Size Control in Animal Development

Review

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"The most obvious differences between different animals are differences in size, but for some reason the zoologists have paid singularly little attention to them."

—J. B. S. Haldane, *On Being the Right Size*, 1927

Some proclaim that the major principles of development are now understood (Wolpert, 1996). Yet one of the most fundamental aspects of development remains as mysterious as ever—how the size of an animal or plant is determined. How is it, for example, that we grow to be larger than mice and our arms grow to be the same length? Although it is clear that genes play the predominant part in determining size, it is largely unknown how they do so. Considering the importance of size control, it is remarkable that so little attention has been paid to it.

The size of an animal, organ, or appendage depends on the number and size of the cells it contains as well as on the amount of extracellular matrix and fluid. We shall ignore these extracellular components in this review and consider only the controls that regulate cell size and cell numbers, as it is mainly these that determine animal size. We begin by listing some basic observations on size control in animals. We then consider the cell behaviors that principally determine animal size—cell growth, cell death, and cell proliferation. Finally, we discuss how animals with abnormally large or small cells can adjust their cell numbers to grow to a normal size.

Observations

The zygote initially divides without growth, using macromolecules laid down in the egg. Once zygotic transcription starts, and cells start to grow and divide, embryonic growth begins.

The parts of an animal are usually first formed in miniature and then enlarge by cell growth and cell division. Whereas most animals, including mammals and birds, stop growing at some point in development, some fish and invertebrates continue to grow throughout life.

Animals and their organs seem to know roughly what total cell mass they should have, rather than how many cells they should contain. The cells in a tetraploid salamander, for example, are twice the size of those in a diploid salamander (see below), yet the corresponding organs in the two animals are the same size because the tetraploids contain half as many cells as the diploids

(Fankhauser, 1952). Thus, the central question of size control is how total cell mass is determined.

Both local and systemic controls determine organ size, but their relative importance can vary greatly. If multiple fetal thymus glands are transplanted into a developing mouse, each grows to its normal adult size, suggesting that their growth is mainly controlled by factors within the thymus (Metcalf, 1963). If the same experiment is performed with fetal spleens, the total mass of the transplanted spleens attains the mass of one normal adult spleen, suggesting that their growth is mainly controlled by factors outside the spleen (Metcalf, 1964). Similar transplantation experiments, as well as tissue culture experiments, indicate that most animal organs grow to a characteristic size under autonomous controls (Goss, 1978; Bryant and Simpson, 1984). Systemic factors such as hormones, however, influence final organ size. Growth hormone (GH), for instance, which the pituitary gland secretes under the control of the hypothalamus, plays a major part in stimulating postnatal mammalian growth: children deficient in GH become dwarfs, whereas those with excessive GH become giants. GH stimulates growth largely by inducing the liver and other organs to produce insulin-like growth factor 1 (IGF1) (Heyner and Garside, 1994, and see below).

Although genes largely determine the size of an animal or organ, environmental factors such as nutrition or functional load also play a part. During development, for example, both animals deprived of adequate nourishment and muscles deprived of tension end up smaller than normal.

Differences in size between animals of the same or different species can reflect differences in cell size, cell number, or both. Cell numbers depend on both cell division and cell death, which, like cell size, depend on both intracellular programs and extracellular signaling molecules that regulate these programs. The signaling molecules can be soluble, cell-bound, or part of the extracellular matrix, and they can act in an endocrine, paracrine, or autocrine mode. They may regulate cell growth, cell division, or cell survival, or some combination of these. Their concentrations are crucial and depend on the amount of the molecule produced and the rate at which it is destroyed; the destruction often occurs in the target cell the molecule acts on. The concentration in vivo is usually limiting, as experimentally increasing it generally leads to a change in the behavior of the responsive cells.

The challenge is to explain how the intracellular programs and extracellular signals work together to determine total cell mass and to coordinate growth in different parts of the animal. We first consider cell growth, as it is this that ultimately limits animal growth; without it, total cell mass cannot increase. Inexplicably, compared to cell division and cell death, animal cell growth has hardly been studied.

Cell Growth

Most proliferating cells grow (i.e., increase their mass and enlarge) and duplicate their contents before they

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divide. (For this reason, the terms cell growth and cell proliferation are often used interchangeably, which is confusing and often misleading.) The intracellular control system that coordinates events of the cell division cycle monitors cell growth and halts progression through the cycle at specific checkpoints in G1 or G2 if the cell has not grown to a minimal threshold size (Nurse, 1985; Gao and Raff, 1997), which is characteristic for each cell type. Thus, cell proliferation usually depends on cell growth. The molecular bases of these cell size checkpoints are unknown, and it remains uncertain how a cell coordinates its growth with cell division. One possibility is that the amount of one or more components of the cell cycle control system, such as a cyclin, serves as a marker of cell size and has to reach a threshold value for the cell to pass through the checkpoint (Polymenis and Schmidt, 1997).

Cell growth, however, does not depend on progression through the cell division cycle, as mutations that block cell cycle progression tend not to arrest cell growth (Johnston et al., 1977; Weigmann et al., 1997; Neufeld et al., 1998). Moreover, some cells, such as nerve and muscle cells, grow mainly after they have permanently withdrawn from the cell cycle and dismantled their cell cycle control system; others, such as *Drosophila* imaginal disc cells, grow before they enter the cell cycle (Madhavan and Schneiderman, 1977).

The growth of animal cells, whether proliferating or not, usually depends on extracellular growth factors produced by other cells. For conceptual clarity we shall distinguish between growth factors, which stimulate cell growth, and mitogens, which stimulate cell cycle progression, even though some signaling molecules can do both (Zetterberg et al., 1984); unfortunately, the two responses are rarely studied separately in proliferating cells. If cell growth is limiting for cell cycle progression, an increase in the concentration of a growth factor will accelerate progress through the cycle, even if the factor has no mitogenic activity on its own.

Growth factors activate intracellular signaling pathways that stimulate protein synthesis and other biosynthetic processes in the cell, so that the rate of macromolecular synthesis exceeds the rate of macromolecule degradation. One of the most important of these pathways operates through phosphatidylinositol (PI) 3 kinase, which activates a branched serine/threonine kinase cascade, one arm of which increases protein synthesis (Figure 1A) (Thomas and Hall, 1997; Vanhaesebroeck et al., 1997; Downward, 1998). Growth factors such as IGF1 that activate the PI3 kinase pathway in vertebrate cells stimulate both cell growth and cell survival and, in some circumstances, cell proliferation as well (see above). Receptors for IGF1-like signals activate the same pathway in *Drosophila* cells (Chen et al., 1996). If the pathway is genetically impaired in a developing fly or one of its imaginal discs, the fly or organ that develops is abnormally small because it contains fewer and smaller cells than normal (Chen et al., 1996; Leevers et al., 1996). Conversely, if the pathway is constitutively active in an imaginal disc, the organ grows to be abnormally large because it contains more and larger cells than normal (Leevers et al., 1996).

Even in adult animals, some postmitotic cells alter their size in response to changes in extracellular growth factors. Sympathetic neurons in adult rodents enlarge if the level of nerve growth factor (NGF) is experimentally increased and shrink if the level is decreased, indicating that the cells adjust their size according to the availability of NGF (Purves et al., 1988). NGF is produced by the tissues that the sympathetic neurons innervate, so that the neurons automatically adjust their size to match the size of the target tissue. In addition to stimulating cell growth by enhancing protein synthesis, NGF maintains the size of sympathetic neurons by ensuring that protein synthesis and degradation are tightly coupled: if cycloheximide treatment is used to block protein synthesis in the cells, the degradation of long-lived proteins ceases as well, and so cell size is maintained, but only if NGF is present; without NGF, cycloheximide treatment inhibits protein synthesis but leaves protein degradation unaffected, and so the cells atrophy (Franklin and Johnson, 1998). How NGF couples protein degradation to protein synthesis is unknown.

In terms of understanding how total cell mass is determined, the mechanisms that stop cell growth during development are as important as those that stimulate it. Yet the stopping mechanisms remain largely unexplored. Even with saturating amounts of growth factors and nutrients, there is a limit to how large a cell can grow, which is determined by the genes the cell expresses. The intracellular mechanisms that limit net biosynthesis, and thereby limit cell growth and maximum cell size, are unknown.

Sympathetic nerve cells grow to their large size without increasing their DNA content. Some cells, however, increase their DNA content in order to grow large enough to function effectively. Myoblasts fuse to form giant multinucleate skeletal muscle fibers. Many hepatocytes undergo mitosis without cytokinesis to become multinucleate. Many myocardial cells go through an extra round of DNA replication without undergoing mitosis, so that they contain a 4N complement of DNA within a single nucleus. Many cells in a *Drosophila* larva use an exaggerated form of the same strategy to grow very large, going through repetitive rounds of DNA synthesis without intervening mitoses. Oocytes grow to their large size while arrested in prophase of the first meiotic division, with two copies of the diploid set of chromosomes; some, in addition, amplify their ribosomal RNA genes.

The relationship between nuclear DNA and cell size was first discovered at the beginning of this century, when it was found that the size of a cell is proportional to its ploidy: a tetraploid cell, for example, is twice the size of a diploid cell of the same type. As the tetraploid cell would be expected to have twice the number of ribosomes and twice the biosynthetic capacity of the diploid cell, this fundamental relationship between ploidy and cell size is not surprising, and it holds for all organisms that have been examined (Nurse, 1985).

As mentioned earlier, the relationship between ploidy and size does not extend to the size of the whole animal or its organs, as polyploid animals usually compensate and have correspondingly fewer cells than the corresponding diploid animals (Fankhauser, 1952; Henery et al., 1992). A similar ability to compensate for an abnormality in cell size is seen in *Drosophila* imaginal discs, where the unit of size control during development is a compartment (Garcia-Bellido et al., 1973). If the cells in
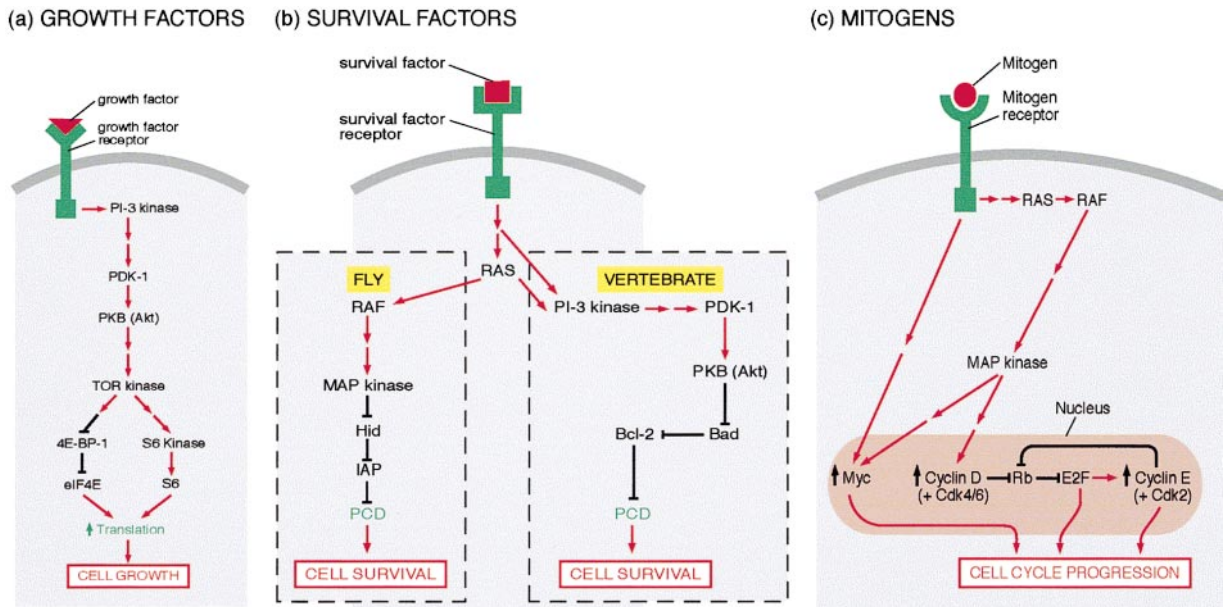
(a) GROWTH FACTORS

growth factor
growth factor receptor

PI-3 kinase
PDK-1
PKB (Akt)
TOR kinase
4E-BP-1   S6 Kinase
eIF4E   S6
↑ Translation

CELL GROWTH

(b) SURVIVAL FACTORS

survival factor
survival factor receptor

RAS

FLY
RAF
MAP kinase
Hid
IAP
PCD

VERTEBRATE
PI-3 kinase → PDK-1
PKB (Akt)
Bcl-2 ⊢ Bad
PCD

CELL SURVIVAL   CELL SURVIVAL

(c) MITOGENS

Mitogen
Mitogen receptor

RAS → RAF

MAP kinase

Nucleus

↑ Myc   ↑ Cyclin D ⊢ Rb ⊢ E2F → ↑ Cyclin E
(+ Cdk4/6)   (+ Cdk2)

CELL CYCLE PROGRESSION

Figure 1. Some Intracellular Pathways Activated by Growth Factors, Survival Factors, and Mitogens; Some Signaling Molecules Such As PDGF Can Apparently Activate All of These Pathways

(A) Growth factors promote cell growth mainly by stimulating protein synthesis. One tentative signaling pathway is shown. Activated PI3 kinase phosphorylates PIP_2 to generate PIP_3 (not shown), which activates PDK1 and allows PKB to be phosphorylated and activated by PDK1. Activated PKB indirectly activates the TOR kinase, which stimulates RNA translation in two ways: it leads to the activation of the S6 kinase, which phosphorylates S6, a ribosomal protein, and it inactivates the 4E-BP-1 protein, an inhibitor of the translation initiation factor eIF4E (Thomas and Hall, 1997).
(B) Survival factors can suppress PCD by activating intracellular signaling pathways that lead to the phosphorylation and inactivation of intracellular death promoters, such as Bad in vertebrate cells (Zha et al., 1996) and Hid in *Drosophila* cells (Bergmann et al., 1998). Unphosphorylated Bad promotes PCD by inhibiting death suppressors such as Bcl2; it can be inhibited by other kinases besides PKB. Unphosphorylated Hid promotes PCD by suppressing the activity of another class of PCD inhibitors called inhibitors of apoptosis (IAPs) (Bergmann et al., 1998).
(C) Mitogens promote cell cycle progression, in part at least, by stimulating the production of Myc and cyclin D. Complexes of cyclin D and CDK 4/6 phosphorylate and inhibit Rb, releasing E2F to stimulate the transcription of genes such as cyclin E that are required for progression from G1 into S phase. It is still uncertain how Myc promotes cell cycle progression. Some signaling molecules such as PDGF can apparently activate all of these pathways.

a compartment are genetically manipulated to accelerate or retard the cell cycle so that the cells become smaller or larger than normal, the compartment usually grows to be normal in size and shape, as cell numbers adjust to achieve a near-normal total cell mass (Weigmann et al., 1997; Neufeld et al., 1998). We shall consider later how this adjustment might occur.

Although differences in cell size often contribute to differences in animal size, differences in cell number usually make a larger contribution. A 70 kg human, for example, contains about 10^{13} cells, whereas a 25 g mouse contains about 3×10^9 cells (Baserga, 1985), so that the fold difference in mass (2800-fold) can be accounted for by the difference in cell number (3333-fold). With the exception of a few specialized cell types such as muscle cells and nerve cells, cell size does not vary much between one mammal and another, even when the animals are very different in size (Tessier, 1939; Altman and Dittmer, 1961).

Cell number depends on cell division, as well as on cell death, which we consider first.

Cell Death

Just as animal cells need extracellular signals to grow (growth factors) and divide (mitogens), so they need extracellular signals to survive (survival factors) (Raff,

1992). If they fail to get enough survival factor, they activate an intracellular death program and kill themselves, a process called programmed cell death (PCD), or apoptosis. The only known exceptions are blastomeres, which do not require extracellular signals either to survive or divide (Biggers et al., 1971). For other cells, the dependence on survival factors may help ensure that a cell survives only when and where it is needed. Survival factors act, in part at least, by regulating the amount or activity of intracellular proteins that control the death program—especially proteins of the Bcl2 family and IAPs (inhibitors of apoptosis) (Figure 1B). The death program itself depends on an intracellular proteolytic cascade mediated by members of the caspase family of cysteine proteases (Nicholson and Thornberry, 1997).

The dependence of animal cells on extracellular survival factors provides a powerful way to regulate cell numbers during development. Some of the best studied examples are in the developing vertebrate nervous system. Many types of nerve cells are generated in excess and are then thought to compete with one another for limiting amounts of survival factors produced by the target cells they innervate; only a proportion of the nerve cells get enough factor to survive, while the rest undergo PCD. Such an arrangement ensures that the number of

nerve cells is appropriately matched to the number of target cells they innervate; it also ensures that nerve cells that project to inappropriate target cells are eliminated (Oppenheim, 1991).

A similar mechanism operates in the development of oligodendrocytes, the cells that myelinate axons in the central nervous system. These cells are overproduced, and at least half of them undergo PCD, apparently in a competition for limiting amounts of survival factors provided by the axons that they myelinate. In this way, the number of oligodendrocytes is automatically matched to the number and length of axons requiring myelination (Barres and Raff, 1994). If the number of axons is experimentally decreased, more of the oligodendrocytes die and their numbers adjust downward (Barres and Raff, 1994); if the number of axons is experimentally increased, fewer oligodendrocytes die and their numbers adjust upward to match the increased number of axons (Burne et al., 1996). Moreover, if the number of oligodendrocytes generated is experimentally increased, PCD eliminates all of the extra oligodendrocytes (Calver et al., 1998). It seems likely that similar mechanisms operate in other developing organs, allowing different cell types in the organ to adjust their numbers automatically when the number of one cell type is perturbed. This strategy for adjusting cell numbers would presumably facilitate both development and evolution.

Not all cell deaths that occur in development, however, are caused by a lack of survival factors. Some are induced by PCD-inducing extracellular signals, which can act either systemically or locally and override the action of survival factors. Bone morphogenic proteins (BMPs), for example, induce PCD in prospective neural crest cells before they migrate from certain rhombomeres (Graham et al., 1994).

PCD can also play an important part in maintaining the appropriate number of cells in an adult organ. If adult rats are treated with phenobarbital, for example, hepatocytes are stimulated to proliferate, causing the liver to enlarge; when the drug treatment is stopped, a large increase in hepatocyte PCD rapidly returns the liver to its normal size (Bursch et al., 1985). Similar results are obtained when cell proliferation is experimentally increased in other adult organs, including the adrenal cortex, kidney, and pancreas. How does such an enlarged organ know what its correct total cell mass should be? One possibility is that the level of survival factors available in the organ helps to determine how much total cell mass there can be: if the mass increases above this value, more cells will die; if it falls below this value, fewer cells will die. This arrangement would automatically match cell death and cell proliferation in the adult organ (Raff, 1992). Presumably, hepatocytes themselves are not the source of the survival factors. Perhaps connective tissue cells are the source; they are present in most vertebrate organs and could, in principle, help determine the size of an organ. If so, it will be important to discover how their numbers are determined.

As mentioned earlier, there are some genetic accidents or manipulations that increase total cell mass abnormally in an animal or organ. Why does cell death not bring cell mass back to normal in these cases? In some, the reason is that survival signaling is excessive. Overproduction of GH during development, for example, produces excessive total cell mass (Palmiter et al., 1983) largely because it stimulates an increased production of IGF1, which is a potent growth and survival factor for many cell types (Heyner and Garside, 1994). In other cases, compensatory PCD is prevented by abnormalities in the intracellular regulation of the death program. Transgenic mice that overexpress the intracellular PCD-suppresser protein Bcl2 in neurons, for example, have enlarged brains with excessive numbers of neurons compared to wild-type mice (Martinou et al., 1994) because the cells expressing the transgene need less survival factor to avoid PCD.

A surprising example of size control that is based on the regulation of cell survival rather than on the regulation of cell proliferation is seen in hydra. In well-fed hydra, cell proliferation greatly outstrips cell death, and new hydra continually bud off from the parent animal. When hydra are starved, however, growth stops, mainly because cell death greatly increases, while the rate of cell division changes little (Bosch and David, 1984). It will be important to determine whether nutritional effects on growth in other animals are mediated by changes in cell growth, cell proliferation, cell survival, or, as seems likely, some combination of these.

Cell Proliferation

Extracellular mitogens and inhibitory molecules regulate cell division to ensure that animal cells only divide when more cells are needed. Mitogens activate intracellular signaling pathways that stimulate the production and/or activity of components of the cell cycle control system that promote progression through the cycle (Sherr, 1994) (Figure 1C); in order for cell division to increase total cell mass, the mitogen, or an independent growth factor, must also stimulate the cells to grow, as discussed earlier. Conversely, proliferation inhibitors usually activate intracellular signaling pathways that stimulate the production or activity of components of the control system that block progress through the cycle, usually in G1 (Sherr and Roberts, 1995). The concentration of a mitogen can influence the rate of cell cycle progression, with the cycle speeding up with increasing concentration of mitogen (Brooks and Riddle, 1988; Gao and Raff, 1997). In the absence of mitogen, cell cycle progression arrests, usually in G1, and the cell enters a modified G1 state (G0) in which much of the cell cycle control system is dismantled.

At the heart of the control system are the cyclin-dependent protein kinases (CDKs), which are cyclically activated to trigger the different phases of the cycle at the right time and in the right sequence (Morgan, 1995). A variety of regulatory proteins control the activity of the CDKs: cyclins activate them and help direct them to their substrates; kinases and phosphatases regulate their activity; CDK inhibitors block their activity or their assembly with cyclins. In addition, ubiquitin-dependent proteases degrade cyclins and other regulatory proteins at crucial points in the cycle. The control system can halt progression at various checkpoints in the cycle, ensuring that the next step is not triggered until the

previous one is completed, conditions inside and outside the cell are favorable, and the cell is of adequate size (Elledge, 1996).

In the standard cell cycle, the cell grows, and both the nucleus and cell divide. The control system can be modified in various ways, however, to alter the standard cycle. Such a modified control system can drive the rapid embryonic cleavage divisions of blastomeres that occur without cell growth so that the cells get smaller with each division; it can produce multinucleated cells through repeated nuclear divisions without cell division; or it can produce giant mononuclear cells that contain several thousand times the normal amount of DNA through repeated rounds of DNA replication without nuclear or cell division.

The concentrations of mitogens and growth factors are often limiting, as increasing them usually leads to an increase in cell proliferation. A dramatic example is seen in mice that have been genetically engineered to express variable numbers of the *PDGF A* gene (Calver et al., 1998). PDGF-AA is the major mitogen (and probably the major growth factor and survival factor) for oligodendrocyte precursor cells. The number of precursor cells produced in the embryonic mouse spinal cord increases with the number of transgenes expressed: in mice with one copy of the transgene, for example, there are three times the normal number of precursor cells, while in mice with two copies there are seven times the normal number. As discussed earlier, even though the number of oligodendrocytes produced is greatly increased in such transgenic mice, the final number in the spinal cord is normal, as all of the extra oligodendrocytes kill themselves (Calver et al., 1998).

Not only can cells within a developing organ compete with one another for limiting amounts of locally produced mitogen (and other signaling molecules), but cells in one developing organ can compete with those in another for systemic growth-inducing signals. A recent example is seen in insects. Although there is abundant evidence from transplantation and regeneration experiments that the size of an insect organ or appendage is mainly determined autonomously, the final size is influenced by hormones that can act, in part at least, as mitogens (Bryant and Simpson, 1984). If the hind wing imaginal discs of a caterpillar larva are removed, the butterfly that develops has abnormally large forewings and forelegs (Nijhout and Emlen, 1998). This enlargement is less if just one hind wing disc is removed rather than both. These findings suggest that the developing wings and legs compete for limiting amounts of some systemic resource, which seems likely to be hormonal. Besides coordinating the growth of the different parts of a developing insect, hormones may also mediate the insect's growth responses to environmental factors, including nutrition (Britton and Edgar, 1998) and temperature; these responses can involve changes in cell size, cell number, or both.

Most signaling molecules that stimulate cell proliferation in animal development, however, act locally rather than systemically. In many cases, they, or the signals that regulate their production, are produced in signaling centers formed at boundaries between different groups of cells and at points where such boundaries intersect within the developing organ or appendage; gradients of these signaling molecules help generate the structure's characteristic size and shape (Serrano and O'Farrell, 1997). The apical ectodermal ridge of the vertebrate limb bud, for example, is a signaling center that secretes the mitogens FGF4 and FGF8, which stimulate the proliferation of cells in the underlying progress zone (Martin, 1998). The patterned expression of various developmental control genes determine which cells make the growth-regulating signaling molecules and which cells respond to them. There is increasing evidence, for instance, that Hox genes play an important part in controlling cell proliferation in a position-dependent manner (Duboule, 1995), but how these genes regulate the cell cycle is unknown.

An even more localized control of cell division has been proposed to operate in regenerating limbs (French et al., 1976) and developing compartments of *Drosophila* imaginal discs (Garcia-Bellido et al., 1973; Bryant and Simpson, 1984). Here, a disparity in positional values between neighboring cells is thought to stimulate cell proliferation; the new cells produced adopt intermediate positional values until a continuous and appropriate pattern of values is established (French et al., 1976). Thus, the final size of a disc compartment might depend on the terminal positional values established at the time the axes of the disc are determined. It is unknown how positional values would be set to give the appropriate final size, how positional disparities are translated into mitogenic signals, or what the mitogenic signals are. It is also unclear whether such intercalation mechanisms operate in developing vertebrate organs or appendages.

Even though they do not usually act directly to stimulate cell proliferation, the Notch and Delta families of cell contact–dependent signaling proteins can play an important part in regulating cell division during animal development. They mediate lateral inhibition, in which differentiating cells inhibit dividing precursor cells from following the same fate (Simpson, 1997). In this way, Notch signaling can prolong precursor cell division and can keep stem cells in a self-renewing pattern of division, thereby indirectly increasing the number of differentiated cells ultimately produced (Henrique et al., 1997).

Stopping Cell Proliferation

To understand how total cell mass is controlled during development, it is just as important to understand what stops cell proliferation at the appropriate time as it is to understand what stimulates the proliferation. In most vertebrate cell lineages, for example, precursor cells divide a limited number of times before they stop and terminally differentiate into specialized postmitotic cells. This is the case for the precursors that give rise to nerve cells, muscle cells, gut cells, skin cells, cartilage and bone cells, some types of blood cells, and so on. In none of these cases is it known why the precursor cells stop dividing when they do. The stopping mechanisms are important, as they influence how many terminally differentiated cells are generated. In most cases, it is uncertain whether cell cycle withdrawal triggers terminal differentiation, or vice versa, or whether the two processes are controlled separately.

Not all vertebrate cells, however, permanently withdraw from the cell cycle when they differentiate. In response to increased functional load, for example, terminally differentiated heart muscle cells can reenter the cell cycle and replicate their DNA, but they are unable to divide (Rumyantsev, 1991). Differentiated mammalian skeletal muscle cells, by contrast, never synthesize DNA or divide, even in response to increased functional load or injury, although if the tumor suppresser gene *Rb* is inactivated, the cells can be induced to synthesize DNA, suggesting that *Rb* normally helps maintain the arrested state (Novitch et al., 1996). During limb regeneration in an adult urodele, however, skeletal muscle cells can dedifferentiate, reenter the cell cycle, proliferate, and redifferentiate into new muscle cells and at least one other cell type (Brockes, 1997).

Hepatocytes retain the ability to proliferate throughout life. If two-thirds of the adult rat liver is surgically removed, proliferation of the remaining hepatocytes increases until the liver returns to its normal size. Although some of the mitogens and growth factors involved have been identified (Michalopoulos and DeFrances, 1997), it is not known how their production is controlled or what stops cell division at the appropriate point.

In principle, the arrest of cell proliferation during development or regeneration can result either from an extracellular mechanism, such as a decrease in extracellular mitogen or growth factor or an increase in extracellular inhibitor, or from an intracellular mechanism, such as the activation of an intracellular stopping mechanism or the dismantling of the cell cycle control system, or from some combination of these.

Extracellular Stopping Mechanisms

Cell contact–dependent cell–cell interactions can play a crucial part in the stopping process. Genetic studies in *Drosophila*, for example, have shown that molecules involved in cell–cell adhesion and in gap junction–mediated cell–cell communication are required to stop imaginal disc epithelial cell proliferation at the right point in development (Bryant and Schmidt, 1990), although it is uncertain how these cell interactions contribute to cell cycle withdrawal.

We have already discussed the importance of limiting amounts of mitogen and growth factor in controlling cell proliferation. As a population of dividing cells increases, for example, it will degrade an increasing amount of these factors. If factor production does not increase to the same extent, its concentration will fall, and cell proliferation will decrease until the population reaches an equilibrium size where factor production and degradation are equal (Calver et al., 1998). Where the factors are produced by a signaling center or result from positional disparity, cell division will decrease as cells proliferate and either move away from the signaling center or acquire appropriate positional values. Thus, the availability of growth factor or mitogen can be as important in limiting cell growth and proliferation as the availability of survival factor can be in limiting cell survival.

Another way of stopping cell proliferation is through the production of extracellular inhibitory signals. The secreted morphogen Wingless, for instance, is required for normal cell proliferation and patterning in the early developing *Drosophila* wing disc but is later involved in
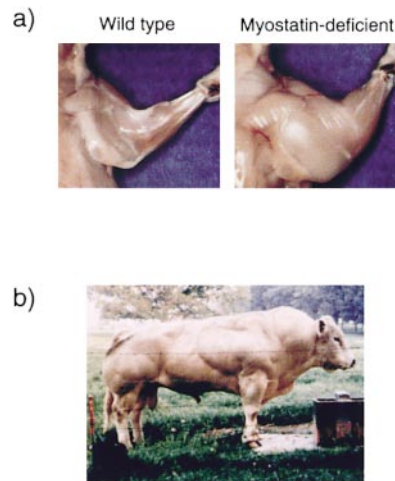
Figure 2. Increased Skeletal Muscle Mass in Animals Deficient in Myostatin

(A) Upper limb muscles in a wild-type and a myostatin-deficient mouse. (From McPherron et al., 1997; reprinted by permission from Nature, copyright 1997, MacMillan Magazines Ltd.)

(B) A full-blood Belgian Blue bull, which is myostatin-deficient. (From McPherron and Lee, 1997; reprinted by permission from Proc. Natl. Acad. Sci. USA, copyright 1997, National Academy of Sciences, USA.)

arresting the cell cycle in specific parts of the same disc; it promotes cell cycle arrest in G2 by inducing the expression of proneural genes, which in turn downregulate the Cdc25 phosphatase, which is required for entry into mitosis (Johnston and Edgar, 1998).

It has long been postulated that the size of some organs is controlled by secreted inhibitory molecules that are produced by the organ and specifically suppress cell division in that organ: as the organ grows, the putative inhibitory substance accumulates until it reaches a level that stops further cell proliferation in the organ (Goss, 1978). Although such a mechanism could explain the results of the liver regeneration experiments discussed earlier, direct evidence for it has been lacking. Recently, however, a dramatic finding suggests that this type of mechanism can operate, at least in skeletal muscles. When the gene encoding the TGFβ superfamily member *myostatin* is inactivated by targeted gene disruption in mice, muscles grow to be up to three times normal size and contain more and bigger cells (McPherron et al., 1997) (Figure 2A). This result suggests that myostatin normally inhibits both muscle cell division and muscle cell growth. Two breeds of cattle that were bred for large muscles (Figure 2B) turn out to be mutant in the same gene (McPherron and Lee, 1997). The myostatin protein is made almost exclusively by skeletal muscle cells (McPherron et al., 1997), but it remains to be determined whether it acts directly or indirectly on these cells to influence their growth and division and whether a similar mechanism operates in other organs.

Intracellular Stopping Mechanisms

Even when cultured in saturating amounts of the mitogen that normally stimulates their division, many types of precursor cells will divide a limited number of times before they stop and terminally differentiate, much as

they do in vivo. These precursor cells seem to have an intrinsic mechanism that limits their proliferation and controls when they terminally differentiate.

In principle, an intracellular mechanism that stops cell proliferation after a period of time or number of divisions could depend on a controlled decrease in a cell cycle promoter such as a CDK or a cyclin, a controlled increase in a cell cycle inhibitor such as a CDK inhibitor, or both. There is evidence for both types of mechanisms operating during animal development. (Neither of these types of mechanisms, however, explains why a cell stops growing; as mentioned earlier, a cell can continue to grow after it exits the cell cycle, and the intracellular mechanisms that stop cell growth are unknown.)

Evidence for the first mechanism has come from null mutations in the *cul-1* gene in *C. elegans*, which result in extra divisions in all larval cell lineages (Kipreos et al., 1996). The gene encodes a protein that is thought to act as an E3 ubiquitin-protein ligase that helps direct the ubiquitination enzymatic cascade to cell cycle control proteins that act in G1, including G1 cyclins, thereby targeting them for destruction (Willems et al., 1996). The finding that inactivation of *cul-1* prolongs cell proliferation suggests that the CUL-1 protein normally helps cells in the worm exit the cell cycle by degrading cell cycle promoters such as G1 cyclins. Similarly, a fall in cyclin E levels may be required for cells in *Drosophila* embryos to exit the cell cycle at the appropriate point in development, as prevention of the fall by cyclin E overexpression causes the cells to go through an extra cycle (Knoblich et al., 1994).

Evidence for the second mechanism came initially from studies of the CDK inhibitor p27/Kip1 (p27) in mice. Mice that are deficient in p27 are about 30% larger than normal, with more cells in all organs that have been examined (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996). Studies of oligodendrocyte precursor cells suggest that p27 is part of a timing mechanism that limits proliferation and helps the precursor cells exit the cell cycle and terminally differentiate at the appropriate time (Casaccia-Bonnefil et al., 1997; Durand et al., 1997, 1998). The evidence that these cells are measuring time rather than counting cell divisions is that they divide more slowly, undergo fewer divisions, and yet differentiate sooner when cultured at 33°C than at 37°C (Gao et al., 1997). Three lines of evidence suggest that the accumulation of p27 is part of the cell-intrinsic timer: (1) the level of p27 protein progressively increases with the expected time course as the cells proliferate in culture (Durand et al., 1997); (2) p27 levels rise faster at 33°C than at 37°C (Gao et al., 1997), as would be expected if p27 accumulation is part of the timer; and (3) oligodendrocyte precursor cells isolated from p27-deficient mice divide with a normal cell cycle time, but many of them go through one or two more divisions than do wild-type cells before they differentiate (Durand et al., 1998). It seems likely that a similar defect in cell cycle withdrawal occurs in other cell lineages in p27-deficient mice and is responsible for the general increase in cell number.

The phenotype of mice deficient in another CDK inhibitor, p18/INK, is remarkably similar to that of p27-deficient mice (Franklin et al., 1998), suggesting that p18 may be another component of the stopping mechanism in many cell lineages. In mice that are deficient in both p27 and p18, some organs are even larger than they are in mice deficient in either protein alone, but the mice still develop normally (Franklin et al., 1998), indicating that precursor cells eventually stop dividing and differentiate without either of these proteins. The two proteins are apparently only part of the mechanism that is responsible for stopping the cell cycle at the appropriate time in development. The advantage of such a multicomponent stopping mechanism is that it is both accurate and robust; if one or two components are defective, the mechanism still works, although less accurately.

Why doesn't cell death bring cell numbers back to normal in p27-deficient and p18-deficient mice? This may be because many cell types are increased in each organ and can therefore support one another's survival. Similarly, some of the increased cell proliferation in these mice may be secondary to an increase in mitogens and growth factors produced by those cells that have proliferated excessively as a direct consequence of the p27 or p18 deficiency.

Genetic studies indicate that the p27-like proteins Dacapo in *Drosophila* (de Nooij et al., 1996; Lane et al., 1996) and CDI-1 in *C. elegans* (Hong et al., 1998) help developing cells exit the cell cycle in these animals. Cells in multiple lineages in these mutants go through one or more extra divisions before withdrawing from the cell cycle. It seems that CDK inhibitors are involved in stopping precursor cell division in all animals.

How Do Cell Numbers Adjust to Attain a Normal Total Cell Mass?

Having considered how total cell mass is controlled both by extracellular signals and by intracellular mechanisms that limit cell growth, survival, and proliferation, we now address a problem raised earlier: how does an animal or organ with abnormally large or small cells grow to be normal in size? How is it, for example, that polyploid animals, which have bigger cells than diploid animals of the same type, usually have correspondingly fewer cells and therefore end up the same size as the diploid animals?

For species such as salamanders that go through a midblastula transition (MBT), where zygotic transcription begins, the intracellular mechanism responsible for the MBT is likely to be part of the answer. The early embryo undergoes rapid cleavage divisions without cell growth until the ratio of nuclear DNA to cytoplasm in the blastomeres reaches a critical value that triggers MBT (Newport and Kirschner, 1982). A tetraploid embryo can be produced by inhibiting cytokinesis at the first cleavage division; the resulting zygote starts off with the same amount of cytoplasm as the diploid zygote, but with twice the amount of DNA. As a result, the tetraploid embryo reaches MBT one division earlier than the diploid; although the tetraploid and diploid are the same size at MBT, the tetraploid contains half the number of cells and its cells are twice as large (Figure 3). When the tetraploid and diploid animals start to grow after hatching, they will grow to the same total cell mass because the extracellular and intracellular mechanisms that control post-MBT growth regulate total cell mass
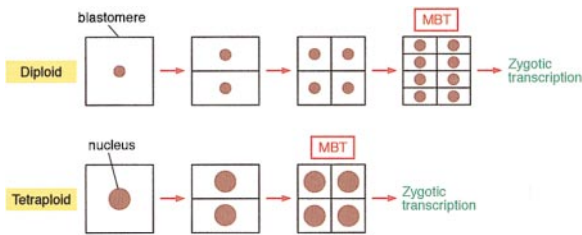
Figure 3. A Tetraploid Embryo Undergoes MBT Earlier Than a Diploid Embryo

MBT occurs when the ratio of DNA to cytoplasm in a cleaving blastomere reaches a critical value. Because the tetraploid blastomeres have twice as much DNA as the diploid blastomeres but the same amount of cytoplasm, they achieve this critical ratio one division earlier than do the diploid blastomeres. Thus, at MBT the tetraploid embryo has half the number of cells, but its cells are twice as large, and so the total cell mass is the same in the two embryos. Note that in these early embryos there is no cell growth, and so the cells get progressively smaller with each division, and the total cell mass remains unchanged.
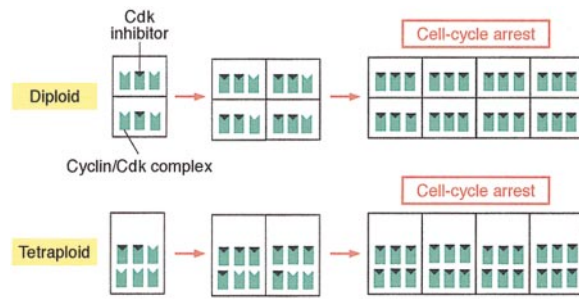

Figure 4. The Operation of an Intrinsic Cell Cycle Stopping Mechanism in Tetraploids versus Diploids

As shown, the mechanism, which involves a progressive increase in the concentration of a CDK inhibitor will help halt cell division at the same total cell mass in tetraploid and diploid animals. As long as the total cell mass of the tetraploid and diploid cell populations start off the same (as is the case, for example, at MBT in Figure 3), the cells will tend to stop dividing around the same total cell mass, as indicated. Note that cell growth allows the cells to maintain their size as they proliferate, and so there is a progressive increase in total cell mass.

rather than cell numbers (see below), and the mass at the start of growth is the same.

As far as extracellular controls on growth are concerned, a tetraploid cell is probably functionally equivalent to two diploid cells, in that it will make roughly twice as many signaling molecules as a diploid cell and will ingest and degrade twice as many signaling molecules that bind to its receptors. Therefore, the levels of extracellular growth factors, mitogens, growth inhibitors, and survival factors, which play such an important part in determining total cell mass, will tend to be similar in the tetraploid and diploid animals as they grow.

An intracellular stopping mechanism that limits cell proliferation by an accumulation of a CDK inhibitor such as p27 will also tend to produce a similar level of growth in the tetraploid and diploid salamanders. In a tetraploid cell, the levels of both the inhibitor and the cyclin–CDK complexes that the inhibitor blocks would be expected to be proportionally increased compared to a diploid cell, and so the cells in tetraploid and diploid populations would stop dividing when they reach about the same total cell mass (Figure 4).

Although mammalian embryos do not go through an MBT, similar principles to those just discussed probably explain why a tetraploid mouse embryo is the same size as a diploid (Henery et al., 1992). As long as they start off at roughly the same total cell mass, the tetraploid and diploid will grow to be about the same size. These principles might also explain the results in *Drosophila* larvae, where the cell cycle is genetically manipulated to generate cells in an imaginal disc compartment that are smaller or larger than normal but still diploid (Neufeld et al., 1998); as mentioned earlier, in these experiments, cell numbers adjust so that organ size is close to normal. Abnormally large or small cells in these circumstances might make and degrade proportionally more or less signaling molecules, respectively, which would tend to keep the levels of these molecules, and therefore the total cell mass, normal.

Limiting levels of extracellular signals might also explain the ability of early embryos to adjust their total cell mass toward normal if it is perturbed. If the number of mouse blastomeres is experimentally increased (Rossant, 1976) or decreased (Tarkowski, 1959), cell numbers adjust, apparently through changes in cell division later in embryogenesis, to produce mice of normal size. Thus, when two 8-cell mouse embryos are fused to produce one large double embryo, the proliferative burst seen in a normal embryo between 5 1/2 and 6 1/2 days gestation is reduced, and the double embryo ends up the same size as the normal embryo from 6 1/2 days onward (Rossant, 1976). This adjustment in total cell mass would be readily explained if the normal proliferative burst depends on growth factors or mitogens that are supplied in limiting amounts by the mother.

Animals and organs, however, do not always adjust their total cell mass toward normal if it is experimentally or accidentally perturbed. As mentioned earlier, excessive or deficient signaling along the PI3 kinase pathway can lead to more and larger cells or fewer and smaller cells, respectively. Signaling along this pathway may be especially important for the determination of total cell mass, as it promotes cell growth, cell survival, and, in some circumstances, cell proliferation. In addition, the amount of extracellular signals produced by a cell may increase with increased signaling along this pathway.

Conclusion

How will we discover why we grow to be larger than mice? The identification of the genes involved in size control will be a great help, and, for this, genetic studies in flies and worms are likely to provide the lead, as they have in so many other areas of developmental biology. But this is unlikely to be enough. We will also need cell biological studies to learn more about how cell growth is controlled from inside and outside the cell and how cell proliferation is regulated in developing animals. It is clear, for example, that, on average, human cells divide more than mouse cells, but it is unclear whether this is primarily because of differences in the intracellular mechanisms that limit cell proliferation, in extracellular

signal production, or in both. We need to discover these intracellular mechanisms and compare them in mice and humans. We also need to understand how the levels of extracellular signaling molecules are determined, which probably means that we need better ways of measuring them in developing tissues. As a start, however, we need more developmental biologists studying size control.

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