be grouped into two major categories: (1) gross changes and (2) cellular or molecular changes.

2.3.1.1 Gross Changes

Osteoarthritis is typically marked by a characteristic deterioration of articular cartilage that is spontaneous or in response to an injury-induced lesion known as a defect. The progression of osteoarthritis on a macroscopic level affects different zones of articular cartilage in different stages of the disease.

During early stages of the disease, minor focal defects on the superficial layer of the cartilage are observed. These changes include surface irregularities that do not penetrate the middle zone and are limited in their thickness to a few cell layers. A few cells can be seen undergoing hypertrophy and cell clustering or cloning which is a result in an increase in mitotic index. There is some thinning of articular cartilage in the middle compartments but is limited to surface layers only, and some fibrillations are usually found.

During mid-level stage of the disease, the defects in the matrix penetrate to the middle layers of the cartilage, and matrix fibrillations are branched and cover a sizeable portion of the articular cartilage surface. There is loss of proteoglycan-specific staining such as Safranin-O, Toluidine blue, etc. The proportion of hypertrophic cells is higher and the surface irregularities are pronounced. In advanced stage of the disease, there is further erosion of the cartilage, and the surface layers are damaged to a large extent such that there is an extensive sclerosis and bone protrusion or osteophytes can be observed as the surface lesions have progressed to the subchondral zone. The loss of proteoglycans is extensive and upon staining, an extensive area not staining with Safranin-O or Toluidine blue is overt. The overall contour of articular cartilage is lost, and there is an appearance of regenerated fibrocartilage at some sites [23].

2.3.1.2 Molecular Changes

Articular cartilage function and maintenance are critically dependent on a tight regulation of anabolism and catabolism that is mediated by the chondrocytes, and any perturbation in this metabolism can lead to pathologies. During osteoarthritis, the tightly regulated metabolism of articular chondrocyte is perturbed which can be the result of a multitude of factors influencing the chondrocytes. On the whole there is a loss of chondrocytes, because of extensive matrix fibrillations or lesions. However, apoptosis has been ruled out as a probable cause of cell loss since cell death has not been observed in multiple animal model-based studies [24].

The matrix synthesis carried out by articular chondrocytes is affected especially during osteoarthritis. The principal change observed during osteoarthritis is a dysregulation of the turnover of the cartilage matrix, especially type II collagen, aggrecan, collagen type IX, etc. A normal articular chondrocyte matrix is arranged into three prominent zones: pericellular, territorial, and interterritorial matrix depending on the distance from the surface of the articular chondrocyte [14]. The pericellular matrix has receptors and interacting molecules, e.g., hyaluronan cognate receptor CD44 and the discoidin domain receptor (DDR) (receptor for collagen type II). In the immediately adjacent territorial matrix, aggrecan and other macromolecular aggregates are found. Interterritorial matrix primarily comprises of proteins such as cartilage oligomeric matrix protein (COMP), fibromodulin, decorin, etc. The initial hallmarks of osteoarthritis include an upsetting of the anabolism to catabolism ratio of chondrocytes for reasons not entirely understood yet. This dysregulation of matrix metabolism manifests as generalized or focal defect in the cartilage which is marred by a loss of matrix components such as collagen type II and aggrecan.

During the initial phases in experimentally induced osteoarthritis models of mice, a spike in matrix synthesis is usually observed [25]. This initial spike in synthesis is followed by matrix turnover dysregulation such as enhanced aggrecan catabolism. The degradation of collagen and aggrecan are major hallmarks of osteoarthritis [26]. The collagen degradation is the result of cleavage by enzymes known as collagenases, which are actually matrix metalloproteinases (MMPs). The major enzymes involved in cleavage of collagen are MMP-1, MMP-4, MMP-8, MMP-13, and MMP-14 which are considered to play critical roles in the development of osteoarthritis [27, 28]. It may be recalled that MMP-13 plays a crucial role in matrix remodeling during endochondral bone formation as well [29]. Aggrecan on the other hand is cleaved by matrix metalloproteinases as well as by another group of enzymes known as disintegrates with integrin motifs (ADAMTS). ADAMTS4 and ADAMTS5 are the principal mediators of aggrecan cleavage in articular cartilage [30, 31]. Loss of aggrecan leads to decreased resistance to compressive stress and deformation, and given the dynamic mechanical loads at the synovial joints, the stress relaxation response of articular cartilage is impaired upon extensive aggrecan depletion.

The other principal molecule that is involved in the pathogenesis of osteoarthritis is the vascular endothelial growth factor (VEGF). Articular cartilage is normally avascular in nature and the blood supply is restricted to the subchondral bone. The avascular nature of articular cartilage is attributed to the expression of anti-angiogenic molecules specific to interterritorial articular cartilage zone such as chondromodulin-I (ChM-I), tenomodulin, troponin, etc. [32, 33]. It is however observed that osteoarthritic cartilage is replete with blood vessels. Interestingly, a high level of VEGF expression is observed in articular cartilage during osteoarthritis and is usually not found in healthy articular cartilage [34, 35]. Injection of VEGF into knee joints in mice induces osteoarthritis-like changes [71]. Moreover, VEGF induces the expression of matrix remodeling enzymes such as MMP-1 and MMP-13 which are known to be important molecules in pathogenesis of osteoarthritis [36]. It is known that VEGF plays a critical role during the later phases of endochondral bone formation and that antibody-mediated inhibition of VEGF activity in postnatal mice leads to a reduction in lengths of forming long bones and an uncoupling of cartilage remodeling, hypertrophic differentiation, and ossification [37]. Defects in bone formation are also observed in mice that are deficient in some isoforms of VEGF [38]. It is established that angiogenesis is a critical aspect of endochondral bone formation and that VEGF is a principal mediator of vascular invasion during bone development.

Osteophytes, or bone spicules, are bony outgrowths that arise in the articular cartilage during the pathogenesis of osteoarthritis. They are characteristic of osteoarthritis development and are often used as diagnostic feature for clinical determination of osteoarthritis as they are prominently visible during radiographic examination of joints [39]. Moreover, experimental models of osteoarthritis in mice, rats, dogs, and rabbits also exhibit development of osteophytes [40]. There is no universally accepted explanation for the appearance of osteophytes as some groups claim that they are an attempt at cartilage repair, while there is a view that

osteophytes develop in order to tackle the mechanical instability at joint surfaces that results due to progressive degradation of articular cartilage. However, the formation of osteophytes has been molecularly characterized, and it is established that it starts with a cartilage template which undergoes endochondral ossification, albeit ectopically, and finally leads to the development of the bone [41]. The osteophytes are composed of hypertrophic cells expressing collagen X, which also express MMP-13 and VEGF, and subsequently secrete matrix rich in Col1a1 and permit ossification though they are also known to express Col 2a1 and aggrecan [42]. The osteophyte extracellular matrix is not conducive for the natural function of articular cartilage, and since osteophytes are bony in nature, this enhances the friction at the joint surfaces and worsens the prognosis of osteoarthritic patients [43]. One may recall that the end point of endochondral ossification is also the formation of Col I-expressing osteoblasts.

2.3.2 Resemblance to Transient Cartilage Differentiation

During endochondral ossification, the cartilage that stays as cartilage, i.e., permanent cartilage, and the cartilage that eventually is replaced by the bone, i.e., the transient cartilage, come from the same population of cells [17]. As discussed earlier, this is brought by a tightly regulated precise spatial domain of BMP and Wnt signaling influence. It has been demonstrated that chondrogenic cells of the developing limb elements which express Col 2a1, and are exposed to Wnt signaling emanating from the interzone region, will finally differentiate as articular cartilage cells, while cells exposed to BMP signaling will end up as transient cartilage eventually getting replaced by the bone [17].

During transient cartilage differentiation, there is a predetermined chain of molecular events that proceeds in a sequential manner. The first step in transient cartilage differentiation is the expression of pre-hypertrophic marker Ihh which is followed by an expression of Col X, a marker of hypertrophic chondrocytes. The next step is the remodeling of cartilage matrix mediated by MMPs followed by vascular invasion mediated by VEGF and subsequent recruitment of osteoblasts expressing type I collagen. There is an uncanny resemblance between the process of transient cartilage differentiation and the order of events leading to pathogenesis of osteoarthritis. A critical aspect of pathogenesis of osteoarthritis is hypertrophic differentiation which is crucial to endochondral bone formation. The articular chondrocytes in healthy cartilage do not show hypertrophic features, but during osteoarthritis, there is an expression of markers such as Col X. The expression of hypertrophic markers has been shown to increase with the severity of the disease [44]. Therefore, hypertrophy is often used as a marker of degradation of cartilage though there is currently no census on this.

Akin to endochondral ossification, matrix remodeling during osteoarthritis is also accompanied by invasion of blood vessels. Osteoarthritic cartilage shows overexpression of VEGF receptors and ligands in both human patients and experimental animal models. The role of VEGF in the pathogenesis of osteoarthritis is not

Table 2.1 Comparison of markers among articular cartilage, transient cartilage, and osteoarthritic cartilage

Marker	Adult articular cartilage	Transient cartilage	Osteoarthritic cartilage
Type II collagen	Yes	No	Degraded
Aggrecan	Yes		Degraded
Ihh	No	Yes	Yes
Type X collagen	No	Yes	Yes
MMP-13, MMP-2, MMP-9	No	Yes	Yes
ADAMTS-4, ADAMTS-5 (aggrecanases)	No	Yes	Yes
VEGF	No	Yes	Yes
Runx2	No	Yes	Yes

completely clear, but it has been linked to osteophyte formation, which is a feature of advanced-stage osteoarthritis [45]. Hypertrophic cells in osteoarthritic cartilage express VEGF which substantiates the hypothesis that osteophyte formation during osteoarthritis could be mediated by VEGF.

During transient cartilage differentiation, matrix remodeling by MMP-13 follows an invasion of hypertrophic cartilage by blood vessels. The matrix remodeling process is a crucial aspect in the development of osteoarthritis because the loss of tensile and compressive properties of articular cartilage in a mechanically active environment leaves little scope for recuperation of damage in a tissue that bears little regenerative potential of its own. MMP-13 has been shown to be especially overexpressed in cartilage of osteoarthritic patients [26].

Similarly, expression of Col I is also observed during osteoarthritis in articular cartilage which is often the result of cartilage repair program that leads to the development of a mechanically inferior fibrocartilage in place of the native cartilage and contributes to osteophyte formation [46]. Moreover, the expression of *Runx2*, which is a marker for osteoblast differentiation, is also routinely observed in osteoarthritic cartilage as well as osteophytes in patients and in experimental models. It is easily observable that much of the progression during disease development in osteoarthritis hints at a recapitulation of transient cartilage differentiation program (Table 2.1). The pertinent question is whether the same regulatory mechanism that drives transient cartilage differentiation also mediates the pathogenesis of osteoarthritis. This may be addressed by combining genetics and surgical manipulations in experimental mouse models of osteoarthritis.

2.3.3 Developmental Biology Perspective on Osteoarthritis

The discussion in the preceding section highlights the similarity between osteoarthritis and transient cartilage differentiation. It has been observed that the development of osteoarthritis closely follows molecular events that are characteristic during the differentiation of transient cartilage. Moreover, multiple studies exist where a