CHAPTER 3

Tissue Formation during Embryogenesis

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LEARNING OBJECTIVES

- To appreciate that tissue engineering strategies rely on the merger of developmental biology with the fields of engineering in a strategy nowadays known as developmental (re)engineering
- To understand the origin of pluripotent stem cell populations in the embryo and to realize that each organ is build out of stem cells from endodermal, mesodermal, ectodermal, and neural crest origin
- Be able to recapitulate some of the basic principles by which cellular differentiation and specification is induced during organogenesis
- To appreciate that the subsequent, timely and proper dosing of signaling events, for example, initiated by cell migration, cell–cell interactions, para- and intracrine signaling of growth factors, epithelial-to-mesenchyme transitions, and environmental factors define cell specification and shape organs
- To understand the importance of paracrine signaling between mesenchymal and epithelial cells, for example, between smooth muscle cell and endothelial cells in blood vessel development or dermal fibroblasts and keratinocytes in skin development and that this paracrine signaling is instrumental to obtain functional tissue
- To understand the role of adaptive signaling in tissue formation in response to environmental factors like pulsatile fluid flow and wet-to-air transitions in cell specification and tissue architecture

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- Be able to recapitulate the sequential steps that can be recognized in skin formation and how these steps are reiterated in skin tissue engineering
- To understand the difference between intramembranous and endochondral bone formation and the role of the osteoblasts, chondrocytes, and osteoclasts in both processes
- To appreciate that members of the Bone Morphogenetic Protein (BMP)/ Transforming Growth Factorβ (TGFβ) superfamily, the Fibroblast Growth Factor (FGF) family, and the Wingless-Int (Wnt) family are involved in organogenesis of almost all organs
- To realize that key concepts from developmental biology such as modularity and robustness can be recapitulated in engineering strategies to generate functional organs.

3.1 INTRODUCTION

Tissues in the human body are the result of millions-of-years of evolution. This process has resulted in the natural selection of a tissue structure that is optimally adapted to its function in the body. Tissue engineering aims at repairing or replacing damaged tissue that cannot or insufficiently be repaired by the intrinsic repair mechanisms present in almost all organs of an individual. In most instances, these intrinsic repair mechanisms recapitulate many of the processes involved in the formation of an organ during embryogenesis. Incorporation of these mechanisms in tissue engineering procedures will significantly contribute to the success of the construct in the body. Thus, important lessons for tissue engineering can be learned from the formation of organs during embryogenesis. For example, on (1) the origin of cells that contribute to the formation of a particular organ, (2) the growth factors and their interrelationship in the formation of an organ, (3) the mechanisms by which undifferentiated stem or precursor cells are induced to specialize into an organ of specific cell type, (4) the subsequent steps in organ formation, and (5) the interaction between cells and their environment consisting of both the extracellular matrix and neighboring cells. Ideally, inclusion of all these aspects in the tissue engineering procedure will provide the best opportunities for success. Indeed, one of the basic concepts of developmental biology, i.e., the modularity of the tissue architecture according to which intermediates in tissue development constitute semiautonomous entities, is nowadays incorporated in many tissue engineering strategies to optimize the product.

The past decade has shown a rapid increase in our understanding of the cellular and molecular basis of tissue formation. In addition, our knowledge about stem cell renewal and maintenance of pluri- and multipotency has substantially grown. In fact, it is now possible to generate pluripotent stem cells from a variety of tissue types in a process called reprogramming. It still remains a challenge to combine this knowledge into successful strategies for the engineering

of functional tissues that can replace lost or worn out organs. The tissue engineering strategy in which the principles of developmental biology are merged with typical engineering disciplines like chemical, biomechanical, and biophysical engineering is nowadays known as developmental engineering or developmental (re)engineering (Lenas et al., 2009; Tonnarelli et al., 2014).

This chapter aims at providing a background on the formation of various organs during embryogenesis that are subject of tissue engineering techniques, like the heart, blood vessels, peripheral nerves, skin, bone, and cartilage. It starts with providing an overview of the formation of pluri- and multipotent cell populations and their positioning in the overall body plan. Subsequently, some of the basic mechanisms by which these cell populations are recruited in organogenesis and are induced to specify are discussed in greater detail.

3.1.1 The Formation of Pluripotent Cells

All tissues in the adult mammal originate from the fertilized oocyte, called zygote. The first divisions of the fertilized egg are not accompanied by cellular growth and are therefore referred to as cleavage divisions and arising cells are called blastomeres. At the early cleavage stages, each blastomere is still totipotent and thus is capable of forming an entire organism.

The first differentiation occurs at the morula stage after the third series of cleavage divisions (8 cell stage). The cells that are on the outside of the morula differentiate into the trophectoderm, a cell layer mediating implantation and formation of extraembryonic structures such as the placenta. The cells on the inside of the morula will give rise to the inner cell mass. In the next stage, the embryo is called a blastocyst and contains besides the trophectoderm and a fluid-filled cavity or

CLASSICAL EXPERIMENT 1: GENERATION OF THE FIRST EMBRYONIC STEM CELL LINES

Already in the ¨50s of the twentieth century, it was recognized that the 129Sv strain of mice exhibited a high incidence in the formation of spontaneous testicular tumors, the so-called teratocarcinomas. Teratocarcinomas can also be induced in mice by the grafting of early embryos into extrauterine sites (Stevens, 1967). Individual cells from these tumors could be transplanted to other mice where they formed new tumors, demonstrating the existence of cells with self-renewal capacity or stem cells in the tumors (Kleinsmith and Pierce, 1964). Teratocarcinomas contain a wide variety of differentiated cells that were derivatives of the stem cells. The exact potency of these stem cells remained unclear until chimeric mice were created by injecting few of the tumor cells into blastocyst stage embryos. The tumor cells started to participate in the development of many cells and tissues, including germ cells. This demonstrated that the teratocarcinoma cells were pluripotent (Mintz and Illmensee, 1975; Papaioannou et al., 1975). The cell lines that were derived from teratocarcinomas were called embryonal carcinoma cell lines. The pluripotency of the embryonal carcinoma cells raised many questions. These cells were after all derived from embryos. It was hypothesized that the early embryo contained cells that divided and remained pluripotent unless they received signals for differentiation, as normally occurred during embryogenesis. It seemed logical to assume that these cells could be directly isolated from the embryo and kept in culture.

Continued...

CLASSICAL EXPERIMENT 1: GENERATION OF THE FIRST EMBRYONIC STEM CELL LINES *Continued*

Martin Evans and Matthew Kaufman, then from the University of Cambridge, were the first to directly generate progressively growing cultures from preimplantation mouse embryos. By delaying the implantation of blastocyst stage embryos, they obtained embryos with a bigger-than-normal inner cell mass. Using their knowledge of culture media obtained from working with embryonal carcinoma cells, they were able to culture cells from these large blastocysts. The cells strongly resembled embryonal carcinoma cells; they too formed teratomas when injected into mice and differentiated in vitro. Evans and Kaufman published their seminal paper on the derivation of pluripotent embryonic cells in 1981 (Evans and Kaufman, 1981). In the same year, Gail Martin from the University of California independently established pluripotent cell lines from mouse blastocysts (Martin, 1981). Instead of using delayed implantation embryos, Martin was able to successfully culture the embryonic cells by using a feeder layer of fibroblasts and conditioned medium from embryonal carcinoma cells. Martin coined the term embryonic stem (ES) cells for these embryo-derived cell lines. The pluripotency of the ES cells was convincingly demonstrated by the formation of healthy germ-line chimaras (Bradley et al., 1985).

The aim of the underlying studies was now at reach, namely to use mutation-carrying cells for the generation of mice bearing these mutations to assess their functional consequence. Gene-targeting by homologous recombination in ES cells was developed (Smithies et al., 1985), which proved very fruitful. Indeed, the production of many knockout mice and the knowledge of gene function by studying these mice would not have been possible without ES cells.

blastocoel also an inner cell mass. The inner cell mass consists of pluripotent cells that will ultimately form the entire embryo. Cells can be isolated from the inner cell mass and under the right conditions kept into culture. They are then called embryonic stem (ES) cells. ES cells have the capacity to self-renew and are truly pluripotent as they can differentiate into every cell type of the embryonic and adult organism. ES cells cannot differentiate into extra-embryonic tissue. Their capacity to self-renew and their pluripotency makes ES cells an excellent cell source for tissue engineering purposes. In mouse genetics, ES cells are an important tool for the generation of genetically modified animals.

The first stages of embryonic development occur when the zygote travels through the oviduct prior to implantation in the uterus. Under the appropriate culture conditions, this process can be mimicked in vitro. Thus, isolated oocytes (human or from other mammals) can be fertilized in vitro and cultured until the early blastocyst stage. From these blastocysts, it is possible to generate ES cells that could be applied in regenerative medicine and tissue engineering. Thus, left over embryos from in vitro fertilization procedures could be used for the establishment of new human ES cell lines. Further development of the embryo requires, however, implantation in the uterus and the formation of the germ layers by gastrulation.

3.1.2 Preimplantation Development and Gastrulation

When the embryo has reached the uterus, at around 3–4 days after fertilization in mice and 5–7 days in humans, it is ready to implant in the uterine tissue.

Subsequent steps in embryonic development aim at establishing the proper structure in which specification of the three germ layers, the ectoderm, endoderm, and mesoderm, can occur by a process called gastrulation.

During gastrulation, the three definitive germ layers that construct the adult organism are established: the outer ectoderm, the inner endoderm, and the interstitial mesoderm. In the mouse, gastrulation starts at about 6.5 days after fertilization and in the human at about 13–15 days. The ectoderm forms the outer part of the skin, brain cells, nerve cells, parts of the eye like the lens, epithelial structures of the mouth and anus, the pituitary gland, parts of the adrenal glands, and pigment cells. The endoderm forms the lining of the gastrointestinal and respiratory tracts, plus the liver, pancreas, thyroid gland, thymus, and the lining of the bladder. The mesoderm gives rise to skeletal muscle, heart and blood vessels, connective tissue, kidney, urethra, gonads, bone marrow, blood, bone, cartilage, and fat. During gastrulation, extensive cell movements take place through which cells acquire new positions and new neighbors with which to interact. These cell movements are accompanied by an increase in cell number and a decrease in cell doubling time so that in the mouse, the cell number increases from about 660 at the start of gastrulation to 15,000 at the end of 24 h.

3.1.3 Establishment of the Body Plan by Morphogen Signaling

During gastrulation, not only are the germ layers specified but cells will also receive instructions on their future position and role in the developing fetus by morphogen signaling. An important factor in this process is Nodal, which is a member of the bone morphogenetic protein (BMP)/transforming growth factorβ (TGFβ) superfamily of growth factors. Nodal is first expressed in the proximal part of the epiblast, the part of the embryo that will give rise to all embryonic structures, and is expressed in the node, hence its name. The node organizes gastrulation. Nodal signaling is essential for the formation of mesoderm and definitive endoderm. The highest levels of Nodal are found in the node and with increasing distance to the node, the concentration of Nodal will gradually decrease establishing a so-called morphogen gradient. Cells along this gradient are exposed to different levels of Nodal, providing the cell with important instructions on its future position and role in the fetus. Morphogen gradients are important and recurrent mechanisms by which undifferentiated cells receive instructions on their future position and role in the body.

3.1.4 Neural Crest Cells

Besides the three germ layers that are established during gastrulation, a fourth pluripotent cell population is required for organogenesis. This population consists of neural crest cells (NCCs) (Le Douarin and Klacheim, 1999). Although NCCs are derived from the ectoderm, they are sometimes called the fourth germ layer because of their importance. NCCs arise relatively late in embryonic

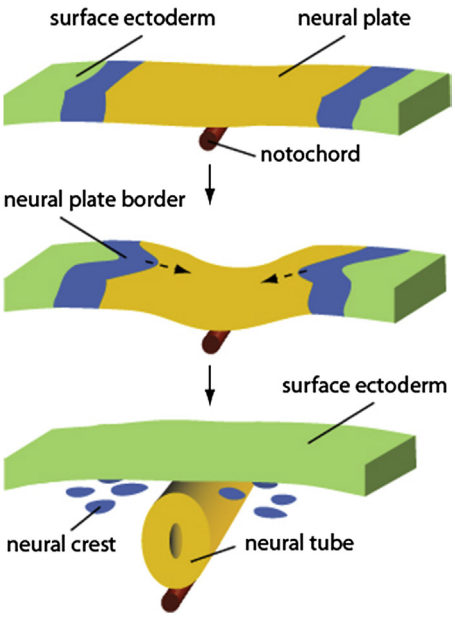

FIGURE 3.1

Formation of the neural crest. Midline ectoderm differentiates into neural cells to form a neural plate (yellow) by signals from the underlying notochord (red). Induction of neural crest (blue) cells takes place at the border between surface ectoderm (green) and the neural plate by signals from the mesoderm and the surface ectoderm. The sides of the neural plate fold together and close to form a neural tube. The neural crest cells delaminate from the ectoderm and migrate to their destinations.

development after establishment of the general body plan and their formation is tightly linked with the development of the central nervous system. The differentiation of the central nervous system starts with formation of the neural tube from the neural plate. The neural plate is a layer of ectodermal cells in the dorsal midline of the embryo. Signals from the underlying notochord (the future nucleus pulposa) instruct cells in the neural plate to proliferate and adapt a tube-like shape that will eventually generate the entire central nervous system. NCCs are generated at the interface of the neuroectoderm with the surface ectoderm, the so-called neural plate border (Figure 3.1).

From their source of origin, NCCs migrate extensively to specific places in the embryo where they generate a diverse group of differentiated cells that can be divided into four distinct subgroups: cranial, cardiac, vagal, and trunk cells that together give rise to sympathetic and parasympathetic neurons, glia cells, fat, cardiac mesenchyme, melanocytes, skin, connective tissue of salivary, thymus, adrenal, thyroid, and pituitary glands, smooth muscle cells (SMCs) of arteries, tooth, and bone and cartilage particularly of the face and cranium. The NCCs therefore have extreme plasticity and are truly pluripotent giving rise to cells

from the endoderm, mesoderm, and ectoderm lineage. Due to this extreme plasticity, pluripotent NCCs would provide an excellent cell source for tissue engineering and regenerative medicine particularly for restoration of cranial and facial birth defects. However, NCCs are extremely difficult to isolate due to their high mobility and to the fact that they are derived from a transient structure. The neural crest disappears soon after the neural tube is closed and the neural plate border has disappeared. Consequently, limited studies have been done with NCC as cell source for tissue engineering purposes.

It is thought that the surface ectoderm together with the underlying mesoderm induces the neural plate to form NCCs by signals that include BMPs, in particular BMP4 and BMP7, fibroblast growth factors (FGFs), and members of the Wingless-Int (Wnt) family of morphogens. As they arise, NCCs undergo an epithelial-to-mesenchymal transition (EMT) characterized by expression of members of the *Snail* family of zinc finger transcription factors. Migration is probably initiated by a decrease in the amount of N-cadherin, which is a cell adhesion molecule typically expressed in epithelial cells. EMT is an important mechanism by which cells can dramatically change their fate. It plays an essential role not only in organogenesis but also during tumorigenesis, e.g., the transformation of a carcinoma in situ to an invasive, metastatic tumor.

3.2 CARDIAC DEVELOPMENT

Rebuilding the injured heart after infarction is a major challenge for tissue engineering. Presently, various cell-based approaches are used. Initially, this work started with committed myoblasts, the precursor cells of cardiac muscle, but now a variety of undifferentiated cells are used, such as endothelial progenitors, mesenchymal stem cells, resident cardiac stem cells, and ES cells. Various cell types from different origins are involved in heart formation. Important lessons can be learned from the complex interplay between these cell types that eventually give rise to the four-chambered cyclically beating heart during embryonic development. The heart, being the first functionally active organ, must continuously adapt to the growing needs of the embryo, whereas in postnatal life it has to support the ever-changing activity levels of the body. The development of the heart is modular in which intermediates in tissue development constitute semiautonomous entities. Figure 3.2 provides a time table of the sequential formation of different parts of the heart during embryogenesis. The proper and subsequent integration of all these processes is of crucial importance. Disturbance will result in embryonic heart failure or congenital heart deformities.

Here a framework is provided for understanding the multiple origins of the cardiac cell populations, including the cardiomyocytes, fibroblasts, and smooth muscle and nerve cells. Furthermore, the increasing complexity of the heart's architecture will be explained, in particular chamber formation, cardiac septa

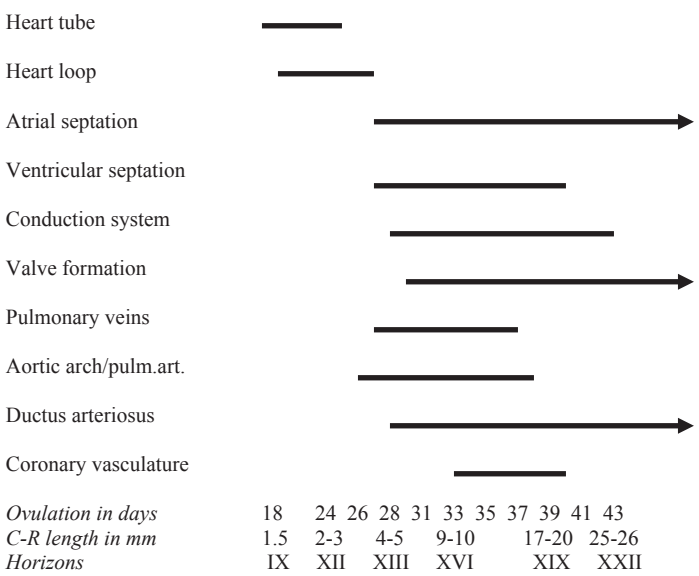

FIGURE 3.2

Time table of human heart development during embryogenesis. Note the different developmental windows for the various parts of the heart. Days since ovulation as a measure of developmental stage is compared with crown-rump length (C-R) and with the human stages of development indicated by Streeter's Horizons.

and valves, the coronary circulation, and the conduction system. Finally, from the myriad of growth factors and transcription factors important during the building of the heart a brief selection will be provided.

3.2.1 Cell Interactions

Cardiac formation starts in the so-called cardiogenic plates. The cardiogenic plates develop at the bilateral anterior tips of the unsegmented splanchnic mesoderm. The splanchnic mesoderm refers to cells of the inner lining of the body cavity. The transcriptional machinery for defining the cardiomyocyte is still largely unclear, and seems more complicated than, for instance, for skeletal muscle, in which the transcription factor MyoD is elemental and sufficient for muscle cell differentiation. Evidence exists that the transcription factor myocardin and signaling via BMPs govern cardiomyocyte differentiation. Myocardin is not specific for cardiac muscle, since it is also present at the onset of SMC differentiation. The cardiogenic plates fuse in the midline (Figure 3.3(a)) and the precursor cells differentiate into cardiomyocytes and endocardial cells (see Gittenberger-de Groot et al., 2005, for a schematic overview). The cardiomyocytes become the contracting part of the heart. The space between myocytes and endocardial cells, which have adapted an epithelial phenotype, becomes filled by cardiac jelly produced by the myocytes. The endocardium gives rise to mesenchymal cushion cells by a

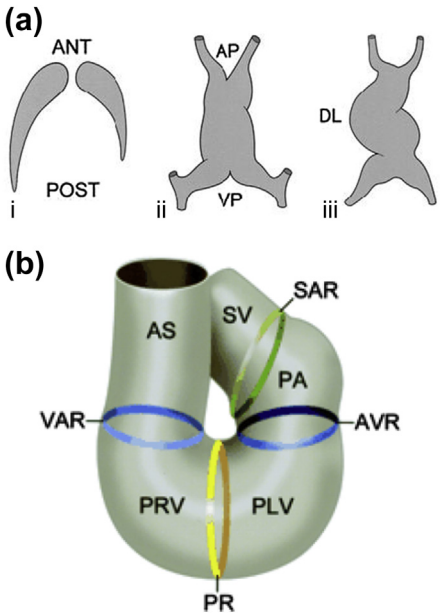

FIGURE 3.3

The first stages in heart development. (a) Schematic representation of (i) the bilateral formation of the mesoderm-derived cardiogenic plates, which are not quite symmetric. (ii) After fusion, these form a straight heart tube with an arterial pole (AP) and a venous (VP) pole. (iii) Thereafter, the tube starts its rightward (dextral) looping (DL). (b) Schematic drawing of the looped heart tube with the cardiac chambers and the transitional zones. Following the blood flow from venous to arterial, we can distinguish the sinus venosus (SV), the sinoatrial ring (SAR), the primitive atrium (PA), the atrioventricular ring (AVR), encircling the atrioventricular canal, the primitive left ventricle (PLV), the primary fold or ring (PR), the primitive right ventricle (PRV), the outflow tract ending at the ventriculoarterial ring (VAR), and the aortic sac (AS). *Adapted from Gittenberger-de Groot et al. (2005).*

process known as EMT (Hay, 2005) and, as a consequence, the cardiac jelly will transform into the thick endocardial cushions containing extracellular matrix and mesenchymal cells. Part of these cells is also recruited from the neural crest (see below). This is a complex process orchestrated by various growth factors and many transcription factors. The atrioventricular cushions reside in the transition zone of the primitive atrium and ventricle, the so-called atrioventricular (AV) canal, whereas the outflow tract (OFT) cushions are found in the conotruncal transition between ventricle and aortic sac (Figure 3.3(b)). Both cushion systems are in close proximity in the inner curvature of the heart. The OFT cushions are important in separation of the OFT into the aorta and the pulmonary trunk, while the AV cushions will play an important role in AV septation by heterologous fusion with the interventricular or primary fold and with the intra-atrial spina vestibuli (see Figure 3.3(b) and Gittenberger-de Groot et al., 2005).

3.2.2 Extracardiac Cell Populations

Two extracardiac cell populations are very important in heart formation: the cardiac neural crest cells (CNCCs) and the proepicardial organ (PEO). Both primordia have in common that their original epithelium (being neuroectoderm and splanchnic epithelium, respectively) transforms by (EMT into migratory mesenchyme. Subpopulations of NCCs migrate into the pharyngeal arches (to form, e.g., the arterial vessel wall: adventitial fibroblasts and SMCs), and into the secondary heart field, a part of the dorsal body wall adjacent to the heart tube (*vide infra*), subsequently migrating into and contributing to the OFT and the AV cushions. The PEO starts as a small grape-like structure extending from the inner lining of the body cavity, the splanchnic mesoderm. It expands and migrates as an epithelial sheet over the outer surface of the myocardium to form the epicardium. After complete coverage of the heart tube, EMT will give rise to subepicardial mesenchyme. A large number of these so-called epicardial-derived cells (EPDCs) migrate into the heart and differentiate into cardiac fibroblasts, SMCs, and adventitial fibroblasts of the coronary vessels (Gittenberger-de Groot et al., 1998). In bulk number the fibroblasts comprise the largest cell population, providing for the extracellular matrix evolving into the fibrous heart skeleton. This system mechanically supports the cardiac valves and the tendinous apparatus during the various phases of life. After cardiac infarction, the EPDC-derived fibroblasts provide for the scar tissue giving integrity to the cardiac wall, although it is, of course, not contractile. However, in early embryonic development, it is responsible for the induction of the architecture of the cardiac wall. Experimental ablation of the PEO results in an aberrant coronary vasculature feeding a paper thin myocardium almost devoid of trabeculations. This resembles a failing heart, eventually leading to embryonic death (Gittenberger-de Groot et al., 2000).

3.2.3 Looping and Chamber Formation

Initially, the heart is formed as a single almost strait tube. By a series of geometrical changes this tube finally transforms into a double pump, separately serving lungs and body. Various mechanisms involve looping of the primary tube, wedging of the OFT between left and right atrium, expansion of the chambers, atrial and ventricular septum formation, valve differentiation, and formation of the pharyngeal arterial system. Recent descriptions of the morphological changes important for the understanding of congenital anomalies have been provided elsewhere (Gittenberger-de Groot et al., 2005), while part of the genomic coding underlying chamber formation has been reviewed (Moorman and Christoffels, 2003) (Figure 3.4). It is evident that one simple transcriptional code for all cardiomyocytes does not exist, as left and right ventricular, atrial, and OFT myocytes present with a variety of different gene expression patterns in successive time windows.

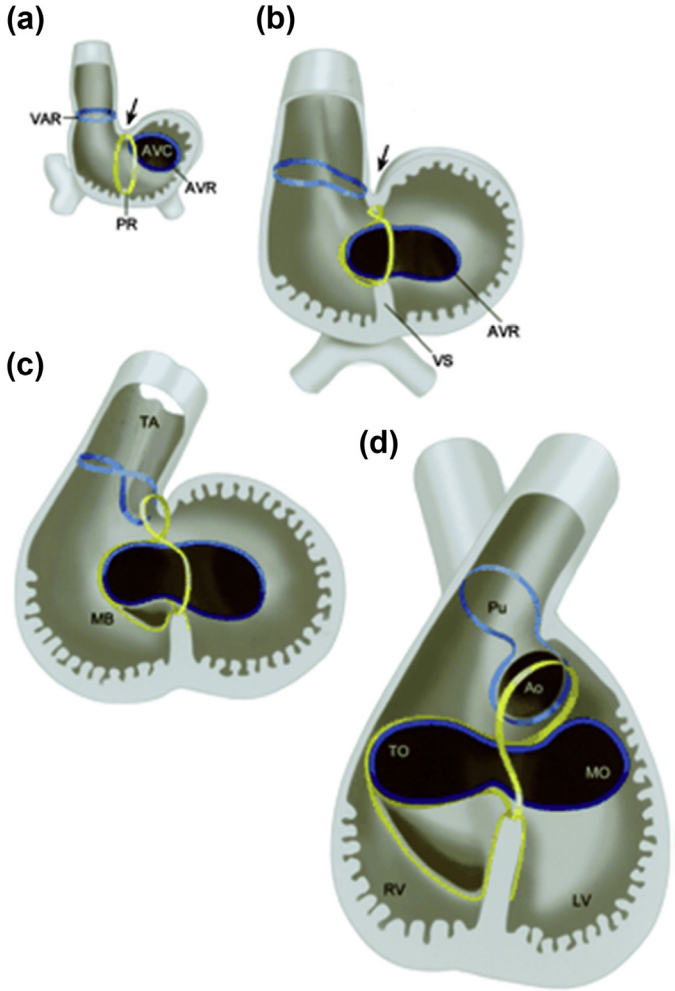

FIGURE 3.4

Transformation of a single heart tube into a four-chambered pumping heart. Schematic representation of the remodeling of the cardiac chambers and the transitional zones at the ventricular level. (a) Internal view (compare with Figure 3.6(b)) of the looped heart tube. The transitional zones are, going from the venous to the arterial pole, the atrioventricular ring (*AVR, dark blue*), the primary ring (*PR, yellow*), and, at the distal end of the myocardial outflow tract, the ventriculoarterial ring (*VAR, bright blue*). (b) During looping, with tightening of the inner curvature (*arrow*), the right part of the AVR moves to the right of the ventricular septum (*VS*). (c) Start of formation of the inflow tract of the right ventricle by excavation of the PR. The lower border is formed by the moderator band (*MB*). (d) Completion of the process with formation of a tricuspid orifice (*TO*) above the right ventricle (*RV*) and the aortic orifice (*Ao*) and the mitral orifice (*MO*) above the left ventricle (*LV*). It is easily appreciated that there is aortic-mitral continuity, whereas the distance between the TO and the pulmonary orifice (*Pu*) is marked. *Adapted from Gittenberger-de Groot et al. (2005).*

3.2.4 Septation and Valve Formation

Septation of the OFT requires the coordinated interactions of the OFT cushions, containing also NCCs, with the surrounding cardiomyocytes. AV canal septation requires fusion of the AV cushions, harboring also NCCs and EPDCs. As a final step in septum formation, cardiomyocytes penetrate parts of the fused cushions. Differentiation of the OFT semilunar valves, and AV mitral and tricuspid valves and their tendinous apparatus, involves coordinated interactions between cells of various origins such as migrated NCCs in conjunction with the surrounding cardiomyocytes (Gittenberger-de Groot et al., 2000). Replacement of malfunctioning valves, which is a highly relevant clinical problem, by tissue-engineered substitutes is an active field of research. The complexity in the cellular origin of the valves next to their unique biomechanical properties makes tissue engineering of these structures challenging.

3.2.5 Conduction

Contractions of the heart tube start as a peristaltic movement (de Jong et al., 1992) with a pacemaker functioning at the venous pole, but this will soon change in a cyclic base-to-apex contraction that in time will be followed by an apex-to-base constriction in a time-specific manner. In yet to establish time windows, these alterations in contraction mode are paralleled by differentiation of the myocardial entity acquiring fast and slow conduction properties. The formation of the conduction system probably requires the involvement of NCC, for the induction or insulation of the central conduction system, and EPDC for the peripheral Purkinje fibers.

3.2.6 Concluding Remarks

The formation of the heart is a complex, modular, and time-consuming process, which is not finished before birth. The multiple transcriptional interactions and molecular and cellular contributions framed in exact developmental windows provide an overwhelming myriad of possibilities, of which only a few combinations are adequate to result in a normally structured and functioning heart. Many combinations prove to be embryonic lethal or result in dramatic congenital malformations. Reconstructing and engineering the heart for major diseases must be based on thorough knowledge of the multiple interactions at all levels of development, function, and maintenance of the heart. The different origin of various cell populations involved in heart formation still provides a challenge for tissue engineering.

3.3 BLOOD VESSEL DEVELOPMENT

A clear need for a suitable arterial replacement has prompted researchers to look beyond autologous and synthetic tissue replacements toward the engineering of vessels using mesenchymal, hematopoietic, and embryonic stem cells. In embryonic development, the formation of the vasculature is tightly

linked with heart development. Thus, when the heart starts to beat (about 4 weeks after conception) a network of endothelial-lined vessels through which the blood will be transported to and from the yolk sac, is in place. This almost two-dimensional extraembryonic plexus will rapidly grow out and gives rise to organ-specific vasculatures within the fast growing embryo. Except for the capillaries each vessel will be enveloped by one or more layers of specialized mural cells, such as SMCs, largely depending on their position and function within the vasculature.

Here the embryonic origin and development of the multiple cell populations involved in vessel formation, such as endothelial cells (ECs), SMCs, fibroblasts, and pericytes, are related to their contribution to the various types of vessels (muscular and elastic arteries, capillaries, and veins). Recent studies lift a tip of the veil clouding the molecular pathways involved in embryonic endothelial and SMC differentiation and the role of environmental factors like hemodynamic forces in the morphogenesis of the vessel wall. Although the combination of these factors will determine the ultimate phenotype of the vascular cells, one has to keep in mind that a single endothelial or a single SMC phenotype does not exist. In fact, there is a large heterogeneity in endothelial and smooth muscle phenotypes reflecting their multiple origins and their site and tissue-specific role in controlling homeostasis of the body.

Endothelial specification is an excellent example of a tissue structure that develops and specifies under blood pressure. For example, the pressure in the great arteries sets specific demands for the architecture of this vessel, which are not needed in small arteries penetrating in areas of a particular organ. To accomplish this, environmental cues activate biochemical pathways in cells which subsequently orchestrate tissue architecture, i.e., the alignment of cells and extracellular matrix.

3.3.1 Origin of the Endothelial Cells

Like the endocardium, the ECs of the blood vessels differentiate in situ within the so-called splanchnic mesoderm that faces the endoderm (Figure 3.5 and Section 3.2.1). This process is called vasculogenesis. The endothelial precursors line up, connect, lumenize, and form a plexus that gives rise to both the embryonic and extraembryonic vessels in the yolk sac and later in the placenta. Vascular endothelial growth factor (VEGF), TGFβ, and FGF signaling pathways are key players in the initial establishment of this endothelial network. This endothelial plexus in the splanchnic mesoderm expands into the somatic mesoderm by the formation of new sprouts. This process is also termed angiogenesis. Recent studies demonstrate that even before the heart starts to beat, an imprinting of arterial and venous identity exists within this capillary plexus. Arterial identity is characterized by the expression of genes like *ephrin-B2 (ephB2)*, *neuropilin-1*, *Notch3*, *DLL4*, and *gridlock*. *EphB4* and *neuropilin-2* are indicative for a venous identity. Blood flow is necessary for

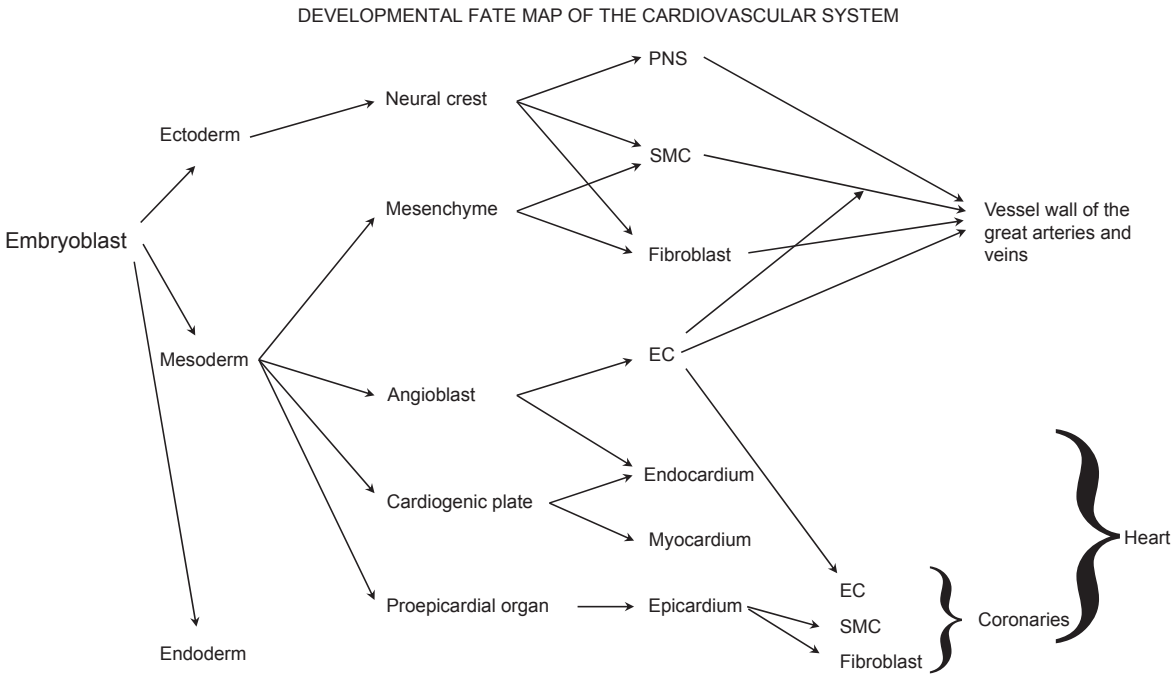

FIGURE 3.5

Developmental origin of cells contributing to the cardiovascular system. The mesodermal compartment of the three-layered embryo gives rise to the major part of the mural cells within the embryo. Angioblasts are the first vascular cells that differentiate by vasculogenesis within the mesoderm. Then the crescent-shaped cardiogenic plate arises giving rise to the endocardium and myocardium of the heart. The remaining mesenchyme will later on differentiate into SMC and fibroblasts of most of the organs. The vascular SMC and fibroblasts in the head and trunk, however, are derived from the mesectodermal neural crest population. Although the endothelial lining of the coronaries are derived by angiogenesis within the septum transversum, the SMC and fibroblasts differentiate from a specialized region within the splanchnic mesothelial lining of the coelomic cavity. Abbreviations: Endothelial cell (EC); Peripheral nerve system (PNS); Smooth muscle cell (SMC).

ongoing differentiation and shaping of the vasculature. Blood flow induces shear stress in as well as cyclic expansion and relaxation of ECs. Both processes are known to activate specific biomechanical signaling pathways that can specify the differentiation process and orchestrate tissue build up. Experimentally changing the blood flow can induce a phenotypic shift of the initial identity demonstrating the plasticity of the ECs in the early stages of vasculogenesis (Le Noble et al., 2005).

3.3.2 Origin of the Smooth Muscle Cells

The next step in vessel wall differentiation is the recruitment of SMCs (arteries and veins) and pericytes (precapillaries) to the preformed endothelial

scaffolding. The location of the vessel within the embryo determines the origin of the SMCs (Figure 3.5). In the greater part of the body the SMCs have a mesodermal origin. In the head and the thoracic region the arteries and veins recruit their SMCs from the NCCs. The SMCs around the coronary vessels are derived from the PEO. Moreover, even differentiated ECs of the dorsal aorta can contribute to SMCs by rapidly changing their cellular phenotype in a process known as transdifferentiation. Sharp borderlines unmark the segments of the vasculature that are composed of SMCs from different origins. Cell tracing studies have demonstrated that within elastic arteries these borderlines coincide with a change in morphology and functionality. Origin-related SMC differences are, e.g., reflected in the differences in glycosylation of the TGFβ type II receptor resulting in a different response to TGFβ1 in the aortic arch and descending aorta. Vascular abnormalities like Marfan (disrupted fibrillin-1), Williams' syndrome (disrupted elastin), Loeys–Dietz syndrome (TGFβ receptor 1 and receptor 2 mutations), and Char syndrome (mutations in the transcription factor AP-2) have preferential sites of occurrence that probably reflect the embryonic origin of the SMCs.

3.3.3 Stabilization of the Vessel Wall

The vessels stabilize with the recruitment of SMCs from the surrounding mesoderm or NCCs. This process is in part governed by the growth factor platelet-derived growth factor (PDGF)-B, which is expressed by the angiogenic ECs. PDGF-B initiates the recruitment of PDGF receptor-expressing mural cells (pericytes and SMCs) toward the newly formed vessel (Hellström et al., 1999). In turn these cells produce angiopoietin 1, which after binding the Tie2 receptor on the ECs downregulates the angiogenic activities of the ECs. This is an example of the intensive communication between the ECs and underlying pericytes and SMCs and vice versa. Cross talk between ECs and underlying mesenchymal cells is a recurrent theme in cell type specification. Pericytes have recently attracted a lot of attention in the field of tissue engineering as they possess stem cell-like characteristics and are considered to be one of the origins of adult mesenchymal stem or stromal cells in the adult organism (Lv et al., 2014).

3.3.4 Hemodynamics and Vessel Formation

From the onset of circulation, the blood exerts mechanical forces on the ECs and the surrounding undifferentiated mesenchyme which appear to be essential epigenetic and environmental factors in vascular differentiation. It is known that the expression of many genes is regulated by shear stress inherently linked to fluid flow inside a blood vessel. Flow depends, among others, on the diameter of blood vessels, a larger vessel diameter allowing for more

flow. Among many others, the expression of TGFβ, endothelial nitric oxide synthase (eNOS), and VEGF is regulated by shear stress. Furthermore, pulsatile and relatively high levels of shear stress protect against apoptosis in which nitric oxide (NO) produced by the enzyme eNOS is a potent intermediate. In contrast, low shear stress areas and turbulent flow promote apoptosis. For these reasons, preconditioning of blood vessels grown in the lab by simulating pulsatile blood flow through the construct is an essential element in tissue engineering of functional blood vessels. The preconditioning helps in defining the identity of the ECs. In addition, it helps in the adaptation and orientation of the underlying SMCs and extracellular matrix in order to withstand the mechanical forces exerted by the pulsatile flow. Such tissue maturation process is needed to avoid rupture and hence failure of tissue-engineered constructs shortly after transplantation.

3.4 DEVELOPMENT OF PERIPHERAL NERVE TISSUE

Driven by an enormous clinical need, peripheral nerve regeneration has become a prime focus within the field of tissue engineering. The various tissue components that make up the peripheral nerve originate from different germ layers. The neurons and glial or Schwann cells are derived from either the neuroectoderm or NCC, while the nerve sheath, which protects the nerves, is derived from the mesoderm (Le Douarin and Kalcheim, 1999). The development and organization of the peripheral nerves with their myelinated and nonmyelinated axonal fibers and their protective epithelial layer involves complex cellular interactions and molecular mechanisms.

3.4.1 Development of the Schwann Cell Lineage

The peripheral nerve contains two types of Schwann cells: myelin-forming Schwann cells that ensheath large caliber axons (diameter larger than 1 μm) and nonmyelinating Schwann cells that accommodate multiple lower caliber axons in cytoplasmic cuffs (Figure 3.6). Both cell types are derived from an immature Schwann cell, which itself is the product of a common precursor cell: the Schwann cell precursor. From studies in the mouse and rat, it was found that Schwann cell precursors are formed early during embryonic nerve development, around embryonic days 12–13 (E12-E13 mouse). At this time, NCCs have populated the outgrowing axon bundles. The cellular interactions and cues that divert the fate of the NCC to the Schwann cell lineage at the eventual exclusion of other fate options are still largely unknown. However, it is known that the survival and ensheathment of axons by Schwann cell precursors critically depend on the axonally derived neuregulin1 protein. Migrating NCCs express the neuregulin1 receptor ErbB3/ErbB2 which depends on the Sry box transcription factor Sox-10. Both Sox-10 and ErbB3/ErbB2 are essential

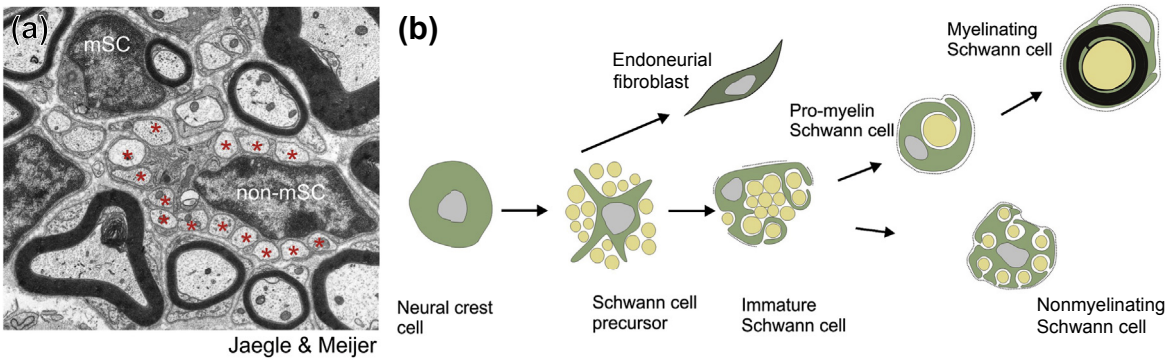

FIGURE 3.6

Development of the Schwann cell. The mature Schwann cell phenotypes that can be distinguished in the nerve differentiate through a number of intermediate stages from the neural crest. (a) This electron micrograph of a transverse section of a typical mixed nerve (containing both sensory and motor fibers) shows myelinating Schwann cells associated with a single larger axon and a nonmyelinating Schwann cell that ensheaths multiple small axons (asterisks) accommodating them in cytoplasmic cuffs. (b) The schematic drawing illustrates the different intermediate stages that can be distinguished in the development of a Schwann cell from the neural crest. Lineage tracing studies in mice have indicated that the endoneurial fibroblasts also are derived from the neural crest cells that populate the embryonic nerves. The basal lamina that surrounds the axon Schwann cell units is produced by the Schwann cell and is here illustrated by a stippled line. Immature Schwann cells have not produced a continuous basal lamina yet. For further details see text.

for survival and proliferation of Schwann cell precursors. Lack of embryonic Schwann cells results in massive sensory and motor neuron cell death in these embryos, demonstrating that neurons not only rely on target-derived trophic support but also critically depend on Schwann cell support.

Schwann cell precursors migrate, proliferate, and ensheath axons and differentiate into immature Schwann cells. Immature Schwann cells populate the fetal nerves and differ from Schwann cell precursors in several ways. While the survival of Schwann cell precursors depends strictly upon axonal contact, immature Schwann cells have acquired the ability to survive in the absence of axonal contact by establishing an autocrine survival pathway (Meier et al., 1999). Insulin-like growth factor (IGF1), Neurotrophin-3 (NT-3), and PDGFβ are important components of this pathway. The survival of immature and mature Schwann cells in the absence of axonal contact is of physiologic importance as successful regeneration of injured peripheral nerves greatly depends on axonal contact with living Schwann cells in the denervated nerve stump. Indeed, these so-called reactive Schwann cells secrete a range of neurotrophic factors and cytokines, such as nerve growth factor (NGF), ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), and interleukin-6 (Il-6), which stimulate and direct axonal growth (Scherer and Salzer, 2001). Consequently, successful tissue engineering or even regeneration of peripheral nerves requires the presence of a healthy Schwann cell population which can support and direct nerve out growth.

3.4.2 Radial Sorting and Myelination of Peripheral Nerves

In mouse embryos from mid-gestation onwards, the migration, proliferation, and ensheathment of axonal fibers continue until around birth, when the number of Schwann cells and axons are eventually matched. The process of radial sorting and ensheathment of axons relies on interactions between the Schwann cell and its basal lamina (Colognato et al., 2005). In particular laminin, which is a major component of the basal lamina, is of importance for proper axonal sorting by Schwann cells. During the process of radial sorting, individual Schwann cells select larger caliber axons in a 1:1 relationship. These Schwann cells will exit the cell cycle and initiate myelin formation. In contrast, groups of lower caliber axons (mainly the fibers of nociceptive and most autonomic neurons) remain associated with a single Schwann cell. These so-called non-myelin-forming Schwann cells accommodate and ensheath the individual axons in cytoplasmic intentions or cuffs. These populations of axons-glia are called Remak fibers (see Figure 3.6(a)).

The fate decision to become either a myelin-forming or a non-myelin-forming cell is governed by axonal cues. The exact nature of these cues has long remained elusive and is still not understood in full detail. The extent of myelination, that is, the number of layers of compact myelin and the length of the internode, the distance between two successive nodes of Ranvier, which play an essential role in action potential conduction, correlates with the diameter of the axon. Thus, thicker axons have thicker myelin and longer internodes.

3.4.3 Structure of the Peripheral Nerve Sheath

While nerve tracts in the central nervous system are protected by the rigid bony structure of the skull and vertebral column, peripheral nerves lack these protective structures. Instead, they have a compact connective tissue matrix which protects the peripheral nerves. Three layers can be distinguished in this compacted tissue, and these have been termed epineurium, perineurium, and endoneurium. The epineurial and perineurial cells of the nerve sheath are derived from the mesoderm (Bunge et al., 1989). The majority of endoneurial cells are derived, like the Schwann cells, from the NCC (Joseph et al., 2004).

While the epineurium is a condensation of connective tissue surrounding the peripheral nerve, the perineurium is a multilayered cellular sheath surrounding individual fascicles of the nerve. The layers consist of concentric sleeves of flattened epithelial cells that interdigitate and are connected by tight junctions at their margins.

The endoneurium mainly consists of a thick layer of collagen fibrils running parallel with the axon—Schwann cell units. Axons, Schwann cells, and fibroblasts are the major cellular components of the intrafascicular space.

3.4.4 Development of the Peripheral Nerve Sheath

The peripheral nerve sheath develops from mesenchymal cells surrounding the embryonic nerves. From E15 onwards, mesenchymal cells assemble concentrically around the developing nerve, and around birth a recognizable perineurium is present. At this stage the perineurium is still leaky, but becomes impermeable a few weeks after birth (Sharghi-Namini et al., 2006).

Genetic experiments have demonstrated that the formation of a structurally and functionally normal perineurium largely depends on the Schwann cell-derived signaling molecule desert hedgehog (Dhh). Dhh is one of three members of the hedgehog family of intercellular signaling molecules that play key roles in embryonic pattern formation and organogenesis (Hammerschmidt et al., 1997). The fact that still a thin layer of perineurial cells is formed in the absence of Dhh signaling suggests that Dhh is not required for the initial recruitment of mesenchymal cells. Since perineurial-like sheaths are formed in genetic mutants that lack Schwann cells, this suggests that the initial signal might be derived from the axon.

3.4.5 Summary and Perspective

The development of the peripheral nerve tissue is orchestrated through a number of cellular and molecular interactions that are now understood in some detail. In addition to providing essential support for neurons, Schwann cells orchestrate the development of the protective perineurial sheath. Ideally, artificial nerve grafts that aim to guide and support the regenerating nerve into a distal, denervated nerve stump should include autologous Schwann cells. However, to obtain autologous Schwann cells healthy nerves need to be sacrificed. Much effort is put in place to obtain Schwann cells from various stem cell populations such as embryonic stem cells, induced pluripotent stem cells, stem cells isolated from the hair follicle bulge, and mesenchymal stromal cells. The successful application of these cells in a clinical setting will require a detailed understanding of the biology of these stem cells and the development of procedures to derive and expand Schwann cells and their subsequent incorporation in a tissue engineering strategy.

3.5 EMBRYONIC SKIN DEVELOPMENT

Tissue-engineered living skin substitutes have found their way into clinical practice for treatment of severe skin defects. While the application of these in vitro generated skin substitutes have greatly improved treatment outcome of severe burn defects, many issues remain to be resolved to end up with an aesthetic and functional equivalent that matches the natural skin. Here the sequential steps and factors involved in embryonic skin development are summarized and correlated with the formation of skin substitutes in vitro. Skin

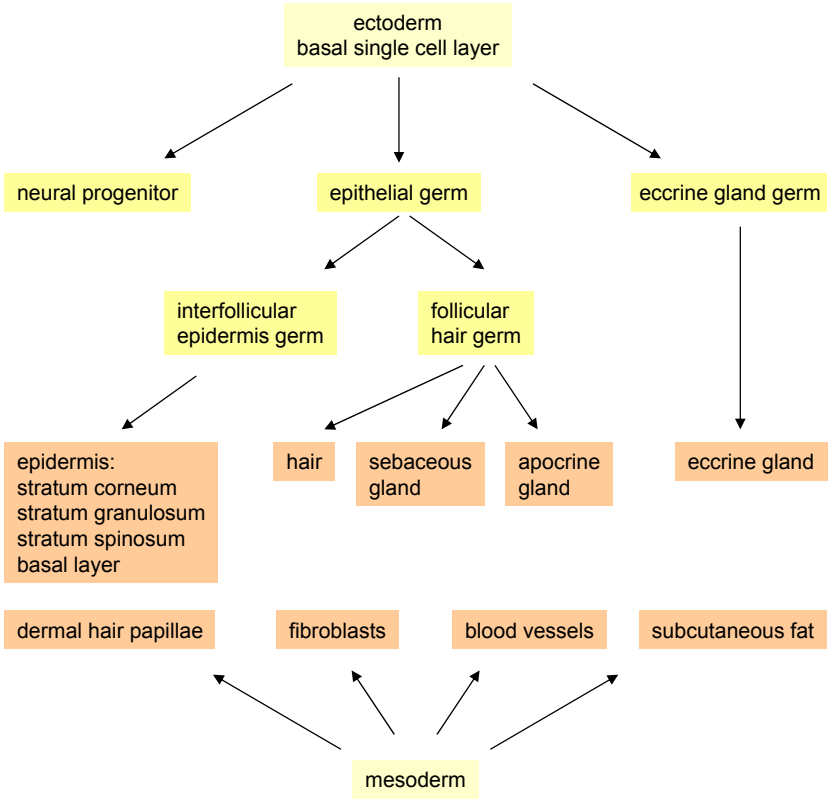

FIGURE 3.7
Schematic diagram of the cellular origin of the skin.

tissue engineering is one of few examples in which developmental engineering has resulted in clinically applicable tissue replacements.

Embryonic skin develops from the ectoderm and mesoderm via a complex process of cell proliferation, differentiation, migration, and apoptosis (Figure 3.7; Table 3.1). The ectoderm is the progenitor of the follicular and interfollicular epidermis and is also the neural progenitor (Moreau and Leclerc, 2004). The mesoderm is the progenitor of the dermis and subcutaneous fat.

3.5.1 Interfollicular Epidermis

In 5-week gestational skin, the epidermis consists of a single layer of ectodermal cells with intermittent areas containing a second suprabasal layer—the periderm (Figures 3.8(a) and 3.9(e)). After 7 weeks, Langerhans cells (immune surveillance cells of the skin), derived from bone marrow cells, begin to appear in the developing epidermis. By the 10th week, the stratum intermedium forms between the basal cell layer and the periderm through upward movement of

Table 3.1 Timelines for Embryonic Skin Development

Gestation (weeks)	Fetal Length (cm)	Developing Structure
5	0.14	Ectoderm: basal single cell layer and intermittent periderm
7		Langerhans cell precursors from hematopoietic origin
8		Mesoderm: loosely arranged mesenchymal cells embedded in ground substance; Merkel cell differentiation from ectoderm
10	5.0	Epithelial germ: basal cell layer, stratum intermedium, periderm
12		Epithelial germ: melanocyte precursors in intermediate layer; early dermis: argyrophilic reticulum fibers (collagen III), abundant fibroblasts
13		Eccrine glands start to develop
17		Hair and sebaceous gland development
19	17	Epidermis: basal layer, 2–3 intermediate layers, flattened periderm
20		Apocrine glands; fat cells mainly in brown fat (very little white fat)
22		Dermis: elastic fibers
23	19	Epidermis: keratinization in stratum intermedium (keratohyalin granules), premature stratum corneum
24		Formation of vernix caseosa
32		Papillary and reticular dermis
36	40–50	Fully developed immune competent skin

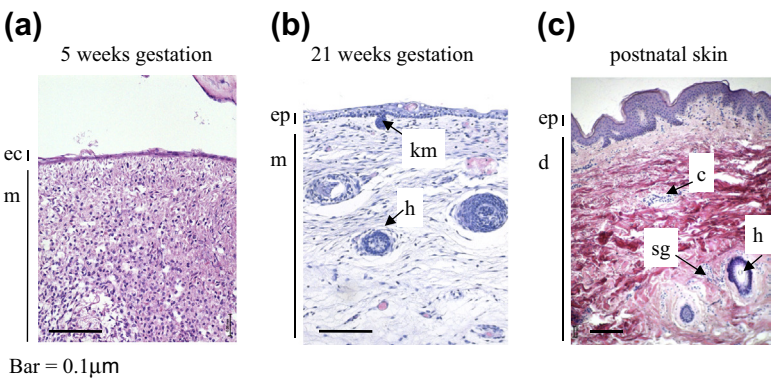

FIGURE 3.8

Developing embryonic skin. (a) Embryonic skin at 5 weeks gestation. Note the single-layered ectoderm, ec, containing sporadic periderm cells, the cell dense mesenchyme, m, and the absence of capillaries. (b) Embryonic skin at 21 weeks gestation. Note the multilayered developing epidermis, ep, lacking a stratum corneum. The epidermal keratinocyte mass, km, that develops into the hair follicle and sebocytes can be seen budding into the dermis. Mesenchymal cells are becoming less dense in the dermal matrix and precursor hair follicles, h, are visible. (c) Fully developed new born skin. Note the stratified epidermis, ep, endothelial capillaries, c, and low density of fibroblasts (single cells) in the dermis, d. Fully developed hair follicles, h, and sebaceous glands, sg, are visible deep in the dermis. Bar = 100 μm.

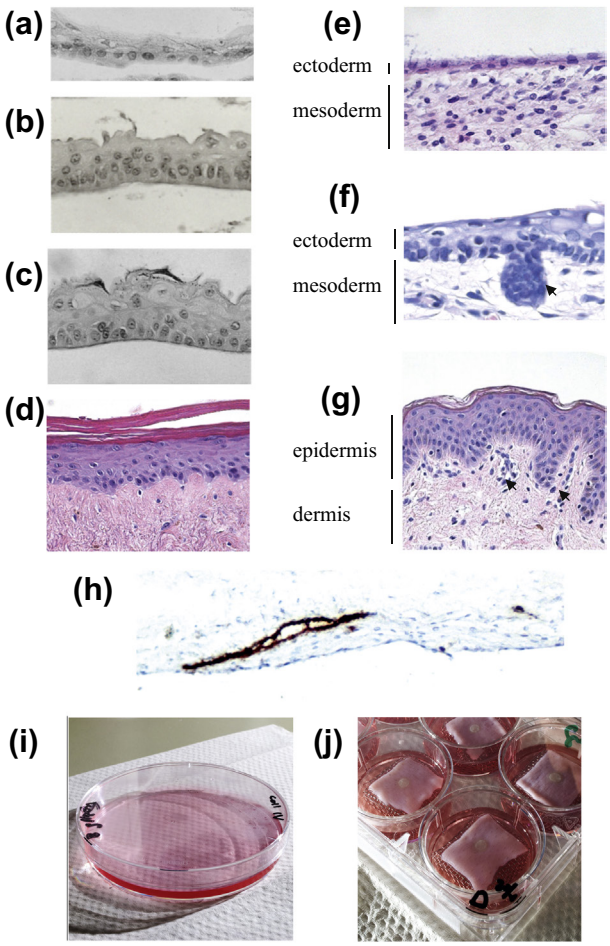

FIGURE 3.9

In vitro versus in vivo developing skin. (a) Cross section of a keratinocyte sheet cultured submerged under the culture medium on a feeder layer of irradiated 3T3 mouse fibroblasts. Fibroblasts are washed away before harvesting and therefore are not visible. Note the similarity with 5-week gestational ectoderm (E). (b) Keratinocyte sheet cultured as described in (a) but with addition of retinoic acid to the culture medium which stimulates keratinocyte proliferation. Note the resemblance to 21-week gestation skin (f), only the follicular bud is absent (arrow). (c) Keratinocyte sheet as described in (a) but with the addition of vitamin D as well as retinoic acid to the culture medium. Retinoic acid stimulates proliferation and stratification whilst vitamin D stimulates differentiation. Note the keratohyalin-like granules in the upper layers making this culture resemble developing epidermis at 23 weeks gestation. (d) Reconstructed epidermis cultured on fibroblast populated human dermis. As the cultures are grown at the air–liquid interface, with nutrients from the medium diffusing in through the dermis, complete epidermal differentiation occurs. Note the fibroblasts populate the dermis at a similar frequency to that observed in (g) but significantly less than in (e). Also, no endothelial capillaries are present. (h) Fibroblast–endothelial cell coculture system stimulates endothelial capillary formation (blue shows hematoxylin staining of fibroblast nuclei; red shows EN4 immunostaining of endothelial cells) and deposition of extracellular matrix from fibroblasts. (i) Petri dish showing experimental setup for culturing under submerged conditions as described in (a), (b), (c), and (h). (j) Experimental setup for culturing at the air–liquid interface as in (d).

cells from the basal cell layer. The cells of the periderm become large and protrude into the amniotic cavity. Merkel cells (nerve/touch cells) differentiate from the ectoderm between weeks 8 and 12 of gestation. Around 12 weeks, melanoblasts migrate from the NCC into the ectoderm (Hirobe, 2005). These cells later develop into melanocytes—the pigment forming cells in the epidermis. The appearance of melanoblasts takes place in a craniocaudal direction following the "Lines of Blaschko" (Figure 3.10) (Bolognia et al., 1994). After 21 weeks of gestation, fetal skin becomes thickened due to increasing numbers of stratum intermedium cells and the periderm becomes flattened (Figures 3.8(b) and 3.9(f)). At 23 weeks, keratinization takes place in the upper cell layers and small keratohyalin granules form. The cells of the periderm are shed into the amniotic fluid, leaving only fragments of degenerated periderm cells above the keratinized cells of the newly formed stratum corneum. The epidermis continues to form by upward movement of differentiating keratinocytes from the proliferating basal layer until a fully differentiated, stratified epidermis is formed (Figures 3.8(c) and 3.9(g)) (Mack et al., 2005). During the last trimester of gestation (>24 weeks), the newly formed stratum corneum of the fetus is protected from the amniotic fluid by a white, greasy biofilm called vernix caseosa. Vernix caseosa consists of water-containing corneocytes embedded in a lipid matrix and the basic structure shows certain similarities with the stratum corneum (Rissmann et al., 2006). After birth, the vernix caseosa is absorbed and the epidermis forms a competent barrier to the environment preventing infection and dehydration of the infant.

3.5.2 Follicular Epidermis

The ectoderm basal cell layer proliferates and differentiates into the keratinizing interfollicular epidermis as described above. Additionally, the ectoderm forms the eccrine glands (sweat) and hair germs which then further differentiate into hair, sebaceous glands, and apocrine glands (Figure 3.7) (Fu et al., 2005). In early gestation (13 weeks), when fetal skin is composed of two or three epidermal layers, eccrine glands begin to form. Eccrine gland islands composed of epidermal cells gradually migrate down into the dermis to form the juvenile sweat glands at weeks 18–20. Hair follicles begin to form at 17 weeks. The formation of these follicles represents a proteotypic interaction between the neuroectoderm and mesoderm that is provided by three different stem cell sources: epidermal, neural crest, and mesenchyme. The epidermal keratinocyte mass that differentiates into hair follicles and sebocytes buds into the deeper layers of the dermis (Figures 3.8(b) and 3.9(f)). Beneath each bud lies a group of mesenchymal cells (fibroblasts) from which the dermal hair papillae and connective tissue sheath are later formed. NCCs give rise to the melanocytes of the hair follicle pigmentary unit. After 21 weeks of gestation, the structures of skin appendages (eccrine glands, hair follicles, and sebaceous

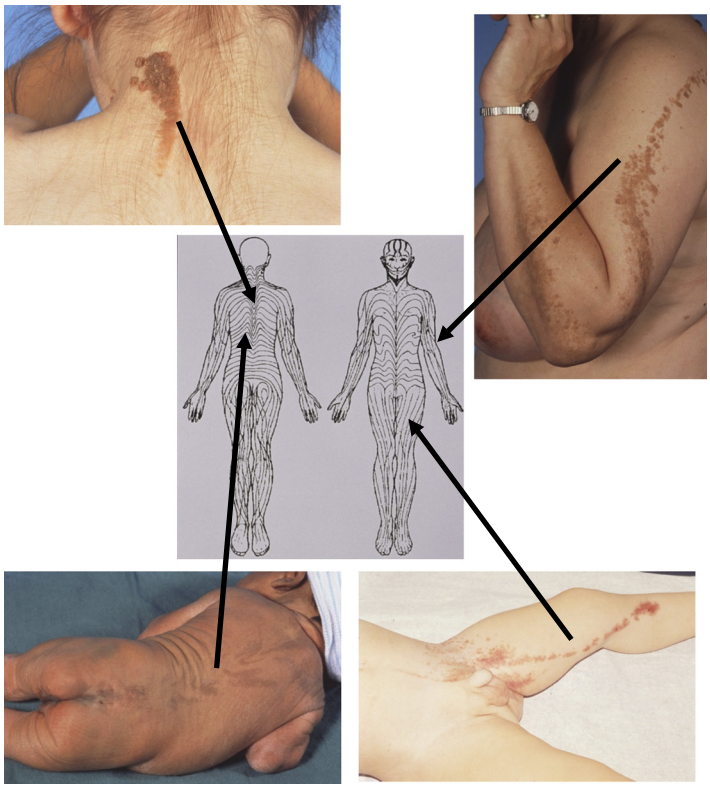

FIGURE 3.10

Lines of Blaschko. More than a century ago, the lines of Blaschko were described by a dermatologist named Alfred Blaschko (Happle and Assim, 2001; Rott, 1999; Traupe, 1999; Bolognia et al., 1994). The lines were based on the observation of patterned skin lesions that were linear on the extremities, S-shaped on the anterior trunk and V-shaped on the back. The lines (or mosaicism) result when a postzygotic mutation occurs leading to a subject with two or more genetically different populations of cells that originate from the genetically homozygous zygote. In most cases, the linear patterns of abnormal skin are surrounded by normal skin. Blaschko's lines are thought to trace pathways of ectodermal development. Several studies have demonstrated that the genotypes of keratinocytes and underlying fibroblasts do not correlate in mosaic skin conditions, confirming the different developmental patterns of ectoderm and mesoderm. The epidermis and its appendageal structures, the melanocytes, the vascular system, and the subcutaneous fat, separately or in combination may be involved in the morphological manifestations which follow Blaschko's lines. The central figure illustrates the Lines of Blaschko originating from the neural crest. Upper left/right and lower right show subjects with epidermal nevi; lower left shows a baby with "linear and whorled nevoid hypermelanosis." *Photographs are supplied by Dr. M. Wintzen, dermatologist, VU university medical center, NL.*

glands) can be detected (Figure 3.8(b)). After 28 weeks of gestation, as fetal skin becomes even thicker, the number of eccrine glands and hair follicles is increased and the structure of the appendages becomes mature. At birth, the sebaceous glands are considerably larger than they are in infancy and secrete the vernix caseosa.

3.5.3 Dermis

In 5-week gestational skin, the dermis consists of loosely arranged mesenchymal cells that are embedded in a ground substance (Figure 3.8(a)). At 12 weeks, argyrophilic reticulum fibers appear. As these fibers increase in number and thickness, they arrange themselves in bundles. Simultaneously, mesenchymal cells develop into fibroblasts and ECs (Figure 3.7). The fetal dermis shows many more fibroblasts than adult dermis (compare Figure 3.8(a) and (b) with Figure 3.9(c)). Also, the dermis of the fetus contains a large amount of collagen type III, in contrast to adult skin which contains a large amount of collagen type I. Elastic fibers appear in the dermis at 22 weeks. As gestation continues, elastic fibers increase in number until at 32 weeks, a well-developed network is formed in the reticular and papillary dermis which is indistinguishable from that found in new born infants.

Fat cells begin to develop in the subcutaneous tissue after 20 weeks. Three types of cell can be found: (1) spindle shaped, lipid-free mesenchymal precursor cells; (2) young type fat cells containing two or more small lipid droplets; and (3) mature fat cells possessing one large lipid droplet.

3.5.4 Tissue Engineering of Embryonic and New Born Skin

The various stages of skin development can be mimicked in vitro by adjusting and finely tuning the culture conditions under which the cells are grown. One should not just consider the tissue itself (in this case skin) but also the environment around the tissue. For example, embryonic ectoderm is in a wet environment engulfed by amniotic fluid on one side and the developing mesoderm rich in fibroblasts on the other side. This provides an extremely wet and nutrient-rich environment. Upon birth, the environment changes significantly. The skin is now exposed to the air. Nutrients reach the epidermis by diffusion from blood vessels in the underlying dermis. Therefore, conditions switch from a wet to a dry environment and also from a nutrient-rich to a nutrient-poor environment. This transition is thought to trigger the final stage in the formation of fully developed skin, thus forming a competent barrier to the environment. Therefore when trying to generate different stages of skin development, for example, the ectoderm transition to epidermis, one should try to copy these environmental changes. Cultures that resemble early ectoderm can be generated from newborn or adult keratinocytes by culturing the keratinocytes on a

dense feeder layer of lethally irradiated fibroblasts completely submerged in culture medium (Figure 3.9(a) and (i)) (Gibbs et al., 1996). The fibroblasts provide a contact point for the initially seeded keratinocytes to nestle up to, but importantly the fibroblasts are metabolically active resulting in the secretion of growth factors and cytokines essential for keratinocyte viability and proliferation. Again this is an example of the importance of cross talk between, in this case, epidermal cells and the underlying mesenchyme which directs tissue formation and maturation. The result is a keratinocyte sheet which consists of approximately two layers of unkeratinized epidermis hardly showing any similarity to new born skin. The keratinocyte sheet cultured in this way visibly resembles early ectoderm at 5 weeks gestation (compare Figure 3.9(a) with (e)).

In order to progress to the next stage of embryonic epidermal development in vitro, additional nutrients are required. Addition of, for example, retinoic acid (a factor known to be involved in healthy skin development) to the primitive ectoderm-like culture stimulates cell division and upward migration of keratinocytes. This increases the number of cell layers and results in cultures which visibly resemble embryonic skin at 21 weeks gestation (compare Figure 3.12(b) and (f)). Addition of vitamin D in combination with retinoic acid further increases the number of layers, epidermal differentiation, keratinization, and the appearance of small keratohyalin-like granules in the upper layers (Figure 3.12(c)) (Gibbs et al., 1996). These cultures visibly resemble embryonic skin at approximately 23 weeks gestation.

In order to mimic the final stage in epidermal development, the culture environment has to be changed significantly—copying the environmental change at birth. The most profound change is exposure of the skin to the air in place of the warm and wet environment of the amniotic fluid. In vitro this can be mimicked by raising the keratinocyte cultures to the air–liquid interface (Figure 3.12(j)) (Gibbs et al., 1997). The transition from submerged culture conditions to air exposed culture conditions stimulates the final step in skin development—the formation of the stratum corneum.

The developing dermis can be copied in vitro by coculturing fibroblasts and ECs (Ponec et al., 2004). Cell–cell interactions and optimal culture conditions stimulate individual ECs to migrate toward each other to form capillary-like tubes (Figure 3.12(h)). Fibroblasts synthesize extracellular matrix components thus forming a human collagen–elastin rich dermal matrix similar to in vivo.

Whereas the development of the interfollicular epidermis and dermis can be mimicked in vitro, development of the follicular epidermis is still challenging although in the past years much progress has been made.

3.5.5 Cell—Cell Interactions and Growth Factors

The skin development and thereafter the maintenance of skin integrity (homeostasis) is dependent on a complex interplay between cell types within the ectoderm (epidermis) and mesoderm (dermis). Cell homing to the correct location, proliferation, and differentiation is regulated by growth factors, cytokines, and chemokines that act in autocrine and paracrine loops. The result is the formation of a fully developed skin consisting of keratinocytes and fibroblasts with constant ratios of keratinocytes:melanocytes (36:1) and keratinocytes:Langerhans cells (53:1) (Hoath and Leahy, 2003). The formation of cell units can be mimicked in vitro and give an insight into embryonic skin development. Melanocytes cocultured with keratinocytes under submerged conditions (ectoderm-like culture) maintain a constant ratio for up to three passages (3 weeks) and when coseeded onto acellular dermis and cultured at the air–liquid interface form a differentiated epidermis interdispersed with melanocytes in the basal layer (Gibbs et al., 2000a). Melanosomes formed in the melanocytes are transferred to keratinocytes and cap the nuclei just as in vivo in order to protect the dividing keratinocyte population from harmful ultraviolet irradiation after birth. It has been reported that fetal keratinocytes possess a greater stimulatory effect on proliferation of melanocytes than neonatal keratinocytes, as fetal keratinocytes produce and release more mitogens than newborn keratinocytes. Similar to melanocytes, Langerhans cell precursors ($CD34^+$ cord blood-derived) can be introduced into submerged keratinocyte cultures which when air-exposed, develop into fully immunocompetent Langerhans cells in the epidermis in ratios similar to those found in fully developed skin (Facy et al., 2005). Cocultures of keratinocytes and fibroblasts (irradiated feeder layer) maintain the keratinocyte stem cell population in vitro and enable large amounts of keratinocytes, including stem cells, to be amplified for transplantation as sheets onto large burns wounds (Green et al., 1979). Upon transplantation, the ectoderm-like culture develops into a differentiated adult epidermis as the environmental conditions change (submerged, nutrient-rich environment changes to air-exposed, nutrient-poor environment). Soluble factors secreted by keratinocyte–fibroblast interactions are involved in formation of the basement membrane (El Ghalbzouri and Ponec, 2004). The basement membrane is essential for the attachment of the epidermis to the dermis and heritable defects can result in blistering (bullous disease). Soluble factors secreted by keratinocytes result in homing of fibroblasts into the dermis (Gibbs et al., 2006), whereas soluble factors secreted by fibroblasts stimulate formation of the epidermal layers (basal layer, spinous layer, granular layer, and stratum corneum) (El Ghalbzouri et al., 2002). One of these factors is keratinocyte growth factor (KGF or FGF7) (Figure 3.11). Supplementation of FGF7 to the culture medium induces keratinocyte proliferation and

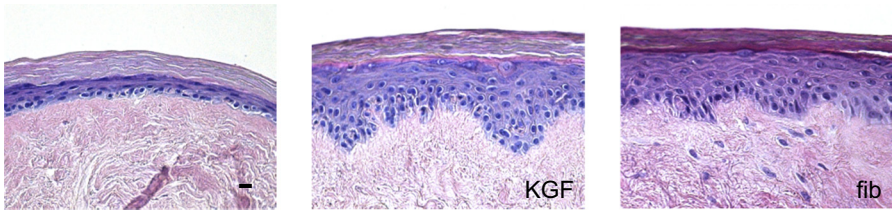

FIGURE 3.11
Keratinocyte growth factor (KGF) can be used to replace fibroblasts (fib) in stimulating epidermal morphogenesis. In the absence of KGF or fibroblasts (fib), the stratified layers of the epidermis are not properly formed. KGF is a growth factor made and secreted by fibroblasts which stimulates keratinocyte proliferation and differentiation and is used as a substitute for fibroblasts (left panel) in coculture experiments with human keratinocytes.

Table 3.2 Growth Factors Secreted by Keratinocytes and Fibroblasts Which Are Involved in Skin Development

Growth Factor	Secreted by	Target Cell	Property
Bone morphogenic protein	?	Ectoderm/KC	Differentiation of ectoderm and epidermis
Noggin	Mesoderm	Ectoderm	Neural differentiation, hair follicle formation, suppression of ectoderm
Epiregulin	KC	KC	Proliferation
Epidermal growth factor	Hair follicle?	KC/Fib	Proliferation, migration
Keratinocyte growth factor	Fib	KC	Proliferation, differentiation
Tumor growth factor-α	KC/Fib	KC/Fib	Differentiation, migration, extracellular matrix synthesis
Vascular endothelial growth factor	KC	Endo	Angiogenesis
Tumor necrosis factor-α	KC/Fib	KC/Fib/LCp	KC/Fib proliferation, LCp differentiation to LC
Tumor growth factor-β	KC	Fib/LCp	Fibroblast differentiation to myofibroblast, LCp differentiation to LC
Granulocyte macrophage-colony stimulating factor	KC	LCp/MC	LCp differentiation to LC, MC proliferation, melanogenesis, dendritogenesis
Basic fibroblast growth factor	KC	MC/Endo/Fib	Proliferation, angiogenesis
Hepatocyte growth factor	KC/Fib	MC	Proliferation
α-MSH	KC	MC	Melanogenesis, dendritogenesis
Nerve growth factor	KC	MC	Melanogenesis, dendritogenesis
Endothelin 1	KC	MC	Proliferation, melanogenesis, dendritogenesis
Stem cell factor	KC/Fib	MC	Proliferation, melanogenesis, dendritogenesis

KC: keratinocyte; Fib: fibroblast; LCp: Langerhans cell precursor; LC: Langerhans cell; MC: melanocyte; Endo: endothelial cell.

differentiation and can be used to replace living fibroblasts in the dermal matrix (Figure 3.14) (Gibbs et al., 2000b). Table 3.2 summarizes the properties of some keratinocyte- and fibroblast-derived growth factors involved in skin development.

3.5.6 Summary

The development of embryonic skin involves environmental, matrix, and cell–cell interactions. Inspired by developmental biology these processes can be mimicked in vitro to engineer skin substitutes. Very important in this engineering process is the fine tuning of differential and sequential secretion of soluble factors which regulate cell growth and differentiation. In vitro, a number of phases of embryonic skin development can be mimicked, such as development of the interfollicular epidermis including keratinocytes, melanocytes, and Langerhans cells. Also a capillary network in a fibroblast-populated dermal matrix can be generated from a mesenchymal cell mix. However, the formation of appendages such as hair follicles and eccrine glands, which involves extensive dermal and epidermal interactions, is still challenging.

3.6 SKELETAL FORMATION

The skeleton is a dynamic living tissue largely consisting of an extracellular matrix combined with matrix forming and degrading cells. The extracellular matrix can be divided into two types, bone and cartilage, which differ in their matrix composition and physical properties in line with their respective functions. Three cell types are involved in skeletal formation: the cartilage producing chondrocyte, the bone forming osteoblast, and the cartilage and bone resorbing osteoclast. Major challenges for tissue engineering of the skeleton are (i) the repair of damaged articular cartilage covering the distal ends of the bone in the joints; (ii) the repair of nonunion fractures, and (iii) the improvement of the union of synthetic prostheses with native bone. The purpose of this section is to provide a general overview of skeletogenesis during fetal development including the origin of the bone forming and degrading cells and the basic principles underlying the maintenance of skeletal integrity during adulthood.

The adult human skeleton contains 213 bones. Depending on their location in the body, these bones have one or more specific functions. They provide structural support and integrity to the body and protect vital internal organs such as the brain, heart, and lungs. Bones play an essential role in body movement by providing attachment sites for muscles. They are also involved in the regulation of mineral homeostasis. Furthermore, they provide the appropriate environment for the development of the hematopoietic system inside the bone marrow cavity. Bones are formed either by endochondral ossification or by intramembranous ossification.

Three cell lineages are responsible for the formation of the skeleton during embryonic development. The mesodermal paraxial and lateral plate mesoderm and the neuroectoderm-derived cranial NCC. The lateral plate

mesoderm will form the long bones in the limbs. The paraxial mesoderm will form the axial skeleton (vertebral column and rib cage) and together with the NCC will shape the craniofacial bones. The formation of bones starts during the fourth week of human development and begins with the formation of the bones at the base of the skull. New bones are formed in an anterior–posterior direction. With exception of the bones in the jaws, the formation of the craniofacial bones begins later. The shape of each bone is specifically adapted to its function, which depends on its place in the skeleton. Even before the onset of skeletogenesis, cells at the site of the future bone already contain information on the shape and structure of the bone they will form.

3.6.1 Skeletal Precursor Cells

Osteoblasts and chondrocytes are both derived from the multipotent mesenchymal stromal cells or also often referred to as mesenchymal stem cells (MSCs). These cells also give rise to a variety of related mesodermal cell lineages such as fibroblasts, muscle cells, tendons, and adipocytes. The MSC is derived from either the mesoderm or the cranial NCC. During fetal development, MSCs can be isolated from virtually any tissues in relatively high quantities. In adulthood, most tissues possess only a limited number of MSCs, except for the bone marrow in which relatively high amounts of MSCs reside throughout life. These cells play an important role in the continuous cycle of bone formation and degradation by providing a source for new osteoblasts. Signaling by the Wnt family of morphogens plays an important role in the maintenance of the undifferentiated stem cell population (Hartmann, 2006). Wnts stimulate stem cell proliferation and renewal and simultaneously block the initiation of differentiation by increasing the nuclear localization and the transcription potential of β-catenin, an essential and critical intracellular mediator of the Wnt signal transduction pathway.

At sites of future bones, the MSC will give rise to a skeletal precursor cell which can differentiate into either an osteoblast or a chondrocyte. The direction of differentiation depends on the activity of two transcription factors: Sox-9 and RunX2. Knockout studies in mice have shown that Sox-9 is indispensable for chondrocyte differentiation, while RunX2 is indispensable for osteoblast differentiation. The skeletal precursor cell expresses both transcription factors. Intracellular levels of β-catenin controlled by Wnt signaling determine the direction of differentiation. High levels of β-catenin inhibit Sox-9 and potentiate RunX2 activity resulting in osteoblast differentiation. In contrast, low levels of β-catenin result in unopposed Sox-9 activity and thus in the formation of chondrocytes (Figure 3.12) (Hill et al., 2005; Day et al., 2005). In embryogenesis, chondrocyte differentiation appears to be the default differentiation route of the MSC.

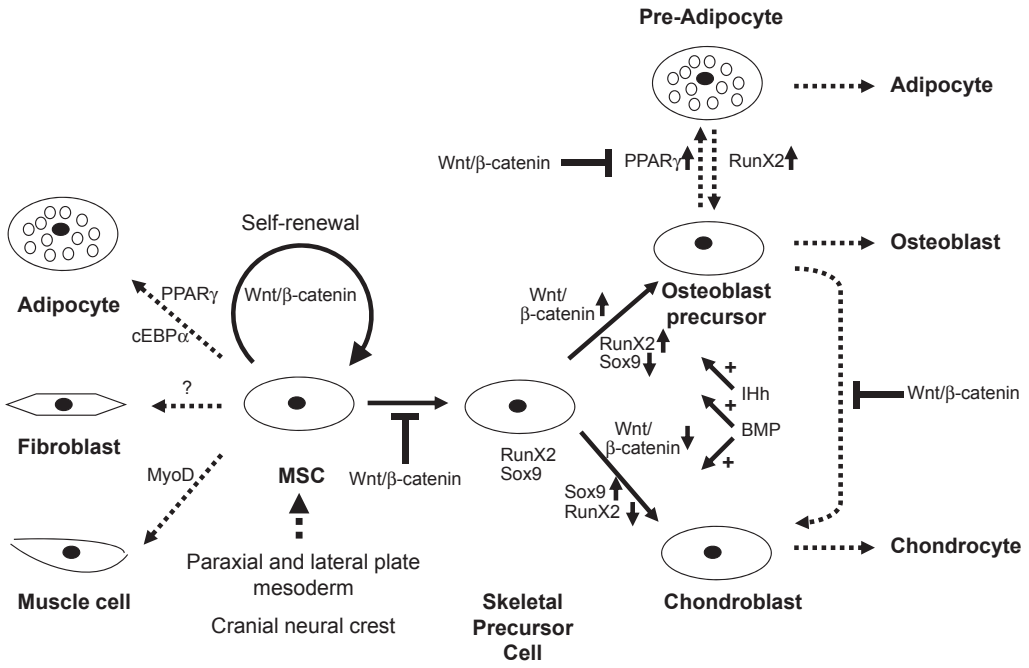

FIGURE 3.12

The formation of skeletal precursor cells. Skeletal precursor cells are derived from the mesenchymal stromal cells (MSCs) that also give rise to adipocytes, tendonocytes, and fibroblasts. Wnt/β-catenin is responsible for stem cell proliferation and renewal. The MSC differentiates into a skeletal precursor cell that expresses the transcription factors for osteoblast differentiation (RunX2) and chondrocyte differentiation (Sox-9). Wnt/β-catenin signaling directs the precursor cell into the osteoblast lineage and prevents transdifferentiation into chondrocytes by augmenting RunX2 activity and repressing Sox-9. Also Indian Hedgehog (IHh) and BMP signaling are involved in this process. The osteoblast precursor is still able to differentiate into adipocytes. The balance between the transcription factors PPARγ (adipocyte specific) and RunX2 (osteoblast specific) determines the fate of the cell. See text for further details.

3.6.2 Endochondral Ossification

The axial and appendicular skeleton is formed by endochondral ossification, i.e., the skeletal elements are preshaped in a cartilaginous mold which is subsequently replaced by bone (Kronenberg, 2003). This process starts with the condensation of loosely connected mesenchymal cells at sites of the future bones (Figure 3.13). This is accompanied with a rearrangement of the vasculature resulting in an avascular condensed mesenchyme surrounded by blood vessels. The transcription factor Sox-9 is responsible for the condensation of the skeletal precursor cells. The combination of increased cell–cell contacts, resulting in signaling of, amongst others, cadherins, low oxygen conditions due to the absence of vessels and low levels of Wnt signaling, is believed to trigger the initiation of chondrocyte differentiation. It is characterized by the production of an abundant extracellular matrix consisting predominantly of collagen type 2a1 and glycosaminoglycans, which are typical cartilage markers. In the middle of the cartilage anlage, chondrocytes start

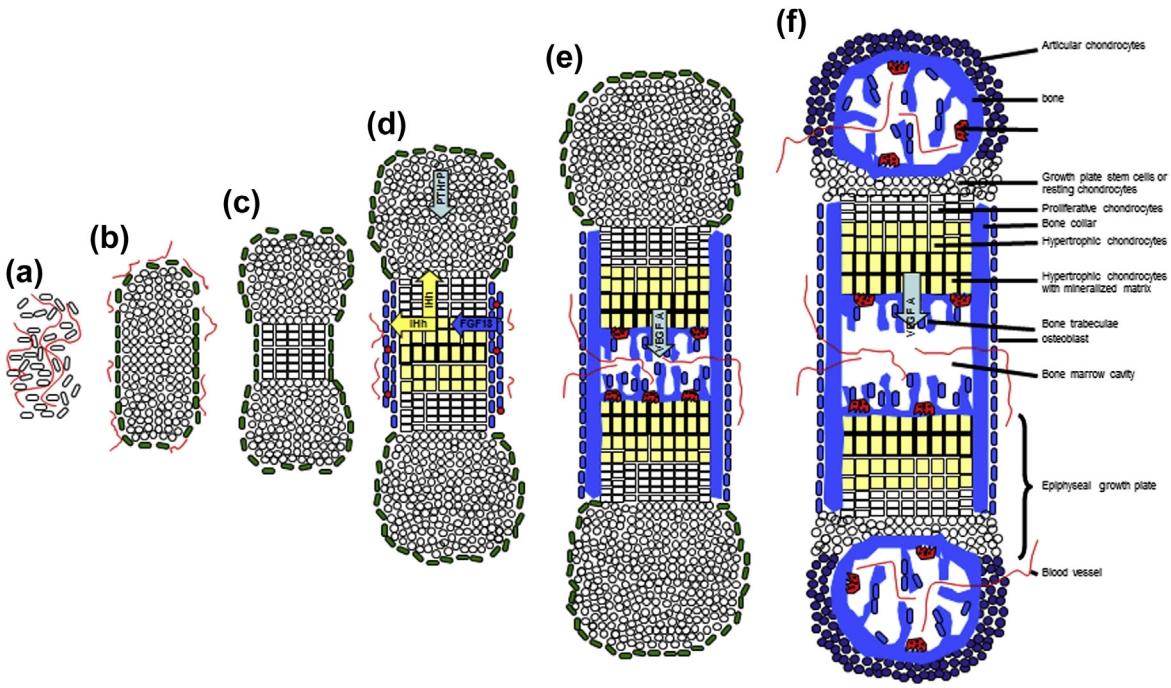

FIGURE 3.13

Endochondral bone formation. Schematic presentation of the subsequent stages of endochondral ossification. It starts with loosely connected mesenchyme invaded with blood vessels (red) (a). The mesenchymal cells start to condense and the vasculature is rearranged. The condensed cells differentiate into chondrocytes that are surrounded by a perichondrium (green) (b). Cells in the middle of the cartilage anlage arrange into columns and start to proliferate (c) and subsequently start to hypertrophy (yellow) (d). Chondrocytes in the transition zone secrete Indian Hedgehog (IHh) that stimulates the formation of a bone collar (blue), chondrocyte proliferation, and the expression of PTHLH in the periarticular region of the long bone. Osteoblasts secrete FGF18 that inhibits chondrocyte proliferation. Terminally hypertrophic chondrocytes start to mineralize their matrix (black lines). At this time point mononuclear osteoclast precursors (red dots) reside in the perichondrium. These cells differentiate into multinucleated osteoclasts that together with blood vessels and osteoblasts (blue) invade the hypertrophic cartilage and start to resorb the mineralized cartilage matrix. VegfA expressed due to the hypoxic conditions in the cartilage plays an important role in the attraction of blood vessels. This results in the formation of a bone marrow cavity. The osteoblasts replace the chondrocyte matrix with a bone matrix (e). Somewhat later this process is repeated in the secondary ossification centers in the cartilaginous heads of the long bones. The formation of the secondary ossification centers divides the cartilage into articular chondrocytes (dark blue) covering the bone surface in the joints and in epiphyseal growth plate cartilage that is responsible for bone elongation (f).

to arrange in typical columns and display high proliferative activity. Somewhat later these chondrocytes stop proliferating and differentiate further into hypertrophic chondrocytes. This is characterized by a dramatic increase of the cell's volume and a reshuffling of the extracellular matrix, which now contains collagen type 10 instead of collagen type 2. The hypertrophic chondrocytes start to mineralize their matrix forming the primary ossification center and subsequently die by apoptosis. At the same time, the chondrocytes signal to the surrounding

perichondrial cells in which osteoblast precursors reside by producing Indian hedgehog (IHh). IHh initiates osteoblast differentiation and the formation of the bone collar. It is the most important coupling factor between chondrocyte and osteoblast differentiation during endochondral ossification (Kronenberg, 2003). The hypoxic conditions in the center of the bone anlage induce the expression of the transcription factor hypoxia inducible factor1α (Hif1α), an essential transcription factor for the expression of VEGF. VEGF recruits blood vessels from the lateral perichondrium (Schipani et al., 2006). With these factors in growing vessels, both osteoclasts and osteoblasts enter the bone resulting in the resorption of cartilage, replacement by newly formed bone and the formation of the bone marrow cavity. In this process a cartilaginous matrix containing collagen type 2, collagen type 10, and glycosaminoglycans is replaced by a mineralized bone matrix predominantly consisting of collagen type 1. Somewhat later, chondrocytes start to hypertrophy in the center of the cartilaginous heads at the distal ends of the bones and mineralize their extracellular matrix resulting in the formation of the secondary ossification center. This is followed by ingrowth of vessels, osteoclasts, and osteoblasts. The secondary ossification center demarcates the separation of two types of chondrocytes: articular chondrocytes that cover the distal ends of the bones and have a relatively low metabolic activity, and epiphyseal growth plate chondrocytes that have a high metabolic activity and are solely responsible for bone elongation. The signaling mechanisms involved in this process are largely unknown. Experimental evidence suggests that specification of the articular versus the growth plate chondrocyte occurs already at the first stages of endochondral ossification.

3.6.3 Intramembranous Ossification and Osteoblast Differentiation

The flat bones of the skull and the flat part of the clavicle are formed by intramembranous ossification. In this process cells directly deposit a mineralized bone matrix without a cartilage intermediate. This process also starts with condensation of mesenchymal cells. In contrast to endochondral bone, these condensations occur in vascularized regions of mesenchyme. The condensed cells directly differentiate into osteoblasts which start to produce a mineralized bone matrix. The sequential activation of two transcription factors is essential for the formation of osteoblasts from skeletal precursor cells. This differentiation route is initiated by RunX2 and requires relatively high levels of Wnt signaling. The subsequent activation of a second transcription factor, Osterix (Osx), is needed for further maturation of the cells and the production of a mineralized bone matrix. Indeed, knockout mice lacking either RunX2 or Osx lack bone formation due to the absence of mature osteoblasts. In Osx knockout mice, osteoblasts are arrested in a somewhat later differentiation stage in comparison to the RunX2 knockout mice (Day et al., 2005; Hill et al., 2005).

Other transcription factors like Msx2, ATF4, deltaFosB, and Fra1 and 2 further facilitate and cooperate with RunX2 and Osx in osteoblast differentiation and production of a mineralized bone matrix (Karsenty and Wagner, 2002; Nakashima and de Crombrugghe, 2003).

The life cycle of the osteoblast can be divided into various phases. In the first phase, the number of osteoprogenitors is increased by rapid proliferation. Subsequently, the cells begin to secrete large quantities of extracellular matrix predominantly consisting of collagen 1 followed by a maturation phase in which the matrix is prepared for matrix mineralization. Each of these phases is characterized by the expression of typical markers (Figure 3.14). At the end of the life span, the osteoblast has three choices: it can die by apoptosis, it can become fully embedded in the extracellular bone matrix and differentiate further into an osteocyte, or it can become a quiescent bone lining cell that covers the bone surface. Osteocytes are single cells fully surrounded by a mineralized bone matrix. They are in close contact with each other and the bone surface with a network of cell extensions. These cell protrusions are localized in small channels, the caniculae. The lining cells are quiescent cells which are no longer involved in bone formation. Upon the appropriate signals these cells can, however, resume their activity and start participating again in the formation of a bone matrix.

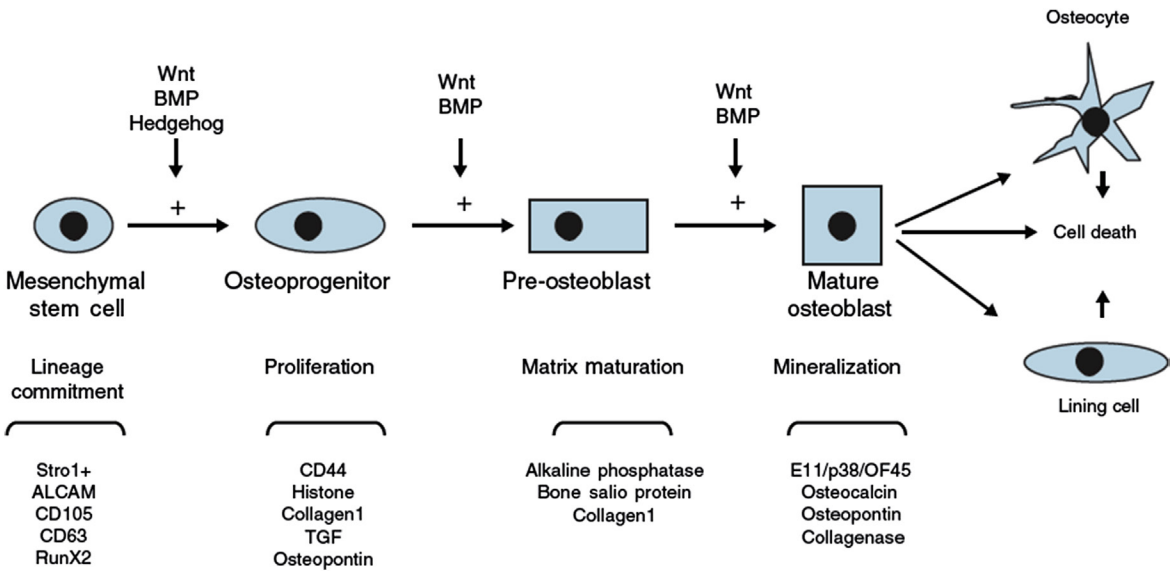

FIGURE 3.14

Osteoblast differentiation. Schematic illustration of osteoblast lineage cells and frequently used markers of the stages of maturation. Wnt and BMPs have positive effects on osteoblast differentiation in all stages of development. The actions of hedgehog are limited to the earliest phases of differentiation from mesenchymal stromal cells.

A large number of growth factors are involved in the regulation of osteoblast differentiation. Members of the TGFβ/BMP superfamily, particularly the BMPs, IHh, and Wnt family members can mediate initiation of osteoblast differentiation from uncommitted precursors. These factors act, amongst others, by inducing the expression of RunX2. Other factors like insulin-like growth factor I (IGF-I), TGFβ, FGFs to name a few play a role in osteoblast proliferation, matrix production, and mineralization. Of particular interest are members of the Wnt family, which are involved in successive stages of osteoblast differentiation such as in the initiation of osteoblast differentiation, matrix production, and osteoblastic cell death. Furthermore, they are involved in the regulation of the coupling between bone formation and bone resorption. Besides control by locally produced growth factors, bone formation by osteoblasts is controlled by systemic factors, such as sex steroids, growth hormone and parathyroid hormone, and by the hypothalamus via the sympathic nervous system (Karsenty and Wagner, 2002; Manolagas, 2000; Ducy et al., 2000).

3.6.4 Osteoclast Differentiation

The osteoclast is a multinucleated highly specialized cell specifically equipped for the resorption of a mineralized matrix (Figure 3.15). Osteoclasts are derived from the hematopoietic stem cell. This cell gives rise to erythrocytes, granulocytes, mast cells, megakaryocytes, lymphocytes, and macrophages. The transcription factor PU-1 and the growth factor macrophage-colony stimulating factor (M-CSF) are required for the establishment of the macrophage lineage. The derivation of osteoclasts from this lineage requires a direct interaction between the osteoclast precursor and the osteoblast involving the expression of receptor activator of nuclear factor kappa β (RANK) by the osteoclast precursor and the membrane associated ligand (RANKL) by the osteoblast. The interaction between RANK and RANKL is required for all subsequent stages of osteoclastogenesis including the fusion of mononucleated precursor cells into a multinucleated functional osteoclast. The subsequent stages of osteoclast development are characterized by specific marker genes (Figure 3.15) (Roodman, 2006).

The interaction between RANK and RANKL can be antagonized by the decoy receptor osteoprotegerin (OPG). The major source of RANKL and OPG is cells from the osteoblast lineage although also T-cells appear to be involved. A large variety of stimuli like cytokines (e.g., IL-1, IL-6, TNFα) and hormones (vitamin D, parathyroid hormone, estrogens) are involved in the regulation of the osteoblastic expression of RANKL and OPG. The balance between these two proteins determines whether osteoblasts can support osteoclastogenesis or not. The formation of osteoclasts is tightly linked with osteoblast differentiation, since the expression of RANKL is controlled by RunX2. Furthermore, the expression of OPG is repressed by Wnt signaling which simultaneously increases osteoblast activity (Glass and Karsenty, 2006).

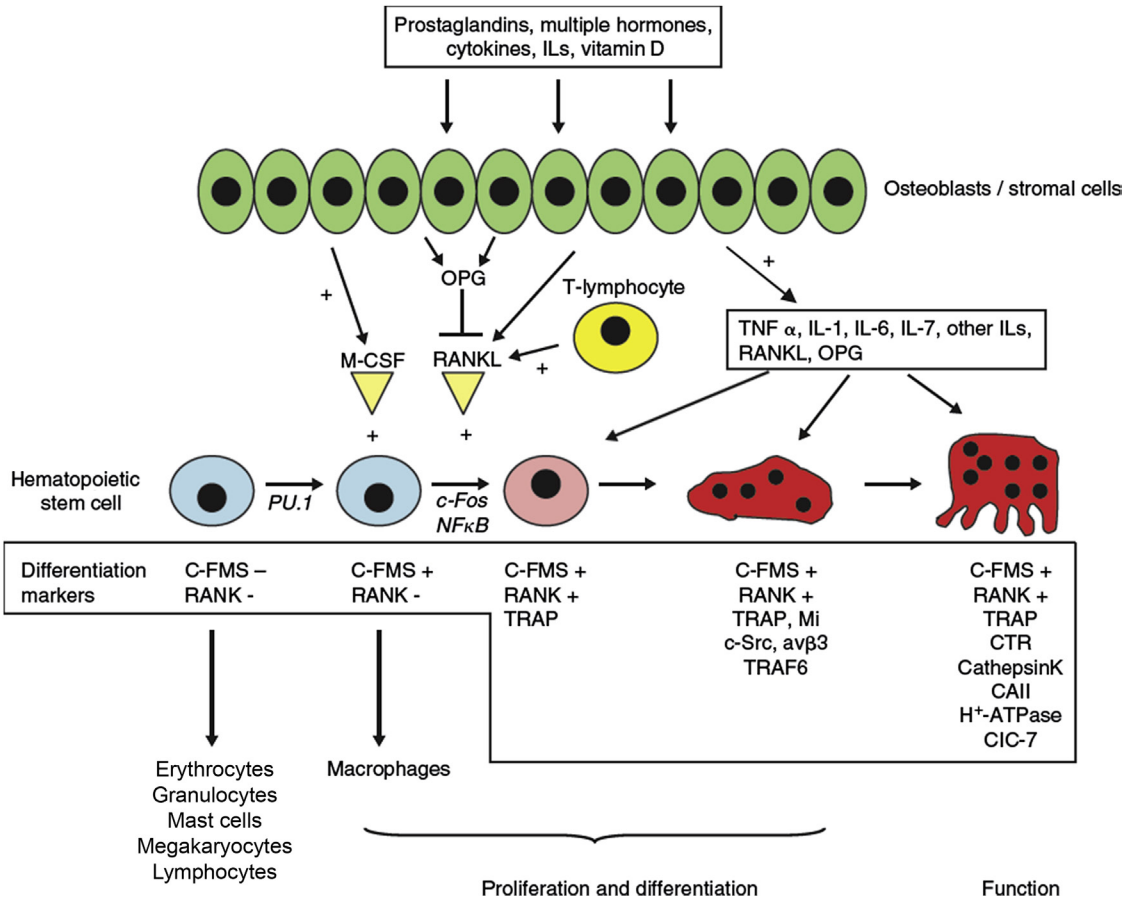

FIGURE 3.15

Osteoclast differentiation. Osteoclasts are derived from the hematopoietic stem cell. These cells become committed to the myeloid lineage under the influence of the transcription factor PU-1 and start to express c-FMS and RANK, the receptors for *m*-CSF and RANKL, respectively. In subsequent differentiation steps, the transcription factors c-Fos and NFκB are of critical importance. Osteoclastogenesis can be divided into various stages, that are characterized by the expression of typical marker genes. The formation of osteoclasts is tightly controlled by osteoblasts and stromal cells that express *m*-CSF and RANKL. Furthermore they express a negative regulator of RANKL, osteoprotegerin (OPG). The RANKL/OPG ratio is a critical component in the regulation of osteoclast formation and activity. Besides RANKL and OPG, osteoblasts and stromal cells express a variety of other growth factors and cytokines that are either pro- or antiosteoclastogenic. The expression of all these factors is tightly controlled by prostaglandins, a variety of hormones like vitamin D3 and estrogens, interleukins (ILs), and other cytokines. Besides osteoblasts and stromal cells, T-lymphocytes also express RANKL and can thus influence osteoclastogenesis and bone resorption.

3.6.5 Bone Remodeling

While the majority of organs in the human body are static, i.e., do not undergo major changes once the formation of the adult organ is completed, bone is continuously renewed by a process called remodeling. It starts as soon as the first bone is formed. In this process, old fatigued bone is replaced by new mechanically sound bone. It is an essential mechanism in the preservation of bone strength and in skeletal adaptation to changing environmental conditions such as in load-bearing of the skeleton. Remodeling is largely orchestrated by the osteocytes and osteoblasts and is controlled by environmental, hormonal, and hypothalamic functions. For example, weight bearing of the skeleton induces a fluid shear stress in the caniculae which is sensed by the osteocytes. The osteocytes signal to the osteoblasts at the bone surface. Amongst others, the secreted Wnt antagonist SOST is an osteocyte expressed gene that appears to play an important role in this process (van Bezooijen et al., 2004). Depending on the signal, osteoblastic bone formation is either activated or repressed. In addition, these signals may influence the balance between OPG and RANKL expression thereby modulating osteoclast formation and bone resorption. Disturbances in the balance between bone formation and resorption are at the basis of all skeletal disorders.

3.6.6 Tissue Engineering of Bone

MSCs derived from adult bone marrow are the most frequently used cell source to engineer bone. In most strategies, their capacity to differentiate into bone forming osteoblasts in a process that resembles intramembranous ossification is exploited. In the treatment of critical size bone defects, limited clinical successes have been achieved. Based on lessons from bone formation in the embryo, this could have been predicted. Bone fractures normally heal, with a few exceptions, in a process resembling endochondral ossification. Recapitulation of this process in a tissue engineering strategy to treat large bone defects implies that one should not aim at directly making bone out of MSCs but one should first make a cartilage anlage (Box State-of-the-Art Experiment). After implantation, this cartilage will be replaced by bone through endochondral ossification which is organized by the tissue itself through interaction with host tissue. Implantation of tissue-engineered cartilage instead of bone has various advantages. For example, cartilage is an avascular tissue and chondrocytes are resilient to low oxygen concentrations. In contrast, bone is highly vascularized and metabolically very active. Lack of oxygen as a consequence of the absence of a vasculature in many tissue-engineered constructs is the main reason for transplant failure. In endochondral ossification, the cartilage self-organizes the ingrowth of blood vessels, which is the first step leading to cartilage degradation, and its replacement by bone. This is an example how tissue engineers have started to exploit

the insights from developmental biology into an engineering strategy using the modularity and self-organizing principles of developing tissue to obtain a robust strategy for treatment of bone defects.

3.7 FUTURE DIRECTIONS

Over the past years the tissue engineering field is gradually changing. While at its infancy, tissue engineering was mainly based on trial and error, this new discipline is rapidly developing into a technology-based discipline comparable to other branches of engineering. Reiteration of processes in developmental biology is now exploited to build in vitro tissues. Concepts such as modularity, robustness, and self-organization, which are at the basis of the formation of organs, will be more and more incorporated in tissue engineering strategies. This trend will continue in the coming years resulting in maturation of a new discipline in the formation of tissues called developmental engineering.

3.8 SUMMARY

- Recapitulation of developmental processes in combination with typical engineering disciplines is known as developmental (re)engineering and forms the basis of most, if not all, tissue engineering procedures.
- Tissue formation depends on the timely and subsequent interaction between multiple stem or progenitor cells derived from various origins. Cell migration, cell–cell contact, auto- and paracrine signaling and also environmental cues like pulsatile flow or exposure to air are some of the processes that are used for the specification of cells into a functionalized tissue.
- The inner cell mass in the blastocyst contains pluripotent cells that give rise to all tissues in the adult body. When brought into culture these cells are called embryonic stem cells that retain pluripotency and self-renewal in vitro.
- In gastrulation, the three germ layers ectoderm, mesoderm, and endoderm are formed by a complex process involving cell–cell interactions, cell movements, and gradients of morphogens by which the body plan is established.
- Pluripotent neural crest cells originate from the neural plate border. They migrate via clearly defined patterns to all tissues in the body where they actively participate in tissue formation. They give rise to cell types of ectodermal, mesodermal, and endodermal origin.
- The heart is formed by mesoderm- and ectoderm-derived cell types. The mesodermal cells are present in the cardiogenic plates and will give rise to cardiomyocytes and endocardial cells. The cardiac neural crest, which

will contribute to formation of, e.g., the arterial vessel wall, and the proepicardial organ, which will form the epicardium, originate from the ectoderm by epithelial-to-mesenchyme transition.

- The formation of the heart is a complex and time-consuming process. Initially the heart is formed as a single beating tube that by a series of geometrical changes transforms into a four-chambered septated beating heart.
- In blood vessel formation, endothelial cells are derived from the splanchnic mesoderm. The smooth muscle cells and pericytes are derived from the mesoderm or the neural crest.
- Blood flow and communication between endothelial cells and surrounding smooth muscle cells or pericytes will shape and define the identity of the vessel. In the absence of blood flow, vessels will not develop properly or even deteriorate.
- In peripheral nerve tissue, the neurons and glial cells (also called Schwann cells) are derived from either the neuroectoderm or neural crest, while the nerve sheath is derived from the mesoderm.
- Neurons can only survive in close contact with Schwann cells. Vice versa, mature Schwann cells can only survive in close contact with neurons. Only immature Schwann cells can survive without axonal contact.
- In adult skin, the follicular and interfollicular epidermis is derived from the ectoderm. The mesoderm is the progenitor of the dermis and subcutaneous fat.
- The development of embryonic skin involves environmental, matrix, and cell–cell interactions. These processes can be mimicked to some extent in vitro by changing the culture conditions from a wet to air-exposed environment and by coculture of dermal fibroblasts and keratinocytes.
- The major bone forming cells, the osteoblast and chondrocyte, are derived from the paraxial and lateral plate mesoderm and the neuroectoderm-derived cranial neural crest. The bone-degrading osteoclasts are derived from the mesenchymal hematopoietic cell lineage.
- Bone is an active tissue that is continuously remodeled. It is formed either by intramembranous ossification or by endochondral ossification. In intramembranous ossification, mesenchymal stem cells directly differentiate into a bone matrix depositing osteoblast. In endochondral bone formation, skeletal elements are preformed in a cartilaginous mold that is replaced by bone.
- Osteoblasts and chondrocytes have a common precursor, the multipotent MSC. The differentiation into either cell lineage is governed by a complex array of signaling molecules including BMPs and Wnts of which the intracellular Wnt target β-catenin appears to play a central role.

STATE-OF-THE-ART EXPERIMENT

Realizing that most bones in our body are formed and healed by endochondral ossification, Martin and coworkers started to explore whether this developmental biological phenomenon could be used to generate bone. They started with human mesenchymal stromal cells. While others focused on directly making bone via intramembranous ossification, Martin started to make cartilage first. The cartilage templates were subsequently implanted ectopically in a mouse. Indeed the cartilage templates were efficiently replaced by functional bone containing a mineralized matrix. The efficacy depended on the maturation state of the cartilage. Efficient replacement by bone was shown only in cartilage constructs containing hypertrophic cartilage. The ectopic bone was vascularized and contained a bone marrow compartment in which hematopoiesis took place. Hypertrophic cartilage was capable to recruit the ingrowth of blood vessels from the host tissue. In addition, the cartilage was able to recruit osteoblast precursors from the host which started to deposit bone. Cells from the bone marrow compartment, such as the osteoclasts, were also derived from the host. Some human MSCs also differentiated into osteoblasts particularly at the pericortical region. The bone in this region was thus derived from both implanted human cells and host cells. Importantly, the underlying morphogenetic process was structurally and molecularly similar to the temporal and spatial progression of long bone development in the limbs. The study by Martin and coworkers provides a model for fundamental and translational studies on bone morphogenesis and regeneration by invoking a developmental engineering paradigm.

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