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Robert Koch: The Grandfather of Cloning?

Essay

Robin A. Weiss*
Wohl Virion Centre
Division of Infection and Immunity
University College London
46 Cleveland Street
London W1T 4JF
United Kingdom

This year marks the centenary of Robert Koch's Nobel Prize for discovering the cause of tuberculosis. Koch was also the first scientist to isolate the anthrax and cholera microbes. Yet perhaps one of his greatest contributions to biology is the least appreciated: his method for propagating individual colonies of bacteria on plates, a technique that came to be called cloning.

As Barry Marshall and Robin Warren are set to collect the 2005 Nobel Prize in Physiology or Medicine next month for "their discovery of the bacterium *Helicobacter pylori* and its role in gastritis and peptic ulcer disease," it is apposite to recall that exactly 100 years earlier Robert Koch traveled from Berlin to Stockholm to receive his Nobel Prize for "investigations and discoveries in relation to tuberculosis." Koch's achievements in identifying other pathogens, such as anthrax and cholera, were equally remarkable. Yet his most original contribution to basic biology is the method he devised in 1881 for propagating single colonies of bacteria on plates, a technique that we would now call cloning. This method led to the second of his famous postulates for identifying the cause of an infectious disease: that the microbe must be isolated in pure form. Since then, many scientists have made major discoveries by adapting the cloning technology developed by Koch. From DNA cloning to monoclonal antibodies and even cloning whole organisms, such advances have helped to spawn at least six more Nobel Prizes.

Koch and His Postulates

Robert Koch (1843–1910) (Figure 1) was a German scientist without mentors in research (Brock, 1999). He began his microbiological investigations as a hobby soon after his appointment in 1872 as a distinct physician in Wollstein (now Wolsztyn in Poland). He laid the groundwork for his great contributions there, moving to Berlin in 1880. As early as 1873, from examining a sheep that had died from anthrax, he proposed that the cause of this disease was rod-shaped "bacilli" that differentiated into long-lived round spores. Louis Pasteur was already advocating the germ theory of disease and was quick to develop an anthrax vaccine following Koch's discovery.

In 1840, Jacob Henle first conceptualized the source of a contagion as being a living agent, with his famous dictum: "It is not the disease which is being transferred but the cause of the disease." But a generation later, Rudolf Virchow in Berlin, Max von Pettenkofer in Munich, and Theodor Billroth in Vienna still doubted the key role of micro-organisms in infectious disease.

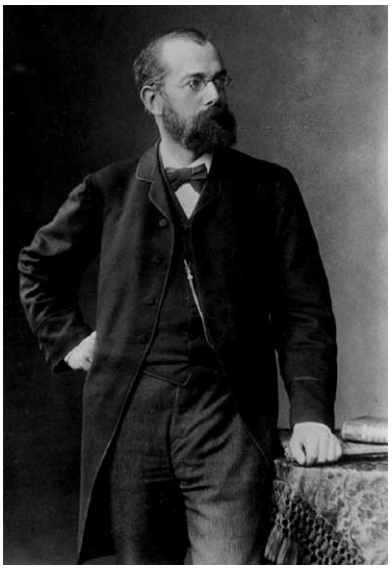

Figure 1. Robert Koch in 1884 (Courtesy of the Robert Koch Institute)

The postulates for which Koch is chiefly remembered today are something of a misnomer because he never clearly enunciated them himself, although he followed their logic in pathogen discovery. It was Edwin Klebs, Professor of Pathology in Prague and a keen champion of Henle's ideas, who hinted at Koch's postulates in 1875: careful microscopic study of the diseased organ, culture of the germ associated with the disease, and production of the same disease by inoculation of this cultured germ into healthy animals. Klebs also rephrased Henle's aphorism to: "It is not the disease, but the cause of the disease, that reproduces."

The version of Koch's postulates most frequently cited was ascribed to Koch by his research assistant, Friedrich Loeffler, in his 1883 paper identifying *Corynebacterium diphtheriae* as the cause of diphtheria.

(1) The microbe must be consistently shown to be present in the diseased tissue and not in healthy tissue;
(2) The microbe must be isolated and grown in culture in pure form;
(3) The pure culture must be shown to induce the disease anew.

Pasteur and Koch

Pasteur and Koch ought to have stood shoulder to shoulder in promoting the germ theory of contagious

*Correspondence: r.weiss@ucl.ac.uk

disease, but instead they and their acolytes actually spent more energy attacking each other. Initially Louis Pasteur treated the younger Koch with haughty disdain; meanwhile Koch attacked Pasteur's studies on anthrax. Joseph Lister's diplomacy in bringing the two together in London in 1881 did little to defuse their antagonism. Pasteur focused on treating individuals by immunization, whereas Koch's venture into this field with tuberculin (PPD) was a disaster; his strengths lay in pathogen discovery and prevention through sanitation. Doubtless their rivalry was fueled by nationalist feelings in the aftermath of the Franco-Prussian war. Pasteur himself wrote "La science n'a pas de patrie, mais le savant doit en avoir une" (Science has no fatherland, but the scientist must have one).

Koch and Pasteur not only bequeathed to us the major principles of microbiology but also the institutes that carry out leading research to this day. Last month the Robert Koch Institute in Berlin held a symposium on "Challenges Posed by Infectious Diseases on the 100th Anniversary of Robert Koch's Nobel Prize Award"; tuberculosis, alas, was still among them. Two of Koch's former assistants themselves won Nobel Prizes for Medicine: Emil von Behring in 1901 (who won the first Prize) for developing antitoxins and Paul Ehrlich in 1908 for developing chemotherapy.

Bacterial Cloning

Why should I call Robert Koch the "grandfather of cloning"? It is on account of his 1881 paper "Zur Untersuchung von Pathogenen Organismen" (On the Examination of Pathogenic Organisms) in which he describes a method for growing isolated colonies of bacteria. He poured a hot mixture of nutrient broth and gelatin onto a glass plate, which was then allowed to set before exposing it to germs. He described how separate colonies developed that eventually merged into a microbial film. This method allowed him to pick cells from individual colonies and to propagate them as "pure" cultures. He could then test their pathogenicity by inoculation into experimental animals. He recognized that different colonies have different morphologies yet breed true on propagation, and he adopted a Linnean nomenclature for bacterial species (Schlich, 2000).

Koch's cloning technique did not come out of the blue. In 1875, Joseph Schroeter had observed discrete, red colonies of the bacterium *Serratia marcescens* on the cut surface of potatoes. Although Koch did not cite Schroeter, he stated that seeing different types of colony growing on potato slices led him to develop the gelatin plate technique. In 1878, Joseph Lister described the limiting dilution method in liquid culture for isolating bacteria in pure form, but Koch exploited bulk culture for the progeny of his clones.

Two further developments in the 1880s established microbiological cloning essentially as we know it today, namely the use of agar as a semi-solid nutrient medium suggested in 1882 by Fannie Hesse (a practical "hausfrau" and a laboratory assistant) and the introduction of the petri dish (Figure 2A). In 1887, Koch's protégé, Richard Petri, described "A small modification of Koch's plate technique" as his paper was titled (in German). He wrote:

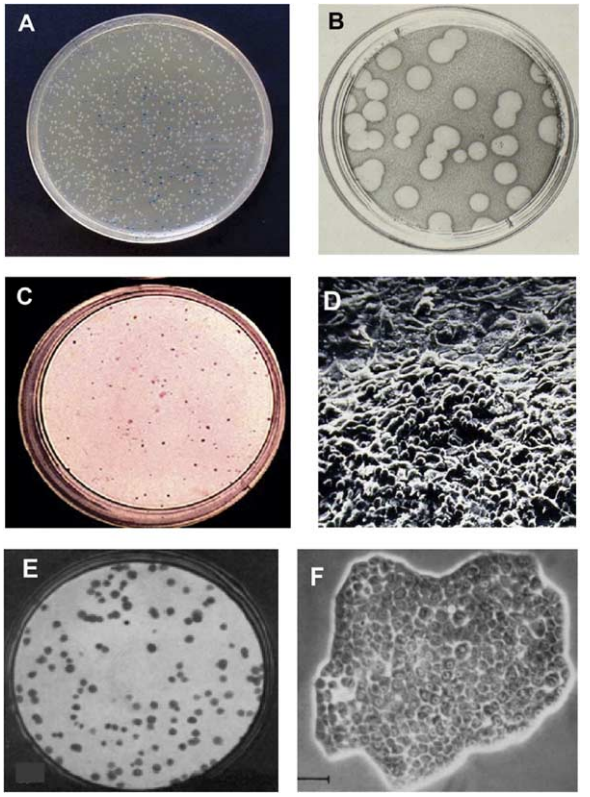

Figure 2. Clones that Koch Would Recognize

(A) Colonies of *E. coli* on agar in a Petri dish. White clones contain recombinant DNA, whereas blue clones are wild-type (S. Nambulli and I. Harrison, Wohl Virion Centre).

(B) Polio virus plaques in a monolayer of HeLa cells under agar (courtesy of J.E. Darnell, Jr).

(C) Rous sarcoma virus assay on a monolayer of quail embryo fibroblasts and (D) scanning electron micrograph of a transformed clone (R.A. Weiss, 1969, PhD thesis, University of London).

(E) Cloning of human HeLa cells by T.T. Puck, P.I. Marcus, and S.J. Cieciura and (F) individual clone showing epithelial morphology (reproduced from The Journal of Experimental Medicine (1956). *103*, 273–283 by copyright permission of the Rockefeller University Press).

> I have been using flat double dishes of 10–11 cm wide and 1–1.5 cm high. The larger diameter dish serves as a lid. These dishes are sterilized by dry heat. After cooling, the upper dish is lifted only slightly and nutrient gelatin is poured in… These dishes are especially recommended for agar-agar plates, since agar-agar sticks poorly to flat glass plates. In addition it is quite simple to count the colonies after they have grown by replacing the lid with a glass plate with squares etched on it viewed over a black background.

Petri's name lives on from this one and only notable contribution.

Cloning since Koch

The word cloning today conjures up images of Dolly the sheep or the contentious ethics of human cloning (Klotzko, 2004). Yet back in 1975, it was the potential hazard of recombinant DNA cloning that was hotly de-

bated at the Asilomar conference. In the same year, Koehler and Milstein published their seminal paper on monoclonal antibodies, made possible by fusing antibody-producing B lymphocytes with immortal myeloma cells. But where did the concept of cloning come from, be it DNA, cells, or whole organisms? When was the term introduced and what is its origin?

I had imagined that the etymology of clone was a corruption of colony, but it is actually derived from the Greek, *klon*, meaning a twig or small branch. This etymology reveals the original use of the word clone to describe vegetative reproduction of plants by taking cuttings. Because higher plants, unlike animals, do not sequester a germline distinct from somatic cells, plant cells remain pluripotent; reproductive cloning of plants, for example potatoes, occurs naturally and has been practiced for millennia by horticulturists in the propagation of vines and fruit trees. The new plants derived from cuttings or grafts are genetically identical to the parent, and the term clone came to be used for any form of artificial, asexual means of replication.

Although the word clone was introduced botanically in 1903, the first use that I have found to describe replication from a single progenitor was in 1954 when Theodore T. Puck and colleagues cloned human cells in culture. However, the word clone did not appear in general dictionaries until later. My 1964 *Oxford Concise Dictionary* restricts the definition to "a group of plants reproduced vegetatively from one original seedling or stock," whereas my wife's 1992 *Chambers Pocket Dictionary* gives two meanings: (1) (anyone of) a group of identical organisms reproduced by a non-sexual process from a single cell of the parent; (2) (colloq. or derog.) a person or thing that looks like a replica of someone or something else. The "single-cell" restriction in *Chambers* implies that it no longer refers to vegetative propagation of plants.

If Koch is the grandfather of cloning, who is the father? I would regard the parents of cloning to be the founders of microbial genetics. The heritable transformation of a bacterial phenotype by Fred Griffith in 1928 led directly to the identification of the genetic material as DNA by Oswald Avery, Maclyn McCarty, and Colin MacLeod in 1944. The study of bacterial genetics through the selection of colonies and of bacteriophage genetics by picking plaques launched molecular biology. This new field gained tremendous impetus from the Nobel laureates Max Delbruck, Alfred Hershey, Joshua Lederberg, Salvador Luria, and André Lwoff (Judson, 1996). The culmination of microbial genetics was the invention of DNA cloning (see below). Koch's biological cloning method for bacteria also led to studies of mutation, DNA repair, and antibiotics. What else was Alexander Fleming's seminal observation other than inhibition of bacterial colonies by a contaminating mold in a discarded petri dish?

Cloning Viruses

In 1917, F. d'Herelle grew bacteriophage on a lawn of *Shigella* bacteria in a petri dish for 50 successive passages; in calculating a titer of over 10^9, he described clear 1 mm diameter plaques. This heralded the cloning of viruses. Plant viruses could be plaque titrated on leaves, but a problem remained for animal viruses, which had to be grown by inoculation into animals or eggs. The first cloning technique for animal viruses was achieved by infecting the chorio-allantoic membrane (CAM) of fertile chick eggs. This is the membrane just under the shell that serves as the embryo's "lung." In 1936, E.V. Keogh used CAM assays to titrate and clone vaccinia virus, and in 1938 he developed a quantitative CAM assay for Rous sarcoma virus.

It was not until the trypsinization of embryonic cells was developed in 1952 that the animal cell equivalent to a lawn of bacteria became available. Renato Dulbecco immediately took advantage of the technique to plate pathogens such as Western equine encephalitis and polio viruses on dispersed cell monolayers. Again agar medium, this time as an overlay to keep lytic plaques discrete (Figure 2B), enabled cloning through recovery of the viral progeny with a pipette. Cell transformation in monolayer culture by oncogenic viruses was achieved in 1955 by Dulbecco for the polyoma virus, for which he received the 1975 Nobel Prize (with David Baltimore and Howard M. Temin, who discovered reverse transcriptase in 1970). In 1958, Temin and Harry Rubin, working in Dulbecco's laboratory, used chick embryo cell monolayers to develop a quantitative assay of cell transformation by Rous sarcoma virus (Figures 2C and 2D).

These lytic and transformation techniques opened the way to animal virus genetics and the discovery of oncogenes. More recently, viral pathogens that could not be cultured in the laboratory were discovered through recombinant DNA and cDNA cloning, for example hepatitis C virus in 1989 and Kaposi's sarcoma herpesvirus in 1994.

Mammalian Cell Cloning

Immortal cell lines were developed from the late 1940s. Mouse L cells were first cloned by Earle in 1948 through single-cell isolation in capillary tubes, and human HeLa cells isolated by G. Gey in 1952 were cloned by Theodore Puck (Figures 2D and 2E). In the 1960s, nontransformed cell lines with a high cloning efficiency (such as hamster BHK21 and mouse 3T3 cells) were developed and used to select transformed colonies after exposure to oncogenic viruses or other carcinogenic agents.

Now that cells could be grown as colonies like bacteria, the science of mammalian somatic cell genetics blossomed (Harris, 1997) and was a boon to the fields of radiobiology and mutagenesis. Fusion of cells of different parentage allowed analysis of chromosome segregation and genetic mapping. Just as Koch labored to find a suitable medium to grow tubercle bacilli in 1882, mammalian cell culture required meticulous studies by Puck and Harry Eagle to establish optimal media for cloning. We still use DMEM (Dulbecco-modified Eagle's medium) with 10% fetal calf serum, which was developed nearly 50 years ago to afford 100% cloning efficiency of HeLa cells.

Monoclonal Antibodies and Reproductive Cloning

Perhaps the most famous example of exploiting the immortal, clonable properties of tumor cells is the derivation in 1975 of monoclonal antibodies by Georges

Koehler and Cesar Milstein, who were awarded the Nobel Prize for this discovery in 1984. By fusing a myeloma cell with a primary B lymphocyte, they created a cloned "hybridoma" that secreted a specific antibody. Myeloma cells had to be used for immortalization because they are differentiated plasma cells, whereas fusion of somatic cells of different types tends to extinguish expression of lineage-specific genes.

The restriction to mammalian gene expression has now been overcome by nuclear transfer into eggs or pluripotent embryonic stem cells, which had its birth in the successful transfer of embryo cell nuclei into frog eggs by R. Briggs and T. King in 1952. This resetting of the transcriptome makes possible reproductive and therapeutic cloning. These techniques hold great promise but require considerable debate to ensure that they are not misused (Klotzko, 2004).

Recombinant DNA and Clonal Amplification

The universality of the genetic code means that DNA can function in any biological setting. This was powerfully exploited by introducing DNA from different sources (e.g., human DNA) into viruses or bacterial plasmids, which were then clonally propagated. Thus, the practice of recombinant DNA cloning was born in 1972–1973 through the pioneering investigations of Paul Berg (a Nobel laureate), Stanley Cohen, and Herb Boyer using restriction enzymes and DNA ligation. DNA cloning has, of course, revolutionized molecular biology and the pharmaceutical industry (Figure 2A).

When the possibilities of DNA cloning first became apparent, however, the leading molecular biologists themselves expressed concern that exchanging genetic information between quite unrelated organisms posed a potential biological hazard. They convened a conference at Asilomar in California in March 1975, which resulted in a voluntary moratorium on DNA cloning until the possible risks could be more precisely assessed. No apparent harm has emerged from DNA cloning, and we now know that horizontal transfer of DNA and plasmids frequently occurs in nature.

The PCR revolution led to the award of a chemistry Nobel prize to Kary Mullis in 1993. The polymerase chain reaction allows the clonal amplification of specific DNA sequences resulting in millions of copies. This version of cloning has transformed diagnostic techniques including forensic science. Short synthetic yet specific DNA sequences are also the stuff of DNA microarrays. Indeed, the promise of DNA microchips in computers illustrates the increasingly sophisticated miniaturization of cloning technology.

If Koch Were Here Today?

What might Koch have thought of modern medical technologies? He would immediately recognize an *E. coli* cloning dish, viral plaques, and mammalian cell cloning as direct derivatives of his and Petri's cloning methods (Figure 2). I imagine that he would be supportive of therapeutic stem cell cloning and reproductive animal cloning yet be nonplussed (as I am) as to why anyone should wish to clone a human.

Koch would surely delight in Marshall's and Warren's 2005 Nobel Prize because the identification of the bacterium *Helicobacter pylori* (characterized and named by Stewart Goodwin) as the cause of peptic ulcers relied on his classical methods of isolation and his postulates. Indeed, in the absence of an animal model, Barry decided to swallow the bacteria himself and he soon developed acute gastritis. Koch would wryly recall Max von Pettenkofer in 1892 swallowing a culture of *Vibrio cholerae* in an attempt to disprove its role in cholera, given that Koch and Georg Gaffky had first isolated the comma-shaped pathogen in Calcutta in 1884. Thus, Robert Koch's contributions to infectious disease and to cloning methods resonate to this day.

Acknowledgments

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