8

Organogenesis

This chapter deals with the development of some organ systems in the mammalian body which are particularly relevant to stem cell research. Their descriptive embryology has been known for both mouse and human for a long time. The molecular mechanisms have been established in recent decades using the mouse as the experimental model, with some assistance from work on chick, *Xenopus* and zebrafish embryos.

In particular, two groups of experimental method have been used to establish the molecular mechanisms of organogenesis. First, mouse knockouts can show the requirement for a particular gene in a process. If the process fails to occur in the absence of the gene it is presumed to be necessary. Often a gene is required several times in development and this may mean that a knockout embryo dies before the stage of interest. In such cases it is necessary to do organ-specific knock outs or overexpression using the techniques described in Chapter 3. Second, organ cultures can be set up in vitro for many of the systems considered here. The organ rudiment is dissected from a mouse embryo and grown in tissue culture medium, often with special substrates or extracellular matrix components (see Chapter 4). This enables inducing factors or specific inhibitors to be added to the culture. Furthermore it is often possible to separate epithelial and mesenchymal components of an organ rudiment by microdissection. This enables investigation of which component of the rudiment is responding to particular stimuli, and investigation of signals passing between epithelium and mesenchyme.

The descriptions given here are necessarily brief and it should be borne in mind that every system is actually much more complex than indicated here. It is usual for several inducing factors and signaling pathways to be involved in each developmental event, although one factor may predominate and be able to bring about appropriate effects on overexpression.

Nervous System

The central nervous system originates from the ectoderm of the embryo and comprises the brain and spinal cord. The region of the ectoderm which ends up overlying, or closely apposed to, the notochord and the prechordal plate becomes exposed to the BMP inhibitors: chordin and noggin. These suppress the formation of epidermis and induce the formation of the neural plate, characterized by expression of S*ox1* and *-2* and of other neural transcription factors. The neural plate is a keyhole shaped structure. The rostral end is wider and becomes the brain, the caudal end is narrower and becomes the spinal cord. As the node regresses, the neural plate folds up to form a tube, the folds starting in the center and proceeding both rostrally and caudally. As a result of neural tube closure the previous lateral edges become joined at the dorsal midline (see Figure 7.8).

The Science of Stem Cells, First Edition. Jonathan M. W. Slack.

Companion website: www.wiley.com/go/slack/thescienceofstemcells

Apart from the morphogenetic movements involved in neural tube closure, the development of the neural tube involves two distinct types of process. There is regional specification, which creates a set of rostrocaudal and dorsoventral zones with different states of commitment. There is also differentiation of neurons and glia from the cells of the early neuroepithelium. The types of neurons and glia vary according to the region although most of the histological types are found throughout the CNS.

The Brain

The main parts of the developing human brain are shown in Figure 8.1a. The early brain has three swellings called the forebrain, midbrain and hindbrain, and these terms are also used to indicate the later anatomical regions arising from each swelling. Within the brain is a system of ventricles, derived from the original lumen of the neural tube, which are continuous with each other and with the spinal canal. The ventricles are lined with ependymal cells, which are cuboidal glial cells bearing cilia. Each ventricle contains a choroid plexus, consisting of capillaries surrounded by ependymal cells, which secrete the cerebrospinal fluid. Unless they arise in the dorsal or ventral midline, brain structures are paired, one on each side. Twelve pairs of cranial nerves arise from the brain. They supply various parts of the head and some parts of the upper thorax and may be motor or sensory or mixed in composition. In neuroanatomy, the term "nucleus" refers to a condensation of neurons, not to the nucleus of a single cell. Mouse and human brains contain generally similar parts but there are also large differences in proportion and scale (Figure 8.1b; Figure 8.C.1).

The forebrain comprises the telencephalon rostrally and the diencephalon caudally. The most rostral part of the telencephalon forms the olfactory bulb, the receptive area for the olfactory nerve (cranial nerve I). The olfactory neurons themselves arise from the olfactory placode, and the axons grow into the olfactory bulb. In mice the olfactory bulb is large, but in humans it is small and tucked below the brain. Rodents also have an additional vomeronasal organ in the nasal cavity connected to an accessory olfactory bulb, not found in humans.

The main structures formed by the telencephalon are the cerebral hemispheres, which arise as lateral evaginations separated by a medial longitudinal fissure, each containing a lateral ventricle. The dorsal part of each hemisphere becomes the cerebral cortex which is responsible for the higher brain functions including consciousness, perception, memory, and, in humans, thought and language. The main part of the cortex in mammals is called the neocortex and consists of six layers of neurons which arise from precursors adjacent to the ventricles. The cerebral cortex is, of course, relatively much larger in humans than in mice and during late fetal growth it increases greatly in surface area and becomes thrown into multiple folds (Figure 8.C.1). On the inner side of each hemisphere, within the medial fissure, forms a sausage-like body called the hippocampus (Figure 8.2a), just above the choroid plexus. This is later important in spatial navigation and in the consolidation of short to long term memories. It is notable in that its caudal portion, the dentate gyrus, is one of a very few areas of the brain containing neural stem cells which remain active through adult life. The ventral part of the cerebral hemispheres, called the striatal region, is much thicker than the dorsal part and contains folds called ganglionic eminences which are a source of migratory cells entering the neocortex (Figure 8.2b). Later, the striatal region forms the basal ganglia: the caudate nucleus, putamen and globus pallidus, which are involved in the selection of behaviors and movements. These have connections to the cortex, the thalamus, and the brainstem. In the adult brain the term striatum is used to refer to the caudate nucleus, putamen and some other structures.

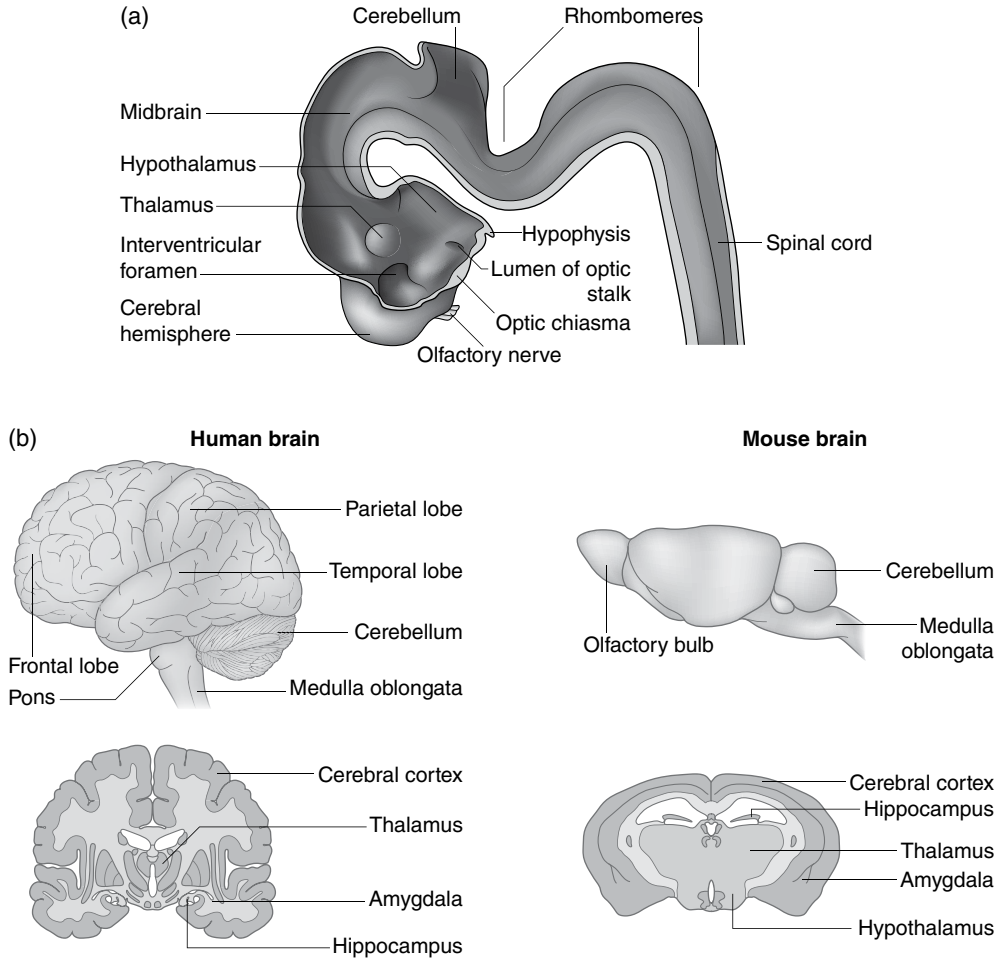

Figure 8.1 (a) Human brain at about 35 days from fertilization. (From: Hamilton Boyd and Mossman (1972) Human Embryology. Reproduced with permission.) (b) Comparison of structure of adult brains of human and mouse. (From: John F. Cryan, J.F. & Holmes, A. (2005) The ascent of mouse: advances in modelling human depression and anxiety. Nature Reviews. Drug Discovery 4, 775–790.)

The caudal part of the forebrain, the diencephalon, surrounds the third ventricle and produces several important structures. The thalamus, which later relays sensory information to the cerebral cortex, arises from the lateral walls. Later, as the cerebral cortex overgrows the diencephalon, the thalamus comes to lie adjacent to the basal ganglia (Figure 8.2b). The optic vesicles are lateral outgrowths of the forebrain that remain attached to the diencephalon. They become the neural parts of the eyes, comprising the sensory retina, pigmented retina and optic nerves (cranial nerves II). In the ventral part of the diencephalon arises the hypothalamus and the neural part of the hypophysis (= pituitary gland). The hypothalamus is later closely involved with control of the hypophysis. From the dorsal part of the diencephalon develops the epiphysis (pineal gland), and the choroid plexus of the third ventricle.

The midbrain cavity becomes narrowed to form the aqueduct, connecting the third and fourth ventricles. The dorsal part of the

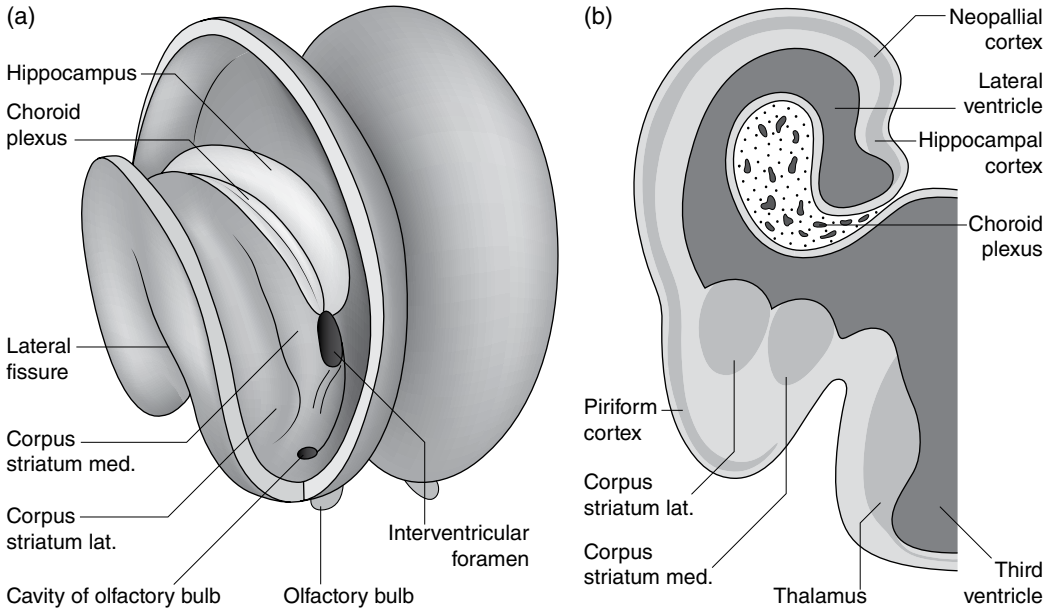

Figure 8.2 (a) Location of the developing hippocampus and corpus striatum within the cerebral hemisphere of a human embryo of about 10 weeks post-fertilization (embryo size 46 mm). (b) The developing basal ganglia viewed in transverse section. (From: Hamilton Boyd and Mossman, Human Embryology.)

midbrain forms the superior and inferior colliculi. The superior colliculus, known as the optic tectum in non-mammalian vertebrates, is the primary area for receipt of visual information from the optic nerves. The inferior colliculus is a center for receipt of auditory and balance information. The ventral part of the midbrain forms the nuclei of the third and fourth cranial nerves and the substantia nigra, an important center for motor control, reward seeking and learning. This is considered as one of the basal ganglia, along with those derived from the telencephalon, and contains GABAergic neurons connecting to the thalamus, and dopaminergic neurons connecting to the striatum.

The hindbrain comprises the cerebellum, later connecting to the thalamus and the brainstem and important in movement control; and the medulla oblongata which contains the remaining cranial nerve nuclei. The hindbrain shows a transient segmentation into seven rhomobomeres, of which the most rostral becomes the cerebellum.

Regional Specification of the CNS

Rostrocaudal

The rostrocaudal pattern of the neural tube and the later CNS depends initially on the gradients of FGF and Wnt which are high at the caudal end and low at the rostral end. These induce expression of *Cdx* genes which in turn induce expression of *Hox* genes. Because of the graded nature of the signals, the *Hox* genes are activated in a nested pattern such that all the genes are on in the tail bud and the rostral frontiers of expression vary such that the more 3′ the gene lies in the chromosome, the more rostal is its boundary (Figure 8.3). There are four Hox clusters in vertebrates which arose in evolution by a double duplication of the original cluster around the time of the origin of vertebrates. The equivalent genes in each cluster are known as paralogs and tend to have similar expression domains and functions.

The *Hox* gene expression domains extend into the future hindbrain. Rostral to this the initial subdivision of the neural plate depends

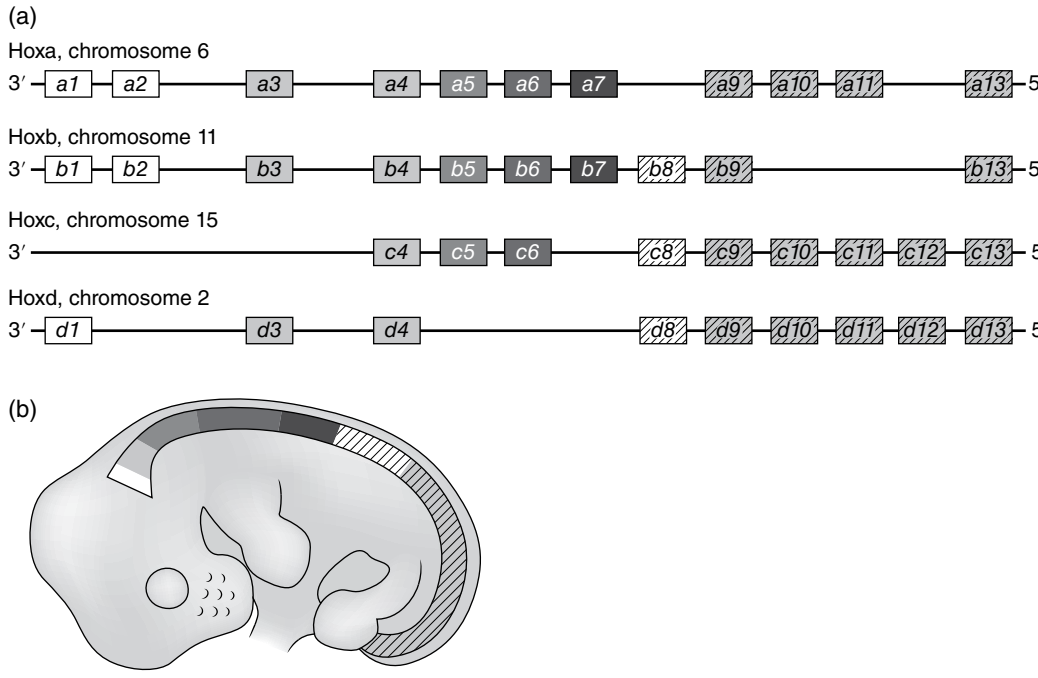

Figure 8.3 Expression pattern of Hox genes in the mouse embryo neuraxis. (a) The 4 HOX gene clusters in the mouse. (b) Anterior expression limits of each paralog group of HOX genes. From: Box 5E p.215 in Wolpert, L. et al. (2015) Principles of Development. 5th edn. Oxford University Press, Oxford and New York. (Reproduced with the permission of Oxford University Press.)

on expression of two genes encoding non-*HOX* homeodomain transcription factors: *Otx2* and *Gbx2*. *Otx2* is expressed in the future fore- and midbrain while *Gbx2*, is expressed more caudally. There is a considerable subdivision of this simple rostrocaudal pattern which occurs during and shortly after neural tube closure. The forebrain is subdivided into telencephalon and diencephalon, in response to FGF from the anterior neural ridge. The rostral cerebral hemispheres are marked by expression of *Pax6* and the caudal part by *Emx2*. Regionalization of the caudal telencephalon also depends on Wnt and BMP from the cortical hem, which lies along the dorsal midline of the developing cortex. These signals are required for formation of the hippocampus.

The border between the midbrain and hindbrain, known as the isthmus, is marked by expression of FGF8 and WNT1 (Figure 8.4). On the rostral side these factors induce expression of the transcription factors Engrailed 1 and 2, defining the future midbrain. The tegmentum arises from the basal plate of the midbrain and requires both SHH from the ventral midline and FGF8 from the midbrain-hindbrain boundary for its formation. It later produces the dopaminergic neurons of the striatum. On the caudal side the FGF8 and WNT1 from the isthmus induce formation of the future cerebellum.

As mentioned above, the hindbrain becomes divided into seven segments called rhombomeres (Figure 8.5) of which the cerebellum forms from the most rostral, rhomobomere 1. The rhombomeres transiently form visible segments and the process of cell condensation depends on expression of Eph and ephrin adhesion molecules in alternate segments. Control of regional specification here is largely due to a local gradient of retinoic acid. This is produced from dietary vitamin A (retinol) by retinaldehyde dehydrogenase

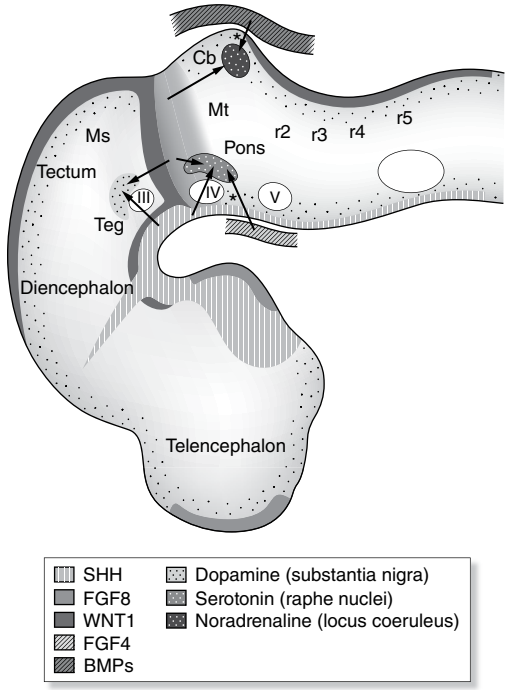

Figure 8.4 Inductive signals patterning regions of the CNS near the midbrain–hindbrain boundary. (Modified from Wurst, W. and Bally-Cuif, L. (2001) Neural plate patterning: Upstream and downstream of the isthmic organizer. Nature Reviews. Neuroscience 2, 99–108. Reproduced with the permission of Nature Publishing Group.) cb = cerebellum, Ms = mesencephalon, Mt = metencephalon, Teg = tegmentum, r2-r5 = rhombomeres, III, IV, V = cranial nerve roots. Signals marked * operate during gastrulation.

(RALDH2), expressed in the somites and the lateral plate of the trunk region. It is destroyed by CYP26, a cytochrome p450 enzyme, located in the fore- and midbrain. This establishes a caudal to rostral gradient of retinoic acid across the future hindbrain which induces expression of a unique combination of transcription factor genes in each rhombomere, including the *HOX* genes *b1-b4* and the gene encoding the zinc finger transcription factor KROX20. The knockout of *Cyp26A1* increases the level of retinoic acid and leads to caudalization of the hindbrain structures. Conversely the knockout of *Raldh2* decreases the level of retinoic acid and leads to a rostralization of the hindbrain.

The rostrocaudal organization of the spinal cord is less apparent in terms of gross morphology than that of the brain, but there are some differences, for example in the presence of certain types of motorneuron, and this is controlled by the nested expression of the *HOX* genes within the spinal cord.

Mediolateral

After about 10.5 days of mouse development, the spinal cord begins to differentiate into layers. The inner layer is the single layer of cells that becomes the ependyma lining the spinal canal. Outside this, the middle layer is called the mantle zone, composed of neuroepithelial

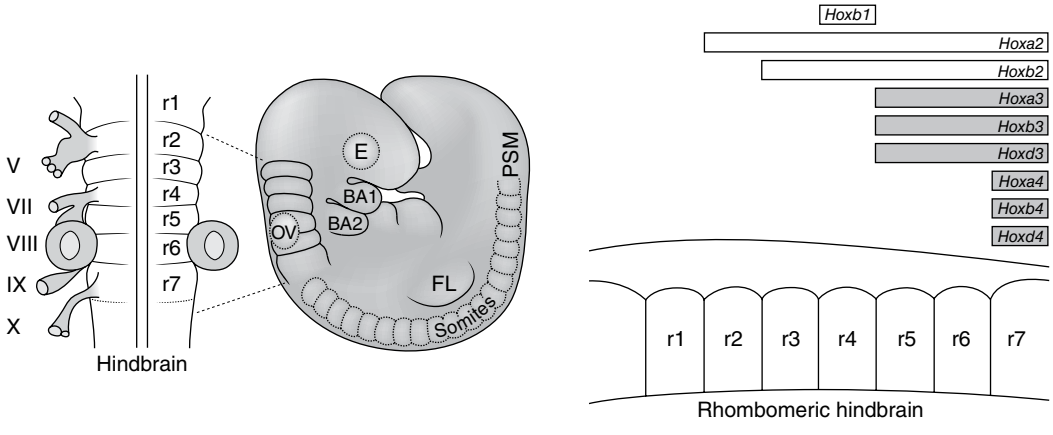

Figure 8.5 The rhombomeres of the hindbrain. Their individual character is determined by the nested expression of *HOX* genes, which is controlled by a local gradient of retinoic acid. (Modified from Alexander, T., Nolte, C. and Krumlauf, R. (2009) *HOX* genes and segmentation of the hindbrain and axial skeleton. Annual Review of Cell and Developmental Biology 25, 431–456.)

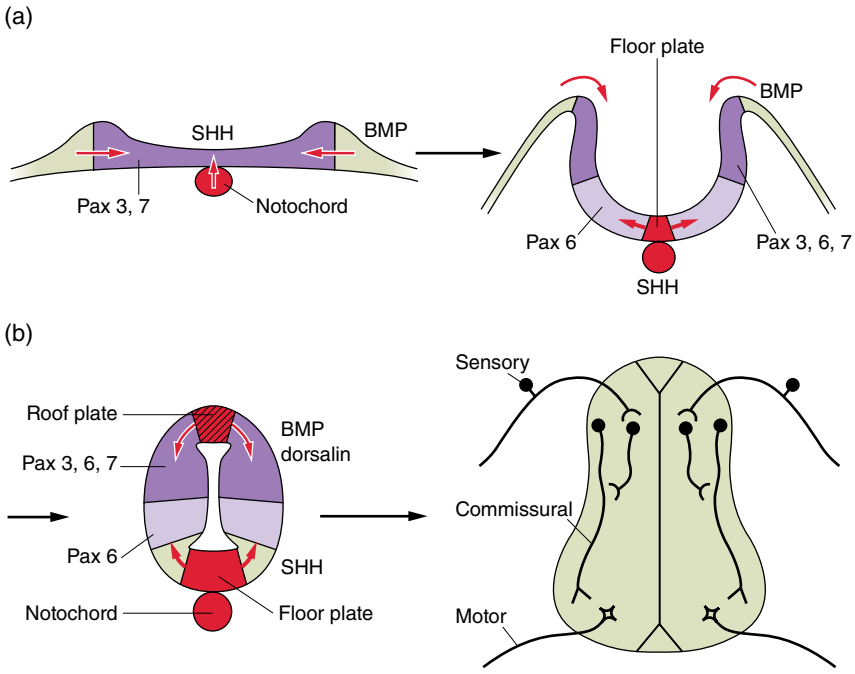

Figure 8.6 Dorsoventral patterning of the neural tube. (a) SHH from the notochord induces the floor plate in the overlying neural plate and this also secretes SHH. BMP from the epidermis induces the roof plate. (b) Inductive signals from the floor and roof plates induce zones of gene expression that later generate specific types of neuron. (Slack, J.M.W. (2013) Essential Developmental Biology, 3rd edn. Reproduced with the permission of John Wiley and Sons.)

cells and newly formed neurons. This will later become the gray matter. The outer layer is the marginal zone, consisting of the fiber tracts growing from the newly formed neurons, which will later be the white matter. In the brain the general arrangement is similar to the spinal cord, except that many cell populations migrate from the mantle zone through the marginal zone toward the surface, thus bringing layers of cells outside of the zone of fiber tracts.

Dorsoventral

Both the brain and spinal cord show considerable dorsoventral pattern. In the spinal cord the dorsal part is called the alar plate and the ventral part the basal plate. These are domains of different neuron types, with motorneurons ventrally and interneurons dorsally connecting to the sensory ganglia (Figure 8.6). This situation arises from a pair of morphogen gradients. Sonic hedgehog (SHH) comes from the notochord and the ventral midline structure called the floor plate. BMPs come from the epidermis flanking the lateral edges of the neural plate, which become dorsal on closure of the neural tube. The gradients initially activate transcription of *Pax6* in the ventral region and *Pax 3, -6* and *-7* in the dorsal region. These broad domains are further subdivided by expression of other transcription factors to produce a series of zones arranged dorsal to ventral. Each of these produces a particular repertoire of motorneurons or interneurons.

The Eye

The eyes are formed as outgrowths from the diencephalon called the optic cups (Figure 8.7). These grow out as vesicles which invaginate to form cups. The inner, invaginated, surface becomes the neural retina, composed of photoreceptors and ganglion cells.

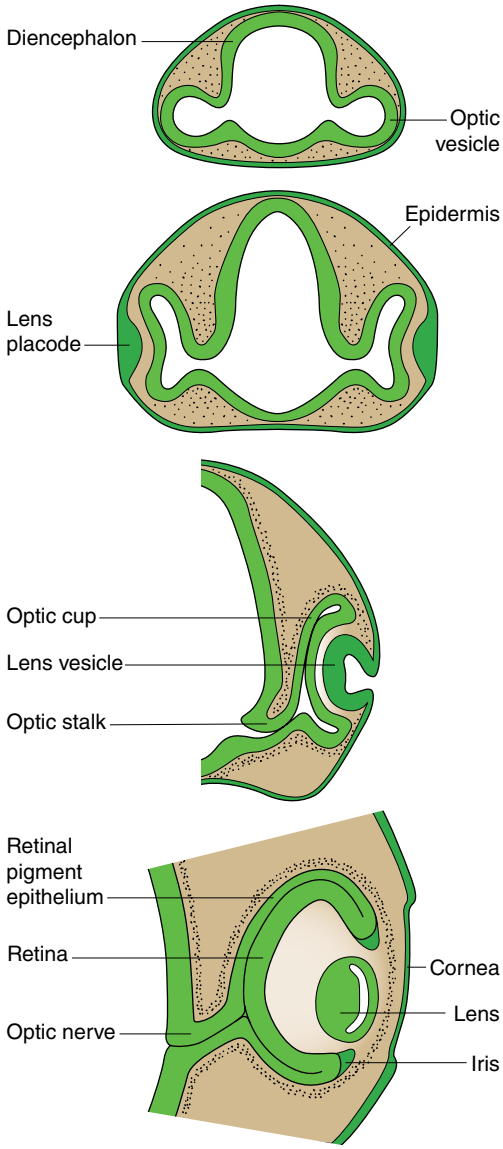

Figure 8.7 Development of the eye. The optic vesicle grows out of the diencephalon while the lens and cornea develop from the epidermis. The inner layer of the optic cup becomes the retina while the outer layer becomes the pigmented epithelium. (Hildebrand., 1995. Reproduced with the permission of John Wiley and Sons Inc.)

This sends axons back down the interior of the optic stalk into the brain making up the optic nerves, which project through the optic chiasma to the lateral geniculate nuclei and superior colliculi in the brain. Much of the optic projection crosses to the opposite side of the brain (contralateral) at the optic chiasma, although there is also some projection to the same side (ipsilateral).

The outer surface of the optic cup becomes the pigmented retina. The capsule of the eye is formed from mesenchymal cells of neural crest origin surrounding the optic cup. The lens and cornea arise from the ectoderm. They are formed from a thickened disc of cells, or placode, arising at the anterior margin of the neural plate. Like the neural crest, the epidermal placodes arise in areas of intermediate BMP signaling between the neural plate (low BMP) and the epidermis (high BMP). The position of specific placodes depends on a balance between FGF signaling from the anterior neural plate, BMP from the lateral region, WNT from the caudal region and SHH from the midline mesoderm. The whole eye territory, comprising optic vesicles and placodes, expresses the transcription factor PAX6, which is, remarkably, involved in eye development in all other types of animal, including lower invertebrates. When the optic cup contacts the optic placode, the lens invaginates forming a vesicle and the surface ectoderm which comes to overlay the lens becomes the corneal epithelium. The remainder of the cornea, comprising the stroma and the endothelium, arise from neural crest mesenchyme. Both the pigmented retina and the epithelium of the cornea are current targets for stem cell therapy.

The Neural Crest

Like the epidermal placodes, the neural crest is formed from the zone around the neural plate where the level of BMP inhibition is intermediate. Transcription factors active in the neural crest include FOXD3 (forkhead type), SNAIL and SLUG (Zn finger) and SOX9 and -10 (SRY related). Following neural

tube closure, the crest comes to lie along the dorsal midline of the neural tube. The neural crest is characterized by an extensive migration of its cells, some of which can range all over the body, and by the wide range of differentiated cell types which they can become. The first step in the migration consists of the expression of SNAIL protein which represses expression of E-cadherin, hence reducing cell adhesion and promoting an epithelial-mesenchymal transition to a migratory behavior. In the course of their migration the crest cells show an affinity for the extracellular matrix components fibronectin and laminin, and they secrete proteases to assist in their progress. The pattern of migration is partly controlled by inhibition from the surroundings, for example the ephrins present in the posterior sclerotome of each segment, and also in rhombomere 3 and 5 of the hindbrain, inhibit crest migration. There are two main migration pathways in the trunk: a dorsolateral route under the epidermis, where the cells normally become pigment cells; and a ventrolateral route through the sclerotome to become the dorsal root ganglia and the other trunk derivatives.

The neural crest normally forms a remarkable range of cell types. These include:

- neurons and glia of the sensory and autonomic system;
- adrenal medulla and the calcitonin cells of the thyroid;
- pigment cells of the skin;
- skull bones and connective tissues of the head;
- part of the cardiac outflow tract.

The normal fate of different parts of the neural crest was originally established by orthotopic (same position in donor and host) grafts of tissue from quail to chick embryos (Figure 8.8a). The quail cells can be distinguished by the presence of a heterochromatin mass in the nuclei, or by immunostaining for species-specific cell surface proteins. More recently studies have used localized marks of the vital dyes DiI or DiO. The cranial neural crest, which later expresses SOX9 rather than SOX10, differs considerably from the trunk neural crest in that it generates a large amount of mesenchyme which becomes most of the skull and the soft tissues of the face. The trunk neural crest does not form skeletal tissues but both cranial and trunk crest form various types of neuron.

When, and how, do neural crest cells become committed to form their specific differentiated progeny? Labeling of single cells in the neural folds both before and during migration indicates that at least some of them generate clones containing several different cell types, in other words at this stage they are multipotent. When crest cells in culture are exposed to specific inducing factors, there is a clear ability to control the pathway of differentiation. BDNF (bone derived neurogenic factor) induces sensory neurons; BMPs induce autonomic neurons; endothelin 3 induces pigment cells and enteric neurons; neuregulin induces Schwann cells and TGFβ induces smooth muscle (Figure 8.8b). A role for these factors is also suggested by the phonotypes of mouse knockouts for the factors concerned, which tend to be defective in specific types of neural crest progeny. It is however also found that labeling of single crest cells at an early stage sometimes only gives rise to a single differentiated cell type, even though the progeny spread out and are exposed to more than one environment. So there is probably also an autonomous element to cell differentiation as well as that controlled by inducing factors.

There has been some debate about whether multipotent neural crest cells persist into postnatal life, in other words whether there is a neural crest stem cell population that survives long term. This is still unresolved, but there are some cells found in the skin (SKPs) which can under some circumstances show multipotency similar to the embryonic neural crest cells.

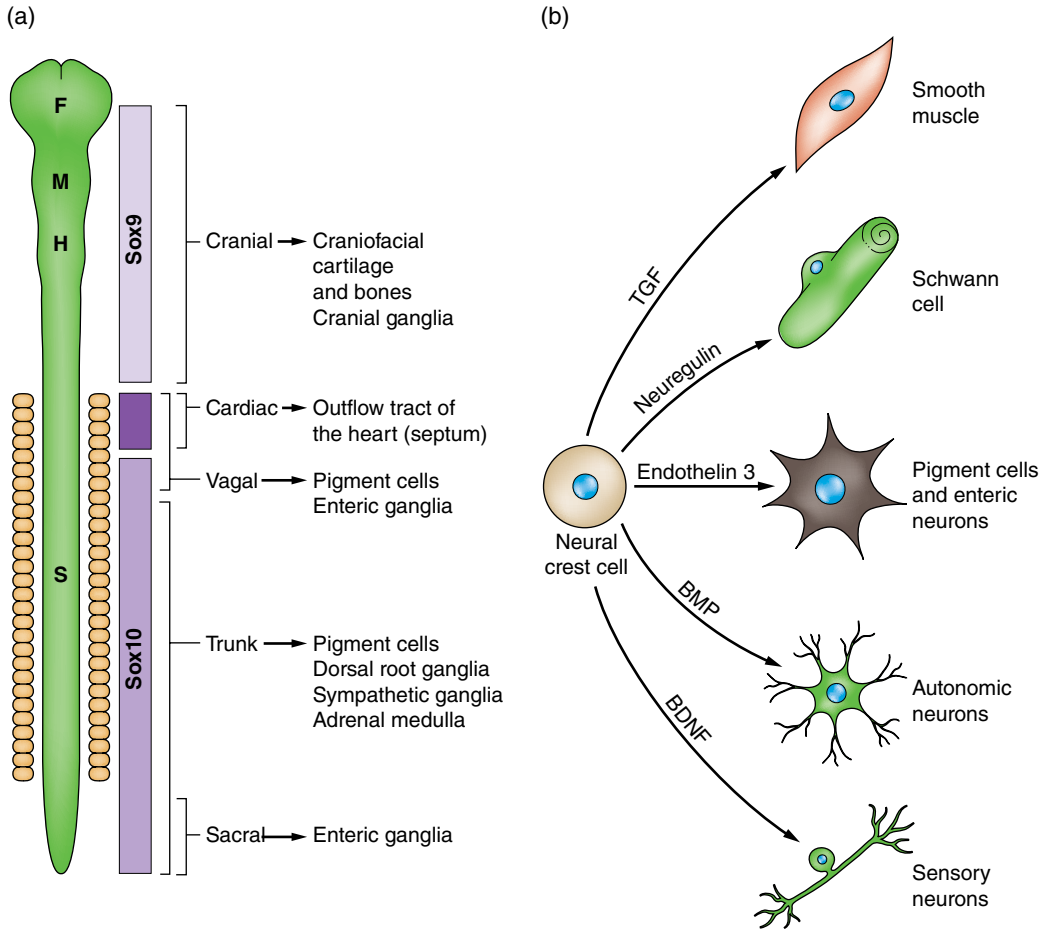

Figure 8.8 The neural crest. (a) Structures and cell types populated by the neural crest. Data from grafts of quail to chick embryos. F: forebrain; M: midbrain; H: hindbrain; S: spinal cord. (b) Differentiation behavior of neural crest cells exposed to different inducing factors. (Slack, J.M.W. (2013) Essential Developmental Biology, 3rd edn. Reproduced with the permission of John Wiley and Sons.)

Epidermis

After the closure of the neural tube the whole embryo is covered by surface ectoderm which will form the epidermal layer of the skin. The surface ectoderm is a simple one cell thick epithelium with a temporary periderm layer on the outside. In later gestation (mouse 14d; human from 11 weeks) this simple epithelium becomes multilayered, with a cuboidal basal layer and a series of progressively more flattened (squamous) upper layers (Figure 8.9). This transition depends on the activity of the *p63* gene, encoding a transcription factor necessary for the formation and maintenance of all squamous epithelia in the body. During the transition the cell divisions change from symmetrical in the plane of the epithelium, to asymmetrical and vertical, i.e. one daughter remains in the basal layer while the other enters the next layer. As with other examples of asymmetric division, this process depends on localization of the PAR complex within each cell (see Chapter 9). As the epidermis matures, the cells become known as keratinocytes, expressing specific keratin genes in different layers. For example, K14 is

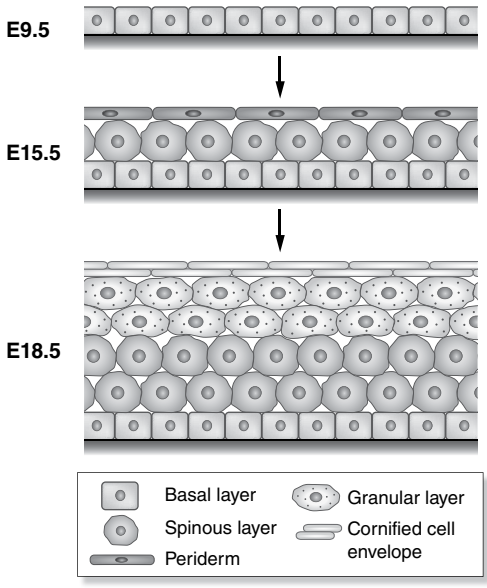

Figure 8.9 Stratification of the mouse epidermis. The first step is the expression of *p63* in the surface ectoderm at about E8.5 and the upregulation by p63 protein of *K14*, encoding keratin 14 which remains expressed in the basal layer. Suprabasal cells initially arise by tangential divisions, express *K1*, cease dividing and mature into granular keratinocytes.

expressed in the basal layer and K10 in the upper layers. Keratins are one of the classes of intermediate filament proteins important in maintaining cell structure. In the postnatal organism the basal layer is the only layer containing dividing cells, and some of these are epidermal stem cells, which still depend on *p63* for their properties.

As well as the stratified epidermis, the surface ectoderm also generates a number of specialized structures: sweat glands, sebaceous glands and hair follicles. In recent years a lot has been learned about these, particularly about the hair follicles.

Hair Follicles

Hair follicles (see Figure 2.C.2) are formed through a set of reciprocal interactions between the surface ectoderm and the

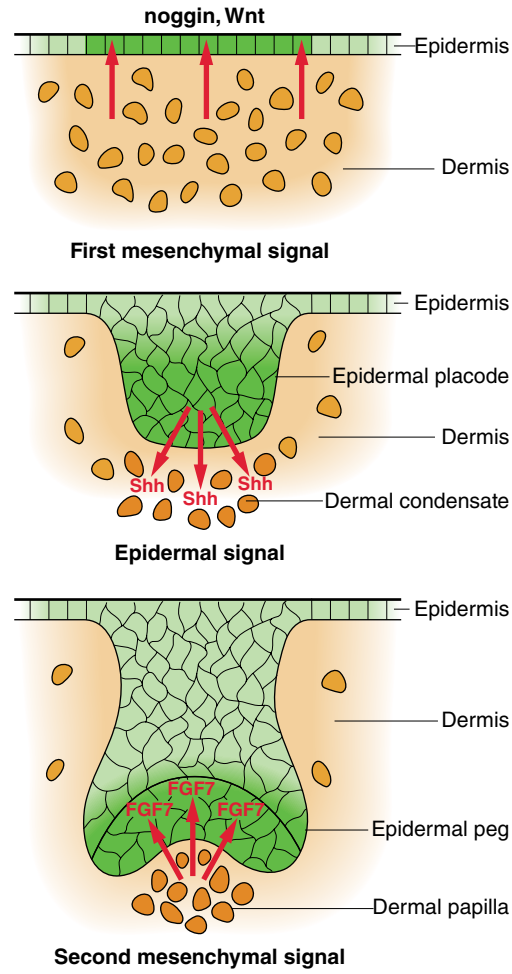

Figure 8.10 Embryonic development of hair follicles. (Slack, J.M.W. (2013) Essential Developmental Biology, 3rd edn. Reproduced with the permission of John Wiley and Sons.)

underlying mesenchyme, which becomes the dermal layer of the skin (Figure 8.10). The first signal comes from the mesenchyme and is a self-organizing pattern of Wnt activation and BMP inhibition set up by a lateral inhibition process (see Chapter 7 for symmetry breaking and Chapter 9 for lateral inhibition). This occurs about E14.5 of mouse development and establishes foci of cells from the surface epidermis committed to become epidermal placodes. In humans the first epidermal hair placodes appear at

7 weeks. The placodes emit sonic hedgehog (SHH) and provoke the formation of condensations of cells in the mesenchyme, which will become the dermal papillae of the later hair follicles. The papillae emit FGF7 and other factors which provoke the downgrowth of cells from the placode to surround the papilla and form the hair bulb. The evidence for this complex process comes from several sources. Mouse knockouts for key components show arrest of hair follicle development at the appropriate stage. Overexpression of the Wnt inhibitor Dickkopf can change the spacing of follicles, providing evidence for a lateral inhibition mechanism for placode formation. Grafting of the dermal papilla into the epidermis can, in favorable circumstances, provoke formation of new follicles.

Although the hair bulb contains the cells that support growth of the fiber, the true stem cells of the hair follicle are located higher up, in a region called the bulge (see Chapter 10). They arise in late gestation (mouse E17.5) as a population of SOX9-positive cells. The hair follicles are closely associated with sebaceous glands, which have their own stem cells and secrete lubricant onto the growing hairs.

Sweat glands arise around E17.5 and depend on BMP and FGF signaling from the mesenchyme and on a suppression of SHH activity.

Mammary Glands

Mammary glands develop from the ventral epidermis (Figure 8.11). The first indication is the formation of "milk lines", a slight thickening of the epidermis running from axilla to groin (mouse E10.5; human 4 weeks). This becomes a set of mammary placodes (5 pairs in mouse, one pair in humans). A mass of dense mammary mesenchyme appears underneath each placode. The epithelial cells grow inwards to form a bud and by E18.5 have formed a rudimentary duct system embedded in a dermal fat pad. The outer part of the placode becomes the nipple. The process, at least for placodes 2 and 3 in the mouse, is initiated by secretion of FGF10 from the somites. This induces Wnt expression in the milk lines and the later placodes, and expression of transcription factors including TBX3. Secretion of PTHrH (parathyroid hormone-related hormone) from the placode is responsible for induction of the mammary mesenchyme. This produces BMP which stimulates the ductal growth and the branching of the epithelial bud.

The mammary gland is unusual among organs in that most of its development occurs postnatally. The embryonic development is the same in males and females, but at commencement of puberty (from 4 weeks after birth in mouse, about 12 years in

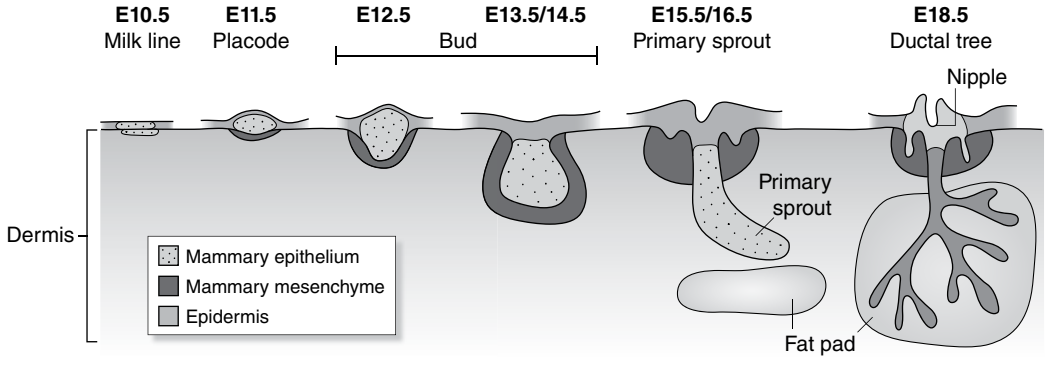

Figure 8.11 Development of the mammary glands in the mouse embryo. (From: Robinson, G.W. (2007) Cooperation of signalling pathways in embryonic mammary gland development. Nature Reviews Genetics 8, 963–972. Reproduced with the permission of Nature Publishing Group.)

human) there is a specific stimulation of further development in females. Increased gonadotrophin from the anterior pituitary stimulates release of estrogens from the ovaries. This causes a renewal of growth and branching activity in the mammary epithelium. The growth of the epithelium at this stage is estrogen-dependent, and in mice knockout of the estrogen receptor α (ERα) prevents further development. One effect is the upregulation of amphiregulin (an EGF-like factor) in the epithelium which stimulates growth in both epithelium and mesenchyme. There is also an effect of growth hormone (GH) via the mesenchyme. This causes release of IGF1 which stimulates growth of the epithelial cells. During this period the growth rate exceeds that of the rest of the body, so that the mammary glands become relatively larger. During pubertal growth, terminal end buds form which burrow into the fat pad, assisted by matrix metalloproteinases from the mesenchyme. These have an outer undifferentiated layer of cells and multiple inner layers. As they grow some of the inner cells apoptose to generate a duct lumen. Trailing cells of the bud become myoepithelial cells on the outer surface of the duct system.

The third phase of mammary development occurs during pregnancy and lactation and is described in Chapter 10.

Somitogenesis

The mesodermal germ layer initially forms the notochord, somites and lateral plate. These are arranged mediolaterally during gastrulation and as the embryo body folds develop they become rearranged to run from dorsal to ventral (Figure 8.12). In the embryo the notochord has an important signaling role as a source of SHH, and also has a morphogenetic function stiffening the embryo and helping to drive its elongation. The somites are segmented structures, discussed here. The lateral plate forms various organs including the limb buds, kidney and gonads,

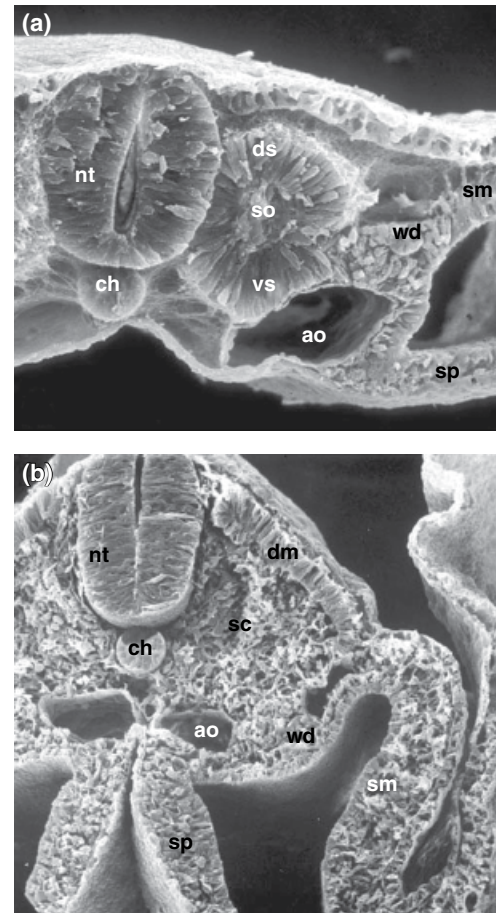

Figure 8.12 Scanning electron micrographs showing the disposition of the main axial structures in the chick embryo. (a) 2 days incubation. (b) 3 days incubation. ao aorta, ch notochord, dm dermomyotome, ds dorsal somite, nt neural tube, sc sclerotome, so somite cavity, sm somatopleure, sp splanchnopleure, vs ventral somite, wd Wolffian duct. (From: Christ, B., Huang, R. and Scaal, M. (2004) Formation and differentiation of the avian sclerotome. Anatomy and Embryology 208, 333–350. Reproduced with the permission of Springer.)

blood vessels and blood cells. Much of the lateral plate becomes divided by a cavity, called the coelom, into an inner splanchnic layer and an outer somatic layer. The somatic mesoderm with its overlying epidermis is often known as the somatopleure.

The presence of segmented somites is a prominent feature of all vertebrate embryos. They develop in the mesoderm in rostral to

caudal sequence on either side of the notochord. Because they are clearly visible and can be counted, the somite number is often used as an indication of the developmental stage of the embryo. The somites are initially formed as cell condensations, they transiently become epithelial vesicles and later become divided into two cell masses. The central mass is the sclerotome and becomes the vertebrae, with each vertebra formed from the anterior part of one somite and the posterior part of the adjacent somite. The outer part is the dermomyotome, which forms the striated muscles of the trunk and the limbs, and the dermal layer of the skin in the dorsal part of the body. In lower vertebrates, such as *Xenopus* and zebrafish, the bulk of the somite becomes the myotome and the other parts are relatively very small.

The Somite Oscillator and Gradient

The development of somites consists of two clear phases: the formation of the segmented pattern, and the development of the sclerotome and dermomyotome within each somite. It was long suspected that the formation of a perfectly regular series of structures might depend on some sort of clock or oscillator and indeed this is the case (Figure 8.13). The cells of the presomitic mesoderm express *Hes* genes, encoding basic helix-loop-helix (bHLH) type transcription factors, whose expression oscillates with a periodicity of a few hours (2 hours in mouse; 6–8 hours in

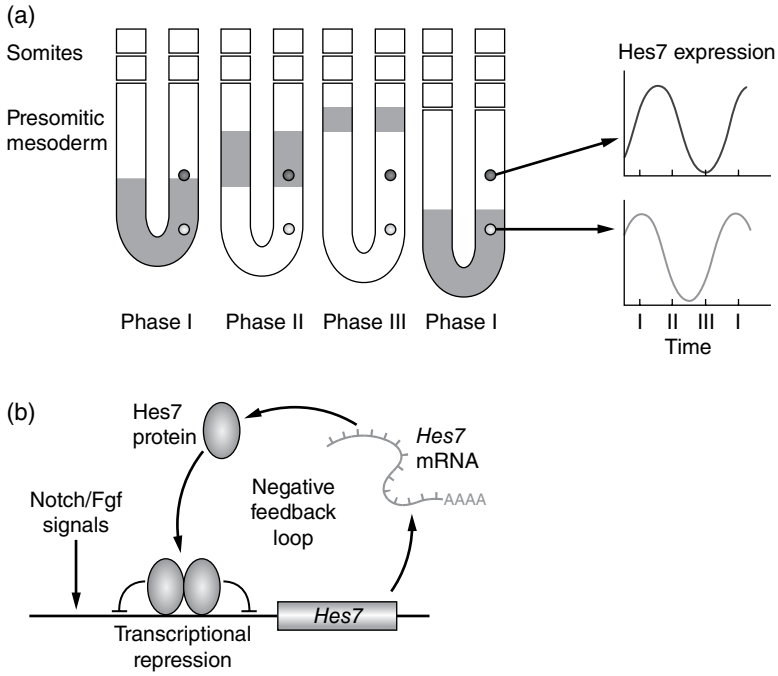

Figure 8.13 Mechanism of segment formation during somitogenesis. (a) The anterior end of the presomitic mesoderm becomes segmented every 2 hours in mouse embryos, forming a bilateral pair of somites. In each cycle, *Hes7* expression is initiated at the caudal end and appears to propagate rostrally because it is following the oscillatory time course illustrated on the right. A caudal to rostral gradient of FGF8 maintains the oscillations and as its overall level declines the oscillations stop in rostral to caudal sequence. When this occurs each group of cells with high *Hes7* increases its cell adhesivity and becomes the next somite. (b) Negative feedback mechanism for the oscillation of *Hes7* expression. (From: Kageyama, R., Niwa, Y., Isomura, A., Gonzalez, A. and Harima, Y. (2012) Oscillatory gene expression and somitogenesis. Wiley Interdisciplinary Reviews – Developmental Biology 1, 629–641. Reproduced with the permission of John Wiley & Sons.)

human). The basic oscillator mechanism depends on a negative feedback and delay. In the mouse, HES7 represses its own transcription, but the delay in transcription and translation, and the short lifetime of both the message and the protein, means that the repression is cyclical. HES7 also represses transcription of *Delta*, thus causing an oscillation of Notch activation in the surrounding cells. Synchronization of the oscillator between cells is achieved through *Lunatic fringe* (*Lfng*), which is upregulated by HES7 and encodes a glycosyl transferase which modifies Notch and facilitates Notch signaling. Notch activation upregulates *Hes7* in neighboring cells thus synchronizing the *Hes7* oscillator between cells.

In addition there is a caudal to rostral gradient of FGF8 which is needed for continuation of the oscillations. The FGF gradient gradually declines so the oscillations stop in a smooth sequence from rostral to caudal as cells fall below the threshold level of FGF signaling. This effectively converts a temporal oscillation of *Lfng* mRNA into a spatial one, such that in each cycle Notch and FGF can drive expression of a transcription factor gene, *Mesp2*, in a one segment wide strip of presomite mesoderm. MESP2 represses *Snail* which represses genes for cell adhesion molecules such as integrins and cadherins. The result of this double inhibition is an increase in cell adhesion and the formation of an epithelial somite from the region of *Mesp2* activity. It is now known that the FGF signaling is also oscillating, due to another negative feedback loop involving dephosphorylation of the ERK-phosphate. But it is still the gradual decline of the FGF8 gradient that confers the polarity and the sequence for the process of somite formation. The initially formed epithelial somite is characterized by expression of the paired-box transcription factor PAX3.

Subdivision of the Somites

The development of the sclerotome, myotome and dermatome from the epithelial somite is controlled by inducing factors from the surrounding structures (Figure 8.14). The sclerotome, which forms the cartilage, and later the bone of the vertebrae, is induced by SHH coming from the notochord and the floor plate of the neural tube. Any part of the epithelial somite can form sclerotome if combined with one of these tissues or exposed to SHH. Expression of *Pax1* and *Pax9* is induced and these upregulate *Nkx3.2* (*Bapx1*) which is a master gene for cartilage differentiation. At the same time, *Pax3* is repressed and a downregulation of N-cadherin causes the cells to undergo an epithelial to mesenchymal transition. The outer part of the sclerotome, formed furthest from the SHH signal, is known as the syndetome. It later generates tendons and is characterized by expression of the bHLH transcription factor Scleraxis. Each vertebra arises from the anterior half of one sclerotome

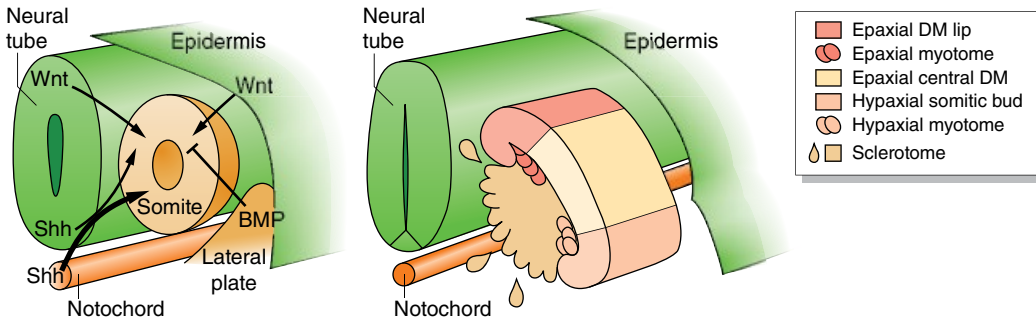

Figure 8.14 Subdivision of the somite by signals from the surrounding tissues. DM = dermomyotome. (Slack, J.M.W. (2013) Essential Developmental Biology, 3rd edn. Reproduced with the permission of John Wiley and Sons.)

and the posterior half of the next. The morphological character of the vertebrae (cervical, thoracic, lumbar etc.) is determined by the expression of *HOX* genes at that particular rostrocaudal level. Loss of function mutants of *HOX* genes tend to convert vertebrae into a more rostral type, and gain of function mutations to a more caudal type.

By the time that the sclerotome has appeared, the remainder of the somite is known as the dermomyotome. The myotome, which forms the skeletal muscle of trunk and limbs, is often depicted in the center of the mature somite, but it actually arises as two distinct primordia, the epaxial myotome on the dorsal side and the hypaxial myotome on the ventral side. Cells from these regions migrate to form layers of myoblasts under the central dermomyotome. The induction of the two myogenic regions arises from different signals. The epaxial myotome requires an early exposure to SHH, and a later exposure to WNT from the dorsal neural tube. The hypaxial myotome requires WNT7A from the dorsal epidermis. Myoblasts from both regions express *Pax7* in addition to *Pax3* and their division is maintained by FGF signaling.

Because of the process of myogenesis is ongoing, there is never really a distinct region of the somite that can be called the dermatome. The dorsal dermis does arise from cells of the dermomyotome, while the remainder of the dermis comes from mesoderm of the ventrolateral body wall.

Myogenesis

The myoblasts are elongated mononucleate dividing cells. Following a reduction of FGF signaling they become committed to form striated muscle by expression of the myogenic transcription factor MYF5. This is one of a group of bHLH type myogenic transcription factors that control expression of the characteristic proteins of striated muscle including actin, specific isoforms of myosin, and the sarcoplasmic reticulum ATPase. The other myogenic factors are MyoD, Myogenin and MRF4 (= MYF6). These are expressed sequentially during myogenesis, but have overlapping biochemical properties. The combined knockout of *Myf5* + *MyoD* + *Mrf4* lacks most embryonic muscle.

In the dermomyotome, the central myoblasts remain in situ to form the muscles of the dorsal body wall while the lateral ones undergo migration, under the influence of hepatocyte growth factor (HGF, =Scatter Factor) from the lateral plate, to form the muscles of the limbs and of the ventral body wall. Later differentiation of multinucleated striated muscle fibers involves cell fusion, described in Chapter 9.

The Kidney

The region of mesoderm ventral to the somites is called the intermediate mesoderm. From this develops both the gonads and the kidney. Because kidney transplantation is so important in human medicine, and there is a perpetual shortage of kidneys, the prospect of using stem cells to repair damaged kidneys, or to make completely new ones, has prompted much interest. Higher vertebrates (the amniotes) have three kidneys: a pronephros, which is vestigial, a mesonephros, which has a segmental structure but very limited function confined to embryonic life, and the metanephros, or definitive kidney. Lower vertebrates, such as *Xenopus* and the zebrafish, do not have a metanephros, and their definitive kidney is the mesonephros.

The metanephros arises from two distinct components of the intermediate mesoderm through a reciprocal inductive interaction. The nephric duct (= Wolffian duct) arises in the trunk region and grows caudally to the cloaca. Near the cloaca, a side bud is emitted, called the ureteric bud. This grows into the surrounding intermediate (nephrogenic) mesenchyme and undergoes repeated branching (Figure 8.15a). This branching behavior depends on an inducing factor called glial derived neurotrophic factor

(GDNF). This factor is not, in this case, derived from glia, but from the nephrogenic mesenchyme. Its receptor, RET, is present on the ureteric bud and stimulation activates both the ERK and the PI3K signaling pathways (see Figure 7.4). The branched ducts arising from the ureteric bud later become the collecting ducts of the kidney. They contain mostly cells called principal cells, responsible for water reabsorption, together with some intercalated cells, which regulate pH. Like many other examples of cell differentiation, formation of the intercalated cells depends on the Notch system (see Chapter 9).

The nephrons of the kidney arise from the nephrogenic mesenchyme, under the influence of the branching ureteric bud (Figure 8.15b). The main inducing factor from the bud is thought to be WNT9B, and the competence of the mesenchyme depends on expression of a Zn finger transcription factor Wilms' Tumor 1 (WT1, named after the tumor which arises from its loss of function mutation). Other important factors from the bud are BMP7 and FGF2. Following the initial steps of tubule induction, further development depends on WNT4 secreted by the nephrogenic mesenchyme itself.

During development of the nephron, a dense cap of cells forms around the bud tip which expresses the homeodomain transcription factor SIX2. The cap undergoes a mesenchymal to epithelial transition and grows to form the Bowman's capsule and the kidney tubule, fused to

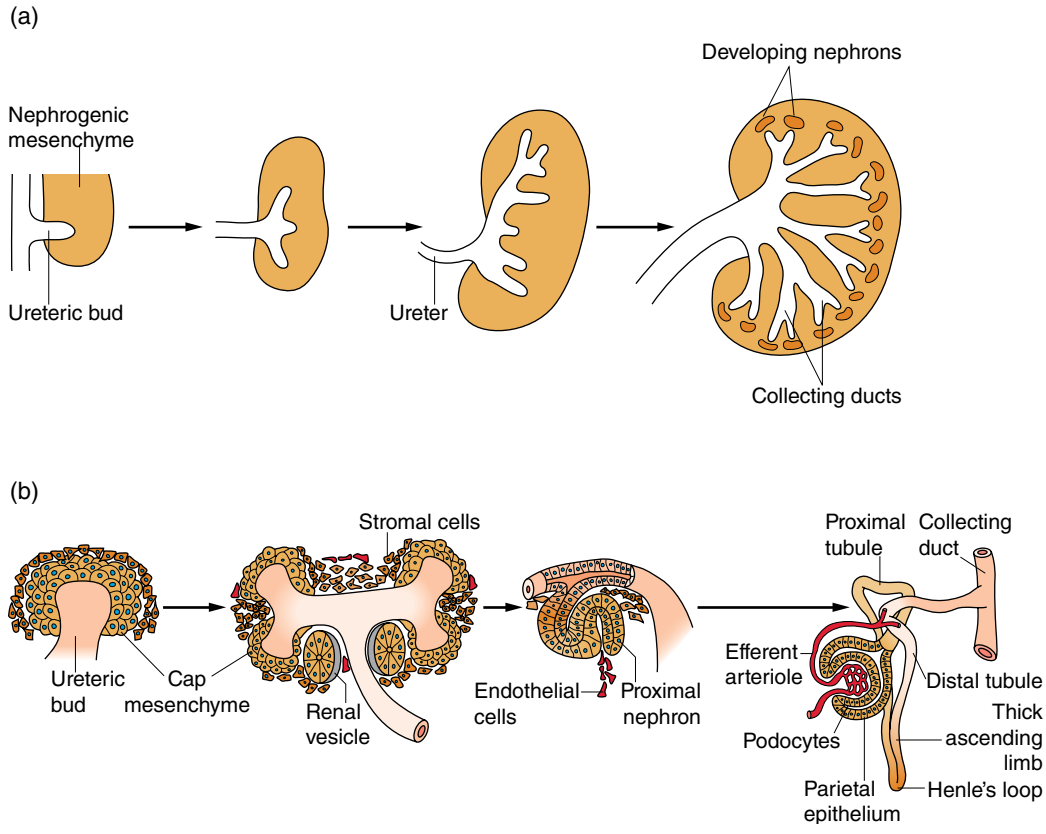

Figure 8.15 Development of the metanephric kidney. (a) Growth and branching of the ureteric bud into the surrounding nephrogenic mesenchyme. (b) Induction and development of an individual nephron. (Adapted from Dressler., 2009. Reproduced with the permission of Company of Biologists Ltd.)

and continuous with, the collecting system arising from the ureteric bud. The capsule contains specialized cells called podocytes that filter the blood. The tubule contains several other cell types with specific functions of reabsorption or secretion. The formation of new nephrons terminates just after birth in the mouse, coincident with the loss of cells expressing *Six2*. These cells are often called stem cells in the kidney literature, but as they only persist for a few cell cycles during embryonic development, they are better considered as progenitor cells.

Blood and Blood Vessels

Blood

Without blood and blood vessels no animal of any size could survive. Furthermore no grafted tissue can survive unless it rapidly acquires a vascular system. Effective tissue-engineered tissues and organs will need to be supplied with a vascular system so this is a topic of particular interest to stem cell researchers.

In the mouse the embryonic development of blood commences in the yolk sac at about 7.5 days gestation when a number of blood islands appear in the mesoderm. They are surrounded by endothelium but initially there is no circulatory system for them to join. These primitive blood islands contain a different repertoire of cells from the later definitive blood. Most of the primitive blood cells are erythrocytes which are larger than the definitive erythrocytes, and retain their nuclei (Figure 8.C.2). There are also some megakaryocytes (the cells that form blood platelets) and a population of primitive macrophages precursors that generate the microglia of the brain and other tissue resident macrophage types.

There has been much controversy about whether the cells of the primitive blood islands contribute progeny to the stem cells of the definitive blood. In the mouse it is difficult to be certain, but in *Xenopus* it is possible to label the primitive and definitive blood by microinjection into different parts of the early embryo and this shows that the two populations are distinct. The definitive blood arises from the endothelium of the dorsal aorta and other central arteries (Figure 8.16). This has been shown by lineage labeling experiments and also directly observed by time lapse filming of the dorsal aorta in both mouse and zebrafish. In mammalian development the main site of hematopoiesis in the mid-gestation embryo is the liver, and in the late gestation and postnatal animal it is the bone marrow. The labeling experiments show that the hematopoietic cells of the fetal liver and the postnatal bone marrow are progeny of the cells originally arising from the central arteries. Definitive blood cells are often said to arise from the "AGM" region. This relates to early experiments which located their origin to a region of the lateral mesoderm including the aorta, gonads and mesonephros, but in fact the origin is just from the aorta and other central arteries.

The first time of appearance of real hematopoietic stem cells (HSCs) in the mouse embryo is known from experiments where cells are transplanted to lethally irradiated mice (see Chapter 10). HSCs are able to lodge in the bone marrow of the host and, in time, produce a new population of all the cell types of the blood and immune system which repopulate the host and prevent it from dying of the radiation. Such experiments indicate that before about E10.5 there are no HSC in the embryo. From E10.5 the first repopulating cells are detected in the region of the dorsal aorta and can be lineage labeled with endothelial cell markers. This shows that the primitive blood islands do not contain HSC, whereas the region of the dorsal aorta does contain them from the time that definitive hematopoiesis is first observed.

The three transcription factors SCL (bHLH type), LMO2 (LIM domain) and GATA1 (Zn finger) are all required both for primitive and definitive hematopoiesis, and form a complex

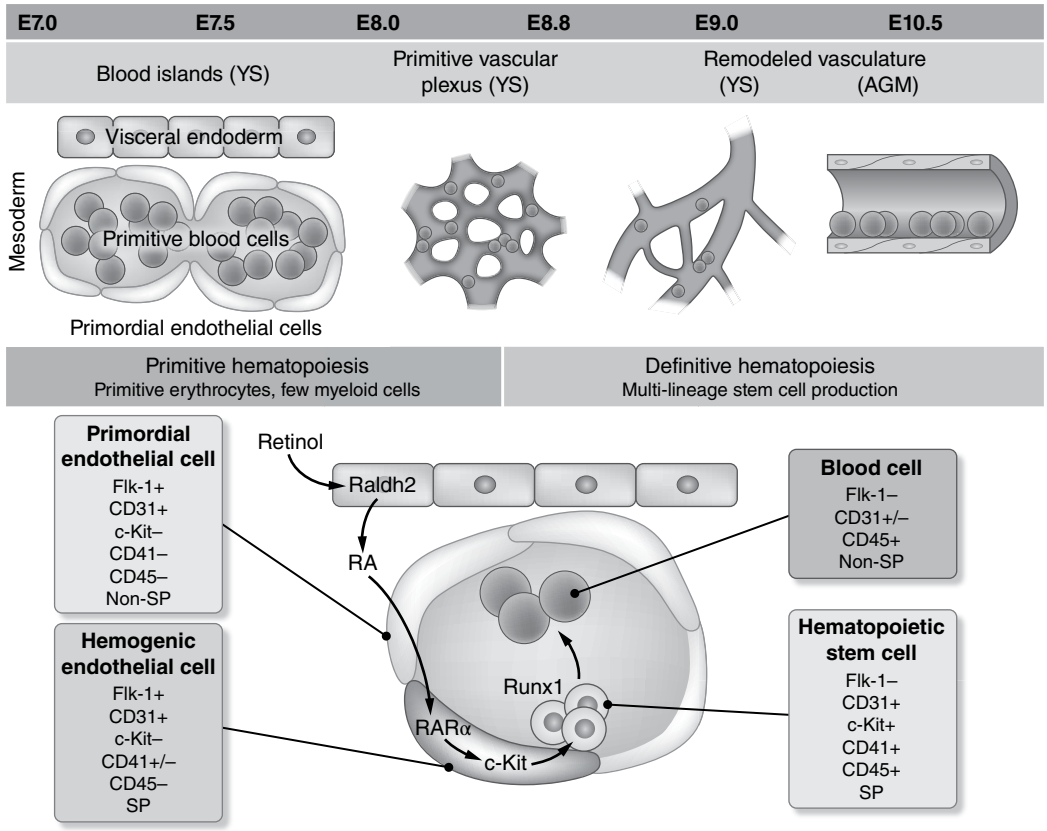

Figure 8.16 The switch from primitive blood island formation to definitive hematopoiesis from central artery endothelium, a process requiring retinoic acid. Surface antigens used to identify the various cell types are shown. (From: Marcelo, K.L., Goldie, L.C. and Hirschi, K.K. (2013) Regulation of endothelial cell differentiation and specification. Circulation Research 112, 1272–1287. Reproduced with the permission of Wolters Kluwer Health, Inc.) RA = retinoic acid, Raldh2 = retinaldehyde dehydrogenase, RARα = retinoic acid receptor, SP = side population.

for regulating gene activity. The mouse knockouts of all three factors die in mid-gestation with hematological defects. The transcription factor RUNX1 (runt domain) is expressed in both primitive and definitive hematopoietic cells, but mouse knockouts are defective only in definitive hematopoiesis, indicating that it is not required for primitive hematopoiesis.

Blood Vessels

Although the primitive blood islands of the yolk sac are surrounded by endothelial cells, the blood vessels of the embryo originate mainly from the splanchnic mesoderm of the lateral plate. Some vessels in the head arise from neural crest mesenchyme and in the heart from the endocardium. The primitive cells from which capillaries are assembled are called angioblasts. They arise from the splanchnic mesoderm in response to a signal from the endoderm, which can be replaced by treatment with FGF2. The angioblasts assemble themselves into a primitive vascular plexus under the influence of vascular endothelial growth factor (VEGF). VEGF-A which is the most active member of this family, binds to a receptor Fetal Liver Kinase-1 (FLK-1, = VEGFR2, KDR), which activates several intracellular transduction pathways. Mice lacking FLK-1 form angioblasts but not

vessels, showing the importance of VEGF signaling for the assembly of vessels.

Soon after their formation it is apparent that the primitive vascular plexi have distinct arterial and venous regions. Arterial capillaries express the ephrin B2 adhesion molecule and venous ones express the complementary Eph B4. Possession of these complementary adhesion molecules enables the arterial and venous capillaries to meet and fuse with each other. High levels of VEGF induce production of the Notch ligand Dll4. The resulting stimulation of Notch induces ephrin B2 and represses EphB4, leading to artery formation. Lower level of VEGF lead to expression of another transcription factor, COUPTFIII, which defines venous development (Figure 8.17). At least in the zebrafish a dorsal-ventral gradient of VEGF arises as a result of SHH signaling from the notochord and floor plate. This determines that the dorsal aorta arises in a dorsal position and has arterial character, whereas the cardinal veins arise more ventrally and have venous character.

Initially capillaries are solid rods of cells. They form a lumen through a symmetry breaking process whereby PAR3 (see Chapter 10) is attracted to the external (basal) side, repelling the protein podocalyxin

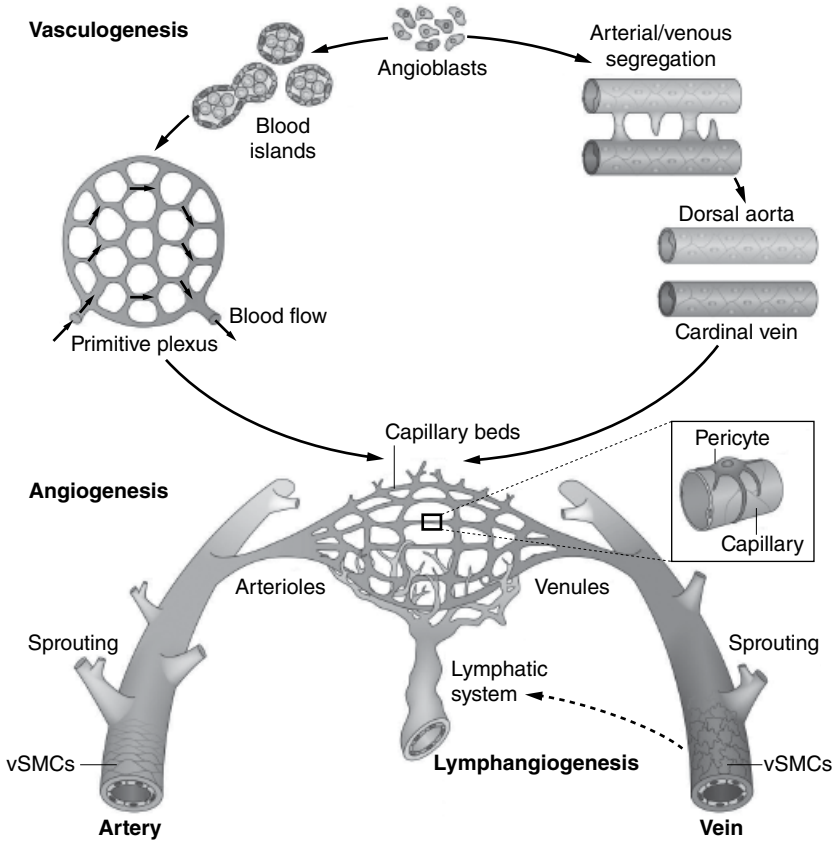

Figure 8.17 Blood vessel development. De novo formation is called vasculogenesis while sprouting from existing vessels is angiogenesis. Complementarity of adhesion molecules between arterial and venous capillaries enables their fusion into capillary beds. The lymphatics originate from the venous system. vSMCs = vascular smooth muscle cells. (From: Herbert, S.P. and Stainier, D.Y.R. (2011) Molecular control of endothelial cell behaviour during blood vessel morphogenesis. Nature Reviews. Molecular Cell Biology 12, 551–564. Reproduced with the permission of the Nature Publishing Group.)

to the inner, apical side. The junctional protein VE-cadherin, specific to endothelia, is shifted basally and the repulsion of podocalyxin molecules opens the lumen internally. Small capillaries remain as simple tubes of endothelium while the larger blood vessels develop outer layers. Pericytes are undifferentiated cells also formed from the splanchnic mesoderm, and recruited to the newly formed vessels, and smooth muscle cells are also recruited to invest the newly formed arteries.

The Heart

The heart arises from mesoderm at the rostral end of the body. Fate mapping experiments on chick embryos show that cells lateral to the node migrate through the primitive streak to form lateral mesoderm territories during gastrulation. These then migrate rostrally on both sides. When the embryo begins to fold, the prospective heart becomes visible as a cardiac crescent at the rostral end of the blastoderm (Figure 8.18). As the head fold forms, the crescent becomes tucked under the head. The actual specification of the heart rudiments occurs just before appearance of the cardiac crescent and depends on inductive signals from the rostral endoderm, especially BMP and FGF8. Evidence that specification occurs at this stage is that extirpation or transplantation of tissue within the crescent leads to subsequent heart defects, whereas this is not the case at earlier stages. The cardiac crescent itself is characterized by expression of several transcription factors including NKX2.5 (homeodomain), GATA4-6 (Zn finger), MEF2c (MADS box) and TBX5 (T box).

The early heart rudiments on each side of the body have the form of tubes. As these become tucked below the head, they fuse into a single midline tube. This early heart tube is the precursor of both atria and the left ventricle. Cells continue to be added at the rostral end to augment the tube and to add territories destined to become the right ventricle and the outflow tract. These cells come from the second heart field: a population lying medial to the cardiac crescent, which is characterized at early stages by expression of ISLET-1 instead of NKX2.5 (Figure 8.18). A third population of cells come from the neural crest. They express the transcription factor TBX1 and contribute to the outflow tract and the septum forming within it that later divides the pulmonary from the aortic circulation. The primitive heart tube has four layers: the endocardium inside, an extracellular layer called the cardiac jelly, the myocardium, which becomes the actual cardiac muscle, and the pericardium which becomes a thin connective tissue layer around the outside.

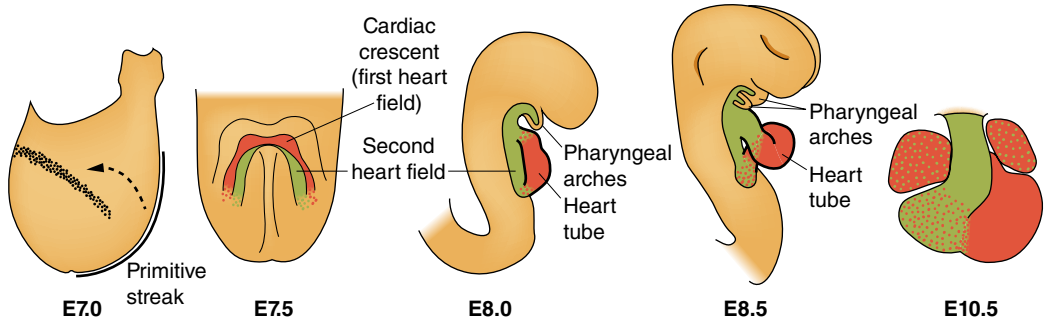

Figure 8.18 Heart development in the mouse embryo. The cardiac crescent, or first heart field, forms mainly the atria and the left ventricle, while the second heart field forms mainly the right ventricle and outflow tract. (Rosenthal and Harvey, 2010. Reproduced with permission of Academic Press.)

The heart commences its physiological functions at an early stage. The primitive heart tube begins autonomous pulsation from E8.5 in the mouse or 4 weeks in human. The heart is asymmetrical because of inherent difference between left and right side dependent ultimately on an asymmetrical expression of Nodal on the left side, which occurs somewhat after the formation of the node itself. Asymmetry initially becomes manifest as a looping of the heart tube to the right side. Different transcription factors are expressed in different territories: the prospective atria preferentially express COUP TFIII and TBX5, the prospective right ventricle dHAND and the prospective left ventricle eHAND. The later development of the heart involves a complex sequence of morphogenetic events that convert the simple tube into the four-chambered mammalian heart. These include the looping, which brings the atria to the rostral side, remodeling, the migration of blood vessel insertion sites on the surface of the heart, and the formation of septa to separate the chambers. Errors in these processes are not uncommon, resulting in a significant number of congenital heart defects in human infants (about 1% of live births). Because of the importance of these, a considerable amount of research has been conducted into the causes, which are, in many cases, mutations in the transcription factors controlling cardiac development.

The Gut

The endoderm becomes the epithelial lining of the gut tube and the respiratory system, plus the various organs that bud off the gut, including the liver and pancreas. The definitive endoderm arises as the bottom layer of the embryo during gastrulation (see Chapter 5), forming a strip of epithelium along the midline, flanked by the extraembryonic visceral endoderm. The definitive endoderm becomes transformed into a tube by the process of body folding (Figure 8.19). It commences as a lifting of the head end of the embryo above the surrounding blastoderm. Since all three germ layers participate, this means that at the rostral end a blind-ended cavity arises lined with definitive endoderm. This is the foregut. The folding continues progressively to more caudal levels bringing more and more of the definitive endoderm into the foregut tube, with less and less of it being open to the yolk sac. At the caudal end, a similar process starts later, resulting in the formation of a hindgut. The body folding continues until the whole embryo has everted from the original blastoderm and the open part of the endoderm is reduced to a small region forming a canal to the yolk sac lumen, called the vitellointestinal duct. This duct, along with the principal blood vessels to the placenta, and the allantois, becomes wrapped up into the umbilical tube.

The formation of the coelomic cavity within the lateral plate mesoderm separates the gut tube from the body wall. The gut tube now consists of a lining of endoderm surrounded by mesenchyme from the splanchnic mesoderm. It is suspended in the coelomic cavity by dorsal and ventral mesenteries, composed of splanchnic mesoderm, of which the dorsal mesentery remains in place permanently. The process of head folding brings the future heart into a midventral position. Between the heart and the vitellointestinal duct is a mass of mesoderm called the septum transversum. This divides the coelomic cavity into thoracic and abdominal regions and later contributes to the diaphragm.

The endoderm of chick and mouse embryos has been fate mapped by applying small marks of DiI to the early endoderm which is the lower layer of the blastoderm, and locating the labeled cells in the gut tube at a later stage. The results are complex but there is an approximate maintenance of rostrocaudal polarity, i.e. the rostral organs of the gut tube arise from the rostral part of the endoderm and vice versa. There is also a very pronounced mismatch between the fate map of the endoderm and its

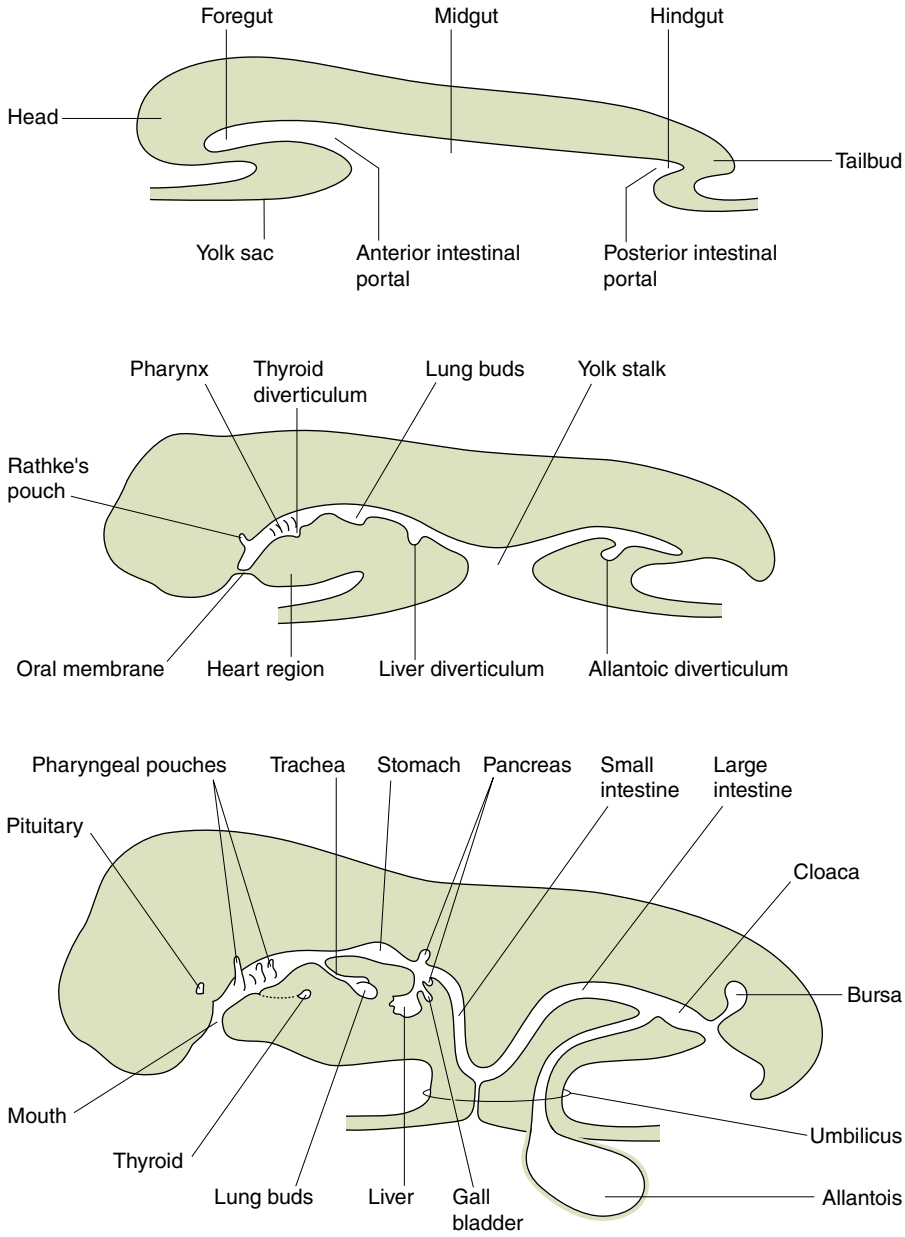

Figure 8.19 Formation of the regions of the gut in a higher vertebrate animal. The bursa is not found in mammals. (Hildebrand, 1995. Reproduced with the permission of John Wiley and Sons.)

associated splanchnic mesoderm. This arises from a progressive displacement between endoderm and mesodermal layers during the formation of the gut tube. This means, contrary to general belief, that the regional pattern of the endoderm cannot be simply "printed" by means of inductive signals from the mesoderm because the mesoderm that ends up as part of, say, the stomach, is initially present at a much more caudal level than the prospective stomach endoderm.

Regional Specification of the Endoderm

The initial formation of the endoderm depends on Nodal signaling, and mouse embryos lacking *Nodal* have no endoderm. Transcription factors important in the early endoderm include SOX17, FOXA1,2, and GATA 4,5,6. The first regional specification occurs during gastrulation and is a response to the caudal to rostral gradients of Wnt and FGF that also pattern the nervous system (Figure 8.20). These signals result in a nested expression of transcription factors, such as FOXA3 caudal to the liver, CDX2 in the prospective intestine, HNF4 in the prospective stomach and intestine. In addition many of the *HOX* genes are expressed, in both endoderm and mesenchyme layers, generally with expression in the caudal region and a boundary of expression at a specific body level. Retinoic acid, from the somitic mesoderm, is required in the foregut region, and the knockout of *Raldh1a* lacks stomach, lungs and dorsal pancreas.

It used to be thought that much of the pattern of the gut was derived from signals from the splanchnic mesenchyme. There are indeed such signals but there is also considerable signaling from the epithelium to the mesenchyme, so the situation tends to be quite complex for each gut region. The mesenchyme itself resolves into four layers, lamina propria, muscularis mucosa, submucosa and smooth muscle. This radial pattern is due to SHH secreted by the early gut tube. In addition to

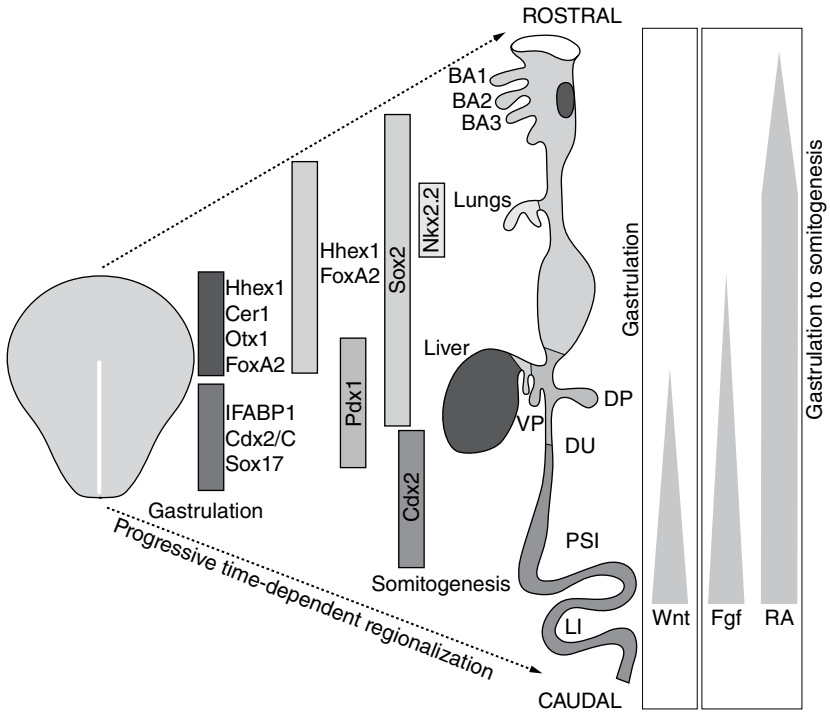

Figure 8.20 Regional specification in the vertebrate gut. Starting from the primitive streak stage the endodermal epithelium becomes subdivided in response to caudal-rostral gradients of Wnt, FGF and retinoic acid. The approximate domains of expression of various transcription factors are shown (IFABP is not a transcription factor but a fatty acid binding protein). BA: branchial arch; DP: dorsal pancreas; DU: duodenum; LI: large intestine; PSI: posterior small intestine; VP: ventral pancreas. (Modified from: Kraus, M.R.C. and Grapin-Botton, A. (2012) Patterning and shaping the endoderm in vivo and in culture. Current Opinion in Genetics and Development 22, 347–353. Reproduced with the permission of Elsevier.)

producing instructive signals for gut patterning, the mesenchyme is also the source of trophic signals such as FGF10, which are needed for growth and morphogenesis of the gut and especially for its outgrowths such as the lung buds, pancreas and cecum.

The Intestine

The mammalian small and large intestine are among the best studied and understood tissue-specific stem cell systems, so it is worth noting how the intestine develops. The mouse gut tube is fully formed by the processes described above by about 9 days of gestation. It is initially lined by a simple epithelium which, over the next 5 days, becomes pseudostratified, then stratified, and then columnar. This change in histology is accompanied by a pronounced elongation of the intestine which, like other cell intercalation processes, requires non-canonical Wnt signaling initiated by Wnt5A. Comparable events in human development occur from about 4 to 9 weeks post-fertilization.

After the epithelium has become columnar, mesenchyme invades to form nascent villi in a rostral to caudal sequence (Figure 8.21). Crypts initially form from cavities in the epithelium. Hedgehog signaling is important for the early establishment of crypts and villi. SHH and Indian Hedgehog (IHH) are both expressed in the early intestinal epithelium, but have opposite effects: loss of SHH causes villous overgrowth while loss of IHH causes formation of fewer, smaller, villi. BMPs are expressed in the mesenchyme invading the villi. Deletion of a BMP receptor, BMPR1A, from the epithelium, causes formation of ectopic crypts, suggesting that BMP signaling promotes villus rather than crypt formation. The transcription factor HNF4α, expressed in early stomach and intestinal epithelium, is needed for intestinal development; knockout in the embryo preventing the crypt-villus pattern from developing at all. The crypt-villus pattern is normally established in mice by E18.5, and in humans by 12–13 weeks post-fertilization. One issue that remains unclear is the precise role of canonical Wnt signaling in the formation of the crypts. This is of critical importance in maintaining the stem cells and proliferative behavior of postnatal crypts (see Chapter 10). However β-catenin activity only appears in the epithelium after villus

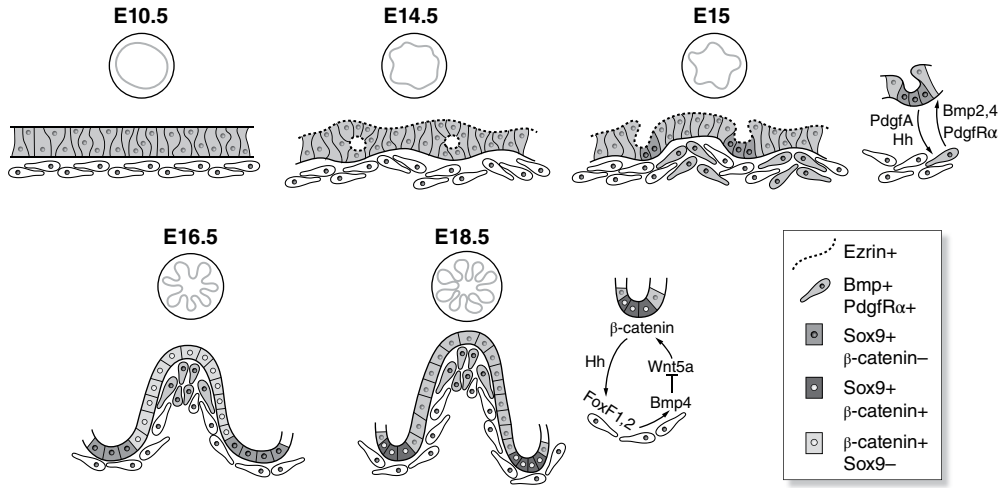

Figure 8.21 Development of crypts in the duodenum of the mouse embryo. Some of the cell interactions are shown. Ezrin (=villin 2) is required for the formation of microvilli on the absorptive cells. (From: Spence, J.R., Lauf, R. and Shroyer, N.F. (2011) Vertebrate intestinal endoderm development. Developmental Dynamics 240, 501–520. Reproduced with the permission of John Wiley and Sons.)

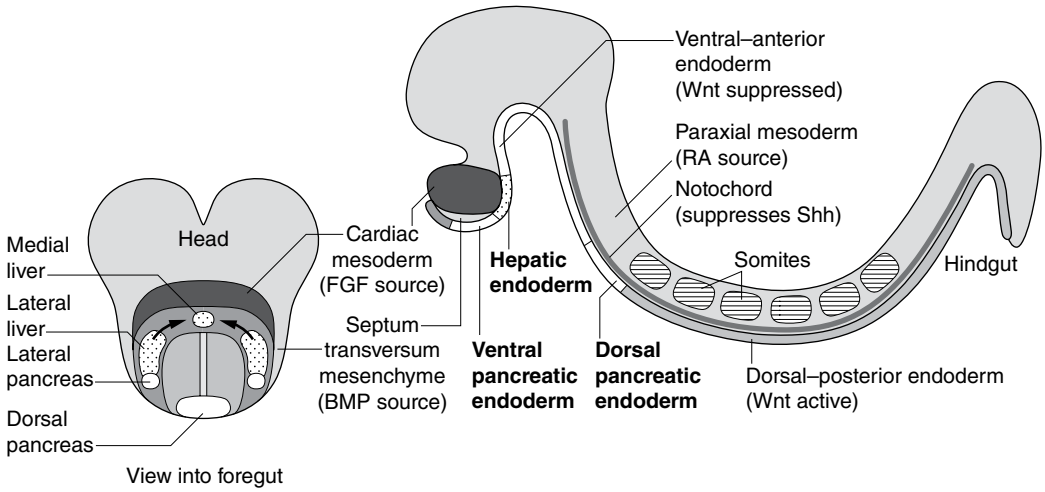

Figure 8.22 Specification of mouse embryo liver and pancreas. On the left is shown a view into the foregut of a mouse embryo at E8.25. The territories indicated are not yet specified. Arrows indicate movement of lateral regions toward the ventral-medial region. On the right is a sagittal view of a later embryo showing the positions of the newly specified liver and pancreas tissue domains. Signals and cell sources that pattern the endoderm are shown.

emergence. By birth, Wnt signaling, and cell proliferation, is confined to the intervillus regions.

The Pancreas

The pancreas derives from two buds which arise in different ways (Figure 8.22). The dorsal pancreatic bud comes from the dorsal midline of the epithelium. It arises at about E9.5 in the region where the notochord contacts the epithelium, and suppresses SHH production locally. This effect can be mimicked by treatment with activin or FGF. The ventral pancreatic bud arises about 2 days later. Its formation also involves suppression of SHH, although here this is not due to the notochord but to something else. Retinoic acid is needed for formation of both the pancreatic buds. Once formed, the buds grow rapidly, stimulated by FGF10 from the mesenchyme, and generate branched structures. The two buds then fuse together to form a single organ, and the duct of the ventral bud becomes the main pancreatic duct.

The best known pancreatic transcription factor is the LIM-homeodomain factor PDX1. This is expressed in a ring around the duodenum encompassing the territories of the two buds. Its knockout does form rudimentary buds but they grow slowly and do not mature. The transcription factor HB9 is expressed a little earlier and is dependent on retinoic acid. The *Hb9* knockout lacks the dorsal bud but the ventral bud is still present. The factor PTF1 has three subunits, of which p48 is needed for formation of the ventral bud and the differentiation of exocrine tissue in the dorsal bud. The terminal differentiation of the exocrine and endocrine cells in the pancreas will be described in Chapter 9.

The Liver

The liver arises as a ventral diverticulum of the foregut endoderm (Figure 8.19). The cells separate from the epithelium and grow as individual cells into the region of mesenchyme called the septum transversum. This is a source of BMP and the adjacent cardiac mesoderm is a source of FGF. Both these factors are needed for the formation of the liver from the endoderm and for the

suppression of formation of the ventral pancreas. The early cells of the liver are hepatoblasts: bipotent cells which can form either hepatocytes or biliary epithelial cells. Important transcription factors active in the early liver are C/EBPα, HNF1α and β, HNF4α, HNF6, and PXR.

Just between the liver and the ventral pancreas lies another bud which forms the extrahepatic biliary system: comprising the gall bladder, cystic duct, and extrahepatic bile ducts. This bud requires persistent expression of SOX17, which is mutually antagonistic to PDX1, ensuring that the ventral pancreatic and biliary buds remain distinct from one another. Expression of SOX17 is maintained by the bHLH factor HES1. Knockout of *Hes1* causes loss of SOX17 and persistence of PDX1, leading to the formation of ectopic pancreas in the biliary system.

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9

Cell Differentiation and Growth

The previous chapters have covered the account of mammalian development from the generation of gametes to the formation of organ rudiments. Developmental biology textbooks often stop at this point, but here we shall continue and look at some selected examples of how organ rudiments become the functional tissues and organs of the body. All tissue types grow and expand during development, but most of those dealt with here do not have real stem cells which persist through adult life. Several bona fide stem cell systems are dealt with in Chapter 10, but in the present chapter the only tissue types which have stem cells are skeletal muscle and the central nervous system. Those of skeletal muscle are a population of muscle satellite cells that can become new myofibers. In the postnatal mammalian brain most of the neurons cannot be renewed, but there are some small stem cell populations in specific areas. Otherwise there are no true stem cells although some tissue types maintain slow cell turnover by division of their functional differentiated cells.

Organs, Tissues and Cell Types

The three concepts of "organ", "tissue" and "cell type" are often confused, as when speaking of "muscle" without specifying whether what is meant is the whole anatomical muscle or just the multinucleated myofibers within a muscle. An actual muscle contains many other tissues and cell types, including connective tissue, blood vessels, nerves and macrophages. Gene expression studies are often conducted on pieces of whole organ despite the fact that these contain multiple tissues and cell types each with vastly different gene expression repertoires. This means that the gene expression patterns of organs are dominated by the most abundant mRNAs from the most abundant cell types in the sample. Worse, these data are then used for various purposes by theoreticians who may not appreciate the limitations of the information arising from the complexity of the initial samples. So it is really worthwhile to be clear about what is meant by organ, tissue or cell type in specific situations.

An organ is a named part of the body familiar from gross anatomy. The stomach, the kidney, or the lungs are all organs. The skin is also an organ although it has a less discrete character. Organs have an identifiable physiological function and always consist of several tissue types which in turn usually contain multiple cell types.

There is no clear definition of a "tissue" in the histological literature, but a tissue may usefully be regarded as the set of cell types originating from a single type of stem cell (or embryonic progenitor cell if the tissue in question does not have stem cells). Under this definition the small intestinal epithelium is a tissue. It is composed of multiple cell types, but they all come from one stem cell

The Science of Stem Cells, First Edition. Jonathan M. W. Slack.

Companion website: www.wiley.com/go/slack/thescienceofstemcells

population. The small intestine as an organ comprises also the connective tissue layers, the blood vessels, the lymphatics, the nerve supply, and some patches of lymphoid tissue. In the liver the hepatocytes and the biliary system comprise one tissue, as they arise in late embryonic development from one type of progenitor: the hepatoblast. The liver as an organ also contains an abundant vascular system and numerous cells of different lineages: the Kupffer cells and hepatic stellate cells. Histology textbooks often classify tissues into five general types: epithelia, connective tissues, nervous tissues, muscle and blood. The last three are dealt with later, in this chapter and in Chapter 10, but some preliminary remarks on the first two are appropriate here.

Epithelia

Epithelia (singular: epithelium) are sheets of cells which may consist of one or many layers (simple or stratified). The cells may be flat (squamous), cuboidal or columnar (Figure 9.1a).

The cells of a simple epithelium have an apical-basal polarity. The apical surface abuts the lumen of the structure formed by the epithelium and often bears specializations such as cilia or microvilli. The basal side abuts a basement membrane (Figure 9.1b). Part of this, the basal lamina, is secreted by the epithelium itself and consists of the extracellular proteins laminin, type IV collagen, entactin and heparan sulfate proteoglycan. This is usually underlain by collagen fibers secreted by the adjacent connective tissue, the whole making up the basement membrane. The lateral surfaces of epithelial cells are attached to each other by means of junctional complexes. The tight junctions form an impermeable barrier, isolating the luminal from the basal side of the tissue. The adherens junctions and the desmosomes bind the cells together via calcium-dependent adhesion molecules called cadherins. The gap junctions, formed from proteins called connexins which contain small pores, enable transfer of small molecules between adjacent cells.

Many organs in the body have an epithelium as their principal component. This is obviously the case for those organs forming the gut (esophagus, stomach, small and large intestine) where the respective epithelia line the luminal surfaces, but is also true of many solid organs including the liver, the kidneys and the salivary glands. Many organs are glands with a secretory function. Glands often originate from a region of an epithelial sheet which invaginates into the surroundings. If it persists, the duct of the later gland shows the original position of this invagination. In the case of endocrine glands, which secrete their products into the bloodstream, the duct disappears in the course of development.

The term "epithelium" is a descriptive one and does not imply anything about embryonic origin. Epithelia may originate from any of the three embryonic germ layers, and from numerous different positions within them. Epithelia derived from the mesoderm are sometimes referred to as "endothelia" or "mesothelia".

Connective Tissues

The term "connective tissue" is used in two very different senses. In its wider usage it refers to all skeletal tissues: bone, cartilage, tendons, ligaments, and also adipose tissue. In its narrower usage it refers just to the fibrous tissue that fills the spaces between other structures. In the latter sense, connective tissue consists of individual cells called fibroblasts, embedded in a loose extracellular matrix. This consists of proteoglycans, hyaluronan, fibronectin, type I collagen, type III collagen (= reticulin) and elastin, which are secreted by the fibroblasts themselves (Figure 9.1c). Connective tissues are often considered to derive from the mesoderm of the embryo, but in fact most of the skeleton and loose connective tissue of the head is derived from the neural crest. In the embryo the tissue filling up the gaps between other structures is called "mesenchyme" (Figure 9.1d). Again, this is a descriptive term

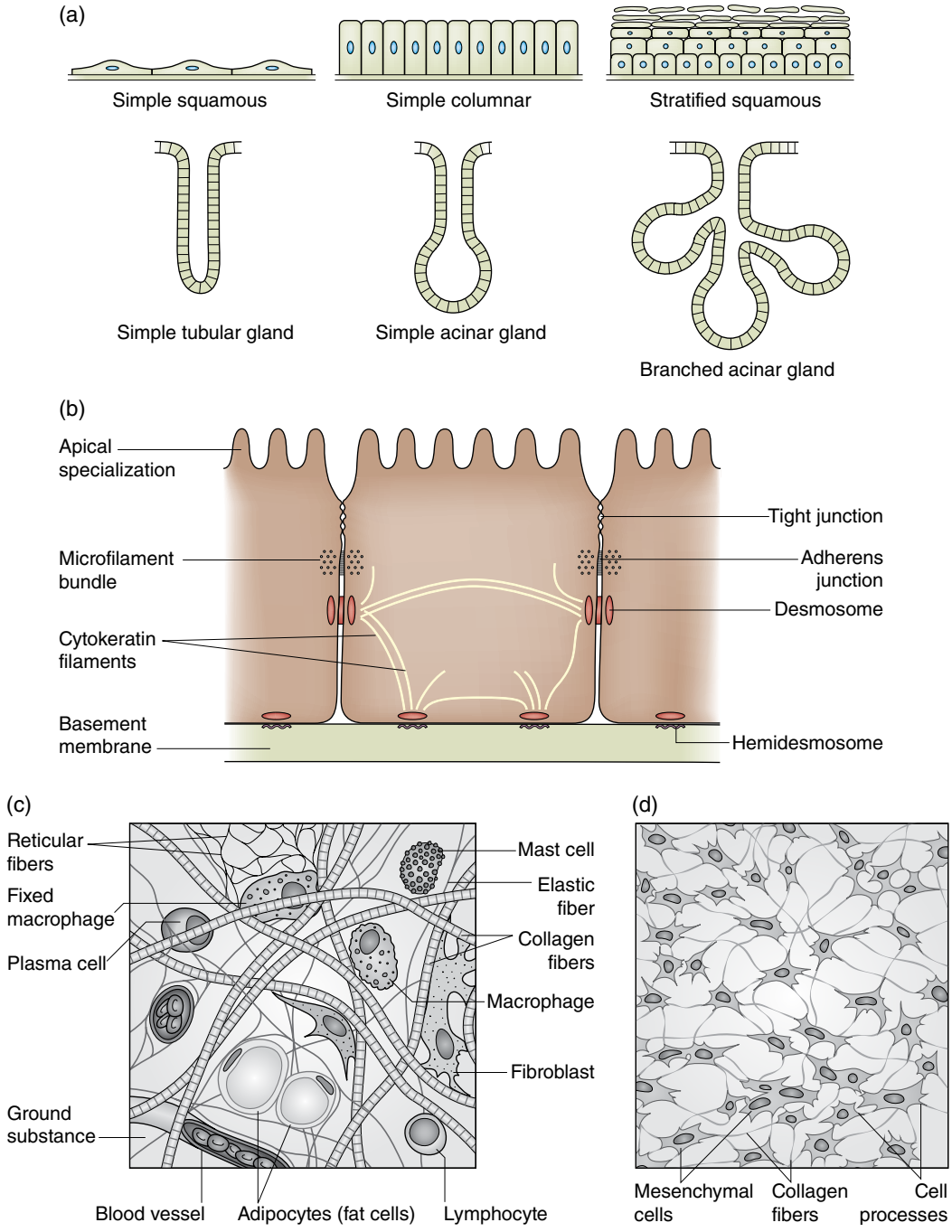

Figure 9.1 (a) Various types of epithelium. (b) Structure of a typical epithelium. (a & b from Slack, J.M.W. (2013) Essential Developmental Biology, 3rd edn. Reproduced with the permission of John Wiley and Sons.) (c) Typical mature connective tissue. (Modified from http://www.mhhe.com/biosci/ap/histology_mh/loosctfs.html.) (d) Loose mesenchyme as found in embryos. (Modified from http://www.mhhe.com/biosci/ap/histology_mh/loosctfs.html.)

and does not designate a specific embryonic origin. Mesenchymal cells have an irregular stellate appearance and their extracellular matrix consists largely of hyaluronic acid and glycosaminoglycans. Although of similar appearance, different regions of the mesenchyme have different developmental commitments, forming the various skeletal structures and the masses of adipose tissue as well as the loose connective tissue of the postnatal organism. Another related term is "stroma". This refers to the non-epithelial part of an organ or a tumor, much of which consists of connective tissue. It is used for adult rather than embryonic tissues.

Cell Differentiation

Regulation of Gene Activity

Different cell types express different repertoires of genes so regulation of gene expression is obviously central to their nature. Regulation of gene expression is mostly exerted at the stage of transcription of DNA to messenger RNA, but there are also some translational controls, exerted by regulatory proteins that bind to mRNAs, and by micro RNAs that impede translation or destabilize mRNA.

Key to the control of transcription are the transcription factors, which are proteins that control the activity of other specific genes. They usually have two important parts: an effector region and a DNA-binding region (Figure 9.2a). The effector region is often rich in acidic amino acids and activates transcription by interacting with the general transcription complexes present in all cells. The DNA-binding region determines the specificity of the transcription factor by binding to specific sequences in the regulatory regions of the target genes. For example, T-box transcription factors like BRACHYURY bind to the sequence TCACACCT in the DNA. The regulatory sequences may be next to the binding site for the general transcription complex, in the region known as the promoter,

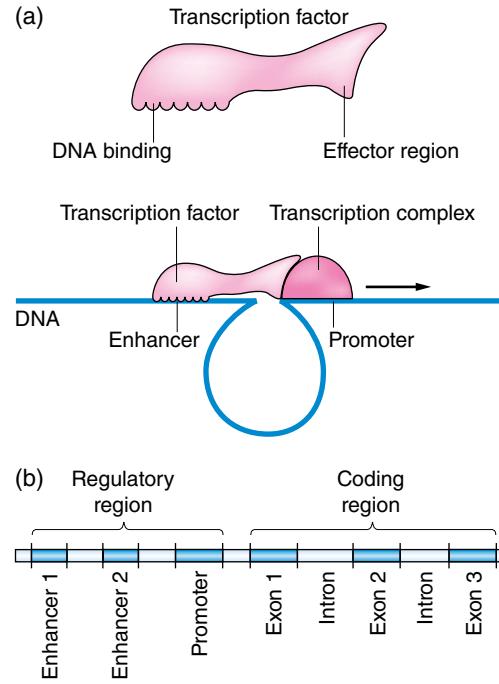

Figure 9.2 (a) Schematic view of a transcription factor binding to an enhancer sequence. (b) Typical gene structure showing multiple regulatory regions. (Slack, J.M.W. (2013) Essential Developmental Biology, 3rd edn. Reproduced with the permission of John Wiley and Sons.)

or they may be distant, then being called enhancers. Genes involved in development tend to have rather complex regulatory regions with a number of binding sites for transcription factors, and specific combinations of factor may be necessary to activate the transcription complex (Figure 9.2b). Because the presence of a specific repertoire of transcription factors in a cell determines the pattern of gene expression, the genes whose activity defines states of developmental commitment, discussed in Chapter 7, mostly encode transcription factors.

In addition there is an important level of gene regulation exerted by the chromatin (Figure 9.3a). Chromatin comprises the genetic DNA plus the many proteins that control its structure and accessibility to regulatory factors. The most important

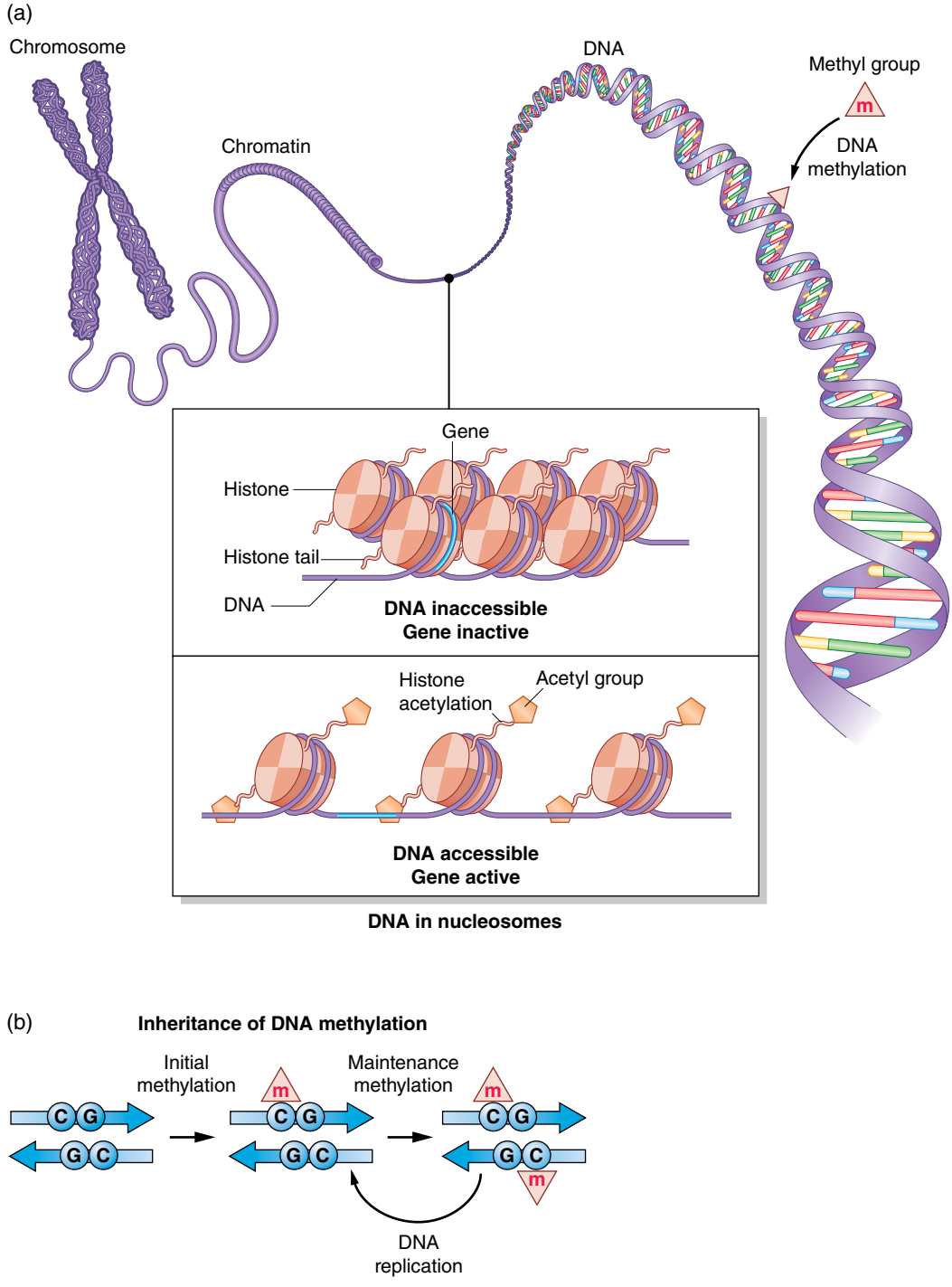

Figure 9.3 (a) Chromatin, showing opening of the structure by histone acetylation. (New drawing based on those by National Institutes of Health.) (b) How DNA methylation patterns are inherited through DNA replication and cell division. (Slack, J.M.W. (2013) Essential Developmental Biology, 3rd edn. Reproduced with the permission of John Wiley and Sons.)

chromosomal proteins are the histones, which are basic proteins highly conserved across all eukaryotic organisms, but there are also many other non-histone chromosomal proteins with critical roles. Most of the DNA is incorporated into structures called nucleosomes, each containing eight histone molecules. The accessibility of the DNA to transcription factors is obviously important for their action and to facilitate it the nucleosomes are in a state of dynamic equilibrium, being continually assembled and disassembled. Opening of the chromatin structure to make the DNA accessible is promoted by the acetylation of lysine groups in the histones, which reduces the number of positive charges displayed and hence weakens the association of the histones with the negatively charged phosphate groups of the DNA. Processes of gene upregulation usually involve the action of histone acetylases to open up the chromatin region concerned. Conversely, the methylation of DNA recruits histone deacetylases which reduce histone acetylation and hence accessibility of the gene. This is why DNA methylation is often associated with repression of gene activity.

DNA methylation is found at the 5-position of cytosine residues in GC sequences. The CG dimer is generally underrepresented in the mammalian genome but higher levels are found in about 40,000 "CG islands" which are generally undermethylated and are often found in the vicinity of gene promoters. Methylation of CG cytosines is achieved by DNA methyl transferase enzymes of which there are several types. Some of them are de novo methylases that can insert methyl groups on a previously unmodified CG. Others are maintenance methylases, that methylate the CG which is paired in the double-stranded DNA molecule with an already methylated CG. DNA methylation offers a very simple mechanism for the inheritance of states of cellular commitment through DNA replication and cell division. This is because when a methylated site is replicated, it becomes a hemimethylated site and is thereby a substrate for the maintenance methylase (Figure 9.3b). So long as maintenance methylase is present, the hemimethylated site becomes fully methylated, with a methyl group on the C of the CGs on both DNA strands. Through this mechanism a methylated CG will remain methylated however many times the cell divides. In the absence of maintenance methylase, passive DNA demethylation will occur to the extent of 50% in each replication cycle. In addition to the process of methylation maintenance, some specific de novo methylation and demethylation events also occur, as with the setting of the sex-specific imprints in developing germ cells (see Chapter 5).

Another class of chromatin modification important in the control of gene expression is the methylation of histone molecules. This occurs on the N-terminal "tails" of the histones exposed on the surface of nucleosomes. Lysines or arginines can be methylated and the modifications are associated either with boosting gene activity (e.g. histone 3 lysine 4 trimethylation), or inhibiting it (e.g. histone 3 lysine 9 dimethylation). The effects of histone methylation are exerted by the recruitment of other proteins that stimulate or inhibit transcription.

There is evidence that the states of histone acetylation and methylation can also be propagated through cell division, although the mechanism is not so simple as that of DNA methylation. Histones remain associated with the daughter DNA strands after replication and it may be that pre-existing modifications can be repeated on the newly inserted histones in the presence of the relevant enzymes.

Because the state of the chromatin can be maintained through DNA replication and cell division, both in terms of DNA methylation and probably also in terms of histone acetylation and methylation patterns, we can regard the developmental history of cells in the adult as being recorded in their chromatin. Erasure of such chromatin states may be achieved by the methods for inducing pluripotency, namely somatic cell nuclear transfer into an oocyte, or overexpression of specific

transcription factors to generate iPS cells. The low efficiency of these procedures show that complete reprogramming is difficult, and, in fact, despite their pluripotent behavior, many iPS cell lines still preserve partial chromatin signatures of their original parent cells and show a preferential tendency to re-differentiate in this direction.

At a macroscopic level, active regions of chromatin are called euchromatin and inactive regions heterochromatin. Heterochromatin is tightly condensed, making access to DNA difficult for transcription factors. The regions around the centromeres and telomeres of chromosomes are heterochromatic, as is the entire X-chromosome, which becomes inactivated in female cells as discussed in Chapter 5.

Lateral Inhibition

One of the most important generic processes of developmental biology is lateral inhibition (Figure 9.4a). This is important in all cases where one type of cell, A, differentiates from a background of another type, B, to generate a scattered population of A embedded in a sheet of B. Lateral inhibition occurs during neuronal development, the formation of secretory cells in the intestine, the formation of endocrine cells in the pancreas, and many other examples. The starting situation is a sheet of cells that are all the same. Because of the small number of molecules of certain types present in a cell, not least the genes themselves, there are always small random fluctuations between apparently identical cells in terms of the levels of gene activity or the amounts of particular gene products present. Imagine that one substance, conventionally called the "activator", stimulates its own production, and also stimulates the production of another substance called the "inhibitor". The inhibitor in turn inhibits activator production. Imagine further that the inhibitor is freely diffusible between cells while the activator is confined to the cell of production. A sight random increase in activator level in one cell will lead to an excess production of both activator and inhibitor in that cell. But the inhibitor level soon falls by diffusion, leading to a situation where the activator exceeds the inhibitor in that cell while, because of its diffusion, the inhibitor exceeds the activator in the surrounding cells. This means that the production of

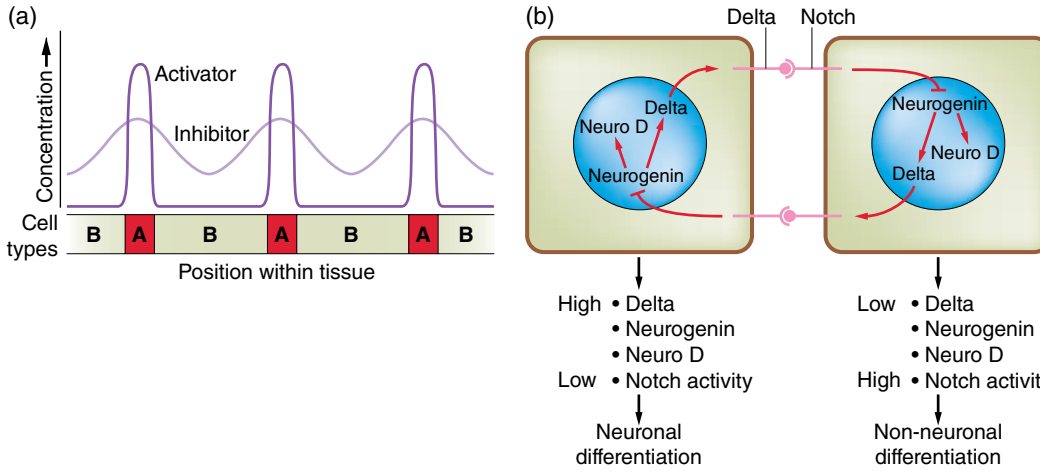

Figure 9.4 (a) The principle of lateral inhibition. The activator promotes synthesis of itself and of the inhibitor. The inhibitor inhibits production of the activator, and is diffusible. (b) Lateral inhibition via the Notch system. (Slack, J.M.W. (2013) Essential Developmental Biology, 3rd edn. Reproduced with the permission of John Wiley and Sons.)

activator and inhibitor will further increase in the original cell, while the production of activator (and consequently inhibitor) is suppressed in the surrounding cells. After a while the situation will reach a steady state where the original cell has high activator and inhibitor and is exporting inhibitor to the surroundings. The surrounding cells have low activator and although they produce little inhibitor themselves, the inhibitor continues to be supplied by diffusion. It only remains to suppose that the activator can regulate specific gene activity to explain the formation of one specific cell type, type A, embedded in a background of another, type B.

In the 1990s it was found that this model did operate to control the differentiation of neurons from the embryonic neuroepithelium. The molecular basis for lateral inhibition is formed by the Notch signaling system. As described in Chapter 7, Notch is a cell surface receptor. Its ligands are also cell surface molecules, called Delta and Jagged. When Notch binds to its ligand, it becomes cleaved by an intramembranous protease to generate a free intracellular domain (NICD). This then combines with transcription factors of the CSL (=Su(H)) class, enters the nucleus, and regulates specific genes. The Notch system resembles the theoretical model except that the components are not freely diffusible but are tethered to the cell surface, so the range of the system is limited by the range of cell contacts. The activator is a transcription factor, in the case of neurogenesis it is NeuroD, a bHLH factor promoting neuronal differentiation. The formation of NeuroD (the activator), and Delta or Jagged (the inhibitor), is repressed by Notch signaling. So the way the system works is that a slight random increase in NeuroD leads to more Delta, this stimulates Notch on adjacent cells and represses formation of NeuroD and Delta in those cells. Eventually scattered cells will express high levels of NeuroD and Delta and the cells in between will express little or none but experience high levels of intracellular Notch signaling (Figure 9.4b). The high NeuroD cells will then become neurons while the others remain as undifferentiated neuroepithelium.

Evidence for this system was first found in *Drosophila* where the loss of function mutation of *Notch* gave 100% neuron formation from the ventral neurogenic region of the embryo. Experiments on *Xenopus* later showed that primary neurogenesis from the neural plate was stimulated by inhibition of Notch and reduced by overexpression of Notch ligands. It has also been found in mice that neurogenesis from the telencephalon is reduced by stimulation of Notch signaling, and the proportion of undifferentiated subventricular cells is increased.

Subsequently it has been found that the formation of secretory cells in the intestine and of endocrine cells in the pancreas occurs by a very similar mechanism. The key transcription factors are ATOH1 (= MATH1) in the intestine and Neurogenin 3 in the pancreas. Some aspects of the Notch system and its role in lateral inhibition remain uncertain. One issue is whether the initial perturbations are really random, or depend on the intrinsic oscillatory character of the system as displayed during somitogenesis (see Chapter 8). Another issue is the range of the Notch ligands, which may be increased through the ramification of fine cellular processes to reach non-neighboring cells.

Asymmetrical Cell Division

Stem cells do not have to divide in an asymmetrical manner but they often do so. The best understood example of asymmetric cell division is not of a stem cell but of the early stages of the nematode worm *Caenorhadbitis elegans*. This organism is one of a small number of "model organisms" commonly used in developmental biology. It is characterized by an invariant sequence of unequal cell divisions in the early stages of development. The mechanism underlying the process was discovered by isolating maternal effect mutations which abolished the asymmetry. The mutations were known as "partitioning defective", and the genes as *par* genes.

The gene products act as two complexes. They are initially uniformly distributed but following fertilization they sort themselves to the anterior and posterior parts of the egg respectively (Figure 9.C.1). The anterior complex is formed from PAR-3 and -6, which are PDZ type proteins, and PKC-3 (= aPKC), an atypical protein kinase C. The posterior complex consists of PAR-1, a kinase, and PAR-2, a RING finger type protein. The complexes diffuse freely in the cell cortex, the region immediately underlying the plasma membrane. However they are antagonistic to each other. PKC-3 phosphorylates PAR-1 and -2, causing them to dissociate from the cortex and thereby reduces their presence in the anterior. Likewise, PAR-1 phosphorylates PAR-3, causing it to dissociate from the cortex and reducing its presence in the posterior. Evidence for the mutual antagonism is found in the behavior of mutants: when either complex is reduced in activity the other expands its domain.

The establishment of polarity in the zygote depends on the position of sperm entry. Initially the PAR-3/6/PKC-3 complex is present all over the cortex while the PAR-1/2 complex is present in the cytoplasm. The sperm introduces a centrosome which establishes a radiating set of microtubules and also initiates a flow of actomyosin away from the point of sperm entry. Both these events help establish the polarization. PAR-2 binds to the microtubules which inhibits its phosphorylation by PKC-3 and enables it to reach the nearby cortex, recruit PAR-1, and eject PAR-3 by phosphorylation. Thus the point of sperm entry becomes the posterior and the opposite side becomes the anterior of the fertilized egg.

How does this cell polarity affect subsequent differentiation? It is because the PAR complexes are associated with determinants and control their localization with respect to the cell division planes (Figure 9.C.1). A determinant is any intracellular substance that can affect the subsequent pathway of differentiation. In early embryos determinants are often maternal mRNAs encoding key transcription factors, but they may also be proteins or miRNAs that can affect gene expression in some less direct way. There are many determinants in *C. elegans* embryos, and as an example consider MEX-5. This is an RNA binding protein which regulates translation of specific mRNAs. Its distribution depends on phosphorylation by PAR-1, and on dephosphorylation by a uniformly distributed phosphatase, PP2A. MEX-5 mobility is increased by phosphorylation and since it is phosphorylated by PAR-1 in the posterior it accumulates in the anterior where it is less mobile. The action of MEX-5 is to repel various components which then end up in the posterior. These include PIE-1, which represses transcription in the early germline; and P granules, which are droplets containing many proteins and mRNAs involved with germ cell formation. The end result is a zygote which contains determinants for somatic development in the anterior and for germline development in the posterior, and when cleavage occurs into an anterior AB cell and a posterior P_1 cell, the two different lineages are established (Figure 9.C.1f).

Does the lesson of *C. elegans* tell us anything about mammalian stem cells? In principle it shows that localization can arise from a symmetry breaking process following a minor asymmetric perturbation, in this case sperm entry. In terms of actual molecular components there is some evidence for the involvement of PAR homologs in asymmetric cell divisions of vertebrates. For example PAR-3 is expressed in the radial glia (neural stem cells) of the zebrafish embryo forebrain. It is localized in an apical (ventricular) position and sequesters an E3 ubiquitin ligase called Mindbomb (Figure 9.5). Mindbomb increases the activity of Delta-like ligands stimulating the Notch pathway. On cell division, the apical daughter cell displays more Delta than the basal one, and so stimulates more Notch signaling in the basal daughter. This causes the basal daughter to persist as a self-renewing stem cell while the apical daughter becomes a neuron. The evidence of the mechanism is that clones of cells without PAR-3 mostly

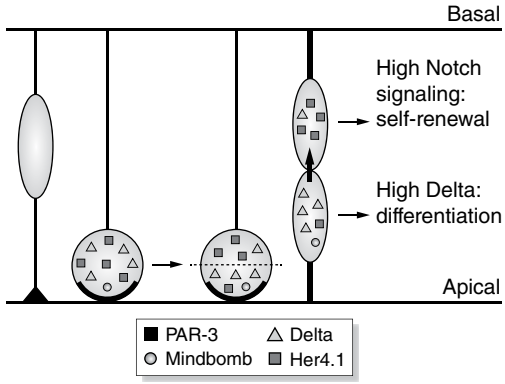

Figure 9.5 Involvement of the PAR system in the unequal division of radial glia cell in the developing zebrafish brain. PAR-3 sequesters Mindbomb on the apical side and this generates an asymmetry of Notch signaling between apical and basal daughters. (Modified from Dong, Z., Yang, N., Yeo, S.-Y., Chitnis, A. and Guo, S. (2012) Intralineage directional notch signaling regulates self-renewal and differentiation of asymmetrically dividing radial glia. Neuron 74, 65–78. Reproduced with the permission of Elsevier.)

divide symmetrically, and that loss of function mutants of *mindbomb* have an early excess of neuron formation. This example nicely brings together the effects of the Notch system leading to lateral inhibition and of the PAR system leading to cellular polarization and unequal division.

Neurogenesis and Gliogenesis

Neurons and Glia

Neurons have a cell body, a dendritic tree and an axon (Figure 9.6). They are electrically excitable and typically connect with many other neurons via their axon, which makes synapses with the dendrites or cell bodies of other neurons. The cell body, or soma, is responsible for most of the protein synthesis, with the products being moved up and down the axon by anterograde and retrograde transport mechanisms. Cell bodies are rich in ribosomes and in rough endoplasmic reticulum (called Nissl bodies in neurons). Neurons contain a special sort of intermediate filament called neurofilaments, composed of class IV intermediate filament proteins. The axons may be myelinated, which increases the conduction rate of action potentials, or unmyelinated. Myelin consists of multiple layers of plasma membrane wrapped around the axon by an oligodendrocyte (in the CNS) or a Schwann cells (in the PNS). The principal types of neuron are the motor neurons, which have a long unbranched axon, the sensory neurons, which have a branched axon, and the interneurons, which have a short axon. However each of these comprise many subtypes, both in terms of morphology, the neurotransmitter deployed, and their gene expression. Neurons typically maintain a membrane potential of about 70–80 mV (negative inside). This arises from the action of ion pumps moving Na, K and Cl ions, together with unequal back-diffusion through ion channels. Excitatory signals received from other neurons via synapses lead to a drop in the magnitude of the membrane potential and, through opening of voltage sensitive ion channels, this initiates a major depolarization or reverse polarization of the cell which propagates along the axon. This is called an action potential and may excite other neurons that are contacted by synapses. There are also inhibitory signals which lead to an increase in membrane potential and make the neuron more refractory to initiation of an action potential. Neurons normally release just one neurotransmitter at their synapses, and the following substances are commonly found as neurotransmitters in the CNS: glutamate (excitatory), γ-amino butyric acid (GABA, inhibitory), acetyl choline (ACh), dopamine and serotonin (5-hydroxytryptamine). The firing patterns of different neuron types are very diverse.

A few neuron types of particular interest in stem cell biology are as follows. Spinal motorneurons are found in the ventral horns of the spinal cord innervating muscles and glands. They secrete acetyl choline at their synapses. α-motorneurons connect to normal muscle fibers and β and

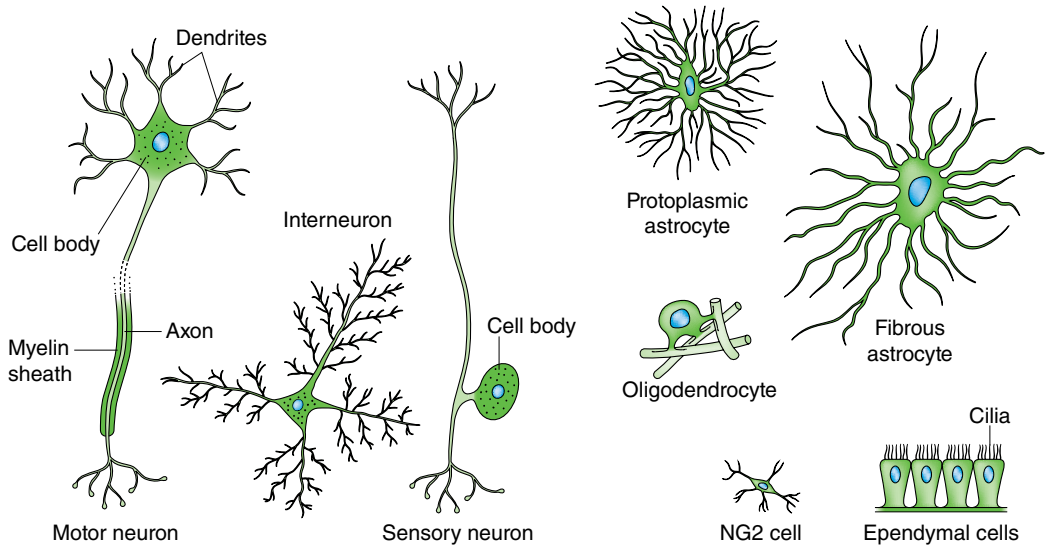

Figure 9.6 Cell types of the central nervous system, neurons are shown on the left and glia on the right. Microglia are not shown as they are not really glia but immune cells of hematopoietic origin. (Slack, J.M.W. (2013) Essential Developmental Biology, 3rd edn. Reproduced with the permission of John Wiley and Sons.)

γ-motorneurons to the intrafusal stretch sensitive fibers. Sometimes the cortical neurons connecting to the spinal motorneurons are also classed as motorneurons. These are pyramidal cells in the motor cortex. Both types of motorneuron, cortical and spinal, may be lost in amyotrophic lateral sclerosis which is a progressive and fatal disorder. Dopaminergic neurons make up 3–5% of the substantia nigra (Latin for "black substance") and project to the basal ganglia and cortex. These cells may have a dark color due to neuromelanin, a by-product of dopamine synthesis, which gives the substantia nigra its name. They express the transcription factors LMX1b, PITX3 and NURR1, which control genes required for dopamine synthesis. Dopaminergic neurons are lost in Parkinson's disease, and their replacement is an important goal of stem cell research. Medium spiny neurons are small cells making up a high proportion of neurons in the striatum. They have a large dendritic tree and contain receptors for glutamate and aspartate. They are GABAergic and innervate cells in the thalamus and elsewhere. These are the first cells to be lost in Huntingdon's disease.

About 50% of cells in the human brain are not neurons but glia (Figure 9.6). Astrocytes provide structural support, control water and ion balance, and can modulate signaling between neurons. There are two main types. The fibrous astrocytes are star shaped with long unbranched processes and mostly located in the white matter. They contain many glial filaments made up of glial fibrillary acidic protein (GFAP), and express aldehyde dehydrogenase 1 L1 (ALDH1L1) among other markers. Fibrous astrocytes can proliferate on injury and form glial scars in the CNS. Protoplasmic astrocytes are of mossy appearance with many short and highly branched processes, and are mostly found in gray matter. They express the complex carbohydrate antigen A2B5. In rodents, the astrocytes are generated in late fetal development and grow their processes in postnatal weeks 1–4.

Oligodendrocytes are the cells which produce myelin and invest the neuronal axons in myelin sheaths. They express myelin basic protein (MBP), myelin gene regulatory factor (MRF) and proteolipid protein 1. There is some plasticity of myelination in adult life,

requiring continuing production and renewal of oligodendrocytes from oligodendrocyte precursor cells.

A fourth type of glial cell are the ependymal cells which line the ventricles. They have cilia and microvilli on the luminal side and secrete cerebrospinal fluid. Certain other types of cell which are called glia are not really glia at all. The radial glia found in the embryo are actually precursor cells of neurons and mature glia. The microglia of the CNS are macrophage-like immune cells derived from the hematopoietic system in early development which are permanently resident in the CNS.

Neurogenesis

Because the generation of neurons from pluripotent stem cells is a very important practical goal, it is worth considering what happens normally during mammalian neurogenesis (Figure 9.7a). The early neuroepithelium is a simple columnar epithelium. In terms of ultrastructure the side which later abuts the brain ventricles (the ventricular surface) is apical while that which abuts the exterior (the pial surface) is basal. As it is conventional to show the ventricular surface at the bottom of a figure, this means that neuroepithelia are normally depicted upside down relative to other epithelia where the apical side is shown at the top.

In lower vertebrates, there is substantial neurogenesis from the open neural plate, but this is minimal in mammals. In the mouse the main periods of neurogenesis are E9–12 in the spinal cord, E11–16 in fore- and midbrain, and E12-postnatal in the hindbrain and retina. Similar to lower vertebrates, experimental procedures that activate the Notch signaling pathway tend to suppress neurogenesis and prolong the period of multiplication of undifferentiated cells. After neural tube closure the cells undergo a process of "interkinetic migration", which involves movement of the nucleus from the basal (pial) side where they reside during G1 and S phases of the cell cycle, to the apical (ventricular) side for the G2 and M phases (Figure 9.7b). This gives the appearance of stratification to the epithelium although for a while the cells remain attached at both ends. Shortly after this the so-called radial glial cells arise, which continue to span the distance from ventricular to pial surface (Figure 9.7c). Although these cells express some glial markers, such as GLAST (Glu-Asp transporter) and GFAP (glial fibrillar acidic protein) they are not really glia, but are progenitor cells for neurons, astrocytes, oligodendrocytes and ependymal cells. They are polarized, with a concentration of prominin (= CD133), which is a cell surface glycoprotein; nestin, which is an intermediate filament protein; and brain lipid-binding protein (BLBP), at the ventricular end. The proteins Numb and Numblike are also present in an apical position and are retained when the radial glial cell buds off new neurons. The fate of radial glia in parts of the CNS can be observed by Cre labeling of reporter mice using adenoviral delivery of Cre to the pial surface. Because only the radial glia contact the pial surface they can be infected and transport the virus to the nucleus where recombination and labeling occurs. Such experiments show that the radial glia are precursors for neurons, intermediate progenitors, adult neural stem cells, astrocytes, oligodendrocytes and ependymal cells.

The neurons that bud off the radial glial cells arise near the ventricular surface and migrate up the radial glia until they reach their definitive position. This means that the formerly single layered neuroepithelium now acquires three layers. In the ventricular zone is the region of neuronal generation. Near the pial surface is a marginal zone devoid of cell nuclei, and in the center is a mantle zone composed of neurons migrating from the ventricular zone. The spinal cord and the brainstem maintain this three-layered appearance, but in some parts of the brain it becomes more complex. In the telencephalon a population of intermediate or "basal" progenitors appears. These are not really basal (i.e. pial) but they form a subventricular zone separated from the ventricular surface.

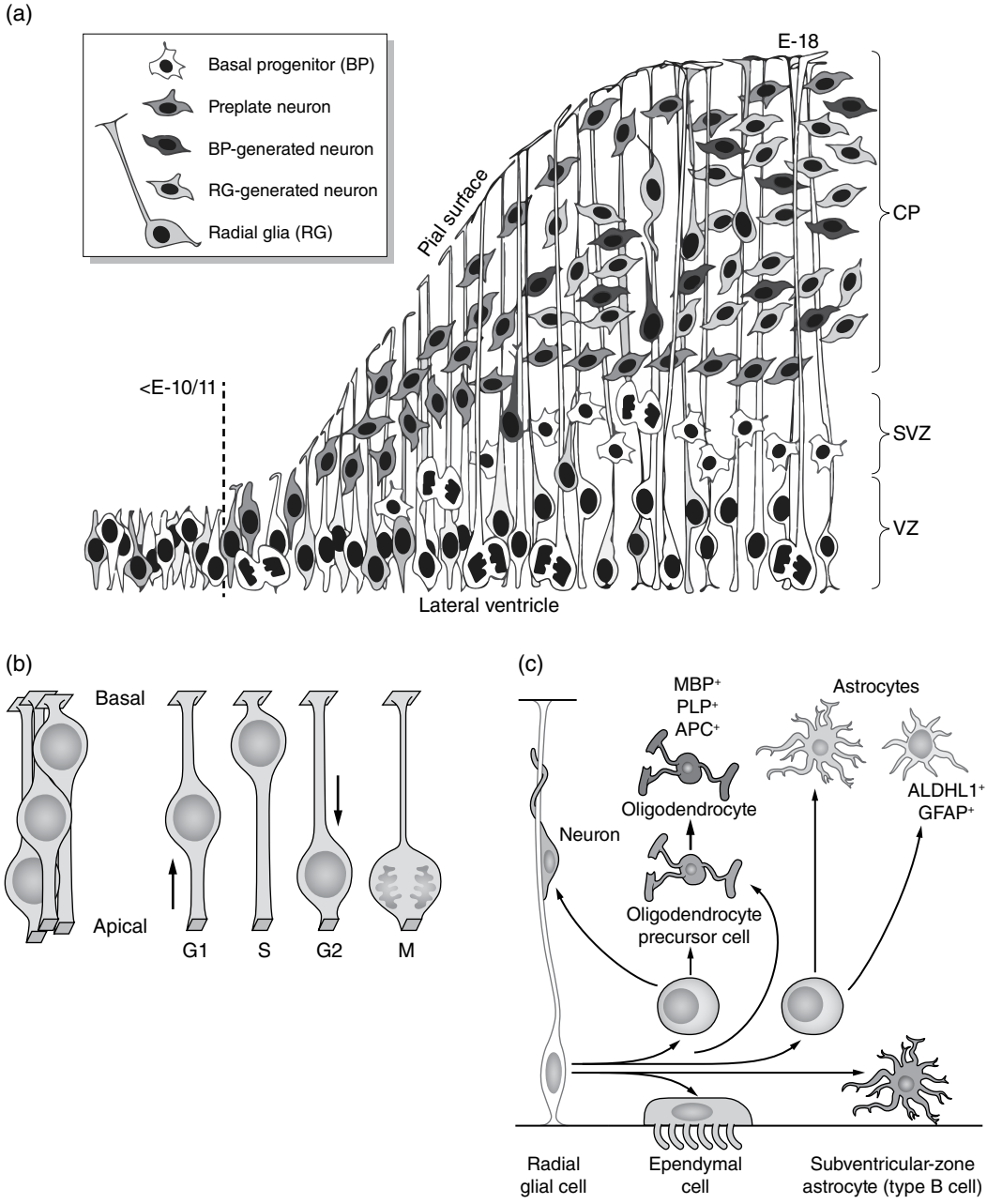

Figure 9.7 (a) Neurogenesis in the mouse. The figure depicts neurogenesis occurring in the future cerebral cortex of a mouse embryo between E10, when the neuroepithelium is just one cell thick, to E18 when the cortical plate is formed. BP = basal progenitor; RG = radial glia; CP = cortical plate; SVG = subventricular zone; VZ = ventricular zone. (b) Interkinetic migration, showing movements of nuclei and change of cell shape through the cell cycle. (c) Products of division of radial glia. Type B cells are considered to be adult neural stem cells. ALDH1L1: aldehyde dehydrogenase 1 family member L1; APC, adenomatous polyposis coli; GFAP, glial fibrillary acidic protein; MBP, myelin basic protein; PLP, proteolipid protein 1. (*Sources:* (a) Malatesta et al., 2007. Reproduced with permission from Springer. (b) From Miyata, T., Okamoto, M., Shinoda, T. and Kawaguchi, A. (2015) Interkinetic nuclear migration generates and opposes ventricular-zone crowding: insight into tissue mechanics. Frontiers in Cellular Neuroscience. 8, 1–11. (c) Modified from Rowitch, D.H. and Kriegstein, A.R. (2010) Developmental genetics of vertebrate glial-cell specification. Nature 468, 214–222. Reproduced with the permission of Nature Publishing Group.)

They express the transcription factor TBR2 (= Eomesodermin) and also Deltalike1 which maintains Notch signaling in the parent radial glia cells, helping to maintain their undifferentiated state. In the cerebral cortex the intermediate progenitors generate neurons which migrate through the mantle zone to form further layers on its pial side. This builds up the cortical plate which eventually consists of six layers of neurons. Additional cells (GABAergic interneurons) enter the cortical plate by lateral migration from the ganglionic eminences at the base of the cerebral hemispheres. In the cerebellum a different process occurs whereby cells migrating from the ventricular surface set up a new generative zone near the pial surface.

The developing human CNS also contains radial glia. They differ slightly from those of the mouse, for example, GFAP is expressed from the time of their first formation at 5–6 weeks gestation. Neurogenesis in the cerebral cortex occurs from then until about 20 weeks gestation, after which glia are formed. Evidence that human radial glia can form neurons has been obtained by sorting them from telencephalon of human fetuses, then culturing them in vitro, and showing that they can produce neuronal, glial or mixed clones. Although in vitro behavior may not be the same as in vivo, this is at least consistent with a similar multipotency to that shown by the mouse radial glia.

Gliogenesis

Radial glia not only generate neurons during development but also glial cells. In each part of the CNS there is a process which controls the switch from neurogenesis to gliogenesis. This is complex, but one important element is the presence of cardiotrophin, secreted by newly formed cortical neurons. This is a LIF-like factor (see Chapter 6 for LIF). Experimental application of cardiotrophin increases the production of glia, and knockout of the receptors gives rise to a glial deficiency. In the neurogenic phase the transcription factors NGN1 and -2 and MASH1 suppress glial genes, while in the gliogenic phase the transcription factor NF1 (nuclear factor 1) suppresses neurogenic genes. A few days after birth in mice the radial glia disappear, mostly differentiating to ependymal cells that line the ventricles, and also to some astrocytes and oligodendrocyte precursors. Cell labeling studies show that glial clones tend to be either astrocytes or oligodendrocytes, but not both.

It should be borne in mind that the control of the cell types formed in different parts of the CNS depends in large measure on the regional specification of those parts, as briefly described in Chapter 8. The intimate association between regional specification and cell differentiation is very clearly shown in the ventral part of the spinal cord where different zones are established in response to the gradient of SHH originating from the floor plate and notochord (Figure 9.8). The p3 domain is defined by expression of NKX2.2 and produces V3 interneurons and oligodendrocytes. Dorsal to this the pMN

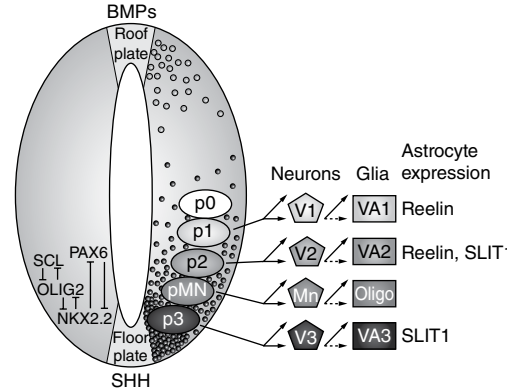

Figure 9.8 Effect of regional specification in the ventral spinal cord on the types of cell differentiation. On the left are shown transcription factors whose expression is induced by the gradient of SHH, together with their mutually inhibitory interactions. On the right are shown five resulting zones of cell differentiation. Mn = motorneurons; Oligo = oligodendrocytes. (From: Rowitch, D.H. and Kriegstein, A.R. (2010) Developmental genetics of vertebrate glial-cell specification. Nature 468, 214–222. Reproduced with the permission of Nature Publishing Group.)

domain is defined by expression of OLIG2 and produces motorneurons and oligodendrocytes. There is a mutual inhibition of expression between NKX2.2 and OLIG2 that sharpens the boundary between these domains. Loss of either factor causes the domain boundary to shift and the cell types produced to change accordingly.

Postnatal Cell Division

Once neurons have differentiated then they remain permanently post-mitotic. If a pulse of BrdU is given to an embryo then those neurons formed shortly after the label has been incorporated into DNA will retain it for life because it is not diluted by further replication and division. By contrast, those that have already differentiated are not labeled because they do not make DNA, and those that continue to divide will dilute out the label so it becomes no longer visible after a few cell cycles. The time of the last S-phase prior to neuronal differentiation is known as the "birthday" of the neuron and much work has been done cataloging the birthdays of different neuronal types in different parts of the CNS. Both neurons and glia may migrate considerable distances after their initial formation and the building of the final neuroanatomy depends on the assembly of various cell layers and the formation of axonal connections between different regions.

Mature oligodendrocytes are also post-mitotic, but there is a population of glial precursor cells, sometimes misleadingly called oligodendrocyte precursor cells (OPCs), which continue to proliferate throughout life. They initially derive from the ventral part of the CNS but later from the dorsal part. They express the cell surface chondroitin sulfate proteoglycan NG2, the receptor PDGFRα, and the transcription factor SOX10. Unlike mature oligodendrocytes, they have some neuron-like properties, being able to respond to glutamate by depolarization and generation of an action potential. Mature astrocytes in the CNS are normally quiescent but can be stimulated to divide by injury.

Adult Neurogenesis

Although most neurogenesis is completed before birth, there is a small amount of new neuron formation throughout life, located in specific parts of the brain. This has been detected by labeling with DNA precursors to detect persistent cell division and also by cell lineage labeling. In the mouse, persistent neurogenesis occurs in the subventricular zone of the lateral ventricles and in the dentate gyrus of the hippocampus (Figure 9.9). The subventricular cells become olfactory

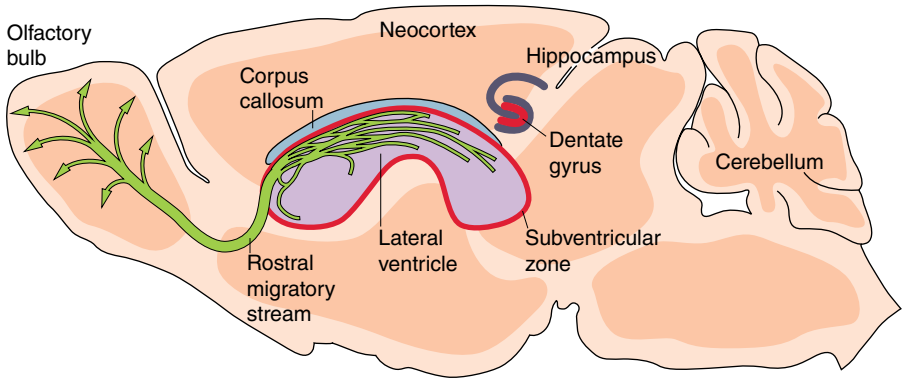

Figure 9.9 Location of adult neurogenesis in the mouse brain. Stem cells are found in the subventricular zone of the lateral ventricles and in the dentate gyrus of the hippocampus. (Zhao et al., 2008. Reproduced with the permission of Elsevier.)

neurons and migrate rostrally into the olfactory bulb. The dentate gyrus cells become glutaminergic granular neurons. Cell lineage labeling experiments show that the adult neural stem cells arise from the radial glia of the embryo. In mice, the experimental inhibition of adult neurogenesis causes a reduction of odor discrimination and of pattern separation ability. These are functions of the brain regions showing adult neurogenesis and indicates that it is of functional importance.

In the subventricular zone the neural stem cells are elongated, extending one process to a blood vessel and another, bearing a cilium, to the ventricular surface (Figure 9.10a). This potentially enables the cells to respond to conditions both in the blood and the cerebrospinal fluid. At least in the mouse, they generate transit amplifying cells, which in

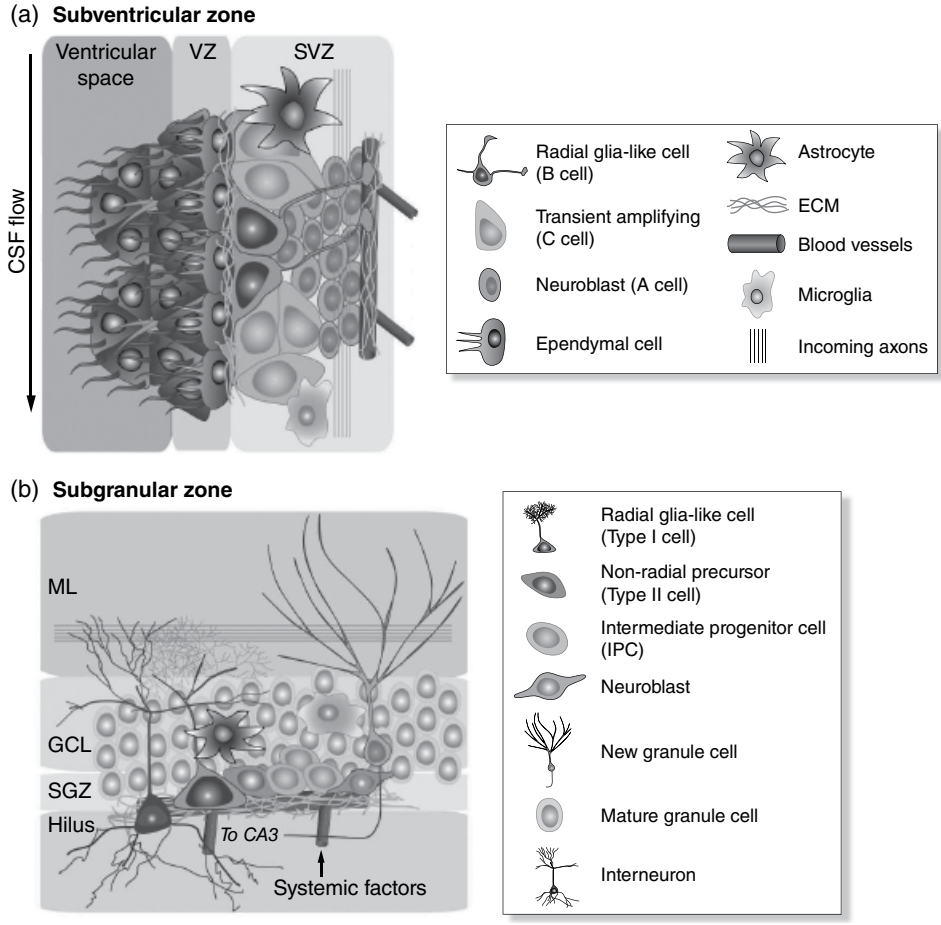

Figure 9.10 Stem cell niches in the adult mouse brain. (a) Subventricular zone. The B cells are considered to be stem cells, with processes contacting both the cerebrospinal fluid and the blood vessels. CSF = cerebrospinal fluid; VZ = ventricular zone; SVZ = subventricular zone. (b) Dentate gyrus of the hippocampus. The Type 1 cells are considered to be stem cells. ML = molecular layer; CGL = granule cell layer; SGZ = subgranular zone; CA3 = cornu ammonis 3 (another region of the hippocampus). (From: Bond, Allison, M., Ming, G.-l. and Song, H. (2015) Adult mammalian neural stem cells and neurogenesis: five decades later. Cell Stem Cell 17, 385–395. Reproduced with the permission of Elsevier.)

turn generate neuroblasts. These migrate to the olfactory bulbs where they migrate radially and eventually differentiate into olfactory interneurons.

The arrangement of the stem cell niche in the hippocampus is different. Here the stem cells are radial glia-like in shape and lie between the inner granule layer and the hilus (Figure 9.10b). They give rise to intermediate progenitors which become neuroblasts. These migrate tangentially along the subgranular zone, then become immature neurons and move radially into the granule layer, differentiating as granule neurons.

In humans there is also ongoing adult neurogenesis in the same regions of the brain, but the situation does differ from that in the mouse. In humans few if any new olfactory neurons are produced. Instead most of the new neurons produced in the subventricular zone end up in the striatum. There may possibly also be some endogenous neurogenesis in the striatum. Estimation of neuronal turnover using the ^{14}C dilution method (see Chapter 2) indicates that in a population of striatal interneurons there is about 2.7% cell replacement per year. In the dentate gyrus of the hippocampus a higher proportion of cells is subject to turnover than is found in mice, the rate according to ^{14}C dilution being about 1.75% per year.

Neurospheres

There is an interesting in vitro model for neuronal stem cells in the form of the neurosphere culture system. This is established from fetal or postnatal explants of CNS which are dispersed and cultured in a medium containing EGF and FGF. Neurospheres are cell clumps of about 0.3 mm diameter which grow and divide and can be cultured indefinitely in suspension. If dissociated into single cells, a few percent of these will reform neurospheres, thus indicating stem cell behavior. When neurospheres are plated onto a laminin surface in the presence of serum they differentiate to form both neurons and glia. Neurospheres may be cultivated from regions of the CNS that are known not to contain stem cells functioning in the intact organism. This again illustrates the fact that stem cell behavior depends on the cell environment and that specific cells in vitro may behave differently from the same cells in vivo.

Skeletal and Cardiac Muscle

Skeletal Muscle

An anatomical muscle has a hierarchical structure (Figure 9.11). The basic cellular unit is the myofiber, which is a highly elongated multinucleated cell surrounded by a

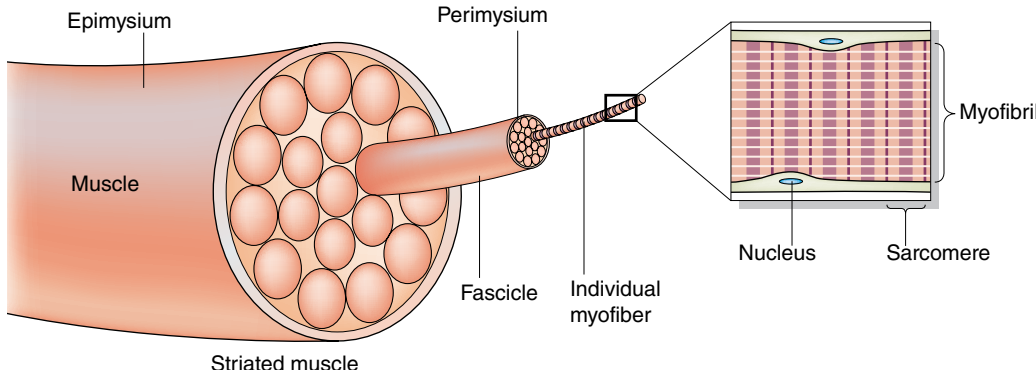

Figure 9.11 Skeletal muscle. The basic units are the myofibers. These are bundled in fascicles which are grouped into a whole muscle. (Slack, J.M.W. (2013) Essential Developmental Biology, 3rd edn. Reproduced with the permission of John Wiley and Sons.)

basal lamina. Myofibers are surrounded by delicate connective tissue called the endomysium. Several myofibers are packed into a bundle (fascicle) surrounded by perimysium, and many fascicles make up one anatomical muscle surrounded by a connective tissue sheath, the epimysium. Muscles are supplied with a vascular system by capillaries investing individual myofibers, and with a nervous system comprising motor axons innervating myofibers and sensory axons innervating the stretch-sensitive muscle spindles, made up of modified myofibers called intrafusal fibers.

The contractile structures found within each myofiber are called myofibrils and each myofiber contains many myofibrils arranged in register to give the characteristic striated appearance. Myofibrils are about 1 μm diameter and consist of repeating units of 2.2 μm called sarcomeres. The ends of the sarcomeres are called Z-discs, and to these are attached thin filaments composed of actin. Thick filaments composed of myosin interdigitate with the thin filaments. Each myosin molecule consists of a dimer of two heavy chains and two pairs of dissimilar light chains. There is a globular head region projecting from the thick filament that makes contact with the adjacent thin filament. Myosin is a motor protein and when supplied with ATP will migrate along the actin filament, driven by cyclic movements of the head region. The myofibrils are invested by a modified endoplasmic reticulum called the sarcoplasmic reticulum and also by a network of T-tubules formed by invagination of the plasma membrane, or sarcolemma. They are aligned with pairs of terminal cisternae of the sarcoplasmic reticulum to form triads. Flanking each complex of myofibril, sarcoplasmic reticulum and T-tubules are mitochondria. Their numbers vary depending on the myofiber type. Slow, type I fibers have an abundance of mitochondria and the oxygen storage protein myoglobin, and function mostly via oxidative metabolism. They tend to be found where recurrent or continuous movement is required. The predominant myosin heavy chain type is MHCIβ. Fast fibers have fewer mitochondria and little myoglobin but abundant glycogen. They function mostly using glycolytic metabolism to generate ATP and are found where short bursts of rapid contraction are required. In rodents fast fibers are classified as IIA, IID and IIB, with predominant myosin heavy chains MHCIIa, d and b respectively. In humans there is no MHCIIb.

Skeletal muscle contraction is initiated by receipt of action potentials from motor axons at specialized synapses between axon and myofiber called neuromuscular junctions. These are rich in acetyl choline receptors. Release of acetyl choline stimulates the receptors and causes depolarization of the myofiber, initiating a muscle action potential. Like the nerve action potential this is short in duration, about 2–3 msec. Because of the structure of T tubules the action potential propagates immediately throughout the whole myofiber. This causes opening of L type Ca channels in the T tubules. In the triad regions these are linked to Ca channels in the sarcoplasmic reticulum (ryanodine receptors), and when these open large amounts of stored Ca^{++} enters the cytoplasm. At rest, the myosin motor is inhibited because of obstruction of interaction with actin by the protein tropomyosin. The Ca^{++} binds to troponin, which is linked to tropomyosin and detaches it from the myosin. This permits the myosin to contact the actin and, so long as enough ATP is present, to drive the contraction.

Development of Skeletal Muscle

Most skeletal, or striated, muscle develops from the dermomyotome region of the somites. As described in Chapter 8, this buds off myoblasts which express myogenic transcription factors such as MYOD and MYF5, and are committed to become skeletal muscle. These continue to proliferate for a while and may also migrate, for example the myoblasts that form the abdominal muscles migrate to the ventral side of the body, while those that form the limb muscles migrate into the limb buds to form dorsal and ventral

muscle masses. The myoblasts are attracted into the limb buds by hepatocyte growth factor (HGF = Scatter factor) secreted by the limb mesenchyme.

Much work on mammalian myogenesis has utilized the cell line C2C12. At least in these cells continued proliferation requires FGF, and FGF signaling maintains expression of the transcription factor MSX1, which inhibits differentiation. On withdrawal of FGF, cell division ceases and the cells become competent to fuse. Fusion requires various components including the integrin types β1, α3 and α9, the cell adhesion molecule M-cadherin, and the protease ADAM12 (Figure 9.12). The actual fusion process involves the actin cytoskeleton and essential components include various GTP exchange proteins and small GTPases.

In the development of the mouse embryo there are two phases of myoblast fusion. Primary myoblast fusion occurs around E11 generating mostly slow fibers, while secondary fusion occurs around E14.5–17.5 generating mostly fast fibers. In embryonic life the myosin heavy chains MHC-emb and MHC-neo are present, becoming replaced by the mature forms after birth. Initially cells of the epaxial and hypaxial myotome express PAX3, although lineage labeling shows that PAX3 positive cells can become capillary endothelium as well as muscle. If these cells are ablated then primary myogenesis fails. PAX7 is necessary for the formation of muscle satellite cells (see below) and thus for postnatal myogenesis, but it is dispensable for secondary myogenesis, presumably because of the overlapping role of PAX3. In the postnatal growth period of the mouse there is continued myoblast fusion to preexisting fibers, increasing their length and girth. In mouse the myofiber number does not increase after P7. But the volume of myofibers does continues to increase to adult size (Figure 9.C.2), and this size may be further augmented by exercise.

In humans there are three phases of myoblast fusion, which occur between about the 7th and 23rd week of gestation. New myofiber formation and myoblast fusion with existing fibers is finished by about 5 months gestation.

Muscle Satellite Cells

Once nuclei have entered a multinucleate fiber, they become post-mitotic. So the cells of striated muscle are post-mitotic, but striated muscle considered as a tissue is still capable of regeneration from muscle satellite cells. These are small cells found within the basal lamina of myofibers (Figure 9.13). They are characterized by expression of the transcription factor PAX7 and are responsible for postnatal muscle regeneration. Satellite cells become visible from about E16.5, as basal laminas form around the fibers. In mouse, satellite cells are dividing and their progeny fusing with myofibers until about P21, after which the remaining satellite cells (about 5 per fiber) become quiescent. Slow fibers have more satellite cells than fast fibers. In adult life satellite cells are normally quiescent and express PAX7, but not the myogenic transcription factors. Following toxic or traumatic damage to their fiber, the satellite cells become activated. They divide, upregulate expression of myogenic transcription factors, and start to migrate. They can fuse with each

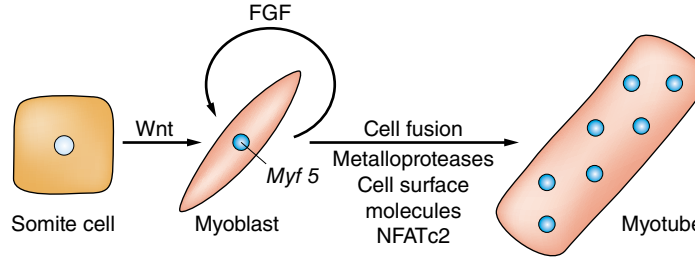

Figure 9.12 Myoblast fusion in the embryo. (Slack, J.M.W. (2013) Essential Developmental Biology, 3rd edn. Reproduced with the permission of John Wiley and Sons.)

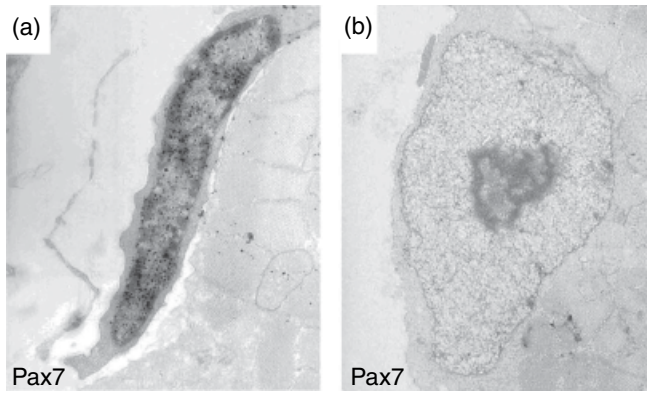

Figure 9.13 Nucleus of muscle satellite cell (a) and myofiber nucleus (b) viewed by transmission electron microscopy. PAX7 is immunostained with gold beads and is only present in the satellite cell nucleus. The specimen is *Xenopus laevis* tadpole tail muscle. (Author's photos.)

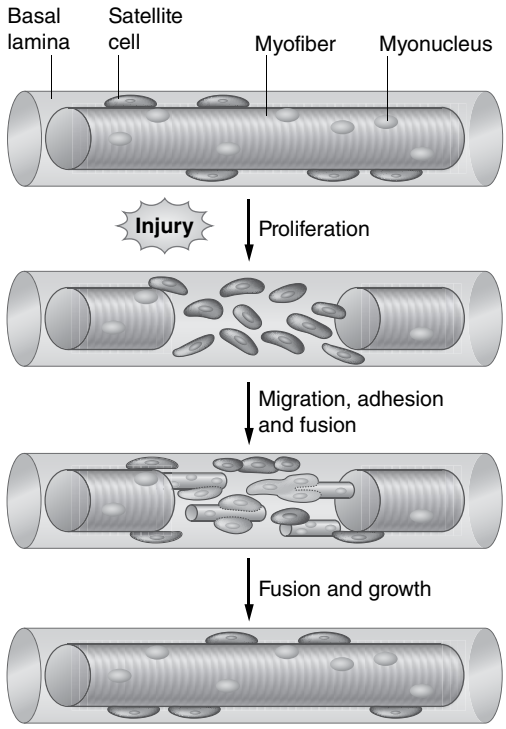

Figure 9.14 Regeneration of adult mouse muscle over about 2 weeks from injury.

other to form small myotubes, which can fuse with each other, and with the ends of the original myofiber, to form a new fiber (Figure 9.14). Regenerated fibers can be identified because they have centrally positioned nuclei when viewed in section, rather than the peripherally positioned nuclei of mature fibers.

Continued Notch activity is required for the maintenance of the quiescent state of muscle satellite cells. It is possible to transplant satellite cells between animals, and if the host is defective in muscle regeneration, for example the *mdx* mouse, which lacks dystrophin and is a partial model for human muscular dystrophy, then the host muscle can be repopulated from the graft.

In mice, the transcription factor PAX7 is not needed for muscle development so long as PAX3 is present, but it is needed for satellite cell function in the young mouse. This is demonstrated by the fact that the *Pax7* knockout mouse is seriously compromised in postnatal muscle growth and regeneration. However, PAX7 is no longer needed after about P21 when the cell recruitment to myofibers has finished. This is shown by ablation of *Pax7* at different times using an inducible knockout system. At the same time, PAX7 does remain expressed permanently in satellite cells. This makes it possible to ablate the cells using controlled expression of diphtheria toxin. The mouse used is *Pax7-CreER/R26R-DTA*. This means that Cre-ER is driven by the *Pax7* promoter. When activated by tamoxifen the CreER will activate expression of *DTA*, encoding diphtheria toxin A, and this will kill the cells in which it is produced. If the cells are destroyed at any stage of life, then muscle regeneration does not occur. So the satellite cells are needed even if the *Pax7* gene itself is not.

Muscle satellite cells are often described as "facultative stem cells" because they persist for the lifetime of the animal and they can generate both myoblasts and further satellite

cells on division, but do not feed continuous ongoing renewal of skeletal muscle. However, the satellite cell numbers do decline with age to about 25% of the starting numbers (mouse), so the regenerative ability in this case is less than perfect.

Cardiac Muscle

The functional cells of the heart are cardiac muscle cells, or cardiomyocytes (Figure 9.C.3). They are responsible for the lifelong rhythmic contraction without which death would be rapid. The heart as an organ contains many cells other than cardiomyocytes, being about 50% connective tissue as well as containing an essential vascular and nervous supply. The cardiomyocytes themselves are surrounded by a delicate connective tissue and have a very abundant blood supply.

The contractile apparatus of cardiomyocytes is similar to that of skeletal myofibers. The sarcomeres form a branching three dimensional network through the cytoplasm and there are numerous mitochondria. Cells have just one or two nuclei. They are joined to each other by intercalated discs which are rich in gap junctions and transmit action potentials between cells, enabling them to behave as a functional syncytium (Figure 9.15). Cardiomyocyte action potentials are much longer in duration than those of skeletal muscle, about 200 msec compared to 2 msec.

Although the process of excitation coupling via T-tubules and sarcoplasmic reticulum is similar to skeletal muscle, a number of the key proteins are derived from different genes. MHCα (human gene *MYH6*) is found in all parts of the heart during development and mostly in the atria in the mature heart. MHCβ (human gene *MYH7*), which is also found in skeletal muscle, predominates in the ventricles of the mature heart. There is a specific cardiac subunit of troponin, cTnT, and a specific myosin light chain MLC2v (human gene *MYL2*) is expressed in the cardiac crescent in early development and then restricted to the ventricles. ALC2 (human gene *MYL7*) is found in the mature atria. The actual channel

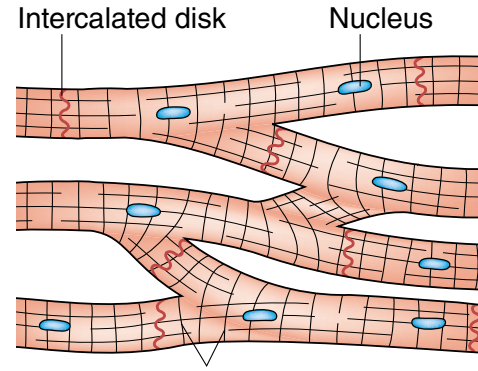

Figure 9.15 Organization of cardiac muscle. Cells may be mono or binucleate. They are joined by intercalated discs to form branched networks. (Slack, J.M.W. (2013) Essential Developmental Biology, 3rd edn. Reproduced with the permission of John Wiley and Sons.)

subunit of the L type Ca channel in the heart is CaV1.2 (human gene *CACNAIC*), which is not found in skeletal muscle.

Cell division among cardiomyocytes does extend after birth, for about one week in mice and 2 months in humans. At least in the mouse there is also a short burst of cell division due to thyroid hormone at about P14. But after this further growth is mostly by cell enlargement which is substantial, comprising about a 30–40 fold increase in volume. Such a large increase in size for a cell normally requires more than a diploid complement of DNA to fuel the necessary transcription and protein production. In mice this is achieved by most cardiomyocytes becoming binucleate during postnatal day 5–10. In humans there are some binucleate cells but most cells achieve the increase of DNA content by the single nucleus becoming polyploid, i.e. replicating the chromosomes without nuclear division, to 4n, 8n or even higher degrees of ploidy. The issue of whether there is any cell division after the postnatal period has been very controversial. The extensive polyploidy in humans, and the existence of DNA repair synthesis following damage, both mean that it is possible to observe DNA synthesis without actual cell multiplication. However the balance of evidence from cell labeling and ^{14}C dilution

studies is in favor of a very small degree of cell multiplication throughout life. In the human heart perhaps half of the cardiomyocytes are replaced by cell division during the course of a whole lifetime. The persistence of cardiac progenitor cells after the embryonic period has also been very controversial. Some examples, such as cells expressing the *c-kit* gene, are probably derived from the blood and are not cardiac cells at all. But calculations from cell numbers in different sized hearts in humans, and from cell lineage labeling following induced infarction in mice, suggest that there may be a limited contribution to heart muscle from non-cardiomyocytes.

In summary, the postnatal growth behavior of skeletal and cardiac muscle is very different. Skeletal muscle fibers are completely post-mitotic but can regenerate from satellite cells. Cardiomyocytes are almost entirely post-mitotic, showing a very low level of proliferation and little or no regeneration from committed progenitors.

Endodermal Tissues

Cell Differentiation in the Pancreas

The pancreas (Figure 9.16) consists mostly of exocrine cells which secrete digestive enzymes into the intestine. The secretions are collected by a system of ducts, also considered to be exocrine cells. In addition, 1–2% of the organ consists of endocrine cells, mostly grouped into the Islets of Langerhans. These comprise α-cells (secreting glucagon), β-cells (secreting insulin), δ-cells (secreting somatostatin), PP cells (secreting pancreatic polypeptide) and ε-cells (secreting ghrelin). Like the exocrine cells they arise from the endoderm of the pancreatic bud. This may be shown by Cre labeling experiments in which the Cre is driven by a promoter active in the endodermal component of the bud, such as that for the *Pdx1* gene, and both exocrine and endocrine cells become labeled.

Exocrine differentiation depends on the transcription factor PTF1, which has three subunits of which one, P48, is pancreas-specific. The *p48* gene is expressed in the whole pancreatic bud in early development but is downregulated in developing endocrine cells. Commitment to ductal differentiation seems to occur just in the narrow time window E8.5–10.5, based on lineage labeling with *Pdx1–CreER*, in which recombination is initiated at different stages by doses of tamoxifen. The transcription factor SOX9 is important for the postnatal ductal phenotype but in early pancreatic bud development SOX9 is more widely expressed and it maintains expression of FGF receptors needed for response to the growth promoting factor FGF10 secreted by the pancreatic bud mesenchyme.

An early population of endocrine cells appears in the mouse pancreas from about E10.5 which produce more than one hormone and do not respond to glucose. Cell lineage labeling indicates that these early-appearing endocrine cells are not the progenitors of the definitive endocrine population. Definitive endocrine cell commitment depends on the transcription factor Neurogenin3 (NGN3). This plays a similar role in a lateral inhibition system to that played by NeuroD in neurogenesis (Figure 9.17a). Notch signaling suppresses formation of NGN3, while NGN3 promotes synthesis of Delta. Starting with tiny random fluctuations of NGN3 levels, the operation of the lateral inhibition system brings about a population of isolated cells expressing NGN3 and Delta, surrounded by other cells in which Notch signaling is active and synthesis of NGN3 and Delta is low. The NGN3-positive cells become endocrine cells while the NGN3-negative cells become exocrine (Figure 9.17b). Overexpression of *Ngn3*, or knockout of components of the Notch pathway, can drive a high proportion of pancreatic cells to become endocrine, while knockout of *Ngn3* suppresses endocrine cell formation. In vivo the maximal expression of *Ngn3* is during the period E13.5–15.5 and the main wave of endocrine cell differentiation occurs a little later.

It is still not entirely clear what causes the diversification of the endocrine precursors into the five types of mature endocrine cell.

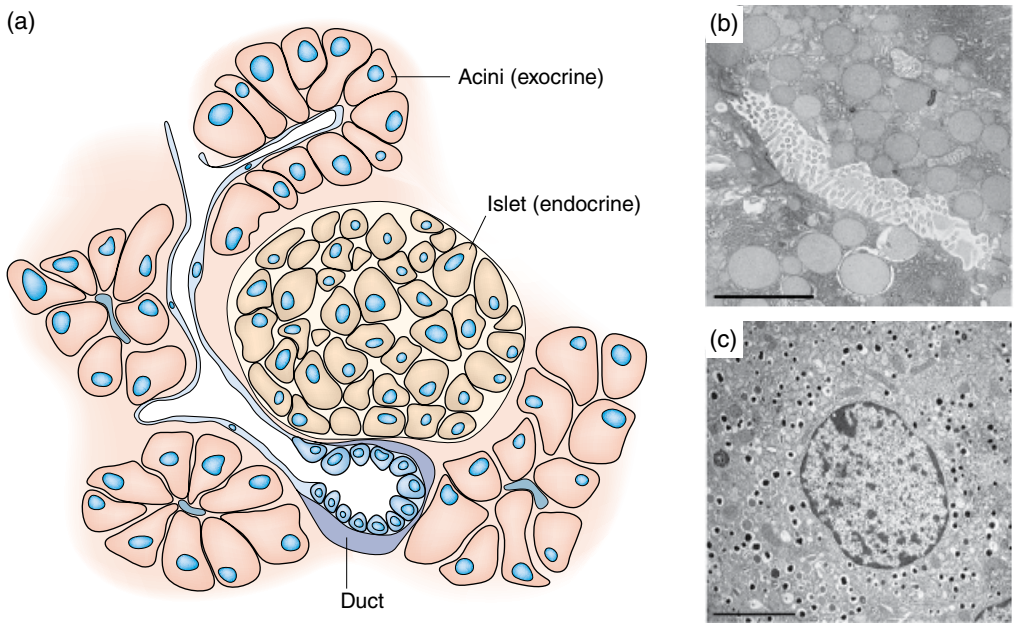

Figure 9.16 The mature pancreas. (a) Diagram of structure, showing exocrine acini and ducts and an endocrine islet. (b) Transmission EM picture of mouse exocrine cells, showing large secretory granules, abundant endoplasmic reticulum, and a luminal space into which project many microvilli. Scale bar 2 µm. (c) Transmission EM picture of a mouse beta cell, showing dense core granules containing insulin. Scale bar 4 µm. (*Sources:* (a): Slack, J.M.W. (2013). Essential Developmental Biology, 3rd edn. Reproduced with the permission of John Wiley and Sons. (b): Behrendorff, N., Shukla, A., Schewiening, E. and Thorn, P. (2009) Local dynamic changes in confined extracellular environments within organs. Proceedings of the Australian Physiological Society 40, 55–61. Reproduced with the permission of John Wiley and Sons. (c): Author's photo.)

However various transcription factors have been identified as essential. Formation of β-cells require the genes *Nkx6.1, Nkx2.2, Pax4, Pax6* and *MafB*. In addition proper maturation requires *MafA* and the re-expression of *Pdx1*. Formation of α-cells requires *Arx* and *Nkx2.2*. δ-cells require *Pax4* but the proportion of δ-cells also increases in the absence of *Arx*. Interestingly, the loss of *Nkx2.2, Pax4* or *Pax6* causes an increase in the proportion of ε-cells. Whether the choice between these various factors arises spontaneously or as the result of some local signaling process is not yet known.

In the normal pancreas the endocrine cells, of which the majority are β-cells, coalesce into islets of Langerhans. During growth of the young animal both exocrine and endocrine cells increase in number as the organ grows, but lineage labeling experiments indicate that this occurs by division of differentiated cells rather than from pancreatic stem cells. In adult life, normal cell turnover is very slow, although the exocrine tissue can regenerate rapidly following toxic damage, and the islets do increase in size during pregnancy. Labeling of the pancreas with DNA precursors indicates that cell turnover is by scattered divisions of differentiated cells, not fed from a stem cell population.

Production of functional β-cells is a major goal of stem cell biology. Although immunostaining for insulin is often used to identify β-cells, the presence of insulin not a sufficient criterion. A mature β-cell has a very high content of insulin, in the range 1–5% of the total cell protein. The insulin is found in secretory granules with a characteristic "dense core" appearance in the transmission electron microscope (Figure 9.16c). The secretion of insulin occurs in response to external glucose concentration. This

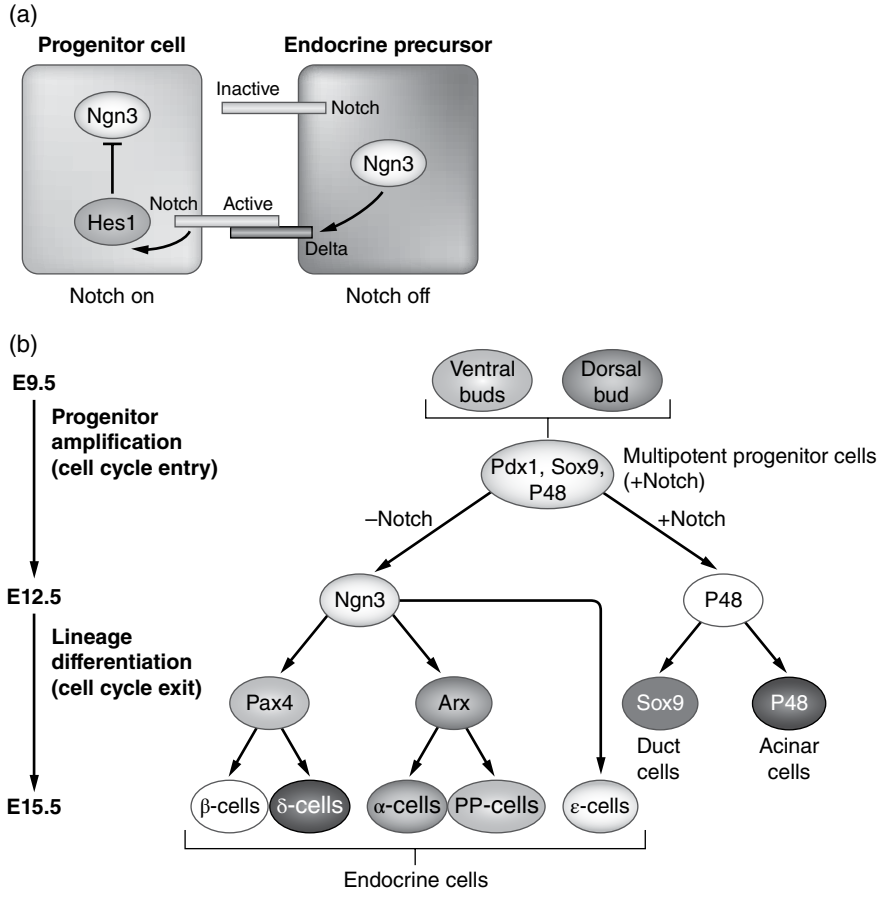

Figure 9.17 Lateral inhibition in the developing pancreas generates the endocrine precursors. (a) Cells with slightly more NGN3 cause more Notch signaling in adjacent cells, with repression of *Ngn3* transcription in these cells. High NGN3 cells become endocrine cells. (b) Putative cell lineage of pancreatic progenitors, showing some of the key transcription factors active at each stage. (From: Li, X.Y., Zhai, W.J. and Teng, C.B. (2015) Notch signaling in pancreatic development. International Journal of Molecular Science, E48. doi: 10.3390/ijms17010048.)

enters the β-cell with the help of the transporter GLUT2, it is then phosphorylated by glucokinase and enters the glycolytic pathway, raising the intracellular level of ATP. The ATP blocks potassium channels, retaining K^+ ions in the cell, and thus decreasing the magnitude of the (negative inside) membrane potential. This opens voltage-sensitive Ca^{++} channels, and the rise of intracellular Ca^{++} causes fusion of the secretory granules with the plasma membrane and the release of insulin. All of this system must be present and working correctly for a cell to be regarded as a β-cell. Immature β-cells, which are the type usually generated following directed differentiation of pluripotent stem cells, have a low insulin content and do not show full glucose sensitivity. Such cells are therefore not suitable for clinical transplantation.

Cell Differentiation in the Intestine

The small intestinal epithelium is lined with columnar absorptive cells. In addition there are several cell types described as "secretory", comprising goblet, Paneth, enteroendocrine and tuft cells. All the cell types are formed from the intestinal stem cells near the base of the crypts, which will be considered in more detail in the next chapter. The formation of

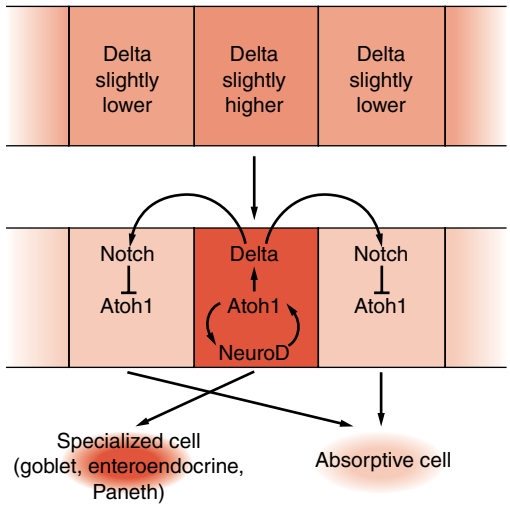

Figure 9.18 Differentiation of the secretory cell types in the intestinal epithelium controlled by lateral inhibition. (Modified from Slack, J.M.W. (2013) Essential Developmental Biology, 3rd edn. Reproduced with the permission of John Wiley and Sons.)

the secretory population is once again controlled by a lateral inhibition system based on Notch signaling. This time the key transcription factor is ATOH1 (= MATH1), a bHLH type factor, whose expression commits the cells to secretory differentiation. ATOH1 promotes the synthesis of Delta, which stimulates Notch in the surrounding cells. Notch-ICD de-represses the transcription of *Hes1,* and HES1 inhibits the transcription of *Atoh1* and thereby of *Delta*. So a small random increase in ATOH1 in one cell will stimulate Notch signaling in adjacent cells, driving down their production of Delta. The original cell will have less Notch signaling and therefore the level of ATOH1 will increase. Eventually this results in a scattered population of high ATOH1 cells (the future secretory cells) surrounded by a larger population of low ATOH1 cells (the future absorptive cells) (Figure 9.18).

Cell Differentiation in the Liver

The liver is a large organ with many vital functions. It is a major center of intermediary metabolism, including the synthesis of cholesterol, urea and glycogen. It is a source of serum proteins, especially of albumin; of hormones including insulin-like growth factor 1 (IGF1), angiotensin and thrombopoietin; and it secretes bile into the intestine. It also detoxifies drugs and toxins using the cytochrome P450 system. All these functions are exerted by hepatocytes, which are the principal cell type. These are epithelial cells whose basolateral surfaces contact the blood and whose apical surfaces contact the bile canaliculi. A histological section of liver shows a rather uniform appearance, but the organ does have a precise substructure composed of plates of hepatocytes arranged in lobules (Figure 9.19). Lobules are roughly hexagonal in shape with a central vein in the middle. At each apex is a portal triad, composed of branches of the hepatic artery, bringing oxygenated blood from the lungs; the hepatic portal vein, bringing nutrient-loaded blood from the intestine; and a bile duct. In between the portal triads and the central vein run blood sinusoids, lined by specialized sinusoidal endothelium. The hepatocytes are arranged along these sinusoids, and because the endothelia have gaps (fenestrations), the hepatocytes are in direct contact with the blood. Other cell types found in the sinusoids are the Kupffer cells, which are a type of macrophage, and hepatic stellate cells, which store vitamin A and are involved in fibrosis. The small bile canaliculi abut the apical surfaces of the hepatocytes but near the portal triad they become lined with biliary epithelial cells, also called cholangiocytes. The biliary system comprises a series of ducts, starting with the small ducts of individual portal triads, which fuse to form larger and larger ducts until they coalesce into the hepatic duct. This leads to the intestine. On the way there is a side branch, the cystic duct, leading to the gall bladder, which is a bile storage organ, and, more distally, fusion with the pancreatic duct to form the common bile duct entering the duodenum. The hepatic duct, cystic duct, gall bladder and common duct are known as the extrahepatic biliary system and originate from a distinct endodermal bud adjacent to the hepatic and ventral pancreatic buds (see Chapter 8).

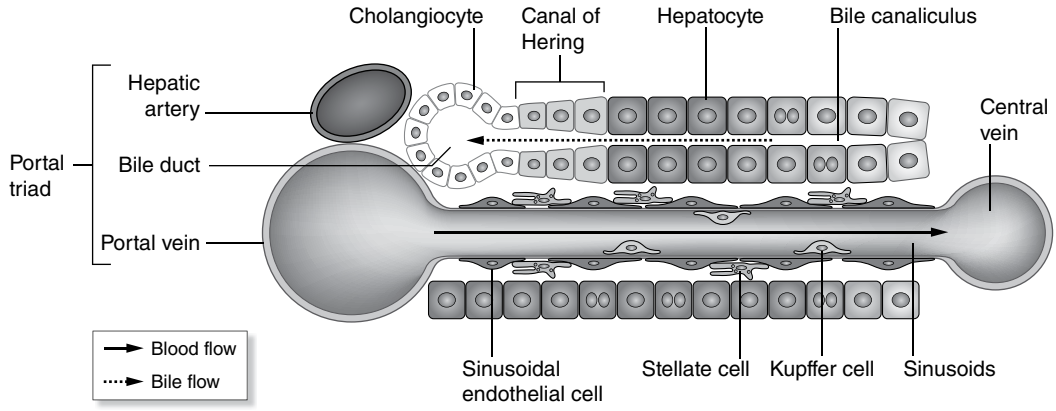

Figure 9.19 Structure of the liver lobule. Hepatocytes are arranged along blood sinusoids running between the portal vein and the hepatic vein. The basal sides of hepatocytes face the sinusoid and the bile canaliculi are formed from apposed apical surfaces. (From: Miyajima, A., Tanaka, M. and Itoh, T. (2014) Stem/progenitor cells in liver development, homeostasis, regeneration, and reprogramming. Cell Stem Cell 14, 561–574. Reproduced with the permission of Elsevier.)

Hepatocytes and Cholangiocytes

The liver bud in the embryo is composed of hepatoblasts, bipotential cells capable of forming both hepatocytes and biliary epithelial cells. Around E15.5 in the mouse some of these congregate around the branches of the future portal vein to form ductal plates, which express the transcription factor SOX9 (Figure 9.20). Clonal labeling using *Sox9-Cre* mice show that the ductal plate cells can form both bile duct cells and hepatocytes. In vitro experiments indicate that TGFβ signaling is needed for the formation of the ductal plates, and knockout studies show that Notch signaling is needed for the formation of cholangiocytes, the signal in this case being Jagged2 from periportal mesenchyme. The transcription factors HNF6(= OC1) and OC2 are needed for biliary and hepatocyte development. In the absence of both, the heptoblasts differentiate as mixed phenotype cells. The function of HNF6 and OC2 is to cause production of TGFβ inhibitors that enable the formation of a gradient of TGFβ activity across the ductal plates such that the high level induces differentiation of cholangiocytes on the portal vein side while the low level allows differentiation of hepatocytes on the parenchymal side.

The Kupffer cells are derived from the hematopoietic system, while the stellate cells come from the mesothelium, which is the mesoderm-derived layer lining body cavities. This is shown by labeling with *Mesp1-Cre,* expressed in the mesothelium. The liver is the main center for hematopoiesis at fetal stages. The hematopoietic cells secrete the cytokine oncostatin M, which may help to promote hepatocyte differentiation. Full differentiation of the hepatocytes is not achieved until after birth, especially in terms of the cytochrome P450 detoxification systems. By this stage the hepatocyte differentiated state depends on a small group of transcription factors which activate each other's expression in a cross-catalytic web: C/EBPα, HNF1β, FOXA2, HNF4α1, HNF6 and LRH1 (Nr5a2).

The hepatocytes of the liver are not all identical but become organized in a pattern of metabolic zonation along the portal triad-central vein axis of each lobule. The cells near the portal triad are specialized for gluconeogenesis and for urea synthesis, thereby exhibiting high levels of glucose-6-phosphatase and carbamoyl phosphatase. The cells near the central vein are specialized for glycolysis and for glutamine

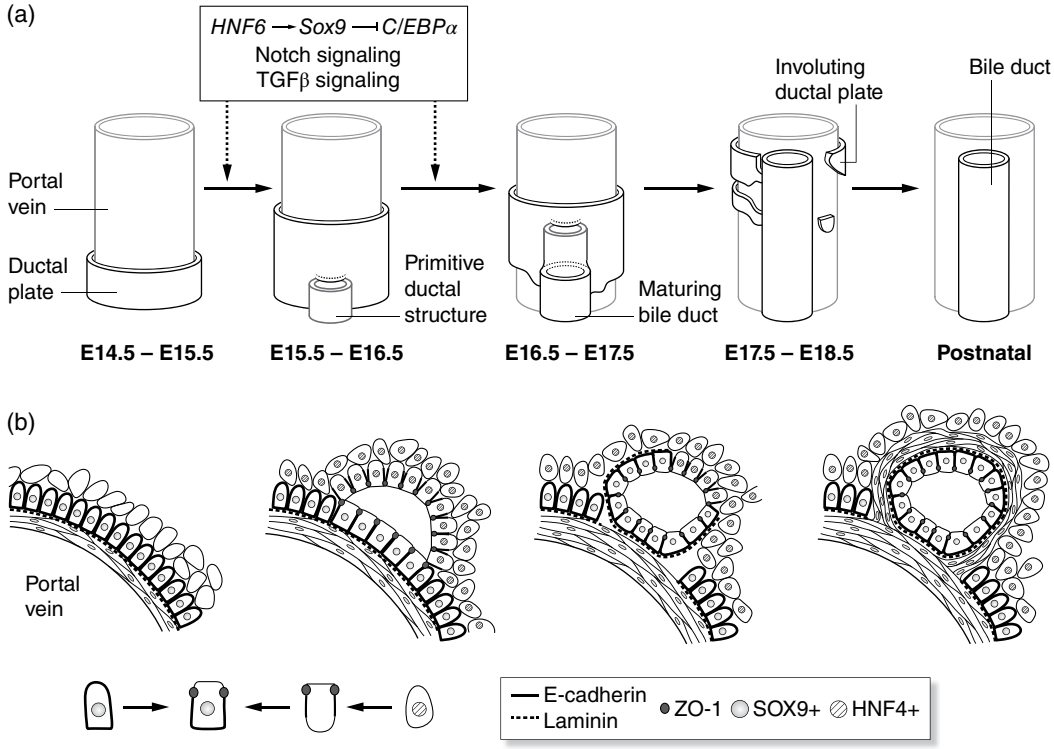

Figure 9.20 Origin of bile ducts during liver development in the mouse. (a) The ductal plates arise as a single layer of hepatoblasts around the portal vein and then bud off tubes that become bile ducts. SOX9 promotes ductal development while C/EBPα and β promotes hepatocyte development. (b) Schematic sections through the ductal plate. E-cadherin is a cell adhesion molecule; laminin is found in basement membranes; ZO-1 is found in tight junctions; HNF4 is one of the hepatocyte transcription factors. (Modified from Antoniou, A., Raynaud, P., Cordi, S., Zong, Y., et al. (2009) Intrahepatic bile ducts develop according to a new mode of tubulogenesis regulated by the transcription factor SOX9. Gastroenterology 136, 2325–2333. Reproduced with the permission of Elsevier.)

synthesis, showing high levels of glucokinase and of glutamine synthase (Figure 9.C.4). This pattern becomes established in late embryogenesis and depends on the Wnt system. There is a gradient of Wnt signaling across the lobule with the high level at the perivenous end and the low level at the periportal end. Upregulation of Wnt signaling by liver specific ablation of the inhibitor APC, using *TTR-CreER* × *floxed APC*, causes perivenous markers to be expressed all over the lobule. Conversely, liver specific inhibition of the Wnt system, by adenoviral expression of the Wnt inhibitor DKK1, causes periportal markers to be expressed across the lobule.

Liver Growth and Regeneration

The liver continues to grow after birth by division of all of its cell types. Most attention has not surprisingly been devoted to the hepatocytes. Saturation labeling with ^{3}HTdR following by long term observation of the subsequent label dilution indicates that in the mouse hepatocytes divide on average about every 100 days, and cholangiocytes about every 50 days. Probably because of the need to maintain a high protein synthesis capability, hepatocytes are, like cardiomyocytes, prone to become binucleate, and also to become polyploid. In the mouse about 60% of cells are polyploid by 3 months and 80% in the adult. About 50–60% cells are

binucleate, some having two diploid nuclei and others having two tetraploid nuclei. In humans the level of polyploidy is 20–45%, and of binuclearity about 30%. Polyploid cells can divide but the daughters are usually aneuploid and incapable of further division, so long term cell renewal inevitably depends on the minority of diploid cells. Proposals for the existence of a hepatic stem cell population living in the portal triad and feeding hepatocytes across the lobule towards the central vein have not been supported by subsequent investigations. There is no concentration of cell division at any position across the lobule, and the shapes of clones visualized by retroviral labeling indicate a random rather than an oriented pattern of clonal expansion.

Although the liver shows very slow normal cell turnover and does not have stem cells, it is capable of vigorous regeneration following damage. Surgical removal of 1–2 liver lobes is followed by a rapid growth of the remaining lobes to restore the missing volume. Classical parabiosis experiments, in which the circulatory systems of two animals were experimentally joined together, showed that the signal for regeneration was present in the blood. In other words an animal with an intact liver starts to regenerate if it shares the circulation and blood supply of another animal with a partially resected liver. The nature of this signal is still unclear but probably includes hepatocyte growth factor (HGF), bile acids, tumor necrosis factor (TNF) and interleukin 6 (IL6) from the damaged liver. Hepatocyte division is elevated within 24 hours, along a portal to venous gradient, and is followed by division of biliary and endothelial cells. HGF is essential for regeneration and it is derived from stellate and endothelial cells within the regenerating liver. The original volume is restored in about 1 week in the rat (2 weeks in human). Initially the size of each liver lobule is increased, but this adjusts back to normal over several weeks, presumably by cell migration and some respecification of the metabolic zonation pattern. The reason for the cessation of regeneration when organ volume is restored is not entirely understood but one component is probably TGFβ from stellate cells, which has an inhibitory effect on hepatocyte proliferation.

Many studies in which hepatocytes are labeled before regeneration show that the new hepatocytes are derived from pre-existing hepatocytes and not from stem cells. In fact the overall growth capability of hepatocytes is very high. As many as five successive partial hepatectomies carried out on a rat are followed by complete restoration of volume. This involves an expansion of the residual liver tissue by a factor of 18. Even more impressive is the behavior of hepatocytes transplanted as cells. There is a very useful mouse transplantation model afforded by mice deficient in the enzyme fumaryl acetoacetate hydrolase (FAH). This enzyme is part of the pathway for tyrosine catabolism. Mice lacking the *FAH* gene die because of liver failure caused by the accumulation of the toxic fumaryl acetoacetate. They can be saved by treatment with a drug called NTBC (or nitisinone) which inhibits a previous step in the pathway leading to accumulation of a less toxic intermediate. Transplantation of normal hepatocytes into an immunodeficient FAH^{-} mouse, followed by withdrawal of NTBC, leads to a rapid colonization of the liver by the graft (Figure 9.C.5). Up to six serial transplants of this type have been carried out with an estimated 86 doublings of the donor hepatocytes.

For many years it has been known that certain toxic and carcinogenic stimuli to the rodent liver can elicit formation of a novel cell population from the portal triad regions called "oval cells". These have some properties of embryonic hepatoblasts such as production of α-fetoprotein. They have been considered as "facultative stem cells" which can regenerate the liver when the division of hepatocytes is blocked, for example after treatment of rats with the toxin 2-acetylaminofluorene. Recent cell lineage labeling with *Sox9-CreER* mice have shown that the oval cells originate from the SOX9-positive small bile ducts. However such studies have

also shown that they do not contribute to regenerated hepatocytes, which arise, as in normal regeneration, from pre-existing hepatocytes. Since the cell damage models available in mice and rats are different, the nature of the "oval cells" may also be different, and so this conclusion might not hold for rats or indeed humans. However in mice it seems clear that in all the regeneration models hepatocytes come from pre-existing hepatocytes.

Transdifferentiation and Direct Reprogramming of Cell Type

In the late 1990s there was a spate of reports to the effect that grafts of hematopoietic stem cells from one animal to another could repopulate a variety of tissue types in addition to the blood. These included the brain, muscles, liver, and many epithelial structures. The results gave rise to some curious theories of continuous cell renewal of all parts of the adult body fed by stem cells in the bone marrow. They also probably helped to establish the myth of the miracle stem cell therapy: the cells that could be grafted into a patient which would seek out any area of decay and rebuild it to restore full function. However further investigation showed that the results were incorrect, either being due simply to lodging of grafted cells in various organs, or to fusion of grafted cells with cells of the host. There are a few genuine examples of transdifferentiation in nature but, within the mammalian body, tissue-specific stem cells generally only produce the cell types of their own tissue. Moreover, as is well-known, grafts of pluripotent stem cells into animals do not repopulate damaged tissues, instead they form the tumors called teratomas which will eventually kill the host (see Chapter 6).

Although the transdifferentiation of normal mammalian cells is generally not possible just by altering their environment, it is possible to force cells to change type by introduction of selected transcription factors. These are usually small subsets of the transcription factors known to be important in normal development. For example, neuron-like cells have been generated by introducing the transcription factors BRN2 (= POU3f2), ASCL1 and MYT1L, into fibroblasts. These have a typical neuronal morphology and gene expression pattern. They can generate action potentials and form synapses in vitro. A more sophisticated cocktail, FOXG1, SOX2, ASCL1, DLX5, and LHX6, can convert fibroblasts specifically into GABAergic neurons resembling telencephalic interneurons. These have firing patterns comparable to cortical interneurons, form functional synapses, and release GABA. Cardiomyocyte-like cells have been generated by introducing GATA4, MEF2c, and TBX5, and undergo spontaneous contraction like bona fide cardiomyocytes. Hepatocyte-like cells have been generated by introducing FOXA3, HNF1A, and HNF4A. These cells display functions characteristic of mature hepatocytes, including cytochrome P450 enzyme activity and biliary drug clearance. Upon transplantation into mice with concanavalin-A-induced acute liver failure and fatal metabolic liver disease due to fumarylacetoacetate dehydrolase (FAH) deficiency, they restore the liver function.

Generally it is harder to do direct reprogramming with human than with mouse cells, and it is harder the further apart the starting cells are from the desired cell type in terms of developmental history. For example, cardiomyocyte-like cells are easier to make from cardiac fibroblasts which are derived from the original heart-forming territory of the mesoderm, than from dermal fibroblasts. However, there are also some examples of cross germ layer transformations such as fibroblast to neuron or hepatocyte. In the cocktails of transcription factor used, at least one needs to be a "pioneer factor" that can locate its target sequences in chromosomal DNA even when these are embedded in closed chromatin. Some studies have incorporated Cre-based markers of known

progenitor cell types to find whether the process of reprogramming involves going "backwards", and thus becoming a normal progenitor, or "jumping sideways", and missing out any normal progenitors. The results indicate that progenitors are not formed so the process really is a "jump" from one cell type to another.

The essence of direct reprogramming is that the process operates a bistable switch. As a result of the transient action of the introduced transcription factors, a whole ensemble of genes is turned on or off and the new state is stable and self-maintaining in the absence of the original stimulus. So studies of this type need to show that the factors causing the transdifferentiation do indeed act in a transient manner and that the new cell phenotype is stable in their absence. This is usually done by using a doxycycline-inducible vector to express the gene products (see Chapter 3), and to show that the new cell types persist after removal of the doxycycline. In cases where the new cell type is dividing such evidence is convincing, but it is less certain where the new cell type is post-mitotic, such as neurons, cardiomyocytes or beta cells. The reason is that proteins can persist for a long time in non-dividing cells so the introduced factors may not be properly removed, and also the differentiation markers used to characterize the new cell type may persist long after their production has stopped.

Detailed studies of gene regulatory networks in transdifferentiated cells have shown that they are never exact copies of the normal cell type. However they may nonetheless be stable, and they may be close enough to the normal phenotype for the purpose intended, for example in vitro studies of drug metabolism, or cell transplantation therapies. One persistent problem, also found with the directed differentiation of cells from pluripotent stem cells discussed below, is the tendency to create immature rather than mature forms of the differentiated cells: for example β-cells that are non-glucose responsive or hepatocytes without full detoxification functions. Overcoming this problem will require further work.

Differentiation Protocols for Pluripotent Stem Cells

What are the lessons of developmental biology for the design of an effective way to make a specific cell type from human pluripotent stem cells? In principle the answer is simple. The cells should be exposed to a sequence of inducing factors at appropriate concentrations and durations of treatment to mimic the series of signals encountered during normal development from the epiblast to the final differentiated cell type required. The reality is more difficult. Pluripotent stem cells grow as aggregates, so even supplying a pure factor to a dish of cells results in different cells receiving different stimuli, depending on the size of the aggregate, and their position within it. This causes a heterogeneity of response to the applied factors, and means that the next cycle of treatment will be of a diverse cell population. Furthermore, once development is commenced by removing pluripotent cells from their normal growth medium, they display a lot of self-organizing potential. They may readily form signaling and responding regions within the aggregate, and this generates further new cellular territories as a result of local interactions. Because of the inevitable presence of endogenous inducing factors generated by such processes, inhibiting these may be just as important as adding new ones. Small molecule agonists and antagonists may be preferred to the protein inducing factors themselves because of better standardization and better penetration into cell aggregates. The monitoring of progress through a protocol is normally carried out by looking for the expression of key transcription factors, or other markers indicative of specific intermediate states of developmental commitment. Some pluripotent cell lines have been made carrying fluorescent protein reporters of useful markers to assist with protocol

development. This makes monitoring very easy because the cells change color with each of the steps for which a reporter is present.

Over the last 10–15 years many new protocols for directed differentiation have been devised and it has become clear that it can be relatively easy to coax the cells into becoming immature or juvenile versions of the cell types required, but much harder to get fully mature cells. This is very important for such targets as cardiomyocytes, or hepatocytes or β-cells, where the immature cells do not display the essential physiological properties that are needed. There has also been considerable difficulty in devising protocols that are robust enough to be repeated successfully in other labs. The existence of many irreproducible protocols has led workers to be very careful to try to ensure robustness when they publish new ones.

Workers in this area have tended not to try to drive differentiation by overexpression of specific transcription factors, and to prefer the use of soluble factors. The reason is a desire not to have to deal with the complexities of introducing new genetic material. Since transient overexpression can now be achieved using the introduction of RNA or protein, it need not involve any heritable change to the cells, and it is likely that such methods will be used more in future.

If the differentiated cells are intended for clinical transplantation the whole process must be carried out under conditions of "good manufacturing practice" (GMP). This means that high standards of sterility and quality control must be adhered to. No animal products may be used as they could be the source of infectious agents. Very high standards of recording and documentation must be met for all materials and procedures used. Moreover the pluripotent cell line itself should have been prepared under GMP conditions. Compared with the normal mode of activity in an academic lab, GMP is very cumbersome and expensive. However it may be worth thinking about GMP well in advance in order to avoid having to go back several years and repeat a lot of preliminary work under GMP conditions. It is also worth noting that the regulations are somewhat different in the USA and Europe.

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10

Stem Cells in the Body

It is now time to consider the nature of the tissue-specific stem cells: namely those stem cells that exist and function throughout life in the animal or human body. Recall the key features of the definition of stem cell behavior propounded in Chapter 1: the stem cell divides to renew itself, it produces differentiated cells, and it persists long term, usually for the lifetime of the organism. This definition means that only tissues undergoing continuous renewal really have stem cells. Those that do not self-renew or renew very slowly, like cardiac muscle or cartilage, do not have stem cells. One could enlarge the definition of a stem cell to include, for example, hepatocytes, which divide to generate further hepatocytes following toxic or surgical damage to the liver; or pancreatic exocrine cells which can rapidly replenish the exocrine population following toxic ablation. However such examples correspond to different types of biological behavior which may reasonably be given different names. They are of course very important and interesting but they are somewhat different from stem cell behavior. The neural stem cells found in the subventricular zone and in the hippocampus are real stem cells, but to avoid disruption to the flow of the text these have already been described in Chapter 9.

The Intestinal Epithelium

One of the best understood stem cell systems is that maintaining the intestinal epithelium. This lines the luminal surface of the gut all the way from the pyloric sphincter of the stomach to the anus. There is a significant morphological difference between the small intestine, responsible for most digestion and absorption of the food, and the large intestine, responsible for absorbing water and accommodating a large population of gut bacteria. The small intestine (Figure 10.1a) is lined with villi which massively expand the surface area for the purpose of absorption. In between the villi are crypts of Lieberkühn in which active cell division takes place (Figure 10.2). Cells differentiate as they leave the crypts, and migrate up the villi, eventually to die by apoptosis and to drop off into the lumen. The flux of cells is considerable and it is estimated that the epithelium, apart from the long lived Paneth cells, is renewed every 4–7 days in the mouse. There are several cell types making up the epithelium. The most numerous are the columnar absorptive cells, or enterocytes. Next most numerous are the goblet cells, containing large vesicles filled with mucins. In the crypt bases lie Paneth cells which contain granules and secrete lysozyme and cryptdins as a defense against infection. In addition there are several types of enteroendocrine cells, secreting hormones such as cholecystokinin, GLP1, PYY and secretin. Although most textbooks only mention these four principal cell types there are also three others: the cup cells, which are a type of absorptive cell; the M cells, which overlie patches of lymphoid tissue; and the tuft cells which bear a tuft of microvilli. Beneath the epithelium lies the

The Science of Stem Cells, First Edition. Jonathan M. W. Slack.

Companion website: www.wiley.com/go/slack/thescienceofstemcells

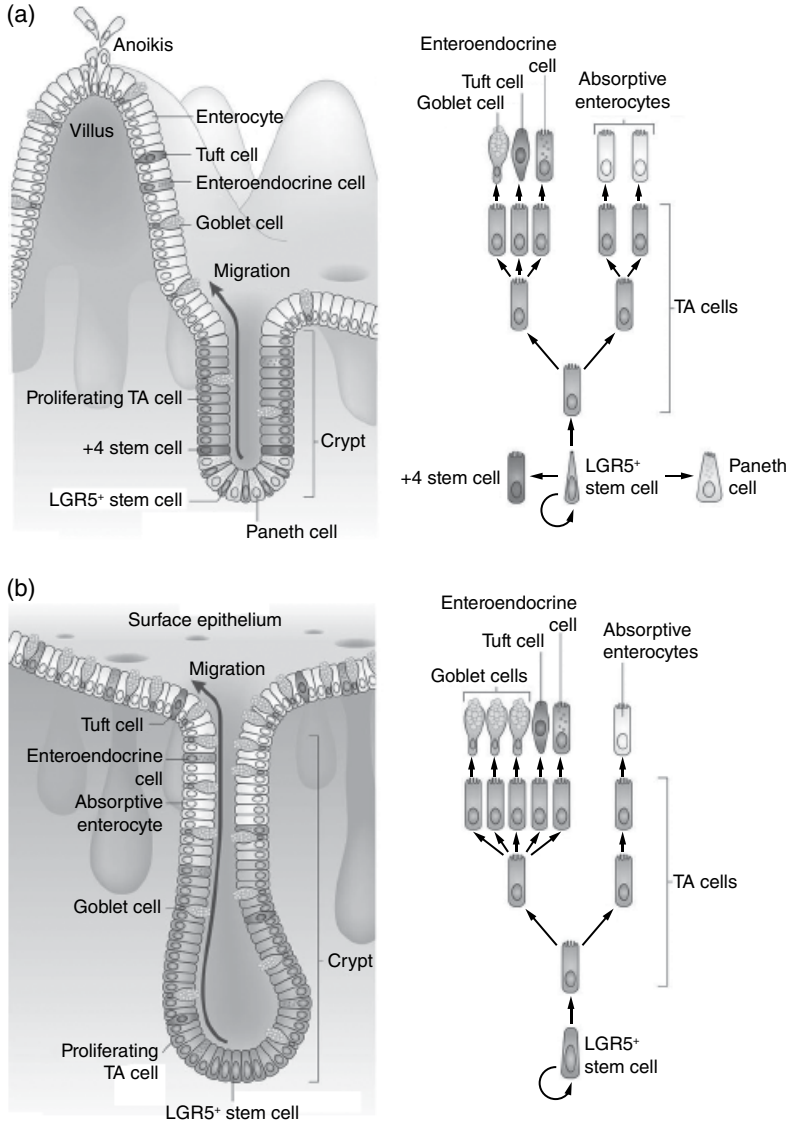

Figure 10.1 Organization of the small and large intestine with putative cell lineage diagrams. (a) Small intestine. (b) Large intestine. TA cells = transit amplifying cells. (From: Barker, N. (2013) Adult intestinal stem cells: critical drivers of epithelial homeostasis and regeneration. Nature Reviews Molecular Cell Biology 15, 19–33. Reproduced with the permission of Nature Publishing Group.)

lamina propria, a stroma containing blood vessels, lymphatics, loose connective tissue and some lymphoid tissue. The core of each villus also contains similar stromal tissues. Outside this lies a thin layer of smooth muscle called the muscularis mucosa. Often all of the tissues lying within the muscularis mucosa are simply described as "the mucosa". Outside the muscularis mucosa lies more connective tissue, substantial layers of smooth muscle and a peritoneal lining on the surface. The small intestine is divided into the duodenum, jejunum and ileum. They are histologically fairly similar although the

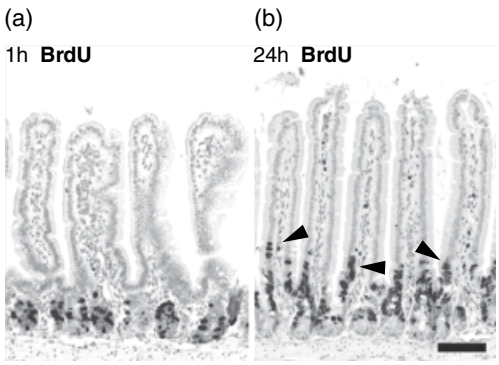

Figure 10.2 Cell division in the intestinal epithelium of a mouse. The mouse has been labeled with an injection of bromodeoxyuridine (BrdU), visualized by immunostaining (dark nuclei). Only epithelial cells in the crypts have incorporated BrdU, plus a few non-epithelial cells in the interior of the villi. (a) 1 hour after BrdU injection. (b) 24 hours after BrdU injection showing migration of labeled cells onto the villi (arrowheads). Scale bar 100 µm. (From: Algül, H., Schneider, M.R., Dahlhoff, M., Horst, D., et al. (2010) A key role for E-cadherin in intestinal homeostasis and paneth cell maturation. Plos One 5, e14325.)

duodenum has Brunner's glands in the submucosa which secrete mucin and bicarbonate, and the ileum contains lymphoid masses called Peyer's patches, associated with M cells in the epithelium. There are also differences in the types of enteroendocrine cells in the three regions.

The large intestine, or colon, is larger in circumference and histologically it differs from the small intestine in that there are no villi and no Paneth cells, the goblet cells are more abundant and the repertoire of enteroendocrine cells is different (Figure 10.1b). As in the small intestine, cell division occurs in the crypts and differentiated cells are exported to the luminal surface. The overall cell renewal rate in the large intestine is somewhat slower than the small intestine.

Intestinal Stem Cells

In many ways the intestinal epithelium currently represents the best understood tissue-specific stem cell system. The stem cells can be identified in vivo, their cell lineage can be visualized, their cell kinetics has been well studied, they can be cultivated in vitro, and their mechanism of differentiation is fairly well understood.

Proliferation in the intestine is dependent on Wnt signaling. Conditional knockouts of Wnt pathway components will bring cell renewal to a halt, and overexpression of Wnt pathway components is associated with intestinal tumors. A key breakthrough came with the discovery of leucine-rich G protein-coupled receptor 5 (LGR5) as a stem cell marker (Figure 10.C.1a,b). This is a co-receptor for Wnt factors, responding to a Wnt agonist called R-spondin, and is expressed on about 12–14 cells at base of each crypt in mouse, interspersed with the Paneth cells. The *Lgr5* knockout is a neonatal lethal, not because of effects on the intestine but because effects on the tongue prevent suckling. The key evidence that the LGR5-positive cells are stem cells comes from CreER labeling experiments. In these, a transgenic mouse is made in which an *Lgr5* promoter drives a *CreER* gene. It also contains a reporter, such as *lacZ* or *GFP*, whose expression is activated by excision of a transcription stop sequence flanked by *loxP* sites. The cells expressing *Lgr5* continuously produce CreER. If tamoxifen is given to the mice, the CreER becomes active and excises the transcription stop sequence in front of the reporter. Those cells will then express the reporter thereafter, regardless of their state of differentiation (see Chapter 3 for a fuller description of this important method). The results in the intestine are as follows (see Figure 1.5 in Chapter 1; also Figure 10.C.1c). Shortly after the tamoxifen dose, some LGR5-positive cells themselves are labeled. A few days later, from each labeled cell at the crypt base a file of labeled cells may be seen extending up the crypt and up the neighboring villus. These files include cells of all the intestinal types found on the villi: absorptive, goblet and enteroendocrine. After a few days, the files extend right to the villus tips where they terminate, and also some Paneth cells at the crypt base become labeled. If the tamoxifen dose is a low single dose, the recombination is inefficient and only a few well separated stem

cells become labeled. If tamoxifen doses are large and repeated then a high proportion of stem cells can be labeled leading to labeling of most of the epithelium. These results reveal several things. First, the stem cells are permanent. Once a clone has become labeled it persists long term. Second, the stem cells lie in the crypt base because this is where labeled cells appear first following the tamoxifen dose. Third, the stem cells can produce all the cell types of the epithelium. Even well-spaced clones contain all the cell types showing that they must arise from single stem cells. It also confirms what was previously deduced from ^{3}HTdR and BrdU labeling studies, that the cells originate in the crypt base, migrate up to the villus tips and then die. For most of the cell types the turnover time is about 5 days, but, unlike the rest of the epithelium, the Paneth cells have a longer lifetime of 6–8 weeks. In the large intestine the situation appears to be broadly similar. Although there are no actual Paneth cells, there are some CD24-positive cells which may serve the same function of providing a niche for the stem cells. CD24, which is also present on Paneth cells, is a glycosyl phosphatidyl inositol (GPI)-anchored sialoglycoprotein with important functions in the immune system.

The transcription factor that controls stem cell character is ASCL1, a bHLH type factor normally expressed in the stem cells, whose targets include the *Lgr5* gene. Its expression is induced by Wnt signaling and its gene is also auto-activating, so that once it has been turned on, it remains on (in other words it is a bistable switch, see Chapter 7). Conditional knockout of *Ascl1* will destroy the stem cells, although in vivo the rate of cell turnover is so high that the intestine rapidly becomes repopulated from the few surviving wild type crypts. Overexpression of *Ascl1* will increase the number of stem cells and give rise to hyperplasia and to crypt fission.

The situation has been somewhat complicated by the discovery of other stem cell markers which behave in a similar way to *Lgr5* in CreER experiments, but may be present on different cell populations. There has been a persistent suggestion of the existence of some radiation-resistant stem cells around the +4 position in the crypt (i.e. about four cells up from the crypt base) but this remains uncertain. These cells have been described as expressing BMI1, a component of the PRC1 polycomb complex regulating chromatin state; also LRIG1, a membrane receptor, telomerase, which rebuilds telomeres after cell division, and HOPX, an atypical homeoprotein. However it is now thought that all these markers are also expressed in the LGR5-positive crypt base cells, so the clonal labeling experiments not surprisingly give similar results. In addition there is CD133 (= Prominin), a transmembrane glycoprotein beloved of those who work with cancer stem cells, but this is actually expressed in the whole dividing population of the crypt.

Analysis of the effects of radiation damage indicate that a larger population of cells per crypt are capable of regenerating the crypt than normally act as stem cells in the steady state. There are about 30–40 regeneration-competent cells as opposed to 6–8 steady state stem cells estimated from cell kinetic measurements or 12–14 cells positive for LGR5. Indeed it is possible to treat transit amplifying cells with WNT3A to mimic normal niche conditions and thereby cause them to produce organoids in vitro, which is a behavior characteristic of stem cells (see below). Once again this fact emphasizes the importance of the local cell environment in determining whether or not specific cell populations do or do not behave as stem cells.

In the human intestine, the current lack of good antibodies for LGR5 makes it impossible to establish that it also serves as a stem cell marker in humans. However, cells from the crypt base isolated by sorting for the Wnt target Ephrin 2 will generate organoids in Matrigel expressing high levels of LGR5, so the situation is probably similar. A clonal labeling study using the loss of cytochrome c oxidase from mitochondria by somatic mutation (see Chapter 3) shows stem cell derived clones similar in behavior to those shown by LGR5 positive cells in the mouse.

The smallest individual clones occupy about 15% of crypt circumference, indicating that there are about six stem cells/crypt, as in mice.

In Vitro Culture

Tissue-specific stem cells are notoriously difficult to culture in vitro, but this has been achieved for intestinal stem cells in the form of organ cultures. Single LGR5-positive cells cultured in laminin-rich Matrigel in the presence of various factors including the Wnt agonist R-spondin, noggin, EGF and Notch ligand, will grow to become organoids resembling a mini-intestine with villi extending inward and crypt-like regions on the outside (Figure 10.3). In vivo it appears that the Paneth cells provide a niche for the stem cells by providing them with WNT3 and other Wnt factors. Removal of Paneth cells reduces the number of functioning LGR5-positive cells, and inclusion of Paneth cells in the organoid cultures enables them to grow in the absence of exogenous Wnt, although R-spondin is still required in the medium. LGR5-positive cells from the mouse colon will also produce organoids in Matrigel. The normal source of R-spondin in vivo is probably from the underlying stromal tissue and not from the epithelium itself.

Clonality of Intestinal Crypts

It first became possible to examine the clonal structure of animal tissues when mouse aggregation chimeras and X-inactivation mosaics were studied. Aggregation chimeras are embryos formed by aggregating together two morulae of different genetic strains to form a single embryo. The resulting mice consist of a mixture of cells from the two strains. X-inactivation mosaics are females heterozygous for a marker on the X-chromosome (described in Chapter 5). In both cases the whole body consists of two cell populations which can be distinguished using suitable antibodies or

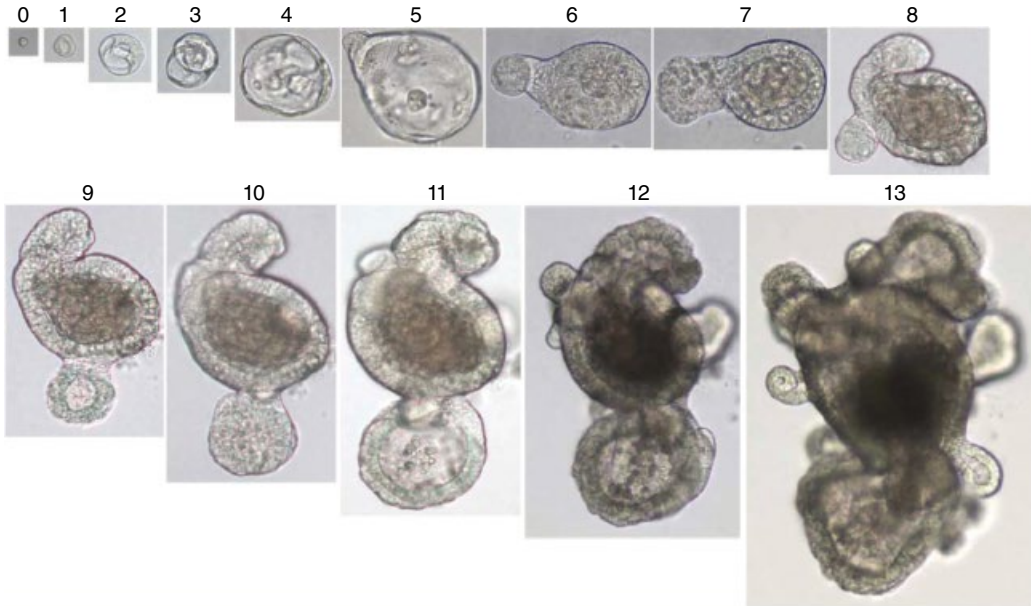

Figure 10.3 Intestinal organoid growing in Matrigel. This colony was founded by a single Lgr5+ stem cell, and growth was recorded each day. The numbers of days of development are shown. (From: Sato, T., Vries, R. G., Snippert, H. J., van de Wetering, M., et al. (2009) Single Lgr5 stem cells build crypt–villus structures in vitro without a mesenchymal niche. Nature 459**,** 262–265. Reproduced with the permission of Nature Publishing Group.)

histochemical methods. A striking finding was that intestinal epithelial crypts were almost always of a single genotype, indicating that they were the clonal progeny of a single cell. The same applies to humans, an example from human colon being shown in Figure 10.4. This specimen was derived from a female heterozygous for a null allele of glucose-6-phosphate dehydrogenase, whose gene lies on the X-chromosome. Due to the process of X-inactivation about half the cells in her body do not express the normal allele and appear negative in histochemical staining. In the specimen the enzyme appears in contiguous patches containing many crypts. All labeled crypts are fully labeled, and there are no partly labeled crypts.

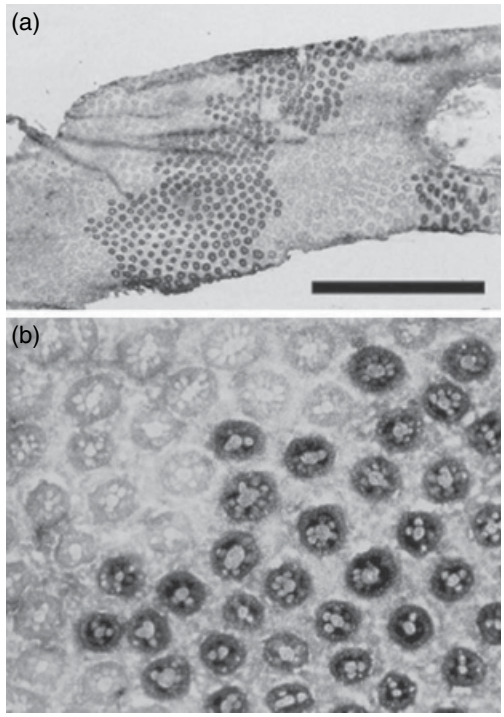

Figure 10.4 X-inactivation clones visualized in the human colon. The specimen is a frozen section from a female heterozygous for the X-linked gene encoding glucose-6-phosphate dehydrogenase, and is stained to reveal presence of the enzyme. (a) Low power, scale bar 2 mm. (b) High power. None of the crypts show mixed clonality. (From: Novelli, M., Cossu, A., Oukrif, D., Quaglia, A., et al. (2003) X-inactivation patch size in human female tissue confounds the assessment of tumor clonality. Proceedings of the National Academy of Sciences of the United States of America 100, 3311–3314. Reproduced with the permission of The National Academy of Sciences.)

The obvious conclusion from such data was that each crypt contained just a single stem cell. But this was out of line with contemporary cell kinetic studies which indicated 6–8 stem cells/crypt, and even more out of line with recent counts of LGR5-positive cells at the crypt base (12–14 cells). How can such divergent findings be reconciled? The answer lies in the mode of division of the stem cells. If individual stem cells are labeled it is possible to observe directly how many of them undergo equal or unequal divisions. For example, stem cells can be labeled using *Lgr5-CreER x R26R-Confetti* (Figure 10.C.1c). In this system the Confetti reporter expresses one of several different colored fluorescent proteins following CRE-induced recombination (see "Brainbow" techniques, Chapter 3). If a tamoxifen dose is given suitable to induce recombination in about 1 crypt in six, then most crypts containing any labeled cells at all have only a single one. After one cell division only about 10% of labeled clones still contain a single LGR5-positive cell, meaning that the original cell divided into one stem cell and one transit amplifying cell. In about 45% of cases the progeny are both LGR5-positive, indicating division to two stem cells, and in the other 45% neither are LGR5-positive, indicating division to two transit amplifying cells. Because only stem cells persist long term, this stochastic mode of division guarantees that, if a crypt remains labeled, the number of labeled cells must progressively increase until the progeny of only one stem cell remains, although the total number of stem cells in the crypt is still the same. In fact after about two weeks, some crypts are becoming monoclonal with respect to the Confetti label, and after 2 months most of them are. A similar situation exists in the newborn mouse, studied using aggregation chimeras or X-inactivation mosaics. Shortly after formation of the crypts they are all polyclonal, but after about 2 weeks, most are monoclonal. In the

newborn mouse the gut is growing rapidly and so crypts are frequently dividing into two. Since the available stem cells will be partitioned at random between the two crypts this accelerates the process of clonal reduction. The mechanism of crypt fission is still not well understood but it is an essential process for normal growth and for regeneration following damage.

The example of intestinal crypt clonality underlines the need for clear thinking about the interpretation of cell labeling data. The fact that a crypt is monoclonal means that it derives from a single cell present at or after the time of labeling. It does not mean that there is any particular difference between crypts, apart from the markers used for labeling. Nor does it mean that the ancestor cell of a particular crypt is any different from the other stem cells in the same crypt before the process of clonal selection. Nor, of course, does it mean that crypts contain only a single stem cell. Cell kinetic calculations indicate that there are about 6–8 stem cells per crypt in the steady state. As mentioned above, this is many fewer than the number of potentially regenerative cells per crypt (30–40); it is also fewer than the number of LGR5-positive cells (12–14); but it is more than the single ancestral cell indicated by clonal marking techniques followed by a period of clonal selection. So at any one time the stem cell population is a subset of the LGR5 positive population and exactly which cells are behaving as stem cells depends on their local environment and interactions. The idea, embedded in the definition of tissue-specific stem cells, that they should persist for life, must of course be taken to refer to the population of stem cells rather than to any one specific cell lineage. This was emphasized in the discussion of in vivo cell labeling presented in Chapter 1.

The Epidermis

In the mouse the epidermis becomes specified at about 9 days of gestation and fully formed shortly before birth. It is a familiar fact that the epidermis of the skin is in a state of continuous cell turnover. It has been estimated that complete renewal occurs in about 9 days in the mouse and about one month in humans, although there is variation in different parts of the body. The epidermis is a squamous epithelium composed of cells called keratinocytes. This means it is multilayered, with cuboidal cells in the basal layer and progressively more flattened cells towards the external surface (Figure 10.5). Histologically the epidermis consists of a stratum germinativum, the basal layer where all cell division occurs; and above this, from inside to outside, the stratum spinosum, stratum granulosum and stratum corneum. In the stratum corneum the cells are dead and consist mostly of flattened sacs of crosslinked cytokeratin proteins. The epidermis lies on a basement membrane and the underlying dermis contains loose connective tissue, adipose tissue, nerves and blood vessels. In humans there is a characteristic pattern of undulations called dermal papillae, a name also given to the dermal component of the hair follicle in mouse and human.

As keratinocytes move up through the layers, the gene expression pattern changes. Basal layer cells express keratin 5 and 14 (K5, K14), while the higher, post-mitotic, layers express K1 and K10. The *K14* promoter is often used in genetic experiments in mouse to drive gene expression in just the basal layer. In the granular layer are found the proteins involucrin and loricrin. These become cross-linked by transglutaminase to form a cornified envelope around the cell. In the stratum corneum the nuclei and other cell organelles are lost. The key gene determining the properties of the epidermis, as well as all other squamous epithelia, is *p63*, encoding a transcription factor structurally related to the famous p53, important for its involvement in human cancer. The direct targets of p63 include the *K14* gene. In mice, the knockout of *p63* prevents formation of squamous epithelia and is lethal.

Because of the continuous cell turnover it is evident that the basal layer must contain

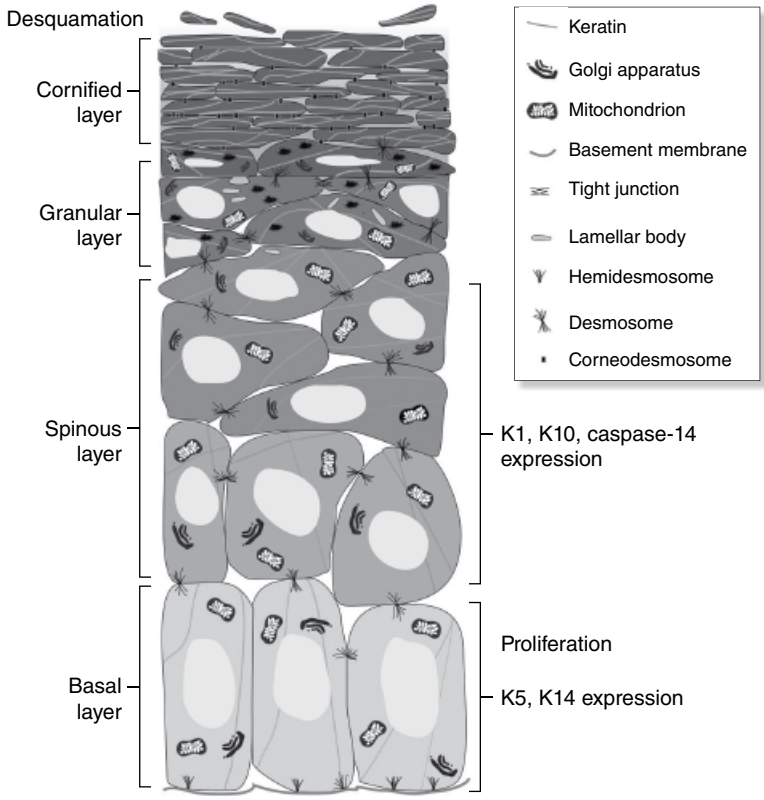

Figure 10.5 Structure of the epidermis. Cell division occurs in the basal layer and progeny are displaced upward. As they move up the keratinocytes differentiate and eventually die and are shed. (From: Eckhart, L., Lippens, S., Tschachler, E. and Declercq, W. (2013) Cell death by cornification. Biochimica et Biophysica Acta (BBA) – Molecular Cell Research 1833, 3471–3480. Reproduced with the permission of Elsevier.)

stem cells. There has been some controversy about whether the stem cells are a small subset of the dividing cells, or whether the basal layer forms a single stem cell compartment with any individual cell having some probability of persisting long term. The basal layer cells express AXIN2, a Wnt signaling target. Wnt signaling does seem to be necessary for normal epidermal homeostasis, as knockouts of pathway components lead to a thin epidermis. The main source of Wnt factors appears to be the basal layer itself, however it is probable that additional factors from the dermis are also needed. Labeling of individual basal layer cells with *K14-CreER* or *Axin2-CreER* shows the expected behavior of stem cells (Figure 10.C.2). Shortly after the tamoxifen dose, there are many small labeled clones in the basal layer. In the long term a few labeled clones persist, spanning the full thickness of the epidermis. Observation of cells shortly after labeling indicate that both symmetric and asymmetric divisions take place, as for intestinal stem cells. There has been disagreement about whether a minority of cells in the basal layer are true stem cells and the remainder transit amplifying cells, or whether they are all equivalent. Mathematical analysis of the labeling studies shows progressive increase of clone size, reduction of clone number, and scaling behavior. As discussed in Chapter 1 this is consistent with

the stochastic model in which all dividing cells are equivalent and they exhibit stem cell behavior as a population.

Epidermal cells can be cultured in vitro. It was discovered in the 1970s that they would form a multilayer tissue similar to the normal epidermis if cultured in the presence of feeder cells. However, like all primary cell cultures these do not grow without limit. It is possible to grow large sheets of cells from a small biopsy of human skin and these have been used on a small scale as autologous grafts to treat severe burns (see online supplement). The grafted skin appears to persist indefinitely but it does not form hair follicles or sweat glands.

In vitro culture also indicates the existence of cells with different propensities for division. Some cells form very large clones while others form only small clones (Figure 1.4). This is one argument for the existence of a hierarchy of dividing cells in the basal layer, as opposed to the stochastic model. It has been considered that the former are the true stem cells while the latter are transit amplifying cells, and in fact the large clone-forming cells do have a higher level of p63 than the others.

Although the keratinocyte is the most abundant cell type in the epidermis, there are some others. Melanocytes are pigment cells derived from the neural crest. They export pigment granules to epidermal cells and to hairs and hence give them their characteristic colors. Langerhans cells are dendritic antigen-presenting cells and are derived from the hematopoietic stem cells of the bone marrow. The Merkel cells are small oval mechanoreceptor cells with connections to the sensory system. Cell lineage labeling using *K14-CreER* suggests that Merkel cells are derived from the epidermal stem cells. Moreover, removal of the transcription factor ATOH1 using *K14-Cre*×*floxed Atoh1* prevents their formation. So although the epidermal stem cell is generally considered as unipotent, forming only keratinocytes, it does actually generate more than one type of differentiated cell. Moreover the need for ATOH1 indicates a possible similarity in cell differentiation mechanism to that of the intestine (see Chapter 9).

Hair Follicles

In addition to the squamous epithelium covering the body, the mammalian epidermis gives rise to various appendages: the hairs, nails, sweat glands and mammary glands. In each case the arrangement of stem cells sustaining these structures is somewhat different from the rest of the epidermis (usually called interfollicular epidermis). Hair follicles have been studied very intensively in recent years although a complete understanding of their component cell types and the relationship between them is still lacking.

Hair follicles are formed from ingrowths of the epidermis during embryonic life, as described in Chapter 8. At the base of the mature hair follicle the epithelium surrounds a specialized clump of dermal cells: the dermal papilla, to form a hair bulb. Within this is the hair germ, a region of dividing epidermal cells that generates the multiple layers of keratinized cells within the hair shaft as well as an inner root sheath around the hair shaft and an outer root sheath lining the cavity (Figure 10.6). Some way up the follicle is a sebaceous gland, continuous with the outer root sheath. Each hair follicle is innervated by a sensory neuron and has a small muscle, the arrector pili (sometimes written erector pili) muscle, which can raise the external hair shaft on contraction.

The dermal papilla is critical as a provider of various extracellular signals needed for hair growth and differentiation. These include FGF7, BMPs and Wnts. New follicles can be induced in interfollicular epidermis by implantation of dermal papillae. They can also be induced by overexpression of Wnt signaling components. It might seem obvious that the epidermal stem cells that sustain hair growth should be found at the base of the hair shaft in contact with the dermal papilla. However there is good evidence that the principal reservoir of stem cells lies further up the follicle in a region known as

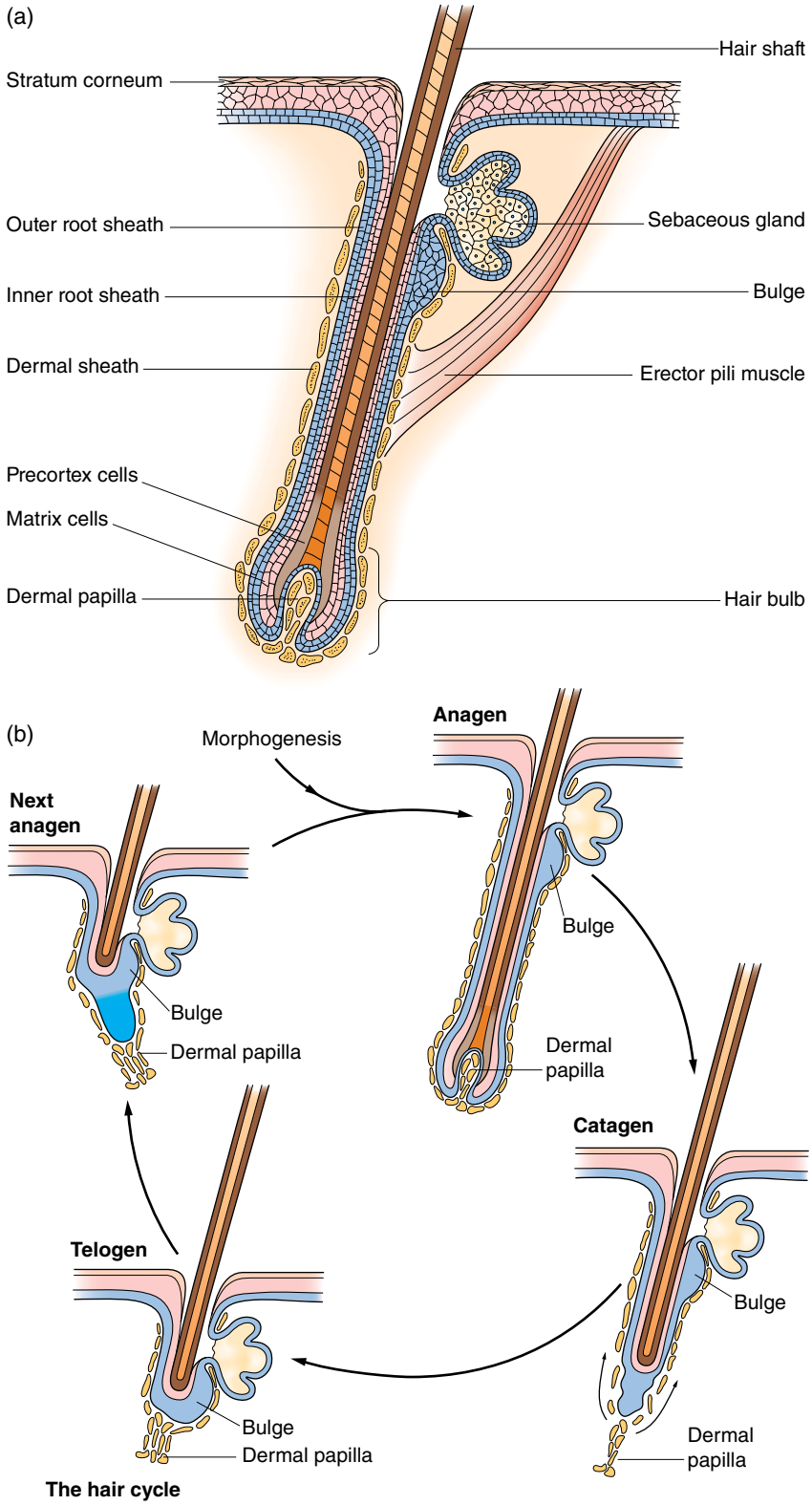

Figure 10.6 (a) Structure of the hair follicle. (b) The hair cycle. (Slack, J.M.W. (2013) Essential Developmental Biology, 3rd edn. Reproduced with the permission of John Wiley and Sons.)

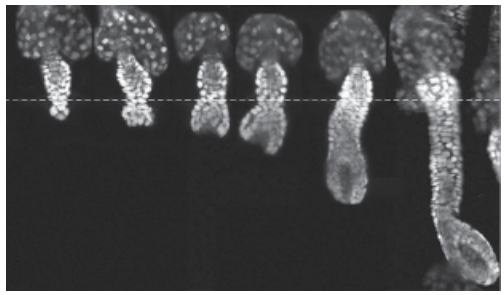

Figure 10.7 Growth of a hair follicle during the hair cycle. These are follicles viewed in vivo by multiphoton microscopy in a mouse expressing a *K14-H2BGFP* reporter. The bulge region lies above the dashed line. As growth commences cells from the bulge move down to surround the dermal papilla. They form a new hair bulb which generates all layers of the hair shaft and the outer and inner root sheaths. (From: Rompolas, P. and Greco, V. (2014) Stem cell dynamics in the hair follicle niche. Seminars in Cell and Developmental Biology 25–26, 34–42. Reproduced with the permission of Elsevier.)

the bulge (which may or may not be an actual physical bulge). This does make sense given that the process of hair growth is cyclical. Individual follicles go through successive phases of growth (anagen), regression (catagen) and dormancy (telogen) (Figures 10.6b and 10.7). In the mouse the first anagen lasts from about postnatal day 6–16, followed by a short telogen and a second anagen to about day 42. There is some regional variation in these timings. During catagen, the cells of the lower part of the follicle are destroyed by apoptosis and the follicle shortens so that the bulge region is brought close to the dermal papilla. The bulge originally attracted attention as a possible location of stem cells because it contained label-retaining cells and because cells derived from the bulge gave rise to long-lived clones in vitro, while those of the hair germ gave rise to shorter lived ones. This last experiment depended upon manual microdissection of the whisker follicles found in rodents which are much larger than other follicles.

Cells of the bulge and the hair germ share various markers including K14, K19, SOX9 and LGR5. However they do also differ in some respects: for example the bulge expresses the cell surface glycoprotein CD34 while the hair germ expresses the cell adhesion molecule P-cadherin. *LGR5-CreER* labeling during telogen indicates that descendants of *Lgr5*-positive cells populate all parts of the follicle below the sebaceous gland in the course of the next anagen (Figure 10.C.3). So it is probable that the bulge is the permanent stem cell niche, and that it provides cells to populate the hair germ during anagen.

Located as they are in the bulge, the hair follicle stem cells are distinct in location and properties from those of the interfollicular epidermis. However, following wounding, cells from the hair follicles can repopulate the interfollicular epidermis, at least for a period.

Human hair follicles are morphologically similar to those of rodents and their stem cells are probably similar. However the timing of the hair growth cycle is quite different: it is asynchronous between follicles, and much longer than in the mouse: anagen may last for a few years and telogen a few months. The morphological sub-types of hairs and hair follicles also differ between rodents and humans.

Cornea and Limbus

The cornea is the transparent structure at the front of the eye (Figure 10.8a). It is responsible for most of the focusing of incoming light onto the retina, although the lens also contributes to focusing and through its shape changes provides accommodation for different distances. The cornea is composed of three cell layers: an outer epithelium, which is a derivative of the epidermis; a central stroma, mostly composed of collagen fibers; and an inner endothelium. Of these components, the outer epithelium is a renewal tissue maintained by a population of stem cells. The stem cells reside in an annular region around the periphery of the cornea called the limbus. This contains cells high in p63, expresses the transporter protein ABCB5, and, like the basal layer of the epidermis,

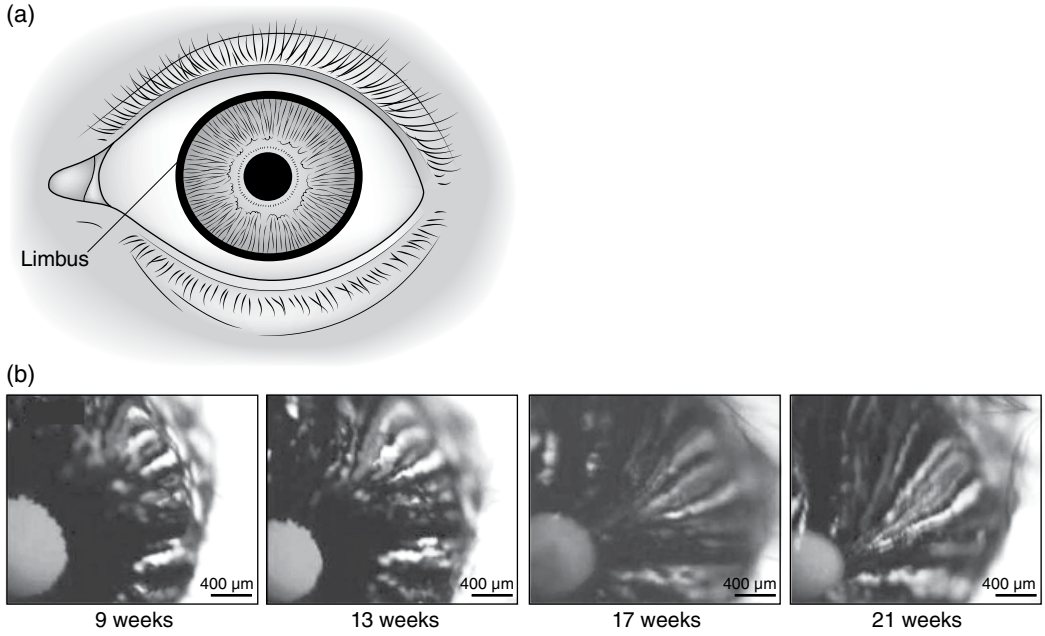

Figure 10.8 (a) Location of the limbus at the periphery of the cornea. (b) Lineage tracing of stem cells in the limbus. Mice were *K14-Cre-ER x R26R Confetti*. Tamoxifen was administered at 6 weeks of age and labeled clones in the cornea visualized after the indicated number of weeks. By 21 weeks clones have reached the center of the cornea. (*Sources:* (a) Modified from Pellegrini, G., Golisano, O., Paterna, P., Lambiase, A., et al. (1999) Location and clonal analysis of stem cells and their differentiated progeny in the human ocular surface. Journal of Cell Biology. 145, 769–782. Reproduced with the permission of The Rockefeller University Press. (b) From Di Girolamo, N., Bobba, S., Raviraj, V., Delic, N.C., et al. (2015) Tracing the fate of limbal epithelial progenitor cells in the murine cornea. Stem Cells 33, 157–169. Reproduced with the permission of John Wiley and Sons.)

expresses cytokeratins 5 and 14. The central cornea does not express these cytokeratins, nor the characteristic K1/K10 found in the upper layers of the epidermis. Instead the central cornea expresses K3 and K12. When a *K14-CreER × R26R-Brainbow* mouse is labeled with a low dose of tamoxifen some limbal stem cells become labeled. These subsequently generate radial streaks of progeny from periphery to the center of the cornea, which is typical stem cell behavior (Figure 10.8b). Limbal stem cells can also be cultured in vitro and are capable of generating large clones.

When a corneal graft is carried out, the epithelium becomes renewed from the limbus of the host. If this does not occur the cornea will become covered with connective tissue and blood vessels and cease to be transparent. Limbal cells can be expanded in vitro and grafts of limbal cells from one eye to the other have been used to support and maintain corneal grafts. The indication for this is usually in cases where the whole front of one eye, including the limbus, has been destroyed (see online supplement).

Mammary Glands

Another derivative of the epidermis is the epithelium of the mammary glands. As described in Chapter 8, the post-pubertal, but virgin, mammary gland consists of a branched duct system connected to the nipple and embedded in a connective tissue stroma. The ducts have a layer of myoepithelial cells around the outside and luminal cells on the inside (Figure 10.9). Although the basic cell types are the same, there are some differences between humans and mice

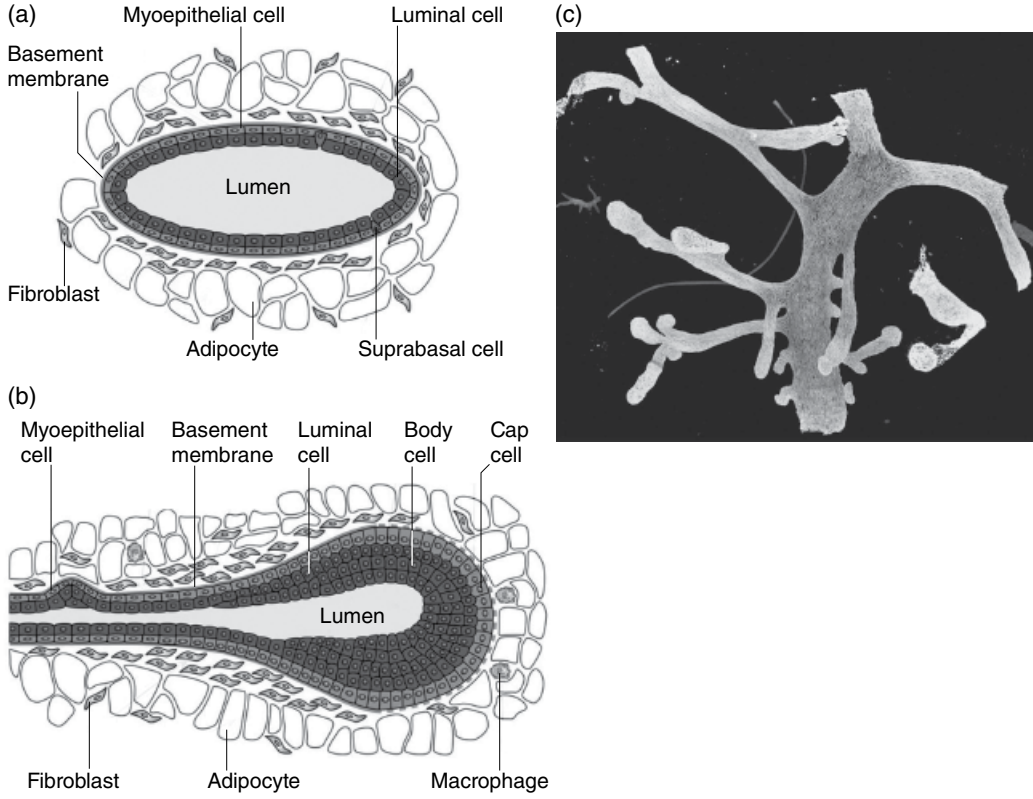

Figure 10.9 Structure of the mammary epithelium in the mouse. (a) Duct showing myoepithelial and luminal cell layers. (b) A terminal end bud. (c) Whole mount of a 3 week old mouse mammary tree, immunostained for keratin 5. (*Sources:* (a&b) from Visvader, J.E. (2009) Keeping abreast of the mammary epithelial hierarchy and breast tumorigenesis. Genes and Development 23, 2563–2577. Cold Spring Harbor. (c) from Rios, A.C., Fu, N.Y., Lindeman, G.J. and Visvader, J.E. (2014) In situ identification of bipotent stem cells in the mammary gland. Nature 506, 322–327. Reproduced with the permission of Nature Publishing Group.)

(Figure 10.10). Human females have just one pair of mammary glands while mice have five pairs. In mice the termini of the duct system form terminal end buds which are growing points, in humans the termini are clusters of small lobules called terminal ductal lobular units. Moreover the mouse stroma is mostly adipose tissue while in humans there is also considerable fibrous connective tissue.

After the post-pubertal maturation of the system, there a limited growth and regression with each estrus/menstrual cycle. But the main developmental changes occur during pregnancy and lactation (Figure 10.11). During pregnancy the epithelium proliferates considerably and generates a large number of secretory alveoli. The alveoli differentiate during mid-late pregnancy and in mice the transcription factor ELF5, specifically expressed in luminal cells, is required for differentiation. After birth of the offspring, the alveoli secrete milk by an apocrine mechanism, meaning that distal portions of cells containing the components of the milk become detached from the cells and enter the duct system. Contraction of the myoepithelial cells helps drive the milk towards the nipple. High level milk secretion continues during lactation. After lactation is finished there is an involution of the organ involving massive cell death, collapse of the alveoli, and remodeling of the epithelium back to the pre-pregnancy appearance.

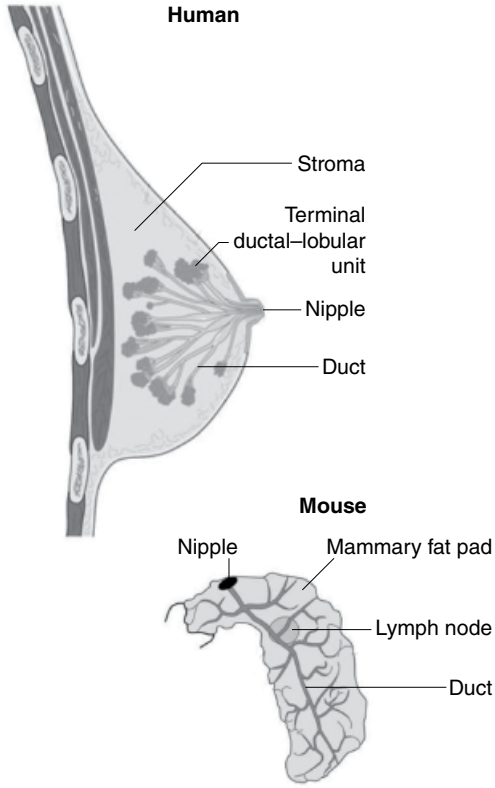

Figure 10.10 Differences between human and mouse mammary glands. (From: Visvader, J.E. (2009) Keeping abreast of the mammary epithelial hierarchy and breast tumorigenesis. Genes and Development 23, 2563–2577. Cold Spring Harbor Laboratory Press.)

The main hormonal stimulus for these changes is prolactin from the anterior pituitary gland. This is a cytokine-type hormone with a cell surface receptor connecting to the JAK-STAT signal transduction pathway (similar to LIF, see Figure 6.1b). In late pregnancy the related hormone placental lactogen, secreted by the placenta, plays a similar role, but after birth prolactin again becomes predominant. STAT5 is the critical intracellular mediator. It is essential for pregnancy in mice and its direct targets include the genes encoding milk proteins and junctional complexes for the secretory cells of the alveoli. Other important signal molecules, which are locally produced in response to the systemic hormones, are RANK-L and members of the epidermal growth factor family.

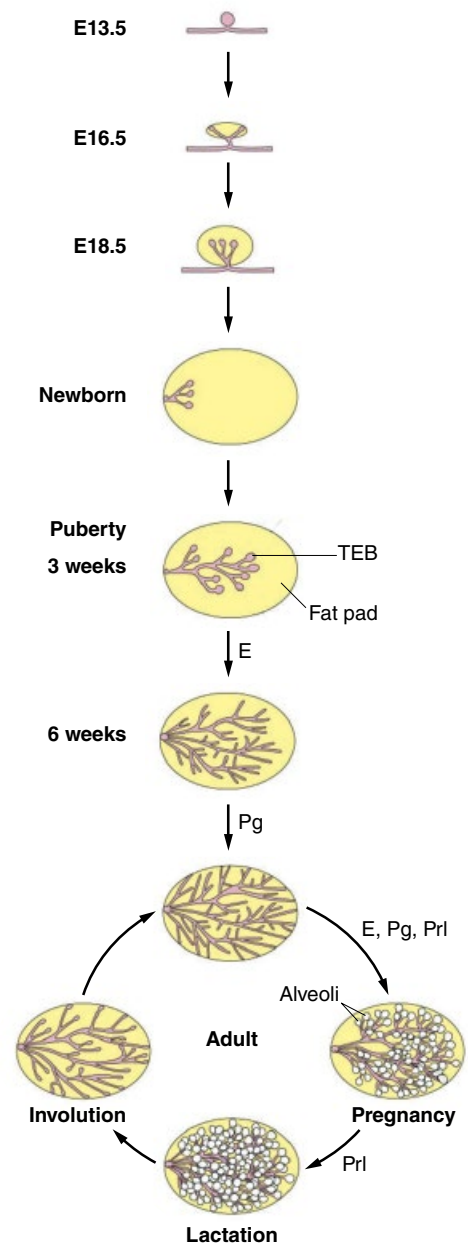

Figure 10.11 The complete development of the mouse mammary gland. At the bottom are shown the phases of growth, secretion and involution accompanying pregnancy and lactation. TEB: terminal end bud; E: estrogen; Pg: progesterone; Prl: prolactin. (From: Visvader, J.E. and Stingl, J. (2014) Mammary stem cells and the differentiation hierarchy: current status and perspectives. Genes and Development 28, 1143–1158. Cold Spring Harbor Laboratory Press.)

Mammary Stem Cells

Although the mammary epithelium is not continuously renewing like the intestinal epithelium or the epidermis, it does obviously require considerable cell renewal to enable the cycles of growth and regression accompanying pregnancy. Modern human females now have few pregnancies in a lifetime, but wild animals such as mice have very many and we should therefore expect to find stem cells in their mammary glands.

The study of mammary stem cells and cell lineage has largely depended on transplantation assays. Because cells may behave in a different way on transplantation, or on in vitro culture, compared to their normal situation in the body, the interpretation of these data requires some care. The usual transplantation system is the "cleared fat pad" of mouse mammary gland. Three week old mice have the nipple region and associated duct system removed from one mammary gland (usually number 4) and this leaves a "fat pad" consisting of adipose tissue, loose connective tissue and blood vessels without an epithelial component. In this environment grafts of pieces of duct system, or even single cells, from genetically compatible mice can develop into a complete duct system (Figure 10.12a). The fact that a single cell from an adult gland can support development of the whole duct system, and undergo differentiation during pregnancy, is considered evidence for the presence of cells with multipotent potential. Serial transplants have been carried out with explants of tissue for as many as seven transplant generations and this indicates that the stem cells are capable of sustaining growth of a mammary duct system for more than a normal mouse lifespan, as is also found for the epidermis and the hematopoietic system. The cleared fat pad system can be modified for the growth of human mammary epithelium by using NOD-SCID (immunodeficient) mice and also by injecting some human fibroblasts into the fat pad to "humanize" the local environment.

There is also a cell culture system in which dissociated mammary cells are cultured in non-adherent dishes in the presence of EGF or FGF. This gives rise to "mammospheres", small cell clusters which can differentiate into ductal and myoepithelial cells and can be serially propagated. These are presumed to contain mammary stem cells, and have an obvious resemblance to the cultured neurospheres which contain neural stem cells (see Chapter 9).

The mouse cells which give good efficiency in transplantation assays are high in α6 and β1 integrins (also called CD49f and CD29 respectively), in the P-selectin ligand (CD24), and they lack Sca1, a GPI-linked cell surface protein found on hematopoietic stem cells. In humans, various cell types have been reported as being enriched in their ability to form mammospheres. These include high α6 integrin cells, aldehyde dehydrogenase 1 expressing cells, "side population" cells which exclude the dye Hoechst 33342, or slow dividing cells which retain the vital dye PKH26.

Because of the uncertain relationship between transplantation, or in vitro culture behavior, and cell behavior in vivo it is also reassuring to have some lineage label results in vivo. These have been hampered by the fact that tamoxifen, used to activate CreER, has deleterious effects on mammary development. However, it is possible to use doxycycline to induce expression with transgenic mice of the type: *promoter-rtTA; TRE-Cre; R26R.* The promoter of interest drives expression of the Tet activator rtTA. In the presence of doxycycline, this binds to the Tet response element (*TRE*) and causes transcription of *Cre*. This excises the stop sequence from the R26R reporter and causes permanent expression of a marker gene. The *keratin 5* promoter is active in basal mammary cells and can be used to drive the expression of *rtTA*. If a doxycycline pulse is administered in adult life the labeled clones are initially basal but extend to the luminal surface after a few weeks (Figure 10.C.4). These multilayer clones can be quite large and they can also label the alveoli that form during pregnancy. If the doxycycline is administered at the start of puberty, small multilayer clones become labeled all over the duct system. If the rtTA is driven by the *Elf5* promoter, active in luminal cells, then clones

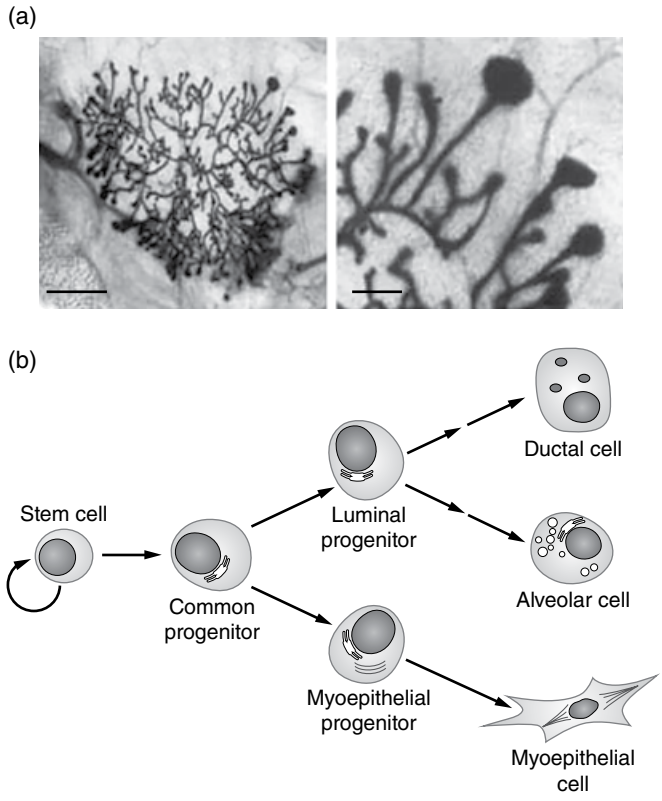

Figure 10.12 Mammary stem cells. (a) Growth of a whole mammary tree from a single stem cell transplanted to a cleared mammary fat pad. Scale bars: 250 µm for low power, 50 µm for high power. (b) Putative cell lineage for mammary stem cells. (*Sources:* (a) from Shackleton, M., Vaillant, F., Simpson, K.J., Stingl, J., et al. (2006) Generation of a functional mammary gland from a single stem cell. Nature 439, 84–88. Reproduced with the permission of Nature Publishing Group. (b) from Visvader, J.E. (2009) Keeping abreast of the mammary epithelial hierarchy and breast tumorigenesis. Genes and Development 23, 2563–2577. Cold Spring Harbor Laboratory Press.)

are confined to the luminal surface and disappear after 20 weeks, indicating that this promoter is active only in progenitor cells and not in stem cells.

The evidence is good from both transplantation and cell labeling studies for the existence of multipotent stem cells that sustain the growth, differentiation, and involution of the mammary epithelium. But at present the system is not as well characterized in terms of cell lineage and cell kinetics as the intestinal epithelium or the epidermis. A plausible model for the cell lineage is shown in Figure 10.12b.

The Hematopoietic System

The hematopoietic system generates all of the cells found in the blood and the immune system. These comprise erythrocytes (red blood cells), granulocytes (neutrophils, eosinophils and basophils), monocytes and macrophages, megakaryocytes (which generate blood platelets), and lymphocytes. The non-lymphocytes are referred to as myeloid cells. The lymphocytes comprise B (antibody secreting), T (thymus derived) and NK (natural killer) cells.

One often reads in stem cell literature that the hematopoietic system is the "best understood" system and stands as a model for investigation of the others. This may have been true before the advent of CreER labeling, but it is not so true today. This is because the hematopoietic system has been investigated mostly by transplantation and by in vitro culture, so a lot is known about these aspects, but rather less about the behavior of the stem cells in the situation of normal steady state tissue turnover, or homeostasis.

Analysis by Transplantation and in Vitro Culture

The standard transplantation assay for hematopoietic stem cells (HSC) was discovered as an offshoot of work on the atomic

bomb in the 1940s. It was found that animals exposed to high doses of radiation would die of bone marrow failure, but that they could be saved with a graft of bone marrow from a healthy donor. By the 1950s it was known that the factor in the marrow consisted of cells rather than a hormone-like substance. It was later found that the recipients of bone marrow transplants developed colonies in the spleen. These consisted of various types of hematopoietic cells, sometimes of one type and sometimes of mixed type. It was concluded that each spleen colony-forming unit was a single cell and that the mixed colonies arose from multipotent cells, considered to be stem cells. In fact the spleen colony assay does not capture the most primitive stem cells, as lymphoid tissues are not formed, but it was important for establishing the concept of a single type of hematopoietic stem cell, resident in the bone marrow, which generated all of the cells of the blood and the immune system throughout life.

The system was later dissected using fluorescence activated cell sorting (FACS) to separate subpopulations of bone marrow cells on the basis of their surface antigens. The identity of the resulting cells could then be established by two methods. One was transplantation into lethally irradiated mice. The radiation destroys the stem cells of the host, and if the graft contains stem cells, they can home to the vacant niches in the bone marrow. All components of the blood and immune system are then restored from the stem cells of the graft and the mice would survive long term. If the graft contained only progenitor cells then a partial spectrum of blood cell types would regenerate, but this would be a temporary effect and the mice would soon die. The other method for characterizing sub-fractions of marrow cells was in vitro culture in soft agar or methyl cellulose supplemented with hematopoietic growth factors, such as GM-CSF (granulocyte-macrophage colony stimulating factor), G-CSF (granulocyte colony stimulating factor), M-CSF (macrophage colony stimulating factor) and erythropoietin. The types of colony formed indicate the nature of the progenitor cells, and in the case of the more primitive progenitors, or of the stem cells themselves, mixed colonies would be formed. In addition to cell isolation and characterization, information about the hematopoietic system came from mouse knockouts for particular genes needed for blood formation. Some of these give rise to animals lacking specific sub-lineages of cells. Putting together the information from these three sources gave rise to the familiar diagrams found in textbooks. The details of these diagrams vary slightly, but they all show a single type of long-lived hematopoietic stem cell giving rise to progenitors of restricted potency and eventually to the various types of blood cell and lymphocyte (Figure 10.13).

The transplantation assay depends absolutely on the radiation given to kill the stem cells of the host. Without this the niches remain occupied and graft HSCs have nowhere to go. A period of 4 months after transplantation is generally considered to be long enough for the progeny of committed progenitors to disappear, so donor-derived cells persisting longer than this time are considered to arise from HSC. Donor cells forming all types of blood and immune cells, but not persisting more than 4 months are considered to be "short-term hematopoietic stem cells" (ST-HSC), a commonly used designation which is rather an oxymoron if the definition of a stem cell is considered to include long term persistence. Limiting dilution studies indicate that there are about 2–8 long-term HSC per 10^5 bone marrow cells. In humans, using limiting dilution transplants into immunodeficient mice, which may be a more demanding criterion, the estimate is 1–4 long term HSC per 10^7 mononuclear cells. If the HSC in a graft are progressively diluted with non-repopulating cells, it is possible to show that just a single HSC is capable of bringing about full repopulation. It is possible to do serial transplants from the recipient of a transplant into further irradiated mice, which makes it possible to investigate the ultimate longevity of stem cells. Serial transplantation of unfractionated bone marrow can be carried out about five times,

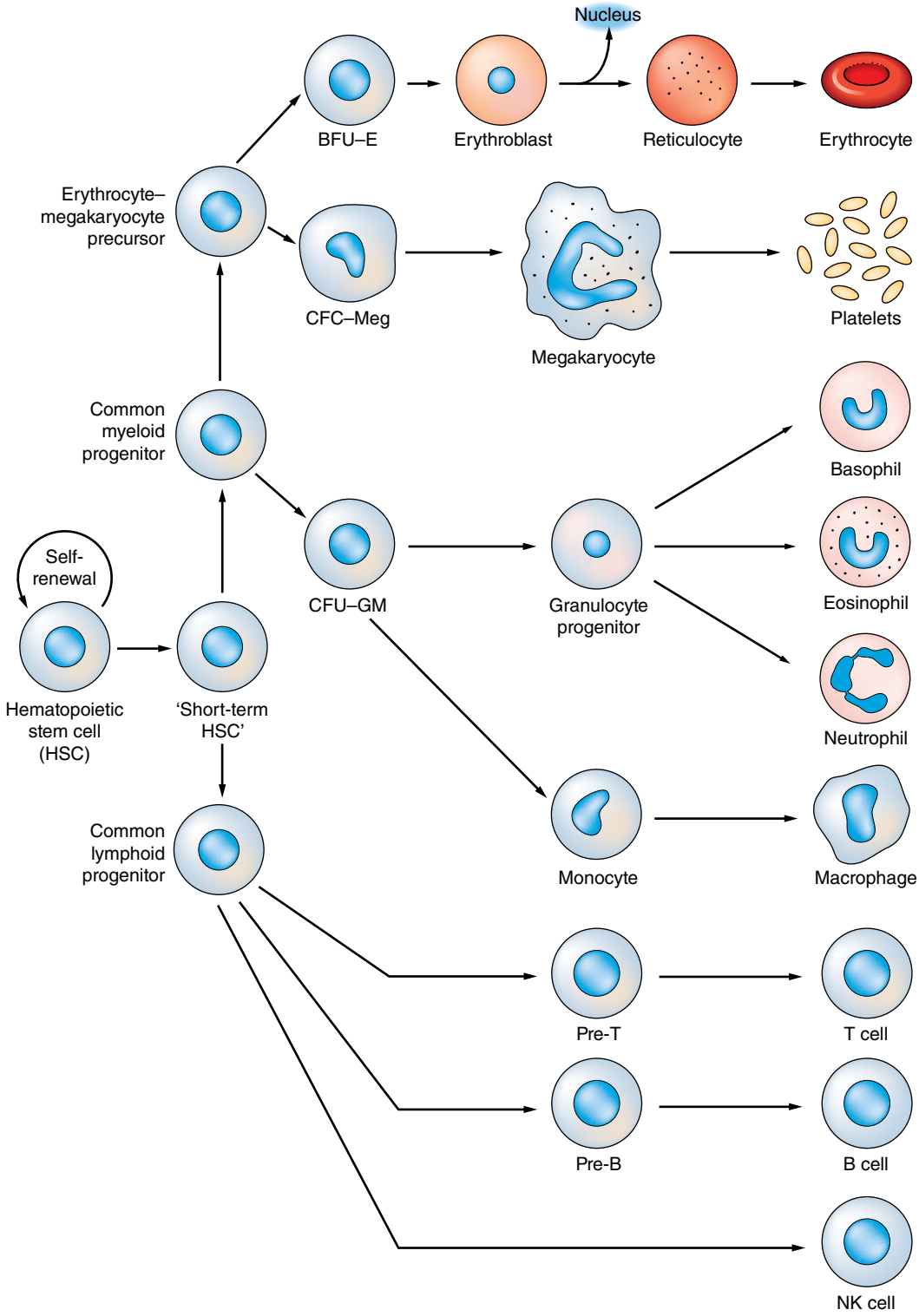

Figure 10.13 Putative hematopoietic lineage. This is a consensus diagram derived from the sort of data described in the text. Other published diagrams may differ in detail. However all have a hierarchical, rather than a reticulate, pattern and a fully multipotent long lived hematopoietic stem cell at the start. (Slack, J.M.W. (2013) Essential Developmental Biology, 3rd edn. Reproduced with the permission of John Wiley and Sons.)

beyond which it fails, at least partly because of telomere erosion. Serial transplantation does show that the HSC population can self-renew for much longer than the lifetime of a single mouse.

It is possible to compare different cell populations for stem cell content or vigor by doing competitive reconstitutions, where two populations of cells are introduced into the same irradiated host. In the blood eventually formed by the grafts, the more vigorous strain will prevail over the less vigorous. For competitive reconstitution assays the two donor strains need to be genetically distinguishable but also equally tolerated by the host.

The hematopoietic stem cells themselves can be isolated by FACS. That of the mouse was first isolated as cells of the constitution Lin^-, $Thy\text{-}1^{lo}$, $Sca\text{-}1^+$. Lin^- indicates the absence of various markers for differentiated blood cells. Thy-1 (= CD90) and Sca-1 are both cell surface GPI-linked glycoproteins, Thy-1 being found at high levels on T lymphocytes. Nowadays mouse HSCs can be isolated by a variety of other criteria, a popular combination being: Lin^-, $Sca\text{-}1^+$, $c\text{-}Kit^+$, $CD150^+$, $CD48^-$. c-Kit (= CD117) is the receptor for the steel growth factor (= stem cell factor, SCF); CD150 (= SLAM) is a cell surface glycoprotein also found on mature T cells; and CD48 (= BLAST-1) is found on mature B lymphocytes. Also the "side population" of cells that rapidly exclude the dye Hoechst 33342, due to the presence of transporter molecules in the membrane, is enriched in HSC (see Figure 1.2 in Chapter 1).

The fact that a single stem cell can repopulate the entire blood and immune system of an irradiated host indicated that the basic hypothesis of the multipotent HSC was correct. The corresponding hematopoietic stem cell in humans is found in the $CD34^+$ fraction. CD34 is a cell surface sialomucin also found on endothelial cells. Not all $CD34^+$ cells are HSCs and human HSCs may be further purified as $CD38^-$, $CD45RA^-$, $Thy\text{-}1^-$. These three components are all found on mature lymphocytes. CD49f (= integrin α6) has also recently been shown to mark human HSC. It may be noted that although the biological properties of mouse and human HSCs are similar, the cell surface markers by which they are identified differ. For example, CD34 is not found on mouse long term HSC. This is because the markers in question, though useful for isolation by FACS, are probably not necessary for the actual stem cell behavior shown by the cells. In vitro experiments with human HSC use soft agar/methyl cellulose culture techniques similar to mouse. In vivo experiments use lethally irradiated immunodeficient mice as hosts. The NOG mouse strain combines the defects of NOD and SCID mice with deficiency of the IL2R γ chain (see Chapter 4). It is highly immunodeficient, lacking B, T and NK lymphocytes, and is currently favored for human grafts.

Recently it has been possible to reprogram committed progenitors and even differentiated blood cells back to HSCs, by introducing a cocktail of transcription factors normally expressed in HSCs. The factors were screened by introducing a large number into the target cells using doxycycline-inducible lentivirus and then grafting them into irradiated hosts. Only if cells have been reprogrammed to HSC status can long term repopulation occur. The transcription factors were identified from those present in the successful grafts and were: HLF, RUNX1t1, PBX1, LMO2, ZPF37 and PRDM5. In cases where the target cells were pre-B cells, their immunoglobulin DNA rearrangements were still present in the donor cells populating the new host, indicating a genuine permanent reprogramming of the pre-B cells arising from the transient expression of the active factors. Furthermore, as with normal HSC, secondary transplantation is possible using grafts from the first hosts, indicating their capacity for long term self-renewal.

Hematopoiesis in the Steady State

There are some difficulties with the standard model for hematopoiesis shown in Figure 10.13, particularly in relation to the immune system. For example, if a skin graft is

given to a newborn mouse, it can render the mouse tolerant to grafts from the same donor strain in adult life. Likewise, if an animal is immunized then antibody producing B-cells and memory T-cells specific to the immunogen may persist for life. Because the functional cells need to undergo specific DNA rearrangements of antibody genes, or of T cell receptor genes, in the course of their maturation, it seems unlikely that they could be continuously renewed from HSCs. It is much more likely that cells which have experienced those specific DNA rearrangements can persist for life. Such examples indicate the existence of permanent committed populations of memory cells within the hematopoietic lineage. Either such populations do not turn over at all, or they possess their own stem cells which remain active for life, even if they were originally derived from HSC.

Labeling of bone marrow for DNA synthesis using BrdU has been extensively carried out to try to estimate the replication rates of various cell subsets in the hematopoietic system. But there are some toxic effects of BrdU that complicate interpretation. Less subject to toxic effects is the H2B-GFP dilution method in which cells are labeled by a burst of synthesis of fluorescent histone 2B (see Chapter 2). This has recently been applied using a human CD34 promoter to drive the Tet activator (this promoter is active in mouse HSC even though the endogenous mouse CD34 is not expressed). In the absence of doxycycline, H2B-GFP is produced, and when Doxycycline is restored the production stops (Tet-Off system). It is then possible to isolate different cell populations by FACS and ask how much GFP label they retain. This shows highest retention in the HSC themselves, less in the "short-term-HSC", the multipotent progenitors and the common lymphocyte progenitors, and none in the common myeloid progenitors, granulocyte-macrophage progenitors and megakaryocyte-erythroid progenitors (Figure 10.14). Modeling of the data plus other studies of HSC cell renewal indicate that the HSC pool is heterogeneous with some fast and some slow dividing cells.

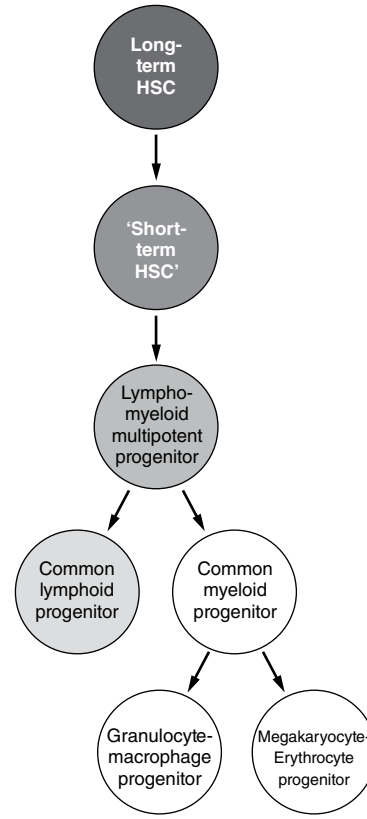

Figure 10.14 Cell division during steady state hematopoiesis. Mouse HSC were labeled by expression of H2B-GFP under the control of a Tet inducible system. The rate of loss of label in each compartment, due to cell division, is indicated by the loss of shading. (From: Schaniel, C. and Moore, K.A. (2009) Genetic models to study quiescent stem cells and their niches. Annals of the New York Academy of Sciences 1176, 26–35. Reproduced with the permission of John Wiley and Sons.)

Until recently there was no suitable promoter known for use in CreER experiments. So genetic labeling of HSC had to rely on induced DNA changes such as insertion sites of applied retroviruses. The most recent such studies use lentiviral libraries of genetic "barcodes" which can be sequenced directly by high throughput machines. Such methods have been used to study the clonal composition of the blood both in a normal turnover situation, and following transplantation. The results tend to indicate many more clones of HSC support normal steady state

(homeostatic) blood formation than do so following transplantation. However the results have been rather variable and it remains uncertain whether clones of HSC remain continuously active or whether one clone succeeds another. It has recently become possible to do CreER experiments using the *Tie2* promoter. *Tie2* encodes an angiopoietin receptor which is present on HSC and also on endothelial cells. A dose of tamoxifen causes irreversible labeling of a fraction of the HSCs and it is possible to trace the formation of their progeny by isolating different cell populations using FACS at different times and determining the percentage of label they contain. The results indicate that the label is slow to enter the system. After 4 weeks some "short-term-HSC" and multipotent progenitors are labeled. After 16 weeks all blood cell types are labeled. When the data are modeled it indicates that the "short-term-HSC" compartment in particular shows a very long residence time and does not come to equilibrium during the lifetime of the mouse.

In conclusion, despite the much vaunted status of the hematopoietic system as a model for all others, it seems that the cell lineage is not really known. The pathway shown in Figure 10.13 may not be entirely correct, as some data suggest the existence of precursors able to produce more than one type of offspring and their presence would make the scheme into a reticular network rather than a bifurcating tree, which would be a significant change to the model. Moreover mouse and human may not be exactly the same, and in each species fetal and adult may not be the same either. However the current evidence does support the idea that in homeostasis a large number of HSC clones feed the system, while after transplantation only a small number does so. Furthermore it seems inescapable that some of the precursor populations derived from the HSC are very long-lived and self-renewing and may reasonably qualify to be regarded as stem cells in their own right.

The Hematopoietic Niche

There has been much controversy about the nature of the niche within the bone marrow occupied by the HSCs. This issue is still not settled although a lot of evidence has been gathered in favor of various possibilities. It is likely that many components of the bone marrow are involved and there may be subniches for different subsets of HSC, such as quiescent cells, rapidly dividing cells, or cells mobilizable into the circulation.

The bone marrow is present in the cavities of the axial and the long bones (Figure 10.15). The endosteal surface of the bone is covered with flat bone-lining cells together with osteoblasts (bone-forming cells, derived from the lateral plate mesoderm) and osteoclasts (multinucleate bone-resorbing cells derived from the hematopoietic system). This lining exists both on the inner surface of the marrow cavity and also around the masses of trabecular bone. The principal artery and vein lie in the center of the cavity and are connected by arterioles and venules with an anastomosing plexus of venous sinusoids. These sinusoids are just one endothelial cell thick and have no basement membrane, hence allow easy entry and egress of cells. There is also a sympathetic innervation to the marrow which may affect hematopoiesis, for instance in terms of circadian variations.

If bone marrow is transplanted to other tissues in the body, hematopoiesis only continues where a bony ossicle is formed, indicating the importance of the marrow environment for the process. Transplantation studies on cells from different parts of the marrow suggest that the sub-endosteal region is richer in transplantable HSCs than the central region. The whole bone marrow has a relatively low oxygen content, about one-third of atmospheric, and low oxygen has been associated with HSC maintenance via stabilization of the hypoxia inducible factor HIF1α.

One problem in locating the HSC niche is the difficulty of identifying HSCs in situ using immunostaining. None of the antibodies used to isolate HSCs are completely

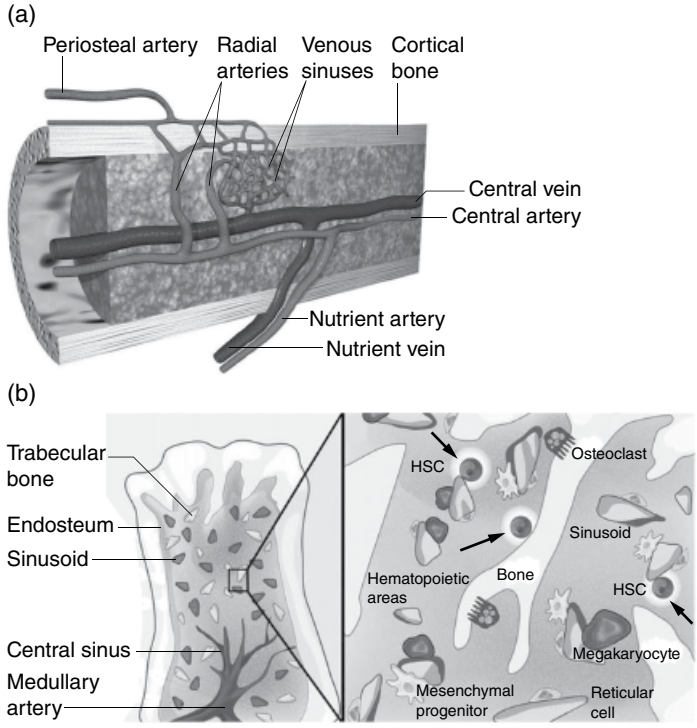

Figure 10.15 Structure of the bone marrow and the putative hematopoietic niches. (a) Overall structure of marrow in a long bone showing the vascular supply. (b) Substructure of bone marrow. The HSC (arrowed) are thought to reside near blood vessels and trabecular bone osteoblasts. (*Sources:* (a) from Travlos, G. (2006) Normal structure, function, and histology of the bone marrow. Toxicologic Pathology 34, 548–565. Adapted from: Abboud, C.N. and Lichtman, M.A. (2001) Structure of the marrow and the hematopoietic microenvironment. In: Williams Hematology, 6th edn. Copyright McGraw-Hill, used with permission. Adaptive drawing by David Sabio. (b) from Morrison, S.J. and Spradling, A.C. (2008) Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. Cell 132, 598–611. Reproduced with the permission of SAGE Publications.)

specific and the FACS method relies on the absence as well as presence of various markers. Studies with CD150 (= SLAM) suggest that the putative HSC may lie adjacent to blood vessels. If HSCs are first purified by FACS they can then be labeled and transplanted to irradiated hosts, whose niches are available for occupation. This method shows that they tend to end up in the sub-endosteal zone adjacent to trabecular bone osteoblasts and probably also to blood vessels (Figure 10.C.5). Mobilization of HSC into the circulation using granulocyte colony-stimulating factor (G-CSF) is associated with the death or flattening of osteoblasts and of marrow macrophages.

Analysis of various mouse knockout strains has indicated certain growth factors and cytokines that are likely to be important components of the niche. These include stem cell factor (SCF), CXCL12 (= SDF1), parathyroid hormone, Notch ligands, and various cell adhesion molecules. Further discrimination has come from the use of transgenic mice in which a promoter active in a specific cell type, such as osteoblasts or subsets of bone marrow stromal cells, are used to ablate or upregulate various factors. Cell ablation studies using Cre-driven diphtheria toxin receptor (see Chapter 2) indicate that subsets of bone marrow stroma expressing nestin or leptin are needed for HSC maintenance.

HSCs have been difficult to expand in vitro. Early methods depended on the use of stroma from bone marrow, which is a complex mixture of fibroblastic cells, blood vessels and adipocytes. More recently some substances have been found that promote division, most notably a compound called stemregenin 1 (SR1), which inhibits the aryl hydrocarbon receptor, a bHLH transcription factor normally resident in the cytoplasm. This has been used to expand the HSCs prior to grafts into human patients.

Spermatogenesis

The process of primordial germ cell migration, spermatogenesis and oogenesis have been described in Chapter 5. But spermatogenesis has another aspect of interest because the dividing spermatogonia constitute a well-characterized tissue-specific stem cell system. As with the hematopoietic system, the stem cells were initially defined by transplantation, but can now be studied in situ using the CreER method of lineage labeling. The transplantation assay uses as recipients mice treated with the alkylating agent busulphan. This destroys the endogenous stem cells, making the mice sterile and opening up the niches for repopulation. Injection of spermatogonial stem cells into the testis leads to the formation of permanent donor-derived colonies of cells at all stages of spermatogenesis (Figure 10.16). Like all transplantation, this assay needs to be conducted with immunocompatible mice, or with immunodeficient mice. Using this method, it is possible to do interspecies grafts of rat spermatogonial stem cells into immunodeficient mouse testis and to recover viable rat sperm.

The first transplantable cells arise in the mouse testis at about 4 days after birth. In the mature testis, the type A spermatogonia undergo divisions to form pairs or lines of cells joined by cytoplasmic bridges (Figure 10.17). These become A1 spermatogonia which divide successively to form A2, A3, A4, intermediate and B spermatogonia. These form spermatocytes which undergo meiosis to form four spermatids, each of which becomes a single sperm. The maximum possible yield of 4096 sperm per type A spermatogonium is not achieved because there is some cell death in the A1-B period. The type A spermatogonia are found near the basement membrane of the tubules, adjacent to Sertoli cells. As differentiation progresses the cell types are located further towards the luminal side, still closely associated with Sertoli cells, until eventually

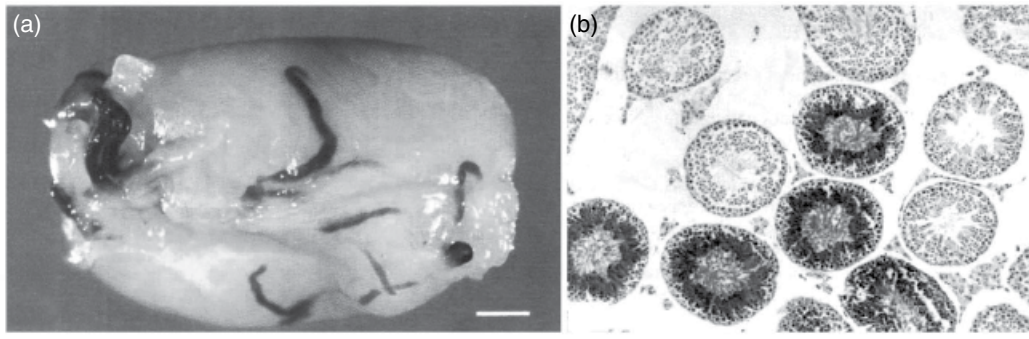

Figure 10.16 Spermatogonial stem cell transplantation. (a) A host testis into which stem cells were injected from a mouse labeled with *lacZ* reporter. The patches of tubule colonized by donor-derived cells are revealed by X-Gal staining (dark). (Slack, J.M.W. (2013) Essential Developmental Biology, 3rd edn. Reproduced with the permission of John Wiley and Sons.) (b) Section through a similar testis. In the colonized patches (dark), all of the germ line, but not the somatic cells, of the tubule are graft-derived. (Reproduced from Brinster and Avarbock (1994) Proceedings of the National Academy of Sciences of the USA 91, 11303–11307, with permission from National Academy of Sciences.)

the mature sperm are released into the lumen (Figure 10.18). It has been known for some time that the spermatogonia depend on Sertoli cells for their survival and multiplication. A key factor is Glial-Derived Neurotrophic Factor (GDNF), which is secreted by Sertoli cells. Its receptors, the tyrosine kinase RET, and the GPI-linked co-receptor GFRα1, are present on the A type spermatogonia. Mice lacking one copy of the *Gdnf* gene have reduced spermatogonial numbers and mice overexpressing *Gdnf* have too many. The Sertoli cell is obviously a key part of the stem cell niche, and it is likely that proximity to blood vessels is also important. The complete process of spermatogenesis takes about 35 days in the mouse and 74 days in human. There is also a "seminiferous epithelial cycle", taking 8.6 days in the mouse and 16 days in human. This is effectively the duration of a cell's residence in any one layer of the tubule. Because division of type A spermatogonia is approximately synchronized, adjacent cells are at about the same developmental stage and this means that there is a particular association of stages for each level from the basement membrane up to the lumen. The phase of this "cycle" varies along the tubule so that different stages are laid out adjacent to each other This arrangement is less apparent in human testis than in those of rat or mouse.

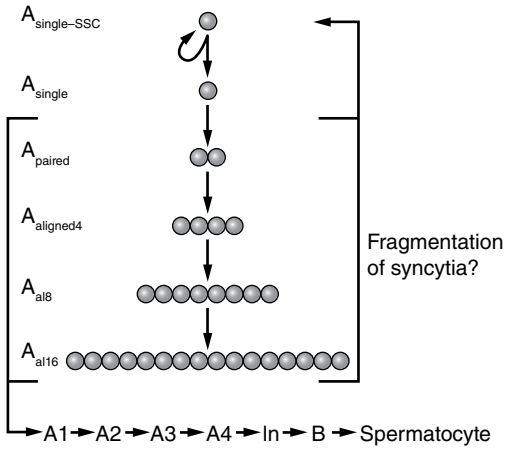

Figure 10.17 Spermatogenesis in the mouse. The spermatogonial stem cells are among the A_{single} population.These divide to form syncytial groups of up to 16 joined cells, which divide through further stages as indicated. The B spermatogonia become spermatocytes each of which undergo meiosis to form four sperm. (From: Griswold, M.D. and Oatley, J.M. (2013) Defining characteristics of mammalian spermatogenic stem cells. Stem Cells 31, 8–11. Reproduced with the permission of John Wiley and Sons.)

The identification of the stem cells has caused some difficulties. It has generally been thought that the true stem cells lie within the A_{single} (A_s) compartment, although lineage

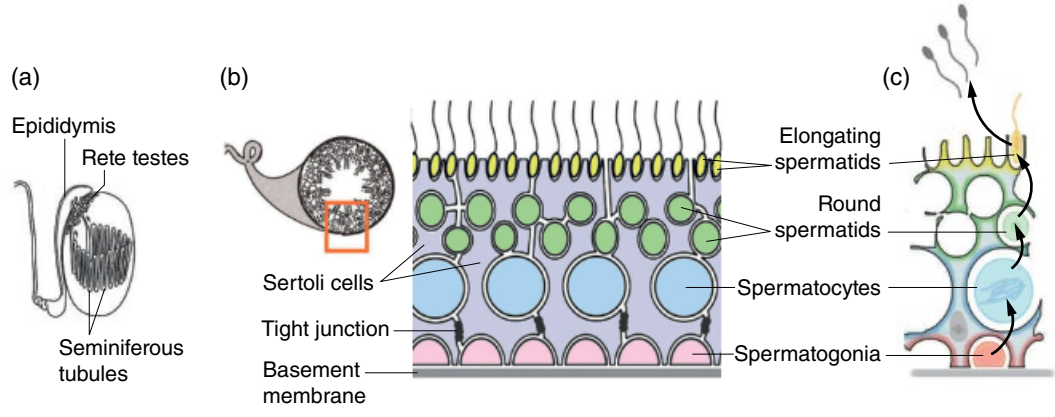

Figure 10.18 Organization of spermatogenesis. (a) Arrangement of the seminiferous tubules in the testis. (b) Structure of a tubule. The spermatogonia lie at the base and cells in different stages of spermatogenesis lie at successively higher levels. (c) Sperm differentiation occurs in very close proximity to the Sertoli cells. (From: Yoshida, S. (2016) From cyst to tubule: innovations in vertebrate spermatogenesis. Wiley Interdisciplinary Reviews:Developmental Biology 5, 119–131. Reproduced with the permission of John Wiley & Sons.)

labeling for *Ngn3*, which is expressed mostly in the syncytial groups, does label some stem cells. Various markers have been proposed for the stem cells, including GFRα1, the GPI-linked receptor for GDNF, and the transcription factors Id4, FOXO, PAX7, BMI1, TAF4b. The cell surface molecule Stra8 is expressed on all the A type spermatogonia and can be used for enrichment of stem cells by FACS. C-KIT, the receptor for the stem cell factor (SCF) is found on all spermatogonia. Cells in the differentiation sequence from A1 spermatogonia onwards are certainly transit amplifying cells. CreER experiments performed with several of these markers yield some clones which are long lived, and include cells at all stages of spermatogenesis, behavior indicative of real stem cells. An example is shown for *Bmi1*, using the multicolor Brainbow reporter system, in Figure 10.C.6. Here, 24 weeks after labeling, each clone covers a whole segment of a tubule.

Considerable analysis has been performed on the stem cells which can be labeled using *Ngn3-CreER*. Most *Ngn3*-expressing cells are transit amplifying cells and yield short lived clones. But some are stem cells and give large, long lived clones. Comparison of the proportion of the testis labeled in the steady state versus the proportion of transplantable cells that are labeled indicates that the transplantable cells are about 30× as abundant as the steady state stem cells. This is similar to the behavior of the intestine and reminds us that transplantation ability, though an interesting and important property, is not the same as being a stem cell in vivo. Furthermore as time goes on the number of *Ngn3* labeled clones decreases, but individual clones get bigger, preserving a roughly equal proportion of the testis labeled. The system shows "scaling behavior" meaning that the frequency distribution of clone sizes remains constant when divided by average clone size. This property can best be explained by the stochastic model in which the individual stem cells may divide to produce two stem cells, one stem cell and one transit amplifying cell, or two transit amplifying cells, such that the overall number of stem cells remains constant (see Chapter 1). This situation resembles that shown in the intestinal epithelium and the skin. As for these systems, it means that the lifetime persistence, which is a key defining feature of a stem cell, is a population property not an individual cell property.

Spermatogonial stem cells can be cultured in vitro. This is achieved by plating enzyme-digested testis, or enriched cell fractions, on a feeder layer of mitotically inactivated cells, with the presence of GDNF in the medium. The stem cells grow as refractile but loose colonies. They resemble gonocytes and are positive for α6- and β1-integrin. They may be frozen and thawed successfully. They can be transplanted to immunologically compatible, busulfan-treated, mice and yield viable sperm. Although they express the pluripotency genes at low levels, they are not the same as ES cells. However, culture of PGC-stage germ cells, or spontaneous transformations of long term stem cell cultures, can give colonies resembling ES cells. Unlike spermatogonial stem cells, these ES-like variants yield teratomas on transplantation.

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