Module 8

Development

Synopsis

Development encompasses the programme of events that begins with fertilization and culminates in complex multicellular organisms like ourselves. Every cell in the adult organism has a distinct life history (Module 8: Figure cellular life history) that can be traced back to the process of maturation that is responsible for the formation of the two gametes that then fuse during fertilization to form the zygote. Fertilization provides a potent mitogenic stimulus, and the zygote enters the cell cycle repeatedly and is rapidly transformed into a multicellular embryo. As development proceeds and the number of cells in the embryo increases, groups of cells are progressively apportioned distinct fates through a process of cell specification. During the final process of cell differentiation, each cell begins to express its unique specialized cell function that enables it to contribute to the operation of different organ systems. This process of differentiation is associated with the cessation of growth, which may be either permanent (e.g. nerves and muscle) or temporary (e.g. lymphocytes, fibroblasts, liver cells, salivary glands, endothelial cells and smooth muscle). In the latter case, the cells that have their growth arrested temporarily retain the ability to return to the cell cycle when stimulated with the appropriate growth factor. Finally, cells die and this can occur either through some catastrophic event, such as necrosis, or through a more controlled programme of cell death known as apoptosis. The typical life history of a cell thus comprises a series of processes:

- Maturation – the formation of mature gametes (spermatozoa and oocytes).
- Fertilization – the fusion of gametes and the initiation of development.
- Cell proliferation – rapid rounds of cell growth and cell division.
- Cell specification – cell determination and pattern formation.
- Differentiation – the formation of specialized cells.

Maturation

Before fertilization can occur, the primordial germ cells must undergo the process of maturation to form the mature gametes (spermatozoa and oocytes). An important aspect of maturation is the development of the signalling processes that are used by the gametes to recognize and fuse with each other. During spermatozoan maturation, a process of capacitation enables the sperm to fuse with the egg. During the process of oocyte maturation, the Ca^{2+} signalling system is strongly developed to enable the oocyte to generate the repetitive Ca^{2+} transients responsible for activating the egg at fertilization.

Spermatozoan maturation

During the maturation process the oval spermatocytes transform into elongated spermatozoa. The structure of the spermatozoon is consistent with its role of delivering a package of DNA to the oocyte. The final event of spermatozoon maturation is the process of capacitation that occurs within the female genital tract during which the sperm becomes competent to undergo fertilization.

Capacitation

During capacitation, there is an increase in sperm motility and chemotaxis, and the sperm acquire the ability to undergo the acrosome reaction. These changes in sperm metabolism and motility are triggered by factors in the fluid within the female reproductive tract. The three factors that seem to be important for promoting capacitation are bicarbonate (HCO_3^-), Ca^{2+} and albumin. Epithelial cells in the uterus and oviduct produce the HCO_3^-, which may enter the genital tract through the cystic fibrosis transmembrane regulator (CFTR), which is defective in cystic fibrosis (CF). It is therefore of interest that infertility has been associated with CF.

The signalling mechanisms that respond to HCO_3^- and albumin are still unclear. The albumin seems to function by leaching cholesterol out of the sperm membrane, thus increasing its fluidity and permeability. The HCO_3^- activates a soluble adenylyl cyclase (AC10) to produce cyclic AMP (Module 2: Figure cyclic AMP signalling), which

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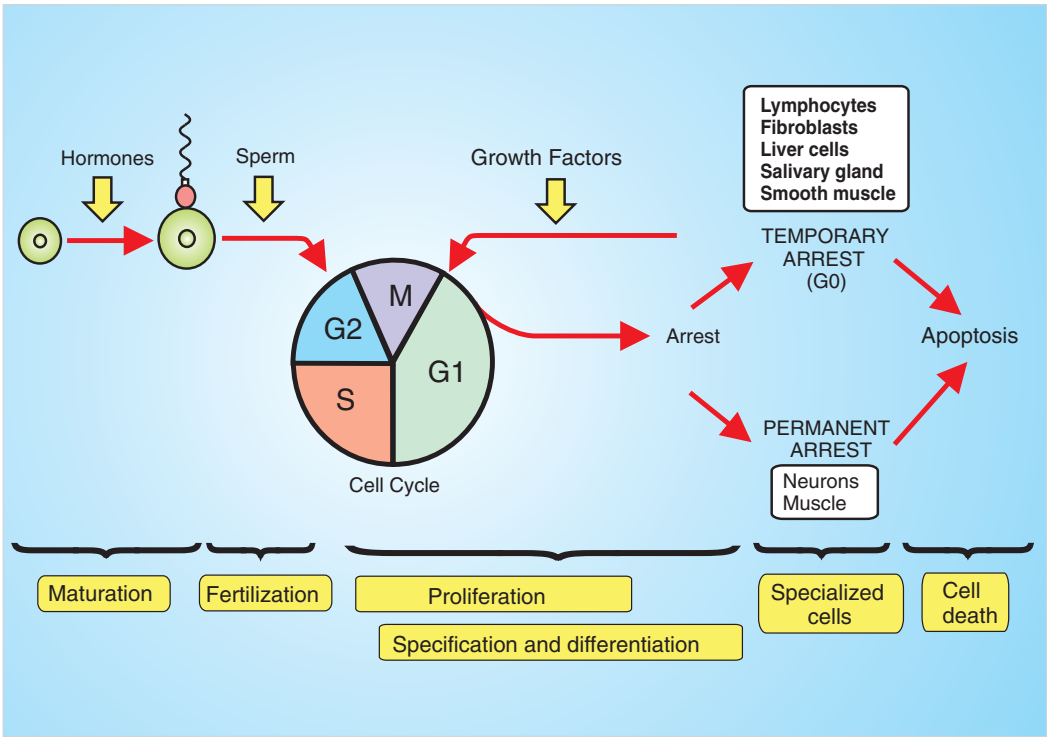
Module 8: | Figure cellular life history



The life history of cells from birth to death.

The typical life history of a cell comprises a series of sequential processes, beginning with maturation of the gametes. A new life begins at fertilization, which provides the stimulus to begin the programme of development. A period of rapid cell growth builds up a large population of cells, which then stop growing and enter a zero growth state (G_0) as they begin to differentiate into different cell types with specialized functions. In some cases, as in nerve and muscle, the G_0 state is permanent, but many other cells retain the ability to proliferate. They return to the cell cycle when activated by growth factors. Therefore there are two main ways of entering the cell cycle. One is the unique event of fertilization, and the other is by the activation of cell proliferation by growth factors. Finally, many cells die through the regulated processes of programmed cell death, otherwise known as apoptosis.

may enhance sperm motility and chemotaxis by inducing the entry of external Ca^{2+}. In human sperm, this process of capacitation seems to correlate with the development of the chemotactic responsiveness observed during sperm motility and chemotaxis.

Structure of the spermatozoon

The sperm is a long cell designed to deliver a packet of DNA to the oocyte during fertilization. It has a vectorial organization with specific functions located in clearly demarcated regions (Module 8: Figure sperm structure). The connecting piece is located between the head and flagellum, which is divided into the middle piece, the principal piece and the end piece. All the mitochondria in the sperm are concentrated in the middle piece, where they form a highly ordered sheath wrapped around the flagellum (Module 8: Figure sperm connecting piece). This region also has an extension of the nuclear membrane, which is where the inositol 1,4,5-trisphosphate ($InsP_3$) receptors might be located.

Oocyte maturation

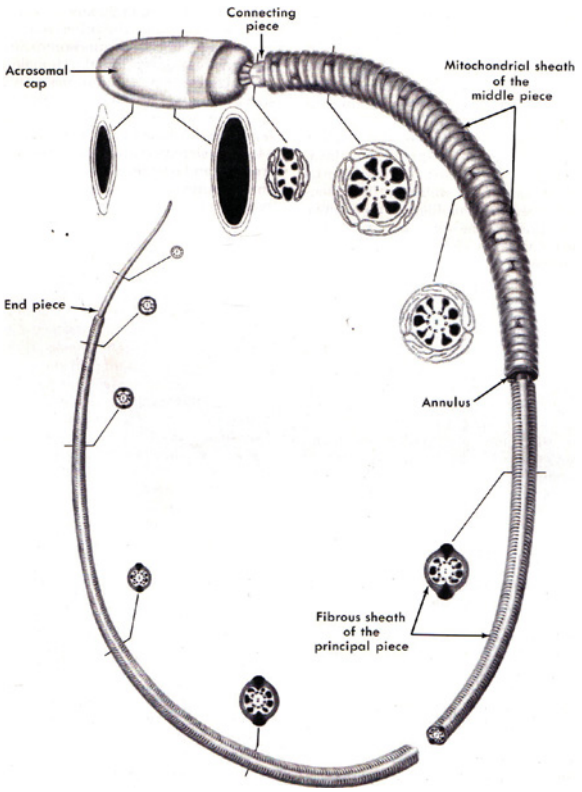
Primordial germ cells that are set aside during embryogenesis remain quiescent within the ovary until puberty, when they begin to fulfil their reproductive function. In the sexually mature female, hormones periodically induce a small number of oocytes to begin the process of maturation, during which they develop the signalling repertoire necessary to undergo fertilization.

The process of maturation takes place in two stages. Firstly, there is the acquisition of meiotic competence during which the small (20 μm), meiotically incompetent oocyte begins to grow, reaching a size of 70 μm by the time it reaches prophase I, when it becomes arrested and is usually referred to as an immature oocyte. It is now meiotically competent and, in response to the preovulatory surge of gonadotropins, such as luteinizing hormone (LH), it begins the second stage of maturation, which is the conversion of prophase I-arrested immature oocytes into the mature metaphase II-arrested oocytes.

Acquisition of meiotic competence: the development of prophase I-arrested immature oocytes

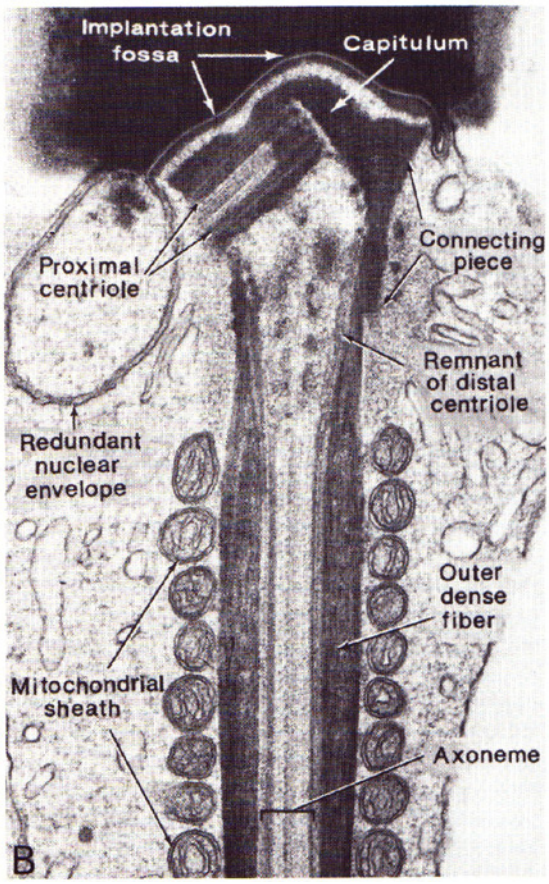
There is relatively little information about the signalling processes that occur during the initial period of maturation, when the oocyte grows from a small (20 μm) precursor to the much larger (70 μm) oocyte. However, there is a progressive acquisition of a Ca^{2+} signalling system that is capable of generating spontaneous Ca^{2+} oscillations (Module 8: Figure meiotic Ca^{2+} signalling). The function of this latent oscillatory activity is unclear, because the surrounding follicle cells that are connected to the oocyte

Module 8: | Figure sperm structure



Structure of a typical mammalian spermatozoon.
The head of the sperm has an acrosomal cap, which contains the acrosome that is released during the acrosomal reaction at the beginning of fertilization. Most of the head is taken up by the large nucleus. The connecting piece (also known as the neck region) attaches the head to the long flagellum that is divided into three sections: the middle piece, the principal piece and the end piece. All the mitochondria in the sperm are concentrated in the middle piece, where they form a highly ordered sheath wrapped around the flagellum (for details of this section, see Module 8: Figure sperm connecting piece). Reproduced from William Bloom and Don W. Fawcett, *A Textbook of Histology*, 10th edition (W.B. Saunders, 1975), © 1975 by W.B. Saunders Company, reproduced by permission of Edward Arnold; see Bloom and Fawcett 1975.

Module 8: | Figure sperm connecting piece



Structural organization of the connecting piece of a mammalian spermatozoon.
A prominent feature of this region is the sheath of mitochondria that surrounds the axoneme. Note the extension of the nucleus (labelled as the 'Redundant nuclear envelope') that extends down into the connecting piece, and which might be the location of some of the inositol 1,4,5-trisphosphate ($InsP_3$) receptors found in the sperm (the location of sperm $InsP_3$ receptors is shown in Module 8: Figure location of sperm $InsP_3$ receptors). Reproduced from William Bloom and Don W. Fawcett, *A Textbook of Histology*, 10th edition (W.B. Saunders, 1975), © 1975 by W.B. Saunders Company, reproduced by permission of Edward Arnold; see Bloom and Fawcett 1975.

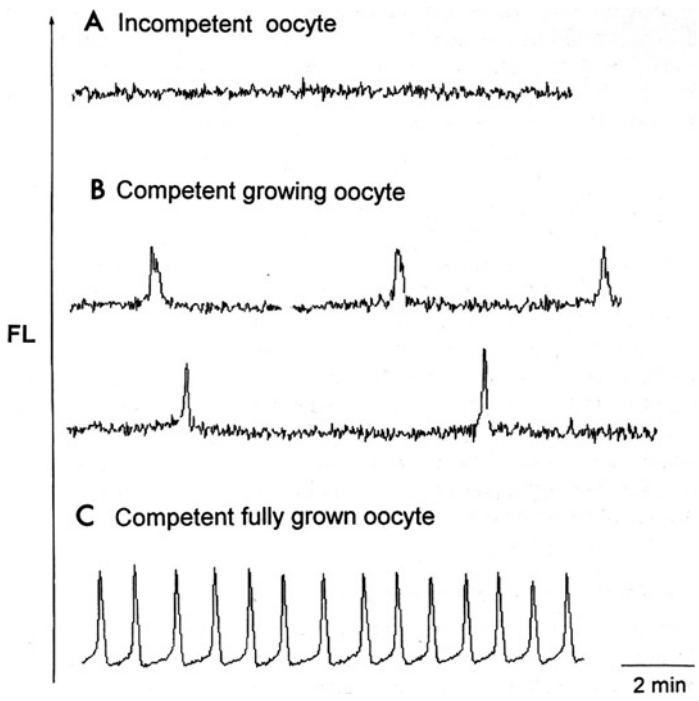
through gap junctions normally suppress it. The follicle cells are thought to suppress these spontaneous Ca^{2+} oscillations by passing cyclic AMP to the oocyte through these gap junctions. The oscillations are revealed when oocytes are separated from their surrounding follicles. Indeed, follicle cells play a crucial role in maintaining prophase I arrest, which effectively separates the two stages of maturation.

Even though the small meiotically incompetent cells fail to show spontaneous oscillations, they do have the capacity to produce repetitive spikes when stimulated with either thimerosal, which acts by sensitizing inositol 1,4,5-trisphosphate ($InsP_3$) receptors, or acetylcholine (ACh). One of the remarkable aspects of certain mammalian oocytes is that they express receptors for neurotransmitters, such as ACh in the mouse and 5-hydroxytryptamine (5-HT) in the hamster. Indeed, immature hamster oocytes are much more sensitive to 5-HT than the mature oocytes. The function of such G protein-coupled receptors (GPCRs) on oocytes is still a mystery, but their ability to generate Ca^{2+} signals clearly shows that even very early oocytes have an intact $InsP_3/Ca^{2+}$ signalling system. It seems that the sensitivity of this signalling system increases during this first stage of maturation so that it gradually develops a capacity to generate spontaneous Ca^{2+} oscillations as the oocyte reaches the stage of prophase I arrest (Module 8: Figure meiotic Ca^{2+} signalling).

Conversion of prophase I-arrested immature oocytes into mature metaphase II-arrested oocytes

The immature oocytes with their inherent capacity to oscillate are arrested at prophase I. This oscillatory activity is normally suppressed in the immature oocyte by cyclic AMP streaming in from the follicle cells. What is in doubt is whether this latent oscillatory activity is ever unleashed to control any of the maturation processes. Following the action of luteinizing hormone (LH), when the connection

Module 8: | Figure meiotic Ca^{2+} signalling



Ca^{2+} signalling during the acquisition of meiotic competence

Ca^{2+} oscillations recorded at different stages of mouse oocyte maturation. (A) Oocytes isolated from 13-day-old mice were quiescent. (B) Oocytes from 16-day-old mice began to show transients, but with very low frequencies. (C) In fully grown competent oocytes from mice at 23 days, regular Ca^{2+} oscillations were recorded that spiked with a frequency of approximately 1 min. Reproduced from Carroll, J., Swann, K., Whittingham, D. and Whitaker, M. (1994) Spatiotemporal dynamics of intracellular $[Ca^{2+}]_i$ oscillations during the growth and meiotic maturation of mouse oocytes. Development 120:3507–3517, with permission from The Company of Biologists; see Carroll et al. 1994.

with the follicle cells is severed, it is possible that the development of such spontaneous oscillations may be responsible for driving some of the later events of meiosis. While the process of germinal vesicle breakdown (GVBD) seems to occur independently of Ca^{2+}, there are indications that Ca^{2+} controls the events of metaphase I associated with extrusion of the first polar body. Such a role for Ca^{2+} would certainly be consistent with its role in mediating some of the cell cycle events following fertilization. As the immature oocyte proceeds past GVBD, it begins to lose this capacity to generate spontaneous oscillations so that the fully mature oocyte arrested at metaphase II is totally quiescent. Paradoxically, this decline in spontaneous activity is matched by a progressive increase in the sensitivity of the inositol 1,4,5-trisphosphate ($InsP_3$)/Ca^{2+} signalling pathway.

This increased sensitivity of the $InsP_3$/Ca^{2+} pathway is associated with a dramatic remodelling of the endoplasmic reticulum (ER). In immature oocytes, the ER consists of large membrane concentrations localized near the surface and around the germinal vesicle, with little evidence of polarity. As maturation proceeds, there is a 3-fold increase in the amount of ER that organizes into a reticular network that extends throughout the cell and forms dense 1–2 μm clusters located in the cortex opposite the spindle. The number of $InsP_3$ receptors increases 2-fold, and immunolocalization of these receptors closely matches the ER network. The polarity of the ER clusters at the pole opposite the spindle coincides with the distribution of the surface microvilli and concanavalin A (ConA)-binding sites. Since the sperm is most likely to fuse with this region, it will be directed towards the pole that has the highest density of $InsP_3$ receptors. This reorganization of the ER is matched by a marked increase in the sensitivity of the oocyte to injected $InsP_3$. There is an apparent logic behind these changes in that the suppression of spontaneity will guard against parthenogenetic activation, while the increase in $InsP_3$ sensitivity will make it easier for the minute sperm to awaken the very much larger egg from its prolonged period of quiescence at the time of fertilization.

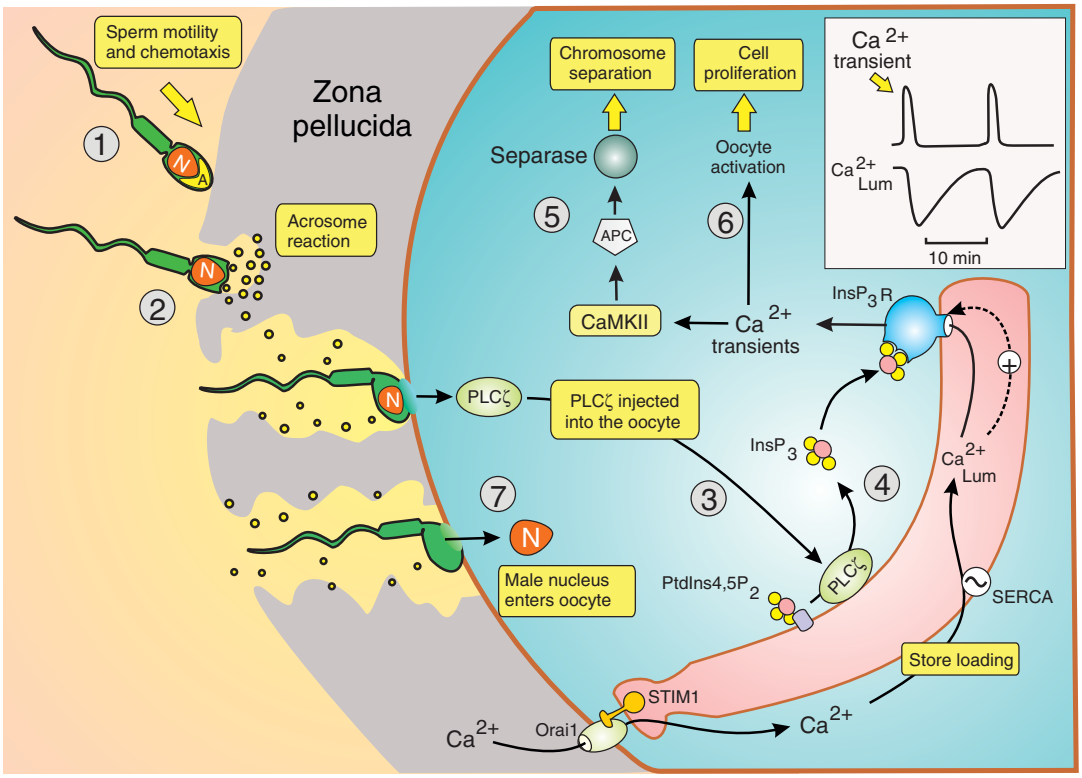
An important feature of oocyte maturation is therefore to put in place a sensitive phosphoinositide signalling pathway that is poised to respond to the sperm by generating the repetitive bursts of Ca^{2+} that initiate the developmental programme at fertilization.

Fertilization

A new life begins when the sperm fuses with the quiescent oocyte arrested at the M_2 phase (Module 8: Figure cellular life history). This process of fertilization proceeds through the following sequence of events (Module 8: Figure mammalian fertilization):

1. First of all, the sperm must find the egg through a process of sperm motility and chemotaxis.

Module 8: | Figure mammalian fertilization



Fertilization of a mammalian oocyte.

The process of fertilization depends on a carefully orchestrated sequence of events as outlined in the text. The Ca^{2+} oscillations that occur during fertilization are shown in Module 8: Figure fertilization-induced Ca^{2+} oscillations . The information used to outline the temporal sequence of Ca^{2+} transients and changes in the luminal level of Ca^{2+} was obtained from Wakui and Fissore (2013). An animated version of this figure is available.

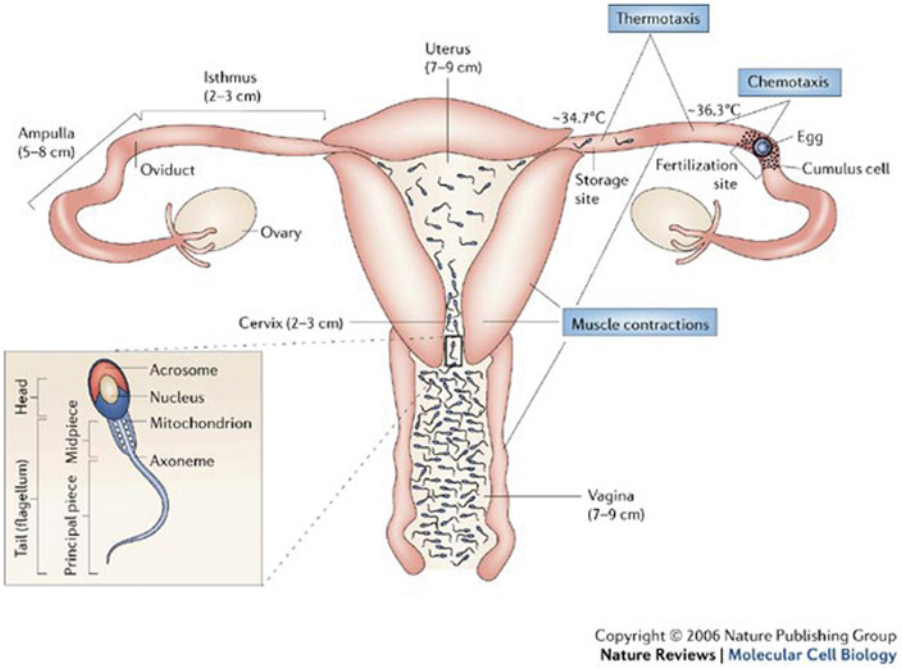
2. When it makes contact with the outer coat that surrounds the oocyte (i.e. the zona pellucida), the sperm undergoes an acrosome reaction that facilitates the movement of the sperm through the zona pellucida so that the sperm can gain access to the oocyte plasma membrane in order for sperm-induced oocyte activation to occur.

   The sperm then fuses with the oocyte and injects a novel phospholipase Cζ (PLCζ) that is then responsible for activating the oocyte. The latter mechanism is duplicated during the process of artificial insemination by intracytoplasmic spermatozoa injection (ICSI), which is used for *in vitro* fertilization. When phospholipase Cζ (PLCζ) enters the cytoplasm, it binds to membranes of the endoplasmic reticulum where it hydrolyses PtdIns4,5P_2 to form inositol 1,4,5-trisphosphate ($InsP_3$) Mutations in phospholipase Cζ (PLCζ) are responsible for some cases of male infertility.
3. The $InsP_3$ triggers sperm-induced Ca^{2+} oscillations. During each spontaneous Ca^{2+} transient there is a rapid decline in the Ca^{2+} concentration in the ER lumen (see white inset). The level of $Ca^{2+}{}_{Lum}$ is gradually replenished through entry by store-operated Ca^{2+}channels that depend on both STIM1 and Orai1. The buildup of Ca^{2+} within the lumen of the ER is an important component of the mechanism of Ca^{2+} oscillations in that it sensitizes the receptor resulting in the activation of the next transient.
4. The sperm-induced Ca^{2+} transients induce chromosome separation (see Module 9: Figure chromosome separation for details of the mechanism).
5. The sperm-induced Ca^{2+} transients are also responsible for oocyte activation that results in cell proliferation.
6. The sperm nucleus (N) moves into the oocyte.

Sperm motility and chemotaxis

An essential early phase of fertilization is sperm motility and chemotaxis, which enables the sperm to find the egg (Module 8: Figure sperm chemotaxis). Sperm deposited in the vagina migrate into the uterus and then into the storage sites in the isthmus at the base of the oviducts. Sperm move by actively swimming and also by being propelled along by muscular contractions of the genital tract. Once they reach the storage site, where they can be held for several days, the sperm undergo the process of capacitation, which enables them to continue their journey towards the egg. Since capacitation occurs asynchronously, small cohorts of sperm are produced continuously over a 2–3 day period to ensure that active sperm are migrating up the oviduct for an extended period. One of the consequences of capacitation is that the sperm become competent to follow the thermal and chemical gradients that will guide them up the oviduct. The thermal gradient seems to provide the initial guidance signal to bring the sperm close to the egg, and the final

Module 8: | Figure sperm chemotaxis



Sperm migration by thermotaxis and chemotaxis in the female genital tract.
This diagram of the human genital tract illustrates the path taken by sperm to reach the egg, which is surrounded by cumulus cells. The temperature gradient down the oviduct was measured in rabbits. Sperm in the vagina swim into the uterus and then into the storage site in the oviduct where they undergo capacitation before migrating up the oviduct to fertilize the egg. This migration is guided by both thermotaxis up the temperature gradient and by chemotaxis. Reproduced by permission from Macmillan Publishers Ltd: Eisenbach, M. and Giojalas, L.C. (2006) Sperm guidance in mammals: an unpaved road to the egg. Nat. Rev. Mol. Cell Biol. 7:276–285. Copyright (2006); http://www.nature.con/nrm; see Eisenbach and Giojalas 2006.

approach seems to be controlled by chemotactic factors that are released from the egg and surrounding cumulus cells. The nature of the sperm chemoattractants is still being determined. Bourgeonal, which might be a physiological analogue of one of these attractants, has been shown to act on the odorant receptor OR17-4, which is coupled through the heteromeric G protein $G\alpha_{olf}$ (Module 2: Table heterotrimeric G proteins) located in the midpiece of the flagellum. The $G\alpha_{olf}$ is known to be coupled to adenylyl cyclase to increase the level of cyclic AMP. In addition, the sperm odorant-like receptors also appear to increase the level of Ca^{2+}, and these second messenger pathways activate chemotaxis through mechanisms that remain to be defined.

In addition, motility plays an important role in enabling the sperm to burrow through the zona pellucida to reach the oocyte plasma membrane. Only a fraction of the sperm is chemotactically competent, and this appears to correlate with the number of sperm that have undergone capacitation, which selects out those sperm that are capable of fusing with the oocyte.

Motility is achieved by flagellar beating that begins in the neck region and propagates through the middle piece and principal piece of the tail. Ca^{2+} plays a central role in the control of sperm motility. Beat frequency is regulated by Ca^{2+} acting on an axonemal site at the base of the flagellum. Hyperactivity of the sperm is induced by an increase in Ca^{2+} that can be derived from both internal and external sources. Release of Ca^{2+} from the inositol 1,4,5-trisphosphate ($InsP_3$)-sensitive store in the neck region of the sperm (Module 8: Figure location of sperm $InsP_3$ receptors) can greatly accelerate motility. Since such stores have a finite capacity, maintenance of the hyperactive state will require entry of external Ca^{2+}. The cation channel of sperm (CatSper) seems to play a major role in regulating sperm motility. The sperm also expresses components of the cyclic nucleotide-gated channels (CNGCs), which may regulate sperm motility by controlling the entry of external Ca^{2+}. The CNGAα subunits, which form the channels, are located over the length of the flagellum. The channels formed from these subunits were found to promote Ca^{2+} entry in response to both cyclic AMP and cyclic GMP, with the latter being somewhat more active.

Cation channel of sperm (CatSper)

The cation channels of sperm (CatSper1–CatSper3) are expressed in sperm only. They have six transmembrane domains closely resembling one of the four repeats found in voltage-operated channels (VOCs). Most information is available for CatSper1, which is localized to the principal piece (Module 8: Figure sperm structure) of the tail. This channel component responds to intracellular alkalinization by an increase in Ca^{2+} entry, which then functions to drive the asymmetrical beating of the flagellum that helps to drive the sperm through the zona pellucida. Ablation of the CatSper gene in mice results in sperm that are poorly mobile and incapable of fertilization. However, the CatSper$^{-/-}$ sperm were able to fertilize zona

pellucida-free oocytes, emphasizing the importance of motility in penetrating the outer layer of the oocyte.

A related CatSper2 has been located on the sperm flagellum, where it may also function in motility.

Acrosome reaction

During the acrosome reaction, the acrosome located in the head of the sperm releases its hydrolytic enzymes through a process of exocytosis (Module 8: Figure mammalian fertilization). Like other membrane fusion events, the acrosome reaction is stimulated by an increase in Ca^{2+} induced by a combination of stimuli that act on the sperm as it approaches the oocyte. The sperm can detect factors such as progesterone contained in the follicular fluid. The action of progesterone appears to sensitize the sperm to the components of the zona pellucida that trigger the acrosome reaction. The zona pellucida is a thick extracellular coat that surrounds the oocyte and has to be breached by the sperm to gain access to the oocyte plasma membrane. This zona pellucida contains three glycoproteins, of which ZP3 interacts with a receptor on the sperm head to induce the acrosome reaction. Increases in Ca^{2+} may occur through both ZP3-induced Ca^{2+} signalling and progesterone-induced Ca^{2+} signalling.

Progesterone-induced Ca^{2+} signalling

In response to a low dose of progesterone, human sperm display a biphasic Ca^{2+} signal: there is an initial transient followed by a sustained plateau (Module 8: Figure progesterone-induced Ca^{2+} signals). Both responses appear to be due to Ca^{2+} entry, but the nature of the channels has not been clearly defined. The plateau phase of the response has all the hallmarks of store-operated Ca^{2+} entry and might be mediated by members of transient receptor potential (TRP) ion channel family that have been identified in sperm.

ZP3-induced Ca^{2+} signalling

The major signal for activating the acrosome reaction is provided by ZP3, a component of the zona pellucida. The thick extracellular coat (zona pellucida) that surrounds the oocyte has to be breached by the sperm before it can fuse with the oocyte plasma membrane (Module 8: Figure mammalian fertilization). Only acrosome-intact sperm are capable of binding the zona pellucida.

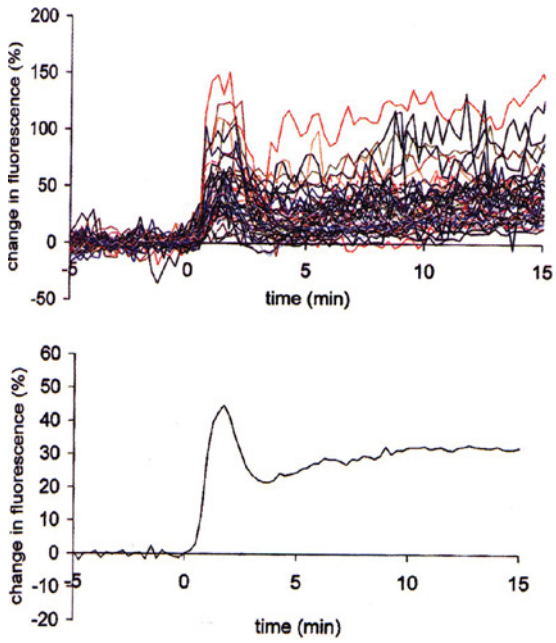
The zona pellucida contains three glycoproteins, of which ZP3 is the component that stimulates the sperm receptors located on the sperm head. The oocyte has receptors such as β-1,4-galactosyltransferase that bind the *N*-acetylglucosamine residues on ZP3. Another candidate is a 95 kDa protein in the sperm membrane, which might possess tyrosine kinase activity.

Just how these sperm receptors function to generate the Ca^{2+} signal that controls the acrosome reaction is still unclear. The Ca^{2+} signal is complex in that there are at least three separate components (Module 8: Figure ZP3-induced Ca^{2+} signals):

1. The first response to binding ZP3 in mice sperm is a very rapid Ca^{2+} transient that lasts for less than 200 ms. The Ca_V3 family of T-type channels, which are low-voltage-activated (LVA) channels, are responsible for this early fast Ca^{2+} transient. In human sperm, there also appears to be a role for a nicotinic acetylcholine receptor (nAChRs). One of the actions of ZP3 is to stimulate glycine receptors (GlyRs), and this causes a small depolarization that contributes to the early signal transduction step during the acrosome reaction.
2. The early transient is followed by a slower elevation of Ca^{2+} that seems to begin with the release of Ca^{2+} from an internal store, most likely to be the acrosome itself, which is known to have inositol 1,4,5-trisphosphate ($InsP_3$) receptors (Module 8: Figure location of sperm $InsP_3$ receptors).
3. Release of Ca^{2+} from the internal store is then responsible for activating store-operated channels (SOCs) to maintain a prolonged elevation of Ca^{2+}, which seems to be important for inducing the acrosome reaction. This SOC might be coded for by the canonical transient receptor potential 2 TRPC2, which is localized in the anterior sperm head adjacent to the $InsP_3$ receptors.

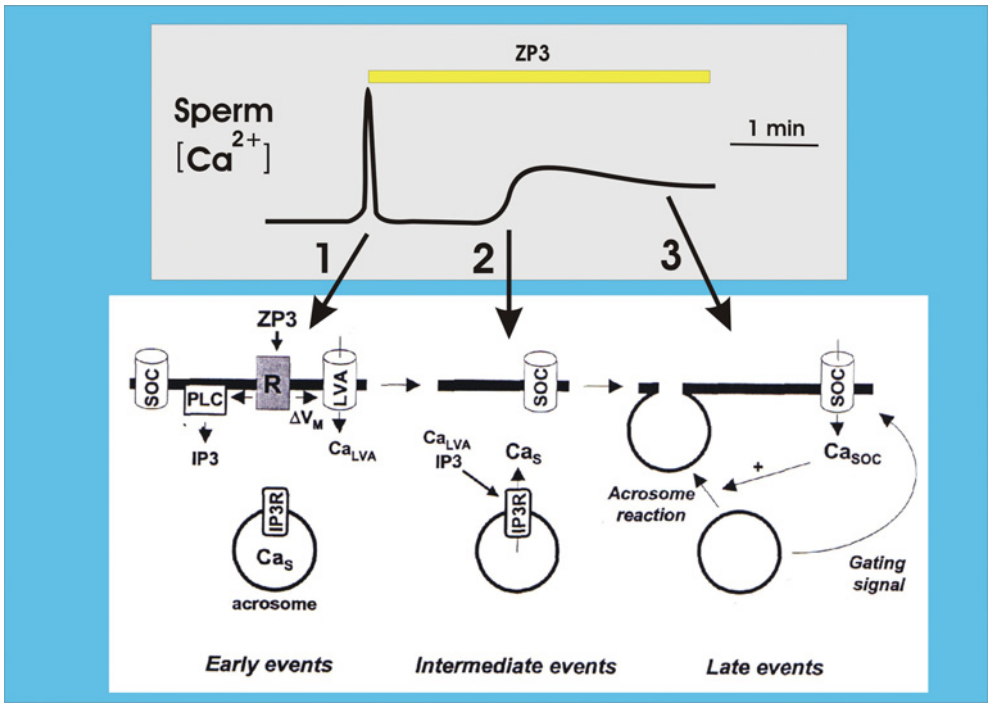
Although much of the Ca^{2+} signal used to drive the acrosome reaction is derived from the outside, there does seem to be a role for internal Ca^{2+} release carried out by the inositol 1,4,5-trisphosphate ($InsP_3$)/Ca^{2+} signalling cassette. ZP3 acts by aggregating sperm receptors on the sperm membrane. These sperm receptors have tyrosine kinase activity that might function by activating

Module 8: | Figure progesterone-induced Ca^{2+} signals



Progesterone-induced Ca^{2+} signals in human sperm.
The top panel represents responses of 40 separate sperm cells stimulated with 3.2 μM progesterone. The average response of 202 cells is shown in the bottom panel. Reproduced from *Dev. Biol.*, Vol. 222, Kirkman-Brown, J.C., Bray, C., Stewart, P.M., Barratt, C.L.R. and Publicover, S.J., Biphasic elevation of $[Ca^{2+}]_i$ in individual human spermatozoa exposed to progesterone, pp. 326–335. Copyright (2000), with permission from Elsevier; see Kirkman-Brown et al. 2000.

Module 8: | Figure ZP3-induced Ca^{2+} signals



Ca^{2+} signalling events during the sperm acrosome reaction induced by ZP3.

When sperm interacts with the zona pellucida, it binds to ZP3 that initiates the Ca^{2+} signal, which has three distinct phases as described in the text. Modified from *Molecular Biology of the Cell* (Mol. Biol. Cell, 2000, 11:1571–1584.) with the permission of The American Society for Cell Biology; see O'Toole et al. 2000.

phospholipase Cγ1 (PLCγ1). In addition, the acrosome itself has a high density of $InsP_3$ receptors (Module 8: Figure location of sperm $InsP_3$ receptors) that may contribute to the onset of the Ca^{2+} signal, which has all the elements of an autocatalytic process in that Ca^{2+} itself is particularly active in stimulating the hydrolysis of $PtdIns4,5P_2$ in sperm to enhance further the level of $InsP_3$.

Sperm-induced oocyte activation

The process of sperm-induced oocyte activation begins when the sperm plasma membrane makes intimate contact with the oocyte plasma membrane (Module 8: Figure mammalian fertilization). Members of the ADAM protease family (Module 1: Table ADAM proteases) have been implicated in this early contact between the sperm and egg plasma membranes. In mice, ADAM-1 and ADAM-2, also known as fertilinα and fertilinβ respectively, form a heterodimer called fertilin that has a role in this early interaction that also seems to require the participation of ADAM-3. In humans, however, the *ADAM1* and *ADAM3* genes are non-functional, but ADAM-2 seems to have a role in an early adhesion step. Since ADAM-2 has its metalloprotease domain removed prior to the acquisition of fertilization competence, it does not act through a proteolytic process but may use its disintegrin domain to bind to integrin receptors on the egg surface.

Close contact between the sperm and egg membranes is followed by sperm–egg fusion, which is a most unusual event in biology and requires a mutual interaction involving special recognition, adhesion and fusion molecules. When the sperm fuses with the oocyte (Module 8: Figure mammalian fertilization), it introduces the sperm-specific phospholipase Cζ (PLCζ), which then begins to hydrolyse $PtdIns4,5P_2$ to generate the second messenger inositol 1,4,5-trisphosphate ($InsP_3$). An unusual feature of this signalling pathway is that the $PtdIns4,5P_2$, which is being hydrolysed to form $InsP_3$, is located on an internal membrane the endoplasmic reticulum and not on the plasma membrane as it is normally for the classical inositol 1,4,5-trisphosphate ($InsP_3$)/Ca^{2+} signalling cassette (Module 2: Figure $InsP_3$ and DAG formation).

The $InsP_3$ is then responsible for setting up the Ca^{2+} oscillations (Module 8: Figure fertilization-induced Ca^{2+} oscillations) that can persist for hours and finally cease at the time the pronuclei form (Module 8: Figure PLC sequestration hypothesis). All mammals studied so far display these sperm-induced Ca^{2+} oscillations that have relatively long periods: 1–10 min in mice and hamsters, 5–12 min in rabbits, 10–35 min in humans and 15–55 min in bovine and pig oocytes. The functional significance of oocyte Ca^{2+} oscillations is 4-fold: these oscillations trigger the release of cortical granules, they provide a block to polyspermy, they complete meiosis, and they initiate the cell cycle programme leading to embryo development. With regard to the completion of meiosis, the pulses of Ca^{2+} act through Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) to initiate the sequence of events that leads to chromosome separation. The Ca^{2+} pulses also activate the oocyte to begin the multiple rounds of cell proliferation that occur at the beginning of development.

Module 8: | Figure location of sperm $InsP_3$ receptors

Location of inositol 1,4,5-trisphosphate ($InsP_3$) receptors in rat sperm. An antibody directed against the inositol 1,4,5-trisphosphate ($InsP_3$) receptor stains both the acrosome (arrowhead) and a small region within the connecting and proximal middle piece (arrow). Vertical bar, 5 μm. Reproduced from *The Journal of Cell Biology*, 1995, vol. 130, pp. 857–869, by copyright permission of The Rockefeller University Press; see Walensky and Snyder 1995.

Sperm-induced Ca^{2+} oscillations

During fertilization, a single spermatozoan can activate a quiescent oocyte to initiate a prolonged series of Ca^{2+} spikes (Module 8: Figure fertilization-induced Ca^{2+} oscillations). Mammalian eggs possess a typical cytosolic oscillator that generates the regular transients that result from the periodic burst of Ca^{2+} release from the internal stores (Module 6: Figure membrane and cytosolic oscillators). The sperm-induced Ca^{2+} oscillations are consistent with a mechanism of Ca^{2+} oscillations that has a number of distinct components (Module 6: Figure Ca^{2+} oscillation model). In particular, an influx of external Ca^{2+} is responsible for recharging the stores during the interval between spikes, which is essential to maintain the oscillator. The initial large release of Ca^{2+} responsible for the early rapid transients (Module 8: Figure fertilization-induced Ca^{2+} oscillations) causes a rapid depletion of the store (see inset in Module 8: Figure mammalian fertilization). This depletion activates a store-operated Ca^{2+} entry mechanism based on STIM1 and Orai1 that remains on for the full period of the oscillation and functions to prime the inositol 1,4,5-trisphosphate ($InsP_3$) receptors to trigger the onset of each transient. It is generally accepted that the $InsP_3$ receptor plays a major role in generating the sperm-induced Ca^{2+} oscillations, although there is some evidence that the ryanodine receptors (RYRs) may function in bovine oocytes.

An important aspect of this Ca^{2+} oscillation model is that the sperm induces an elevation of $InsP_3$. While such measurements of $InsP_3$ are difficult to do in small mammalian oocytes, the mass of $InsP_3$ was found to increase 5-fold following fertilization of the much larger *Xenopus* oocytes. In addition, oscillations can be induced by artificially introducing $InsP_3$ into oocytes either by injection or flash photolysis of caged $InsP_3$.

As with other oscillatory systems, the increase in $InsP_3$ stimulates the periodic release of Ca^{2+} by converting the oocyte cytoplasm into an excitable medium. An indication of this increased excitability is that Ca^{2+} is much more effective when injected into oocytes once they have been fertilized. All of this evidence indicates that $InsP_3$ receptors are responsible for periodically releasing Ca^{2+} during each spike.

The sperm induces oscillations by introducing a sperm factor that functions to sensitize the $InsP_3$ receptors. This sperm factor appears to be the sperm-specific phospholipase: phospholipase Cζ (PLCζ). When the sperm fuses with the egg, it releases PLCζ that stimulates the production of $InsP_3$, which then increases the sensitivity of the $InsP_3$ receptors such that they begin to release the periodic pulses of Ca^{2+} (Module 8: Figure mammalian fertilization).

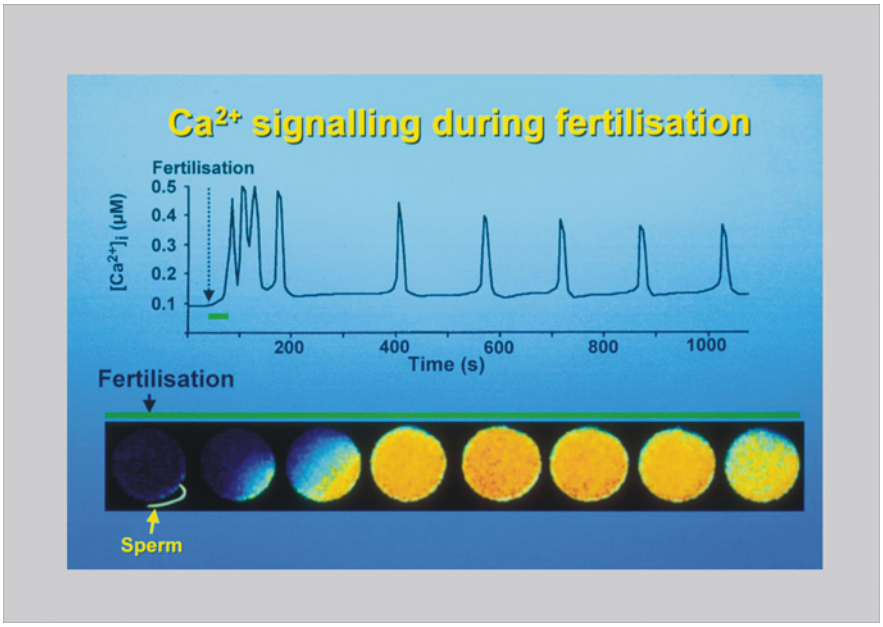
Such a mechanism is also consistent with the process of artificial insemination by intracytoplasmic spermatozoa injection (ICSI) in that the sperm would carry with it the PLCζ that is necessary to activate the egg.

Intracytoplasmic spermatozoa injection (ICSI)

Human *in vitro* fertilization techniques for treating infertility have been transformed by a remarkable new process of injecting a single sperm directly into the egg called intracytoplasmic spermatozoa injection (ICSI) (Module 8: Figure intracytoplasmic sperm injection). After a long latency, the oocyte containing the spermatozoon begins to generate Ca^{2+} oscillations very similar to those seen during normal fertilization (Module 8: Figure ICSI-induced Ca^{2+} oscillations). The fact that the normal sperm-induced oocyte activation processes occur, even though the usual sperm–egg interaction and fusion events have been bypassed, argues strongly for the sperm factor hypothesis. Once the sperm is injected into the oocyte, the sperm membrane must disintegrate to release the phospholipase Cζ (PLCζ), which then initiates the remaining sequence of fertilization events (i.e. Events 3–6 in Module 8: Figure mammalian fertilization). It is clear that this release of PLCζ from the injected sperm takes some time, because, following ICSI, there is a long latency (e.g. 20 min in Module 8: Figure ICSI-induced Ca^{2+} oscillations). In some cases, this latency can extend to 2–10 h before Ca^{2+} oscillations begin. However, this latency can be greatly reduced if oocytes are treated first with a Ca^{2+} ionophore (A23187), which presumably will enhance the level of intracellular Ca^{2+}, thereby helping to sensitize the $InsP_3$ receptors to the point that they will begin to generate Ca^{2+} oscillations.

This ICSI approach to *in vitro* fertilization has been extended as a therapy for male sterility in cases of both

Module 8: | Figure fertilization-induced Ca^{2+} oscillations



Ca^{2+} signalling during fertilization of a hamster oocyte.

Soon after the sperm makes contact with the oocyte at fertilization, the intracellular level of Ca^{2+} begins to oscillate. At the beginning, there are a series of rapid transients, before the oscillator begins to generate a regular rhythm with a periodicity of about 3 min. The series of images shown at the bottom illustrate the spatial organization of the transients that initiate at the point of sperm–oocyte fusion and then spread through the oocyte as an intracellular wave. Reproduced with permission from Miyazaki, S., Yuzaki, M., Nakada, H., Shirakawa, H., Nakanishi, S., Nakade, S., Mikoshiba, K. (1992) Block of Ca^{2+} wave and Ca^{2+} oscillation by antibody to the inositol 1,4,5-trisphosphate receptor in fertilized hamster eggs. Science, 257:251–55. Copyright (1992) American Association for the Advancement of Science; http://www.sciencemag.org; see Miyazaki et al. 1992.

obstructive and non-obstructive azoospermia. Patients with defective spermatogenesis may also benefit by using spermatozoan precursors such as the spermatids. Not having undergone the normal maturation processes, such spermatids cannot induce fertilization following ICSI because they fail to trigger Ca^{2+} oscillations. Various treatments designed to increase the level of Ca^{2+}, such as high-voltage pulses or co-injection of adenophostin B, which is an activator of $InsP_3$ receptors, have been tried to assist fertilization by injected spermatids. In the case of mouse oocytes, co-injection of a spermatid and adenophostin B produced regular Ca^{2+} oscillations (Module 8: Figure adenophostin-enhanced ICSI), and fertilization was normal. What is particularly interesting about ICSI is that successful *in vitro* fertilization requires the same sequence of Ca^{2+} oscillations that occur when oocytes are fertilized through the normal sperm–oocyte fusion process.

Functional significance of oocyte Ca^{2+} oscillations

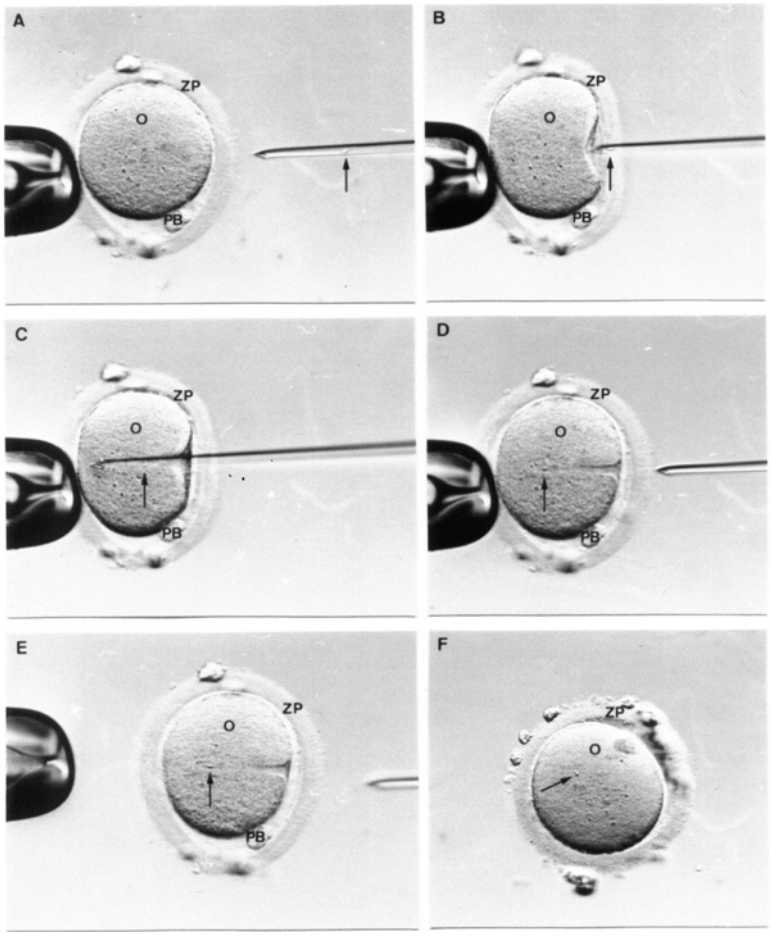
Both normal and *in vitro* fertilization appears to be driven by Ca^{2+} oscillations, raising obvious questions as to the functional role of oscillatory activity. It has been known for a long time that the parthenogenetic activation of eggs can be achieved by a variety of treatments (Ca^{2+} injection, electropermeabilization or addition of Ca^{2+} ionophores or ethanol), which have in common the ability to produce a prolonged monotonic increase in Ca^{2+}. However, such a presentation is not very effective, especially in freshly ovulated eggs, and usually results in limited development, whereas repetitive spiking is much more effective. It is therefore clear that the Ca^{2+} oscillations that occur at fertilization are the most effective way of activating the egg.

Ca^{2+} performs a number of functions during this process of egg activation. It triggers the release of cortical granules to form a further membrane around the fertilized egg. In some cases, it activates K^+ channels, giving rise to regular hyperpolarizations of the membrane that take place in phase with the Ca^{2+} oscillations. Finally, the prolonged Ca^{2+} oscillations are responsible for activating the cell cycle events that complete meiosis and initiate the programme of proliferation and development. Firstly, there is the completion of meiosis at fertilization, which depends on Ca^{2+} interacting with the cell cycle machinery to bring about extrusion of the second polar body to form the pronucleus. Secondly, Ca^{2+} functions in the control of mitosis that occurs at the end of each cell division cycle. Some of this control may depend upon cell cycle and Ca^{2+} oscillator interactions.

Completion of meiosis at fertilization

At the time of fertilization, the regular bursts of Ca^{2+} awaken the egg by acting on the cell cycle machinery that function to maintain the egg in its arrested state at the M_2 phase of meiosis. The onset of these spikes, which last for 2–4 h, are responsible for activating various cell cycle events (Module 8: Figure PLC sequestration hypothesis) that are carried out by a family of cyclins and their associated cyclin-dependent kinases (CDKs) that function in

Module 8: | Figure intracytoplasmic sperm injection



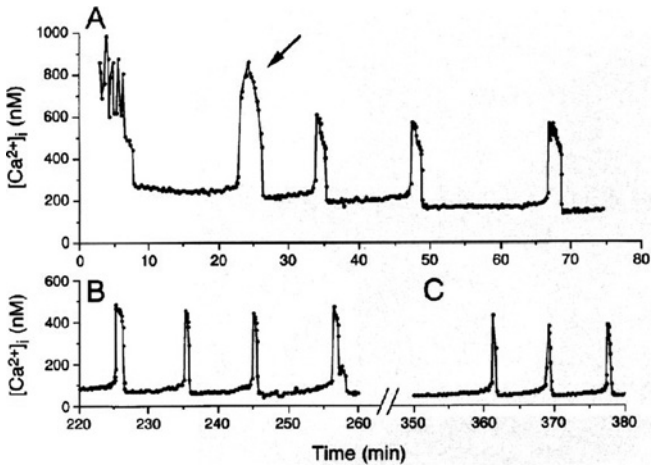
Artificial insemination by intracytoplasmic spermatozoa injection (ICSI) of a human oocyte.
A. A blunt electrode on the left holds the oocyte in place ready to be penetrated by a sharp microinjection needle containing a single spermatozoon (arrow). B. The needle is pushed against the oocyte and causes it to dimple before it penetrates. C. The spermatozoon is injected into the cytoplasm of the oocyte. D and E. The needle is withdrawn, leaving the spermatozoon in the cytoplasm. F. At 2 min after injection, the intact spermatozoon is clearly visible within the cytoplasm of the oocyte. Reproduced from Tesarik, J. and Mendoza, C. (1999) *In vitro* fertilization by intracytoplasmic sperm injection. BioEssays 21:791–801, with permission from John Wiley and Sons, Inc.; Copyright 1999 Wiley-Liss, Inc., A Wiley Company; see Tesarik and Mendoza 1999.

cell cycle signalling (Module 9: Figure cell cycle signalling mechanisms). Some of the cyclin/CDK complexes control events at either meiosis or mitosis. During the completion of meiosis in oocytes, the cell must undergo a mitotic event in order to extrude the second polar body. This requires the oocyte to enter mitosis and to activate chromosome separation.

The Ca^{2+} spikes that occur during fertilization act through calmodulin (CaM) to stimulate Ca^{2+}/CaM-dependent protein kinase II (CaMKII), which then activates the cell cycle machinery responsible for the entry into mitosis (Module 9: Figure mitotic entry). The Ca^{2+} spikes that occur during fertilization are responsible for activating the Cdc25 protein phosphatases that initiate the process of nuclear envelope breakdown and spindle assembly. Once this phase is complete, Ca^{2+} is also required to activate chromosome separation (Module 9: Figure chromosome separation). Although a single Ca^{2+} spike can rapidly degrade cyclin B, this effect is transitory, since the cyclin B level recovers. It seems that multiple spikes are necessary to bring about the prolonged degradation that is necessary to complete meiosis. This completion of meiosis is marked by extrusion of the polar body, and this is then followed by fusion of the male and female nuclei to form the pronucleus, which marks the time when the Ca^{2+} oscillations suddenly cease. It has been proposed that the oscillations stop at this time because the pronuclei sequester the phospholipase Cζ (PLCζ), causing the level of inositol 1,4,5-trisphosphate ($InsP_3$) to fall (Module 8: Figure PLC sequestration hypothesis).

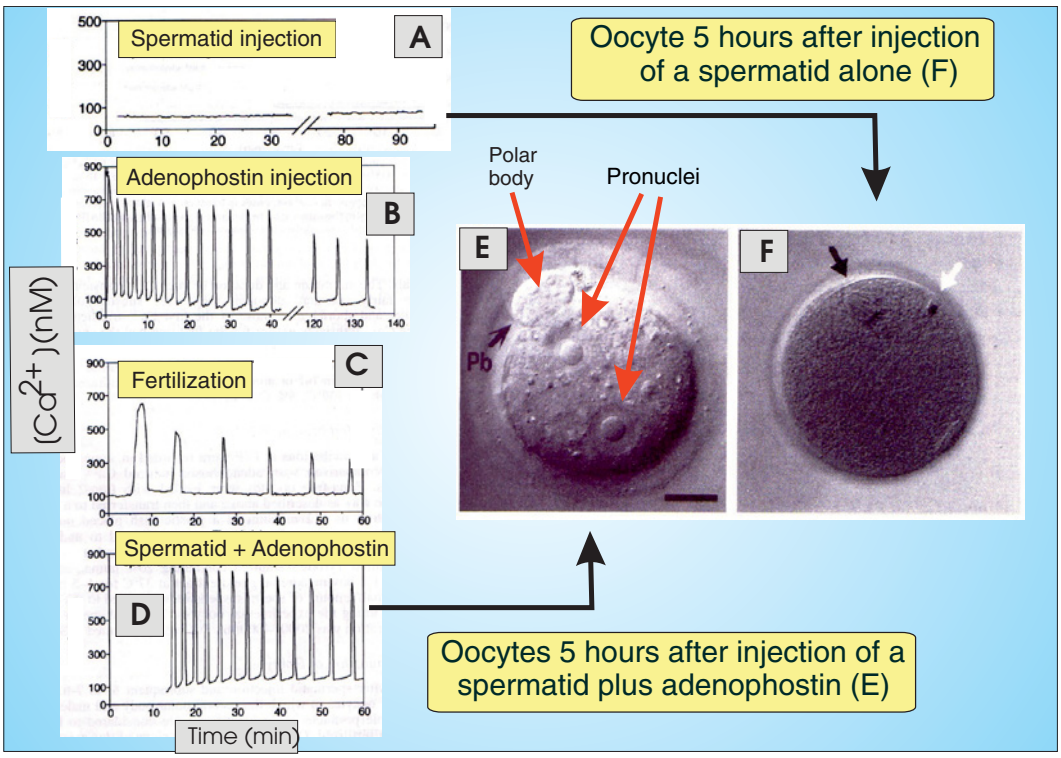
The Ca^{2+} signalling system has now completed its first task in the early embryo. Having initiated the programme of development, the Ca^{2+} signalling system enters a quiescent phase as the zygote moves through the cell cycle, but it is soon called into play again because Ca^{2+} spikes spontaneously reappear as the zygote prepares for its first mitosis. For example, as the one-cell mouse embryo approaches its first mitosis, there is a spontaneous Ca^{2+} transient at the time of nuclear envelope breakdown (NEBD) (Module 8: Figure mitotic Ca^{2+} transient). In some cases, there are a

Module 8: | Figure ICSI-induced Ca^{2+} oscillations



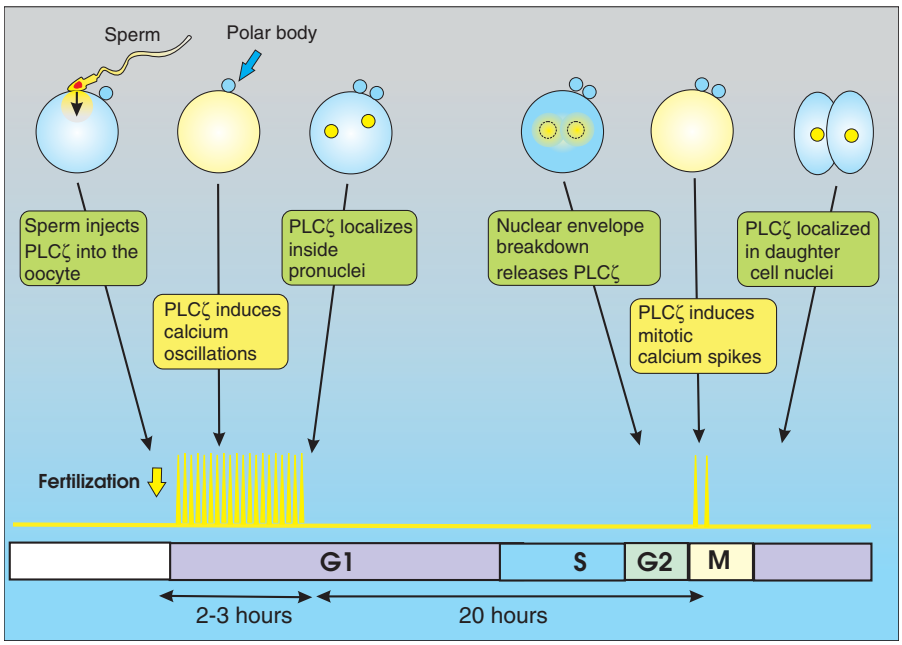
Ca^{2+} oscillations following artificial insemination by intracytoplasmic spermatozoa injection (ICSI).
A. A single spermatozoon was injected into a mouse oocyte at time zero. After recovery from the initial injection transient, there was a latency of 10 min before the onset of the first transient (transient), followed by a series of transients similar to those observed during normal fertilization, which lasted for 6 h as shown by the continuation of the recording shown in traces B and C. Reproduced, with permission, from Nakano, Y., Shirakawa, H., Mitsuhashi, N., Kuwabara, Y. and Miyazaki, S., Spatiotemporal dynamics of intracellular calcium in the mouse egg injected with a spermatozoon, *Mol. Hum. Reprod.*, 2001, vol. 3, pp. 1087–1093; Copyright 2001 European Society of Human Reproduction and Embryology; http://humrep.oxfordjournals.org/; see Nakano et al. 2001.

Module 8: | Figure adenophostin-enhanced ICSI



Enhancement of spermatid intracytoplasmic spermatozoa injection (ICSI) by adenophostin.
A. Injection of a spermatid had no effect on Ca^{2+} signalling, and there was no fertilization in that the oocyte chromosomes remained in metaphase II (black arrow in F) and the spermatid nucleus remained intact (white arrow in F). B. Injection of adenophostin to give a final concentration of 300 nM triggered regular Ca^{2+} oscillations that persisted for over 2 h. C. Normal oscillations resulting from sperm-induced fertilization. D. Oscillations in Ca^{2+} induced by fertilization of a spermatid together with adenophostin. This combination initiated a normal fertilization response, as shown by the extrusion of the second polar body and the appearance of two large pronuclei. Reproduced, with permission. Copyright © 1998, Biol. Reprod. Online by Society for the Study of Reproduction; see Sato et al. 1998.

Module 8: | Figure PLC sequestration hypothesis



The role of phospholipase Cζ (PLCζ) in controlling Ca^{2+} signalling and early cell cycle events following fertilization: the PLCζ sequestration hypothesis.

At the time of fertilization, the sperm inject phospholipase Cζ (PLCζ) into the oocyte to initiate the Ca^{2+} oscillations that function to complete meiosis by extruding the second polar body and forming the pronuclei. The latter marks the point where Ca^{2+} oscillations cease. As the zygote approaches the first mitosis, nuclear envelope breakdown releases PLCζ, which once again initiates the Ca^{2+} signalling that may drive certain aspects of mitosis. When the nuclei reform within the two daughter cells, they resequester PLCζ, which is taken out of circulation causing the spikes to cease. (Summary of work described in Marangos et al. 2003.)

series of transients during the process of mitosis leading up to cleavage to the two-cell stage. As described above, these oscillations may be driven by the periodic release and sequestration of PLCζ to give a pulse of $InsP_3$ at each mitotic event (Module 8: Figure PLC sequestration hypothesis). Direct measurements have shown that the level of $InsP_3$ increases during both the first and second mitotic phases of *Xenopus* embryos. These spontaneous $InsP_3$-dependent Ca^{2+} transients may be responsible for initiating specific cell cycle events, particularly those associated with the entry into mitosis and the separation of the chromosomes at anaphase as described earlier.

In effect, there is a long period Ca^{2+} oscillation that runs in phase with the cell cycle oscillator. Indeed, cell cycle and Ca^{2+} oscillator interactions seem to be important for controlling both mitosis and subsequent phases of the cell cycle.

Cell cycle and Ca^{2+} oscillator interactions

Perhaps the most remarkable aspect of the early embryo is its rapid progression through a number of cell cycles apparently independent of external stimulation. Apart from the initial Ca^{2+}-dependent kick-start provided by the interaction and fusion of the sperm, the developing embryo appears to be driven by a powerful endogenous oscillator(s) capable of driving repeated rounds of cell proliferation. Most attention has focused on the cell cycle responsible for periodic mitotic phases, which are the most visible events of this oscillator. However, this orderly cell cycle programme may be controlled by a 'master clock' that consists of two coupled oscillators: a Ca^{2+} oscillator and a cell cycle oscillator (Module 8: Figure coupled cell cycle/Ca^{2+} oscillator).

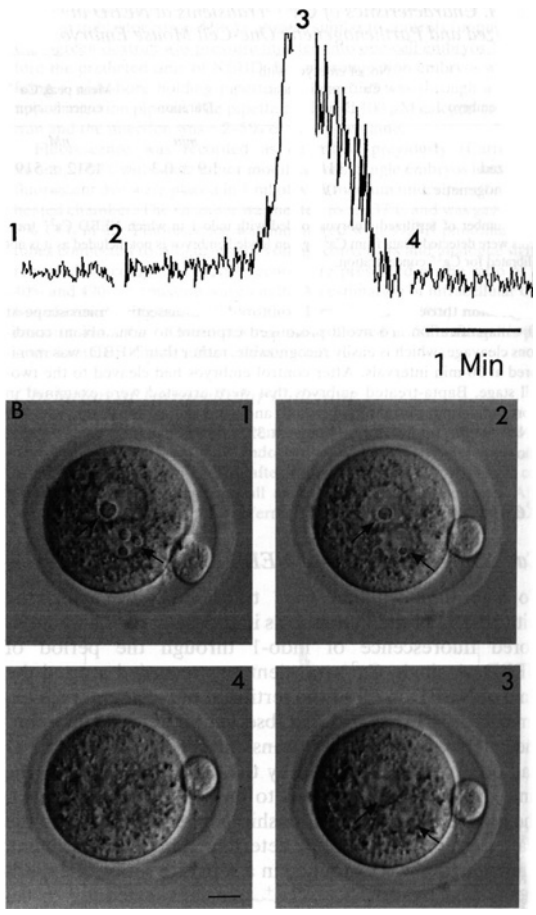
The two oscillators can be dissociated from each other. The Ca^{2+} oscillator appears to be the driving oscillator, since it can persist when dissociated from the cell cycle oscillator, whereas the opposite does not apply:

- Ca^{2+} oscillations persist when cell cycle events are blocked using colchicine to inhibit microtubules or emetine to block the resynthesis of proteins such as the cyclins. Despite this inhibition of the cell cycle oscillator, the Ca^{2+} oscillator in *Xenopus* oocytes persists for at least 5 h, with a periodicity of about 30 min, which coincides exactly with the length of each cell cycle. However, when the Ca^{2+} oscillator is free-running, the individual spikes are not as sharp, suggesting that there is a reciprocal interaction between the cell cycle and the Ca^{2+} signalling system.
- Inhibiting Ca^{2+} oscillations using Ca^{2+} chelators, such as 1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid (BAPTA) or heparin to inhibit the inositol 1,4,5-trisphosphate ($InsP_3$) receptor, arrests the cell cycle oscillator.

Cell specification

The process of embryonic development depends on two parallel processes. Firstly, the single cell zygote grows

Module 8: | Figure mitotic Ca^{2+} transient



Spontaneous Ca^{2+} transients precede nuclear envelope breakdown at the first mitosis of the developing mouse embryo.
The numbers identify gaps in the recording when the four photographs were taken. Note that the nuclear envelope has disappeared in photograph 3 at the peak of the Ca^{2+} transient. Reproduced from *The Journal of Cell Biology*, 1996, vol. 132, pp. 915–923, by copyright permission of The Rockefeller University Press; see Kono et al. 1996.

through rapid rounds of cell proliferation to build up a large mass of cells and, secondly, the processes of cell specification and cell differentiation result in the emergence of the specialized cell types that make up the adult organism (Module 8: Figure cellular life history). During the process of specification, cells acquire the genetic instructions to enable them to differentiate into different cell types. This specification process goes through a series of stages, during which the set of instructions becomes increasingly precise as the embryo moves from a ball of near-identical cells to the fully differentiated organism (Module 8: Figure summary of development). An important feature of specification is axis formation, which begins with the establishment of the dorsal–ventral and anterior–posterior axes. The process of dorsoventral specification has been studied in some detail in amphibians and zebrafish. This early pattern formation also includes the establishment of left–right asymmetry.

Once these axes are established, groups of cells begin to separate into compartments where cells are given a specific cell fate and where their development can proceed without becoming mixed up with other cell types. Once general instructions are laid down for all the cells within each compartment, the final specification process is to provide each cell with its final set of instructions as to its particular function. This final specification process is still very mysterious and remains one of the major unsolved problems in developmental biology. There is increasing interest in the idea that this final specification process might be carried out by diffusible morphogens that set up gradients within the specified compartments. Individual cells may then use the co-ordinates of this gradient (i.e. its concentration and direction) to determine their final fate. An example of such a final specification process is planar cell polarity (PCP), during which individual or groups of cells establish their polarity within the two-dimensional plane of a developing epithelium, such as the epidermis or cochlea. Once these processes of cell determination and pattern formation are complete, the final form of the animal begins to appear as each cell begins to express its specific set of instructions during the process of cell differentiation. This process of differentiation can be reversible in some cases, and such an example of de-differentiation occurs during the phenotypic remodelling that occurs during cardiac hypertrophy when the ventricular cells begin to express foetal genes (Module 12: Figure hypertrophy working hypothesis).

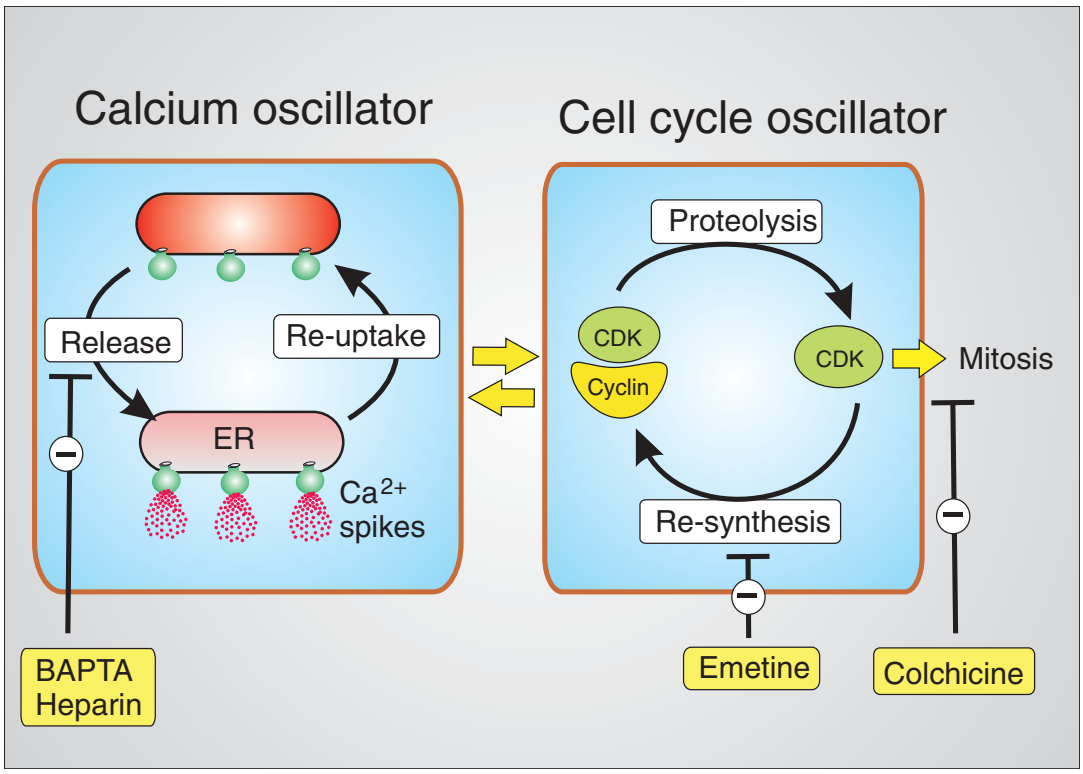
The role of cell signalling pathways in controlling and co-ordinating this complex developmental sequence is still being worked out. The Wnt signalling pathway and the Hedgehog signalling pathway appear to be particularly important. In addition, it is clear that there is a central role for Ca^{2+} in embryonic development.

Ca^{2+} in embryonic development

There have been numerous reports of Ca^{2+} having a role to play in development. The most convincing evidence for such a role in orchestrating development has been provided using aequorin to monitor changes in the transparent zebrafish embryo continuously throughout a 10 h period (Module 8: Figure embryonic Ca^{2+} transients). After an initial quiescent period, the embryo begins to display a remarkable sequence of Ca^{2+} transients with periodicities that range from 5 to 15 min. What is equally remarkable about these transients is their spatial distribution. The Ca^{2+} signalling system is used in different spatial and temporal domains at different stages during development (Module 8: Figure developmental Ca^{2+} signalling).

A role for Ca^{2+} begins at the time of fertilization when intracellular Ca^{2+} waves spread through the cytoplasm to activate the oocyte to begin the process of development (Module 8: Figure mammalian fertilization). It comes in to play again at the time of the first cell division, where it appears to have a role in the activation of contraction during cytokinesis. Ca^{2+} has also been implicated in the trafficking and insertion of membrane vesicles (Module 9: Figure cytokinesis). In the case of the zebrafish embryo, a highly localized microdomain of Ca^{2+} is responsible for activating the process of cleavage (see panel b in Module 8: Figure developmental Ca^{2+} signalling). As development proceeds, there are further localized Ca^{2+} signals, but their

Module 8: | Figure coupled cell cycle/Ca^{2+} oscillator



The periodic activation of mitosis might be driven by an interaction between a Ca^{2+} and a cell cycle oscillator.
The Ca^{2+} oscillator depends upon the periodic release of Ca^{2+} from the endoplasmic reticulum (ER) that occurs spontaneously at the time of mitosis. The cell cycle oscillator depends upon the periodic proteolysis of cyclin through a process controlled by Ca^{2+}. These two oscillators interact with each other, as described in the text.

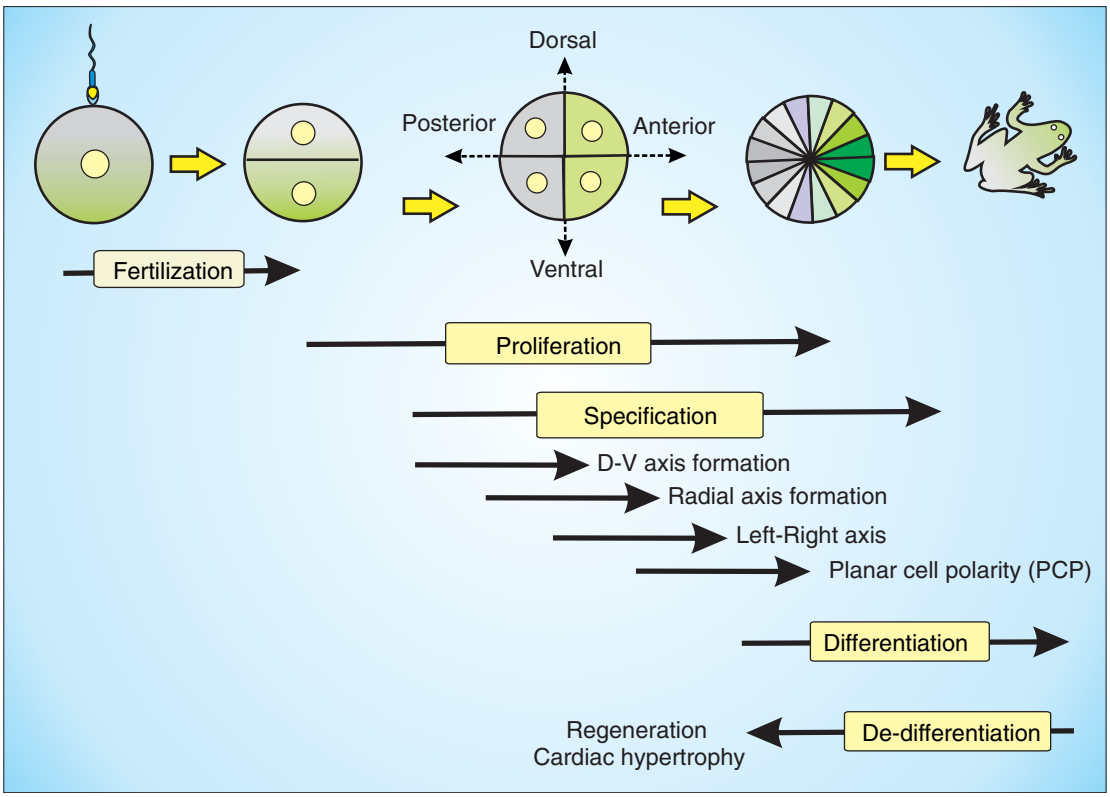
exact function is still being worked out. One dramatic sequence occurs during the late gastrula when there are pan-embryonic intercellular Ca^{2+} waves that sweep out around the blastoderm margin (Module 8: Figure embryonic Ca^{2+} wave). This Ca^{2+} could play some role in controlling the large-scale migration of cells that occurs during gastrulation. Similar intercellular waves have been recorded in the amphibian embryo during the process of convergent extension. Later in development, there are localized pulses during the process of segmentation, but their function remains to be determined.

Dorsoventral specification

Specification of the dorsoventral axis is one of the fundamental processes of animal development. For the vertebrates, it is best understood in amphibia, where the unfertilized egg begins with only the animal–vegetal (A-V) axis of polarity, which already segregates some key signalling components. For example, the mRNA that codes for Vg1 is concentrated in the vegetal region and encodes a growth factor resembling transforming growth factor-β (TGF-β). On the other hand, the inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$) are highly expressed in the animal hemisphere, which gives rise to the skin and nervous system. The differential distribution of this key Ca^{2+} signalling component may play an important role in setting up the dorsoventral axis at the time of fertilization. The dorsal side of the embryo forms opposite the site of sperm entry. The microtubule reorganization associated with formation of the sperm asters causes the cortical cytoplasm to rotate 30° relative to the inner endoplasm. The resulting interaction of components from the vegetal and animal hemispheres leads to the formation of a dorsalizing region (Nieuwkoop centre), which then induces the overlying mesodermal cells to become the Spemann's organizer. This dorsalizing region then orchestrates the patterning of axial structures that emerge during the process of gastrulation by employing a variety of signalling molecules such as Vg1, activin and noggin. By contrast, levels of these signalling molecules are low on the opposite side of the embryo, resulting in the establishment of ventral components. In effect, the dorsoventral axis may be considered as a gradient of signalling components able to specify the developmental fates of cells lying within the gradient.

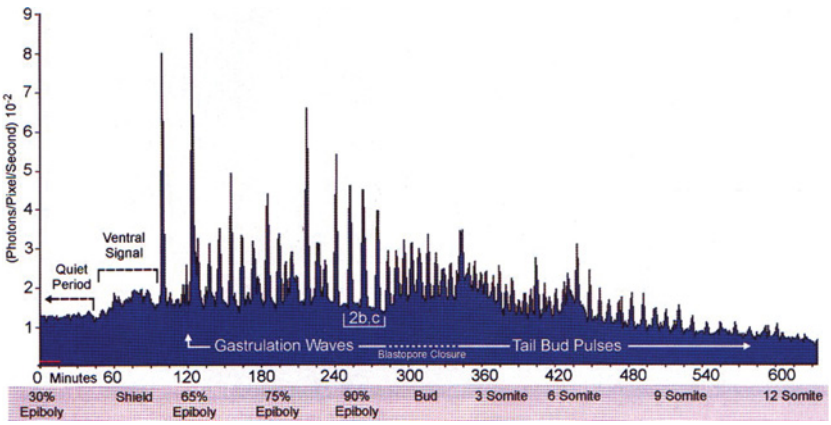
The Wnt signalling pathways appear to have a primary role in the establishment and maintenance of this dorsoventral axis (Module 8: Figure dorsoventral specification). There appear to be important roles for both the canonical Wnt/β-catenin pathway and the Wnt/Ca^{2+} signalling pathway. The latter is responsible for setting up ventral specification, whereas the former controls dorsal specification. The canonical Wnt/β-catenin signalling pathway may contribute to dorsoventral specification by inhibiting expression of miR-15 and miR-16 resulting in an

Module 8: | Figure summary of development



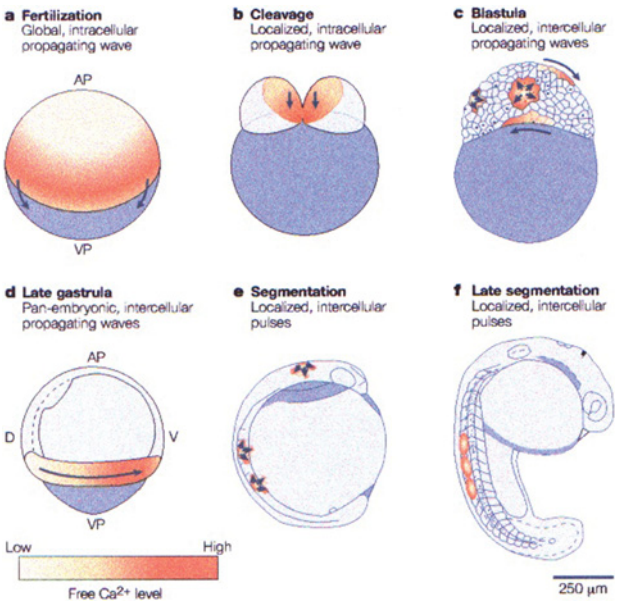
Summary of the major processes occurring during development.
The sequence of developmental events is triggered at fertilization. Initially, the cells proliferate rapidly, and, as the embryo grows, a process of specification begins to apportion cells to specific cell fates. An important feature of specification is the establishment of the different axes beginning with the dorsal–ventral (D-V) axis and culminating with planar cell polarity (PCP). During specification, cells are apportioned into compartments to continue their development. The final form of the animal is revealed through a process of differentiation. A process of de-differentiation can reverse some of the events of differentiation as occurs during regeneration and cardiac hypertrophy.

Module 8: | Figure embryonic Ca^{2+} transients



Pattern of Ca^{2+} transients in a developing zebrafish embryo.
Aequorin was used to record Ca^{2+} in the transparent zebrafish embryo over a period of 10 h, during which most of the developmental events occur. Many of the major Ca^{2+} transients have a precise spatial organization (see the text for details), indicating that they may have a role to play in driving specific developmental processes. Some of the transients that occur at 75% epiboly occur as intercellular Ca^{2+} waves that sweep around the blastoderm margin (see Module 8: Figure embryonic Ca^{2+} wave). Reproduced from Gilland, E., Miller, A.L., Karplus, E., Baker, R. and Webb, S.E. (1999) Imaging of multicellular large-scale rhythmic calcium waves during zebrafish gastrulation. Proc. Natl. Acad. Sci. U.S.A. 96:157–161. Copyright (1999) National Academy of Sciences, U.S.A.; see Gilland et al. 1999.

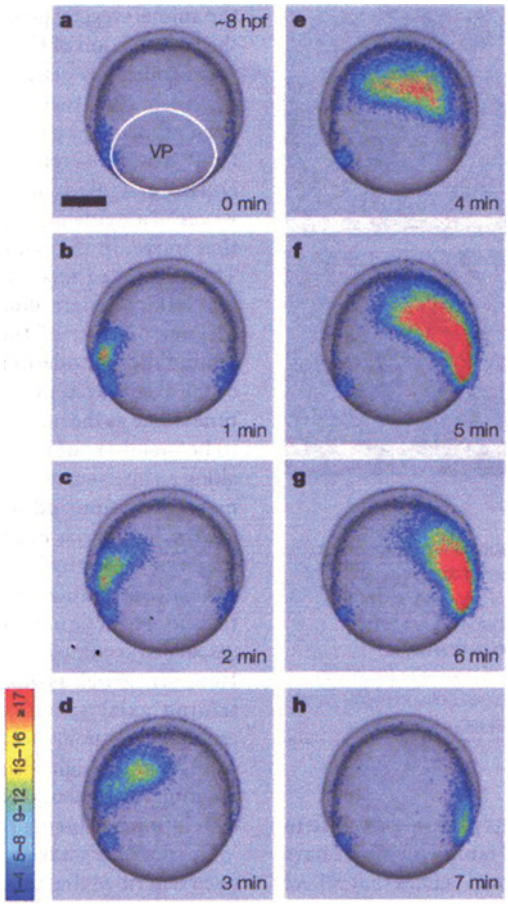
Module 8: | Figure developmental Ca^{2+} signalling



Localized Ca^{2+} microdomains and waves during zebrafish development.

These drawings illustrate that Ca^{2+} signals are highly localized during different stages of zebrafish development (see the text for further details). Reproduced by permission from Macmillan Publishers Ltd: *Nat. Rev. Mol. Cell Biol.*, Webb, S.E. and Miller, A.L. (2003) Calcium signalling during embryonic development. 4:539–551. Copyright (2003); http://www.nature.com/nrm; see Webb and Miller 2003.

Module 8: | Figure embryonic Ca^{2+} wave



Pan-embryonic intercellular wave in the blastoderm margin of a zebrafish embryo.

A zebrafish embryo loaded with aequorin reveals the presence of an intercellular wave that travels around the blastoderm margin in a clockwise direction at a rate of about 5 μm/s. The first gastrulation wave began at about 65% epiboly (see timings on Module 8: Figure embryonic Ca^{2+} transients), and subsequent waves were repeated at about 10 min intervals for about 2 h until blastopore closure. Reproduced by permission from Macmillan Publishers Ltd: *Nat. Rev. Mol. Cell Biol.*, Webb, S.E. and Miller, A.L. (2003) Calcium signalling during embryonic development. 4:539–551. Copyright (2003); http://www.nature.com/nrm; see Webb and Miller 2003.

increase in the activation of the Nodal-ACVRIIA signalling pathway.

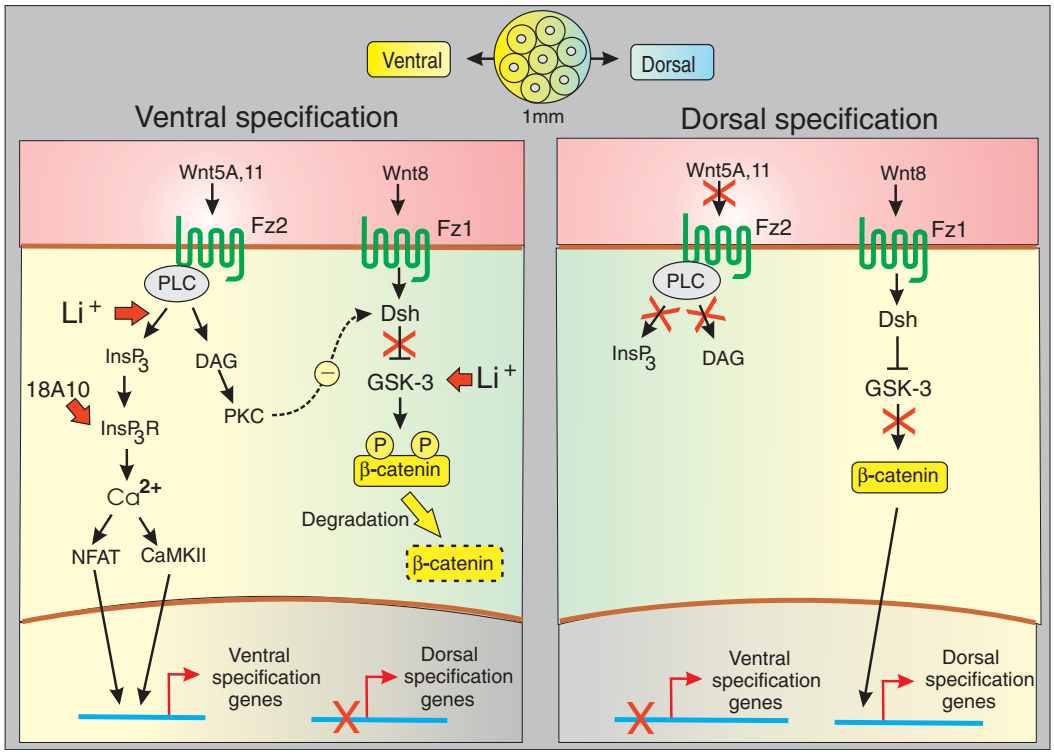
The high level of phosphoinositide and Ca^{2+} signalling on the ventral side is driven by Wnt5A/11, which acts by stimulating PLC (phospholipase C) to produce the two second messengers $InsP_3$ and diacylglycerol (DAG). The $InsP_3$ acts on $InsP_3Rs$ to release Ca^{2+}, which then acts through the transcription factor nuclear factor of activated T cells (NFAT) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) to switch on the ventral specification genes. By contrast, the canonical Wnt pathway that is activated by Wnt8 is relatively inactive in this dorsal region. The critical event seems to be the high level of the enzyme glycogen synthase kinase-3 (GSK-3), which functions to phosphorylate β-catenin to target it for degradation (Module 2: Figure Wnt canonical pathway). One of the key components of axis specification therefore is the enzyme GSK-3, which is high in the ventral region resulting in a decrease in β-catenin and the absence of the expression of dorsal genes such as Goosecoid. It has been suggested that DAG acting through protein kinase C (PKC) may contribute to this inactivation by preventing Dishevelled (Dsh) from inhibiting GSK-3. If the activity of GSK-3 is suppressed by expressing a kinase-dead enzyme, the ventral cells become dorsalized.

Ventral specification depends upon a reversal of the two Wnt signalling pathways described above (Module 8: Figure dorsoventral specification). The $InsP_3/Ca^{2+}$ pathway is suppressed, whereas the canonical pathway is active; the low level of GSK-3 activity means that the degradation of β-catenin is prevented, thus enabling this transcription factor to activate dorsal specification genes.

Axis formation in zebrafish seems to be controlled by a specification system very similar to that described above for *Xenopus*. Some of the evidence for the phosphoinositide and Ca^{2+} signalling systems playing a role in dorsoventral specification is summarized below:

The teratogenic agent Li^+ can respecify the axis by converting ventral cells into dorsal cells, thus forming a secondary axis that can result in embryos with two heads. Two mechanisms have been proposed for this dorsalizing action of Li^+ (Module 8: Figure dorsoventral specification). Firstly, it may act directly to inhibit the activity of GSK-3. Secondly, it may act indirectly to flatten out the proposed $InsP_3/Ca^{2+}$ gradient by preventing the recycling of inositol. This is the same inositol depletion hypothesis that was introduced to explain the action of Li^+

Module 8: | Figure dorsoventral specification



A model for dorsoventral specification in the early *Xenopus* embryo.
Dorsoventral specification of the *Xenopus* embryo is determined by the Wnt signalling pathways. Ventral specification is regulated through Wnt5A/11, which acts through a Ca^{2+} signalling pathway to activate the ventral specification genes. The Wnt8 pathway, which acts through the canonical Wnt pathway, is switched off via a mechanism that may depend upon diacylglycerol (DAG) acting through protein kinase C (PKC). In the dorsal regions, the activity of these two pathways is reversed. The pathway driven by Wnt5A/11 is inactivated, whereas the pathway activated by Wnt8 increases the level of the transcription factor β-catenin, which then switches on the dorsal specification genes.

in controlling manic depressive illness (Module 12: Figure inositol depletion hypothesis). The basic idea behind this hypothesis is that Li^+ inhibits signalling by inhibiting the supply of inositol required to resynthesize the lipid necessary for signalling. Indeed, the dorsalizing action of Li^+ can be reversed by supplying embryos with inositol, thus enabling them to maintain the dorsoventral gradient of this signalling pathway.

This observation has some interesting implications for human development, because inositol has been found to reduce neural tube defects in *curly tail* mice that are a model of folate-resistant spina bifida in humans.

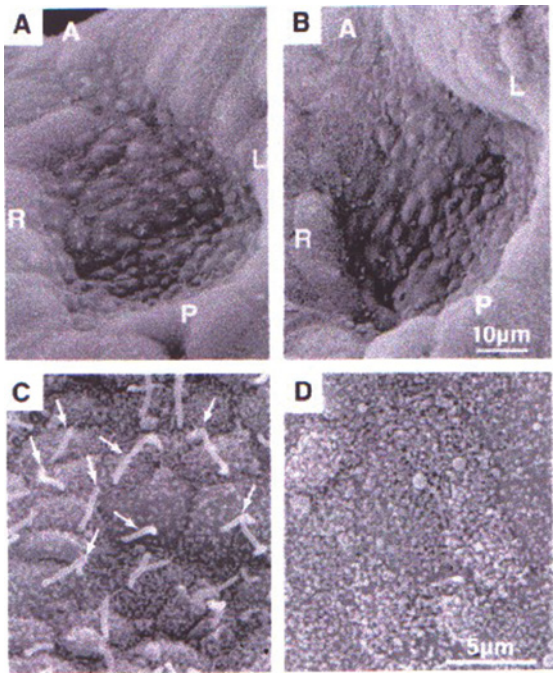
- Dorsalization also occurs following injection of the 18A10 antibody that inhibits Ca^{2+} release by *Xenopus* $InsP_3Rs$.
- Artificially elevating the level of phosphoinositide signalling on the dorsal side by applying 5-hydroxytryptamine (5-HT) to embryos that have been induced to express $5\text{-}HT_{1C}$ receptors results in ventralization. On the basis of these experiments, it was proposed that the activity of the phosphoinositide signalling pathway was graded along the dorsoventral axis.
- The concentration of $InsP_3$ increases significantly from the 32- to the 512-cell stage in zebrafish at the time the dorsoventral axis is being established. This increase in the level of $InsP_3$ coincides with the appearance of Ca^{2+} spikes, which are restricted to cells of the outer enveloping layer. Spike periodicity was somewhat irregular, and varied between a few seconds up to several minutes. The frequency of these transients was doubled in embryos expressing Xwnt-5A, and this could be duplicated if embryos were made to express the $5\text{-}HT_{1C}$ receptor that is known to generate $InsP_3$.

All of this evidence seems to indicate that a high level of phosphoinositide signalling on the ventral side may contribute to pattern formation by helping to define the all-important dorsoventral axis in amphibians and in zebrafish.

Left–right asymmetry

One of the three primary body axes is left–right asymmetry, which is set up within a node, which is a transient structure formed early in embryogenesis (e.g. at embryonic day 7.5 in mice). The node is a triangular depression of cells, each of which is lined with a layer of ciliated cells (Module 8: Figure nodal structure). Each cell contains a primary cilium, which plays a crucial role in setting up the asymmetry by establishing a right-to-left flow of fluid over the surface of the node (Module 8: Figure nodal flow hypothesis). The node has two populations of cilia; those in the centre are motile, whereas those on the periphery are immotile. The basic idea is that the central cilia have a rotary

Module 8: | Figure nodal structure



Structural organization of the node where left–right asymmetry is determined.
These scanning electron micrographs illustrate the triangular organization of the node, with its apex towards the anterior (A). Panels A and C are from a wild-type mouse, showing that the surface of the nodal cells have a single primary cilium (white arrows in the higher magnification picture in C). Panels B and D are from a kif3B$^{-/-}$ embryo, where the nodal cells lack cilia. Reproduced from *Cell*, Vol. 95, Nonaka, S., Tanaka, Y., Okada, Y., Takeda, S., Harada, A., Kanai, Y., Kido, M. and Hirokawa, N., Randomization of left–right asymmetry due to loss of nodal cilia generating leftward flow of extraembryonic fluid in mice lacking KIF38 3B motor protein, pp. 829–837. Copyright (1998), with permission from Elsevier; see Nonaka et al. 1998.

motion that sets up a flow of fluid that is directed from the right to the left. As it flows leftwards, it deforms the cilia on the side that have mechanosensitive polycystin-2 channels, which gate Ca^{2+} to establish a standing Ca^{2+} gradient that is high on the left and low on the right (Module 8: Figure nodal Ca^{2+} gradient). The ability of such primary cilia to generate a Ca^{2+} signal in response to fluid flow has been established in kidney epithelial cells (Module 3: Figure flow-induced Ca^{2+} signals).

Such a nodal Ca^{2+} gradient may then be responsible for activating genes such as *lefty-1*, *lefty-2*, *nodal* and *Pitx2* responsible for establishing the left–right axis. The *nodal* gene codes for nodal, which is a member of the TGF superfamily of ligands that act through the Smad signalling pathway (Module 2: Table Smad signalling toolkit). As the left–right asymmetry emerges, nodal is located on the left side of the developing embryo.

Planar cell polarity (PCP)

Many cell types acquire a distinct polarity that emerges during the final stages of development. In the case of epithelia, an apical–basal polarity enables cells to carry out directed transport functions. In addition, some cells also develop a polarity within the two-dimensional plane of an epithelial layer of cells and this is known as planar cell polarity (PCP). A number of examples of PCP have been described in both insects and vertebrates (Module 8: Figure PCP in insects and vertebrates):

- Insect bristle polarity
- *Drosophila* ommatidial polarity
- Convergent extension (CE)
- Orientation of hair cell stereociliary bundles
- Mammalian hair follicle orientation

Insect bristle polarity

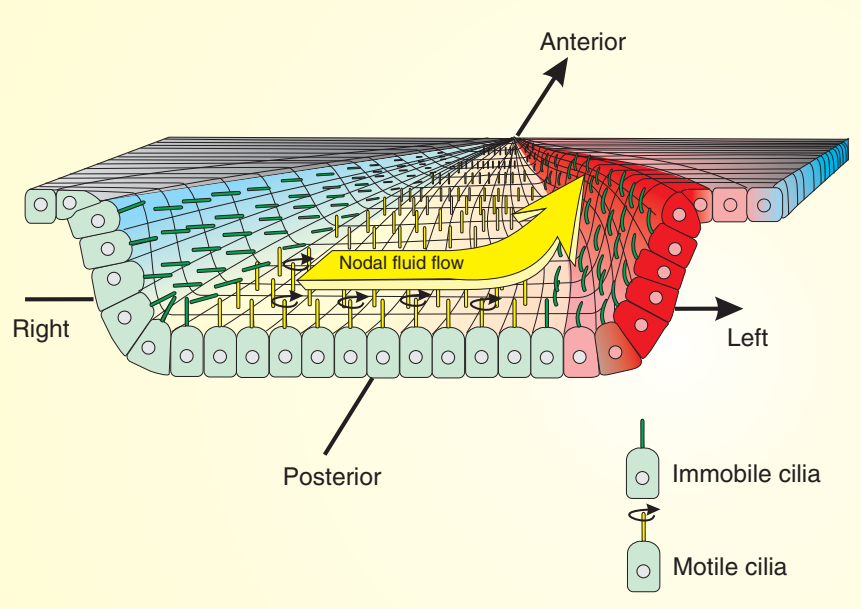
Planar cell polarity (PCP) was first described in insects where the hairs and bristles on the wings and abdomen have a precise polarity. In the case of the abdomen, they have an anterior–posterior orientation (Module 8: Figure *Drosophila* planar cell polarity), whereas on the wing, they lie along the proximal–distal axis (see panel a in Module 8: Figure PCP in insects and vertebrates). Many of the genes responsible for this polarity have been identified in *Drosophila*, and many of these also play a role in setting up PCP in vertebrates (Module 8: Table PCP signalling components and effectors). The challenge is to work out how all of these signalling components interact with each other to orchestrate the internal effector system responsible for establishing PCP. These different signalling components appear to be organized into two parallel planar cell polarity (PCP) signalling pathways (Module 8: Figure planar cell polarity signalling).

Planar cell polarity (PCP) signalling pathways

The signalling pathways that control planar cell polarity remain to be fully characterized. In the case of *Drosophila*, there appear to be two pathways. The Frizzled (Fz)/Flamingo (Fmi) polarity signalling pathway and the Dachsous (Ds)/Fat (Ft) polarity signalling pathway (Module 8: Figure planar cell polarity signalling).

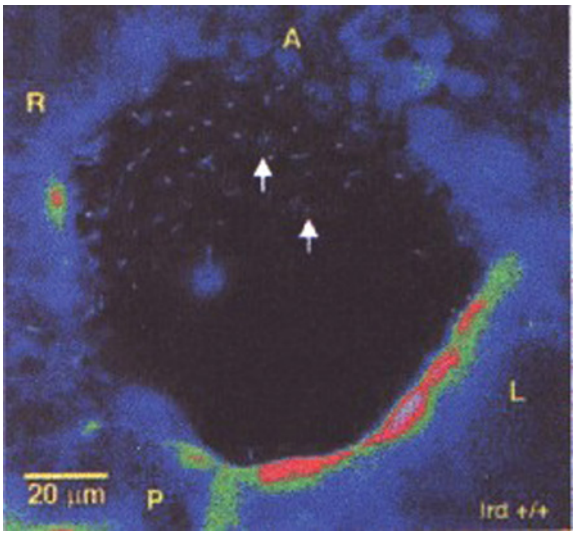
An interesting feature of these polarity signalling pathways is that they both seem to be linked to elements of the cytoskeleton, which is reasonable, since at some point they have to interact with downstream effectors responsible for polarizing the biosynthetic pathways that give bristles and hairs their distinctive orientation. In the case of Frizzled (Fz)/Flamingo (Fmi) polarity signalling pathway, cytoskeletal contraction is activated through the DAAM1/Rho/Rho kinase (ROK)/myosin regulatory light chain (MRLK)/myosin II pathway (Module 8: Figure planar cell polarity signalling). Less information is available for the Dachsous (Ds)/Fat (Ft) polarity signalling pathway, but studies on vertebrate Fats have shown that the cytoplasmic tail can interact with the Ena/vasodilator-stimulated phosphoprotein (VASP) family, and this may suggest a link to the cytoskeleton. During wound healing of vertebrate cells, Fat also seems to play a role in the orientation of the Golgi apparatus, and such a role may be highly significant since the four-jointed (Fj) protein, which is an important component of this signalling pathway, is thought to be located on the Golgi.

Module 8: | Figure nodal flow hypothesis



The nodal fluid flow hypothesis for establishing left–right asymmetry.
The triangular depression that is the node contains cells, each of which has a single cilium. The node has two populations of cilia: there are motile cilia in the central region (yellow) that are surrounded by non-motile cilia (green). The central motile cilia are distinguished from the non-motile cilia by having the motor protein left–right dynein (lrd) that enables them to set up a rotary counter-clockwise motion that produces a directed flow of nodal fluid. This nodal fluid flow causes the non-motile cilia to bend over, and this mechanical deformation activates the mechanosensitive Ca^{2+} channel polycystin-2. The idea is that the left-to-right flow deforms the cilia to activate polycystin-2, thereby setting up a Ca^{2+} gradient specifically in the cells on the left side of the node (red cells). This is the symmetry-breaking event responsible for activating the genes such as lefty-1, lefty-2, nodal and Pitx2 that then go on to establish the left–right axis.

Module 8: | Figure nodal Ca^{2+} gradient



A standing Ca^{2+} gradient in the node of a mouse embryo.
The Ca^{2+} indicator Fluo3 was used to detect the high level of Ca^{2+} located in a band of cells on the left (L) side of the node. Reproduced from *Cell*, Vol. 114, McGrath, J., Somlo, S., Makova, S., Tian, X. and Brueckner, M., Two populations of node monocilia initiate left–right asymmetry in the mouse, pp. 61–73. Copyright (2003), with permission from Elsevier; see McGrath et al. 2003.

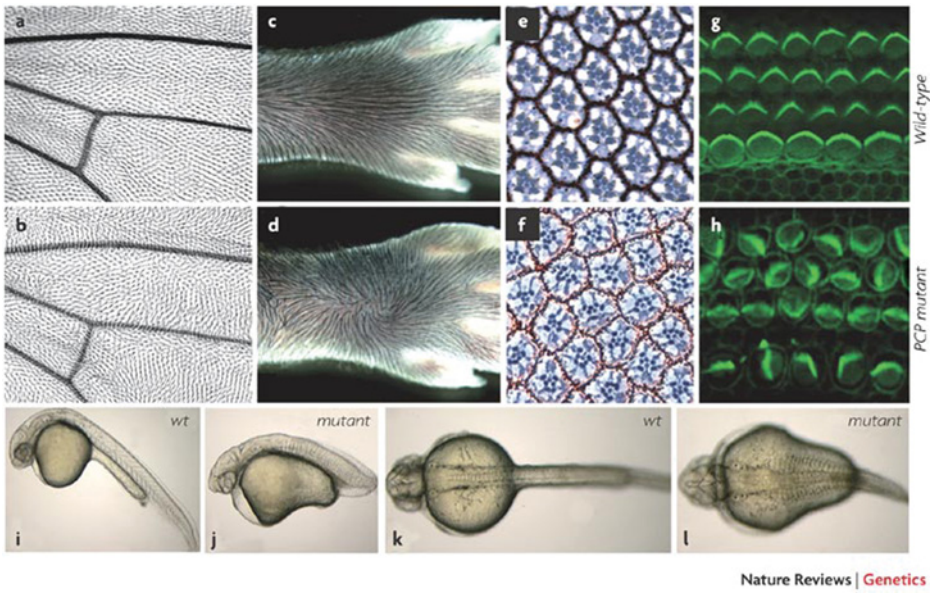
Frizzled (Fz)/Flamingo (Fmi) polarity signalling

The Frizzled (Fz)/Flamingo (Fmi) polarity signalling pathway contains all the elements of the Wnt/planar cell polarity (PCP) pathway (Module 2: Figure Wnt signalling pathways). An important additional component of this pathway is the atypical cadherin flamingo (Fmi), which forms homophilic junctions between cells and seems to be intimately connected to the role of Fz in regulating polarity. A number of other polarity signalling components (Module 8: Table PCP signalling components and effectors) such as strabismus (Stbm), Diego (Dgo) and Prickle (Pk) seem to be part of a signalling complex that ties together Fz and Fmi (Module 8: Figure planar cell polarity signalling).

The evidence that two separate signalling pathways exist is based on experiments where bristle/hair polarity is analysed following mutations of individual components of these two putative signalling pathways. Since each cell has a clear identifier of its polarity (i.e. the orientation of a single hair or bristle), there is an unambiguous way of determining the outcome of genetic manipulations designed to alter these putative PCP signalling candidates. A functional assay based on producing a small clone of mutated cells within the main body of cells, which are either wild-type or have an altered genotype, has proved most instructive (Module 8: Figure *Drosophila* planar cell polarity).

The abdomen of *Drosophila* has bristles that all point backwards, indicating that the individual cells have an anterior–posterior polarity (yellow arrows in panel A in Module 8: Figure *Drosophila* planar cell polarity). If a clone of cells, which have a null mutation in the frizzled gene

Module 8: | Figure PCP in insects and vertebrates



Examples of planar cell polarity (PCP) in insects and vertebrates.
a. The orientation of hairs in a wild-type *Drosophila* wing. b. A wing carrying a mutation in Frizzled (Fz). c. Normal hair orientation on a mouse paw. d. Hair swirls in a mouse carrying a mutation in Fz6. e. Normal ommatidial orientation in the eye of *Drosophila*. f. Ommatidial orientation disrupted in a fly carrying a mutation in strabismus (Stbm). g. Normal orientation of stereocilia on hair cells in mouse cochlea. h. Pattern of stereociliary bundles disrupted in Vangl2 mutants. i. and j. Lateral and dorsal views of the zebrafish embryo after convergent extension. j. and l. The same views of a Stbm mutant that failed to extend. Reproduced by permission from Macmillan Publishers Ltd: Seifert, J.R.K. and Mlodzik, M. (2007) Frizzled/PCP signalling: a conserved mechanism regulating cell polarity and directed motility. Nat. Rev. Genet. 8:126–138. Copyright (2007); http://www.nature.com/nrg; see Seifert and Mlodzik (2007).

(Fz^-), is introduced into such a wild-type background, there is a dramatic change in the polarity of bristles at the back (posterior) of the clone (blue arrows in panel B in Module 8: Figure *Drosophila* planar cell polarity). Instead of pointing backwards, the bristles now point anteriorly towards the clone. Conversely, if cells in the clone overexpress frizzled (Fz^+), the wild-type cells in front of the clone point their bristles away from the clone. Experiments of this kind indicate that the Fz receptor plays an important role in establishing polarity. Furthermore, these Fz^+ and Fz^- clones were not able to influence polarity if they occur within a background of cells where Fmi was inactivated. Fmi is an atypical cadherin, which forms homophilic interactions between neighbouring cells. It has been suggested that Fmi may function as a comparator to relay information about the relative levels of Fz activity (Module 8: Figure planar cell polarity signalling).

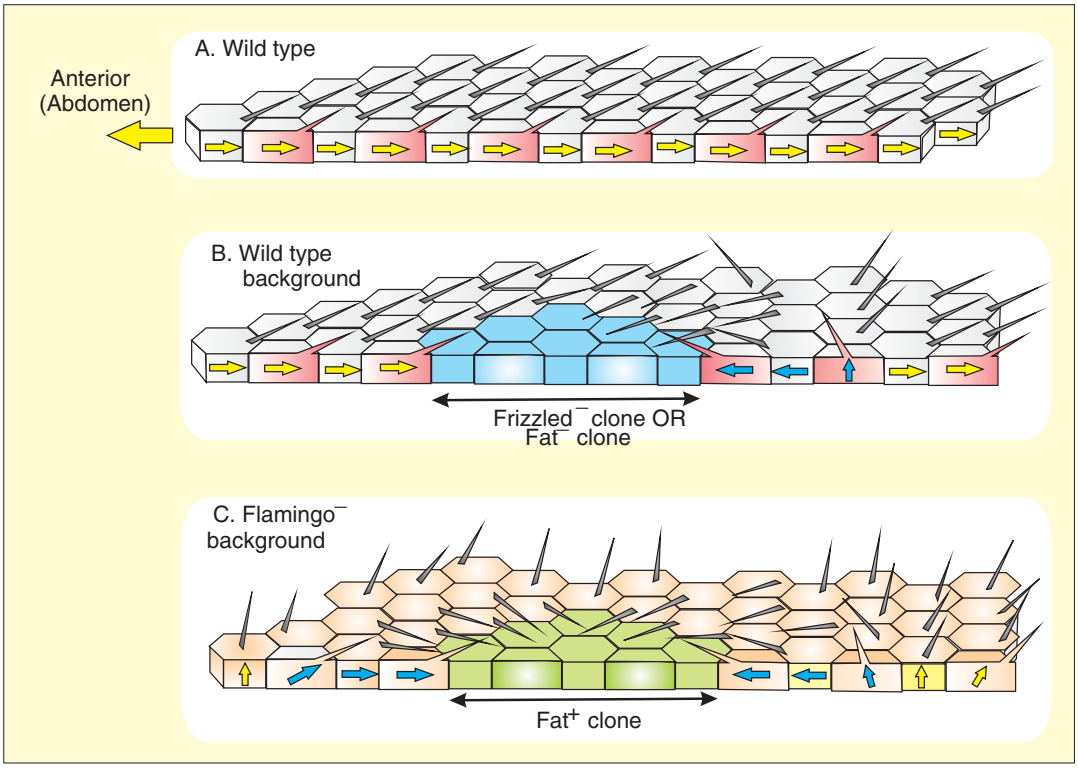
A number of important properties of the polarity mechanism are revealed by these experiments. Firstly, each cell somehow measures the difference in Fz activity between itself and its neighbours and then points the hair in the direction where the Fz level is lowest. Secondly, the polarity reversals are not restricted to the cells immediately in contact with the clone, but they spread out to affect cells three or four cells away. This so-called non-autonomous behaviour indicates that the polarity signalling mechanism in one cell might be capable of releasing a morphogen to influence polarity decisions in cells further afield.

Dachsous (Ds)/Fat (Ft) polarity signalling

The Dachsous (Ds)/Fat (Ft) polarity signalling pathway is still being characterized. It depends on the atypical cadherins Ft and Ds (Module 6: Figure cadherin superfamily), which are known to form heterophilic interactions at cell–cell contacts. There are indications that cell polarity decisions depend upon Ds on one cell interacting with Ft on a neighbouring cell (Module 8: Figure planar cell polarity signalling). The operation of this Ds/Ft polarity pathway seems to require four-jointed (Fj), which is a single-membrane spanning protein that appears to be located on the Golgi.

This putative Ft/Ds/Fj polarity signalling pathway seems to operate in parallel with that of the Frizzled (Fz)/Flamingo (Fmi) polarity signalling pathway. For example, a clone of cells containing defective Fat (Ft^-) has an effect on the polarity of neighbouring wild-type cells that is similar to that of Fz^- (blue arrows in panel b in Module 8: Figure *Drosophila* planar cell polarity). If Ft is overexpressed, it causes wild-type cells in front of the clone to reverse their polarity in exactly the same way as does the overexpression of Fz. A major difference emerges when the effect of Fz and Ft are compared when cells are expressed in a background where Fmi is inactivated. The removal of Fmi is not particularly severe: the bristles point in a posterior direction, but polarity is not quite as regimental as in the wild-type. A clone overexpressing Fz in this Fmi^- background has no effect, which would be expected as the action of Fz requires Fmi. On the other

Module 8: | Figure *Drosophila* planar cell polarity



Functional assay of planar cell polarity in *Drosophila*.
A. In the wild-type abdomen, all the bristles are orientated in an anterior–posterior direction as illustrated by the yellow arrows. B. A small Frizzled$^-$ clone reverses the polarity of cells posterior to the clone (blue arrows). C. A small clone overexpressing Fat (green cells) located in a background of cells deficient in Flamingo. The somewhat haphazard bristle orientation of the Flamingo$^-$ cells near the clone is altered so that several layers have their bristles pointing towards the clone both in the anterior and posterior regions (blue arrows). Information contained in Figure 2 from Lawrence et al. (2007) was used to construct this figure.

hand, a clone overexpressing Ft has a remarkable effect in that it can polarize cells both in the anterior and posterior regions of the Fmi$^-$ cells (blue arrows in Module 8: Figure *Drosophila* planar cell polarity). These experiments seem to indicate that Ft and Ds may represent a separate polarity signalling system acting in parallel with the Fz/Fmi polarity signalling pathway.

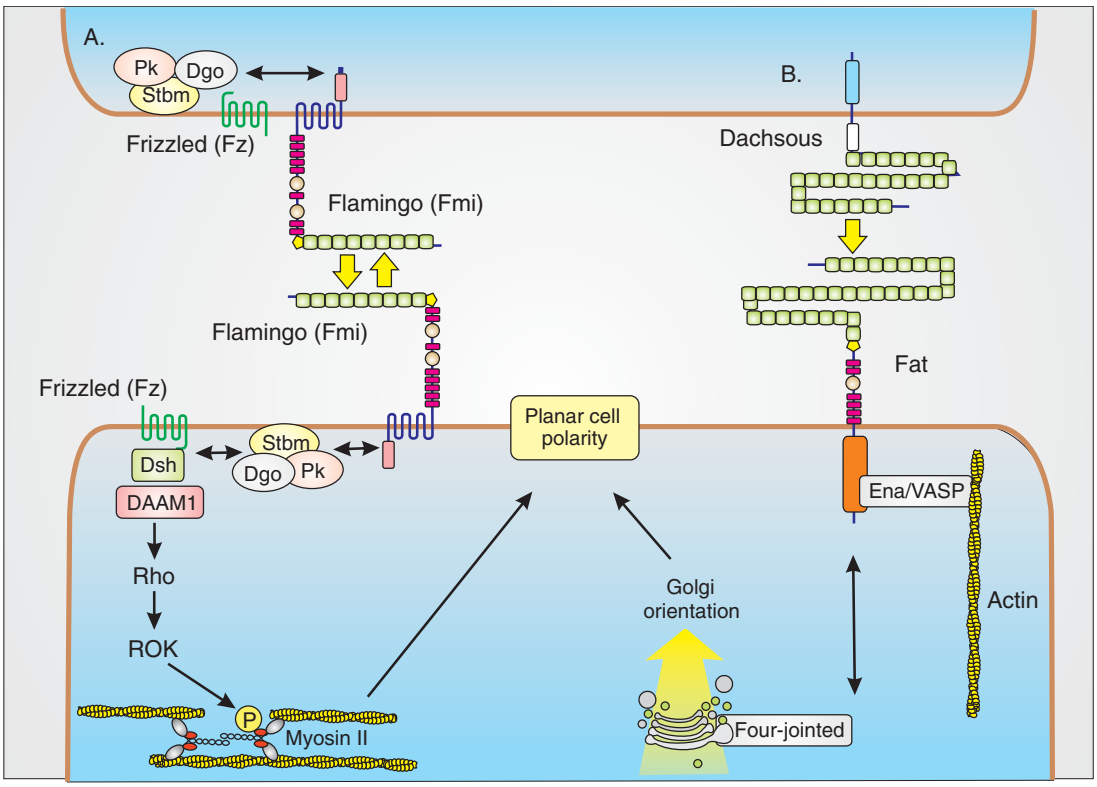
Drosophila ommatidial polarity

During the development of the *Drosophila* eye, the individual photoreceptors have a precise orientation within each ommatidium (see panel e in Module 8: Figure PCP in insects and vertebrates). In addition, each ommatidium is polarized within the eye relative to the midline. This example of planar cell polarity is somewhat complicated, because the orientation mechanism is controlling groups of cells rather than the individual cells just described for the wing and epidermis. Nevertheless, the same planar cell polarity (PCP) signalling pathways seem to be operating. For example, mutation of strabismus (Stbm) (Module 8: Figure planar cell polarity signalling) randomizes the positions of the photoreceptors within the ommatidia.

Convergent extension (CE)

Convergent extension (CE) relates to the mass migration of cells that occurs during gastrulation and neurulation in both zebrafish and *Xenopus*. During CE, cells polarize and then interact with each other to lengthen the developing tissue along the anterior–posterior axis as illustrated for zebrafish (Module 8: Figure PCP in insects and vertebrates). This is an example of planar cell polarity (PCP) because the cells polarize in the medial–lateral axis by protruding lammellipodia and filopodia at each end (Module 8: Figure convergent extension). Convergence occurs as cells attach to each other at their ends and begin to pull on each other. The cells lengthen and begin to intercalate resulting in the anterior–posterior extension. Mutations of PCP signalling components such as Wnt11, Fz and Dvl interfere with this process of CE. It therefore seems that the Wnt/Ca^{2+} signalling pathway, which closely resembles many aspects of the Wnt/planar cell polarity (PCP) pathway in insects (Module 2: Figure Wnt signalling pathways), plays a role in this process of CE. CE is also regulated by the single membrane-spanning tyrosine kinase-linked receptor called protein tyrosine kinase 7 (PTK7), which is also known as colon carcinoma kinase-4 (CCK-4) (Module 1: Figure tyrosine kinase-linked receptors). PTK7/CCK-4

Module 8: | Figure planar cell polarity signalling



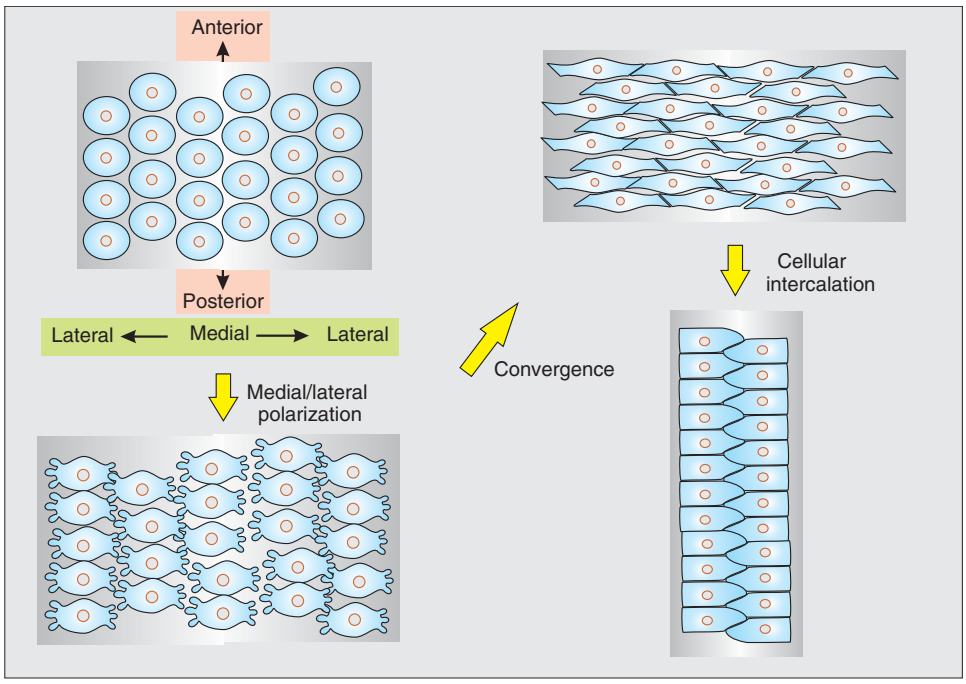
Planar cell polarity signalling mechanisms in *Drosophila*.
A working hypothesis concerning how the putative planar cell polarity signalling components might be organized in *Drosophila* through the operation of two parallel signalling pathways. A. The Frizzled (Fz)/Flamingo (Fmi) polarity signalling pathway may contribute to planar cell polarity by activating the actin/myosin contractile system. B. The Dachsous (Ds)/Fat (Ft) polarity signalling pathway depends on heterophilic interactions formed between the atypical cadherins Ds and Ft. This figure is based on information contained in Lawrence et al. (2007).

Module 8: | Table PCP signalling components and effectors
Planar cell polarity (PCP) signalling components and downstream effectors.

Drosophila	**Vertebrates**	**Function**
Wnt	Wnt5a Wnt11	The external ligands for Frizzled (Fz) receptors The cell-surface receptors for the Wnt ligands
Frizzled (Fz)	Fz6	
Dishevelled (Dsh)	Dvl	Scaffolding protein containing DIX, PDZ and DEP domains; interacts with Fz, Pk, Stbm and Dgo
Strabismus/van Gogh (Stbm/Vang)	Vangl2	A four-pass transmembrane protein that binds Pk, Dsh and Dgo
Prickle (Pk)	Prickle-1, Prickle-2	Possibly a scaffolding protein with three LIM domains and a PET domain; interacts with Dsh, Stbm and Dgo
Flamingo (Fmi)/Starry night (Stan)	Celsr1, Celsr2, Celsr3	Atypical cadherin with a seven-pass transmembrane region (Module 6: Figure cadherin superfamily)
Fat (Ft)	Fat1, Fat2, Fat3 and Fat-j	Atypical cadherin (Module 6: Figure cadherin superfamily)
Dachsous (Ds)	Dchs1	Atypical cadherin (Module 6: Figure cadherin superfamily)
Four-jointed (Fj)	Fjx1	Type II transmembrane domain; located in Golgi and may regulate Ds
Diego (Dgo)	Ankrd (Diversin)	Cytoplasmic protein with ankyrin repeats
Spaghetti squash (Sqh)	Myosin regulatory light chain (MRLC)	The regulatory subunit that controls myosin II
Zipper (Zip)	Myosin heavy chain	Catalytic component that interacts with actin to cause contraction
	Protein tyrosine kinase 7 (PTK7)/colon carcinoma kinase-4 (CCK-4)	A single membrane spanning tyrosine kinase-linked receptor (Module 1: Figure tyrosine kinase-linked receptors)

These planar cell polarity (PCP) signalling components were first identified in *Drosophila*, but for many of these, there are now vertebrate homologues. The way in which some of these components are arranged to form PCP signalling pathways is illustrated in Module 8: Figure planar cell polarity signalling.

Module 8: | Figure convergent extension



Convergent extension during vertebrate gastrulation and neurulation.
During gastrulation, the developing masses of cells polarize in the medial–lateral plane and then begin to converge on each other. By attaching and pulling on each other, they become elongated, and then through a process of intercalation, the embryo begins to lengthen. The appearance of the zebrafish embryo at the end of this process of convergent extension is shown in panels i and j in Module 8: Figure PCP in insects and vertebrates.

seems to interact with Vangl2, the mammalian orhthologue of insect Strabismus/van Gogh (Stbm/Vang), suggesting that it may be a part of the vertebrate PCP signalling mechanism.

A similar medial lateral intercalation of the neuroepithelium is responsible for neural tube closure.

Orientation of hair cell stereociliary bundles

In the mammalian organ of Corti, the sensory hair cells are organized into four rows: a single row of inner hair cells (IHCs) that face the medial region and three rows of outer hair cells (OHCs) that face the lateral region (Module 10: Figure organ of Corti). The apical surface of each hair cell has a bundle of stereocilia (Module 10: Figure hair cell), that carry out the process of hair cell mechanoelectrical transduction responsible for hearing. Since this transduction process detects the motion of endolymph within the cochlear channels, it is critical that the stereociliary bundles are precisely orientated to detect the slightest movement. When viewed from the surface, each bundle is seen to be V-shaped, with all their points polarized in the medial–lateral direction and is a good example of planar cell polarity (Module 8: Figure PCP in insects and vertebrates).

During early development of the organ of Corti, a process that resembles convergent extension (CE) transforms a primordial mass of cells into a long thin epithelium that constitutes the developing cochlea. In the next step, the IHCs and the OHCs begin to differentiate and display planar cell polarity (PCP) as each cell organizes its stereociliary bundle in a precise medial–lateral orientation. The polarity of this bundle seems to depend on the position of a single kinocilium that is located in the middle of the hair cell as it begins to differentiate. The kinocilium is a typical microtubule-containing cilium, which is surrounded by small microvilli (presumptive stereocilia) containing actin filaments. PCP becomes apparent when the kinocilium/microvilli complex migrates to the lateral side of the hair cell. The microvilli begin to transform into stereocilia that organize into their characteristic V-shaped medial–lateral pattern.

The orientation of these bundles is disturbed by mutations in a number of the PCP signalling components, such as Fz, Vangl2, Celsr and Dvl (Module 8: Table PCP signalling components and effectors). The signalling molecules are part of the Wnt/Ca^{2+} signalling pathway, which closely resembles many aspects of the Wnt/planar cell polarity (PCP) pathway in insects (Module 2: Figure Wnt signalling pathways). The single membrane-spanning tyrosine kinase-linked receptor called protein tyrosine kinase 7 (PTK7), which is also known as colon carcinoma kinase-4 (CCK-4) (Module 8: Table PCP signalling components and effectors), also plays a role in hair cell orientation. PTK7/CCK-4 seems to interact with Vangl2, which is the mammalian orhthologue of insect Strabismus/van Gogh (Stbm/Vang), suggesting that it may be a part of the PCP signalling mechanism.

Mammalian hair follicle orientation

The patterning of hair in mammals is another example of planar cell polarity (PCP) (see panels c and d in

Module 8: Figure PCP in insects and vertebrates). In the wild-type mouse, hairs on the paw all point distally. When the gene for the frizzled 6 (Fz6) receptor is deleted, the hair pattern of the $Fz^{-/-}$ mutant animals is disorientated and appears as swirls and waves. The Fz6 receptor is part of the Wnt/Ca^{2+} signalling pathway (Module 2: Figure Wnt signalling pathways), which contains many of the signalling components normally associated with the insect PCP signalling system (Module 8: Table PCP signalling components and effectors).

Differentiation

The body is composed of a diverse repertoire of cell types (Module 7: Table cell inventory) that emerge during the process of differentiation towards the end of development. Many cells develop from specific stem cells such as the haematopoietic stem cell (HSC) and the mesenchymal stem cell (MSC). The cell lineages that develop from such stem cells gradually differentiate into specific cell types as illustrated by the following examples:

- Differentiation of brown fat cells
- Differentiation of bone cells
- Differentiation of B-cells
- Differentiation of cardiac cells
- Differentiation of intestinal cells
- Differentiation of skeletal muscle cells
- Differentiation of keratinocytes
- Differentiation of smooth muscle cells
- Differentiation of neurons
- Differentiation of white fat cells

When differentiation occurs, cells usually stop proliferating and begin to express cell-type-specific genes. For example, muscle cells begin to express unique contractile proteins, whereas secretory cells encode specific secretion products. This proliferation–differentiation switch is a carefully orchestrated sequence of events that is tightly regulated by various cell signalling mechanisms. Some other general processes that occur during early development are the epithelial-to-mesenchymal transition (EMT) and the converse process of mesenchymal-to-epithelial transition (MET).

This process of differentiation is not fixed in stone, but is a dynamic state that has to be maintained. There are examples of de-differentiation where cells loose their differentiation markers and revert back to a stem cell-like state.

An important component of differentiation is signalsome expression, whereby each particular cell type expresses those signalling pathways that are suited for its particular function.

The Ca^{2+} signalling pathway seems to play a critical role in the process of differentiation of many cell types, such as terminal differentiation of skeletal muscle and differentiation of keratinocytes.

Proliferation–differentiation switch

Growth factors activate the proliferation signalling network to initiate the process of cell proliferation (Module 9: Figure proliferation signalling network). When the daughter cells exit from the cell cycle, they face different options: they can proliferate again, die by apoptosis, enter a state of senescence or they can stop growing and begin to differentiate. The switch from proliferation to differentiation depends on both an inhibition of proliferation and the activation of the cell-specific genes that result in the emergence of different cell types (Module 8: Figure proliferation–differentiation switch). The inhibitor of DNA binding (Id) proteins, which are also known as inhibitor of differentiation proteins, play a critical role in this switch from proliferation to differentiation. One of the functions of Myc, which is activated by growth factors, is to increase the expression of the Ids that help promote proliferation by switching off the cyclin-dependent kinase (CDK) inhibitor $p16^{INK4a}$. Conversely, transforming growth factor β (TGF-β) reduces the Ids and this contributes to the inhibition of proliferation.

An important component of the switch from proliferation to differentiation is the removal of Ids. In the case of neurons, the destruction of Id2 is carried out by the anaphase-promoting complex (APC), which normally functions to degrade cyclin B, during chromosome separation at anaphase (Module 9: Figure chromosome separation). The removal of Id2 then frees E47 to begin the transcriptional events that lead to neuronal differentiation.

A similar role for the Ids in controlling differentiation has been described for the transcriptional activation of MyoD during skeletal muscle differentiation. The Ids bind to the helix–loop–helix (HLH) transcription factor E2A and prevents it from binding to MyoD to begin the transcription of muscle-specific genes. When Id is destroyed, E2A binds to MyoD to initiate transcription (Step 10 in Module 4: Figure MyoD and muscle differentiation). The expression of the Ids is repressed by the methyl-CpG-binding protein 2 (MeCP2) during neuronal differentiation.

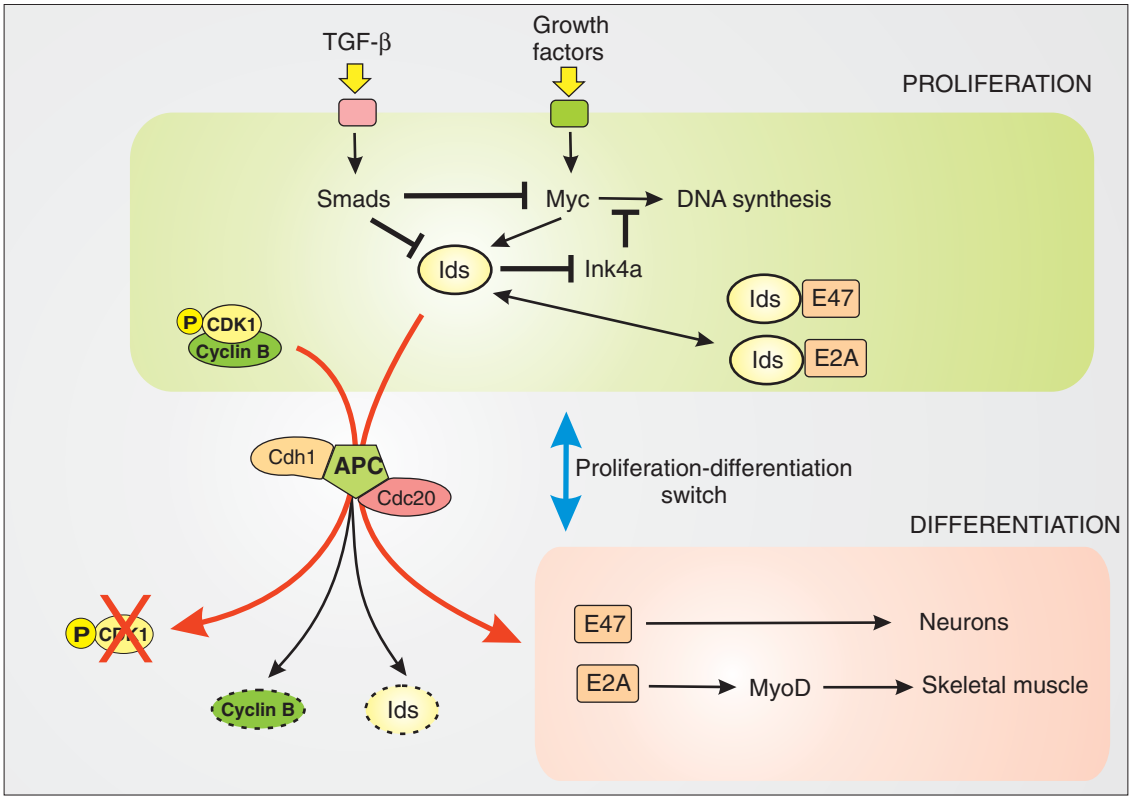
Inhibitor of DNA binding (Id) proteins

The inhibitor of DNA binding (Id) protein family contains four members (Id1–Id4). They have a helix–loop–helix (HLH) motif, which is found in many transcription factors. Unlike these HLH transcription factors, however, the Ids lack a DNA-binding domain. These Ids prevent differentiation by inhibiting tissue-specific transcription factors and thus help to maintain cells in a proliferative state (Module 8: Figure proliferation–differentiation switch). Destruction of Ids is an important step for the proliferation–differentiation switch.

Epithelial-to-mesenchymal transition (EMT)

During epithelial-to-mesenchymal transition (EMT), cells lose their epithelial properties and take on the characteristics of mesenchymal cells. This transition occurs early in development when the primary mesoderm forms from the epithelium of the epiblast. It also occurs when the neural crest forms from the ectoderm. EMT recurs again in adulthood when the placenta is formed or during wound healing. Carcinogenesis is often associated with EMT as the tumour cells develop their metastatic potential.

Module 8: | Figure proliferation–differentiation switch



Function of inhibitor of DNA binding proteins (Ids) in proliferation–differentiation switch.
The Ids proteins promote proliferation by decreasing expression of the cyclin-dependent kinase (CDK) inhibitor Ink4a and prevent differentiation by sequestering transcription factors such as (E47 and E2A). Destruction of Ids by the anaphase-promoting complex (APC) liberates these transcription factors to begin the process of differentiation.

The profound changes that occur during EMT are caused by changes in the expression levels of as many as 4000 genes resulting in the cells taking on more stem cell features and thus is an example of the process of de-differentiation. There is a loss of cell adhesion resulting from a decrease in expression of E-cadherin, and a number of genes are expressed that result in increased motility and invasiveness. The induction of EMT by the TGFβ signalling pathway is associated with a down-regulation of the miR-200 family that act by suppressing the transcription factors ZEB1 and ZEB2 that orchestrate this transition. One of their actions is to regulate the expression of E-cadherin.

Mesenchymal-to-epithelial transition (MET)

The mesenchymal-to-epithelial transition (MET) plays a role in certain developmental processes such as formation of the nephron epithelium from the nephric mesenchymal cells.

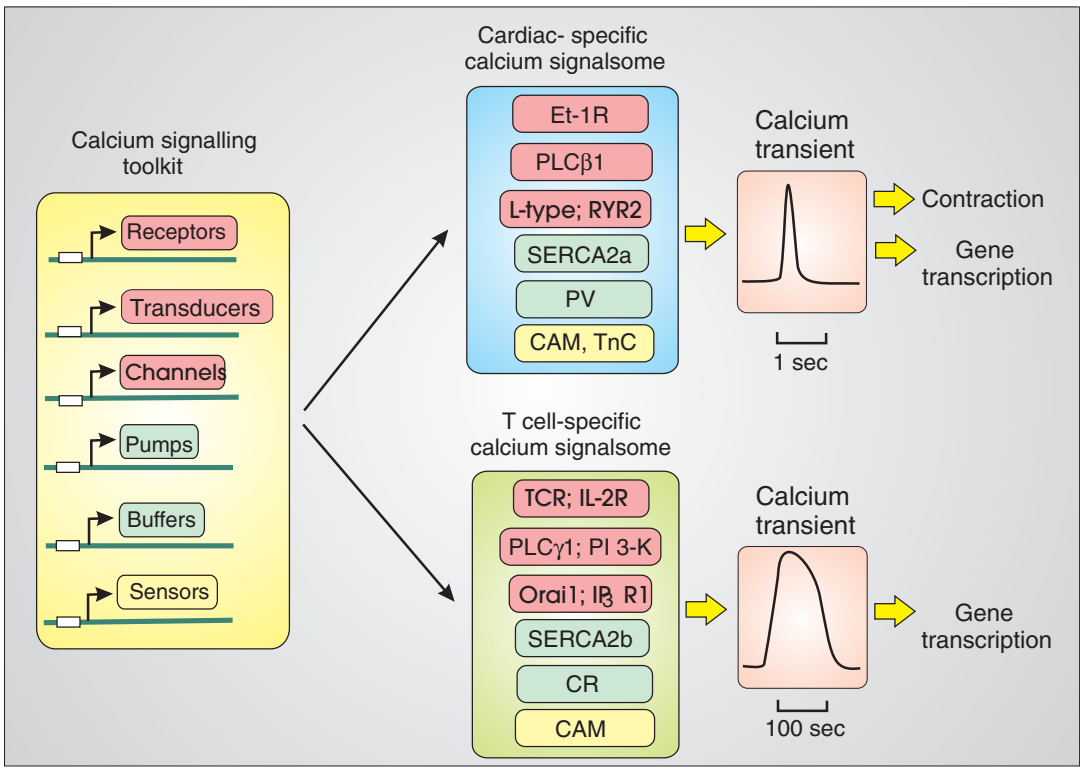
Signalsome expression

During the process of differentiation, each cell type puts in place a specific signalsome that will provide the signalling mechanisms required to control its particular function. Cells can have very different signalsomes, as is well illustrated for the Ca^{2+} signalling system (Module 2: Figure cell-specific Ca^{2+} signalsomes). The development of such cell-specific signalsomes requires the selective transcription of specific signalling components, as is illustrated for the development of cardiac-specific and T cell-specific Ca^{2+} signalsomes (Module 8: Figure signalsome expression). The cardiac cell signalsome delivers a very rapid pulse of Ca^{2+} necessary for contraction (Module 7: Figure ventricular cell E-C coupling), whereas the T cells produce a much longer transient required to stimulate gene transcription and cell proliferation (Module 9: Figure T cell Ca^{2+} signalling). There is a considerable degree of signalsome stability in that cells have an internal assessment system that ensures that the signalling system continues to deliver the appropriate signals. However, within this stability, there is some degree of plasticity that ensures that the signalling system can be remodelled to adapt to changing circumstances. This phenotypic remodelling of the signalsome has both beneficial and pathological consequences.

Stem cells

Stem cells have two unique characteristics. They are capable of self renewal while retaining the ability to differentiate into different cell types. Much interest has focused on embryonic stem cells and adult stem cells. The

Module 8: | Figure signalsome expression



Selective expression of a cell-specific signalsome.
Cells have access to a very large toolkit of signalling components, from which they select out and express specific components to create a cell-specific signalsome. The examples used here show how the selection of different components create cardiac- and T cell-specific signalsomes. The former produces a fast Ca^{2+} transient that occurs in less that 1 s, whereas the T cell transient lasts very much longer. CaM, calmodulin; CR, calreticulin; Et-1R, endothelin-1 receptor; IL-2R, interleukin-2 receptor; IP_3R1, inositol 1,4,5-trisphosphate receptor 1; L-type, an L-type Ca^{2+} channel; PI 3-K, PtdIns 3-kinase; PLCβ1, phospholipase β1 (PLCβ1); PV, parvalbumin; SERCA, sarco/endo-plasmic reticulum Ca^{2+}-ATPase; TCR, T cell receptor; TnC, troponin C.

embryonic stem (ES) cells function during development to produce the mass of cells needed to form the adult organism. Within many of the developing tissues and organs, some of these ES cells fail to differentiate and are set aside and held in reserve as a source of new cells for tissue repair and regeneration. This small population of adult stem cells are nurtured within a stem cell niche where their ability to spawn new cells is carefully controlled. Stem cells spend much of their time in a quiescent state, but when new cells are required they are stimulated to divide to spawn a new population of progenitor cells that go on to differentiate into specialized cells (Module 8: Figure stem cell function). This is a highly regulated process where there is a balance between factors that promote quiescence, proliferation and differentiation.

Another important feature of many stem cells is their low resting levels of reactive oxygen species (ROS), which greatly reduces DNA damage and thus helps to maintain their stem cell function. The low ROS levels are achieved by having an increased level of the free radical scavenging system such as glutathione and thioredoxin.

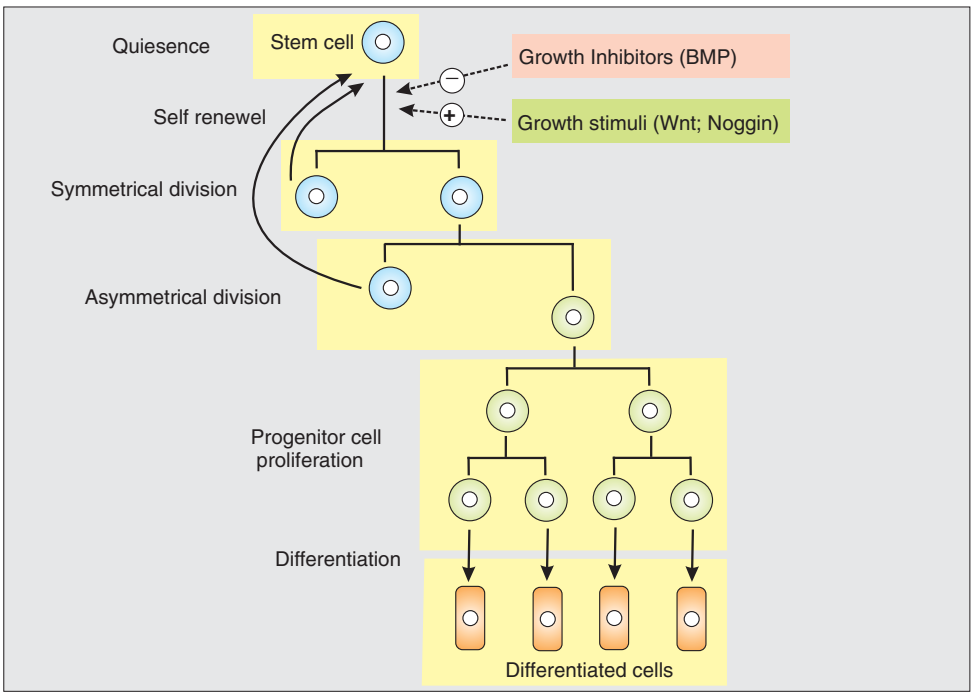
Embryonic stem cells

As their name implies, embryonic stem (ES) cells are found in the inner cell mass of the early embryo where they give rise to the primary germ layers (ectoderm, endoderm and mesoderm). These ES cells thus have the potential to differentiate into any one of the multitude of specialized mammalian cell types found in tissues and organs of the adult body (Module 7: Table cell inventory). This pluripotency is maintained by a limited number of pluripotency regulatory transcription factors such as Oct4, Sox2, Nanog and Krüppel-like factor 4 (Klf4) (Module 8: Figure ES cell miRNAs). The way in which these master regulators operate together with microRNAs to maintain ES stability depends on a complex web of inter-related signalling processes that have been divided into four main groups (Module 8: Figure ES cell miRNAs):

- Pluripotency autoregulatory transcriptional loops
- ES cell cycle miRNA regulatory mechanisms
- miRNA modulation of cell-cycle regulatory mechanisms
- MicroRNA regulation of differentiation

Once cells have differentiated, they can return to a stem cell state through a process of de-differentiation that drives cells back to the pluripotent state. This reprogramming can be achieved experimentally by expressing a cocktail of genes such as Oct4, Sox2, Nanog and Lin28 to produce induced pluripotent stem cells (iPS)

Module 8: | Figure stem cell function



Stem cell proliferation and differentiation.
Proliferation of quiescent stem cells is determined by the balance between growth stimuli and inhibitors. When induced to proliferate, the final process of cell division can be symmetrical or asymmetrical. Symmetrical divisions result in self renewal to maintain the stem cell population. Asymmetrical divisions create two different cells: a stem cell capable of self renewal and a progenitor cell that goes on to divide rapidly to form a population of new cells capable of differentiating into specialized cells with different functions depending on where they are located.

During development, some ES cells are set aside to form the germ line cells, whereas the remaining cells proliferate rapidly to produce the large mass of cells required to construct the internal tissues and organs. During this final process of organogenesis, most of the cells differentiate into specialized cells, but a small number are set aside to become the adult stem cells that remain in organs where they function to replenish dying cells and regenerate damaged tissues.

ES cells can be removed from the embryo and grown in culture where they retain their stem cell characteristic of self renewal. Since they can be induced to differentiate into specialized cells if provided with the right cocktail of factors, they are attracting a lot of attention as a way of regenerating damaged tissues and organs.

Pluripotency autoregulatory transcriptional loops

The pluripotency regulatory transcription factors (Oct4, Sox2, Nanog and Klf) are connected to each other through tightly controlled autoregulatory loops in that they feedback to regulate each other's expression. For example, the expression of Oct4 is controlled by a heterodimer consisting of Oct4 and Sox2. While these core transcriptional factors are active, the ES cells maintain their pluripotency and can continue to proliferate. These master regulators control the expression of a large number of genes that can be either activated or silenced. The net effect is to ensure that cell proliferation is maintained while differentiation is suppressed.

Much of this regulation is exerted through the ES cell cycle miRNA regulatory mechanisms.

ES cell cycle miRNA regulatory mechanisms

The ability of the core pluripotency regulatory transcription factors (Oct4, Sox2, Nanog and Klf) to ensure self-renewal is achieved through the activation of a number of ES cell cycle miRNAs, such as miR-195, miR-372, miR-290 and miR-302 (Module 8: Figure ES cell miRNAs). The primary action of this group of miRNAs is to participate in the microRNA modulation of cell-cycle regulatory mechanisms.

MicroRNA modulation of cell cycle regulatory mechanisms

The core pluripotency regulatory transcription factors (Oct4, Sox2, Nanog and Klf4) function to maintain proliferation by controlling many of the components that regulate the cell cycle (Module 8: Figure ES cell miRNAs). One of their primary actions is to increase the expression of Myc, which is a key activator of cell proliferation (Module 4: Figure Myc as a gene activator). Myc also acts to prevent differentiation by increasing the expression of Lin28 that suppresses expression of the miRNA let-7 that promotes differentiation.

The pluripotency genes also use the ES cell cycle miRNA regulatory mechanisms to control many components of the cell cycle. For example, miR-195 reduces the expression of the Wee1 kinase that phosphorylates and

inactivates the cyclin B/cyclin-dependent kinase 1 (CDK1) complex during nuclear envelope breakdown and spindle assembly during G2 (see step 1 in Module 9: Figure mitotic entry). Other examples are the inhibition of the cyclin-dependent kinase (CDK) inhibitors p57 and p21 by miR-372 and miR-290 respectively.

Somewhat paradoxically, the core pluripotency regulators activate the transcription of a number of proteins that can inhibit the cell cycle. In order for the ES cells to continue proliferating, these various inhibitors are repressed. The Large tumour suppressor-2 (Lats2) is a serine/threonine protein kinase that acts in the hippo signalling pathway (Module 2: Figure hippo signalling pathway) as a tumour suppressor by inhibiting the G1/S transition. Retinoblastoma-like protein 2 (Rbl2) is one of the pocket proteins capable of repressing the cell cycle. The DNA methyltransferases 3a and 3b (DNMT 3a and DNMT 3b) enables differentiation to occur by repressing Oct4. However, the activity of these cell-cycle regulators is reduced by the miR-290/miR-295 cluster and by miR-302 that are components of the ES cell cycle miRNA regulatory mechanism (Module 8: Figure ES cell miRNAs).

MicroRNA regulation of differentiation

The pluripotency regulatory transcription factors not only control the self-renewal process that enables them to proliferate, but they also set the stage for subsequent differentiation by putting in place a number of gene products that will be required if they are called upon to differentiate to form different cell lineages. During the proliferation/differentiation switch, the cessation of proliferation by the self-renewing ES cells depends on inhibition of the pluripotency regulatory factors that have such profound effects on both the cell cycle and differentiation programs. A decrease in the expression of these regulatory factors is enhanced by an increase the activity of various miRNAs such as miR-21, miR-134 and miR-145. In ES cells, the activity of miR-21 is normally repressed by RE1-silencing transcription factor (REST). The miR-145 is also suppressed in ES cells by Oct4, but when expression of Oct4 declines during differentiation, there is an increase in the level of miR-145 that then facilitates the onset of differentiation by further suppressing Oct4 and some of the other regulatory factors such as Sox2 and Klf4 (Module 8: Figure ES cell miRNAs). This decline in the expression of the pluripotency regulatory factors will reduce the level of the ES cell cycle miRNAs that maintained the cell cycle by silencing the numerous inhibitory cell cycle regulatory mechanisms.

The large numbers of latent differentiation gene products, which are induced by the pluripotency regulatory factors, are silenced in ES cells through a number of mechanisms. One of these latent components is the miRNA let-7. The level of let-7 in pluripotent ES cells is silenced by expressing the RNA binding protein Lin28, which acts to inhibit both the Drosha-mediated and Dicer-mediated processing of pre-let-7 to mature let-7. One of the many functions of let-7 is to inhibit cMyc to inhibit the cell cycle. Many of these latent gene products are silenced by Polycomb group proteins (PcG).

Adult stem cells

Adult stem cells are found in many locations throughout the body (Module 8: Figure stem cell locations). Most of the organs and tissues of the body seem to have a dedicated population of stem cells that function to regenerate damaged tissues or to replace dying cells. The origin of these adult stem cells is still not absolutely clear. The most likely scenario is that some of the embryonic stem cells that function to produce specialized cells during development are set aside and reside within a stem cell niche from which they can be called upon to produce new cells for tissue repair and to replace dying cells.

Many adult stem cells spend much of their life in a quiescent state and are activated to divide only when there is a need for new cells either to replace dying cells or to repair tissue damage. There are two types of stem cell division: symmetrical divisions that result in two identical daughter stem cells responsible for self renewal and asymmetrical divisions that create two different daughter cells (Module 8: Figure stem cell function). One of these daughter cells resembles the parent and thus functions in self renewal while the other cell becomes a progenitor cell that divides rapidly to form the large clone of cells that differentiate into specific cell types as required for tissue regeneration. From a signalling point of view, interest has focused on the mechanisms that control the balance between quiescence, proliferation and differentiation. These processes are regulated by a wide range of stimuli that operate within the stem cell niche.

A large number of mammalian organs and tissues are now known to harbour adult stem cells:

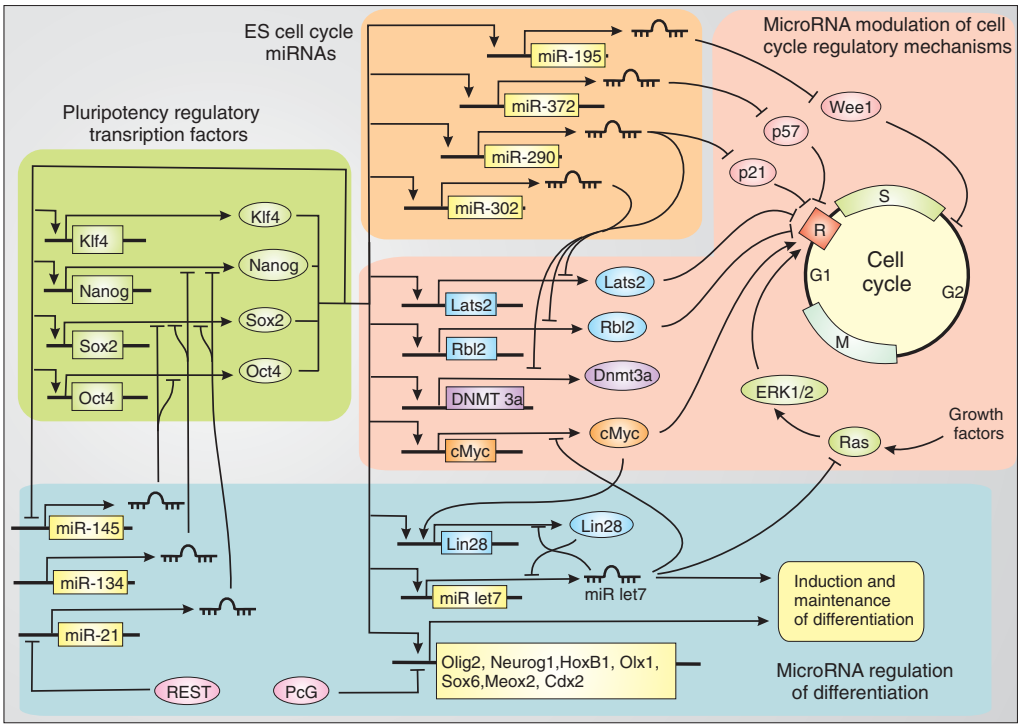
- Intestinal stem cells
- Satellite cells
- Haematopoietic stem cells (HSCs)
- Epidermal stem cells
- Melanocyte stem cells
- Mesenchymal stem cells
- Mammary gland stem cells
- Male germ cells
- Neural stem cells

Stem cell niche

The stem cell niche is a specialized microenvironment where a range of stimuli function to regulate the balance between stem cell proliferation (self renewal) and differentiation. Although the nature of the niche varies considerably between tissues, there are some basic features that are summarized in the generic niche shown in Module 8: Figure stem cell niche:

1. Some stem cells are closely associated with the extracellular matrix (ECM) where they receive stimuli that activate various pathways such as integrin signalling.
2. Some stem cell niches receive a neural input that provides various neurotransmitters.
3. Many niches have a variety of supporting cells that make close enough contact with the stem cells to transmit information through juxtacrine signalling pathways

Module 8: | Figure ES cell miRNAs



Embryonic stem (ES) cell miRNAs.
The survival and self-renewal of embryonic stem (ES) cells is maintained by a limited number of pluripotency regulatory transcription factors such as Oct4, Sox2, Nanog and Krüppel-like factor 4 (Klf4) (green panel). These regulatory factors use a number of ES cell microRNAs (orange panel) to control the expression of key proteins responsible for the cell-cycle regulatory mechanisms (pink panel). The blue panel at the bottom illustrates some of the microRNA regulatory processes that control differentiation.

such as those operated by the cadherins or the Notch signalling pathway.

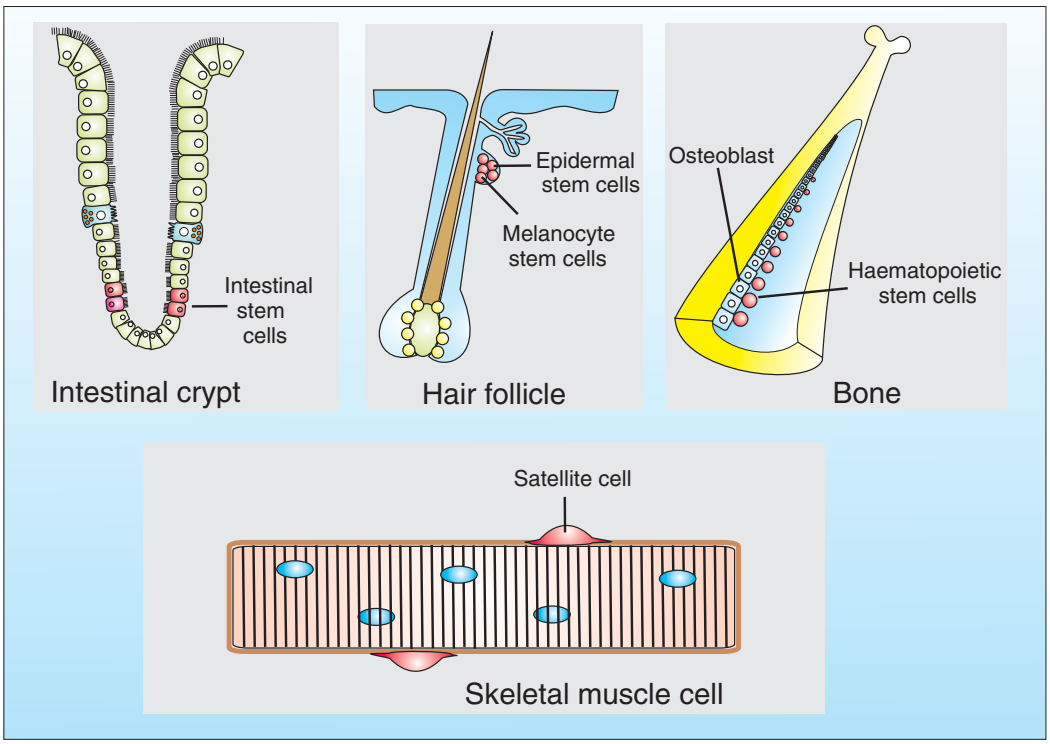
4. Cells located either within or outside the niche release factors such as the Wnts or fibroblast growth factor (FGF) that regulate stem cell proliferation.
5. Stem cells can respond to hormones released from blood vessels.

Satellite cells

Satellite cells are skeletal muscle stem cells that function in muscle repair and regeneration (Module 8: Figure Satellite cell function). The stem cell niche occupied by these satellite cells is located between the inner surface of the basal lamina and the outer surface of the sarcolemma of the muscle fibre. The main aspects of satellite cell function are defined by the four states shown in Module 8: Figure Satellite cell function:

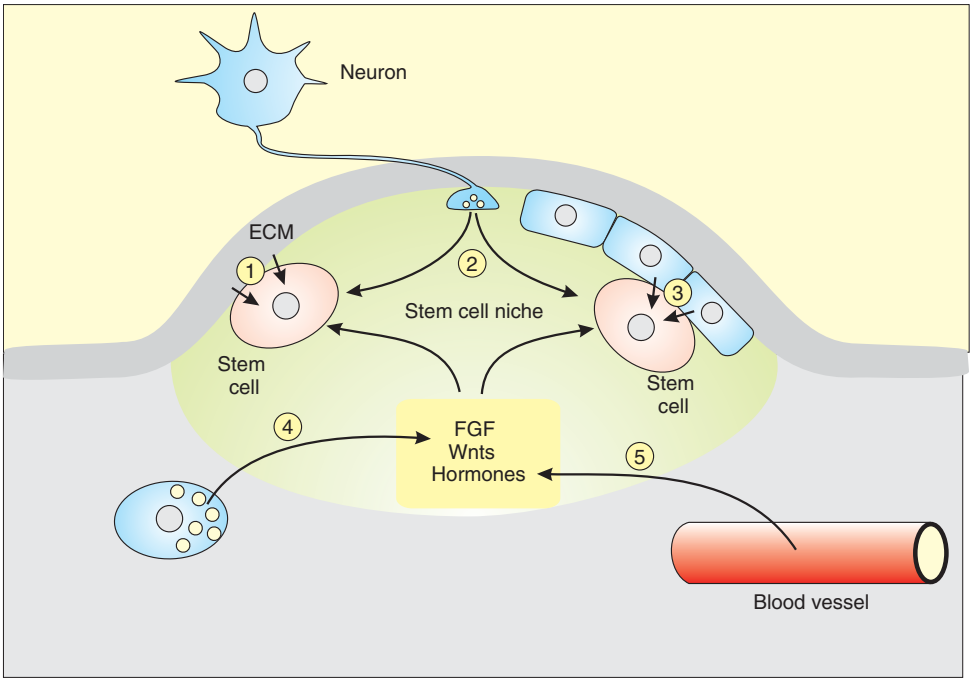
1. Satellite cells spend most of their time within their niche in a quiescent G0 state. They are characterized by expression of the paired box 7 (Pax7) transcription factor and a number of signalling components such as c-Met, M-cadherin, syndecan-3 and syndecan-4 that function during the activation phase.
2. Exercise, muscle damage or various disease states provide the stimuli that activate proliferation. The main proliferative stimuli are hepatocyte growth factor (HGF), which is also known as scatter factor, fibroblast growth factor (FGF) and the Notch signalling pathway stimulus Delta-1 that is expressed on the surface of the myofibre (Module 8: Figure Satellite cell activation).
3. The plane of division plays an important role in defining the subsequent fate of the daughter cells. The simplest hypothesis is that an asymmetrical division creates daughters with separate cell fates (Module 8: Figure Satellite cell function). One cell retains the stem cell characteristics and returns to a quiescent state to maintain the pool of stem cells, whereas the other daughter becomes a progenitor cell that continues to proliferate to provide the pool of myoblasts responsible for repair and muscle regeneration. Soon after activation, the progenitor cells reveal their commitment to a muscle lineage by expressing the myogenic regulatory factors (MRFs) such as MyoD and Myf5, which are typical muscle markers.
4. The progenitor cells stop proliferating and begin to differentiate into myoblasts that have two fates. They can participate in muscle repair by fusing with the existing myofibre. Alternatively, they can migrate through the basal lamina and then fuse with each other to form a new myofibre. The regeneration of a new myofibre recapitulates the process of skeletal muscle myogenesis that occurs during embryonic development (Module 8: Figure skeletal muscle myogenesis).

Module 8: | Figure stem cell locations



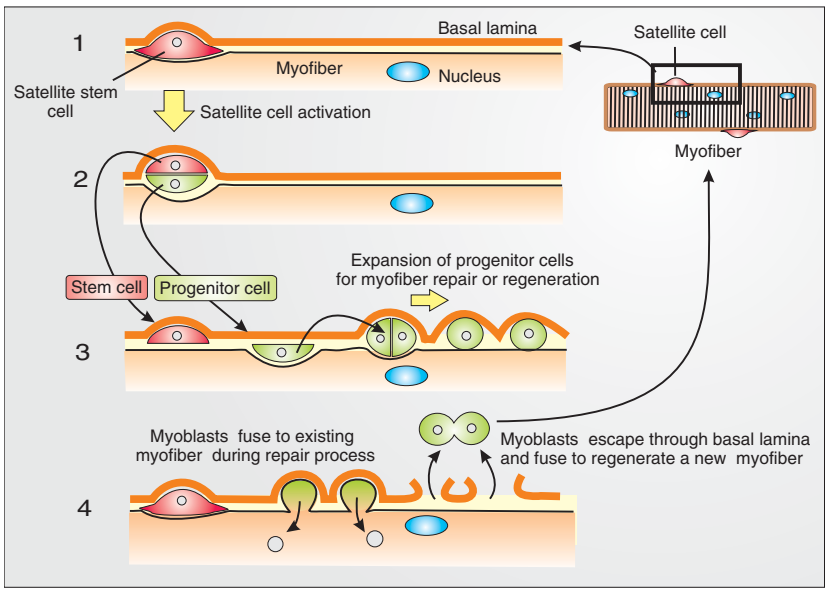
Location of adult stem cells in different tissues.
The adult stem cells (shown in red) are located in a specialized tissue region known as the stem cell niche. Within these niches, stem cell proliferation is regulated to provide the progenitor cells that differentiate into the adult cells used for tissue repair and regeneration (Module 8: Figure stem cell function).

Module 8: | Figure stem cell niche



Summary of the signalling mechanisms operating within the stem cell niche.
Adult stem cells are located in specialized regions that are privileged environments that provide the stimuli to regulate the balance between stem cell proliferation and differentiation. See the text for details of the stimuli labelled 1–5. ECM, extracellular matrix; FGF, fibroblast growth factor.

Module 8: | Figure Satellite cell function



Satellite cells function in skeletal muscle repair and regeneration.
Satellite cells located beneath the basal lamina function in muscle repair and regeneration: 1. Following exercise or muscle damage, satellite cells are activated and begin to proliferate (Module 8: Figure Satellite cell activation). 2. Some of the divisions are asymmetrical such that one cell remains as a stem cell (red) while the other becomes a progenitor cell (green). 3. The progenitor cell continues to proliferate. 4. The progenitor cells stop proliferating and begin to differentiate into myoblasts that either fuse with the existing muscle or escape across the basal lamina and then fuse to form a new myofibre.

Satellite cells have a number of signalling pathways that cooperate with each other in an orderly sequence to control their stem cell function. The cells are maintained in a quiescent state for prolonged periods and are then launched into sequential phases of proliferation, growth inhibition and then differentiation to provide the myoblasts necessary for muscle repair and regeneration.

Exercise or muscle injury promotes the release of growth factors such as HGF and FGF. The HGF, which is bound to the basal lamina, is thus in a position to activate the HGF receptor (HGFR), also known as the MET receptor (Module 1: Figure tyrosine kinase-linked receptors). Another important proliferation stimulus is FGF that is released from both muscle and non-muscle cells and acts through the tyrosine kinase-linked fibroblast growth factor receptors 1 and 4 (FGFR1 and FGFR4), which are expressed on satellite cells. One of the hallmarks of satellite cells is their expression of the heparin sulphate proteoglycans syndecan-3 and syndecan-4, which play an important role in facilitating the receptor activation and transduction events used by c-Met and FGFR to induce proliferation. During muscle damage, there is an increase in the expression of Delta-1 on the surface of the myofibre and this activates the Notch signalling pathway to stimulate proliferation.

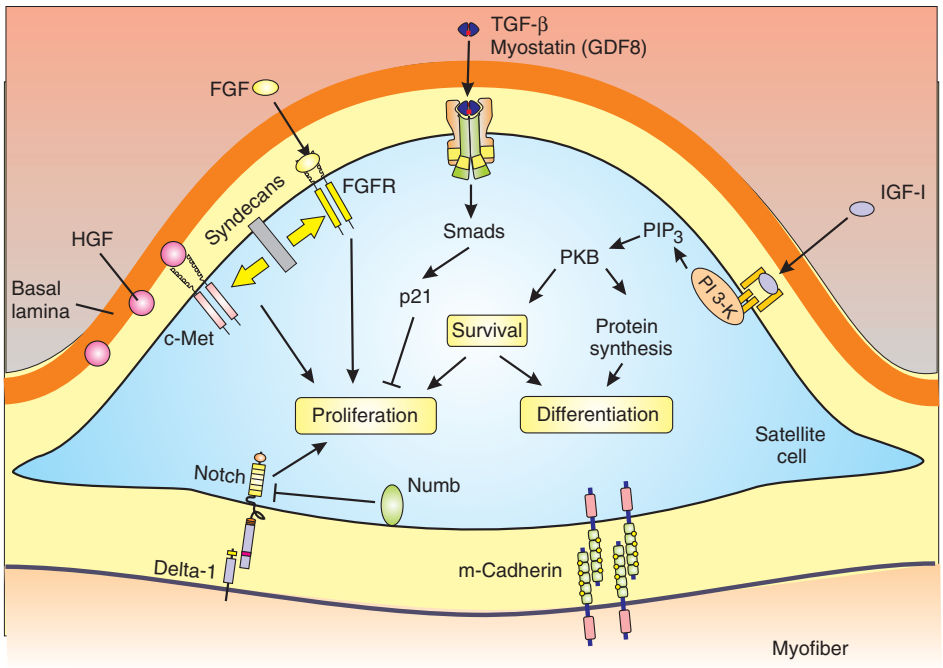
After the proliferative phase to increase the progenitor cell population, cell growth is inhibited as a prelude to the onset of myoblast differentiation. The transforming growth factor β (TGF-β) superfamily (Module 2: Table Smad signalling toolkit), which includes myostatin, also known as growth and differentiation factor 8 (GDF8), plays a critical role in this inhibition of growth (Module 8: Figure Satellite cell activation). This TGF-β inhibition of cell proliferation depends upon the activation of the Smad signalling pathway (Module 2: Figure Smad signalling). One of the functions of Smad transcription factors is to increase the expression of p21, which is one of the cyclin-dependent kinase (CDK) inhibitors, that acts to switch off the cell cycle (Module 9: Figure cell cycle signalling mechanisms). This inhibition of growth is facilitated by an increase in the expression of Numb, which inhibits the Notch signalling pathway (Module 8: Figure Satellite cell activation).

Once growth has stopped, the progenitor cells continue to differentiate into myoblasts. Insulin-like growth factor-I (IGF-I) contributes to differentiation by stimulating protein synthesis. It employs the PtdIns 3-kinase signalling pathway to stimulate protein synthesis by activating both ribosomal S6 protein kinase 1 (S6K1) and the eukaryotic initiation factor 4E binding protein (eIF4E-BP1) (Module 9: Figure target of rapamycin signalling).

Satellite cells express M-cadherin, which is one of the classical cadherins, which may play a signalling role during the developmental sequence. There are indications that it might act through the Rho signalling mechanism.

During aging, there is a decline in the ability of the satellite cells to repair muscle. One suggestion is that there is a decline in the ability of the niche to provide the signals responsible for satellite cell activation. One consequence of this alteration is a decline in the Notch signalling pathway.

Module 8: | Figure Satellite cell activation



Control of Satellite cell proliferation and differentiation.
Activation of the quiescent satellite cell to begin its rounds of proliferation and subsequent differentiation is driven by a number of stimuli. Hepatocyte growth factor (HGF), fibroblast growth factor (FGF) and Delta-1 play a primary role in promoting proliferation. Proliferation is inhibited by TGF-β growth factors including myostatin (GDF8) and by Numb, which inhibits the Notch signalling pathway. Insulin-like growth factor (IGF) has complex actions on both proliferation and differentiation. M-cadherin contributes to differentiation by promoting the expression of both IGF and protein kinase B (PKB).

Skin development

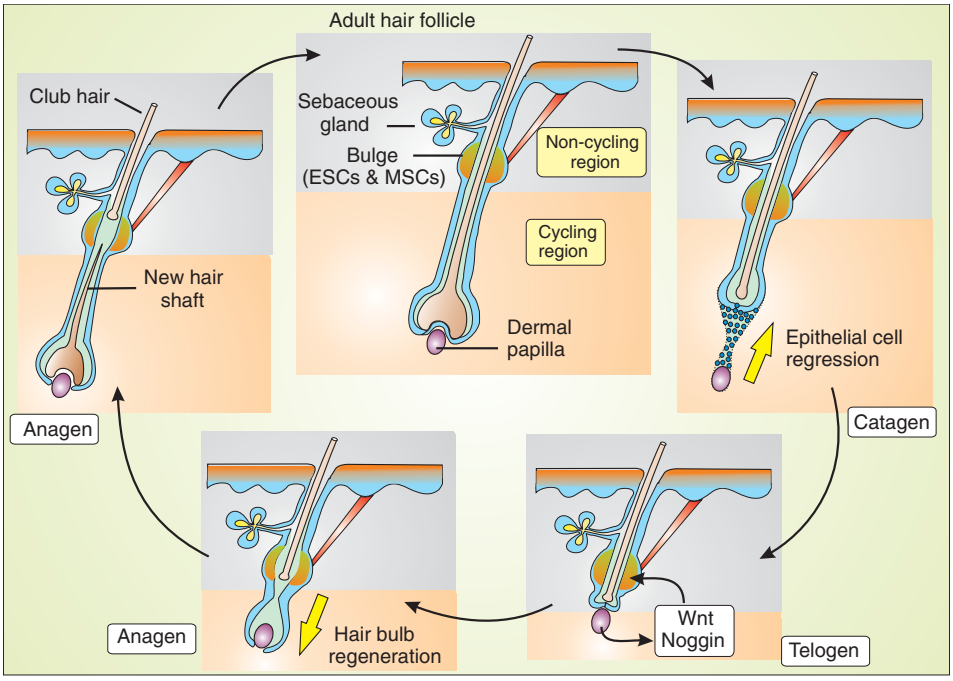
During early embryogenesis, the developing embryo is surrounded by a single layer of neuroectoderm, which gives rise to both the nervous system and the skin. The canonical Wnt/β-catenin pathway controls this lineage divergence by preventing the presumptive epidermis from responding to fibroblast growth factor (FGF) that drives neurogenesis. The lack of responsiveness to FGF results in the expression of bone morphogenic proteins, which further enhance the fate of the epidermal cells, which become the basal layer that differentiates into the keratinocytes that form the different layers of the skin (Module 7: Figure skin cells).

Development of the epidermis begins with the basal keratinocytes that proliferate rapidly to give rise to the intermediate cells, which divide several times before they stop proliferating and transform into the spinous cells. As they stop growing, the spinous cells begin to differentiate into the granular cells (Module 7: Figure skin cells). Growth factors such as epidermal growth factor (EGF) seem to play a role in driving proliferation by activating Myc. There appears to be an important role for the TRPV3 channel that gates Ca^{2+} that may help to promote the activation of the EGF signalling pathway by stimulating ADAM17 that cleaves pro-TGF-α to release the TGF-α that acts on the EGF receptor. One of the actions of the latter is to stimulate phospholipase γ (PLCγ) to produce DAG and $InsP_3$. Release of Ca^{2+} from internal stores by $InsP_3$ acts by stimulating transglutaminase, which is a Ca^{2+}-dependent enzyme that crosslinks proteins to form the cell envelope.

During growth of the epidermis, therefore, the keratinocytes gradually change from a proliferating basal cell to the terminally differentiated cells that gradually transform into the cornified layer. Transforming growth factor β1 (TGF-β1) has an important role in arresting the proliferation of the keratinocytes. TGF-β1 acts through the SMAD signalling pathway, which in the keratinocytes results in the phosphorylation of Smad 2 and Smad3, which then combine with the inhibitor of nuclear factor κB (IκB) kinases α (IKKα) to induce the transcription of Mad1 and Ovol1. The Mad1 and Ovol1 function to repress the activity of Myc, which is an important regulator of keratinocyte proliferation. An increase in the extracellular level of Ca^{2+} seems to be an important differentiation signal. The concentration of Ca^{2+} is low in the basal region, but increases continuously towards the cornified layer. It is this increasing level of Ca^{2+} that initiates keratinocyte differentiation. The increase in extracellular Ca^{2+} appears to increase the formation of the lipid messenger $PtdIns3,4,5P_3$, which then activates phospholipase γ1 (PLCγ1) to form $InsP_3$ and diacylglycerol (DAG). Just how these two messengers promote differentiation is not clear.

Groups of developing mesenchymal cells in the dermis change the fate of local epidermal cells, which then begin to transform into hair follicles. These epidermal cells begin to invaginate into the dermis to form the hair placode. The mesenchymal cells at these placodes provide

Module 8: | Figure hair follicle cycle



The hair follicle cycle.
The hair follicle is divided into two regions. An upper permanent non-cycling region (grey box) contains the sebaceous gland and bulge. The latter contains the epidermal stem cells (ESCs) and the melanocyte stem cells (MSCs). The lower cycling region (red box) contains the hair bulb that produces the pigmented hair fibres. The hair cycle consists of an initial phase of epithelial cell regression (catagen), a quiescent phase (telogen) followed by growth of a new hair follicle (anagen). See the text for further details of this hair follicle cycle.

inductive signals such as FGF and BMP-inhibitory factors. As the epidermal cells grow into the placode they organize the mesenchymal cells to form the dermal papilla, which then becomes a permanent feature of the hair follicle and functions in all subsequent hair follicle cycles (Module 8: Figure hair follicle cycle).

Hair follicle cycle

The lower region of the hair follicle is constantly being remodelled. The hair follicle cycle consists of three clearly recognizable phases called catagen, telogen and anagen (Module 8: Figure hair follicle cycle).

Catagen

During catagen, most of the epithelial cells that make up the hair bulb die through a process of apoptosis resulting in a phase of epithelial cell regression. The mesenchymal cells that make up the dermal papilla remain and are drawn upwards towards the bulge region of the non-cycling region.

Telogen

Telogen is a relatively quiescent period that marks the transition between the catagen and anagen phases. This transition period may be the time when the dermal papilla release the stimuli such as Wnt and Noggin that are responsible for activating the epidermal stem cells and the melanocyte stem cells in the bulge to provide the progenitor cells that move down to begin the anagen phase of hair bulb regeneration.

Anagen

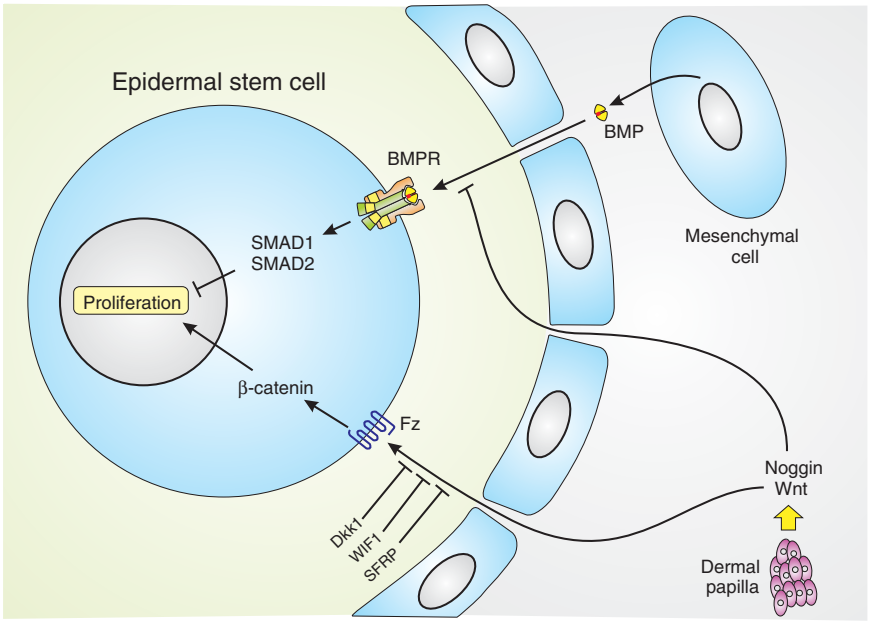
Regeneration of a new hair bulb occurs during the anagen phase. The progenitor cells that emerge from the melanocyte and epidermal stem cells in the bulge combine to form the new hair matrix and inner and outer root sheaths responsible for constructing a new hair shaft.

Skin regeneration and repair

The skin has a remarkable capacity for regeneration and repair, which is determined by the different skin stem cells. Regeneration and repair are considered together because they seem to depend on similar stem cell functions. Renewal of the epidermis depends on the activity of the basal layer of cells, which consists mainly of progenitor cells but some of these may behave like stem cells. These progenitor stem cells sit on an extracellular matrix (ECM), which probably acts as the niche that maintains these cells (see stimulus 1 in Module 8: Figure stem cell niche). During the renewal process, these progenitor cells divide to provide the constant stream of cells that move outwards to differentiate into the multiple layers of the epidermis (Module 7: Figure skin cells). It is likely that many of the divisions are asymmetrical (Module 8: Figure stem cell function) such that the one daughter that sits near the ECM remains as a progenitor, whereas the other daughter that loses its contact with the ECM stops growing and is committed to the differentiation programme.

The renewal of the hair follicle during skin regeneration depends on the activity of the epidermal stem cells and the

Module 8: | Figure epidermal stem cell



Control of epidermal stem cell proliferation.
Proliferation of epidermal stem cells located in the bulge region of the hair follicle (Module 7: Figure skin cells) are inhibited by both the SMAD signalling pathway, which is activated by bone morphogenetic protein (BMP) and by the presence of various inhibitors (Dkk1, WIF1 and sFRP) that block the Wnt signalling pathway activated by the frizzled receptor (Fz). As the dermal papilla are drawn up towards the bulge during the follicle hair cycle they release Noggin and Wnt that act to drive proliferation by reversing these inhibitory mechanisms.

melanocyte stem cells located in the bulge region (Module 7: Figure skin cells).

Epidermal stem cells

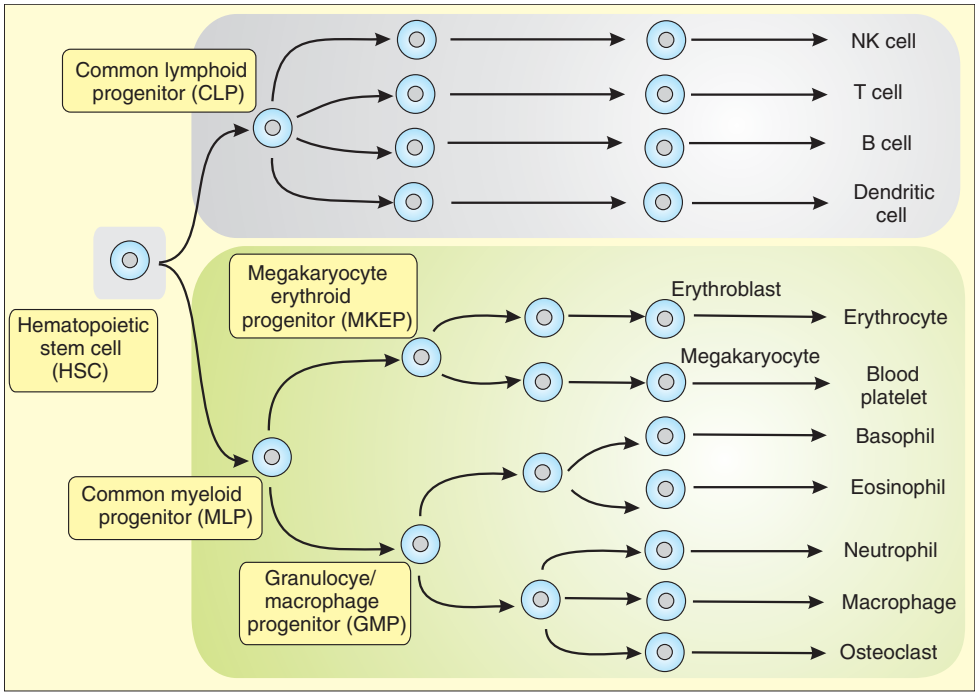
Epidermal stem cells (ESCs) are located in the bulge region of the hair follicle (Module 7: Figure skin cells). The bulge provides the stem cell niche that maintains these stem cells together with the melanocyte stem cells. The epidermal stem cells have two functions. First, they provide the progenitor cells that move downwards to form the hair matrix progenitors in the hair bud during the hair follicle cycle (Module 8: Figure hair follicle cycle). Secondly, they provide epidermal progenitor cells that migrate outwards to reform the basal layer during wound healing. The bulge provides the niche to maintain these stem cells in a quiescent state until they are required either for wound healing or the periodic hair follicle cycle.

There are two main inhibitory mechanisms responsible for keeping the epidermal stem cells in a quiescent state (Module 8: Figure epidermal stem cell). Mesenchymal cells in the dermis release bone morphogenetic protein (BMP), which is a component of the Smad signalling pathway (Module 2: Table Smad signalling toolkit). In addition, the bulge region contains a number of the inhibitors of Wnt signalling such as Dickkopf (Dkk), secreted frizzled-related protein (sFRP) and Wnt inhibitory factor 1(WIF-1) that suppress the ability of Wnt to stimulate proliferation (Module 8: Figure epidermal stem cell). During the telogen phase of the hair follicle cycle (Module 8: Figure hair follicle cycle), the dermal papilla comes to lie close to the bulge and begins to supply various factors such as Noggin and Wnt. These secreted factors then enter the bulge and reverse the various inhibitory effects that normally keep the stem cells quiescent (Module 8: Figure epidermal stem cell). Noggin alleviates the inhibitory effect of BMP whereas the increased formation of Wnt overcomes the inhibitors Dkk1, SFRP and WIF-1 thus enabling the epidermal stem cells to proliferate to provide the progenitor cells that migrate out to begin growing another hair.

Melanocyte stem cells

Melanocyte stem cells (MSCs) are located in the lower region of the bulge together with the epidermal stem cells (Module 7: Figure skin cells). Like the latter, the MSCs remain quiescent until they are required to provide the progenitor melanoblasts that migrate down to populate the hair matrix where they differentiate into the melanophores that provide hair pigments. Hair greying in humans, which is an obvious sign of aging, seems to result from a loss of MSCs due to a decline in the self-maintenance mechanisms that retain viable stem cells in the bulge. The MSCs express the transcription factors paired box 3 (Pax3), sex-determining region Y (SRY)-box 10 (SOX10) and microphthalamia-associated transcription factor (MITF), which not only play a role in self-maintenance but are used again when these stem cells differentiate and begin to produce melanin (Module 7: Figure melanogenesis). The loss of MSCs may result from an increase in apoptosis caused by a decrease in the survival factor Bcl-2, which is one of the genes switched on by the MITF that controls melanocyte differentiation and survival (Module 7: Figure melanogenesis).

Module 8: | Figure haematopoiesis



Development of haematopoietic cells from a common haematopoietic stem cell (HSC).
A haematopoietic stem cell (HSC) gives rise to two main cell lineages. A common lymphoid progenitor (CLP) produces the main cells of the immune system, whereas a common myeloid progenitor (MLP) gives rise to the megakaryocyte erythroid progenitor (MKEP), which produces erythrocytes and blood platelets, and the granulocyte macrophage progenitor (GMP), which produces a number of cell types.

Since the MSCs occupy a similar niche as the epidermal stem cells, it is likely that the former may share some of the same pathways to regulate their switch from quiescence to active proliferation. Indeed, there is evidence to show that the Wnt signalling pathway plays a role in switching the MSCs into the melanoblasts that go on to differentiate into the melanocytes that populate the matrix of the hair follicle (Module 7: Figure skin cells).

Haematopoietic stem cell (HSC)

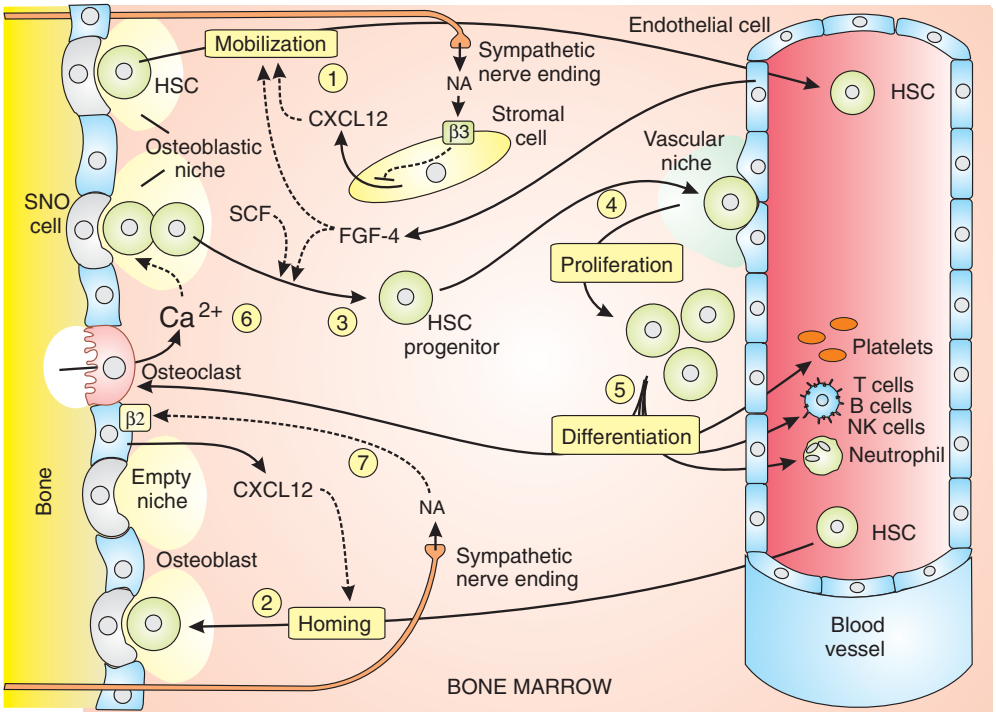
Haematopoiesis is the developmental process responsible for producing the cells of the blood system (Module 8: Figure haematopoiesis). The haematopoietic stem cell (HSC), located in the bone marrow spawns two major progenitor cell lines. The common lymphoid progenitor (CLP) gives rise to the different lymphocytes such as the natural killer cells (NK cells), T cells, B cells and dendritic cells. The common myeloid progenitor (MLP) gives rise to both the megakaryocyte erythroid progenitor (MKEP) and the granulocyte macrophage progenitor (GMP). The former goes on to form the erythrocytes and the blood platelets, whereas GMP is the precursor of a number of cells, including basophils, eosinophils, neutrophils, macrophages and osteoclasts. The differentiation of these haematopoietic cells is regulated by the haematopoietic cytokines (Module 8: Figure haematopoietic cytokines).

The HSC plays an important role in differentiation of bone cells, since it is the precursor of the osteoclast. A process of osteoclastogenesis, which is carefully orchestrated by a number of signalling pathways, is responsible for producing these bone resorptive-osteoclasts.

The haematopoietic stem cell (HSC) is located in the osteoblastic and vascular niches located within the bone marrow on the endosteal surface of bone (Module 8: Figure bone marrow). The HSCs physically interact with the spindle-shaped N-cadherin positive osteoblastic cells (SNOs), which are a subset of the osteoblasts responsible for bone formation (Module 7: Figure bone cells). These SNOs provide many of the instructive signals that control the proliferative activity of the HSCs (Module 8: Figure HSC regulation). Other regulatory signals are derived from other cells within the bone marrow such as the stromal cells and the endothelial cells. The bone marrow is also innervated by the sympathetic nervous system that releases noradrenaline (NA). The activity of these neurons is controlled by the circadian clock and this may explain the oscillatory nature of various bone marrow functions such as the release of HSCs and the proliferation of the osteoblasts (see below). The HSCs integrate all these stimuli and undergo a variety of responses as outlined in the various events illustrated in Module 8: Figure bone marrow:

1. The HSCs are not permanent residents within the osteoblastic niche, but are periodically mobilized and find their way into the blood vessels to be carried around in the blood supply. This movement towards the blood vessels may be directed by the chemokine CXCL12 [stromal-derived factor-1 (SDF-1)] released from the stromal cells. The synthesis of CXCL12 is regulated

Module 8: | Figure bone marrow



Location and function of haematopoietic stem cells (HFCs) in bone marrow.

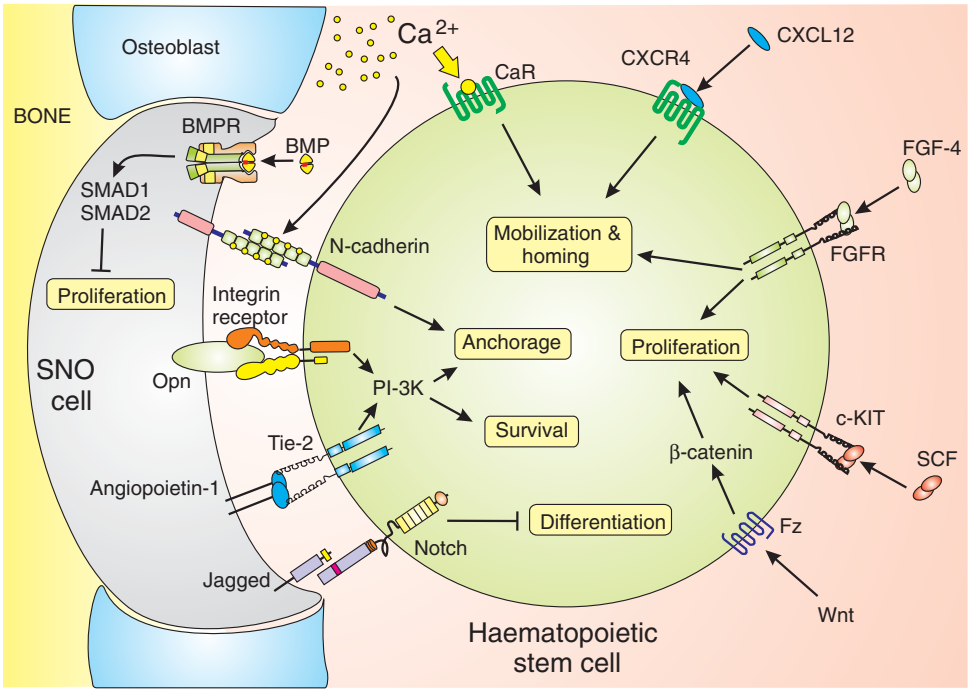
The haematopoietic stem cell (HSC) occupies two main niches within the bone marrow. The quiescent HSCs reside with the osteoblast niche where they associate with the spindle-shaped N-cadherin positive osteoblastic (SNO) cell. They can leave this niche (mobilization) to enter the blood and they can return (homing) to take up residence in empty niches. The HSCs also produce the progenitor cells that reside in the vascular niche where they proliferate to produce the cells that then differentiate into the haematopoietic cells (Module 8: Figure haematopoiesis). FGF-4, fibroblast growth factor-4; NA, noradrenaline; SCF, stem cell factor

by noradrenaline (NA) released from the sympathetic nerve endings that innervate the bone marrow. These sympathetic nerves are regulated by the circadian clock resulting in a periodic release of NA, which then act through the β3-adrenoceptors on the stromal cells to decrease the content of the Sp1 transcription factor that controls the expression of CXCL12. The circadian oscillation of HSCs circulating in the blood may be explained by the daily fluctuation of this chemokine in the bone marrow.

2. The HSCs circulating within the blood can leave the blood vessels and through a homing response they locate an empty niche where they can settle down to carry out their stem cell function.
3. The primary function of the HSCs is to provide the common lymphoid and myeloid progenitors that go on to differentiate into a wide range of cells (Module 8: Figure hematopoiesis). The close association with the SNOs provides both an anchor to hold the cells within the osteoblastic niche, but also the stimuli that regulate their quiescence (G0 state). When required, these stem cells are activated to provide the HSC progenitors that migrate towards the vascular niches surrounding the blood vessels.
4. HSC progenitors within the vascular niche continue to proliferate to provide the large numbers of cells required for haematopoiesis.
5. The HSC progenitor cells differentiate into haematopoietic cells that either enter the circulation or, in the case of the osteoclasts, they migrate to the endosteal surface to begin their role in bone resorption (Module 7: Figure bone cells).
6. The high extracellular Ca^{2+} concentration in the vicinity of the endosteal surface is recognized as an important component of the osteoblastic niche. An increase in the supply of osteoclasts will increase the rate of bone resorption and will thus increase the Ca^{2+} concentration within the osteblastic niche. Since the HSCs express the Ca^{2+}-sensitive receptor (CaR), they will be able to adjust their activity by responding to the change in Ca^{2+} concentrations during bone resorption and this could contribute to bone cell coupling.
7. Noradrenaline (NA) released from sympathetic nerve endings, which occurs with a circadian periodicity, acts on β2-adrenoceptors on the osteoblasts and this may help to entrain the peripheral circadian clock that controls the proliferation of osteoblasts.

These different activities of the HSC are regulated by a large number of signalling pathways. Some of these control HSC anchorage to the SNO cells within the niche whereas others regulate HSC mobilization/homing or HSC self renewal (Module 8: Figure HSC regulation).

Module 8: | Figure HSC regulation



Regulation of haematopoietic stem cell (HSC) function.
The haematopoietic stem cell (HSC) spends most of its time within the osteoblastic niche where its activity is regulated by a host of stimuli, many of which are held on the surface of the spindle-shaped N-cadherin positive osteoblastic (SNO) cell. Some of these stimuli act to regulate proliferation whereas others determining the mobilization and homing responses (Module 8: Figure bone marrow). BMP, bone morphogenetic protein; CaR, Ca^{2+}-sensing receptor; FGF-4, fibroblast growth factor-4; FGFR, fibroblast growth factor receptor; SCF, stem cell factor;

HSC anchorage

Anchorage to the SNO cells within the osteoblastic niche is carried out by various adhesion molecules (Module 8: Figure HSC regulation). There are homophilic links between N-cadherin molecules that are held together by Ca^{2+}. Indeed, the high concentration of Ca^{2+} located near the endosteal surface appears to be an important component of the osteoblastic niche. There also are interactions between the integrin receptor and osteopontin (OPN). In addition, some degree of adhesion is provided by the interaction between angiopoietin-1 (Ang1) and its TIE2 receptor, which is protein tyrosine kinase-linked receptor. Both the integrin and TIE2 receptors are known to be linked to the PtdIns 3-kinase signalling pathway, which also will contribute to stem cell survival by switching off apoptosis.

HSC mobilization/homing

The chemokine CXCL12 [stromal-derived factor-1 (SDF-1)] is particularly important in regulating the mobilization and homing responses of HSCs. SDF-1 is released by different cells (stromal, endothelial and osteoblast cells) within the bone marrow (Module 8: Figure bone marrow). The directionality of the chemotactic response may depend on which of these cells is releasing the largest amounts of SDF-1 at any given time. Mobilization and movement towards the blood vessels (Step 1 in Module 8: Figure bone marrow) depends on the endothelial and stromal cell release of SDF-1, whereas the homing response is driven by release of this chemokine by the osteoblasts (Step 2 in Module 8: Figure bone marrow). Release of fibroblast growth factor-4 (FGF-4) may also contribute towards the movement of HSCs towards the blood vessels.

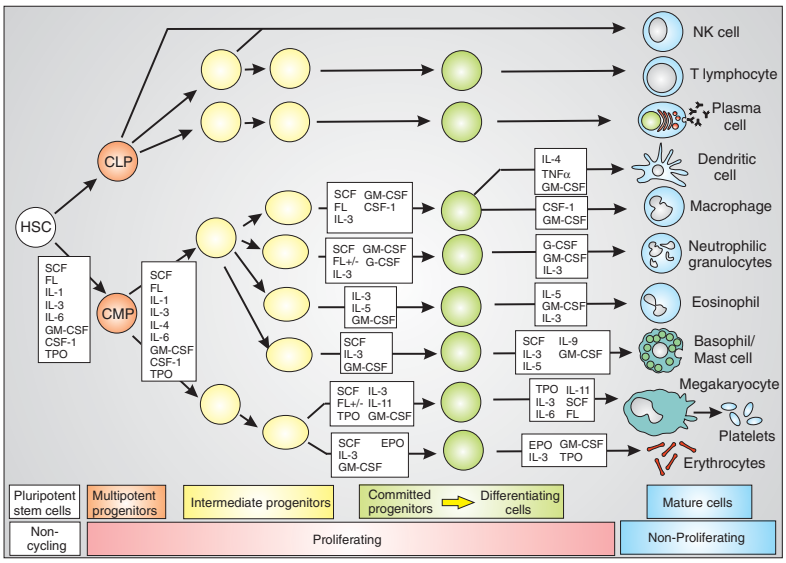
HSC self renewal

As for other stem cells, self renewal of the HSC population is determined by a balance between signalling systems that determine the HSC quiescent state and those that trigger proliferation. With regard to the former, many of the systems that are responsible for anchorage to the spindle-shaped N-cadherin positive osteoblastic (SNO) cell also function to maintain quiescence (Module 8: Figure HSC regulation). For example, angiopoietin-1 (Ang-1) acting through its tyrosine kinase-linked receptor TIE2 seems to be essential for quiescence. Similarly, osteopontin (Opn) that acts through integrin signalling also prevents proliferation.

The switch from quiescence to proliferation is activated by growth factors such as fibroblast growth factor 4 (FGF-4) and stem cell factor (SCF). The canonical Wnt/β-catenin pathway also functions to induce HSC proliferation. During this activation of proliferation, the notch signalling pathway contributes to self renewal by preventing the HSCs from differentiating thus enabling them to maintain their stem cell phenotype.

HSCs appear not to express the receptor for bone morphogenetic protein (BMP), which acts through the Smad signalling pathway (Module 2: Table Smad signalling toolkit) to inhibit proliferation in other stem cells.

Module 8: | Figure haematopoietic cytokines



Cytokine control of haematopoiesis.
The proliferation and development of haematopoiesis is controlled by a battery of cytokines. They operate in different proportions to guide the early common myeloid progenitor (CMP) along different developmental pathways. Redrawn from *Handbook of Cell Signaling*, Vol. 3 (edited by R.A. Bradshaw and E.A. Dennis), Pixley, F.J. and Stanley, E.R., Cytokines and cytokine receptors regulating cell survival, proliferation, and differentiation in hematopoiesis, pp. 615–623. Copyright (2003), with permission from Elsevier; see Pixley and Stanley (2003).

However, BMP acts indirectly by controlling proliferation of the SNO cell, which in turn determines the number of osteoblastic niches available to maintain HFCs.

Haematopoietic cytokines

There are a large group of cytokines that orchestrate the complex process of haematopoiesis (Module 8: Figure haematopoietic cytokines). These cytokines are polyfunctional in that they regulate a number of the essential processes that occur during haematopoiesis. For example, cytokines such as colony-stimulating factor (CSF), stem cell factor (SCF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) function throughout haematopoiesis by promoting survival of the non-cycling haematopoietic stem cells (HSCs), they then stimulate the proliferation of the multipotent progenitor cells and then guide cells during the commitment process when they differentiate into specific cell types (Module 8: Figure haematopoietic cytokines). Finally, these cytokines can continue to function by controlling the activation of specific cell types.

Intestinal stem cells

Intestinal stem cells are located in the crypt region of the intestine (Module 12: Figure colon cancer). Each crypt has approximately 4–6 stem cells, which are arranged in a ring immediately above the Paneth cells at the base of the crypt. These intestinal cells divide periodically to give rise to the progenitor cells that proliferate rapidly as they move up the crypt. When these progenitors reach the junction with the villus, they stop proliferating and the differentiation of intestinal cells begins as the cells migrate up the villus. This orderly sequence of proliferative events within the crypt and differentiation along the villus is carefully orchestrated by signals emanating from surrounding mesenchymal cells that are a part of the intestinal stem cell niche.

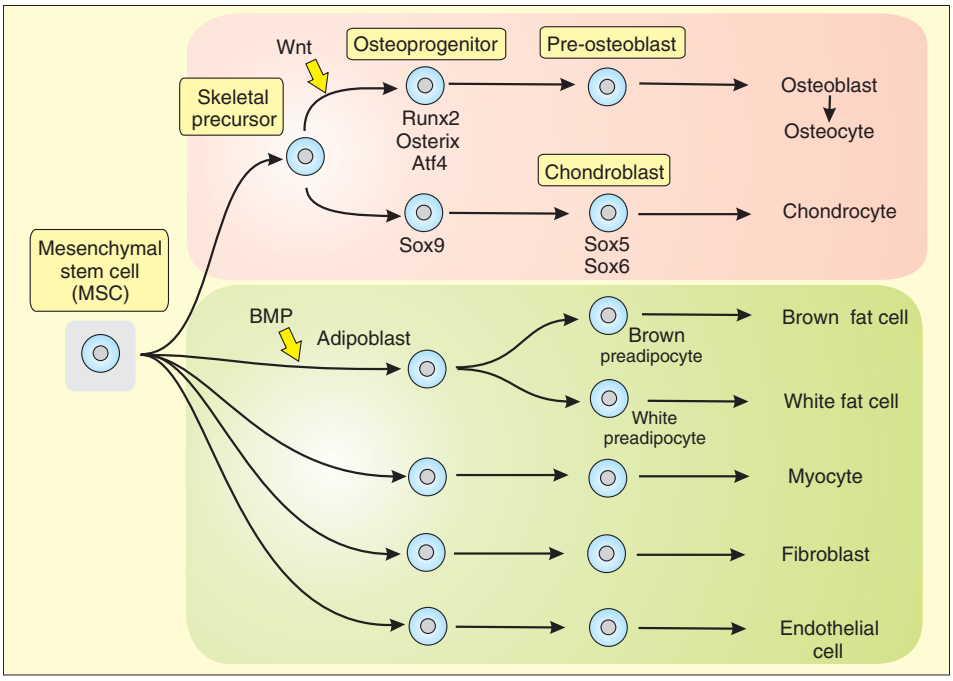
The slow rate of proliferation by the intestinal stem cells is controlled by bone morphogenetic protein (BMP) (Module 2: Table Smad signalling toolkit) that acts through the Smad signalling pathway to inhibit proliferation (Module 12: Figure colon cancer). The shift from quiescence to proliferation that occurs occasionally is carried out by the canonical Wnt/β-catenin pathway as it is for so many other stem cells (Module 8: Figure stem cell function). The Wnt signalling pathway continues to operate in the progenitor cells to drive their high rate of proliferation.

The balance between quiescence and proliferation normally favours the former and the question arises as to how this balance occasionally switches in favour of proliferation. The transient release of Noggin, which is an inhibitor of BMP signalling, seems to be one of the mechanisms to switch off the inhibitory pathways to allow Wnt signalling to initiate proliferation.

Mesenchymal stem cell (MSC)

Mesenchymal stem cells (MSCs) split into two cell lineages early in developmental (Module 8: Figure MSC differentiation). The skeletal precursor line then undergoes two separate processes: chondrogenesis, which results in the formation of the chondrocytes, and osteoblastogenesis, which gives rise to the osteoblasts. Separation into these two developmental pathways appears to be regulated by the canonical Wnt/β-catenin pathway. Activation of Wnt signalling with the release of the transcription factor β-catenin switches the skeletal precursor towards an osteoblast lineage. The absence of β-catenin allows the

Module 8: | Figure MSC differentiation



Differentiation of a mesenchymal stem cell (MSC) into different cell types.
The mesenchymal stem cell (MSC) develops into two main developmental lineages that give rise to a number of cell types. See the text for further information.

precursor to develop into a chondrogenic lineage characterized by the formation of the specific transcription factor Sox9 and its two targets Sox5 and Sox6.

Mutations in components of the Wnt signalling pathway such as lipoprotein receptor-related protein 5 (LRP5) and scelerostin (SOST) cause inherited bone diseases such as osteoporosis pseudoglioma (OPPG) and Van Buchem disease respectively.

MSCs also give rise to the two main cells of adipose tissue: the white fat cells and the brown fat cells. The adipoblast appears to be the common precursor that then diverges towards the white and brown preadipocyte precursors of the white and brown fat cells respectively. A large number of hormones control the differentiation of white fat cells.

Differentiation of bone cells

The three major bone cells are the osteoclasts, osteoblasts and osteocytes (Module 7: Figure bone cells). The osteoclasts are derived from the haematopoietic stem cell (HSC). The process of osteoclastogenesis consists of a series of discrete steps during which this precursor is gradually converted into the mature multinucleated osteoclast (Module 8: Figure bone cell differentiation). A process of preosteoclast chemotaxis controls the trafficking of osteoclast precursors towards the bone surface. The osteoblasts and osteocytes have a different origin in that they are derived from the mesenchymal stem cell (MSC) (Module 8: Figure MSC differentiation). The process of osteoblastogenesis consists of a series of discrete steps during which the MSC precursor is gradually converted into the mature osteoblast (Module 8: Figure bone cell differentiation). The osteocyte is derived directly from the osteoblast. As the latter begin to form bone, they gradually become buried in the mineralized matrix and transform into the star-shaped osteocytes (Module 7: Figure bone cells).

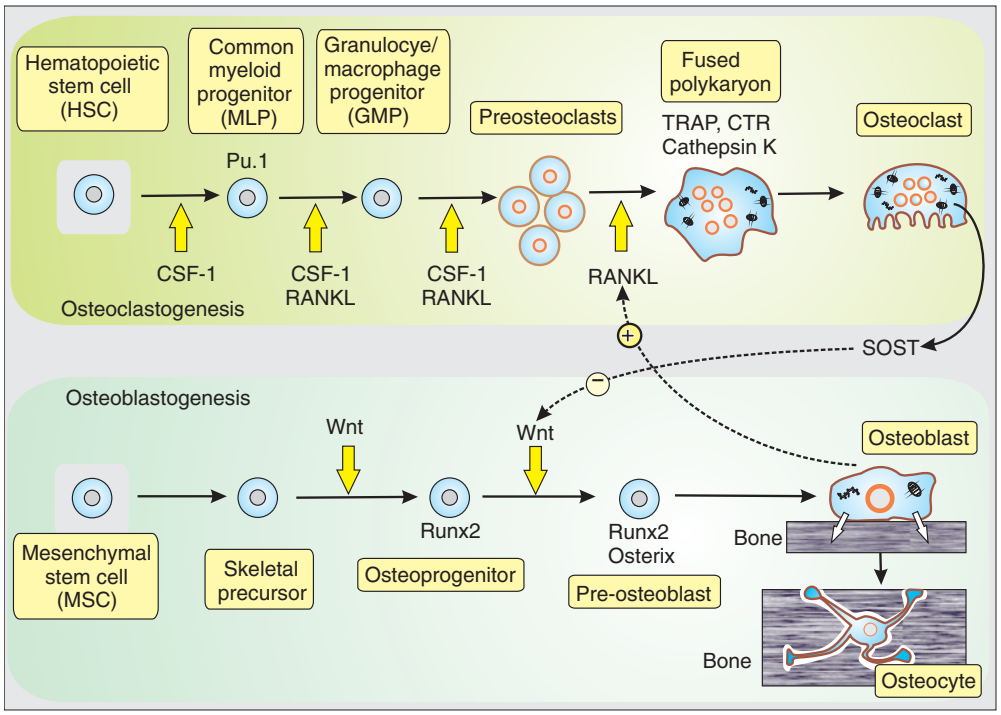
Osteoclastogenesis

The developmental process responsible for forming mature osteoclasts plays a critical role in bone remodelling because it contributes to the bone cell coupling mechanism that maintains the balance between bone formation and bone resorption.

Osteoclasts originate from a haematopoietic stem cell (HSC) line located in the bone marrow close to the osteoblasts on the bone surface. Osteoclastogenesis depends upon an orderly sequence of steps that begin with proliferation of the common myeloid progenitor (MLP), which then transforms into a granulocyte progenitor (GMP) (Module 8: Figure bone cell differentiation). The latter then give rise to the preosteoclasts that fuse to give the polykaryon that differentiates into the multi-nucleated osteoclast. This sequence of proliferation and differentiation is controlled by a number of signalling pathways and is also dependent on interactions with both the osteoblasts and T cells (Module 8: Figure osteoclastogenesis).

The stromal cells release colony-stimulating factor-1 (CSF-1), which acts on the colony-stimulating factor-1 receptor (CSF-1R). Another key component is the tumour necrosis-related factor called receptor activator of nuclear factor κB (NF-κB) ligand (RANKL) acting on the RANKL receptor (RANK). RANKL is expressed on the

Module 8: | Figure bone cell differentiation



Control of bone cell differentiation.
Differentiation of osteoclasts from haematopoietic stem cells (HSCs) occurs through a series of steps (osteoclastogenesis) controlled by colony-stimulating factor-1 (CSF-1) and receptor activator of nuclear factor κB (NF-κB) ligand (RANKL), which is located on the surface of the osteoblasts. Differentiation of the osteoblasts from mesenchymal stem cells (MSCs) occurs through a series of steps controlled by the Wnt signalling pathway. The osteoclasts release sclerostin (SOST), which inhibits the Wnt signalling to reduce osteoblastogenesis. As the osteoblasts secrete bone (white arrows), they gradually become buried in the mineralized matrix where they are transformed into star-shaped osteocytes.

surface of both T cells and osteoblasts, and a close interaction between these cells types and the developing osteoclasts is a key feature of osteoclastogenesis. Osteoclast differentiation also depends on a Ca^{2+} signalling pathway activated by a number of immunoglobin-like receptors such as osteoclast-associated receptor (OSCAR), paired immunoglobin-like receptor (PIR-A), triggering receptor expressed in myeloid cells (TREM-2) and signal-regulatory protein (SIRPβ1). These receptors interact with adaptors such as the DNAX-activating protein 12 (DAP12) and FcRγ that contain a typical immunoreceptor tyrosine-based activation motif (ITAM) that recruits signalling components that activate a Ca^{2+} signalling pathway.

Osteoclasts and osteoblasts also interact with each other through the ephrin (Eph) receptor signalling pathway as part of the bone cell coupling mechanism that provides a two-way communication system (for details see Step 6 in Module 7: Figure osteoclast function).

In summary, osteoclastogenesis is driven by the co-operative activity of an array of receptors as outlined in more detail in (Module 8: Figure osteoclast differentiation:

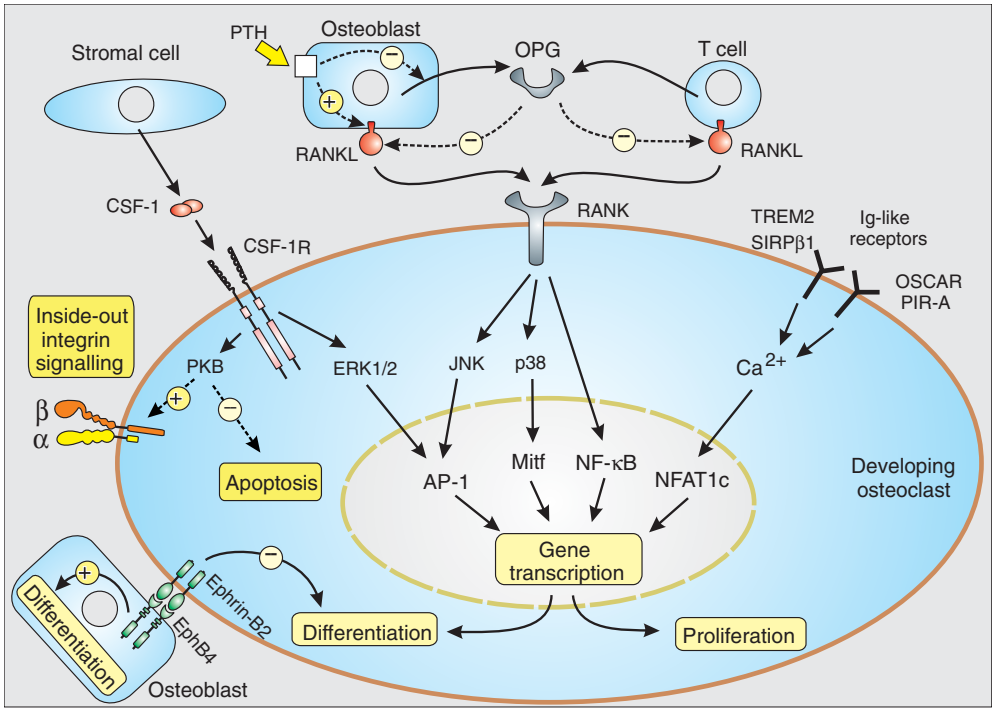
- Colony-stimulating factor-1 receptor (CSF-1R)
- Receptor activator of nuclear factor κB (NF-κB) ligand receptor (RANK)
- Osteoclast-associated receptor (OSCAR)
- Paired immunoglobin-like receptor (PIR-A)
- Triggering receptor expressed in myeloid cells (TREM-2)
- Signal-regulatory protein (SIRPβ1)

Preosteoclast chemotaxis

Preosteoclast cells are the osteoclast progenitor cells that fuse to form the polykaryon that then differentiates into the functional osteoclasts (Module 8: Figure bone cell differentiation). These preosteoclasts are constantly trafficking between the bone surface and the blood. This trafficking in and out of the bone marrow appears to be driven by two chemotactic factors as illustrated in the following sequence of events (Module 8: Figure preosteoclast chemotaxis):

1. The haematopoietic stem cells (HSC) that enter the cell can differentiate into a number of different cell types including the preosteoclasts (Module 8: Figure haematopoiesis). These preosteoclasts then migrate either towards the bone surface or back into the blood depending on the presence of two chemotactic factors.
2. The chemokine CXCL12, which is synthesized and released by both the stromal cells and the osteoblasts directs the cells towards the bone surface where they fuse to form the polykaryons (Module 8: Figure bone cell differentiation) that then go on to differentiate into osteoclasts through the process of osteoclastogenesis (Module 8: Figure osteoclastogenesis).

Module 8: | Figure osteoclastogenesis



Summary of osteoclastogenesis.
Osteoclastogenesis is the developmental process responsible for forming osteoclasts. A number of cells contribute by providing various ligands. The stromal cells provide colony-stimulating factor-1 (CSF-1). The osteoblasts and T cells present receptor activator of nuclear factor κB (NF-κB) ligand (RANKL). They also release osteoprotegerin (OPG), which is a soluble RANKL-binding decoy receptor that inhibits the action of RANKL receptor (RANK). Parathyroid hormone (PTH) promotes bone resorption by inhibiting the release of OPG and enhancing the expression of RANKL. Another group of immunological-like receptors [osteoclast-associated receptor (OSCAR), paired immunoglobin-like receptor (PIR-A), triggering receptor expressed in myeloid cells (TREM-2) and signal-regulatory protein (SIRPβ1)] provide co-stimulatory signals. The CSF-1 receptor (CSF-1R) not only inhibits apoptosis, but also induces *inside-out* integrin signalling, which facilitates integrin binding to bone. The osteoblasts and osteoclasts communicate with each other through the ephrin (Eph) receptor signalling pathway. The way in which these receptors function to regulate apoptosis, proliferation and differentiation are described in Module 8: Figure osteoclast differentiation.

3. The chemotaxis driven by CXCL12 is regulated by the sympathetic nervous system. The release of CXCL12 by the stromal cells and osteoblasts appears to be inhibited by the release of noradrenaline (NA).
4. The blood contains relatively high levels of sphingosine-1-phosphate (S1P), which provides a chemotactic gradient to direct cells back into the blood stream (Module 8: Figure preosteoclast chemotaxis).

Colony-stimulating factor-1 (CSF-1)

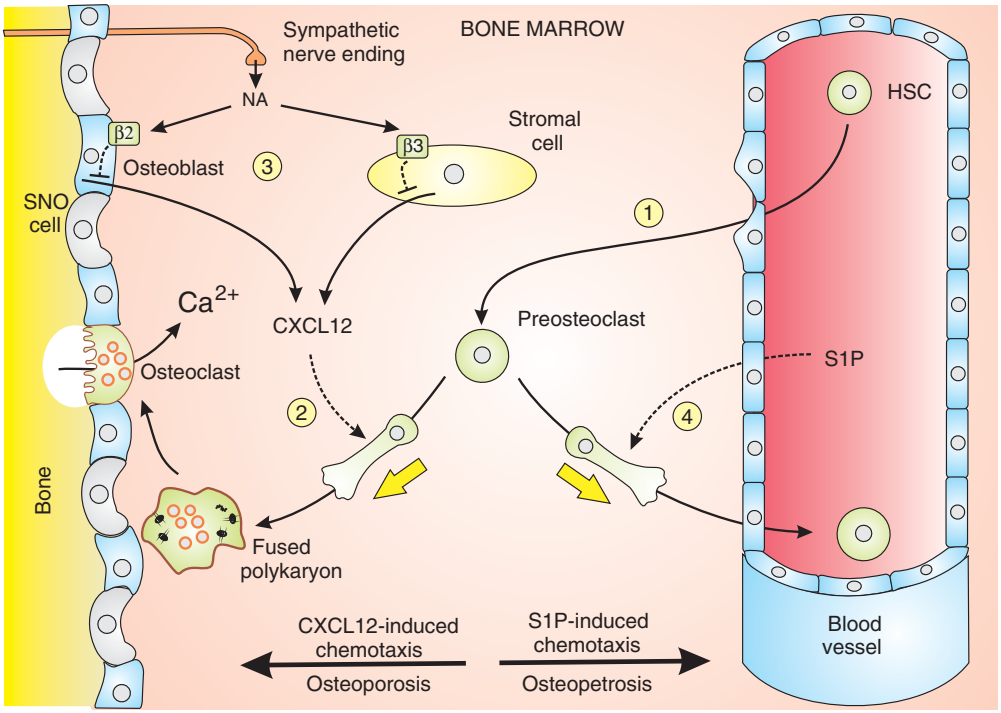
Colony-stimulating factor-1 (CSF-1), which is also known as macrophage colony-stimulating factor (M-CSF), is released from a variety of cells (endothelial cells, fibroblasts, uterine epithelium, macrophages and bone marrow stromal cells). One of its main functions is as a haematopoietic cytokine to regulate the survival of progenitor cells and to orchestrate the emergence of macrophages (Module 8: Figure haematopoietic cytokines).

The CSF-1 released from bone marrow stromal cells also operates in the process of osteclastogenesis (Module 8: Figure osteoclastogenesis). CSF-1 acts through the colony-stimulating factor-1 receptor (CSF-1R), which is a typical protein tyrosine kinase-linked receptor (PTKRs) (Module 1: Figure tyrosine kinase-linked receptors).

Colony-stimulating factor-1 receptor (CSF-1R)

One of the key factors in controlling osteoclastogenesis is colony-stimulating factor-1 (CSF-1), which is released from the bone marrow stromal cells to act on the CSF-1 receptor (CSF-1R) (Module 8: Figure osteoclastogenesis). This CSF-1R, which is also known as FMS, is a typical protein tyrosine kinase-linked receptor (PTKRs) (Module 1: Figure stimuli for enzyme-linked receptors). Activation of the CSF-1R is particularly important early in osteoclastogenesis because it not only helps to drive cell proliferation of the osteoclast precursor cells, but also promotes their survival by inhibiting apoptosis (Module 8: Figure osteoclast differentiation). Like other PTKRs, the CSF-1R relays information down different signalling pathways. It recruits the extracellular-signal-regulated kinase (ERK) pathway, which is one of the mitogen-activated protein kinase (MAPK) signalling pathways that is responsible for stimulating cell proliferation (Module 2: Figure MAPK signalling). The activated ERK1/2 enters the nucleus, where it increases the expression of the D cyclins, which is a key event in the way growth factors activate the cell cycle (Module 9: Figure cell cycle signalling mechanisms). In addition, ERK1/2 also activates transcription factors such as PU.1, activating protein 1 (AP-1) (Fos/Jun) and the microphthalamia transcription factor (Mitf) family

Module 8: | Figure preosteoclast chemotaxis



Chemotaxis of preosteclasts.
The migration of preosteoclasts in the bone marrow is controlled by two chemotaxis gradients. CXCL12 produced by stromal cells and osteoblasts directs cells towards the bone surface where they differentiate into osteoclasts whereas sphingosine-1-phosphate (S1P) directs them back into the blood stream.

of transcription factors, which are responsible for initiating the expression of the genes that define the osteoclast phenotypes such as tartrate-resistant acid phosphatase (TRAP), cathepsin K, integrin β_3 and the calcitonin receptor (CTR) (Module 8: Figure osteoclast differentiation). Another role for ERK1/2 is to promote the expression of the receptor activator of nuclear factor κB (NF-κB) ligand (RANKL) receptor (RANK), which thus makes the cells sensitive to the RANKL.

The CSF-1R also plays an important role in promoting the survival of the osteoclast precursor cells by inhibiting apoptosis. Just how this inhibition is carried out is still unclear, but the CSF-1R is known to activate the PtdIns 3-kinase signalling pathway, which is known to promote cell survival (Module 2: Figure PtdIns 3-kinase signalling). The CSF-1R also recruits the non-receptor tyrosine kinase Src, resulting in its autophosphorylation at position 416 (Module 8: Figure osteoclast differentiation). The phosphotyrosine residue at 416 provides a binding site for c-Cbl, which functions as an adaptor to recruit a number of signalling components. In addition, c-Cbl has E3 ubiquitin ligase activity that carries out the polyubiquitination of the pro-apoptotic factor Bim, which is a key regulator of apoptosis (Module 11: Figure Bcl-2 family functions). The subsequent destruction of Bim by the proteasome prevents apoptosis.

Another role for the CSF-1R, which might be mediated through the PtdIns 3-kinase signalling pathway, is to transactivate the integrin receptor through the *inside-out* integrin signalling pathway (Module 8: Figure osteoclastogenesis). Through this mechanism, the integrin receptors have an enhanced sensitivity and will thus be able to bind to bone to begin the process of osteoclast activation (Module 7: Figure osteoclast function).

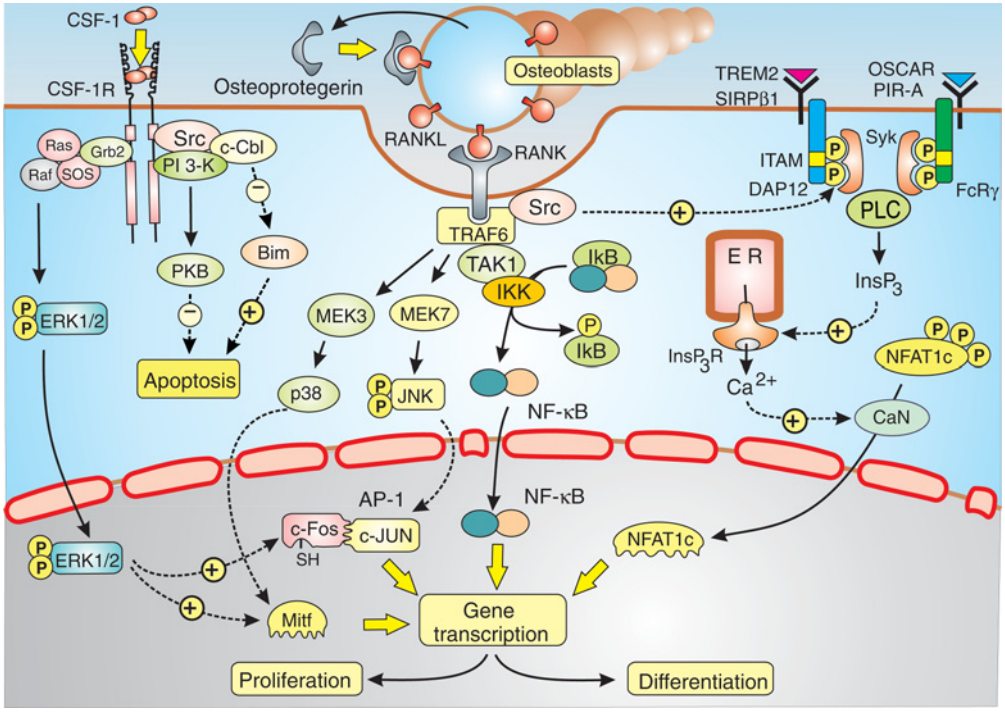
Microphthalamia transcription factor (MITF)

The microphthalamia transcription factor (MITF) belongs to a family of basic helix-loop-helix-zipper type transcription factors that bind to a canonical E-box promoter sequence CACGTG either as a homodimer or as a heterodimer with other closely related factors such as TFE3, TFEB and TFEC. The activity of MITF can also be modulated by phosphorylation. In melanocytes, for example, SCF enhances the activation of MITF by acting through the mitogen activated protein kinase (MAPK) signalling pathway to phosphorylate MITF on Ser-73 (Module 7: Figure melanogenesis). This phosphorylation assists in transcriptional activity of Mitf by enabling it to recruit the transcription coactivator p300.

MITF is an important regulator of developmental events in osteoclasts, melanocytes, mast cells and retinal pigmented epithelium:

- In developing osteoclasts, MITF is activated during CSF-1 receptor (CSF-1R) signalling during which ERK1/2 phosphorylates MITF, which then activates the transcription of the tartrate-resistant acid phosphatase (TRAP) and cathepsin K genes (Module 8: Figure osteoclast differentiation).
- A variety of signalling pathways cooperate to activate MITF transcription in developing melanocytes where it

Module 8: | Figure osteoclast differentiation



Control of osteoclastogenesis.
A number of signalling pathways co-operate to activate the gene transcriptional events responsible for controlling osteoclastogenesis. A simplified version of the main events that occur during osteoclastogenesis is shown in Module 8: Figure osteoclastogenesis. The different signalling pathways function to control apoptosis, proliferation and the differentiation of the precursor cells into the mature osteoclasts responsible for bone resorption. See the text for further information.

acts to increase the expression of the proteins responsible for melanogenesis (Module 7: Figure melanogenesis).

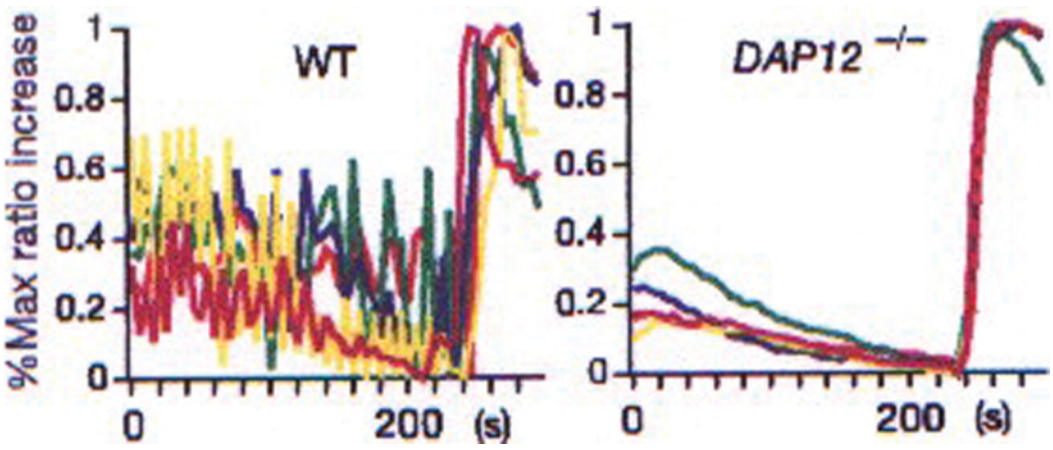
Mutations in MITF are responsible for Waardenburg syndrome 2a (WS2a).

Receptor activator of nuclear factor κB (NF-κB) ligand (RANKL)

An important feature of osteoclastogenesis is the close association between the developing osteoclasts and the surrounding T cells and osteoblasts (Module 8: Figure osteoclastogenesis). Indeed, these two supporting cells have a molecule called receptor activator of nuclear factor κB (NF-κB) ligand (RANKL) embedded in their surface membrane that activates the RANKL receptor (RANK) on the osteoclasts that is critical for their differentiation (Module 8: Figure osteoclast differentiation).The origin of the name of the receptor and its ligand comes from the fact that the RANK/RANKL system acts through the nuclear factor κB (NF-κB) signalling pathway (Module 2: Figure NF-κB activation). Hence, RANK responds to RANKL, which is a transmembrane protein that belongs to the tumour necrosis factor (TNF) family of cytokines (Module 1: Figure cytokines). Likewise, RANK belongs to the TNF receptor (TNF-R) superfamily and, in osteoclasts, it uses the adaptor TRAF6, which is a member of the tumour necrosis factor (TNF)-receptor-associated factor (TRAF) family, to relay information through a number of signalling pathways, including the NF-κB signalling pathway, the c-Jun N-terminal kinase (JNK) pathway and the p38 pathway. These signalling pathways contribute to differentiation by activating transcription factors such as activating protein 1 (AP-1), microphthalamia transcription factor (Mitf) and NF-κB, which switch on the osteoclast-specific genes (Module 8: Figure osteoclast differentiation).

The influence that the osteoblasts and T cells exert on osteoclastogenesis is highly dynamic in that they can have both positive and negative effects. As described above, they present RANKL, which facilitates osteoclast differentiation. On the other hand, these supporting cells can also synthesize and release osteoprotegerin (OPG), which is a soluble RANKL-binding decoy receptor. OPG is attracting a lot of attention because it is a naturally occurring inhibitor of bone resorption. Therefore the expression of RANKL and OPG are critical in regulating the delicate balance between bone formation and resorption. If there is too much OPG, the balance tips in favour of bone formation (osteopetrosis) by the osteoblasts, by reducing the number of osteoclasts. On the other hand, depletion of OPG and increased expression of RANKL accelerates bone remodelling towards bone resorption (osteoporosis). A variety of bone-resorbing hormones [parathyroid hormone (PTH), parathyroid hormone (PTH)-related peptide (PTHrP) and prostaglandin E_2 (PGE_2)] and cytokines [oncostatin M (OSM), leukaemia inhibitory factor (LIF), interleukin-1 (IL-1), IL-6 and IL-11] act by altering the expression of RANKL and OPG by the supporting cells. For example, PTH acts on osteoblasts to increase the

Module 8: | Figure osteoclast Ca^{2+} oscillations



Osteoclast precursor cell Ca^{2+} signalling.
Wild-type (WT) osteocyte precursor cells that have been stimulated with receptor activator of nuclear factor κB (NF-κB) ligand (RANKL) and colony-stimulating factor-1 (CSF-1) display oscillatory Ca^{2+} signals. Such oscillations are completely absent in cells from mice, where the adaptor protein DNAX-activating protein 12 (DAP12) has been knocked out ($DAP12^{-/-}$). Reproduced by permission from Macmillan Publishers Ltd: Koga, T., Inui, M., Inoue, K., Kim, S., Suematsu, A., Kobayashi, E., Iwata, T., Ohnishi, H., Matozaki, T., Kodama, T., Taniguchi, T., Takayanagi, H. and Takai, T. (2004) Costimulatory signals mediated by ITAM motif cooperate with RANKL for bone homeostasis. Nature 428:758–763. Copyright (2004); http://www.nature.com; see Koga et al. 2004.

expression of RANKL, while reducing that of OPG, and this creates conditions that will enhance osteoclast differentiation.

Mutations in the gene that encodes RANK have been linked to familial expansile osteolysis.

Osteoclast-associated receptor (OSCAR)

The osteoclast-associated receptor (OSCAR), which is expressed on developing osteoclasts, plays an important role in osteoclastogenesis by switching on the Ca^{2+} signal that activates the transcription factor nuclear factor of activated T cells (NFAT) (Module 8: Figure osteoclastogenesis). The related receptor paired immunoglobin-like receptor (PIR-A) has a similar role, and their actions will thus be considered together. When activated, OSCAR and PIR-A interact with FcRγ, which is an adaptor that has a typical immunoreceptor tyrosine-based activation motif (ITAM) (Module 8: Figure osteoclast differentiation). This ITAM region has tyrosine residues that are phosphorylated by the non-receptor tyrosine kinase Src. These specific phosphotyrosine residues recruit Syk that then activates phospholipase Cγ1 (PLCγ1) to switch on the inositol 1,4,5-trisphosphate ($InsP_3$)/Ca^{2+} signalling cassette. The resulting Ca^{2+} signal appears as a series of repetitive Ca^{2+} transients (Module 8: osteoclast Ca^{2+} oscillations) resembling a typical Ca^{2+} oscillation. This Ca^{2+} oscillation is responsible for activating the transcription factor NFATc1 by the well-established mechanism that depends upon the Ca^{2+}-dependent activation of calcineurin (Module 4: Figure NFAT activation).

Paired immunoglobin-like receptor (PIR-A)

The paired immunoglobin-like receptor (PIR-A), which is expressed on developing osteoclasts, contributes to osteoclastogenesis by switching on the Ca^{2+} signal that activates the transcription factor nuclear factor of activated T cells (NFAT) (Module 8: Figure osteoclastogenesis). The action of PIR-A closely resembles the action of the osteoclast-associated receptor (OSCAR) (Module 8: Figure osteoclast differentiation).

Signal-regulatory protein (SIRPβ1)

The signal-regulatory protein (SIRPβ1), which is expressed on developing osteoclasts, functions in osteoclastogenesis by switching on the Ca^{2+} signal that activates the transcription factor nuclear factor of activated T cells (NFAT) (Module 8: Figure osteoclastogenesis). The action of SIRPβ1 closely resembles the action of the triggering receptor expressed in myeloid cells (TREM-2) (Module 8: Figure osteoclast differentiation).

Osteoblastogenesis

Osteoblast are derived from the mesenchymal stem cells (MSCs) (Module 8: Figure MSC differentiation). The canonical Wnt/β-catenin pathway plays a central role in regulating the events that occur during osteoblastogenesis beginning with the induction of the skeletal precursors (Module 8: Figure bone cell differentiation). The significance of the Wnt signalling pathway is evident from the fact that the administration of Li^+, which inhibits the glycogen synthase kinase-3 (GSK-3) that is an essential component of Wnt signalling (Module 2: Figure Wnt canonical pathway), increases the rate of bone formation and the number of osteoblasts in mice. The appearance of the osteoblast lineage is characterized by the expression of transcription factors such as runt-related transcription factor 2 (Runx2), Osterix and ATF4. These processes of osteoblastogenesis can be inhibited by sclerostin (SOST), which is released from the osteoclasts.

Osteoporosis pseudoglioma (OPPG) is a bone abnormality cause by a mutation in lipoprotein receptor-related

protein 5 (LRP5), which functions as a co-receptor during Wnt signalling (Module 2: Figure Wnt canonical pathway).

Differentiation of B-cells

B-cells are derived from the haematopoietic stem cells (HSC) that provide the common lymphoid progenitors (CLPs) (Module 8: Figure haematopoiesis). These progenitors occur in the bone marrow (See step 5 in Module 8: Figure bone marrow). B-cell differentiation occurs in a series of discrete steps that occur in different locations (Module 8: Figure B-cell differentiation). B-cell differentiation in the bone marrow is the first step during which the HSC progenitors are converted into immature B-cells. B-cell differentiation in the lymph node completes the final developmental step as transitional B-cells leave the bone marrow and enter lymph nodes where they differentiate to mature B-cells.

A feature of these developmental stages are the changes that occur in the quality of the antibody the cells produce. Each antibody has two identical light (L) and two identical heavy (H) chains. The genes that code for these chains have variable ('V') and constant ('C') regions. The 'V' region of the H chain has V, D and J segments that can recombine randomly through a process known as VDJ recombination. The 'V' region of the L chain has V and J segments that can also be rearranged. These rearrangements produce a great variety of 'V' domains in the immunoglobulin of each B-cell. In other words, each B-cell has a unique ability to interact with a specific antigen, and it is this interaction that drives the process of B-cell differentiation in both the bone marrow and within the lymph node. Millions of such B-cells are formed each day and they express a unique B-cell receptor (BCR) capable of detecting a specific antigen. When a foreign antigen arrives, the B-cell with the unique receptor capable of binding to this specific antigen initiates the adaptive immune response that has a number of features. First of all, the cell is stimulated to proliferate to create a clone of cells all capable of detecting the foreign antigen. As each B-cell proliferates during this phase of clonal expansion, it can improve the affinity of the antibody they produce through the recombination processes mentioned earlier, together with a process of somatic hypermutation (SHM) to create receptors with a higher affinity. This is a risky strategy because these mutational events can give rise to B-cell clones expressing autoantigens, but there is a selection processes that eliminate such autoantigens while promoting the survival of those B-cells best capable of detecting the antigen. This process of proliferation and clonal selection occurs repeatedly as the cells proceed through the different stages of B-cell differentiation in the bone marrow and B-cell differentiation in the lymph node.

B-cell differentiation in the bone marrow

One of the earliest steps in B-cell differentiation is the expression of Igα (CD79a) and Igβ (CD79b) in the pro-B-cell (Module 8: Figure B cell differentiation). These two signalling components play an important role in providing the information to begin to express Igμ, which is one of the key components of the IgM components of the developing B-cell receptor (BCR). Initially, the two transmembrane Igμ chains are associated with surrogate light chains in the form of VpreB and λ5. At this stage of its development, the putative BCR is not able to respond to antigen, but it seems that the Igα, Igβ and Igμ complex is constitutively active to provide the signals necessary to drive the early development from the pre-B-cell to the immature B-cell stage (Module 8: Figure B-cell differentiation). This early signalling system has many of the components that are present in the mature B-cell receptor (Module 9: Figure B-cell activation). For example, the Src kinase Lyn is recruited to the complex where it phosphorylates the ITAMs on both Igα and Igβ to provide binding sites to recruit Syk, BLNK and PtdIns 3-kinase. The B-cell stimulatory co-receptor CD19 also appears at this stage (Module 8: Figure B-cell differentiation) and contributes to the activation of the PtdIns 3-kinase signalling pathway, which drives proliferation and prevents apoptosis. The development of the BCR signalling components is mirrored by the appearance of the B-cell inhibitory co-receptor CD22, which modulates BCR activation (Module 9: Figure B-cell activation).

The final stage of B-cell differentiation within the bone marrow is the conversion of pre-B-cells into immature B-cells, which is characterized by the formation of the B-cell receptor that depends on a light-chain rearrangement during which the surrogate light chains are replaced with the Igκ and Igλ light chains (Module 8: Figure B cell differentiation). It is during this transition that the emerging immature B-cell carries out its first immunological function in that it can discriminate between external antigen and self-antigen. Since the latter would lead to autoimmune diseases, such auto-reactive B cells have to be either removed or re-educated. Such auto-reactive B-cells usually stop further development, and in this arrested state they have time to alter the specificity of their IgM receptors to avoid responding to self antigens while maintaining an ability to detect foreign antigens. If this B-cell receptor editing process is successful, the level of the newly edited IgM increases and this is followed by the expression of IgD and the emergence of the transitional B-cells that leave the bone marrow and enter the circulation.

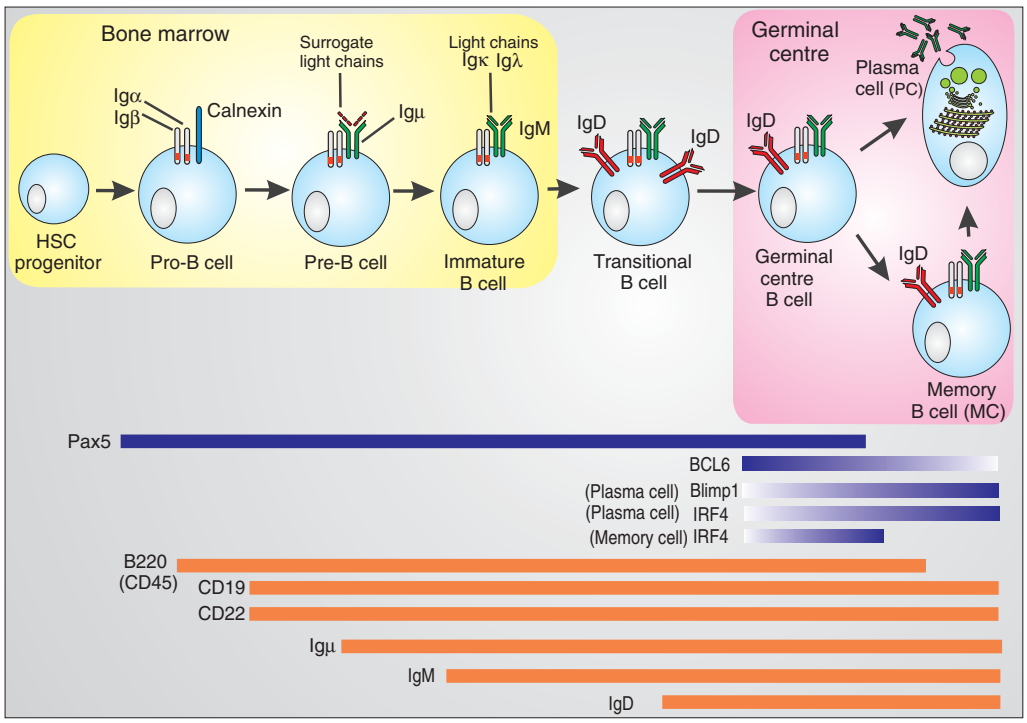
The next stage of B-cell differentiation in the lymph node occurs in the lymphoid organs, such as the lymph node, spleen, tonsils and Peyer's patches.

B cell differentiation in the lymph node

The lymph node creates a special environment where the T-cells and B-cells can interact with each other to complete the adaptive immune response. The primary function of the node is to provide a local environment where antigen can be presented to the somewhat rare antigen-specific B-cells. The following sequence traces out the final stages of B-cell differentiation within the lymph node (Module 8: Figure lymph node):

a. The antigens that enter the node through the afferent lymphatic vessels pass into the subscapular sinus (SCS) and then percolate through the outer ring of macrophages that trap the soluble and particulate

Module 8: | Figure B-cell differentiation



Differentiation of B-cells.
The haematopoietic stem cell (HSC) progenitors, which are formed within the bone marrow, are the precursors that differentiate into mature B-cells through a series of discrete steps that take place in different locations. The initial conversion of HSC progenitors to immature B-cells occurs in the bone marrow. The transitional B-cells leave the bone marrow and make their way to the germinal centres within the lymph nodes where differentiation to mature B-cells takes place. Passage through each step is carefully orchestrated by a signalling system that is based on the Igα and Igβ subunits, and gradually evolves by the addition of other signalling components to form the B-cell receptor (BCR) as part of the differentiation process. The time lines at the bottom illustrate the points where different components appear. See the text for further details.

antigens and function to present these antigens to the B-cells.

b. The B-cells are constantly circulating through the lymph node. The T-zone region has numerous high-endothelial venules (HEVs) through which the T-cells and B-cells enter and leave the lymph node. When the naïve B-cells enter the node they migrate into follicles where they reside for approximately 24 h. This temporary sojourn within the node provides the B-cells with ample time to survey the current circulating antigens. Their subsequent fate depends on whether or not they detect any of the antigens being presented by the macrophages. If no antigen is detected, the B-cells leave the follicle and circulate back in to the HEVs.

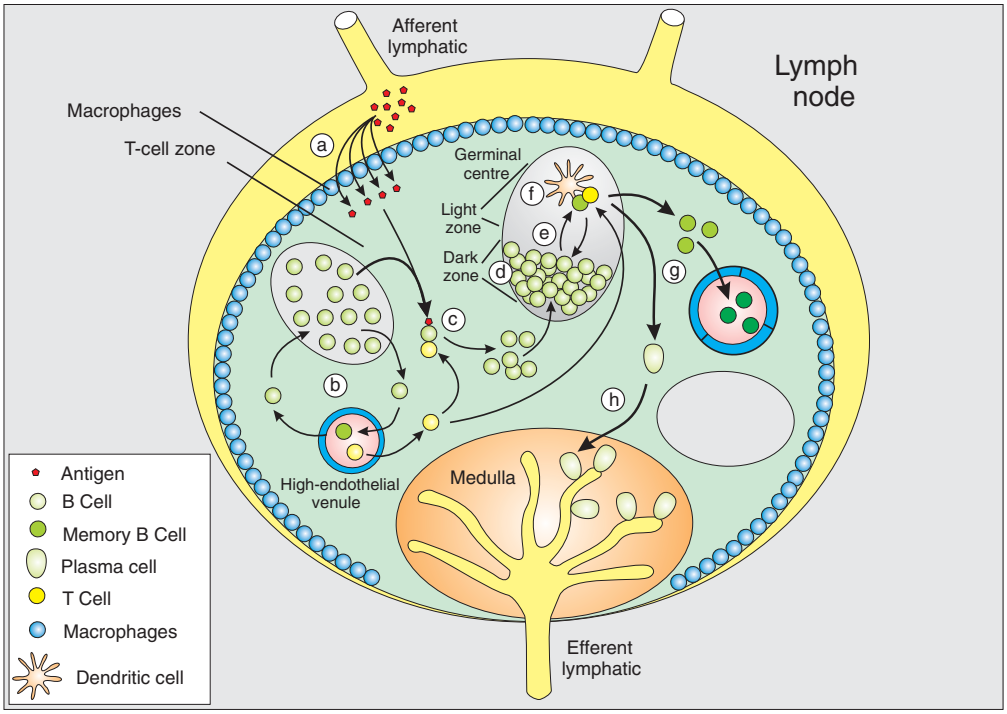
c. A different scenario unfolds if a specific antigen on the antigen-presenting cells (APCs) is detected. This initial interaction with the APCs begins to activate the B-cells and this process is enhanced by interaction with $CD4^+$ T helper cells (T_H cells). This results in the rapid expansion of a specific clone of germinal centre (GC)-precursor B-cells, some of which differentiate to short-lived plasma cells. The development of these plasma cells depends on the up-regulation of B-lymphocyte maturation protein 1 (BLIMP1) and Epstein–Barr virus-induced gene 2 [EBI2, which is also known as the orphan G protein-coupled receptor 183 (GPR183)]. However, the majority migrate into a follicle, which is transformed into a germinal centre (Module 8: Figure germinal centre).

As a result of the interaction with the B-cells, the T_H cells differentiate into follicular helper T-cells (T_{FH} cells) during which they express the chemokine receptor CXCR5 that enables them to migrate into the germinal centre where they participate in the final stages of B-cell maturation. In order to carry out its functions within the germinal centre, the T-FH cells express B cell lymphoma 6 (BCL-6), which appears to control the expression of molecules such as ICOS and the CD40L that define the T_{FH} cell phenotype as described below.

The germinal centre is a transient compartment located in lymphoid organs, such as the lymph node, where a subset of B-cells that are responsive to a particular antigen can be selected and amplified to create a clone of cells that are highly efficient in antigen detection. The germinal centre takes its name from the fact that the B-cells proliferate rapidly into centroblasts that form a dense population of cells within the dark zone.

d. During this rapid proliferative period, there is an enhanced period of genomic remodelling processes, such as somatic hypermutation (SHM) and class-switch recombination (CSR), that introduces a high rate of

Module 8: | Figure lymph node



Organization of a typical lymph node.
Lymph nodes provide a special environment where T-cells and B-cells can interact to carry out the adaptive immune response. See the text for details of the different processes.

mutation in the immunoglobulin variable region ('V' mentioned earlier) to generate modified antibodies that then undergo a selection process in the next phase of differentiation that occurs in the light zone (Module 8: Figure germinal centre).

This very fast rate of centroblast proliferation depends upon a marked up-regulation of genes that stimulate cell growth whereas the negative growth regulators, such as the cyclin-dependent kinase (CDK) inhibitor p21, are suppressed. These changes that allow for such rapid expansion is orchestrated by the transcription factor B cell lymphoma 6 (BCL-6) that is expressed specifically during the expansion of the centroblast clone. One of the functions of BCL-6 is to switch off the p53 tumour-suppressor gene, which would have been activated as a result of the mutational events associated with the SHM mentioned earlier to activate the processes of p53-induced cell cycle arrest and p53-induced apoptosis (Module 9: Figure cell cycle network).

In order to prevent telomere shortening and the loss of replicative potential during the very rapid clonal expansion of centroblasts, there is an increase in the expression of telomerase.

e. The B-cells in the dark zone that have acquired modified antibodies are known as centrocytes that then migrate into the light zone where the quality of their new antibody is assessed. If there is no improvement, the centrocytes can either be sent back to the dark zone to be re-educated by undergoing further rounds of SHM or these non-functional and auto-reactive B-cells are eliminated and removed by tingible body macrophages. The constant trafficking of cells between the dark and light zones is controlled by chemokine gradients (Module 8: Figure germinal centre). In the light zone, there is a source of CXC-chemokine ligand 12 (CXCL12) that acts through the CXCR4 receptor on the centroblasts to move them out of the dark zone, whereas a CXCL13 gradient emanating from the dark zone moves centrocytes that have CXCR5 receptors in the opposite direction.

f. The quality-control selection process, which occurs in the light zone, is carried out by both follicular dendritic cells (FDCs) and the follicular helper T-cells (T_{FH} cells) (Module 8: Figure B-cell maturation signalling). Information is passed between the three interacting cells through both juxtacrine and paracrine mechanisms. For example, FDCs use both their antigen receptor (IgG) and the complement system to capture antigen that is then presented to the B-cell to induce B-cell receptor (BCR) activation (Module 9: Figure B-cell activation). The signals generated by the BCR act to increase proliferation and survival. The immune synapse that forms between these two cells is held together by lymphocyte function-associated antigen 1 (LFA1) on the FDCs binding to the integrin intercellular adhesion molecule 1 (ICAM1). Dedicator of cytokinesis 8 (DOCK8) plays an important role in concentrating the ICAM1 in the immunological synapse and is essential for B-cell maturation. Loss-of-function mutations in DOCK8 have been linked to the immunodeficiency

variants of hyper-IgE syndromes (HIES) an autoimmune disease.

There are a number of interactions operating between the B-cells and the T-$_{FH}$ cells. Immune complexes, which are provided by the FDCs, are taken up and processed by the B-cells before being presented on MHCII molecules to activate the T-cell receptors on the T-$_{FH}$ cells (Module 8: Figure B cell maturation signalling). This TCR activation then stimulates the T-$_{FH}$ cells to provide a variety of signals to promote the survival, proliferation and maturation of the B-cells. For example, the activated T-$_{FH}$ cells begin to express the CD40L that activates the B-cell CD40 receptor that interacts with TRAF adaptors and signals through the nuclear factor κB (NF-κB) signalling pathway to help activate proliferation and to promote survival by increasing the expression of cellular FLICE-inhibitory protein (cFLIP). Another important juxtacrine mechanism is the expression of inducible costimulatory molecule (ICOS), which respond to the ICOS ligand (ICOSL) on the B-cell membrane. ICOS is coupled to the PtdIns 3-kinase signalling pathway to activate the transcription factor c-Maf that promotes the production of interleukin-4 (IL-4) and interleukin-21 (IL-21). The IL-4 and IL-21 are cytokines that are released from the T-$_{FH}$ cells and act on cytokine receptors on the B-cell where they activate the JAK/STAT signalling pathway that promotes proliferation. Finally, there is an important interaction between the signalling lymphocyte activation molecules (SLAMs) that are located on both the B-cells and T-$_{FH}$ cells (Module 8: Figure B-cell maturation signalling). The homophilic interactions between these SLAMs seem to be important in maintaining the functional adhesion between the two cell types. The SLAM embedded in the T-$_{FH}$ cell membrane recruits the SLAM-associated protein (SAM) that can recruit various signalling pathways by activating Fyn and this may contribute to the activation of cytokine formation.

If the developing B-cells fail to illicit the necessary survival and proliferative signals from the T-$_{FH}$ cells, as described above, they are induced to die through activation of the extrinsic pathway of apoptosis (Module 11: Figure TNFα apoptotic signalling). The cell surface of the T-$_{FH}$ cells contains the Fas ligand that can rapidly activate the death receptor Fas on the B-cells (Module 8: Figure B cell maturation signalling). The activity of Fas is switched off by cellular FLICE-inhibitory protein (cFLIP), whose expression is enhanced by the nuclear factor κB (NF-κB) signalling pathway activated by the CD40 receptors.

g. If the B-cells have improved their receptor affinity for antigen, they then go on to complete the process of differentiation to form either memory cells or plasma cells (Module 8: Figure B-cell differentiation), both of which can survive for many years. It is the up-regulation of B-lymphocyte maturation protein 1 (BLIMP1) and the decline in Pax5 that defines the development of the antibody secreting plasma cells (Module 8: Figure B-cell differentiation). On the other hand, the memory B-cells maintain Pax 5.

h. The plasma cells migrate into the medulla and take up positions on the medullary strands (Module 8: Figure lymph node).

Somatic hypermutation (SHM)

Somatic hypermutation (SHM) is the process that introduces single nucleotide exchanges into the rearranged immunoglobulin variable region ('V') during B-cell differentiation in the lymph node germinal centre (GC) (Module 8: Figure germinal centre). The mutational process depends on DNA strand breaks that is driven by activation-induced cytidine deaminase (AID), which deaminates the cytidines

CD40

CD40 is a co-stimulatory protein located primarily on antigen-presenting cells. It belongs to the TNF-receptor superfamily and interacts with the adaptor protein TNFR2 to form a signalling complex that includes adaptors such as TRAF1, TRAF2 and TRAF6. CD40 is activated through a juxtacrine mechanism in that it responds to the CD40 ligand (CD40L), which is also known as CD154, embedded in the membrane of neighbouring cells.

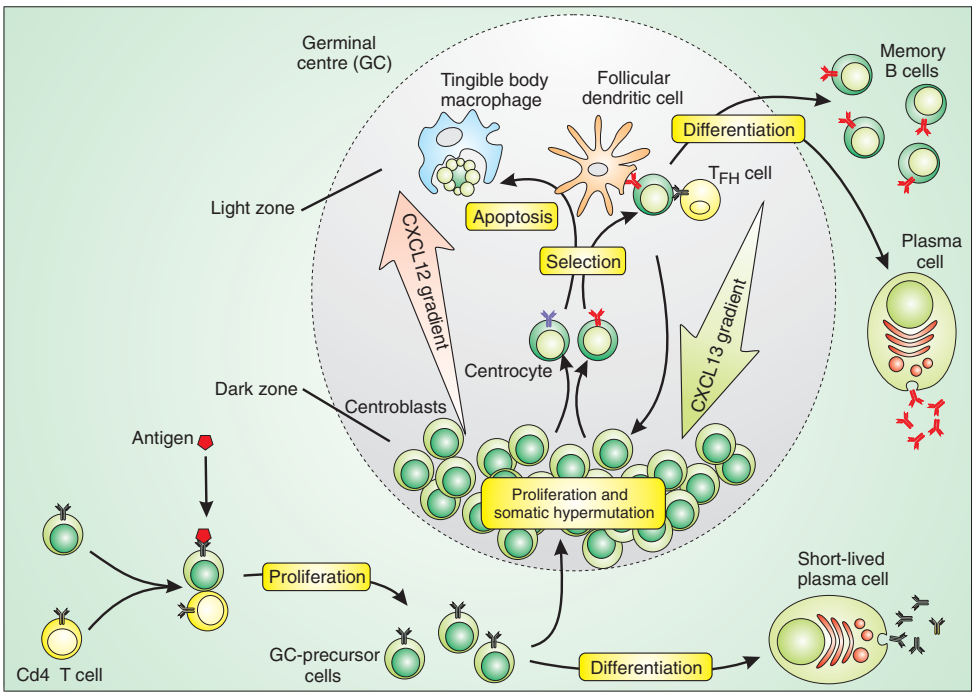
CD40 functions in a number of immune and inflammatory responses such as T-cell class switching and B-cell differentiation in the lymph node that occurs in the germinal centre (Module 8: Figure B-cell maturation signalling). CD40 has been implicated in Alzheimer's disease because it may play a role in amyloid-dependent microglial activation.

Differentiation of intestinal cells

The intestine, which appears during the late blastula to gastrula phase, is derived from the endoderm to form the foregut, midgut and hindgut. These three regions also give rise to other special regions, such as the thyroid, lungs, liver and pancreas. As the intestine and stomach develop, the epithelium folds to produce the crypts and villi. The former contains the proliferating stem cells that generate the progenitor cells that then differentiate into the intestinal cells as they migrate up the villi (Module 12: Figure colon cancer). These new cells constantly replace the cells at the tip that die by apoptosis and are sloughed off into the intestinal lumen. The extent of intestinal proliferation is enormous. It has been estimated that the human intestine produces approximately 100 billion cells every day. Each cell that emerges out of the crypt undergoes the orderly sequence of events that results in the formation of the intestinal cells.

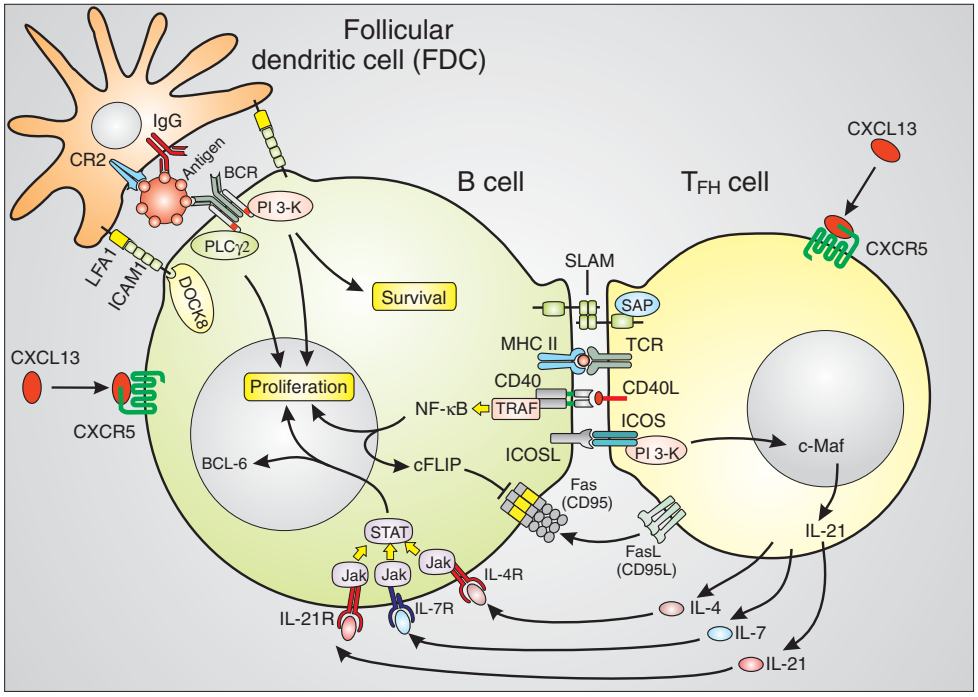
The splanchnic mesoderm that surrounds the intestine develops into the smooth muscle cells and connective tissue layers that surround the absorptive cells. Some of these connective tissue cells contribute growth factors such as the Wnts and transforming growth factor-β (TGF-β) that control the programme of intestinal cell development that goes on continuously during adult life. The Wnts acting through the Wnt signalling pathway (Module 2: Figure Wnt canonical pathway) control the proliferative events in the crypts, whereas TGF-β acts through the Smad signalling pathway (Module 2: Figure Smad signalling) to promote differentiation as cells move up the villi. Wnt

Module 8: | Figure germinal centre



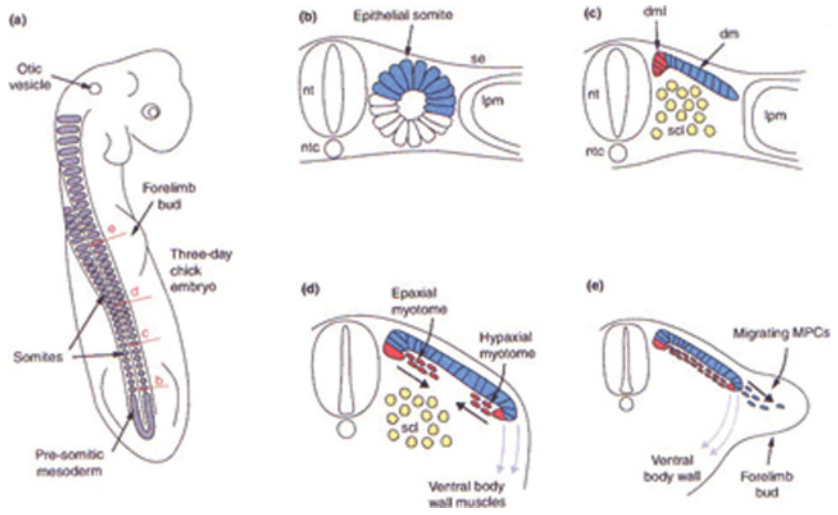
Organization of a germinal centre.
The lymph nodes contain germinal centres (Module 8: Figure lymph node) where the final steps of B-cell differentiation are carried out. See the text for details of the intricate interactions that occur between the B-cells, T_{FH} cells and the follicular dendritic cells.

Module 8: | Figure B cell maturation signalling



Affinity maturation of germinal centre B cells.
Affinity maturation of B-cells within the germinal centre (Module 8: Figure germinal centre) depends on interactions with both follicular dendritic cells and follicular helper T-cells (T_{FH} cells). Information is passed between these interacting cells through juxtacrine and paracrine mechanisms as described in the text. This Figure is based on Figure 2 in Vinuesa et al. (2009).

Module 8: | Figure chick myogenesis



Sites of myogenesis in the chick embryo.

a. Apart from the muscles in the head, all skeletal muscles arise from the somites. Panels b–e represent stages in the development of the muscle progenitors within these somites. b. A newly formed somite receives signals from the notochord (ntc), neural tube (nt) and lateral plate ectoderm (lpm), which are responsible for switching on the myogenic factors (e.g. MyoD, Myf-5) that begin to define the myogenic cell line. The dorsal cells (marked in blue) are destined to become the dermomyotome. c. A later stage somite, where a region of the dermomyotome (dm) has begun to differentiate into a dorsomedial lip (red), which expresses high levels of both MyoD and Myf-5. The ventral region of the somite forms the sclerotome that will begin to form vertebrae and ribs. d. Further on in development, cells at either end of the dermomyotome migrate out to form the epaxial and hypaxial myotome. The lateral region of the myotome continues to grow outwards, where it will form the muscles of the ventral body wall. e. In the region where the limb buds begin to form, muscle progenitor cells (MPCs) slough off from the hypaxial myotome and migrate into the ventral body and the limb bud, where they will begin to differentiate into skeletal muscle cells. Reproduced from *Curr. Opin. Cell Biol.*, Vol. 13, Bailey, P., Holowacz, T. and Lassar, A.B., The origin of skeletal muscle stem cells in the embryo and the adult, pp. 679–689. Copyright (2001), with permission from Elsevier; see Bailey et al. 2001.

activity declines as cells move away from the crypts and is replaced by TGF-β, which comes into play in the villus to switch off proliferation and to promote the differentiation of the intestinal cells. Mutations in the signalling pathways used by the Wnts and TGF-β feature prominently in the development of colorectal cancer (CRC).

Differentiation of skeletal muscle

The differentiation of skeletal muscle is a classical example of the final phases of development, where muscle progenitor cells begin to express their muscle phenotypes and are transformed into one of the specific skeletal muscle myofibres. To understand the various processes, it is necessary to consider both the location and the temporal events that occur during skeletal muscle embryology. The appearance of muscle fibres depends upon a process of skeletal muscle myogenesis during which myogenic regulatory factors (MRFs) initiate a transcriptional cascade that begins by converting the mesodermal precursor cells within the somite into myoblasts, which acquire a myogenic identity. Differentiation begins with a process of myoblast fusion to form the myotubes. The neural control of differentiation is responsible for activating the genes that determine the terminal differentiation process that culminates in the appearance of the different skeletal muscle phenotypes (slow- and fast-twitch myofibres). Skeletal muscle provides a good example of phenotypic remodelling of the signalsome, because these skeletal muscle phenotypes can switch from one to the other when provided with the appropriate stimuli. This skeletal myofibre conversion seems to depend upon changes in the Ca^{2+} signalling pathway.

Skeletal muscle embryology

The muscle precursor cells, which are derived from the mesoderm, first make their appearance in the somites, where they are transformed into myoblasts by signals emanating from neighbouring axial structures (the notochord and neural tube) and the ectoderm. These myoblasts continue to proliferate within the somite, where they congregate within the dermomyotome. As development proceeds, this dermomyotome segregates into the epaxial and hypaxial myotome. Once the population of myogenic cells has built up sufficiently, they then migrate out to different parts of the embryo, where they then differentiate into skeletal muscle fibres (Module 8: Figure chick myogenesis). It is during this embryological sequence that the process of skeletal muscle myogenesis occurs.

Skeletal muscle myogenesis

During this multistep developmental process, there is a progressive expression of sets of proteins such as the contractile proteins, metabolic enzymes, cell signalling components and transcription factors that define the different skeletal muscle phenotypes. There is a clearly defined sequence of events during which the final form of the functioning muscle gradually emerges (Steps 1–6 in Module 8: Figure skeletal muscle myogenesis):

1. Determination of a myogenic cell line. Mesodermal cells, which proliferate rapidly to form the somite, are

induced to become myoblasts by signals (e.g. the Wnts and Sonic Hedgehog) emanating from the axial tissues (notochord and neural tube) and from the ectoderm. These inducing signals activate transcription factors such as Pax3 and Pax7, which control the expression of myogenic regulatory factors (MRFs) such as MyoD and Myf-5. The Myf-5 and MyoD provide the myoblasts with their myogenic potential, which they retain as they continue to proliferate. Once sufficient cells have been set aside, the myoblasts stop proliferating and begin the first process of differentiation, which is myoblast fusion to form the multinucleated myotubes. The proliferation–differentiation switch is regulated by the inhibitor of DNA binding (Id) proteins, which regulate the activity of MyoD (Module 8: Figure proliferation–differentiation switch). The expression of MyoD within the myoblasts plays a critical role in controlling the balance between proliferation and differentiation (Module 4: Figure MyoD and muscle differentiation).

2. Initiation of the proliferation/differentiation switch is a critical developmental phase when the myoblasts stop proliferating and begin to develop a muscle phenotype. MyoD helps to orchestrate this transition by increasing the expression of miR-1 and miR-133, which have two separate roles. The miR-133 inhibits the serum-response factor (SRF) that functions to maintain myoblast proliferation. At the same time, miR-1 acts to inhibit histone deacetylase 4 (HDAC4) that initially prevents the onset of differentiation by inhibiting myocyte enhancer factor-2 (MEF2). Once proliferation ceases and the differentiation factors such as MEF2 are activated, the myoblast are converted into myotubes as the first step in skeletal muscle differentiation.

   This process of terminal differentiation begins with myoblast fusion, when small groups of myoblasts stop proliferating and fuse to form a multinucleated myotube. The inhibition of proliferation depends upon the up-regulation of cyclin-dependent kinase (CDK) inhibitors such as p16 and p21, which act to inhibit the cell cycle (Module 9: Figure cell cycle signalling mechanisms). These myotubes are then innervated by different types of motor neurons whose firing patterns then determine whether they differentiate into slow- or fast-twitch fibres. This next phase, which is terminal differentiation, depends upon the neural control of differentiation.
3. Terminal differentiation of slow-twitch fibres. Myofibres innervated by tonically active neurons have high sustained levels of Ca^{2+}, and this drives the calcineurin (CaN)/nuclear factor of activated T cells (NFAT) transcriptional cascade to induce the slow-twitch fibre phenotype.
4. Terminal differentiation of fast-twitch fibres. Myofibres innervated by phasically active neurons receive brief bursts of Ca^{2+}, which fails to activate the CaN/NFAT pathway, and results in the appearance of the fast-twitch fibres.
5. Slow- into fast-twitch fibre conversion. Following denervation or treatment with the calcineurin inhibitor cyclosporin A (CsA), the slow fibres switch to fast fibres.
6. Fast- into slow-twitch fibre conversion. If the neurons innervating fast-twitch fibres are stimulated at the rates of tonically active neurons, the fast-twitch fibres switch to become slow-twitch fibres.

Myoblast fusion

The terminal differentiation of skeletal muscle is represented by the fusion of the undifferentiated myoblasts to form the multinucleated skeletal muscle cell. A member of the Ca_V3 family of T-type channels carries out this fusion process. The undifferentiated myoblasts have a very low membrane potential, but they become fusion-competent as they begin to express K^+ channels. Firstly, they express *ether-a-go-go* ($K_v11.1$) followed by $K_{ir}2.1$, which causes the membrane potential to gradually hyperpolarize towards a potential of -65 mV. This potential allows the $Ca_V3.2$ T-type channel containing the α_{1H} subunit (Module 3: Table VOC classification) to open and to develop a 'window current', so-called because the channels are poised between activation and inactivation states, and can thus continue to generate a small but persistent inward flux of Ca^{2+} that is sufficient to trigger the fusion process.

Neural control of differentiation

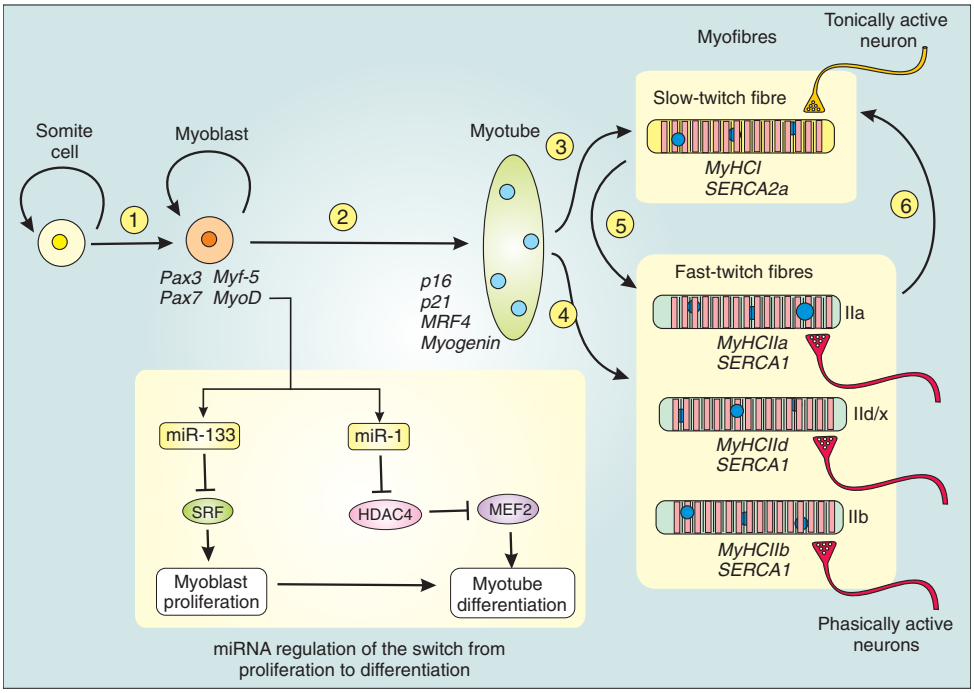
Terminal differentiation represents the final phase of development, resulting in the emergence of the different skeletal muscle phenotypes (Module 8: Figure skeletal muscle myogenesis). Some of the genes that define these phenotypes are summarized below:

- Type I: slow-twitch fibre (oxidative): myosin heavy chain I (MHCI); sarco/endo-plasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a); myoglobin; troponin I slow
- Type IIa: fast-twitch fibre (oxidative/glycolytic): MHCIIa; SERCA1
- Type IIx/d: fast-twitch fibre (glycolytic): MHCIId/x; SERCA1
- Type IIb: fast-twitch fibre (glycolytic): MHCIIb; SERCA1

The Type I and Type IIa phenotypes are strongly oxidative, and are thus well suited for prolonged muscle contraction. One of the most noticeable differences between these fibre types lies in the expression of the MHCs. However, there are many other components, such as those that define both the metabolic and signalling systems. These different phenotypes are defined by the nature of the neural input.

This neural control of differentiation is initiated by the neurons as they innervate the myotubes, which then translate the electrical signals they receive from the neurons into pulses of intracellular Ca^{2+}. The frequency of these Ca^{2+} pulses determines the muscle phenotype. Myofibres innervated by tonically active neurons become slow-twitch fibres, whereas phasically active neurons turn myofibres into fast-twitch fibres. The slow-twitch fibre phenotype is critically dependent on the calcineurin (CaN)/nuclear factor of activated T cells (NFAT) signalling pathway that appears to act together with myocyte enhancer factor-2 (MEF2) (Module 4: Figure NFAT activation), not only to

Module 8: | Figure skeletal muscle myogenesis



Developmental programme of skeletal muscle myogenesis.
The development of skeletal muscle myofibres from embryonic mesodermal cells within the somite provide the precursor cells that pass through a clearly defined sequence of events, during which there is progressive activation of muscle-specific genes (shown in italics at the bottom of the different stages). The different steps of myogenesis are described in the text.

induce this phenotype, but also to maintain it. A characteristic feature of this signalling pathway is its sensitivity to the temporal patterning of Ca^{2+} signals, and this is particularly evident in the way that NFAT accumulation in the nucleus responds to tonic but not phasic stimulation (Module 8: Figure nuclear import of NFAT). Canonical transient receptor potential 3 (TRPC3) is one of the proteins induced by the NFAT transcriptional cascade, and entry through this channel plays a role in maintaining NFAT within the nucleus.

The different muscle phenotypes can undergo skeletal myofibre conversion in response to external signals as part of an adaptation process.

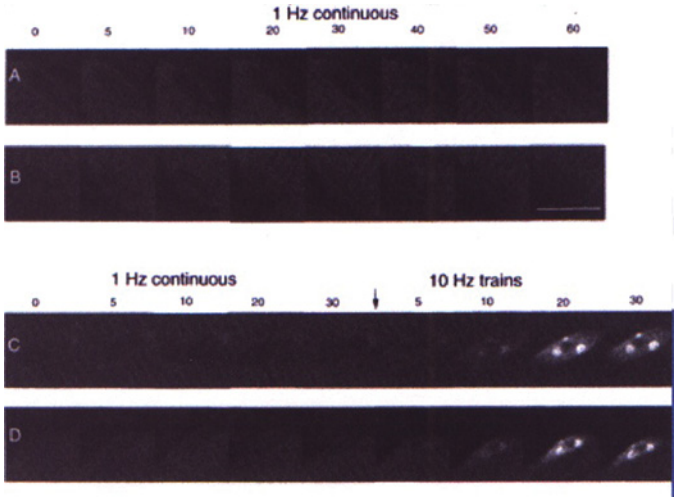
Skeletal myofibre conversion

The different skeletal muscle phenotypes can undergo myofibre conversions as part of an adaptive response to changes in load (e.g. exercise training) or hormonal shifts. It is possible to switch slow-twitch fibres into fast-twitch fibres and vice versa (Module 8: Figure skeletal muscle myogenesis). Such skeletal muscle fibre conversion is an example of how cells can alter their specific phenotype to adapt to changing conditions. In response to prolonged stimulation as occurs during intense exercise, there are changes in the expression of numerous genes resulting in an increase in mitochondrial biogenesis, oxidative capacity and changes in the contractile protein composition such that fast twitch type II fibres are switched to type I slow twitch fibres. The elevation in Ca^{2+} that drives contraction during intense exercise activates the transcription factor MEF2 that is normally inactivated by an inhibitory complex containing HDAC, Cabin 1 and SIN3 (Module 4: Figure MEF2 activation). If the high levels of Ca^{2+} are reduced, these slow-twitch fibres convert back into fast-twitch fibres and this conversion seems to depend, at least in part, on a decrease in the calcineurin (CaN)/nuclear factor of activated T cells (NFAT) signalling pathway, because a similar conversion can occur if mice are treated with the immunosuppressant drug cyclosporin A (CsA), which acts to inhibit calcineurin.

Differentiation of cardiac cells

Cardiac cells are some of the first functional cells to appear during development. A limited number of myogenic differentiation transcription factors such as serum-response factor (SRF), together with its coactivator myocardin, and myocyte enhancer factor 2 (MEF2) are responsible for activating the expression of the cardiac genes that emerge during the process of myoblast differentiation (Module 8: Figure cardiac development). Some of the earliest markers of the cardiac lineage are the Nkx2-5 gene and myocardin. In addition to activating cardiac-specific genes, these early transcription factors also activate the bicistronic miR-1 and miR-133 clusters, and these two miRNAs contribute to the process of differentiation by helping to suppress cardiomyocyte proliferation and to fine-tune the expression of many cardiac components such as those functioning in cardiac conduction. There are multiple loci responsible for encoding miR-1 (*miR-1-1* and *miR-1-2*) and miR-133 (*miR-133a-1*, *miR-133a-2* and *miR-133b*). The genes

Module 8: | Figure nuclear import of NFAT



Frequency-dependent nuclear import of nuclear factor of activated T cells (NFAT) in skeletal muscle fibres.
Fibres injected with nuclear factor of activated T cells (NFAT)/green fluorescent protein (GFP) were stimulated electrically at either 1 or 10 Hz. A and B. At 1 Hz stimulation, the NFAT/GFP, which is diffusely distributed throughout the fibre, is not evident. C and D. Fibres were stimulated for 30 min at 1 Hz and then for a further 30 min at 10 Hz. Following the faster stimulation, the NFAT/GFP gradually began to appear in the nucleus, where it was concentrated in distinct foci. In separate experiments, the half-time ($t_{½}$) for import was approximately 15 min, whereas the export was very much slower, with a $t_{½}$ value of 90 min. For another example of how varying the frequency can alter the nuclear transcription of NFAT, see Module 6: Figure NFAT nuclear translocation. Reproduced from *The Journal of Cell Biology*, 2001, vol. 155, pp. 27–40, by copyright permission of The Rockefeller University Press; see Liu et al. 2001.

located at these different loci are highly related and genetically redundant and will thus be referred to as miR-1 and miR-133 for simplicity. The organization of the *miR-1-1/miR-133a-2* cluster is illustrated in Module 8: Figure cardiac development. The myogenic transcription factors act at both upstream and intronic enhancers to increase the expression of these two miRNAs that contribute to the emergence of the cardiac phenotype. One of the actions of miR-1 is to operate in a feed-forward regulatory loop to inhibit histone deacetylase 4 (HDAC4), which is a transcriptional repressor of MEF2.

Both miR-1 and miR-133 help to reduce cardiomyocyte proliferation, with miR-1 acting to inhibit translation of the heart and neural crest derivative-2 (Hand2) protein that promotes proliferation of the early myocyte population. The miR-133 also inhibits proliferation by reducing the expression of cyclin D, which has a critical role in activating the cell cycle (Module 9: Figure proliferation signalling network).

Cardiac microRNAs also play a role in regulating development of the conduction system, which consists of numerous ion channels. For example, miR-1 regulates the gap junction protein connexin 43 (Module 8: Figure cardiac development), which provides the ionic communication necessary for the action potential to spread throughout the heart to drive each contraction. Expression of $K_{ir}2.1$, which provides the I_{K1} current that holds the cardiac resting potential in its hyperpolarized state (Module 7: Figure cardiac action potential), and the HCN2 and HCN4 members of the hyperpolarizing-activated cyclic nucleotide-gated (HCN) channel family, is also regulated by miR-1. The miR-133 also plays a role in the development of the conduction system by modulating the expression of the HCN2 and the $K_v11.1$ channel [also known as the human *ether-a-go-go* (hERG) channel].

Differentiation of keratinocytes

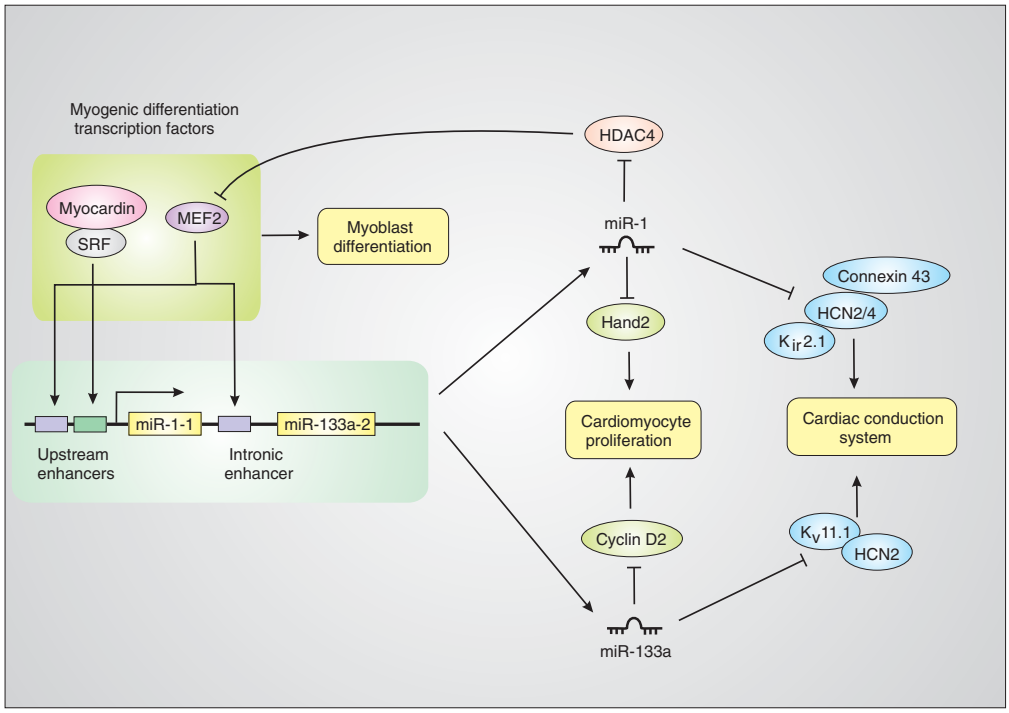
Extracellular Ca^{2+} plays a critical role in the differentiation of keratinocytes. There is an extracellular gradient of Ca^{2+} that is low in the basal epidermis, but then increases approximately 4-fold towards the periphery. This increase in Ca^{2+} may play a role in stimulating keratinocyte differentiation.

Differentiation of smooth muscle

Smooth muscle cells (SMCs) are unique among the muscle cells in that they are able to switch rapidly between differentiation and proliferation. Smooth muscle cell proliferation plays an important role in wound healing, but also occurs during vascular remodelling, which can have serious pathophysiological consequences when it leads to pulmonary vasoconstriction and hypertension (Module 9: Figure SMC proliferation). Proliferation is activated by Ca^{2+} and by the MAP kinase signalling pathways (Module 8: Figure smooth muscle cell differentiation). In the latter case, MAP kinase acts through ERK1/2 to phosphorylate the E twenty-six (ETS) transcription factor Elk-1 (Module 4: Figure ETS), which then begins to activate the genes responsible for proliferation. The Krüppel-like factor 4 (Klf4) also plays a role in stimulating proliferation and it also acts to inhibit myocardin, which is a major SMC differentiation factor.

A key component of the proliferation/differentiation switch is an increase in the level of serum-response factor (SRF) that binds to the CarG box, where it recruits the transcriptional coactivator myocardin and this

Module 8: | Figure cardiac development



Development of cardiac cells.
The myogenic differentiation transcription factors serum-response factor (SRF), myocyte-enhancer factor 2 (MEF2) and MyoD activate the expression of the cardiac genes that emerge during the process of myoblast differentiation. These transcription factors also activate the miR-1 and miR-133 clusters and these two miRNAs contribute to the process of differentiation by helping to suppress cardiomyocyte proliferation and to fine-tune the expression of many cardiac components such as those functioning in cardiac conduction.

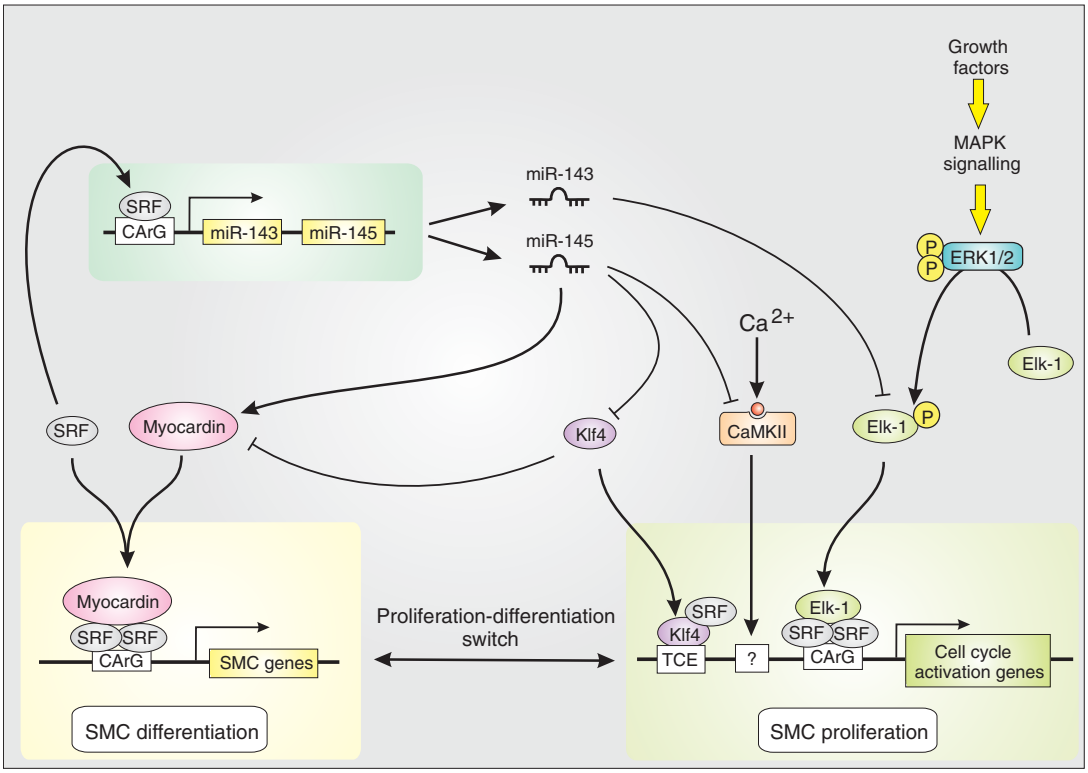
complex is responsible for the expression of SMC genes. The switch to differentiation coincides with a cessation of proliferation and this process is orchestrated by SRF-induced activation of the miR-143/miR-145 cluster, which act to inhibit many of the components that promote proliferation (Module 8: Figure smooth muscle cell differentiation). The miR-143 inhibits the stimulatory action of Elk-1, whereas miR-145 enhances the activity of the differentiation factor myocardin while simultaneously removing the inhibitory action of Klf4. Inhibition of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) also reduces the Ca^{2+}-dependent activation of proliferation.

Differentiation of neurons

Development of the brain proceeds through a number of distinct phases that begin early in development and continue for a number of months after birth (Module 8: Figure brain development). First, the embryonic cells, which are set aside to form the brain, begin to proliferate rapidly to form a large population of neuronal precursors. In the next step, these neuronal progenitors begin to differentiate into the specific neuronal cell types that constitute the fully functional neuronal circuits.

An important consequence of this differentiation process is the expression of different neurotransmitters, which will define neuronal functions such as the emergence of either excitatory or inhibitory neurons. The specification seems to be determined by the generation of spontaneous Ca^{2+} transients. All the cells become active and those with the highest oscillation frequency act as pacemakers that then recruit neighbouring cells giving rise to a Ca^{2+} wave that creates oscillating hubs of cells. Studies on embryonic *Xenopus* neurons revealed that the frequency of spontaneous Ca^{2+} transients determines the specification of neurotransmitter expression. Neurons that have low frequency oscillations express excitatory transmitters such as acetylcholine (ACh) and glutamate, whereas those that have more frequent Ca^{2+} transients express inhibitory transmitters such as glycine and γ-aminobutyric acid (GABA). This differential activation of neurotransmitter genes is an example of how information is transmitted through the encoding and decoding of Ca^{2+} oscillations. It is likely, therefore, that the pacemaker neurons in the centre of these hubs will become inhibitory neurons (red cells), whereas the majority of cells in the hub periphery with the slower oscillations will differentiate into excitatory (green cells) neurons (Module 8: Figure brain development). Such a mechanism is consistent with the fact that in most neuronal circuits there are many more excitatory neurons compared to the inhibitory neurons (Module 10: Figure network gamma oscillations). A unique feature of these circuits is that each inhibitory neuron controls the activity of many excitatory neurons all of which send axon collaterals back to the inhibitory neuron. This anatomical arrangement is a central feature of the network gamma oscillatory mechanism responsible for setting up fast gamma oscillations.

Module 8: | Figure smooth muscle cell differentiation



Control of smooth muscle differentiation.
Smooth muscle cells (SMCs) are highly plastic in that they can readily switch from a highly differentiated contractile smooth muscle phenotype to a more fibroblast-like proliferative phenotype. Serum-response factor (SRF), working together with the miR-143/miR-145 cluster, controls this proliferation/differentiation switch.

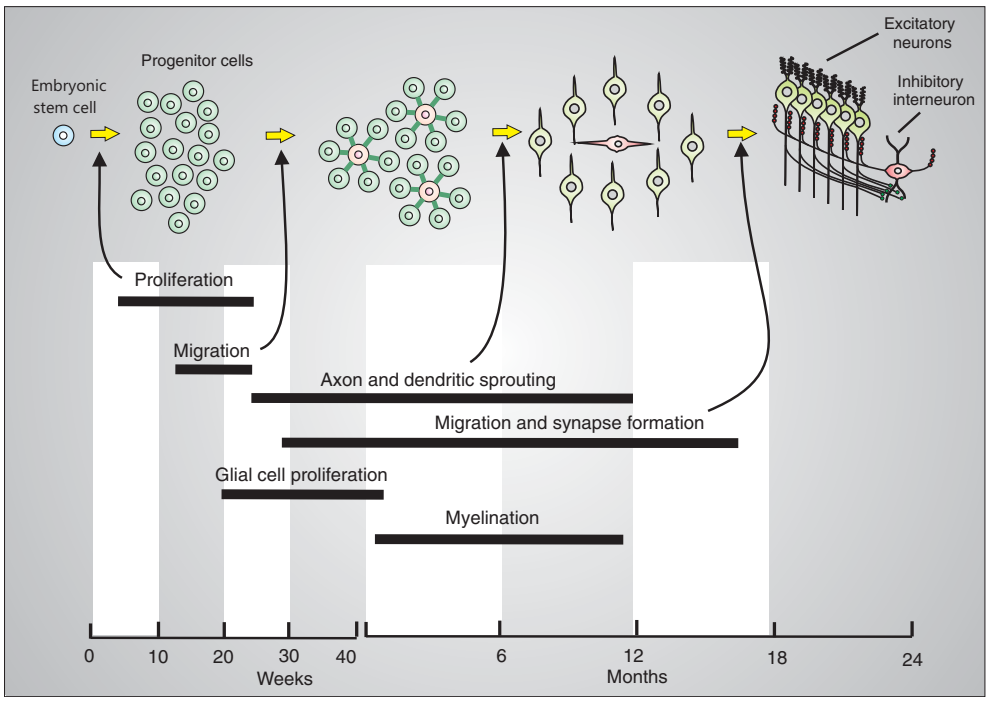
As the neuronal progenitors begin to differentiate, they migrate and are guided by the radial glial cells to their final positions where synapse formation begins to constitute the fully functional neuronal circuits.

Differentiation of white fat cells

White fat cells are derived from mesenchymal stem cells (MSCs) (Module 8: Figure MSC differentiation). Adipogenesis of white fat cells is driven by a transcriptional cascade that depends on two main transcription factors. Some of the earliest genes to be activated belong to the CCAAT/enhancer-binding protein (C/EBP) family, which set the stage for the activation of the transcription factor peroxisome-proliferator-activated receptor γ (PPARγ). A number of stimulatory and inhibitory signalling pathways are responsible for orchestrating this transcriptional cascade as outlined in (Module 8: Figure white fat cell differentiation):

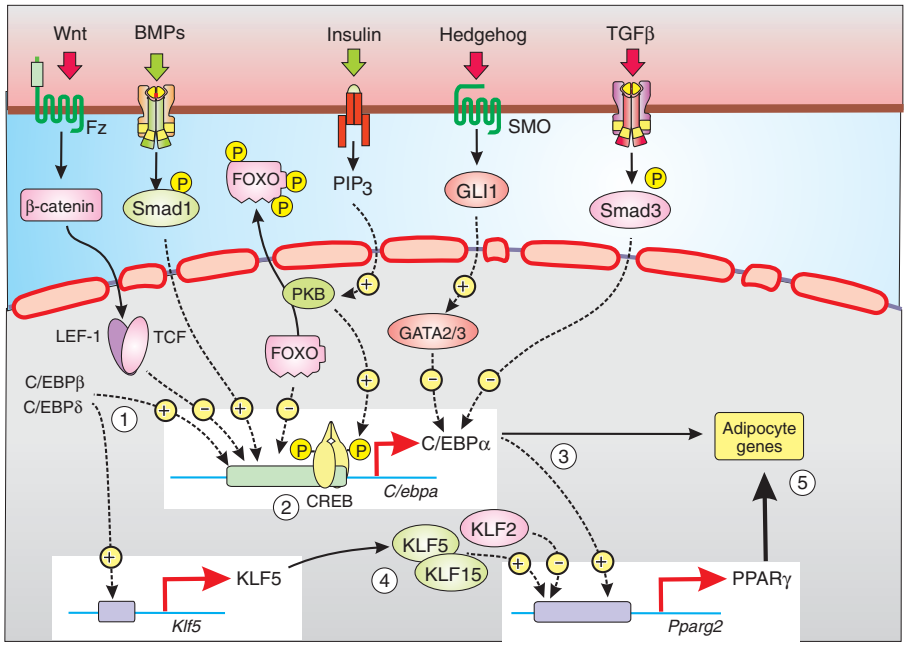
1. The CCAAT/enhancer-binding protein (C/EBP) family members C/EBPβ and C/EBPδ appear early in the cascade and are one of the main signals responsible for initiating the cascade of transcriptional events that drive differentiation. They act to induce the transcription of C/EBPα and KPF5, which is one of the Krüppel-like factors (KLFs).
2. The inhibitory influence of the canonical Wnt/β-catenin pathway, which blocks the differentiation of white fat cells, has to be removed in order for differentiation to proceed. Bone morphogenetic protein (BMP), which is a major inducer of differentiation, acts through the Smad signalling mechanism to phosphorylate Smad1 that enters the nucleus to induce the transcription of C/EBPα. The forkhead box O (FOXO) transcription factors FOXO1 and FOXOA2, which are resident nuclear factors that suppress transcription, are removed through the action of insulin acting through the PtdIns 3-kinase signalling pathway.
3. The expression of C/EBPα then continues the transcriptional cascade by activating the *Pparg* gene that codes for peroxisome-proliferator-activated receptor γ (PPARγ).
4. Expression of the *Pparg* gene is also controlled by a cascade of Krüppel-like factors (KLFs). KLF2 is anti-adipogenic but is replaced by the stimulatory KLF5 isoform, which is induced early by C/EBPβ and C/EBPδ (see step 1). KLF15 may then replace KLF5 in order to stabilize the differentiated state.
5. The peroxisome-proliferator-activated receptor γ (PPARγ) transcription factor is responsible for switching on the expression of the adipocyte genes that function in the mature white fat cell, such as those for fatty acid-binding protein, phosphoenolpyruvate carboxykinase (PEPCK), lipoprotein lipase and glycerol kinase (Module 4: Figure PPARγ activation). The activity of

Module 8: | Figure brain development



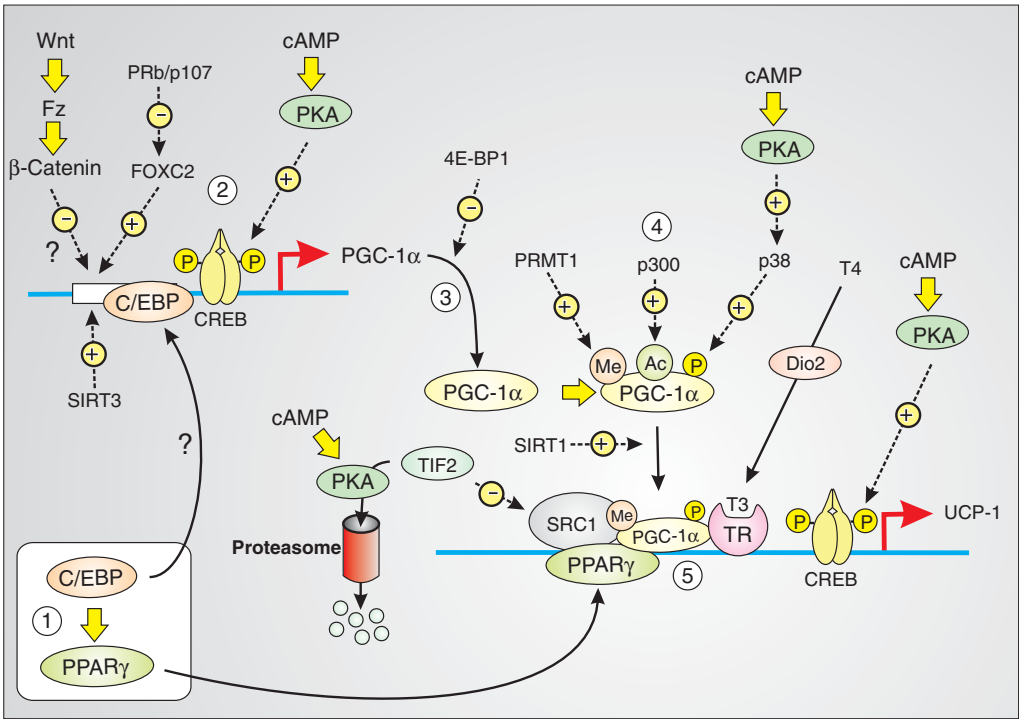
Stages of brain development.
Brain development begins within a few weeks when embryonic cells begin to proliferate to generate the large numbers of progenitor cells. These progenitors become connected together through gap junctions and begin to generate Ca^{2+} signals that initiate at a pacemaker cell and then spread out to their neighbours to produce large numbers of hubs. Neuronal precursor cells within these hubs then differentiate into excitatory (green cells) and inhibitory neurons (red cells) as evident by the axonal and dendritic sprouting. These neurons then begin to form synapses with each other to form recognizable neural circuits. Some of the information used to draw this Figure was obtained from de Graaf-Peters and Hadders-Algra (2006).

Module 8: | Figure white fat cell differentiation



Control of white fat cell differentiation.
The differentiation of white preadipocytes into white fat cells depends on a sequence of gene transcriptional events that are controlled by a number of stimulatory and inhibitory stimuli. See text for further details.

Module 8: | Figure brown fat cell differentiation



Differentiation of brown fat cells.
Summary of some of the main signalling events that control the differentiation of brown fat cells, which is defined by the expression of the uncoupling protein-1 (UCP-1). Differentiation proceeds through a cascade of transcriptional events that are described in more detail in the text. Based on information contained in Hansen and Kristiansen (2006).

PPARγ has to be maintained otherwise the white fat cell will begin to de-differentiate.

A contributory factor to the onset of obesity is an increase in the white fat cell population. These new cells appear when an excess of dietary fatty acids begin to activate fibroblast-like pre-adipose cells to initiate the same PPARγ-dependent white fat cell differentiation programme. An increase in the number of white fat cells then greatly enhances the ability of the body to store fat.

Differentiation of brown fat cells

Brown fat cells, which function in non-shivering thermogenesis (Module 7: Figure brown fat cell), have a similar lineage to that of the white fat cells (Module 8: Figure MSC differentiation). The brown fat cells differentiate early during foetal development and are fully functional at birth but begin to decline during infancy and are retained in a few isolated areas in adults. Both white and brown cells have similar patterns of gene expression during the early process of differentiation but they then diverge into their distinctive cell types. One of the major differences is the expression of uncoupling protein-1 (UCP-1), which defines the brown fat cell phenotype. As for other cell types, the process of brown cell differentiation depends on a sequential series of transcriptional events that result in the emergence of the distinctive genes such as UCP-1 that define the brown cell phenotype (Module 8: brown fat cell differentiation):

1. Some of the earliest genes to be activated, which are similar to those found during the differentiation of white fat cells (Module 8: Figure white fat cell differentiation), belong to the CCAAT/enhancer-binding protein (C/EBP) family, which initiate the transcriptional cascade by activating some of the early transcription factors such peroxisome-proliferator-activated receptor γ (PPARγ). Just how the different C/EBP isoforms function during the early events are still uncertain in brown fat cell differentiation but it would seem likely that they may play a role in inducing the transcription of peroxisome-proliferator-activated receptor γ (PPARγ) coactivator-1α (PGC-1α), which plays a key role in controlling some of the later events.
2. Apart from a possible role for C/EBP, little is known about how the transcription of this PGC-1α gene is regulated. In keeping with other cell types, there may be a role for the transcription factor CREB, which is activated by the cyclic AMP signalling pathway. In addition, the expression of PGC-1α seems to be inhibited by the canonical Wnt/β-catenin pathway.
3. Translation of the PGC-1α mRNA into protein is regulated by 4E (eIF4E)-binding protein (4E-BP1), which is a translation initiation factor that regulates protein synthesis (Module 9: Figure target of rapamycin signalling).

4. The role of PGC-1α as one of the main transcriptional regulators of UCP-1 expression depends on a number of post-translational modifications (Module 8: brown fat cell differentiation). First, protein methylation on arginine residues by protein arginine methyltransferase 1 (PRMT1) can enhance the ability of PGC-1α to activate UCP-1 transcription. Secondly, protein acetylation on multiple lysine residues by p300 reduces the activity of PGC-1α. This acetylation can be reversed by sirtuin 1 (SIRT1), which is a member of the sirtuin family of deacetylases. Finally, three amino acid residues are phosphorylated by the p38 pathway, which in turn is activated by the cyclic AMP signalling pathway.
5. The modified PGC-1α binds to the UCP-1 promotor region where it helps to orchestrate transcription by interacting as a cofactor for both PPARγ and the thyroid hormone receptor (TR), which is one of the nuclear receptors. The enzyme type 2 deiodinase (Dio2) converts thyroxine (T4) into the active 3,5,3'-tri-iodothyronine (T3) that functions to activate TR. The transcription factor CREB, which is regulated by the cyclic AMP signalling pathway, also plays a major role in switching on the expression of UCP-1. PGC-1α also has a role in recruiting members of the p160 family of nuclear receptor co-activators such as p300, SRC-1 and transcriptional intermediary factor 2 (TIF2). SRC-1 facilitates transcription through its histone acetylase activity. This SRC1 function is inhibited by TIF2 and one of the actions of the cyclic AMP signalling pathway is to facilitate removal of this inhibitory effect by phosphorylating and consigning TIF2 to destruction by the proteasome.

Since the cyclic AMP signalling pathway controls many of the transcriptional events that occur during the later stages of brown fat cell differentiation, it is clear that expression of the toolkit components of this pathway must be put in place early in the differentiation programme. As yet there seems to be little information on how or when this pathway develops.

De-differentiation

De-differentiation is a process whereby cells lose there differentiated state and begin to revert back towards a simpler stem cell-like state.

- If the peroxisome-proliferator-activated receptor γ (PPARγ) transcription factor that drives the differentiation of white fat cells (Module 8: Figure white fat cell differentiation) is inhibited, the cell lose there adipocyte markers and their lipid store.
- During the onset of heart disease, there is a remodelling of the cardiac signalsome such that the ventricular cells lose adult genes and begin to express foetal genes (Module 12: Figure physiological and pathological hypertrophy).

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