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Cell Culture and Somatic Variation

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Preface

This book deals with the newly emerging field of somatic cell heredity, broadly interpreted to include developmental, genetic, and neoplastic changes in differentiated cells and tissues. It is intended as a synthesis and summary of the research literature bearing on these topics, much of which presently exists in widely scattered form. The discussion has been developed with cell culture, and to a lesser extent, tissue transplantation, as the principal experimental approaches. Through these techniques, isolated cell populations can be created for the analysis of somatic change. Considered in such a framework, somatic variation can be expressed by a series of specific processes—for example, differentiation and dedifferentiation in cell groupings, the evolution of growth forms and chromosome patterns, nutritional shifts, antigenic changes, the development of resistance to drugs or other extrinsic agents, and carcinogenesis *in vitro*. The concepts and patterns that emerge from these studies require for their interpretation a background of more general information. For this reason, the treatment of population dynamics in cell cultures and transplant systems is here preceded by an outline of developmental genetics, tumor biology, and microbial variation.

The present account cuts freely across established disciplines in describing mechanisms that operate in somatic variation at the cellular level. No claim can be made for completeness in this regard, or for adequacy in relating separate approaches. There can be little question, however, that a more unified picture is desirable. Somatic cell heredity, like growth, is still a frontier field. Some of the most significant developments will continue to arise in borderline areas, through links created between focal points of research. The establishment of cross-correlations can be facilitated by increased communication between embryologists, geneticists, pathologists, and other students of cellular change. This book attempts to supply in part the necessary information and to bridge cer-

tain gaps that have arisen through parallel rather than complementary studies on somatic cells and their information systems.

In its final form, the volume at hand reflects the generous assistance of friends and associates. I am particularly grateful to Dr. Curt Stern and Dr. Frank Ruddle, both of whom read the entire manuscript and offered many helpful suggestions. Additional criticism and comment were provided by the following individuals, to whom I am indebted for their willingness to review chapters appropriate to their special fields: Dr. E. A. Adelberg, Dr. R. E. Billingham, Dr. R. W. Briggs, Dr. Harry Eagle, Dr. Glenn Fischer, Dr. T. C. Hsu, Dr. George Klein, Dr. Harry Rubin, Dr. K. K. Sanford, and Dr. W. Szybalski. In assembling the illustrations, I have made use of numerous photographs that were provided by individual investigators from original sources. It is a pleasure to acknowledge their generosity, and credit in each case is specified with the corresponding text figure. The line drawings that accompany the text were prepared directly or adapted from published sources by Mrs. Emily Reid, with patience and skill. To Dame Honor B. Fell I wish to express my appreciation for courtesies extended during a sabbatical year at the Strangeways Research Laboratory, Cambridge, England, and to the John Simon Guggenheim Foundation for a fellowship provided during the same period. Lastly, my wife has provided continuous advice and assistance in the preparation of this book. Much of the bibliographic labor as well as the typing of the manuscript became her responsibility, and without this effective collaboration the work would still be far from complete.

M. H.

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Three types of somatic variation

chapter 1

Cellular transformation is a familiar event in multicellular organisms, and assumes many forms. Somatic variation is implicit, for example, in the ordered sequences of embryonic development, and local foci of differentiation persist into later stages. The implementation of these processes is distinctive, for morphogenetic conversions of cellular phenotypes seem to proceed without modification of hereditary determinants. However, genetic changes are by no means excluded at the somatic level, and can be documented on occasion during developmental stages. Isolated mutations or chromosome changes within the cells of embryonic rudiments sometimes occur, and may give rise to patches of cells with a variant phenotype. By inference, similar alterations may take place in the genetic apparatus of differentiated cells, although their detection in this case is more difficult. More spectacular examples of unprogramed variation within somatic cells can be found in the origin and evolution of tumors. The basis for neoplastic conversion is uncertain, and it is not clear whether transformation in this case rests on genetic alteration, an aberrant sequence of development, or some more distinctive process. Biologically, however, tumors represent a unique degree of freedom in multicellular systems. Evidently the autonomy of somatic units is a persistent, if largely potential, property within organized cell groupings. Developmental, genetic, and neoplastic changes thus constitute distinct modalities of somatic variation when viewed at a purely operational level. Their closer identification in terms of basic mechanisms presents an outstanding series of problems.

Conceptually, the partial processes of somatic variation can for convenience be assigned to alternative categories. Those that depend on a recasting of hereditary determinants stand in contrast to shifts

which take place against a constant cellular genome. In this respect, a useful distinction can be made between genetic and epigenetic control systems (Nanney, 1958). Truly genetic mechanisms are concerned with the preservation and replication of information in structural form; for example, the molecular configurations of deoxyribonucleic acid or the individuality of self-duplicating cytoplasmic organelles. Epigenetic mechanisms, on the other hand, regulate the expression of genetic information. They serve to translate structural symbols into phenotypic reality. Since control systems at the effector level may be modulated to yield alternative or multiple end products, it follows that epigenetic as well as genetic changes can provide a basis for heritable variation. Accordingly these two patterns form the central themes of somatic cell inheritance. Much effort has been expended, directly or indirectly, to give these distinctions a substantial meaning in experimental terms. The relevant data are to be found in a broad spectrum of studies of embryonic differentiation, developmental genetics, and experimental carcinogenesis, with which the present discussion begins. In brief perspective, this information provides a backdrop for more detailed descriptions of variation in isolated cell systems.

THE ESTABLISHMENT OF CELLULAR SPECIFICITY

The emergence of cell lineages in specific form from more general precursors is one of the hallmarks of embryonic development. These products often display remarkable stability. While the reversibility or irreversibility of differentiation has long been debated, it is obvious that *in situ* each cell type perpetuates a characteristic pattern. The constancy of end products is seen with particular clarity in those instances where differentiation is not accompanied by the cessation of cell division. In embryonic cartilage, for example, the chondrocytes can fix inorganic sulfate and elaborate extracellular matrix, and these potentials are transmitted in stable fashion to mitotic derivatives. Aside from their predictability and occurrence in organized patterns, developmental changes may thus on occasion simulate genetic change. Nevertheless, it is clear that cellular specificity in embryonic differentiation originates largely, if not entirely, by epigenetic means. Evidence for this well-known generalization has been accumulating for many years, and some of the critical proofs are classics in the biological literature.

Types of Developmental Patterns The early history of experimental embryology has as one of its most colorful concepts the "germ plasm"

theory of August Weismann (1892). This explanation sought to explain the facts of heredity as well as the basic mechanisms of development. According to Weismann, the chromosomes of the zygote contain a large number of determinants, which correspond to the parts of the embryo ultimately to be formed. These were assumed to persist as an intact array within the line giving rise to the germ cells, a concept still preserved in the phrase "continuity of the germ plasm." A very different fate, however, was depicted for the developing somatic cells. At each division of these units, a segregation of determinants was thought to occur. Finally, only one kind of determinant remained, in accordance with which the end products of differentiation emerged.

Weismann's idea of "unpacking" the nucleus was an attractive mechanism to explain the multiplicity of cell types as races of cells in developing embryos. It did not, however, stand up to experimental tests. One of the earliest of these was based on a separation of the embryo into parts. Driesch (1891), in a historic paper, described the results of dissociating sea urchin embryos at the two-cell stage by means of shaking. The isolated blastomeres were allowed to develop further, but did not differentiate into part embryos as expected. Instead, both formed complete and normally proportioned individuals. Thus, in the two-cell stage at least, the sea urchin nuclei are equivalent and totipotent; that is, each nucleus in the presence of competent cytoplasmic systems can give rise to a complete embryo. Many experiments have confirmed these results and have shown that isolated blastomeres from the early stages of a number of other embryos retain a full range of formative potentialities. Somatic cells, however, do not remain equipotential indefinitely. With continuing development, there is a progressive restriction in capability. In the sea urchin, for example, occasional cells at the 8-cell stage may form complete individuals, but cells isolated from the animal or vegetal regions of later cleavage stages give rise to partial larvae only (Hörstadius, 1939).

Evidence that the nuclei are undifferentiated during early development can also be found in other types of experiments. Among these are a number of investigations performed by pioneer workers to test the effect of pressure on cleavage. If the eggs of sea urchins (Driesch, 1892) or of frogs (Hertwig, 1893) are placed between two glass plates under pressure, cleavage continues to occur. A unidimensional aggregate of cells arises under these conditions, since all cleavage planes are oriented perpendicular to the glass surfaces. If the pressure is then relieved, however, the eggs regulate to form normal embryos. This result is obtained despite the fact that the topographic relations of individual nuclei are quite different from what would be the case in an intact

embryo. The interchangeability of nuclei in early stages is further documented by the studies of Seidel (1932), made on embryos of the dragonfly *Platycnemius pennipes*. In the eggs of insects, the cleavage nuclei multiply initially in an undivided cytoplasmic mass and migrate only later to various positions in the elongate embryo, where cell boundaries are established and further development occurs. Seidel showed that there is no regional specialization of the nuclei during this process. If one of the first two cleavage nuclei is destroyed by local application of ultraviolet light, descendants of the other proceed to nucleate the entire egg, and differentiation occurs normally. An equally typical pattern of development is observed if the migration of nuclei into the posterior part of the embryo is delayed, so that this region is provided with the products of the eighth rather than the fourth cleavage.

Perhaps the most striking demonstration of nuclear equivalence in early stages is to be seen in Spemann's "noose" experiment (Spemann, 1928, 1938). Using a fine loop of hair, Spemann constricted the egg of the European newt, *Triturus* (*Triton*), shortly after fertilization had taken place (see Figure 1.1). Initially the noose was passed around animal and vegetal poles and was oriented so as to bisect symmetrically the gray crescent. This structure, a region of cytoplasmic specialization, appears as a pale area on one side of the amphibian zygote. The embryo constricted in this way becomes dumbbell-shaped, with the egg nucleus on one side of the noose. The constriction in Spemann's experiment was left incomplete, however, so that an open cytoplasmic bridge remained between the two halves of the original egg. Under these conditions, cleavage occurs normally on the nucleated side, but the other half of the original egg remains unsegmented. Spemann noted, however, that occasionally a cleavage nucleus at the 16- or 32-cell stage might pass across through the persisting cytoplasmic bridge to the adjoining blastomere. When this took place, cleavage was initiated in the previously undivided cytoplasm. The noose could then be tightened so as to separate the two original halves of the egg completely, and under these conditions, both halves continue to develop. In such cases, twin larvae are eventually produced. These experiments show unmistakably that even as late as the 16- or 32-cell stage, nuclei of the newt embryo are still totipotent and can initiate the full range of embryonic differentiation.

Information of many kinds thus demonstrates that the establishment of specificity in the developing embryo depends on cytoplasmic differentiation, rather than on the progressive segregation of nuclear determinants as Weismann had supposed. It is equally obvious, however, that development is closely determined by nuclear factors and must be viewed as a function of gene actions, ordered in time and space. Nucleocytoplasmic

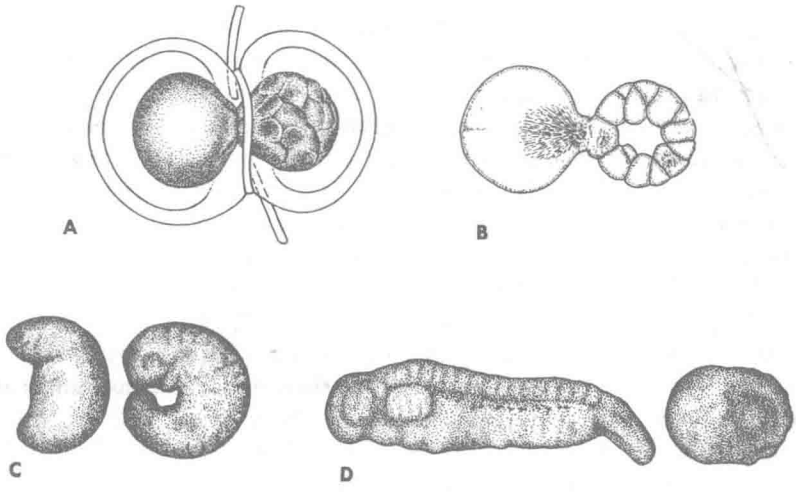

FIG. 1.1. Spemann's noose experiment. (*A*) Egg of European newt, *Triturus*, constricted soon after fertilization by a loop of hair in the median plane. Cleavage has occurred on one side only. (*B*) Diagram of a similar embryo in section, showing eventual passage of a cleavage nucleus across the persistent cytoplasmic bridge. (*C*) Later stage, showing the formation of twins. Embryo at left is normal in appearance, but retarded in development, owing to delayed nucleation of egg cytoplasm. (*D*) Two embryos resulting from a frontal constriction of the fertilized egg. The dorsal half forms a normally proportioned embryo, whether provided initially or later with a nucleus. The ventral half, lacking gray crescent material, forms only a rounded mass without external differentiation. (Redrawn from Fankhauser, 1930, and Spemann, 1938.)

interactions thus hold the key to morphogenetic change, a conclusion reached by experimental embryologists well before the turn of the century. Among others, Driesch (1894), Morgan (1934), and Waddington (1956) have pointed out that the sequential activation of genes in development can be visualized as a feedback relationship between nucleus and cytoplasm. Within the fertilized egg, or early embryo, a differential distribution of cytoplasmic substances occurs, and elicits a complementary pattern of gene action in the cells concerned. The cytoplasmic systems are further modified in different directions, calling forth a new individuation in operation at the genic level. This process may be mediated by activators and repressors for specific genetic loci, which come into being, disappear, or undergo modification as the developmental prospectus unfolds. "Operator" genes may turn on or turn off particular sequences of gene action in accordance with the constellation of factors present at the cytoplasmic level (see Chapter 2). Con-

ceptually, at least, it is thus possible to visualize the processes of cytoplasmic localization and specialization taking place against the background of a constant genotype.

Regardless of the exact mechanism of cytoplasmic specialization, there are wide differences in the extent and timing of this process among different embryos. A substantial part of the embryological literature during the early part of the present century was devoted to the analysis of these differences. The classic studies of E. B. Wilson, Conklin, and others showed that a precocious localization of morphogenetic substances occurs in the eggs of a number of invertebrate groups such as the ctenophores, annelids, molluscs, and tunicates. In these embryos, regional specialization is pronounced even at the time of fertilization and early cleavage. Such germinal localizations, as they were called, can be illustrated by the investigations of Conklin (1905, 1931) on the ascidian tunicate, *Cynthia* (*Styela*). In this embryo, as in a number of others, visible differences exist in the various regions. These can be described in terms of granules, mitochondria, or other inclusions of different color and appearance. Thus, in *Cynthia*, four cytoplasmic regions are evident in an egg that has completed the first cleavage (Conklin, 1905). The animal hemisphere consists of a colorless cytoplasm, whereas in the region of the vegetal pole, the cytoplasm is a contrasting gray. In addition, a light-gray crescentic area appears on one side of the egg and a yellow crescent on the opposite side. Because these cellular inclusions can be distinguished for some time in development, it is possible to show that the nervous system and notochord originate from the gray crescent and mesoderm from the yellow crescent. Ectoderm is derived from the clear cytoplasm of the animal hemisphere and the gray vegetal region produces endoderm. The colored granules or mitochondria are thus useful "markers" for the prospective developmental fates of specific regions, although the inclusions as such seem to have little significance. Conklin (1931) showed that the relative position of these particles can be altered by centrifuging eggs of *Cynthia* without seriously deranging development. However, he felt that the underlying ground substance of the cytoplasm did possess regional specializations, for if part of the early embryo was injured or removed, the remainder formed only those parts corresponding to its normal developmental fate. Complete individuals are not ordinarily obtained from isolated blastomeres. The egg of *Cynthia* thus seemed to represent a patchwork of morphogenetic substances, distributed during cleavage in orderly fashion to give rise to various parts of the future embryo. A similar picture emerged from the studies of E. B. Wilson (1904) on the mollusc *Dentalium* and from the work of various investigators on a number of other eggs.

As a result of a number of such studies, the concepts of mosaic and regulatory eggs came into being. The latter category is typified by the sea urchin embryo, in which differential distribution of cytoplasmic components does not occur in early cleavage stages, or is easily reversible. By contrast, mosaic eggs such as *Cynthia* or *Dentalium* can be pictured in terms of an early segregation of morphogenetic substances into specific cytoplasmic regions, which are thereby correspondingly limited in developmental potentialities. This apparent dualism in organization of the egg was intriguing to early investigators, and discussions of the difference dominated morphogenesis for a number of years. On the basis of more recent studies, however, it does not appear that any qualitative distinction can be made between the patterns of mosaic and regulatory eggs (Grobstein, 1959; Brachet, 1960). It is now known that twinning or other examples of regulation can be observed in some mosaic eggs by variation of the techniques used earlier. On the other hand, even the blastomeres of the sea urchin assume a mosaic character after the first few cleavages (Hörstadius, 1939). Cytoplasmic localization is thus a universal feature of differentiation. Although the time relationships of this process may vary among different embryos, it seems doubtful that these distinctions are of any fundamental significance.

Cytoplasmic localization and the segregation of morphogenetic substances by cleavage were the first mechanisms of embryonic differentiation to be demonstrated in clear-cut fashion by experimental means. These were soon followed by the discovery that developmental patterns can also be established by a more dynamic process, involving the interaction of embryonic rudiments. This phenomenon, termed embryonic induction, is associated particularly with the name of Spemann, although many investigators have joined in establishing its broad validity as a developmental principle. The nature of inductive processes became known largely as a result of studies by Spemann and others on the formation of the eye in amphibian embryos (see Spemann, 1938). In amphibia, as in other vertebrates, differentiation of the lens of the eye is a sequential process, involving outgrowth of the optic vesicle from the embryonic brain and contact with the overlying ectoderm. The question posed initially was whether the association of optic cup and lens-forming ectoderm is fortuitous or represents a causal relationship. In some embryos the lens proved to be self-differentiating, but the more typical finding was a correlative relationship with the optic cup. In *Rana fusca* or *R. palustris,* for example, removal of the optic vesicle prevents formation of the lens, whereas if the optic cup is implanted beneath the trunk ectoderm, a lens is elicited from ectodermal cells that would not otherwise form such a structure (Lewis, 1904; Spemann,

1912). Other induction systems were subsequently discovered in the embryos of amphibia and other types of animals. It was not until later, however, that the fundamental role of organizers in development was perceived. The basic discovery that opened up this new vista was the induction of secondary embryos in amphibia by the implantation of embryonic rudiments from the early gastrula stage (Spemann and Mangold, 1924; Spemann, 1938). In these experiments a piece of chordamesoderm was excised from the dorsal lip of the blastopore in an egg at the early gastrula stage. This block of cells was then grafted to the lateral marginal zone of another embryo and became covered over with host ectoderm. In this location, a new system of embryonic organization subsequently developed, complete with nervous system, notochord, somites, and other axial structures (see Figure 1.2). By making use of two species of newts whose cells differ in degree of pigmentation, Spemann and Mangold were able to distinguish the cells of host and graft. Rather than merely undergoing transformation, the implant had served as an organizer to implement a far-reaching reorganization within adjacent host tissues. Some parts of the secondary embryo, such as the nervous system, were derived entirely from host cells; other structures were composite in nature, derived partly from the graft and partly from the host.

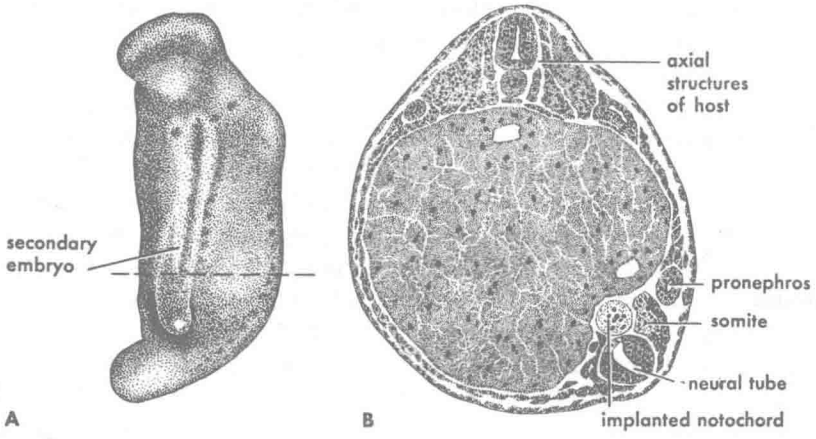

FIG. 1.2. Induction of a secondary embryo in *Triturus* by means of grafted chordamesoderm. (*A*) Host embryo bearing an accessory set of axial structures on the left side. Note two rows of somites, and ear vesicles at anterior end of neural tube. (*B*) Cross-section along plane indicated by dotted line in (*A*). Differences in pigmentation of cells show that secondary embryo is derived from tissues of both graft and host. (Redrawn from Spemann and Mangold, 1924.)