disease, juvenile diabetes, and so on: For each of these illnesses stem cells might be able to produce a cure by replacing cell function. This area of hope was termed regenerative medicine, because of the emphasis on regenerating lost function. With much at stake, different sides became polarized in their positions. In the midst of debate, we lost track of what was at issue. And we lost track of the fact that what was far more likely than magical therapies was that the research would teach us a great deal about development.

Lessons Learned. We have learned that development and differentiation is far more complex than we once thought. Cells do not just neatly become differentiated and stay that way forever. Under some conditions, they can be de-differentiated and re-differentiated as a different kind of cell. In addition, many epigenetic factors contribute alongside gene expression so that methylation and many other considerations that we are only beginning to understand play a role. Even when researchers can take those human embryonic stem cells, culture them, and get them to differentiate in what looks like just the right way, this does not mean that the new nerve or heart muscle or whatever will stay exactly that same way after it is transplanted to a new environment in the diseased body. It also does not guarantee that the cells will not become cancerous, or will not revert to a pluripotent stage and develop teratomas as Stevens's mouse strain 129 did, because the cells are in the wrong place at the wrong time.

As a result of such uncertainties, one line of research has proved very exciting. James Thomson and the Japanese medical researcher Shinya Yamanaka (1962–) independently developed ways to produce induced pluripotent stem cells that seem to be like the normal cells but started out as already differentiated body cells that were reprogrammed by adding a mix of genes (Takahashi et al. 2007; Thomson 2007). Called "ethically pure" pluripotent stem cells, the resulting cells did not require killing or discarding any embryos, yet they seem to have the same capacity. Yamanaka provided proof of principle with success in mice before his team and Thomson's both succeeded in humans as well (Takahashi and Yamanaka 2006). Research continues along these lines.

In addition, research continues on cancer stem cells. It seems clear that some kinds of stem cells are involved in some kinds of cancer, though whether these stem cells are related to embryonic stem cells or to more differentiated stem cells remains under examination. Embryonic stem cell research remains a vibrant and fast-moving field, full of surprises and discoveries.

SEE ALSO *Cell Division Molecular Dynamics; Cell Signaling; Dolly the Sheep; Regenerative Medicine; Stem Cells, iPS Cells.*

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STEM CELLS, iPS CELLS

The fountain of youth is an enduring story about the search for substances that can reverse aging. Some biologists believe that these substances may lie within the human embryo. As the embryo develops, molecules start to mature and age the cells of the organism, and biologists reason that they can manipulate these same

molecules to turn back the clock after maturation. Over the last sixty years, pioneering "reprogramming" experiments have identified some of the key molecules needed to turn back the clock on cells—making adults cells acquire embryonic capabilities. At their core, reprogramming experiments derive from a curiosity about how the human body develops and ages.

As this article describes, a set of experiments that started nearly sixty years ago culminated in a breakthrough experiment in 2006 that generated embryonic-like stem cells from adult skin cells. These cells, termed *induced pluripotent stem cells,* or iPS cells for short, are being used to advance biomedical care in fundamentally new ways. This article will first describe the breakthrough experiment. Next, we will move onto discussing several fundamental questions about the design and interpretation of this experiment. The exciting journey of going from hypothesis to successful experiments is chronicled within this field of regenerative and stem cell biology.

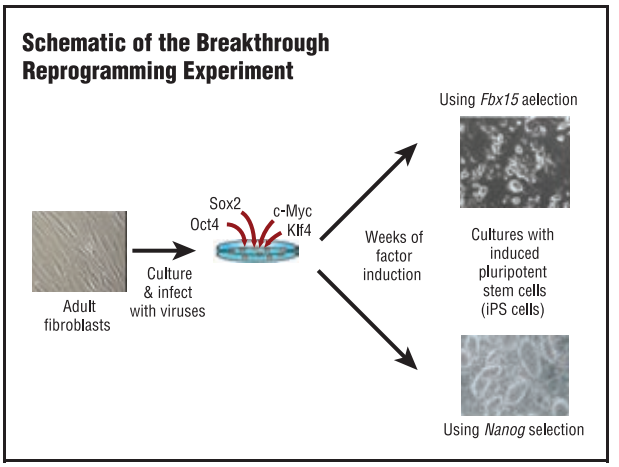

Figure 1. *The top image shows heterogeneous culture with reprogrammed iPS cells when using Fbx15 transgenic mice. The bottom image indicated more homogenous iPS cells when using Nanog transgenic mice.* **ADAPTED FROM TAKAHASHI AND YAMANKA 2006.**

THE BREAKTHROUGH EXPERIMENT

In 2006 a laboratory in Kyoto, Japan—headed by the Japanese physician and cell biologist Shinya Yamanaka (1962–)—first reported the induction of pluripotent stem cells from mouse skin fibroblasts (Takahashi and Yamanaka 2006). (Pluripotent stem cells have the potential to develop into nearly any other type of cell, such as heart cells, neural cells, and bone cells; by contrast, fibroblasts are specialized cells restricted to forming various types of connective tissues.) A postdoctoral fellow in the lab, Japanese cell biologist Kazutoshi Takahashi, set up an experiment where he used pools of retroviruses to infect fibroblast cultures. The pools consisted of up to twenty-four candidate transcription factors taken from the human and mouse genomes. Transcription factors are proteins that bind to DNA. Takahashi and Yamanaka culled the developmental biology literature to find reports of transcription factors that were highly expressed in the inner cell mass structure of the embryo. This embryonic structure is the source of embryonic stem cells. They reasoned that artificial induction of the factors that are naturally expressed in this stage would reprogram adult cells. This route of expressing molecules found in the embryo without having to manipulate human embryos themselves would be useful, because embryo manipulation and destruction were main points of contention in the politics surrounding stem cell research in that decade (i.e., the 2000s). They also culled the literature to identify transcription factors that were highly expressed in tumors, because they hypothesized that oncogenic (tumor-causing) factors would help with transformation processes during reprogramming.

For his experiments, Takahashi utilized a transgenic mouse wherein an embryonic marker gene, *Fbx15*, had been modified to express a drug selectable enzyme called the neomycin resistance gene. The drug, neomycin, normally kills all human cells. In this transgenic system, only cells that express Fbx15, presumably embryonic cells, would express the neomycin resistance enzyme and therefore survive when neomycin was added to cell culture media. This strategy allows for researchers to isolate specific rare cells, such as one cell in ten million cells. Takahashi dissected the transgenic mice at two stages of development. He isolated fibroblasts from midway through embryonic development, and from the tips of tails of newborn mice. In these fibroblast cell populations, Fbx15 is not expressed and therefore the neomycin resistance gene is not expressed.

Using the isolated mouse fibroblasts, Takahashi infected different cell cultures with different combinations of candidate factors. He first infected with all factors and allowed these cells to grow for several weeks in culture. Under the microscope, he observed changes in cell shape and cell clustering (see Figure 1; i.e., changes in cell morphology). However, these changes did not occur in all of the infected fibroblasts. Only small portions of the cell culture dish seemed to be transformed into a state that was not fibroblast-like. Since he was searching for pluripotent cells, he added neomycin after two weeks of culture. This killed nearly all of his cells in the dish, but a few rare colonies remained that withstood the neomycin drug selection. These cells grew and formed colonies that looked like embryonic stem cell colonies. He isolated these colonies one-by-one into separate cell culture dishes and started analyzing them.

IDENTIFYING THE VITAL TRANSCRIPTION FACTORS

When Takahashi looked to see whether any of his factors were inside the colonies, he found several copies of the retroviruses. These viruses had been integrated into the genome. He noticed different numbers and patterns of viral integration in the different colonies that he isolated. To identify exactly which factors seemed to be responsible for the reprogramming event, he divided the pool of retroviruses in half and repeated the experiment with each half of the pool. He discovered that one pool gave rise to many more colonies that the other half. Then he divided the pool further by half and repeated the experiment. Again, he noticed different numbers of colonies using different pools with various subsets of the reprogramming factors. He decided to remove each factor individually from the pool and then repeat his experiments. He noticed that four transcription factors had the largest effect on the final number of reprogrammed colonies: Oct4, Sox2, c-Myc, and Klf4. When each of these factors was taken away from the pool, no colonies were observed. Then he repeated the experiment with only this combination of factors—with Oct4, Sox2, c-Myc, and Klf4 only. He consistently observed colonies every time he attempted this experiment. Takahashi and Yamanaka concluded that they had identified a cocktail of transcription factors that could generate reprogrammed cells.

The isolated reprogrammed colonies were next compared to embryonic stem cells. Gene expression profiling of thousands of genes indicated that iPS cells were extremely similar in expression pattern to embryonic stem cells, and extremely dissimilar from fibroblasts. They monitored molecular changes in DNA and the proteins that surround and package DNA within the nucleus. They found several copies of the retroviruses in the DNA sequence and found methyl groups taken away from promoter sequences near key pluripotency gene sequences compared to fibroblasts. These changes in DNA methylation in iPS cells mirror the difference between embryonic stem cells and fibroblasts. Importantly, they injected iPS cells into mice to see if cancerous tissue called *teratomas* would form. Teratomas contain cells from the three main germ layers of mammalian organisms—ectoderm, mesoderm, and endoderm. Pluripotent stem cells like embryonic stem cells readily form teratomas after injection into mice. Therefore, injection of iPS cells is a stringent functional test of the cells to see if they have the potential to mature into the three principal germ layers of the body. For many of the iPS cell lines, they obtained teratomas that contained all three germ layers. From these experiments, Yamanaka and Takahashi concluded that they had obtained pluripotent stem cells and that their cells were very similar to embryonic stem cells.

In the following year, Yamanaka and Takahashi reported that this same cocktail of factors could reprogram human fibroblasts (Takahashi et al. 2007). The American developmental biologist James Thomson (1958–), based at the University of Wisconsin-Madison, reported that a variant of Yamanaka's approach could also be used for reprogramming (Yu et al. 2007). Like Yamanaka, he utilized Oct4 and Sox2, but added two different transcription factors—Lin28 and Nanog—to reprogram human fibroblasts. Several groups rapidly replicated these results in both human and mouse cells.

Significance. Stem cell research has historically been performed with model organisms, because obtaining human material for research purposes was a bottleneck. This was particularly true for human embryonic stem cell research in the 2000s. While working at the University of Wisconsin-Madison, Thomson first isolated human embryonic stem cells from human embryos in 1998 (Thomson et al. 1998). Subsequent to Thomson's initial findings, about twenty or so stem cell lines had been derived from other human embryos, when in 2001 President George W. Bush (1946–) announced a moratorium on federal funding for the derivation of human embryonic stem cells. His policy aimed to stop the destruction of human embryos, and chilled activity in the field. Political pressures to avoid destruction of human life limited the ability of the stem cell research community to readily experiment with human pluripotent stem cells.

Yamanaka's experiment provided a way forward around this bottleneck of working with human embryos. No longer were stem cell biologists waiting on fertilization clinics to move forward with experimentation. Instead, any human skin sample could be infected with Yamanaka's cocktail to yield reprogrammed cells.

ARE IPS CELLS USEFUL?

Since Yamanaka's discovery, iPS cells have been used to study human disease in human cells. Any cell of the body can be made with iPS cells in theory, and researchers have been able to generate cells of the heart, lung, brain, liver, and eyes readily from these cell lines. Because these cells lines are suspended in the embryonic state, they can be grown for years and decades—forever in theory. By performing this process on skin or blood samples isolated from diseased patients, the biomedical research community is able to investigate pathological processes in unlimited amounts of patient-matched human cells. Tissue engineers are able to use these resources to generate mini-organs or organoids that are useful for disease modeling efforts. The biotechnology and pharmaceutical industries are able to use these cells to test and discover drugs for specific patient populations. The list of

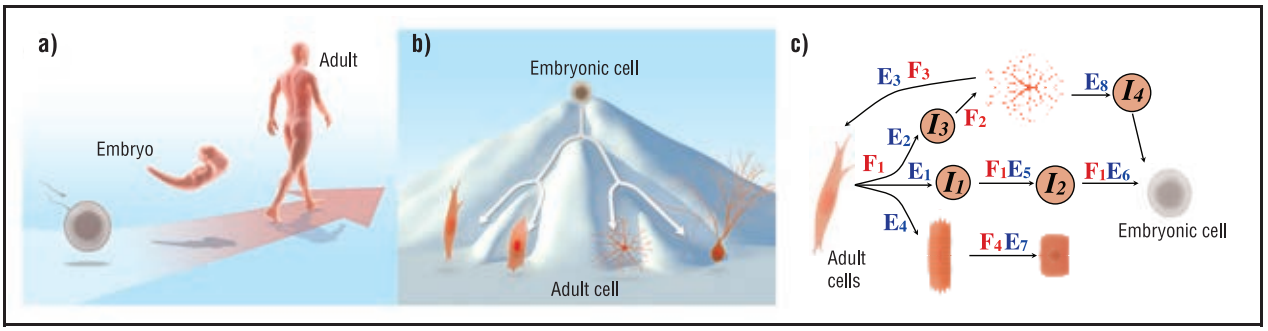

Figure 2. *a) Human development as a one-way street. b) Cell development as a marble rolling down an epigenetic landscape (Frisén et al. 2012; Waddington 1940, 1957). C) Complex and nonlinear trajectories of cells dynamically responding to reprogramming factors (F_n) and extracellular signals (E_n) to adopt various fates. I_n: intermediate cell.*

diseases that have been modeled with iPS cells includes Parkinson's disease, Alzheimer's disease, cystic fibrosis, Down's syndrome, hepatitis, and many rare genetic disorders; and the list is rapidly growing (Saha and Jaenisch 2009). Clinical trials are also underway wherein mature cells generated from iPS cells are being injected into the patient's eye to alleviate macular degeneration.

Yamanaka's experiment also prompted biologists to return to an old question in developmental biology: How plastic (i.e., adaptable) are the mature cells of mammalian organisms? In the 1950s, the English developmental biologist Conrad Hal Waddington (1905–1975) developed an epigenetic landscape to visually characterize the maturation process of human embryonic cells during development (see Figure 2b) (Waddington 1957). This landscape consisted of mountains and valleys as a metaphor for development where embryonic cells, represented as marbles, reside on a mountaintop. During differentiation they roll down into more stable valleys, where they come to rest as differentiated cells. This framework led biologists to accept the theory that it would be difficult to revert the differentiated cells back to the embryonic state by moving them back to the mountaintop (see Figure 2b). Along with the cloning of Dolly the sheep in 1997, Yamanaka's experiment provides experimental proof that this landscape needs to be revised to reflect our experimental ability to take cells back to the mountaintop.

Yamanaka's experiments did not stop in 2007. A number of questions have been posed and resolved through further experimentation since then. Below we discuss a number of questions about why Yamanaka's experiment succeeded and what limits there may be to his strategy of reprogramming.

Why Oct4 and Sox2? Prior cloning experiments indicated that proteins in the female eggs, oocytes, could reprogram adult cell nuclei. The English developmental biologist John B. Gurdon (1933–) used eggs from frogs (*Xenopus laevis*) and physically took out their nucleus. These "enucleated" eggs were then transplanted with nuclei from mature tadpole intestinal cells. This process was termed *nuclear transfer,* and Gurdon was able to generate a small number of swimming tadpoles using this technique (Gurdon 1962). These experiments indicated that mature cell nuclei had the potential to revert to pluripotency. The process relied critically on the cytoplasmic milieu of an egg cell. The English embryologist Ian Wilmut (1944–) later used these results as inspiration to clone Dolly the sheep through nuclear transfer (Wilmut et al. 1997). Biologists also directly fused the cytoplasmic milieu between an egg and a mature cell through use of a chemical called polyethylene glycol. They also fused embryonic stem cells with mature cells as well and found similar reprogrammed cells. These results together indicated that the cytoplasmic milieu of eggs and embryonic stem cells have factors that could reprogram mature cells. Yamanaka importantly used this insight to look for transcription factors, such as Oct4 and Sox2, that were highly expressed in these cell types.

Why c-Myc and Klf4? Both c-Myc and Klf4 are involved in cancer and are termed *oncogenes.* Cancer cells and stem cells share the ability to self-renew. Plus, pluripotent stem cells develop cancerous teratomas when they do not differentiate within a host embryo. Finally, cancer cells in some cases have broad potential to mature into various cell types. The process by which normal cells become cancerous involves oncogenic transformation, where normal cells lose their normal characteristics. Yamanaka reasoned that these processes could be important for reprogramming in order for mature cells to lose their somatic identity. Thus, he included factors highly expressed in cancer cells like c-Myc and Klf4.

Do iPS Cells Function Just Like Embryonic Stem Cells? While the Fbx15 selection procedure utilized in Yamanaka's protocol generated cells that could make all three germ layers in teratoma assays, they failed other standard assays of pluripotency. They could not integrate into embryos when injected into natural embryos. Therefore, researchers began to experiment with other drug selection techniques, because the Fbx15 selection procedure utilized in Yamanaka's protocol may have selected poorly for pluripotency. Fbx15 marks cells outside of the early stages of embryonic development, and a more stringent marker of pluripotency is the gene Nanog, which marks only the inner cell mass stage of embryonic development from which bona fide embryonic stem cells are derived. Reprogramming experiments utilizing fibroblasts from a Nanog-neomycin transgenic mouse yielded iPS cells that had enhanced pluripotency characteristics (see Figure 1) (Wernig et al. 2007). These cells could be injected into mouse embryos. Upon further development of these embryos and birth of young mice, iPS cells were found in all of the tissues of the adult mice.

Further experimentation demonstrated that one could even take an iPS cell and derive an entire mouse from it, through a variant experiment of injecting iPS cells into embryos called tetraploid complementation (Boland et al. 2009). The resulting mice generated through tetraploid complementation lived normal lives and could breed like regular mice. These experiments demonstrate that reprogramming is complete through Yamanaka's protocol and do not carry "memory" of their original fibroblast state that interferes with the pluripotent functions of these cells. Finally, iPS cells could be generated from mice and humans simply by identifying iPS colonies during reprogramming by eye and without any drug selection. Through rigorous experimentation, these visually-selected cells have been deemed functionally similar to those derived with drug selection.

Why Does Reprogramming Take Multiple Weeks? The multi-week long process of reprogramming is much slower than nuclear transfer and cell fusion approaches, which take only a few days at most to complete reprogramming. This observation indicated that the process may be gradual and involve multiple cell divisions. To address this question the German physician and biologist Rudolf Jaenisch (1942–), as head of a laboratory at the Whitehead Institute for Biomedical Research, conducted experiments for extended periods of time to see whether reprogramming would continue with further growth. They found that more colonies appear with more growth, indicating that the reprogramming process could continue upon further cell division (Hanna et al. 2009). This reprogramming process could be accelerated by growing the cells faster, and slowed down by conditions that made the cells grow more slowly. Further, they found that the time to see the first reprogrammed event could not be predicted and would vary anywhere from two to eighteen weeks after induction of the reprogramming factors. This indicated that there is a stochastic component to reprogramming that seemed to depend on how fast the cells divided. They concluded that cell division provides the four transcription factors an opportunity to access the genome and exert their reprogramming actions.

What Changes Occur within the Cells during Reprogramming? Several studies have probed which molecules are involved in reprogramming once transcription factors are expressed in the cell. The first variants of defined-factor reprogramming further modified the Yamanaka cocktail, using a wide variety of reprogramming factors. Each of the factors could be replaced by other factors. For example, Klf4 could be replaced by Klf2, and N-Myc could be replaced by c-Myc. Further, alternate combinations without any of the four factors could be utilized (Buganim et al. 2012) with various efficiencies of producing reprogrammed colonies. In addition, the viruses could be replaced by directly putting proteins into cells and also small molecules could be used to activate various pluripotency pathways to generate iPS cells. This is still an active area of research, as a clear molecular mechanism for reprogramming within mouse and human cells has yet to be elucidated.

Are the Other Molecules around the Cell Important? In addition to the identity of the transcription factor cocktail used in reprogramming, the culture environment can dictate how reprogramming occurs. Drugs that block important signaling pathways, such as glycogen synthase kinase 3 beta (GSK3-beta) and fibroblast growth factor (FGF), can produce pluripotent stem cells with different characteristics than those derived without them. Two types of pluripotent cells have been produced by varying these conditions, corresponding to different stages of embryonic development called *naïve* and *primed* stages of development (Hanna et al. 2010). Naïve cells are thought to be more primitive and rely on a different set of molecules compared to primed cells. Somehow the molecular signals outside the cell connect with molecular processes inside the cell involving the reprogramming factors. This interface between the inside and outside of the cells is still an active area of interest.

Can All Adult Cells Be Reprogrammed? One hypothesis that surrounded Yamanaka's experiment involved the idea that only specialized, "elite" cells of the skin could be reprogrammed. These elite cells had characteristics that made them more amenable to reprogramming. Such cells could be adult stem cells in the source skin that

contaminated the fibroblast preparation. This hypothesis was disproved when nearly all lineage-committed B cells from the blood could be reprogrammed (Hanna et al. 2008). B cells harbor a genetic mark that indicates loss of an adult stem cell state and gain of a mature state. After reprogramming, the B-cell-derived iPS cells retained these genetic marks indicating that mature cells could be reprogrammed. Further, nearly all of the B cells placed in a Petri dish could be reprogrammed after long-term culture. In addition to B cells, many other parts of the mouse have been reprogrammed including liver and brain. Plus, human cells from the hair follicle, renal epithelium, and blood have been reprogrammed successfully into iPS cells.

Could the Strategy Make Other Types of Cells? Researchers have been inspired to use Yamanaka's strategy to reprogram cells to other cell types. The Austrian neuropathologist Marius Wernig at Stanford University reported in 2010 that skin cells could be reprogrammed directly into neurons (Vierbuchen et al. 2010). Like Yamanaka, he scanned prior reports of gene expression in the final cell type—neurons—for transcription factors that were highly expressed. Then, he used viruses to deliver these transcription factors into transgenic fibroblasts. The fibroblasts were harvested from mice which had the Tau neuronal gene modified to express green fluorescent protein (GFP). After infecting with pools of candidate transcription factors, Wernig looked for cells that had a neuronal morphology and expressed GFP. The resulting GFP-positive induced neurons, dubbed iNs, had electrophysiological properties of primary neurons harvested directly from mouse brains. Others have also used this strategy to generate heart components like induced cardiomyocytes (iCMs), liver components like induced hepatocyte-like cells, and induced astrocytes.

Revisiting Waddington's Landscape. A common way of conceptualizing mammalian development is as a one-way street (Waddington 1940, 1957): cells mature unidirectionally from an embryonic to adult cell state (see Figure 2a–b). Reprogramming experiments that moved cells in the reverse direction disrupted this thinking (Frisén et al. 2012). However, reprogramming does not simply reverse the direction of the arrow, nor is it linear. Reprogramming can be noisy, complex, and subject to many factors: the street itself needs to be reconceptualized (see Figure 2c). For example, cells undergoing reprogramming can be directed away from the embryonic state and towards hematopoietic lineages by adding soluble growth signals (Szabo et al. 2010). Also, developing embryonic cells interact with neighboring cells—a mechanism that can be used to suspend cells in an intermediate progenitor state (Sneddon, Borowiak, and Melton 2012). Cell-extrinsic, ''niche'' signals (Lutolf and Blau 2009, p. 3,255) thus can act effectively as turn signals or stoplights. Scientists hope to systematically control such signals to elucidate the role of cell-extrinsic signaling mechanisms in reprogramming.

Deeper mechanistic understanding would build on trailblazing work that has already identified cell-intrinsic factors and genes important for reprogramming (Yamanaka and Blau 2010) and help us rationally produce diverse cells for regenerative medicine (Yamanaka and Blau 2010) and human disease modeling (Saha and Jaenisch 2009).

SUMMARY

With the advent of reprogramming, we have a set of molecules that can reverse the age of mature cells. This is not exactly like finding the fountain of youth, because reprogramming cannot be easily applied to one part of the body to cause instant rejuvenation. Rather the process is slow, noisy, and involves careful experimentation in the lab. Still, biologists now have a better understanding of the molecules that could lead to regeneration. In essence, one bold experiment opened the door to a new era in stem cell biology. Yamanaka had a unique vision that questioned established theories of developmental biology. His careful reading of prior experiments involving reprogramming through nuclear transfer and cell fusion allowed him to narrow down a wide range of potential molecules into a tractable set of reagents that could be used for his experiments. The resulting experiments inspired others to replicate his work and utilize it to generate new types of cells. His contributions to biology and medicine have been deep and significant, inspiring the future generation of experimentalists to blend heterodoxy with careful experimental design.

SEE ALSO *Cell Signaling; Dolly the Sheep; Neurogenesis, Adult; Regenerative Medicine; Stem Cells, Embryonic; Transplantation.*

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STRING THEORY AND QUANTUM GRAVITY

The theories of quantum mechanics and General Relativity (Einstein's theory of gravity) grew out of scientific revolutions in the early twentieth century. They assert that as one moves away from everyday scales to extremes of large and small, the familiar properties of space, time, and matter cease to hold, and one instead encounters new and seemingly paradoxical phenomena. Each theory has now been verified in a host of precision experiments, and so must form part of a more complete theory of quantum gravity that incorporates both. The search for such a theory has been an active field of research, which has been explored in many directions. There is still no complete or generally accepted solution, but the search has led to many new concepts and surprising connections between different areas of physics.

The methodology in this field is primarily theoretical. In most natural situations, only one of quantum mechanics and gravity will have significant effects. Quantum mechanics is important for the behavior of very small systems, while gravity dominates in very massive systems. Thus, there are few experimental clues as to the unification of quantum mechanics and gravity. Nevertheless, one can make progress by considering extreme situations in which both would enter, and in particular by looking for conflicts between the predictions of the two theories. A model for this is Maxwell's completion of the equations of electromagnetism, revealing the nature of light, to which he was led by similar theoretical reasoning.

Some approaches to this problem consider quantum mechanics and gravity in isolation from the understanding of the other forces and the elementary particles. String theory is an attempt to derive all the laws of physics from a single principle.

QUANTIZATION AND ITS SHORTCOMINGS

As a first attempt, one can treat the gravitational field by the same method of second quantization that was successfully applied to the electromagnetic field. This approach implies that gravitational radiation comes in quantized packets of energy, gravitons, analogous to the photons of the quantized electromagnetic field. It also implies that the emission and absorption of virtual gravitons affects the properties of atoms, similar to the well-understood effects of virtual photons. However, these quantum gravity effects are very small, far below the level that could yet be detected in experiment. One measure of this effect is given by the Planck length, $l_P = \sqrt{\hbar G/c^3} = 1.6 \times 10^{-32}$ cm. This is the unique length scale that can be formed from the basic constants of quantum mechanics and relativity: Planck's constant $\hbar$, the gravitational constant G, and the speed of light c, and so it represents a fundamental distance scale in nature. It was discovered in