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4 A HISTORY OF IN VITRO MODELS

Anything that is true of *Escherichia coli* must be true of elephants, only more so.
—JACQUES MONOD, 1967 (SEE FRIEDMANN, 2004, P. 49)

Instead of working with intact animals or plants, many biologists perform their research on single-celled microbes or on cells and tissues that are grown in petri dishes or culture flasks. That is, they work with *in vitro* systems (meaning "in glass" in Latin). In this chapter, we first discuss the major microbial models, which comprise select bacteria, viruses, and fungi. Then we move on to tissue explants, primary cell cultures, immortal cell lines, stem cells, and the creation of complex cell cultures, including organoids. These discussions are followed by a brief review of how microbial and cell culture models have been used in toxicology. The chapter ends with a discussion of how and why the popularity of the various model systems, be they in vitro or in vivo, waxes and wanes. As we shall see, this dynamic involves both biological and sociological factors.

4.1 MICROBIAL MODELS

Microbes are often defined as any organisms that are too small to see with the unaided eye, but for present purposes they comprise viruses and unicellular organisms. Because multicellularity evolved independently from unicellular life at least 25 times, today's microbes are scattered across the phylogenetic tree (figure 4.1). Most microbes are prokaryotes, which means that they lack a distinct nucleus, intracellular organelles, and several other features typical of cells in the eukaryotes, which include the fungi (e.g., yeasts and mushrooms) and all animals. Viruses are often considered inanimate because they cannot replicate without the help of their host cells, and their phylogenetic relationships remain a major puzzle because viruses can evolve rapidly and engage in extensive horizontal gene transfer (Nasir & Caetano-Anollés, 2015).

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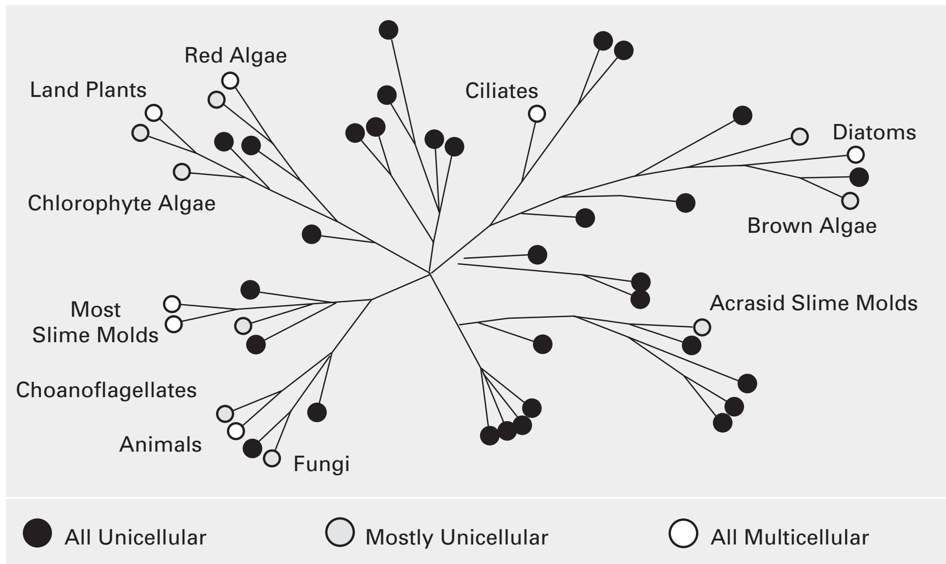

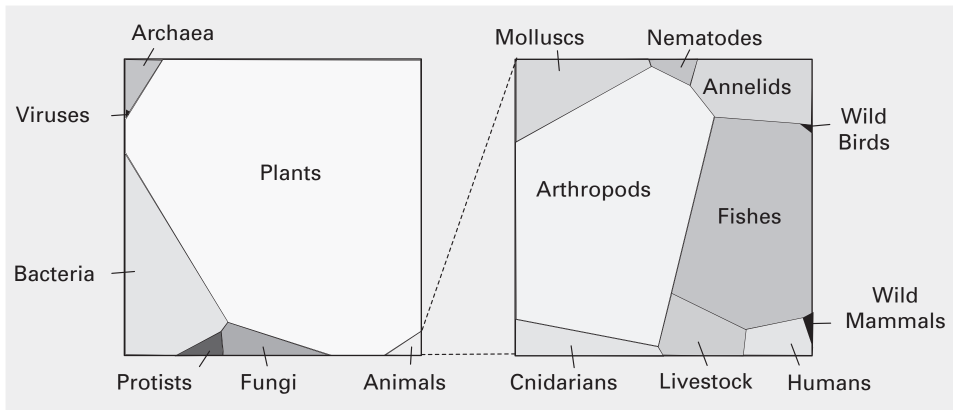

Figure 4.1
Unicellular life forms are ancestral to multicellular life and account for more biomass than animals. (*A*) Multicellularity evolved repeatedly. A phylogeny of eukaryotes indicates the distribution of multicellularity; the most parsimonious conclusion is that it evolved repeatedly from unicellular life. Multicellularity also evolved a few times among prokaryotes, which are not shown here. (*B*) Relative distribution of global biomass. For reference, bacteria account for about 15% of global biomass, whereas humans account for 0.01%. Adapted from (A) Grosberg & Strathmann (2007); (B) Bar-On et al. (2018).

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4.1.1 Bacteria

Bacteria are typically less than 10 micrometers long (for elongate bacteria) and 0.5–2.0 micrometers in diameter. They are estimated to comprise about 1 million species (aka operational taxonomic units) and constitute 15% of the globe's biomass (Bar-On et al., 2018; Louca et al., 2019) (figure 4.1B). They have been studied for many years, especially after Louis Pasteur, Robert Koch, and others in the second half of the 19th century discovered their causal role in numerous infectious diseases (see chapter 5). Biologists have studied many different bacteria, but a few species have attracted disproportionate attention. One of these heavily studied bacteria is *Salmonella typhimurium*, which causes food poisoning. It was in this bacterium that researchers first described the process of transduction, defined as the virus-mediated exchange of genetic material between bacteria (Zinder & Lederberg, 1952). However, the bacterium that has had the most profound effect on biological research is *Escherichia coli*. As one enthusiast put it, "It is not hyperbole to say that *E. coli* is now the most important model organism in biology" (Blount, 2015, p. 1).

E. coli is named for Theodor Escherich, who first cultured it from human feces, and for the lowest part of the intestine, the colon, where *E. coli* lives roughly half the time (the remainder being spent in water, sediment, or soil, waiting to infect another warm-blooded vertebrate host) (Savageau, 1983). Most strains of *E. coli* are benign and actually help humans obtain vitamins K and B12; they also make it easier for other bacteria to thrive in the human gastrointestinal tract by consuming any oxygen there that might harm anaerobic bacteria. As you probably know, however, some strains of *E. coli* are toxic. The O157:H7 strain, for example, normally lives in cattle but can cause severe diarrhea and other complications in humans. Fortunately, the most widely used laboratory strain of *E. coli* (K-12) has lost the ability to thrive in the human colon and is, therefore, quite safe.

Aside from being relatively safe, *E. coli* is well suited for laboratory research because it can synthesize all amino acids and most other organic essentials, which means that it can grow in very simple culture media. Moreover, *E. coli* cells divide every 20 to 60 minutes under ideal conditions. This generation time is not the shortest among bacteria—that distinction goes to *Vibrio natriegens*, which can divide every 10 minutes—but it still allows researchers to grow cultures containing billions of *E. coli* overnight (Sezonov et al., 2007). Such a high rate of population growth facilitates the search for mutations and many other kinds of experiments. It even allows for studies in experimental evolution, where changes in specific traits are followed over thousands of generations (Tenaillon et al., 2012). Another factor that simplifies research with *E. coli* is that its genome contains only 4.6 million base pairs, including approximately 4,300 protein-coding genes (Blattner et al., 1997). By comparison, the human genome contains 3 billion base pairs and at least

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20,000 genes. Therefore, *E. coli*'s genome should be easier to unravel. An interesting complication is that, in a comparative study of 61 *E. coli* strains, only 20% of genes were found to be conserved across all strains (Lukjancenko et al., 2010). Given this observation, it is not surprising that humans and *E. coli* share relatively few homologous genes (O'Brien et al., 2004).

An early obstacle to genetic studies with *E. coli* was the belief among many biologists that bacteria exhibit Lamarckian inheritance, with mutations appearing in response to environmental needs rather than by chance. In the words of a leading *E. coli* biologist, "bacteria could not be adopted as models for genetic research until there was some substantiation of the view that they had a genetic system like other organisms" (Lederberg, 1987, p. 23). This problem was overcome in the 1940s when biologists demonstrated that mutations in *E. coli* do arise stochastically. Another key advance was the discovery that bacteria have evolved a variety of ways to exchange genetic material, even though they do not engage in sexual reproduction as it is typically defined (i.e., two cells joining to combine their DNA). Most influential was the discovery of bacterial conjugation, during which one bacterium inserts a piece of its DNA directly into another bacterium (Lederberg, 1947). In hindsight, it was fortuitous that this research was carried out with the K-12 strain of *E. coli*, because only about 5% of all *E. coli* strains are capable of conjugation (Davis, 2003, p. 91).

Once researchers were able to create *E. coli* mutants and crossbreed different strains, the use of *E. coli* as a model species gathered speed. It accelerated further after the discovery of DNA's double-helical structure in 1953, which immediately suggested many new lines of investigation. Researchers created thousands of *E. coli* mutants and developed ever more powerful techniques for manipulating *E. coli*'s genome. Along the way, they uncovered numerous molecular and cellular processes, ranging from gene transcription and regulation to metabolic pathways. Most of these processes were thought to be broadly conserved, prompting Monod's famous dictum that anything true of *E. coli* must also be true of elephants (see this chapter's opening quotation). A few dissenters argued that "the steadily growing recognition of the existence of alternate pathways, of qualitative and quantitative differences in enzymatic patterns, of differences in submicroscopic cell structure, permeability and rate of cell division [argue for] a 'disunity in biochemistry'" (Racker, 1954; quoted in Friedmann, 2004), but such comments were largely ignored at the time.

Research on *E. coli* slowed down after the 1970s, as biologists turned to new questions and other model systems, notably yeast and mice. In the words of François Jacob, who had performed Nobel Prize–winning work on *E. coli* with Monod (see the appendix):

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> At the end of the 1960s, it was clear that the center of gravity in biology was shifting. Although the study of bacteria and viruses still had much to teach us, it was slipping to second place. If we didn't want to stand around rehashing the same questions, we needed the courage to abandon old lines of research and old models, to turn to new problems and study them with more suitable organisms. (1998, p. 6)

Jacob went on to study mice. However, *E. coli* has continued to be important for diverse forms of research, including comparative genomics, experimental evolution, and the study of toxic strains (Fux et al., 2005; Ferens & Hovde, 2011; Tenaillon et al., 2012). *E. coli* has also been used to synthesize therapeutic compounds, such as insulin or human growth hormone, as well as perfume fragrances and biofuels (Liu & Khosla, 2010; Koppolu & Vasigala, 2016; Idalia & Bernardo, 2017).

4.1.2 Viruses

Biologists in the early 1900s realized that some infectious diseases were caused by particles much smaller than bacteria. These particles, which we now call viruses, range in diameter from 5 to 300 nanometers. The first photographs of viruses were taken with an electron microscope in 1939. Since those early days, biologists have worked with many different viruses, including for example the Rous sarcoma virus, which causes cancer in chickens (see chapter 5). One of the first viruses to be studied in detail was the tobacco mosaic virus, which causes mosaic disease in tobacco and some other plants. It served as the subject for early studies on the structure and chemical composition of viruses and is still widely used in plant biology (Creager, 2002). However, the viruses that have had the greatest, broadest impact on biology are viruses that attack bacteria; they are known as bacteriophages or, more simply, phages.

With an estimated 1 million species and a total of 10^{31} individuals (Keen, 2014), phages are the most diverse and numerous organisms on earth. They infect specific strains or species of bacteria and then use the host's intracellular machinery to make additional phage particles. Their rate of replication is extremely rapid, with infection by a single virus generating up to 100 viral copies in 45 minutes (Davis, 2003). Eventually, the infected bacterium breaks down and releases the new virus particles, which are then ready to infect additional bacteria. In addition to these so-called virulent phages, evolution has fashioned temperate phages, which integrate their genome into that of the infected bacterium. The viral genes then replicate when the bacterium divides but do not create additional virus particles within the host until some triggering event causes the bacteria to enter the "lytic phase," during which the bacteria produce large numbers of phage particles. Either way, viral replication is so rapid that most of the classic phage experiments could be conducted within a single day.

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Early studies with phage focused on the T series of virulent phage. These viruses were selected mainly because they infect *E. coli*, which (as reviewed earlier) is an experimentally convenient bacterium. Coupling the high replication rate of *E. coli* with that of the T phages, researchers were able to detect phage mutants as rare as 1 in 100 million. Such experiments allowed high-resolution mapping of mutations inside of single genes (Benzer, 1959), which in turn facilitated the discovery of the triplet nature of the genetic code (figure 4.2) that converts DNA sequences to a succession of amino acids (Crick et al., 1961; Yanofsky, 2007). Work with T phages also led to other important findings, such as the discovery of messenger RNA (mRNA) being an intermediary between DNA and proteins, and of phages injecting their DNA into host cells while leaving their protein coat outside (Stent, 1963; Brock, 1990; Summers, 2004). An interesting fact related to the topic of this book is that Max Delbrück, who led much of the earliest phage research, pressured his colleagues to work with the same T phages and specific *E. coli* strain (strain B) that he had been using because he felt that standardization of the research organisms would allow studies to build on one another more reliably and, hence, more rapidly.

	U	C	A	G	
U	Phe	Ser	Tyr	Cys	U
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	Stop	Stop	A
	Leu	Ser	Stop	Trp	G
C	Leu	Pro	His	Arg	U
	Leu	Pro	His	Arg	C
	Leu	Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G
A	Ile	Thr	Asn	Ser	U
	Ile	Thr	Asn	Ser	C
	Ile	Thr	Lys	Arg	A
	Met	Thr	Lys	Arg	G
G	Val	Ala	Asp	Gly	U
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val	Ala	Glu	Gly	G

Leu, Ala, or Gln
Pyrrolysine
Stop
Gln
Stop
Thr
unassigned
Ser
Met or unassigned
Trp, Cys, or unassigned
Seleno-cysteine
unassigned
unassigned
Ser
Stop or Gly
Asn or unassigned

Figure 4.2
Variation in the standard genetic code. This table shows how triplets of the RNA nucleotides uracil (U), cytosine (C), adenine (A), and guanine (G) specify which amino acids should be translated or whether translation should stop. The first, second, and third positions within each triplet (i.e., codon) correspond to the letters along the left, top, and right edges of the table. Although this "universal" genetic code is widely conserved, modifications are found (as indicated) in mitochondria and in a handful of bacteria, fungi, ciliates, green algae, and diplomonads. Adapted from Sengupta & Higgs (2015); Koonin & Novozhilov (2017).

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Despite Delbrück's efforts, several very influential biologists preferred to work with temperate phages, especially one called *lambda* (Casjens & Hendrix, 2015). This phage infects the K-12 strain of *E. coli*, and, together, these two microbes became the preferred model system for a number of research areas. Especially important was research on how phage DNA becomes integrated into the host cell's DNA. This work led to the discovery of restriction enzymes and eventually to techniques for inserting foreign genes into host DNA—and thus to gene cloning and recombinant DNA technology more generally. Research with the *lambda* phage also led to the discovery of a repressor protein that prevents viral DNA from being transcribed, which ultimately led to detailed models of transcriptional regulation (i.e., the lac operon model). Overall, research on *E. coli* and its viruses revealed, within a few decades, a long list of molecular mechanisms underlying gene replication, recombination, regulation, and function (Davis, 2003). Many of the principles embodied by those mechanisms have turned out to be conserved across a wide array of species and cell types. In that sense, what is true for *E. coli* really is true for elephants.

However, not all the processes and mechanisms found in *E. coli* and its phages are universally conserved. For example, the *lambda* genome is linear while that of many other viruses is circular; some viruses inject only their DNA into host cells, while others inject some of their protein as well; and most animal viruses do not inject their DNA into host cells at all but are instead endocytosed. Similarly, the lac operon model of gene regulation tends not to hold within eukaryotes. Even the triplet genetic code is not as universal as most biologists assume (figure 4.2). The molecular techniques that emerged from research on *E. coli* and its viruses also had to be fine-tuned. In words of one author, "the technical reasons that initially led to the choice of lambda as experimental material have long since been superseded either by new techniques or by advances that allowed old techniques to be applied to a wider range of material" (Campbell, 1986, p. 280).

None of this imperils the idea that research on bacteria and phage served as "crucibles of the molecular revolution" (Davis, 2003, p. 129). Nor has such research ceased to be fruitful. It has led, for example, to the discovery of the CRISPR/Cas9 gene editing system, which probably evolved as a bacterial defense against phage infections (Stern & Sorek, 2011) and is now used by biologists to edit the genomes of many different organisms. I mostly wish to make the point that not everything that is true of elephants is true of *E. coli* or phage. Indeed, some of those very differences are what made *E. coli* such an ideal organism for the research questions that occupied biologists in the second half of the 20th century.

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4.1.3 Yeast

Yeasts are unicellular fungi and, as such, belong to the eukaryotes (see figure 4.1). Thus, in contrast to bacteria, yeasts have distinct cell nuclei, a complex cytoskeleton, intracellular organelles (notably mitochondria), and intracellular membrane systems (e.g., an endoplasmic reticulum). They also divide by means of mitosis and meiosis, rather than the simple binary fission found in prokaryotes.

The most intensively studied species of yeast is *Saccharomyces cerevisiae*, also known as budding yeast, brewer's yeast, or baker's yeast (Greig & Leu, 2009). Like *E. coli*, it is not pathogenic, can live on simple media, and does not form clumps, which makes it relatively easy to estimate the concentration of cultured cells. Budding yeast cells tend to be only 4–6 microns in diameter and can divide every 90 minutes, roughly half as fast as *E. coli*. Importantly, *S. cerevisiae* has a complex life cycle that includes an asexual phase, during which haploid cells divide by budding, and a sexual phase in which cells of different "mating types" fuse their genetic material and then divide by two successive rounds of meiosis (Barnett, 2007). Over the years, biologists have learned to control this process so that they can isolate recessive mutations in haploid, asexually dividing strains and then perform planned crosses using strains of different mating types. They have also figured out how to freeze and store mutant strains for long periods of time, thus creating immense strain libraries for later use. Another major technical breakthrough was the creation of the first transgenic yeast strain in 1978, using an *E. coli* plasmid (a small extrachromosomal piece of DNA) to insert DNA from one yeast strain into another (Hinnen et al., 1978). Soon it became easier to move genes in and out of yeast than any other species.

An especially powerful approach was gene cloning by complementation, which allows researchers to identify the genes underlying any particular mutation in yeast (Elledge et al., 1993). In essence, the approach involves transfecting a mutant yeast strain with a library of many different plasmids that each carry a different snippet of normal yeast DNA and then asking which plasmid is able to convert the mutant to a wild-type phenotype. The successful plasmid can then be replicated in *E. coli* to produce large amounts of the gene and protein of interest. Ultimately, researchers can use restriction enzymes to determine the gene's nucleotide sequence and the protein product to generate useful antibodies. Using these and related techniques, researchers identified many genes that function in critical aspects of yeast biology, including cell cycle control, metabolic pathways, DNA repair, protein targeting and degradation, and cell death (Botstein & Fink, 2011).

Surprisingly, many yeast genes were found to have homologs in the genomes of humans or other mammals. Even more surprising was that many of the human genes could substitute for their yeast homologs in the previously described complementation

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assays (Tugendreich et al., 1994). These findings supported the idea that most of the "basic" molecular functions of genes are broadly conserved across species. Based on this assumption and the ease with which gene functions could be studied in yeast, findings obtained in yeast came to dominate gene ontology databases, which were originally conceived as species-independent summaries of gene-function associations for "a generic eukaryotic cell" (Ashburner et al., 2000, p. 26). One should note, however, that one of the few studies to explore systematically the conservation of eukaryotic gene-function associations revealed that fewer than one-third of 621 examined yeast genes can be functionally replaced by their human homologs (after accounting for the likelihood of false-negative results) (Hamza et al., 2015). These data indicate a substantial amount of functional divergence between yeast genes and their human homologs.

Considerable divergence is also apparent when one compares the entire genome of *S. cerevisiae* to that of humans or other mammals. The yeast's nuclear genome is 12.8 megabase pairs in size and contains only about 5,600 genes. Roughly 30% of these genes have clearly identifiable mammalian homologs (Botstein et al., 1997); conversely, approximately 30% of human disease genes have yeast homologs with very similar nucleotide sequences (Foury, 1997). These data indicate both a significant amount of genetic conservation and substantial divergence. In this context, it is interesting to observe that the fission yeast *Schizosaccharomyces pombe* shares about 80% of its protein-coding genes with *S. cerevisiae* (Wood et al., 2002), from which it diverged roughly 1 billion years ago (Hedges, 2002). Similarly, it is intriguing that the network of genetic interactions in these two species exhibits substantial differences, even when one considers only homologous genes (Roguev et al., 2008). Whether these genetic differences are surprisingly large or surprisingly small depends on one's expectations, as it does for the yeast-human comparisons.

In any case, most yeast biologists surely agree that "yeast offers invaluable guidance for approaching human disease-associated gene functions. In contrast to humans, the *S. cerevisiae* genes can be easily deleted, mutated and reintroduced into yeast cells, overexpressed, tagged and thoroughly studied, very quickly providing a considerable amount of information useful for understanding the molecular basis of diseases" (Foury, 1997, p. 8). That said, most would also agree that "every major conclusion [reached in yeast] . . . will have eventually to be tested directly in higher organisms" (Botstein & Fink, 1988, p. 1442).

4.2 CELL AND TISSUE CULTURE MODELS

Many in vitro models do not involve microbes but instead entail cells or tissues that were originally isolated from multicellular organisms. The following sections briefly

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review the major types of models based on animal cells, focusing on their principal advantages and limitations.

4.2.1 Hanging Drop Cultures

The first cultures of explanted animal tissue were created by Ross Harrison in 1910, using tissue from the peripheral nervous system of frogs. Adapting a method previously used to observe living bacteria under the microscope, Harrison attached a fragment of frog tissue to a glass coverslip by means of coagulated lymph, which also provided the explanted cells with nutrients. The coverslip was then inverted and laid over an indentation that had been ground into a standard microscope slide so the "drop" of tissue could hang suspended from the coverslip (Millet & Gillette, 2012) (figure 4.3A). After sealing the contraption to prevent desiccation, Harrison was able to watch individual cells under the microscope. Most importantly, he observed individual neuronal cell bodies extend axons that grew longer over time and were tipped with distinctive growth cones. These observations helped to establish the neuron doctrine, which holds that neurons are discrete cells connected via synapses (Guillery, 2007).

Harrison's hanging drop technique was later modified by switching from lymph to clotted plasma, which was easier to work with, and extended to tissues from warm-blooded animals (Carrel & Burrows, 1911). The main advantage of the hanging drop technique remained the ability to observe the behavior of living cells, but the cultures also facilitated the design of elegant experiments. For example, Levi-Montalcini et al. (1954) explanted pieces of spinal ganglia together with bits of mouse tumors and found that the tumor cells promoted the outgrowth of axons from the explanted neurons. Subsequent research then revealed the molecular identity of the diffusible axon growth-promoting substance, which we now call nerve growth factor (Cohen, 1960).

A major limitation of the hanging drop technique was the tissue's relative inaccessibility, which made it difficult to supply the tissue with fresh nutrients and oxygen, or to create subcultures (i.e., starting new cultures from portions of the original culture). These issues were addressed by the development of special culture flasks (figure 4.3A) that were not as convenient for direct microscopic observation of the cultured cells but made it easier to change culture media, create subcultures, and generally maintain cultures for long periods of time.

4.2.2 Organotypic Slice Cultures

In early tissue culture experiments the explants consisted of tissue chunks that had been cut rather haphazardly. However, experimenters in the 1970s and 1980s began to make more deliberate cuts, sectioning the tissue into thin slices along carefully selected planes so that each slice would optimally reflect the tissue's internal organization. These organotypic slices were then cultured in clotted serum "drops" using a variety of

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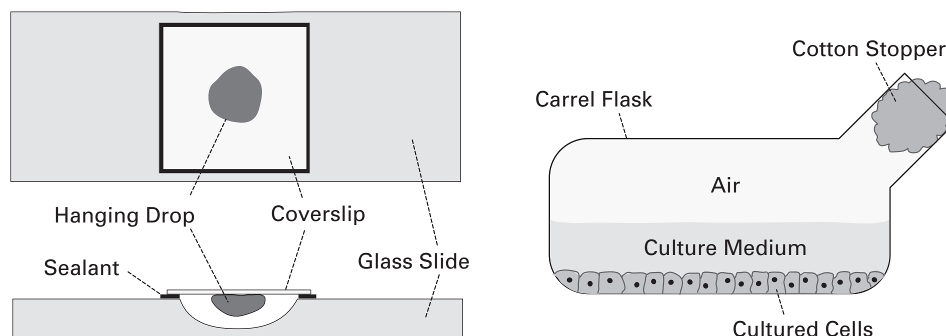

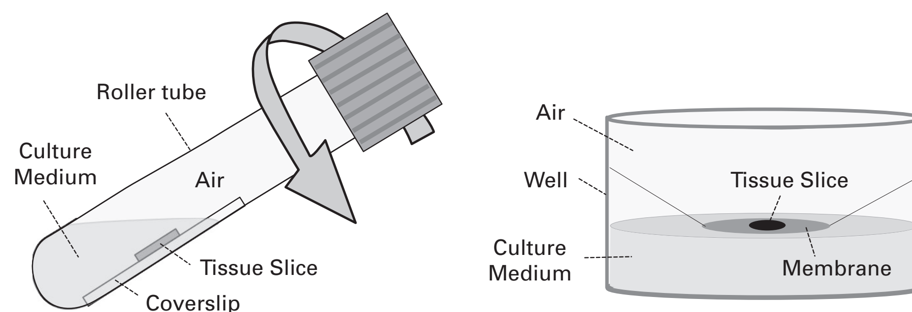

Figure 4.3
Cell and tissue culture methods. (*A*) Hanging drop cultures (top and side views on the left) are convenient for observing cells under a microscope, but so-called Carrel flasks (right) are better for long-term cultures because they facilitate the exchange of culture medium. (*B*) In organotypic slice cultures, thin slices of tissue can be cultured in rotating test tubes (left), where they are exposed alternately to culture medium and air, or on a semipermeable membrane that floats on the surface of culture medium (right). Adapted from (A) Wellbourne-Wood & Chatton (2018); (B) Cavaliere & Matute (2011).

protocols that exposed the slices to both culture medium and air, either alternately or at the interface between the two (figure 4.3B) (LaVail & Wolf, 1973; Gähwiler, 1981; Stoppini et al., 1991; Humpel, 2015).

Using these methods, and keeping slices to less than 500 micrometers in thickness, it is possible to maintain the cultures for several weeks or even months. By now, biologists have developed protocols for maintaining organotypic slice cultures of many different organs, including the kidney, lung, liver, and brain. They have also created organotypic cultures of structures like the retina that do not need to be sliced because they are inherently planar and thin.

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The principal advantage of organotypic cultures is that they maintain much of the tissue's natural organization while allowing experimenters to access the tissue in ways that are not possible or are extremely difficult in vivo. Particularly convenient is that the clot surrounding the tissue gradually disappears in culture and the slices themselves become much thinner over the course of two to three weeks, approaching single-cell thickness with some protocols. These changes make it relatively easy to see individual cells under a microscope and to record their electrical activity with microelectrodes, which is especially useful for elucidating neuronal functions. The use of voltage-sensitive molecules and fluorescence microscopy further allows investigators to monitor patterns of electrical activity within a slice (Phillips et al., 2015). Such experiments are aided by the fact that organotypic slices tend to be relatively healthy because, after one to two weeks in culture, most of the slicing-induced tissue damage has been cleared (Gähwiler, 1981, 1988). It is even possible to monitor some aspects of normal development in organotypic slices made from immature organs (LaVail & Wolf, 1973).

Of course, tissue slices cannot retain an organ's full complexity whenever important structural features extend over long distances in all three dimensions. Slices through the brain, for example, are bound to destroy some long-range connections. The culturing process may also induce some new, unnatural features. It is well established, for example, that organotypic slices through the hippocampus sprout some novel connections (Coltman et al., 1995). Yet another potential problem is that long-term cultures of brain slices may be distorted by the uncontrolled proliferation of glial cells. This problem is usually prevented by blocking all cell divisions in the slice either with radiation or pharmacologically.

Although brain slices can be cultured for many weeks, researchers often study brain slices in the first few hours after they have been cut. Such acute brain slices are maintained in elaborate slice chambers that continuously perfuse highly oxygenated medium across the slice, thereby minimizing cell death (Schwartzkroin, 1975). Such preparations have been used extensively in neurobiology to study intracellular and circuit-level processes. Their main advantage is that they do not depart from the natural, in vivo anatomy as much as the long-term slice cultures do. However, acute brain slices do exhibit significant damage near the cut surfaces, as well as extensive molecular and synaptic changes that can profoundly affect neuronal function (Kirov et al., 1999; Taubenfeld et al., 2002). Thus, they clearly are imperfect, albeit very useful, models.

4.2.3 Dissociated Cell Cultures

One major limitation of explanted tissue cultures is that the tissue tends to contain multiple cell types, which can make it difficult to gather detailed information about specific cells. Biologists have solved this problem by using mechanical forces and enzymes

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that destroy connections between cells to "dissociate" the explanted tissue. They then use cell type–specific antibodies to isolate the desired cells and start new, more homogeneous cultures. Many of the cells in such cultures will divide repeatedly (neurons being a major exception, since most mammalian neurons become postmitotic after embryogenesis). At some point the original (aka primary) cultures become overpopulated and risk deterioration, unless the experimenters take a subset of the cells to seed new subcultures.

Many cell types can be subcultured repeatedly, sometimes for years, but it is now generally agreed that normal somatic cells eventually become senescent, which means that they stop dividing and die. Fibroblasts (i.e., connective tissue cells) isolated from normal human fetuses, for example, can only divide approximately 50 times in ideal culture conditions (Hayflick & Moorhead, 1961; Witkowski, 1980). A major mechanism underlying cellular senescence involves the telomeres, which cap individual chromosomes and shorten with each cell division; when those telomeres become too short, DNA damage results. An interesting (albeit tentative) observation is that the doubling potential of primary cultures tends to increase with the life span and size of the species from which those cells originate (Hayflick, 1975), which in turn correlate with the average number of divisions those cells undergo in vivo (see the discussion of Peto's paradox in chapter 7, section 7.1.4).

Although explanted cells of many tissues can divide in vitro, explanted neurons from postembryonic individuals are usually postmitotic; the study of such primary neuronal cultures can nonetheless provide important results. In particular, dissociated neurons may be diluted to such an extent that individual neurons can be observed in relative isolation, independently of interactions with other cells. Such studies have shown, for example, that different types of neurons tend (to a large degree) to adopt their distinctive in vivo morphologies as they extend new processes in vitro (Bray, 1973; Banker & Goslin, 1998). Other studies have revealed the adhesion- and tension-based mechanisms through which axons elongate (Lamoureux et al., 1989), as well as some of the molecular cues that they use for guidance (Zheng et al., 1994).

4.2.4 Continuous Cell Lines

Because primary cell cultures and their subcultures have a limited life span, working with them requires taking samples from animals that likely suffer some distress as a result. This is true especially for cultured neurons, which become postmitotic as they differentiate. Another issue is that primary cell cultures tend to vary with the genotype of the donor animal (or human), and this variability can reduce the replicability of the experiments. All these problems are overcome by the creation of continuous cell lines, which are also known as immortal or immortalized cell lines. Many of these

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continuous cell lines are derived from tumor biopsies. HeLa cells, for example, were taken from the cervical tumor of a woman called Henrietta Lacks in 1951 (without informed consent, which was not standard at the time), and her cells have been dividing since then in laboratories across the world (Skloot, 2010). More than 60,000 published papers describe work on these cells (Bhatia et al., 2019), and the cells themselves are so hardy and proliferate so rapidly that they have contaminated or completely displaced numerous other cell lines (Gartler, 1968; Hughes et al., 2007; Capes-Davis et al., 2010). Indeed, some biologists have suggested that HeLa cells form a new species that is highly adapted to the laboratory niche (Van Valen & Maiorana, 1991).

Continuous cell lines can also arise spontaneously from primary cultures that have been subcultured repeatedly; presumably this kind of transformation occurs via mutations that would cause cancer if they occurred in vivo. Intriguingly, spontaneous immortalization appears to be extremely rare for chicken cells, more common for human cells, and most common for cells from mice (Macieira-Coelho et al., 1977). A third way to obtain continuous cell lines is to transform cultured cells with viruses that can insert cancer-causing genes into the genomes of their hosts (Cepko, 1989). Some of these genetically engineered cell lines proliferate in one set of culture conditions, but cease dividing and differentiate (e.g., into neurons) when the conditions (e.g., temperature) are changed; that is, they are conditionally transformed. Altogether, biologists now have at their disposal more than 4,000 continuous cell lines that they can order from various cell line repositories (e.g., the American Type Culture Collection at ATCC.org).

The principal advantage of dissociated cell cultures, especially continuous cell lines, is that researchers can examine the properties of specific cell types in tightly controlled contexts and do so repeatedly, using virtually identical (isogenic) cells. Thus, they can examine the effects of various genetic mutations or environmental manipulations and compare them across cell types. Continuous cell lines can also be used to synthesize therapeutic compounds. For example, a cell line derived from a hamster's ovary has long been used to synthesize tissue plasminogen activator, which helps dissolve blood clots (Rahimpour et al., 2016). Similarly, mouse and human cancer cells have been used to synthesize therapeutic antibodies by fusing them to antibody-producing spleen cells to create "hybridoma" cell lines (Köhler & Milstein, 1975; Olsson & Kaplan, 1980) (see chapter 5). Although mammalian cell lines tend to be less efficient at high-volume synthesis than *E. coli*, they contain more of the cellular machinery required for proper folding and post-translational modification of the desired, mammalian proteins (Wurm, 2004).

A key limitation of dissociated cell cultures is that cells tend to change their phenotype as they acclimate to culture conditions. For example, cultured lung endothelial

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cells express only about 42% of the membrane-associated proteins that they express in intact rats (Durr et al., 2004) (figure 4.4A). Similarly, placing microglia from human or mouse brains into primary cultures causes almost 4,000 genes in the explanted cells to be up- or down-regulated more than fourfold, relative to the in vivo condition (Gosselin et al., 2017). Transplantation experiments (with microglia derived from stem cells) suggest that many of these culture-induced changes in gene expression can be reversed when the cultured microglia are brought into their natural, in vivo environment (Hasselmann et al., 2019) (figure 4.4B), but this merely underscores that the culture environment can have profound effects on cellular phenotypes (Matarese et al., 2012). Because culture conditions may vary across laboratories and with different batches of culture medium ingredients (e.g., animal-derived serum), this form of phenotypic plasticity reduces replicability. A related problem plaguing primary cultures is that the more rapidly proliferating cell types may come to dominate the cultures, thereby causing experimental results to vary with time in culture.

A major problem with continuous cell lines is that they often exhibit significant genetic mutations. The aforementioned HeLa cells, for example, exhibit genetic abnormalities on 20 chromosomes, and four of their chromosomes are shattered into multiple pieces (Mittelman & Wilson, 2013). These abnormalities are probably extreme, but tumor-derived cell lines often exhibit genetic mutations and copy number variations that exceed those observed with in vivo tumors (Domcke et al., 2013). Spontaneously transformed cells are subject to analogous problems because the senescence-induced telomere shortening tends to cause chromosomal aberrations (Hornsby, 2007). In addition, continuous cell lines that are maintained as separate populations tend to diverge over time, as their cells acquire random mutations (via genetic drift) and adapt differentially to the cell culture environment. This has been well documented in a comparative analysis of various cancer cell lines (Ben-David et al., 2018). In the words of one commentator, “different stocks of widely used cancer cell lines—a staple of cancer research over many decades—are highly heterogeneous in terms of their genetics, transcriptomics and responses to therapies” (Hynds et al., 2018, p. 1). Many of these molecular changes probably relate to the previously mentioned selective pressure for increased proliferation rates in culture conditions (Auman, 2010).

For all of these reasons, many biologists agree that key experiments performed on continuous cell lines should be replicated in primary cells, at least if the intent is to obtain knowledge that applies also to in vivo conditions (Kaur & Dufour, 2012). Nonetheless, continuous cell lines are often selected as “model cells” for their availability, hardiness in culture, experimental tractability, and accumulated knowledge of their properties. PC12 cells, for example, have been widely used as a model for neurons and, in particular, for the study of transmitter release. This cell line was originally derived

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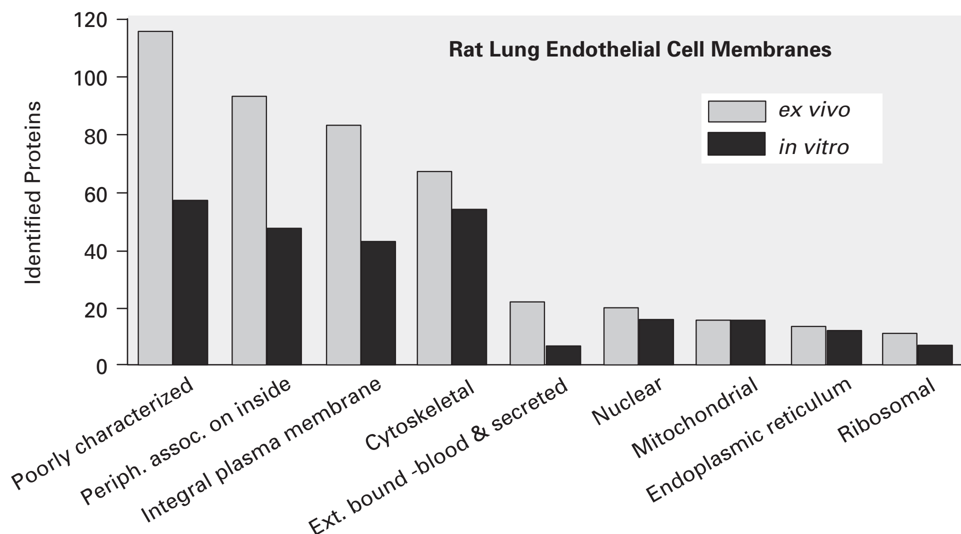

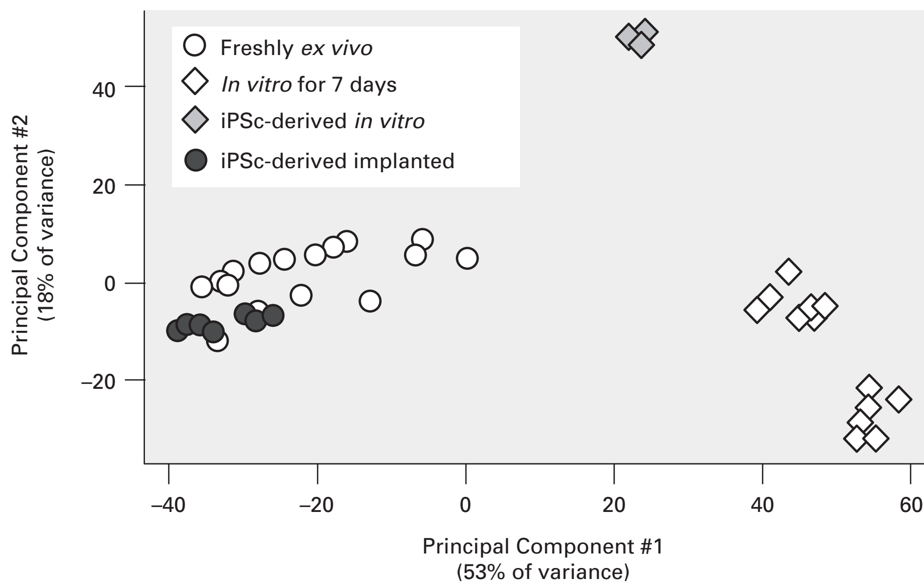

Figure 4.4

Cells are different in vitro versus in vivo. (*A*) Fresh ex vivo versus in vitro. Durr et al. (2004) isolated microvascular endothelial cells from rat lungs and then examined the proteins associated with their cell membranes, either right after isolating the cells (ex vivo) or after they had been cultured for some time. They found that many proteins were expressed ex vivo but not in vitro (varying somewhat with the presumed location of the proteins; shown along the *x*-axis). (*B*) Hasselmann and colleagues derived microglia from human induced pluripotent stem cells (iPSCs) and transplanted them into mouse brains. They found that the gene expression profile of the implanted microglia was quite similar to that of freshly isolated human microglia, but significantly different from that of the cells before implantation and from isolated human microglia cultured for seven days (as visualized in a principal components analysis). Adapted from (A) Durr et al. (2004); (B) Gosselin et al. (2017); Hasselmann et al. (2019).

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from a rat's adrenal gland tumor (Greene & Tischler, 1976), but its cells synthesize and release several neurotransmitters that are also found in postganglionic sympathetic neurons, which are developmentally related to the adrenaline-secreting cells of the adrenal gland. Treatment with nerve growth factor also causes PC12 cells to grow long processes that resemble axons. Therefore, these cells are clearly neuron-like. However, they do not develop dendrites or proper synapses, and their phenotype does not precisely match that of any in vivo neurons (Greene et al., 1998; Banker & Goslin 1998; Westerink & Ewing, 2008).

Thus, PC12 cells are also an example of "*in vivo* veritas—*in vitro* artificia" (Matarese et al., 2012). Fortunately, most cell biologists are keenly aware of this issue and usually perform critical experiments on multiple cell types and, not infrequently, on primary cells and intact animals as well.

4.2.5 Stem Cells

In the last two to three decades, some of the most commonly cultured cell types have been stem cells, which we can define as self-renewing proliferating cells that, given various specific treatments, can differentiate into a broad range of cell types. Stem cells vary in the range of potential cell fates they may pursue, but most of the in vitro research has focused embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). The former are derived from very early (preimplantation) embryos and can develop into virtually all types of adult cells. Mouse ESCs were first created in 1981, but efforts to culture human ESCs were unsuccessful until researchers began to "feed" them with factors derived from cultured mouse cells (Thomson et al., 1998). It also helped to recognize that mouse and human ESCs respond differently to a few critical molecules (Yu & Thomson, 2008). For example, the transcription factor c-Myc is used to promote self-renewal in mouse ESCs, but it causes human ESCs to differentiate or die (Sumi et al., 2007; Watt et al., 2008).

In contrast to ESCs, iPSCs are derived from adult somatic cells (e.g., skin cells) that have been engineered to express, or otherwise treated with, several transcription factors that collectively "induce" the cells to dedifferentiate and then become capable of redifferentiating into a wide variety of different cell types. Essentially, iPSCs are adult somatic cells that have been reprogrammed in vitro to become similar to ESCs. They even resemble ESCs in terms of their "landscape" of epigenetic modifications (Guenther et al., 2010), which change systematically across development and regulate cellular differentiation (Atlasi & Stunnenberg, 2017). However, closer analyses have revealed some significant differences between the epigenomes of iPSCs and ESCs, as well as between different iPSC lines (Lister et al., 2011). These differences imply that the reprogramming of the iPSCs is often incomplete, which causes the cells to have a

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"memory" of their somatic origin (Efrat, 2021). The reprogramming also tends to be somewhat aberrant, which causes iPSC lines derived using identical protocols to be notoriously variable (Ortmann & Vallier, 2017; Bar & Benvenisty, 2019). The first human iPSCs were created in 2007, one year after the discovery of mouse iPSCs (Takahashi et al., 2007).

Mouse ESCs and iPSCs can be used in place of mouse primary cell cultures so long as protocols have been developed to differentiate the stem cells into the desired cell types (McQuade et al., 2018); their principal advantage is the reduced animal sacrifice. Similarly, human ESCs can be derived from unwanted preimplantation embryos containing just 200 to 300 cells, and human iPSCs can be derived from easily harvested tissue (e.g., skin or connective tissue). Especially useful is that these stem cells can be derived from human subjects with known genetic diseases, thus creating cellular disease models that are "genetically precise" (Kaye & Finkbeiner, 2013). The patient-derived cells can then be studied in vitro to gain a better understanding of the molecular disease mechanisms or to test which of several possible therapies is most effective on a patient's own cells. Patient-derived stem cells can also be differentiated into a desired cell type and transplanted back into the patient without risking the immune rejection that usually accompanies transplantation of foreign cells. The implanted cells may be able to replace cells that were lost as part of the disease or they may secrete various factors that can slow or reverse disease progression (Drago et al., 2013; Grade & Götz, 2017; Sharma et al., 2019). If necessary, scientists can "correct" the mutations of the stem cells before the transplantation by use of genome editing techniques (An et al., 2012). Genetically corrected cells can also serve as an ideal control group in experiments on disease mechanisms because they are isogenic with the patient's cells except for the disease-causing defects.

Despite their enormous promise, stem cells have significant limitations. For human ESCs, the principal concern is that their use requires the destruction of human embryos. Although those embryos are usually less than two weeks old and left over after in vitro fertilization (IVF) (Hyun et al., 2016), they have the potential to develop into full-fledged human beings. Many people—including many with pro-life views (Lo & Parham, 2009)—find such a use of human IVF embryos acceptable, but public attitudes on this subject are varied and complex, as illustrated by the extended debates over the tight restrictions on research with human ESCs that were imposed on US researchers while George W. Bush was president (Connolly, 2005). Fortunately, those ethical concerns can be sidestepped entirely by working with human iPSCs because they are derived from tissue humans can spare. Nonetheless, the donors of the original cells must be fully informed and then consent to how their cells are going to be used (Lo & Parham, 2009).

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A second limitation of stem cells is that they tend to acquire significant genetic abnormalities during their time in culture, ranging from extra chromosomes to insertions, deletions, and point mutations (Draper et al., 2004; Gaztelumendi & Nogués, 2014; Martin, 2017). Some of these aberrations may increase the fitness of the cells in the culture environment, and not all of them would necessarily be harmful in vivo, but they clearly make the behavior of extensively cultured stem cells less predictable for purposes of experimentation or therapy. The fact that stem cell cultures vary with the genotype of the organism from which they were derived, together with the "aberrant" epigenetic reprogramming mentioned previously, further increases this unpredictability. Given this variability, one wonders whether regulators charged with ensuring the safety and efficacy of clinical products might have to approve each batch of stem cells separately (Anderson & Cummings, 2016). A related problem is that the differentiation of stem cells before transplantation is rarely complete, which means that some of the transplanted cells may remain proliferative and, hence, potentially tumorigenic (Kaye & Finkbeiner, 2013; Burns & Verfaillie, 2015).

A third significant complication is that human stem cells intended for transplantation into humans must first be tested in animals. However, transplanting human cells into mice tends to trigger a strong immune response that kills the foreign cells unless the mice are genetically modified to lack crucial parts of the immune system (e.g., Marsh et al., 2017). This is potentially problematic because immune responses are integral to many diseases, either as part of the pathology or in response to it (Doty et al., 2015) (see chapter 5). Therefore, modeling such diseases in immunodeficient mice may not accurately predict the human response. A potential solution to this dilemma is to work with mice that have a "humanized" immune system (L. D. Shultz et al., 2007; Yong et al., 2018), but this humanization can only be partial. As a result of all these uncertainties, it currently remains unclear to what extent stem cell therapies will ultimately live up to their enormous potential (Marcheque et al., 2019). The most promising result to date is that the transplantation iPSC-derived cells into patients with macular degeneration stabilized the vision of one patient for at least one year (Mandai et al., 2017). However, the US Food and Drug Administration has not yet approved any human stem cell therapies other than the transplantation of hematopoietic (i.e., blood cell producing) stem cells from human umbilical cords (FDA Commissioner, 2020).

Thus, despite their promise, stem cell therapies should be regarded cautiously (Singh et al., 2020). Unfortunately, this caution is often drowned out by hope and greed, which is why poorly regulated for-profit centers around the world are offering a wide variety of unapproved stem cell therapies, many of which are probably ineffective, and some of which can cause real harm (Cyranoski, 2013; Turner & Knoepfler,

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2016). Debates about how best to regulate these therapies and then enforce those regulations are ongoing (Marks et al., 2017; Laurencin & McClinton, 2020). It is not an easy problem to solve, especially because the diversity of stem cell types and treatment procedures is still expanding rapidly.

4.2.6 Complex Culture Systems

Dissociated and stem cell cultures are usually grown with the goal of culturing a single cell type, but sometimes it is useful to co-culture two or more cell types that interact. For example, the previously mentioned discovery of nerve growth factor involved the co-culture of explanted neurons and a separate set of growth factor–secreting cells (Levi-Montalcini et al., 1954). In those experiments the different cell types were placed next to one another within a single hanging drop, but later co-culture experiments often employed culture chambers with multiple compartments that were interconnected in ways that allow cells in different compartments to interact, either through the culture medium or, in some cases, via axons that grow from one compartment into another (Campenot, 1977).

Starting in the early 2000s, culture chambers with multiple compartments were miniaturized and outfitted with pumps that cause the culture medium to flow along channels that are only a few hundred microns wide (Beebe et al., 2002; Whitesides, 2006). These "microfluidic" devices minimize the quantities of cells and culture medium, thereby reducing costs. They can also be augmented by depositing molecular cues, growth factors, or extracellular matrix onto specific surfaces in one or more of the compartments. Furthermore, they may be equipped with miniature motors that create dynamic physical forces similar to those found in vivo, such as heart muscle contractions or gut peristalsis (Huh et al., 2010; Balijepalli & Sivaramakrishan, 2017; Haring et al., 2017). Because microfluidic devices typically contain multiple cell types and are manufactured using methods similar to those used in the production of microchips, they are often called organs-on-a-chip. Compared with traditional culture chambers, the on-chip systems provide the cultured cells with a far more physiological, in vivo–like environment (Li et al., 2005). This verisimilitude clearly facilitates the modeling of both normal and disease-related processes. However, organs-on-a-chip are commonly populated with cells from continuous cell lines (Marx et al., 2012), which keeps costs low but brings with it the genetic abnormalities and other problems (as mentioned in section 4.2.4).

Another way to generate complex cell cultures is to let the cells grow in three dimensions (3D), rather than the two dimensions (2D) of traditional cell culture plates. Except for epithelia, most cells of the body grow in complex 3D environments that are filled with other cells and extracellular matrix. These conditions can be

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mimicked in vitro by culturing cells in biodegradable scaffolds that resemble extracellular matrix. Such scaffolds may be created in diverse ways, but they typically involve natural or synthetic polymer networks that contain a lot of water. Cells can be mixed into these hydrogels prior to hardening, or they can deposited on top of the matrix and migrate into it (Merceron & Murphy 2015; Funaki & Janmey 2017; Lovett et al., 2020). Such 3D cultures can also be integrated with the on-chip devices described previously, and perfusable channels can be designed into the 3D matrices using micromolds or 3D printing (Xie et al., 2020). Overall, work on these systems has shown that many cells behave differently when grown in 3D versus 2D environments and that they are responsive to the physical and chemical properties of the surrounding matrix. In general, the 3D cultures more closely mimic the in vivo condition (e.g., Pickl and Ries, 2009). This is true especially for the culture of stem cells, which tend to exhibit some "spontaneous morphogenesis" (Pașca, 2018) when cultured in 3D systems, often forming an organoid (Lancaster & Knoblich, 2014).

Organoids result when stem cells self-aggregate in culture and then begin to differentiate into several different cell types, which in turn self-organize to create morphologies that bear some similarity to in vivo organs. By now, scientists have managed to create organoids of many different tissues, including blood vessels, kidneys, intestines, retina, and brain (Kretzschmar & Clevers, 2016; Boutin et al., 2019). The latter are particularly impressive, as they exhibit multiple types of neurons arranged in layers that resemble those of the cerebral cortex (Lancaster et al., 2013). These cerebral organoids, affectionately known as mini-brains, make it possible to study human brain development in vitro. They can also be used to create and study models of various diseases. For example, cerebral organoids have been used to develop potential treatments against Zika virus infection (Y.-P. Xu et al., 2019) and to create a model of Alzheimer's disease that resembles the human disease more precisely than any other in vitro system (Choi et al., 2014). However, mini-brains and other types of organoids typically lack a functioning vasculature, which means that they must be relatively small and frequently contain dead or dying cells in their center. Furthermore, an analysis of many different mini-brains revealed that their constituent neurons do not fully mimic the in vivo cell types and, in general, exhibit symptoms of significant cellular stress (Bhaduri et al., 2020).

An interesting approach to overcoming some of these problems is to implant organoids into live animals. Kidney organoids derived from human iPSCs, for example, have been transplanted into mouse kidneys, where they survive for at least six weeks and become at least partially vascularized (Nam et al., 2019). Similarly, cerebral organoids become vascularized upon transplantation into mouse brains; they even develop active neuronal connections and are infiltrated by microglia, which were not

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present in the original organoids (Mansour et al., 2018). Ultimately, researchers aim to transplant organoids into humans to replace or repair damaged organs. For now, however, this goal remains a distant one. The aforementioned kidney organoids, for example, remain immature after transplantation and develop some abnormal features, such as cartilage and cysts. Similarly distant are dreams (or nightmares) of mini-brains attaining consciousness (Reardon, 2018) or becoming capable of suffering. Still, the mere existence of mini-brains would have been considered science fiction a few decades ago. Indeed, when one recalls the humble beginnings of cell culture as hanging drops under Harrison's microscope, one must marvel at how far this field has come.

4.3 ALTERNATIVE MODELS IN TOXICOLOGY

Humans generally want to know which pesticides, cosmetics, pharmaceutical products, or other substances are likely to make them sick. Historically, this need was met by examining the effects of these chemicals on a variety of mammals, especially rodents, rabbits, and dogs. Such toxicity testing was mandated by governments in response to major disasters in which humans were exposed to highly toxic compounds. The prime example is elixir of sulfanilamide (i.e., sulfanilamide dissolved in diethylene glycol), which had been marketed as an antibiotic (see chapter 5) but not been tested for toxicity; it killed at least 100 people in 1937 before it was pulled from the market (Ballentine, 1981). An even more influential disaster was the drug thalidomide, which was widely taken by pregnant women in the 1950s to prevent morning sickness. It ended up causing at least 10,000 stillbirths and birth defects worldwide because, sadly, it had not been tested for detrimental effects on fetal development (Rehman et al., 2011).

By now, scientists and regulatory agencies have developed a broad array of animal toxicity tests that include diverse forms of chemical exposure (e.g., inhalation versus ingestion) and look for various kinds of toxicity (National Research Council, 2006). In 2019 roughly a quarter of all experimental procedures conducted on vertebrates in the United Kingdom were performed to satisfy regulatory requirements. However, the ability of these animal tests to predict human toxicity is far from perfect, and differences among test species are relatively common (Calabrese, 1988; Bailey et al., 2005) (figure 4.5A). Aspirin, for example, is substantially more to toxic to cats than to dogs or humans, mainly because cats are slower to clear it from their circulation (Court, 2013). The sensitivity to various carcinogens likewise varies across species, with the concordance (i.e., rate of agreement on both positives and negatives) between rats and mice being just 74% (Haseman, 2000; Smith & Anderson, 2017; Smith & Perfetti, 2018). Given this prevalence of species differences, regulatory agencies usually request toxicity testing in two different species, one of which should be a nonrodent; the latter

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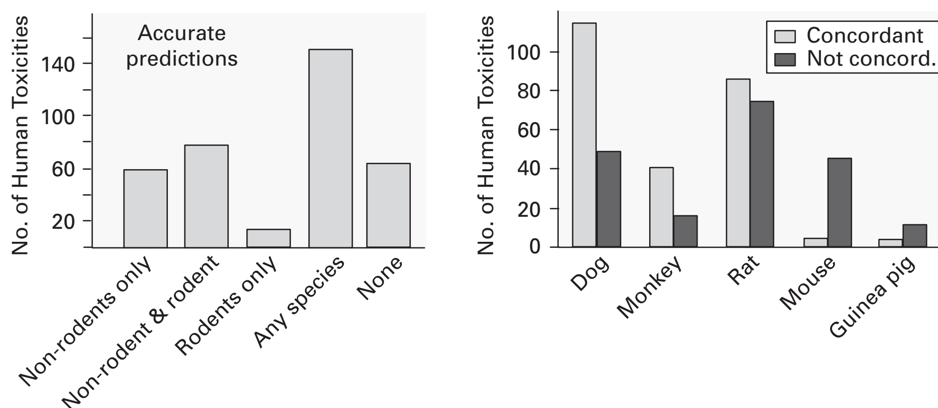

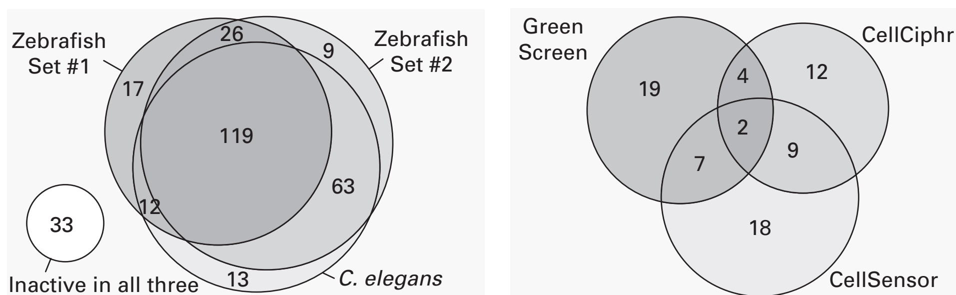

Figure 4.5

Concordance and discordance in toxicity testing. (*A*) Mammalian toxicity testing. Olson et al. (2000) gathered human and animal toxicity data for 150 compounds and asked how frequently the data from various species were concordant with the human data. *Left:* Tests on nonrodents (mainly dogs) are better than tests on rodents (mainly rats) at predicting human toxicity, and combining the results from rodents and nonrodents increases test sensitivity. *Right:* The ratio of correct positives to false predictions (i.e., nonconcordance) is higher in dogs and monkeys than in rodents. (*B*) Non-mammalian and in vitro toxicity screens. *Left:* Results of developmental toxicity tests for 292 compounds in two sets of zebrafish and one set of nematodes (*Caenorhabditis elegans*). Roughly half of the compounds were correctly identified as being either toxic or inactive in all three assays. The results from the two zebrafish assays were 65% concordant. Additional analyses (not shown) revealed that the zebrafish and *C. elegans* data predict rabbit and rat toxicity with a balanced accuracy of approximately 50% (averaging the true-positive and true-negative rates). *Right:* Results of geno- and cytotoxicity testing for 309 compounds in three different in vitro assays based on human cell lines. The overlap between the three data sets is low. Additional analyses indicated that the concordance rates between the individual assays and the Ames test or rodent tumorigenicity data lie between 50% and 60%. Adapted from (A) Olson et al., 2000, in part by recategorizing rabbits as nonrodents; (B) Boyd et al. (2016); Knight et al. (2009).

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is usually a dog, a monkey, or, increasingly, a pig (Brown et al., 2013; Monticello et al., 2017). Although this approach increases the likelihood of detecting true positives, it can also promote false positives. A more general problem with this approach is that it entails a significant amount of animal pain and suffering; it is also expensive and slow. As a result, many compounds have yet to be tested, and the backlog is growing.

In hopes of solving this problem, biologists have begun to perform toxicity tests in diverse non-mammalian animals, notably zebrafish (Cassar et al., 2020) (see also chapter 3, section 3.7.3). This species is well suited for high-throughput testing and raises relatively few ethical concerns, at least for the first five days after fertilization (before the larvae begin hunting for food; Strähle et al., 2012). Moreover, test results in zebrafish are positively correlated with at least some of the analogous mammalian data (Milan et al., 2003; Sipes et al., 2011). However, two different zebrafish toxicity studies examining the same large set of substances yielded a concordance rate of just 65% (figure 4.5B), and both studies predicted the analogous rabbit and rat data with only about 50% accuracy (calculated as the average of true-positive and true-negative rates); analogous screens on the roundworm *Caenorhabditis elegans* yielded quantitatively similar results (Boyd et al., 2016). Whether such concordance/accuracy rates are disappointing or promising depends on your perspective and interests, but they do appear to be lower than those usually reported for nonhuman primates or rodents. Nonetheless, a high degree of discordance could result from various factors other than species differences. One might note, for example, that exposing zebrafish to potential toxins dissolved in ambient water is very different than having rats inhale or ingest the same compounds. This might explain, at least in part, why "the zebrafish was on average 180 times more sensitive than the mammalian system for inhalation [of the same compounds]" (Ducharme et al., 2015).

A promising alternative to toxicity testing in animals is to perform it in vitro. A significant advance in this direction was the development of the Ames mutagenesis test, which uses a mutant strain of the bacterium *Salmonella typhimurium* to quantify a test compound's ability to reverse the effect of the original mutation, indicating that the substance is a mutagen and, by implication, a likely carcinogen. Early studies suggested that the Ames test identifies 90% of known carcinogens (McCann et al., 1975), but later research with larger libraries of test compounds found its rate of true-positive results to range from just 45% to 60%, with a false-positive rate of 14% to 25%, respectively (Tennant et al., 1987; Kirkland et al., 2005). Moreover, roughly 60% of 331 compounds that induce tumors in mice or rats fail to indicate mutagenicity in the Ames test, indicating either that the rodent assays overestimate carcinogenicity (Smith & Perfetti, 2018) or that many carcinogens induce tumors by something other than mutagenesis, such as increasing cellular proliferation or impairing the body's ability

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to fight incipient tumors (Shaw & Jones, 1994). Both possibilities are likely true, and species differences are certainly involved as well. Thus, although the Ames test is often hailed as the gold standard for in vitro testing, its ability to predict mammalian carcinogenesis is not as strong as researchers initially believed (Walmsley & Billinton, 2011).

A more recent development is the push for a "new paradigm" in toxicology that calls for in vitro testing on human cells (National Research Council, 2007). This idea is appealing because the use of human cells removes the specter of species differences; it also eliminates most ethical concerns, reduces costs, and in all likelihood accelerates testing. Although such tests do not allow researchers to quantify toxicity at the whole organism level, signs of cellular toxicity (e.g., DNA damage) can certainly be quantified. Researchers can also use modern molecular techniques to examine whether a test compound perturbs specific molecular and cellular pathways, especially those deemed to be "adverse outcome pathways" (Villeneuve et al., 2014). Although this strategy has been widely hailed and adopted (Kleinstreuer et al., 2013), serious questions remain. Particularly concerning is that the use of many different tests, assembled into test batteries, may lead to numerous false positives (figure 4.5B). In the words of Cox et al. (2016) "the predictive power of the in vitro test batteries is not much better than would be achieved by simply assuming that all chemicals are rodent carcinogens, thus creating excellent sensitivity (no false negatives) but poor specificity (many false positives)" (p. 55).

Another issue with the in vitro tests is that one must decide which human cells ought to be used and how they are to be cultured. The cells most frequently used for in vitro toxicity testing are primary human hepatocytes. These cells are a good choice because most toxins affect the liver, and hepatocytes are the dominant liver cell type, performing most liver functions. In addition, many substances do not become toxic until they are metabolized by liver cells, which would lead to false-negative results if cells from other organs were used. It can be difficult to obtain healthy human hepatocytes for primary cultures, but samples obtained from liver surgeries are sometimes available.

Sadly, explanted hepatocytes tend not to proliferate in vitro, even though liver cells in vivo can regenerate. An additional problem is that hepatocytes rapidly dedifferentiate in culture and undergo profound metabolic alterations (Cassim et al., 2017). Indeed, a proteomic comparison between fresh livers and hepatocytes that were cultured under standard conditions for two days found 457 proteins to be differentially expressed (Bell et al., 2016). The expression changes were less dramatic when the hepatocytes were cultured as small 3D aggregates (i.e., spheroids), but they were nonetheless significant. Scientists are trying to overcome these limitations by making the culture conditions (e.g., oxygen concentrations and substrate stiffness) more naturalistic, but obstacles remain (Ruoß et al., 2020).

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Aside from primary hepatocytes, toxicologists have used a variety of other human-derived cells. Some have used hepatocyte-derived continuous cell lines, which solves the challenge of obtaining the cells but decreases the model's similarity to the in vivo condition. Others use stem cell–derived hepatocyte-like cells (Vinken et al., 2012). Yet other groups combine primary hepatocytes with nonliver cells, such as iPSC-derived heart muscle and motor neurons derived from fetal stem cells, in multicompartment body-on-a-chip cultures (Oleaga et al., 2019). Such systems have enormous potential, but their ability to predict in vivo toxicities is just starting to be examined (Herland et al., 2020). They are also neither simple nor cheap.

Many in vitro toxicity tests are designed specifically to replace the Draize eye test, which uses rabbits to test compounds for their tendency to cause eye irritation or damage. These Draize tests, which had been used extensively in the cosmetics industry, predict the human reaction roughly 85% of the time (Wilhelmus, 2001). However, they have long been criticized for the distress they cause rabbits. In response, many scientists have sought suitable in vitro alternatives. An exciting recent advance is the use of human corneal epithelium cells to create a 3D in vitro epithelium that closely resembles the human cornea. These cutting-edge in vitro tests can predict eye irritation with 80% to 86% accuracy (Organisation for Economic Co-operation and Development [OECD], 2019; Lim et al., 2019). Unfortunately, they cannot distinguish between eye irritation and serious eye damage, and regulators admit that "in the foreseeable future, no single in vitro test method will be able to fully replace the *in vivo* Draize eye test" (OECD, 2019, p. 2). Still progress on this front has been remarkable, which is timely considering that the European Union in 2013 banned the marketing of all new cosmetic products developed with animal testing.

In general, we can conclude that no single test, be it in vitro or in vivo, can fully predict human toxicity. Cell culture artifacts and species differences stand in the way. The most widely accepted solution to this dilemma is to employ multiple tests, such as a rodent and a nonrodent species, or a whole battery of in vitro tests. But what are we to do when test results are discordant? A positive in vitro result is usually used to prioritize the test compound for further testing, including in vivo tests; alternatively, it might halt product development. This seems reasonable and safety conscious, but how many products are prematurely abandoned on the basis of false-positive results? Nobody knows. By contrast, false negatives seem easier to guard against so long as a sufficient number of different tests are conducted and any positive results trigger warnings—but how many different tests are sufficient? Moreover, as the number of tests goes up, so will the number of false positives. To address at least some of these issues, scientists are using computer algorithms and machine learning techniques to analyze many different kinds of toxicity-related data, ranging from chemical structure

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analysis to in vivo testing. Not surprisingly, they find that integrating all of the available data provides the best results (Guan et al., 2018). Unfortunately, this does not tell us which tests are "best." Perhaps that question is ill-posed.

Instead of seeking a single test that perfectly predicts human toxicity, we should probably employ a variety of tests and then seek to understand the variation between them so that we can weigh the evidence accordingly (Adriaens et al., 2018). In addition, it will be important to collect more data on what is toxic to humans; we currently seem to have more detailed information on toxicity in rodents than humans. In that context, it is interesting to note that toxicity testing historically developed after scientists discovered that humans tended to get sick after engaging in certain activities, such as chimney sweeping, or being exposed to certain chemicals (Adami et al., 2011). The putative toxins were then tested in animals to confirm the hypothesized effects and elucidate the toxic mechanisms of action. This kind of epidemiological research still plays an important role in toxicology, and it is likely to persist.

4.4 MODEL ECOLOGY REVISITED

Some animal and in vitro models became widely used but then waned in popularity; others are still ascendant. It is tempting to view these fluctuations as simple fads. As Erwin Chargaff, who made significant contributions to our understanding of DNA, put it in 1976,

> At one time, you could get money only for work on animal organs; bacteria were out. Then suddenly bacteria were in, everything else was taboo. A little later, all this changed again. Bacteriophage workers, once the kings of the coven, now populate the debtors' prisons. Procaryotes are proscribed, eucaryotes acclaimed. Animal viruses, long an object of suspicion, commiseration, and neglect, are highly quoted and oversubscribed. . . . It is the style of a period that conditions, or even compels, us to accept and adopt its various fashions or fads. (Gest, 1995)

Extending Chargaff's view, we might say that mice now rule the day, together with stem cells. It is simplistic, however, to view the historical fluctuations in model popularity as mere fashions. It is more instructive to view them as the outcome of a competition between models, creating a sort of ecology in which only the fittest thrive (see chapter 3, section 3.8). In addition, it is important to realize that the models themselves are hierarchically organized, with the major types having subtypes (e.g., different mouse strains or cell types), which in turn may be composed of multiple variants (e.g., different housing or culture conditions). From this perspective, model systems can be viewed as evolving at multiple levels, so that major model types may

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well be popular for an extended period of time while also changing in their specifics. The fascinating question then becomes: What factors govern the success or demise of the individual, competing models?

One critical factor, enshrined in Krogh's principle (see chapter 2), is experimental convenience. This term embraces many dimensions, including animal/materials costs and availability, housing requirements, and experimental tractability. For genetic studies and many in vitro experiments, the rate of organismal reproduction and cell proliferation, respectively, are also key variables—the faster the better, other things being equal. The availability of highly standardized animals and cell types is another significant aspect of convenience, as variability among research subjects makes it more difficult to obtain statistically significant results. In general, researchers tend to prefer to study models that allow them to obtain statistically significant, replicable results in relatively short amounts of time. Given the importance of research productivity in academic promotions and grant reviews, this makes good sense.

A second major factor is that researchers often want their findings to be generalizable, or, more formally, they want their model to have broad "representational scope" (Ankeny & Leonelli, 2011). A model's scope is difficult to determine ahead of time, however, since even closely related species and cell types may differ in some unexpected respects. Still researchers tend to be aware that some of the experimentally most-convenient organisms—dubbed "Krogh organisms" by Green et al. (2018)—possess at least a few features that are unlikely to be found in other species. Owls, for example, are exceptionally good at localizing sounds, and the study of the mechanisms underlying this capacity has yielded beautiful results (Carr & Konishi, 1988), but some of the specific mechanisms employed by owls do not generalize to mammals (Grothe et al., 2010). Therefore, research on sound localization with owls has now been supplemented with extensive studies in mammalian species (Grothe & Pecka, 2014). Similarly, paramecium had been a heavily studied microbe in the 1950s and 1960s, but it fell out of favor because many aspects of its genetics did not generalize to other species (Preer, 1997).

In short, for a model system to attract practitioners, it should be "experimentally tractable yet at the same time typical enough so that lessons learned in the model have a good likelihood of still being true in many other organisms" (Botstein and Fink, 1988, p. 1440). This does not mean that the study of unusual features cannot be justified. For one thing, it can reveal or confirm general principles, which is how August Krogh himself approached the study of extreme organisms (see chapters 2 and 7). For another, discoveries made in unusual species can be very useful for specific applications. The polymerase chain reaction (PCR) technique employed in DNA sequencing, for example, was made possible by the discovery of an exceptionally heat-resistant DNA polymerase only found in a bacterium adapted to hot springs (Chien et al., 1976).

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The third principal factor influencing model selection is the concern for animal welfare. As reviewed in chapter 3, the history of animal models demonstrates an overall movement away from nonhuman primates and pets toward food animals, pests, and other organisms that elicit few ethical concerns. This movement peaked in the early days of molecular biology, when bacteria, viruses, and yeast reigned supreme. Then, as investigators turned their attention to more complex aspects of biology, multicellular animals were once again ascendant, although worms, fish, rats, and mice took center stage. Nonhuman primates were generally used only for studies where similarity to human is paramount, such as late-stage safety testing. The general calculus is this: "What animals are enough like us to make laboratory results obtained from them generalizable to humans, but not so much like us that we ethically prohibit their being the subjects of experiments?" (Rader, 2004, p. 22). As discussed further in chapter 7, different people tend to answer this question differently, based largely on the research questions that interest them and on their rather subjective notion of how similar the various animals are to humans.

One of the attractions of in vitro biology is that it circumvents the ethical concerns over animal welfare almost entirely, as long as no animals or embryos are killed to harvest cells. Historically, in vitro models experienced a shift in prevalence from tissue explants to continuous cell lines and, more recently, to human cells, especially human stem cells and their derivatives. Clearly, the principal attraction of working with human cells is that they eliminate the concern over species differences that plagues animal research. However, in its place we find the challenge of making the in vitro models complex enough to answer questions about intact humans. To paraphrase Rader (from the previous paragraph), the question is, Which in vitro systems are enough like us to make laboratory results obtained from them generalizable to humans in vivo? The question is clearly troublesome, as demonstrated by the efforts of so many researchers to create ever more complex in vitro systems, be they co-cultures, organs-on-a-chip, or organoids. Unfortunately, as the complexity of these systems goes up, their experimental convenience (in terms of costs and required experimental expertise) tends to decrease.

The fourth major factor governing model selection is sociological. As many science historians have noted, the success of a particular model can often be attributed to one or a few particularly brilliant and charismatic leaders who fostered the development of a collaborative community centered around their model of choice (Kohler, 1994; Rader, 2004; Endersby, 2007). These communities share animals, technical resources, and the latest data, and they generally make their members feel that their research has an eager audience. By comparison, developing a new model is lonely, often frustrating work. Indeed, the mere fact that a model has attracted a number of fans and resources is likely to increase its popularity—and grant funding—further. It is an example of

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the Matthew effect, according to which "to him who has will more be given, and he will have abundance; but from him who has not, even what he has will be taken away" (Matthew 13; quoted in Merton, 1968). Similarly, the accumulation of knowledge about a model system stimulates further research on that model, as researchers can build on the existing information (Matthews & Vosshall, 2020). In the words of Joshua Lederberg, who pioneered much of the early work on *E. coli*, "The very accumulation of knowledge, mostly concentrated on a single strain, 'K-12,' made it more likely that it would be a prototype for still further studies" (Lederberg, 1998, p. 231). That said, scientists sometimes do develop new models (e.g., consider Benzer's switch from *E. coli* and phage to flies), regardless of the obstacles and personal investments they may have already made in established models (i.e., sunk costs). It is a risky but often effective way to open new frontiers and make one's name.

Finally, models wax and wane in popularity because the questions of interest to scientists change over time. Some questions in biology lose their fascination before they are fully resolved, but many do get answered to the satisfaction of the scientific community. Either way, new questions arise, and often they require new models to answer them. The fundamentals of the genetic code, for instance, were worked out in bacteria, but scientists then turned to eukaryotes for the study of more complex processes. Similarly, fruit fly genetics was largely replaced by mouse and zebrafish genetics as research interests shifted from the basics of Mendelian inheritance to the control of vertebrate development and cancer biology.

These observations are consistent with the commonly expressed idea that the selection of a model system should be driven by the question that is asked. This is certainly true insofar as scientific progress generally depends on selecting an appropriate model. However, as knowledge about a model system accumulates and the associated research community grows, it becomes increasingly likely that the model itself will determine the research questions rather than the other way around. When this happens, scientists risk learning more and more about the model but losing sight of how the model relates to other models and its presumed targets (Horrobin, 2003). Such concerns should not be overblown, as model-specific knowledge is often necessary to answer general questions. However, the fact that one observes a relatively high degree of discordance among the various models and their target (e.g., in toxicology) suggests that some caution is warranted.

In particular, it is good to remember that generality should never be assumed. Fortunately, having detailed information about one model makes it easier to examine other, related models and, thus, to ascertain how general the findings are (Striedter et al., 2014). That is, once a model has been studied in detail, the next step is—or ought to be—determining its representational scope. The narrower a model's scope, the less likely it is to remain popular for long.

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Model Systems in Biology

History, Philosophy, and Practical Concerns

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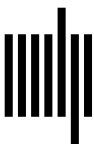

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