latter component is referred to as noise [C. E. Leith, *J. Appl. Meteorol.* **12**, 1066 (1973); R. A. Madden, *Mon. Weather Rev.* **104**, 942 (1976); _____ and D. J. Shea, *ibid.* **106**, 1695 (1978)]. This distinction need not be made here, since the possible effects of increasing CO_2 must be isolated from the total interannual variability regardless of its ultimate origin.
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9. T. Augustsson and V. Ramanathan [*J. Atmos. Sci.* **34**, 449 (1977)] show that the various radiative-convective model studies yield values for enhanced CO_2 radiative heating that agree with each other within 10 percent. On this basis, we conservatively assume an uncertainty of approximately ± 20 percent due to radiation model errors.
10. The upper limit for λ_0 ($\approx$4 W m^{-2} K^{-1}) is obtained by assuming that the climate system is devoid of all feedbacks other than infrared radiative damping and that it emits infrared radiation like a blackbody with an equilibrium temperature of about 255 K. One-dimensional radiative-convective models yield values of λ_0 ranging from 1.25 to 2 W m^{-2} K^{-1} [V. Ramanathan and J. A. Coakley, *Rev. Geophys. Space Phys.* **16**, 465 (1978)]. Energy balance climate models yield λ_0 values ranging from 1 to 2 W m^{-2} K^{-1} [for example, see M. S. Lian and R. D. Cess, *J. Atmos. Sci.* **34**, 1058 (1977)], from which we obtain the lower limit of λ_0 shown in Eq. 4. The principal reason why the model λ_0 values are significantly smaller than 4 W m^{-2} K^{-1} is the inclusion of relative humidity feedback [see the Ramanathan and Coakley reference cited above] and the ice-albedo feedback [see the Lian and Cess reference cited above]. The effects of these two positive feedbacks may be compensated by as yet unidentified negative feedbacks involving cloud-radiative interactions and ocean-atmosphere interactions.
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15. From Fig. 2, we saw that annual averages have larger noise than summer averages. We also computed 9-month averages from March through November, excluding the winter months. The resulting variance is slightly less than that for the 3-month summer averages (0.13 compared to 0.14 K^2); however, the spectrum of the 9-month averaged data has relatively more variance at low frequencies than that of the summer-averaged data, so that averaging several summers together decreases the noise variance faster than averaging several 9-month means together. As a result, we believe that the 3-month summer seasons provide the data set with the least noise.
16. J. E. Kutzbach and R. A. Bryson, *J. Atmos. Sci.* **31**, 1958 (1974).
17. Results did not appear to be particularly sensitive to assumed starting date. For example, when averages are computed from 1930, the positive-feedback signal crosses the $2\sigma_N$ noise line in 1960 (30-year average) and the zero-feedback signal is still below the line in 1990 (50-year average). By beginning averages in 1956, we can avoid some of the uncertainties which bias in the near-zero frequency spectral estimates introduce into longer time averages with virtually no change in the result.
18. In this case, since we have assumed that the ΔT_s amplification due to ice-albedo feedback is manifested during winter, the summer signal is that given by the positive-feedback models without the ice-albedo feedback. The λ_0 for the positive-feedback models without ice-albedo feedback is approximately 2 W m^{-2} K^{-1}, and hence Eq. 9 is obtained by combining Eqs. 2 and 4 with $\lambda_0 = 2$ W m^{-2} K^{-1}. Also, the results for this case closely resemble those obtained by S. Manabe and R. J. Stouffer [*Nature (London)* **282**, 491 (1979)]. In their coupled GCM-simple mixed-layer ocean model, Manabe and Stouffer found, at 60°N for $g = 4$, a winter warming of about 10 K and a summer warming of 4 K. Equations 7 and 9 give similar values of 8.3 and 4.2 K.
19. Several unpublished GCM studies of the CO_2 climate problem are summarized in a recent report [Ad Hoc Study Group on Carbon Dioxide and Climate, *Carbon Dioxide and Climate: A Scientific Assessment* (Climate Research Board, National Research Council, Washington, D.C., 1979)]. As seen from this report, the high-latitude surface warming predicted by the GCM's is of the same magnitude or larger than the upper limit for ΔT_s given in Eq. 8.
20. From Fig. 5 the positive-feedback signal is about 0.70 K in 1977. Since the signal is nearly a linear function of time, it would contribute (0.7 K)^2/12 to the variance.
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22. We thank S. Schneider and H. van Loon for their helpful comments on an earlier version of this article.

Teratocarcinomas and Mammalian Embryogenesis

Gail R. Martin

Teratomas have always intrigued pathologists because they contain a grotesque array of diverse tissues, sometimes including highly organized structures such as teeth, fingers, and hair. Thanks to the pioneering work of Stevens (*1*), these rare tumors have become more than a curiosity of human pathology. Stevens observed that mice of certain inbred strains frequently develop tumors analogous to human teratomas. His subsequent studies provided a clear understanding of how teratomas arise in mice and perhaps in man as well. As cell lines were established from the mouse tumors that Stevens made available, it became apparent that these cultured cells (known as embryonal carcinoma cells or teratocarcinoma stem cells) are remarkably similar to the cells of the early embryo. The use of these tumor cells as a model system for the study of mammalian development in vitro circumvents many of the difficulties of working with embryonic material. Interest was further stimulated by the demonstration that embryonal carcinoma cells taken from the tissue culture dish can participate in the formation of a normal mouse. Such results have raised the hope that teratocarcinoma cells will be useful in creating mouse models of human genetic diseases. The discussion in this article centers on what teratocarcinoma stem cells are and on some of the ways in which their potential uses have been exploited.

Pluripotency and origin of teratocarcinoma stem cells. A typical mouse teratoma or teratocarcinoma contains a mixture of differentiated cell types (Fig. 1A). This complexity is accounted for by the fact that these tumors arise from stem cells that are pluripotent (*2*); that is, they are capable of forming derivatives of all three primary germ layers, namely, endoderm, mesoderm, and ectoderm. The random array of differentiated tissues forms as some stem cells differentiate into cartilage, and others into nerve, muscle, glandular tissue, or other cell types. It is not yet known what triggers such differentiation, but in general the differentiated derivatives of the stem cells are normal, nonmalignant cells (*3*).

Some stem cells, instead of differentiating, continue to proliferate in the undifferentiated state. They thus form nests of pluripotent embryonal carcinoma cells interspersed in the disorganized mixture of differentiated derivatives (Fig. 1B). The continued proliferation of this undifferentiated stem-cell population is responsible for the malignant properties of teratocarcinomas such as progressive growth and transplantability, but not metastasis (*4*). In cases where the stem cells cease to proliferate because they differentiate or die, the tumors become benign and are known as teratomas. Strictly speaking, "teratocarcinoma" refers to a malignant tumor, and "teratoma" refers to a benign one. The latter term, however, is often used to designate either type of tumor.

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Summary. In the last decade there has emerged an appreciation of the remarkable similarity between the cells that give rise to teratocarcinomas in mice and the cells that give rise to the developing mouse embryo. The resemblance is so close that in certain instances the tumor stem cells can join with their embryonic counterparts and develop into a completely normal mouse. The availability of stem cell lines isolated from mouse teratocarcinomas has made possible a number of new biochemical, immunological, and genetic approaches to the study of early mammalian development.

The pluripotency of embryonal carcinoma cells is a reflection of their fundamental similarity to normal early embryonic cells. This close resemblance is not surprising since the tumor cells can be directly derived from early embryonic cells. Embryonal carcinoma cells arise, proliferate, and differentiate—and thus form a teratomatous tumor—each time an early mouse embryo is transplanted to an extrauterine site (such as the kidney or testis of an adult). Under appropriate conditions as many as 50 percent of such experimentally induced tumors are malignant teratocarcinomas that retain a proliferating population of undifferentiated stem cells (*5*).

Tumors can also arise spontaneously in either the ovary or testis (*1*). In these cases, extrauterine embryo-like structures appear to be the progenitors of the tumor stem cells. In the ovary, tumor formation occurs when oocytes undergo spontaneous parthenogenesis in situ (that is, they behave as if they had been fertilized) and develop as normal embryos for a brief period. These parthenogenetic embryos subsequently become disorganized and form a tumor in the same way as embryos experimentally transplanted to an extrauterine site. In the testis, abnormal proliferation of primordial germ cells leads to the formation of embryonic ectoderm-like structures that subsequently become disorganized and form tumors. Most of these spontaneous tumors are benign teratomas, although occasionally they retain a proliferating population of undifferentiated stem cells.

Studying Mammalian Embryogenesis Without Embryos?

Stem cells can be isolated from teratocarcinomas and cultured in vitro (*6*). Although most mouse tumor cell lines are grossly aneuploid (defined here as having abnormal chromosome number or structure), embryonal carcinoma cell lines generally retain a chromosome constitution that is relatively close to that of normal mouse cells, even after long periods of culture in vitro. The pluripotency and other properties that they have in common with their embryonic progenitors are also generally retained by embryonal carcinoma cells in vitro. These established teratocarcinoma stem cell lines thus provide an alternative to pluripotent mammalian embryonic cells in cases where it is difficult to obtain adequate quantities of normal embryonic material. This is particularly important since it has not yet been possible to establish pluripotent embryonic cell lines in vitro.

Embryonic equivalent of teratocarcinoma stem cells. If embryonal carcinoma cells are to be used as an in vitro model for pluripotent mammalian embryonic cells, it is important to know which of the various types of early embryonic cells the tumor cells most closely resemble. During early embryogenesis the pluripotent cell population in the normal embryo undergoes many biochemical and morphological changes (Figs. 2 and 3). Differences among pluripotent cells at various stages of development are reflected in the cell types to which they give rise. Thus, at the time of the first visible differentiation, pluripotent cleavage stage cells form two cell types: (i) the pluripotent inner cell mass (ICM) which will ultimately form the fetus and (ii) the differentiated, extraembryonic trophectoderm, which functions in implantation and establishment of the fetal relationship with the mother. At the time of the second visible differentiation, the pluripotent cells of the ICM give rise to two other cell types: (i) the pluripotent, fetus-forming primary ectoderm and (ii) the differentiated, extraembryonic primary endoderm (Figs. 2 and 3).

Some embryonal carcinoma cell lines give rise to primary endoderm-like cells as their first differentiated derivative in vitro (*6*). This suggests an analogy between embryonal carcinoma cells and the cells of the embryonic ICM. Consistent with this analogy is the observation that certain embryonal carcinoma cell lines display a pattern of differentiation, known as embryoid body formation, that resembles the behavior of ICM's that have been isolated and cultured in vitro (*7, 8*) (Figs. 3 and 4A). However, comparison of the proteins synthesized by embryonal carcinoma cells and early embryonic cells suggests that embryonal carcinoma cells may be more similar to pluripotent cells in the embryonic ectoderm than to those in the ICM (*9*). Consistent with this concept is the observation that isolated embryonic ectoderm grafted to an extrauterine site will give rise to a teratomatous tumor (*10*).

There is thus some uncertainty about the normal embryonic equivalent of embryonal carcinoma cells, and whether pluripotent embryonal carcinoma cells isolated from different tumors are all derived from the same embryonic cell type. Nevertheless, these tumor cells, particu-

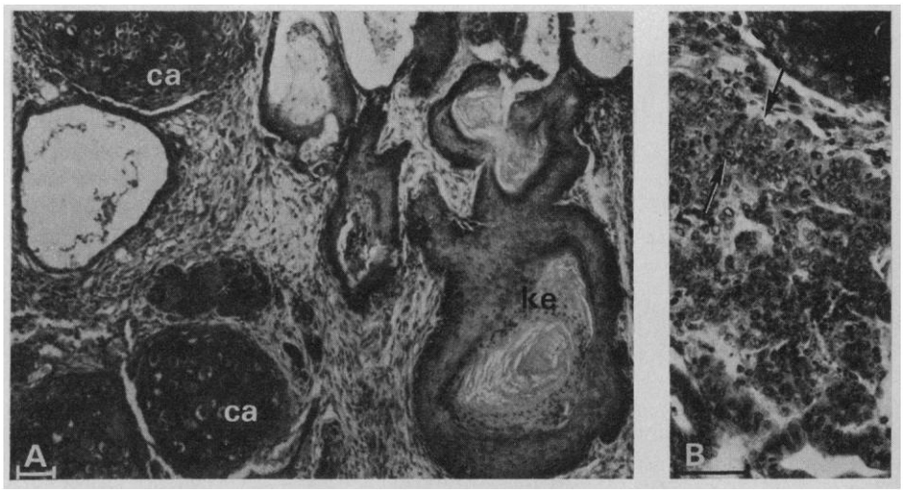

Fig. 1. Sections of a mouse teratocarcinoma. Scale bars indicate 100 μm. (A) The tumor contains a variety of differentiated cell types. Cartilage (*ca*) and skin (keratinizing epithelium, *ke*) are readily distinguished in this section of the tumor. (B) A group of embryonal carcinoma cells adjacent to differentiated derivatives [cartilage (upper right) and neuroepithelial tubules (lower right)]. The arrows point to particularly clear examples of the undifferentiated stem cells, which have relatively little cytoplasm and a large nucleus often containing a single distinct nucleolus.

larly those cell lines that synchronously form embryoid bodies, can provide a model system for studying differentiation during the early postimplantation period (see Fig. 3). Such a model is especially useful not only because this is the period during which the embryo is least accessible, but also because it is the time when critical steps in cell determination and differentiation are occurring. In contrast, embryonal carcinoma cells are apparently not similar to pluripotent cleavage stage embryonic cells and therefore are not likely to be directly useful for studying the earlier stages of preimplantation embryonic development such as trophectoderm formation.

Differentiation of embryonal carcinoma cells in vitro. Most of the early studies in vitro described the isolation and differentiative capacity of the various established embryonal carcinoma cell lines (*6*). One important conclusion from this work was that changes leading to a loss of differentiative capacity can occur in culture. This is consistent with the earlier observations of Stevens that stem cells can lose pluripotency when teratocarcinomas are passaged in vivo (*1*). Embryonal carcinoma cells that are no longer pluripotent do, however, retain many properties in common with their undifferentiated pluripotent counterparts in vivo and in vitro. Cells that show the most extreme type of restriction, known as "nullipotency," appear incapable of differentiation (Fig. 4B). A less extreme form of restriction is the ability of the cell to differentiate into only one or two types. For example, the F9 cell line apparently can spontaneously form endoderm-like cells, but not other cell types (*11*). Cells with such limitations in developmental potential are likely to be useful in studies of how pluripotency is maintained.

An important factor in the differentiation of pluripotent teratocarcinoma stem cells is cell aggregation. Spontaneous differentiation of embryonal carcinoma cells to a wide variety of cell types occurs when the cells are cultured at a high local density (Fig. 4A). In some cases, this is achieved by culturing single cells or small clumps of cells on a tissue culture surface until they become large, tightly rounded colonies (*12*); alternatively, some cell lines give rise to numerous differentiated derivatives when cultured as dense monolayers (*13*). Still other cell lines form embryoid bodies when cultured as aggregates in suspension (*7*); differentiated derivatives not formed in suspension (such as cartilage, keratinizing epithelium, or striated muscle) can be obtained by allowing the

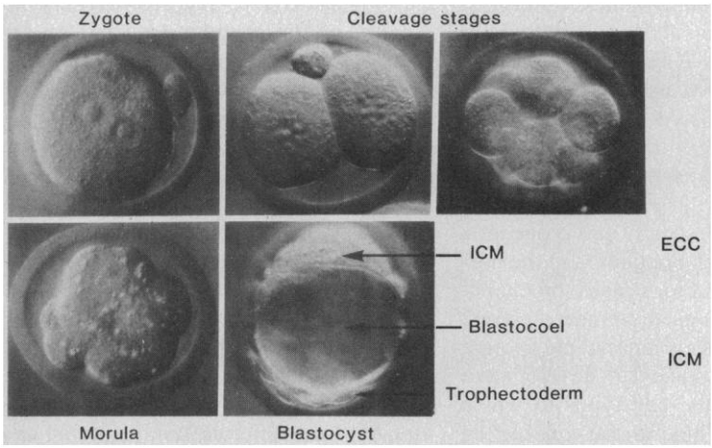

Fig. 2. The preimplantation stages of mouse embryogenesis (*56*). The fertilized egg or zygote undergoes the first cleavage within 24 hours of fertilization, giving rise to the two-cell embryo. The interval between cell divisions becomes shorter, and by approximately 55 hours after fertilization the embryo consists of eight pluripotent cells or "blastomeres." The blastomeres compact to form the "morula" and cell division proceeds. Subsequently, the first visible differentiation occurs, leading to the formation of the trophectoderm by the outer cells of the morula. This cellular layer encloses the inner cell mass (*ICM*), and is responsible for the formation of a fluid-filled cavity (*Blastocoel*). By approximately 4.5 days after fertilization, this 64- to 128-cell "blastocyst" is ready for implantation in the uterus. Throughout these stages the developing embryo and its persistent polar body are surrounded by a protective, acellular matrix, the "zona pellucida," which it sheds just prior to implantation. Around that time the second differentiation in the developing embryo occurs as the cells on the "outer" or blastocoelic surface of the ICM form the primary endoderm. The embryos shown are approximately 100 μm in diameter. [Photographs courtesy of Dr. Patricia Calarco]

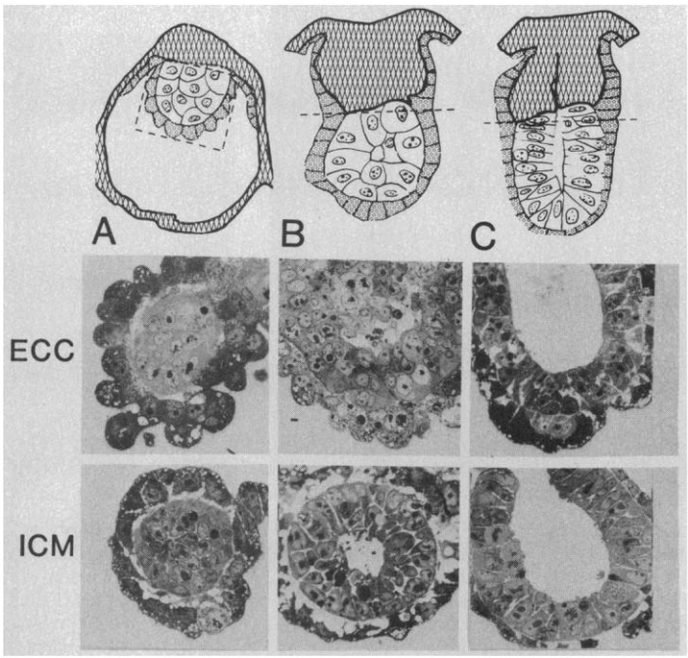

Fig. 3. The similarity between embryoid body development and the early postimplantation development of the mouse embryo (*57*). At the time of implantation (A) the embryo is surrounded by the trophectoderm (cross-hatched area) which is responsible for implantation in the uterine wall and formation of extraembryonic structures, such as the placenta. The inner cell mass or fetal portion of the embryo (delineated by dotted lines) consists of an outer layer of endoderm (stippled cells) enclosing the embryonic ectoderm (clear cells). By approximately 5 days of development (B) the fetal portion of the embryo (below the dotted line) develops a "proamniotic cavity" in the center of the ectoderm. By approximately 6 days of development (C) the proamniotic cavity has enlarged and the embryonic ectoderm is now organized into a columnar epithelium. Below each schematic representation of the developing embryo are sections of the structures formed by certain embryonal carcinoma cell lines (*ECC*) when they are cultured as aggregates. Within 36 hours of being placed in suspension, the outer cells of these aggregates differentiate to form a layer of endoderm. Subsequently these two-layered "embryoid bodies" undergo internal changes that parallel the development of the fetal portion of the embryo. For comparison, sections are shown of ICM's that have been isolated from the blastocyst prior to implantation and cultured in vitro. [Sections of ICM's courtesy of Roger Pedersen]

embryoid bodies to attach to a tissue culture surface (*14*). Thus, embryonal carcinoma cell differentiation occurs in dense, multilayered cultures that are exceedingly heterogeneous in both variety and quantity of differentiated cell types present.

The need became apparent for a simple culture system in which the differentiation of the pluripotential cells to a single type of cell could be studied. Attempts to direct the formation of specific cell types such as nerve, cartilage, or muscle from undifferentiated embryonal carcinoma cells by the addition of various compounds including growth factors have not as yet been successful. Recent studies have concentrated on the process of endoderm formation by embryonal carcinoma cells. This differentiation is of interest for several reasons. (i) Endoderm cells are usually the first type to appear during the differentiation of aggregated embryonal carcinoma cells, and may have some inductive role in subsequent differentiation. (ii) Endoderm formation by embryonal carcinoma cells parallels that of primary endoderm formation by pluripotent embryonic ICM cells. (iii) Its formation by embryonal carcinoma cells can occur relatively synchronously and in the apparent absence of other differentiative changes.

Cells that form embryoid bodies when cultured as aggregates are useful for the study of endoderm formation. In such cultures, cells that are in an "outside" position differentiate to endoderm, whereas those completely surrounded by other cells remain pluripotent. This differentiation mimics the way in which normal embryonic cells respond to their position within the embryonic mass and thus provides a means of studying the nature of positional information in early mammalian development. The transition from undifferentiated embryonal carcinoma cell to endoderm can also be studied in the absence of positional effects. For example, Strickland and Mahdavi (*15*) have shown that the addition of low concentrations of retinoic acid to monolayer cultures of F9 cells stimulates the formation of endoderm-like cells. This method of obtaining endoderm formation is amenable to biochemical analysis since virtually all of the cells in the culture are stimulated to differentiate, and thus a relatively homogeneous population of endodermal cells is formed.

In order to study differentiation, it is necessary to have markers that are specific to each of the cell types in the differentiative sequence. For this reason, many of the studies of endoderm formation by embryonal carcinoma cells have dealt with identifying proteins or other markers specific to each of these two cell types. Several endoderm-specific proteins not present in significant quantity in undifferentiated embryonal carcinoma cells have now been identified. These include α-fetoprotein (*16*), plasminogen activator (*17*), basement membrane proteins (*18*), and certain intermediate filament proteins (*19*), as well as other unidentified proteins that are visualized by gel electrophoresis. Since differentiation is presumed to be the consequence of changes in gene expression, future studies are likely to focus on the genes and messenger RNA's that encode these markers.

A start has already been made in studying the control of gene expression in embryonal carcinoma cells at the level of nucleic acid synthesis and processing. When F9 embryonal carcinoma cells are infected with SV40 virus, viral T antigen is not expressed, whereas similarly infected cultures of endoderm or other differentiated cell types do express this viral protein (*20*). Recent studies (*21*) suggest that the reason SV40-encoded T antigen is not detected in undifferentiated F9 embryonal carcinoma cells is that the cells are unable to carry out the processing (splicing) that is a prerequisite for transport of the viral RNA transcripts from the nucleus to the site of translation in vivo. In contrast, splicing and subsequent translation of SV40 viral RNA occur if F9 cells are infected with SV40 virus after they have been stimulated to form endoderm by treatment with retinoic acid. These results suggest that the differentiation of F9 cells is accompanied by a change in splicing of viral RNA. However, it remains to be determined whether the inability to splice viral RNA is characteristic of most or all embryonal carcinoma cell lines or if it is specific to F9 cells. Nevertheless, this study does introduce the intriguing possibility that changes in splicing may be involved in some aspect of endogenous cellular as well as viral gene expression during differentiation of pluripotent cells.

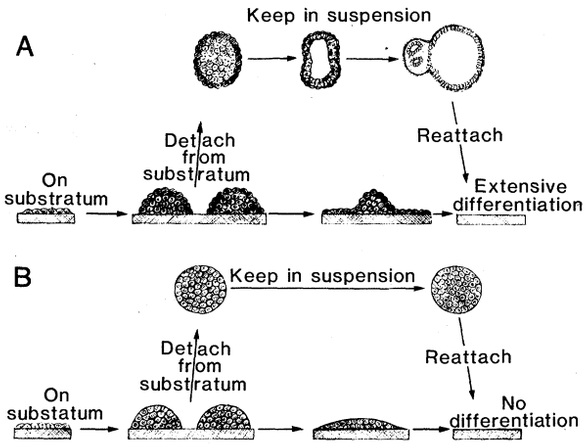

Fig. 4. Schematic representation of the behavior of embryonal carcinoma cells in vitro. (A) Pluripotent cells. Many embryonal carcinoma cell lines will differentiate when cultured at high local density on a tissue culture substratum. In some cases it is apparent that the first differentiated cell type to appear in these attached cultures after several days is an endoderm-like cell. Over the span of several weeks, other cell types such as nerve, muscle, and pigmented epithelium are observed in these dense, multilayered cultures. Certain embryonal carcinoma cell lines will differentiate in an embryo-like manner when cultured as clumps in suspension. If these embryoid bodies are allowed to reattach to a tissue culture substratum, various cell types form, such as cartilage and keratinizing epithelium, which are not observed in the suspension cultures. (B) Nullipotent cells. Certain embryonal carcinoma cell lines apparently lack the capacity to differentiate spontaneously in vitro, regardless of whether they are attached to a tissue culture substratum or cultured in suspension. [Courtesy of *Cell* (*6*)]

Expression of cell surface molecules. Since cell surface molecules are thought to have a role in mediating the differential gene expression that characterizes early embryonic development, teratocarcinoma cell cultures are being used as models of the embryonic cell surface. As might be expected, the numerous similarities of embryonal carcinoma cells to normal embryonic cells include the expression of common cell surface molecules.

Various conventional antiserums and monoclonal antibodies have been produced with the use of embryonal carcinoma cells as the immunogen; these antibodies usually recognize cell surface antigens common to teratocarcinoma stem cells, early embryonic cells, and germ cells (*22*). Such antibodies to embryonal carcinoma cells are generally unreactive against various "adult" cell types, including differentiated derivatives of the teratocarcinoma stem cells themselves. Most of the available data deal with defining the specificity of each antiserum or monoclonal antibody. Particular emphasis has been placed on determining what cells in the developing embryo express the antigens recognized by each of these serological reagents. Such information on the topographic and temporal pattern of antigen expression is important for understanding the function of these cell surface molecules.

Many of these studies have been concerned with the F9 antigen, which is detected by antibodies to the F9 embryonal carcinoma cell line (*22, 23*). First apparent a few hours after fertilization, F9 antigen increases in amount on the surface of cells in the developing embryo (see Figs. 2 and 3) up to the morula stage. In the blastocyst it is present on both the trophectoderm and ICM and, after implantation, is expressed by the embryonic ectoderm. It disappears from the embryo at about day 9 of development, and does not appear to be expressed by any cells in the adult except the male germ cells (*24*). Since the antigens recognized by antibodies to F9 are expressed by cells from human teratocarcinomas and by sperm of all mammalian species tested, it appears to have been conserved through mammalian evolution.

Although reagents such as antiserum to F9 should facilitate the isolation of cell surface molecules from embryonal carcinoma cells, early embryos, or germ cells, relatively little is known about the molecular characteristics of these antigens or their function. There is some evidence that the antigen or antigens recognized by one antiserum to F9 may have an important, stage-specific function in early development. Kemler *et al.* (*25*) found that monovalent Fab fragments of rabbit antibodies to F9 can reversibly inhibit cell compaction and subsequent blastulation in preimplantation mouse embryos, although this antibody apparently has little effect on cell division. Also, Fab fragments from this antibody inhibit adhesion of cells in ICM's isolated from early blastocysts as well as of cells in embryonal carcinoma cultures, without inhibiting their division (*23*).

It has been suggested that the stem cell antigen detected by antiserums to F9 is the product of a gene or genes in the T/t locus (*26*). This genetic complex affects early embryogenesis, as demonstrated by the observation that embryos homozygous for certain T/t mutants die at specific stages of early development (*27*). The concept that the F9 cell surface antigen might be coded for by the T/t complex is based on the hypothesis that the products of the T/t complex are cell surface antigens that mediate critical steps in early development. Although some data indicate that genes in the T/t locus specify cell surface antigens, and suggest that the F9 antigen may be among these, the evidence is by no means conclusive (*28*). It has further been suggested that T/t coded products, possibly including the F9 antigen, are part of an embryonic recognition system that is evolutionarily related and biochemically homologous to the adult major histocompatibility complex (MHC) (*29*). Consistent with this idea is the observation that cells that express the F9 antigen generally do not express MHC antigens (H-2 antigens of mice) and vice versa (*22–24*). Recent studies, however, indicate that an extensive structural homology between F9 and H-2 antigens is unlikely (*30*).

Cell surface molecules expressed by embryonal carcinoma cells have also been studied by methods other than the immunological ones discussed above. For example, using labeled sugars, Muramatsu and his collaborators (*31*) have shown that undifferentiated embryonal carcinoma cells and early embryonic cells synthesize a class of large fucosylglycopeptides that are not synthesized by differentiated cells. These were subsequently found to be a constituent of the cell surface molecules recognized by antiserum to F9 (*30*). Further studies, with teratocarcinoma stem cells as a source of experimental material, should lead to a characterization and understanding of the function of these molecules during early development.

An alternative approach has been to choose a particular cell surface function and then attempt to identify molecules that are responsible for it. Since cell aggregation is an important factor in the differentiation of teratocarcinoma stem cells, Grabel *et al.* (*32*) have begun a characterization of cell surface molecules that are responsible for the intercellular adhesion of undifferentiated cells. Starting from the premise that carbohydrate-binding molecules mediate cell-cell interaction, they obtained evidence that a cell surface molecule that recognizes and specifically binds to certain mannose-rich molecules is involved in the intercellular adhesion of embryonal carcinoma cells. Some evidence suggests that the sugar specificity of this carbohydrate-recognition molecule changes during differentiation of the cells. Whether these or similar molecules play a role in the intercellular interactions that occur during normal embryonic development is not yet known.

X chromosome inactivation. Embryonal carcinoma cells may also provide a means of studying another aspect of genetic regulation in early mammalian development, X chromosome inactivation (*33*). This process, which occurs in the cells of female embryos around the time of implantation, alters one of the two X chromosomes so that it no longer produces functional gene products. This change is apparently irreversible, except perhaps in germ cells. As a consequence of X inactivation, female cells, which have two X chromosomes, and male cells, which have only one, produce the same amount of X-linked gene products.

It has now been shown that certain undifferentiated female teratocarcinoma stem cells with two X chromosomes contain twice the level of X-linked enzyme activity as do comparable cells with only one X chromosome (*34*). Only when the cells are allowed to differentiate in vitro does the X-linked enzyme activity decrease in the cells with two X chromosomes to the level found in cells with only one. The conclusion from these biochemical data is that such female embryonal carcinoma cells can provide an in vitro model system for molecular studies of X inactivation.

Creating Mutant Mice at Will?

With the use of appropriate culture conditions it is possible to select embryonal carcinoma cells that carry specific mutant genes. In addition, with various methods of gene transfer, including intra- or interspecific somatic cell hybridization, genetic information can be introduced into embryonal carcinoma cells. Studies of such altered cells can provide valuable information about the control of gene expression in teratocarcinoma stem cells and the effect of the altered genotype on their differentiation (*35*). Such studies need not necessarily be limited to behavior of the cells in vitro since it has now been shown that teratocarcinoma stem cells can participate in the development of completely normal adult mice.

The precedent for these experiments was the demonstration that pluripotent cells from two or more early embryos can be combined in vitro; after being transferred to pseudopregnant foster mothers (females mated with sterile males), the composite embryos can develop into normal individuals with cells derived from each of the "parental" embryos. Such animals with tissue contributions from two or more genetically different embryos are termed chimeras (*36*). The proportion or distribution of cells derived from each of the component embryos can be assessed by means of genetic markers. For example, when an embryo that is homozygous for a "pigment gene" is combined with an embryo that is homozygous for a different allele, an estimate of the relative contribution by cells from each embryo to the coat can be obtained by examining the hair pigmentation pattern of the chimera. Internal tissues can be analyzed by means of genetically determined elec-

trophoretic variants of intracellular proteins such as glucosephosphate isomerase (GPI) (*37*).

As discussed below, embryonal carcinoma cells are able to substitute for normal embryonic cells in the formation of chimeric mice. Teratocarcinoma stem cells whose genetic information has been altered in vitro thus provide a potential vehicle for introducing this new genetic information into mice. If descendants of the teratocarcinoma cells were to form part of the germ cell population, the mutant genes or genes from other species could be passed to successive generations. A practical application would be to create mice carrying a mutation that is known to cause defects in humans. These mutant mice could then be used as animal models for studying ways to alleviate the deleterious effects of the mutation. Attempts to implement this scheme, although promising, have revealed critical limitations of the approaches employed.

Production of teratocarcinoma-embryo injection chimeras. Using cells taken directly from a transplantable tumor, Brinster (*38*) was the first to show that teratocarcinoma cells can participate in the formation of a chimeric mouse. The teratocarcinoma cells were combined with normal embryonic cells with the aid of a micromanipulator (Fig. 5). This and other blastocyst injection procedures had previously been used as a means of introducing embryonic cells of one genotype into the ICM of another (*39*).

By injecting cells from the same donor tumor into inbred host blastocysts, Mintz and Illmensee (*40*) obtained at least 30 teratocarcinoma-embryo injection chimeras in which donor and host contributions could be distinguished by a variety of genetic markers (*40, 41*). One of these chimeras, "Terry Tom," has provided the best demonstration to date of the ability of embryonal carcinoma cells to complete normal embryogenesis. At autopsy, GPI analyses revealed a predominance of teratocarcinoma-derived cells in all of its tissues. Even more remarkable was the report that the injected tumor cells had developed into functional sperm, as evidenced by the fact that all of the numerous male and female offspring that Terry Tom produced inherited the genetic markers carried by the donor embryonal carcinoma cells.

Papaioannou and co-workers (*42*) were the first to demonstrate the chimera-forming capacity of embryonal carcinoma cells that had been cultured in vitro. Subsequently, studies by others have focused on chimera formation by

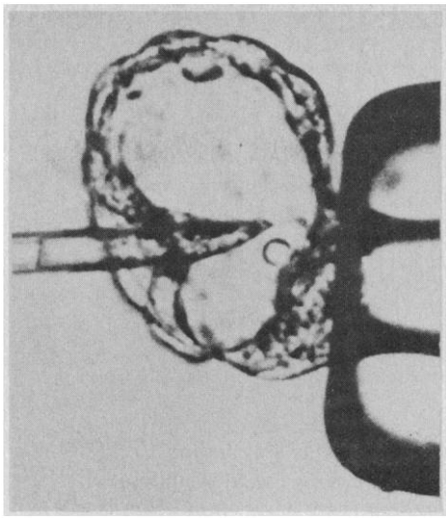

Fig. 5. Injection of a teratocarcinoma stem cell into a blastocyst. The host blastocyst, which is approximately 100 μm in diameter, is held by suction to the blunt pipette on the right. The trophectoderm is pierced with a specially sharpened injection pipette that carries the donor cell. The single teratocarcinoma stem cell is deposited near the ICM and the injection pipette is withdrawn. [Courtesy of Dr. Karl Illmensee and Plenum Press]

embryonal carcinoma cells whose nuclear or cytoplasmic (mitochondrial) genotypes have been altered by genetic manipulation in vitro (*43–46*). For example, embryonal carcinoma cells deficient in hypoxanthine phosphoribosyl transferase (HPRT) activity were selected in vitro and injected into blastocysts (*43*). Cells with this mutation were chosen for study with the expectation that the chimeric mice and any mutant offspring they might produce would serve as models of the Lesch-Nyhan syndrome (*47*), a human genetic disease caused by a severe deficiency of HPRT activity for which there is no animal model. In the chimeras that were obtained, teratocarcinoma-derived cells apparently expressed the mutant phenotype. However, none of the animals produced mutant offspring, nor did they show any spasticity or other clinical correlates of this enzyme deficiency in man. This is probably because the mutant cells in these chimeras were able to interact with large numbers of nonmutant host embryo-derived cells in all tissues.

Another experimental series was aimed at introducing genes from other species into mice. Mouse teratocarcinoma cells were fused with either human fibroblasts (*45*) or rat hepatoma cells (*46*) and cultured under conditions that permit the growth of only those somatic cell hybrids that retain specific human or rat chromosomal material. In the animals obtained by blastocyst injection of embryonal carcinoma cells carrying human chromosome 17, there was no unequivocal evidence that the human genetic material was retained or expressed by teratocarcinoma-derived cells in the chimeric tissues (*45*). However, blastocysts injected with interspecific hybrid (mouse × rat) cells developed into chimeras in which rat-specific electrophoretic variants of several enzymes were clearly demonstrated (*46*). Among these were gene products not expressed by the hybrid cells in vitro, suggesting that differential regulation of the rat genes occurred during development of the chimeric mice.

Taken together, the results of these injection experiments led to some remarkable conclusions. The first is that teratocarcinoma stem cells, whether taken directly from tumors or cultured in vitro, can participate in the formation of chimeric mice. However, embryonal carcinoma cells do differ in their frequency of chimera formation (*48*); for example in some experiments as many as 30 percent of all mice produced were chimeric (*40, 41, 43*), whereas in other studies none of the animals that developed had any teratocarcinoma-derived cells (*49*). A second important conclusion from these studies is that some embryonal carcinoma cells have the potential of contributing to virtually every tissue in the animal.

From the foregoing studies it is also apparent that the chromosomal constitution of a cell is not a good indicator of its developmental potential in chimeras. For example, one embryonal carcinoma cell line with a completely normal karyotype, as assessed by banding techniques, failed to make contributions to fetal tissues in any of several hundred mice analyzed (*49*). In contrast, cells with gross alterations in chromosome number and structure can still participate in the formation of normal somatic tissues (*46*). This is particularly surprising since aneuploidy in embryos generally results in failure or impairment of development. It thus appears that interaction with normal host embryonic cells may allow the full differentiation and normal function of some aneuploid embryonal carcinoma cells.

Most mice produced by blastocyst injection of teratocarcinoma stem cells do not develop tumors. It has been suggested that the few tumors that do form arise from cells that fail to integrate into the ICM, whereas chimeric tissue arises from cells that integrate successfully (*41*). A corollary of this idea is that the probability of both tumor and chimeric tissue formation occurring in the same animal will be a function of the number

Table 1. Tissue contributions by teratocarcinoma-derived cells in chimeric mice formed by blastocyst injection. The numbers indicate the percentage of each tissue that was derived from donor HPRT-deficient embryonal carcinoma cells, as estimated by GPI analysis. The minus sign indicates no detectable teratocarcinoma-derived contribution in the tissue. [Data abstracted from (*43*)]

Case, sex	Autopsy age	Liver	Spleen	Thymus	Heart	Lungs	Kidneys	Gut	Pancreas	Salivary glands	Muscle	Brain	Gonads
1 ♀	5 weeks	15	10	30	55	5	50	40	5	10	25	10	25
2 ♂	2 weeks	30	10	10	50	10	20	40	5	—	40	15	15
3 ♀	3 days	5	15	—	60	10	40	20	—	—	20	—	20
4 ♂	6 weeks	10	5	—	—	—	—	—	—	—	—	10	—
5 ♂	5 days	—	—	—	20	—	5	—		—	—	—	—
6 ♂	3 weeks	—	5	—	—	—	—	—	—	—	—	5	—
7 ♀	3 weeks	—	—	—	—	—	—	—	—	—	—	15	—
8 ♀	3 weeks	—	—	—	—	—	—	—	—	—	—	15	—
9 ♂	3 weeks	—	—	—	—	—	—	—	—	—	—	10	—

of cells injected. This hypothesis could explain why Papaioannou and co-workers (*42*) found that a large proportion of chimeras developed tumors when relatively large numbers of embryonal carcinoma cells were injected, whereas fewer chimeric mice had tumors when fewer cells were injected.

Alternative approaches. In view of the success of these experiments, it is not surprising that many scientists anticipate using such teratocarcinoma-injection chimeras to introduce various types of altered genetic information into mice. This approach may be productive in two ways.

1) The chimeras themselves could be used to gain new insight into how gene expression is controlled during embryonic development and what the effects of such gene expression are on tissue differentiation and function. For example, chimeras with significant numbers of teratocarcinoma-derived HPRT-deficient cells in some tissues but not in others might be useful in identifying the tissue functions responsible for particular clinical correlates of the Lesch-Nyhan syndrome in man. Analysis of the available data on teratocarcinoma-injection chimeras suggests, however, that the enthusiasm for this approach should be tempered with a cautious skepticism. The expectation that teratocarcinoma-injection chimeras may be useful is based on the fact that studies of embryo-embryo injection chimeras have provided important information about early mammalian development. In such cases, the injected embryonic cells usually make substantial contributions to many or all tissues including the germ line (*50*). In contrast, embryonal carcinoma cells injected into blastocysts generally make small and sporadic tissue contributions (cases 4 to 9 in Table 1), possibly because they are dividing more slowly than the host embryo ICM cells and are thus at a competitive disadvantage. It will be difficult to carry out a systematic investigation of teratocarcinoma gene expression in such chimeras unless a method can be found for increasing the frequency of the rare animals with large teratocarcinoma-derived tissue contributions (for example, Terry Tom, and animals 1 to 3 in Table 1). This problem may be overcome if new cell lines that have a higher capacity for chimera formation are established.

2) The genetic information introduced into chimeric mice by teratocarcinoma cells might be passed on to successive generations. This expectation is based on the report that, in at least three cases, embryonal carcinoma cells taken directly from transplantable tumors have formed functional germ cells in chimeras (*40, 41, 51*). In the last 5 years, however, attempts to obtain similar results with cultured cells have failed and none of the chimeras produced (*42–46, 48, 49*) have yielded teratocarcinoma-derived offspring.

The most likely explanation for this lack of germ line contribution by cultured cells is that all embryonal carcinoma cell lines used to create chimeras have been aneuploid (abnormal chromosome number or structure) or genotypically female (XX or XO, that is, containing one X chromosome and no Y chromosome). Because of such aneuploidy, the probability that they would generate germ cells that have a chromosome constitution capable of supporting normal development is significantly reduced. In addition, since embryonic cells of one sex rarely, if ever, form germ cells in chimeric animals that are phenotypically of the opposite sex (*36*), the female embryonal carcinoma cells are likely to form germ cells only in chimeric mice that are phenotypically female. This further decreases the probability of detecting a small teratocarcinoma-derived contribution to the germ line because females produce a considerably smaller number of progeny than do males.

It thus seems likely that the goal of creating mice whose gametes carry genes introduced via teratocarcinoma cells will not be accomplished until there are embryonal carcinoma cell lines, preferably male, that have a completely normal karyotype and are capable of forming chimeras. A logical source of such cells is the subline of the OTT6050 tumor that was used to create Terry Tom, the mouse that had teratocarcinoma-derived cells with a normal male mouse karyotype—namely, 40 telocentric chromosomes, including a Y chromosome (*52*). Unfortunately, numerous attempts to isolate euploid male cell lines from this tumor have failed.

While blastocyst injection with new cell lines may circumvent the problems discussed above, other methods of chimera production are being tested. For example, cultured embryonal carcinoma cells have been found to adhere to cleavage stage embryos or morulae. These aggregates can develop into blastocysts with teratocarcinoma-derived cells in the ICM (*53*). By combining the tumor cells with host embryos at an earlier stage than that in injection experiments, a longer period of association is achieved. This could allow more time for the tumor cells to make necessary adjustments before the critical steps of tissue determination occur, and thus might ultimately lead to a larger contribution by cells of the teratocarcinoma lineage to both somatic tissues and the germ cell population.

A radically different approach would be to try to derive the whole fetus from teratocarcinoma cells. Theoretically, this could be accomplished by surgically replacing the fetus-forming inner cell mass of a blastocyst with embryonal carcinoma cells. This would provide the embryonal carcinoma cells with the trophectoderm they need for implantation. An alternative method is nuclear transplantation. By replacing the embryonic nucleus of a fetilized egg with that of an embryonal carcinoma cell, the nuclear genes in all cells of the animal that develops would be solely teratocarcinoma-derived. This appears to be technically feasible since Illmensee and Hoppe have

obtained normal mice from enucleated zygotes injected with the nucleus of an ICM cell (*54*). Future application of this method to the production of teratocarcinoma-derived mice requires that the egg cytoplasm reprogram the nuclei of the embryonal carcinoma cells to form trophectoderm (rather than endoderm) as the first step in their differentiation.

Both methods of producing mice derived entirely from embryonal carcinoma cells eliminate the possibility of interaction between teratocarcinoma-derived cells and pluripotent embryonic cells; this makes the requirement for a normal chromosome constitution in the teratocarcinoma stem cells very stringent. Neither of the methods may be successful because interaction between the teratocarcinoma stem cells and pluripotent embryonic cells in chimeras might be required either to "normalize" the tumor cells or simply to rescue the developing embryo from the effects of a chromosomal imbalance in the embryonal carcinoma cells.

Reversing Malignancy?

The experiments discussed above are predicated on the close resemblance of teratocarcinoma stem cells to normal embryonic cells. Since embryonal carcinoma cells also have properties in common with transformed cells the question arises as to whether they are early embryonic cells that have undergone a neoplastic conversion. If so, the apparent reversibility of the malignant transformation during differentiation is strong evidence for an epigenetic, as opposed to a genetic, malignant transformation in the embryonic progenitors of the tumor stem cells.

This view of teratocarcinoma stem cells is consistent with the idea that the primary events in tumorigenesis occur only in relatively undifferentiated cells. According to this stem cell hypothesis of tumor formation (*3*, *55*), malignant stem cells continue proliferation because neoplastic conversion has reduced their efficiency of response to the normal signals for differentiation. The expression of gene products characteristic of fetal rather than adult tissues is due to the presence in tumors of transformed stem cells that retain the capacity to differentiate, albeit at a reduced rate. This hypothesis thus provides an alternative to the idea that neoplastic conversion occurs in terminally differentiated cells and that the fetal gene products observed in tumors are produced as a consequence of dedifferentiation.

There is, however, an equally plausible alternative explanation for the continual proliferation of mouse embryonal carcinoma cells, which is the basis for their classification as malignant cells. This idea is that continual proliferation is a normal property of certain early embryonic cells; embryonal carcinoma cells are not, therefore, transformed, but rather are like normal embryonic cells that are programmed to divide until they receive the appropriate signals for embryonic differentiation and development. In a nonembryonic environment, such as the subcutaneous space or a tissue culture dish, the cells may respond to stimuli for differentiation, although these may be inappropriate for normal embryonic development. In contrast, when the cells are placed in an embryonic environment they may complete normal embryogenesis.

At present it is difficult to know how to distinguish between the possibilities that (i) embryonal carcinoma cells are reversibly transformed embryonic cells or that (ii) they are cells that express a normal embryonic program of continued proliferation until stimulated to differentiate. Any evidence for the former hypothesis must, however, account for two distinctive characteristics of teratocarcinomas, namely, the exceptional ease with which they are induced each time an embryo is grafted to an extrauterine site and the stability and relative normalcy of the stem cell karyotype.

The ambiguity about the nature of teratocarcinoma stem cells and their relation to other kinds of tumor cells does not diminish their potential usefulness. Whether or not their ability to differentiate in vitro or in vivo represents a reversal of malignancy or normal gene expression, the cells are particularly suitable for studying mammalian development. The availability of teratocarcinoma stem cell cultures has already made possible new biochemical, immunological, and genetic approaches to the study of early embryonic differentiation. In addition, the production of teratocarcinoma-embryo chimeras may ultimately lead to the creation of strains of mice with novel genotypes, some of which may serve as animal models of human disease.

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Geomorphic Reconstructions in the Environs of Ancient Troy

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Summary. Sea level rise, deltaic progradation, and floodplain aggradation have changed the landscape in the vicinity of ancient Troy during the past 10,000 years. With the waning of the last major world glaciation and resultant sea level rise and fluctuation, a marine embayment protruded nearly 10 kilometers south of the site of Troy at Hisarlik in the Troad of northwest Turkey. As the sea approached its present level approximately 6000 years ago, fluvial and marine deposition caused a northerly migration of the delta and floodplain of the Scamander and Simois Rivers past the site of Troy toward the present-day coast about 6 kilometers north of the site. In view of these major changes in morphology, interpretations of ancient geographies related to historical or historical-mythological settings must be changed. A number of paleogeographic maps have been reconstructed with the use of subsurface data that records the continuing landscape change since the first occupancy of the site at Troy 5000 years ago. These show that ancient Troy was located on an embayment of the sea. If the Trojan War occurred, then the axis of the battlefield and associated events must be relocated to the south and west of Troy.

The Greeks and Romans of Classical, Hellenistic, and Roman times located ancient Troy south of the mouth of the Dardanelles at the western end of the cuesta-like ridge at Hisarlik tell (Figs. 1 and 2). Here they built a city, New Ilium. To these people, there never was a question of where Troy of the *Iliad* and the *Odyssey* was located. However, by the time of the Renaissance, scholars were uncertain as to the location of Troy. Indeed, by the 18th century many historians doubted that Troy had ever existed and considered the stories of the *Iliad* and *Odyssey* to be mythological. Yet, some scholars continued to insist on the historical fact of Troy (*1*, *2*). Most notable was Heinrich Schliemann (*2*). In his excavations at Hisarlik, in the late 19th century, he identified a series of fortified sites approximately 5 kilometers south of the Dardanelles at the same site identified by the ancients as Troy and New Ilium. On the basis of his readings of Homer and other ancient Greeks and Romans such as Strabo (*3*), he correctly identified the location of Troy and made a series of major excavations (*2*, *4*, *5*). Schliemann identified up to 12 separate occupation layers in the tell at Hisarlik. With his absolute faith in the historic validity of the *Iliad*, he attempted to relate various strata of his excavations to ancient Troy of the *Iliad*. We now know that the site at Hisarlik was occupied from approximately 5000 years before the present to Roman times, when the relatively large city of New Ilium was constructed over the site of ancient Troy and the surrounding hill and plain.

Arguments as to the nature of the ancient geography of the region of Troy and the relation of this geography to features described in the *Iliad* are legion. Blegen (*6*) correlated Troy of the *Iliad* with layer VIIa. Even today, however, other archaeologists argue that layer VIh may in fact be the city of the Trojans of the *Iliad* (*7*). Further, some scholars insist that the stories told in the *Iliad* and the *Odyssey* are purely mythological and that there is no evidence that these stories have any definite relation to any known archaeological sites or to historic events (*8*). The arguments regarding the historicity of the *Iliad* and the *Odyssey* are in part emotional and in part empirical. Regardless, an archaeological site with occupation layers including a large

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