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Transdifferentiation and nuclear reprogramming in hematopoietic development and neoplasia

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Acknowledgements
The authors acknowledge support from the National Institutes of Health (T32CA009120 T32CA09056, CA74929), the Lymphoma Research Foundation, and the Amgen/U.C. BioStar Project (S98-35). We also thank Randolph Wall, Cindy Malone and David Dawson for comments in review of this manuscript.

Immunological Reviews 2002
Vol. 187: 22–39

Immunological Reviews
0105-2896

Summary: Cell transplantation and tissue regeneration studies indicate a surprisingly broad developmental potential for lineage-committed hematopoietic stem cells (HSCs). Under these conditions HSCs transition into myocytes, neurons, hepatocytes or other types of nonhematopoietic effector cells. Equally impressive is the progression of committed neuronal stem cells (NSCs) to functional blood elements. Although critical cell-of-origin issues remain unresolved, the possibility of lineage switching is strengthened by a few well-controlled examples of cell-type conversion. At the molecular level, switching probably initiates from environmental signals that induce epigenetic modifications, resulting in changes in chromatin configuration. In turn, these changes affect patterns of gene expression that mediate divergent developmental programs. This review examines recent findings in nuclear reprogramming and cell fusion as potential causative mechanisms for transdifferentiation during normal and malignant hematopoiesis.

Introduction

In early mammalian embryonic development, a commitment is made to ectoderm, endoderm and mesoderm cell layers, followed by dedication to distinct lineages and differentiation into functional cell types. This gradual process of fate restriction of cells committed to distinct germ layers has been referred to as the germ layer model of developmental organization (*Table 1*; *Fig. 1*). In this model neurons invariantly descend from ectoderm, intestinal glandular cells originate from endoderm and monocytes always arise from mesoderm. For many years the germ layer theory has provided the guiding principle for describing progressive changes in whole organism or lineage-specific differentiation during development. This traditional concept of differentiation can be likened to a branching tree, with terminal effector cells arising from multipotent precursors through progressive stages that irreversibly restrict their fates.

Studies of mammalian hematopoiesis have generally sup-

Table 1. **Definition of terms used in this review**

Term	Definition as used in this review
Germ layer model	Fate restriction of cells committed to distinct germ layers
Commitment	Rigid dedication of a cell to a particular lineage
Differentiation	Sequential execution of a developmental program within a lineage
Transdifferentiation	A switch from one committed cell type to another by nuclear reprogramming
Nuclear program	A temporal, spatial and geometric chromatin configuration of the genome

ported the germ layer scheme of hierarchical development. Hematopoiesis originates during gastrulation in the ventral mesoderm of postimplantation embryos with the production of pluripotent hematopoietic stem cells (HSCs) (1). During life, circulating blood cells and platelets are continually generated in response to physiologic demands and to replenish effector cells that senesce or are lost from pathologic states. Dedicated fetal- or adult-type HSCs meet these requirements

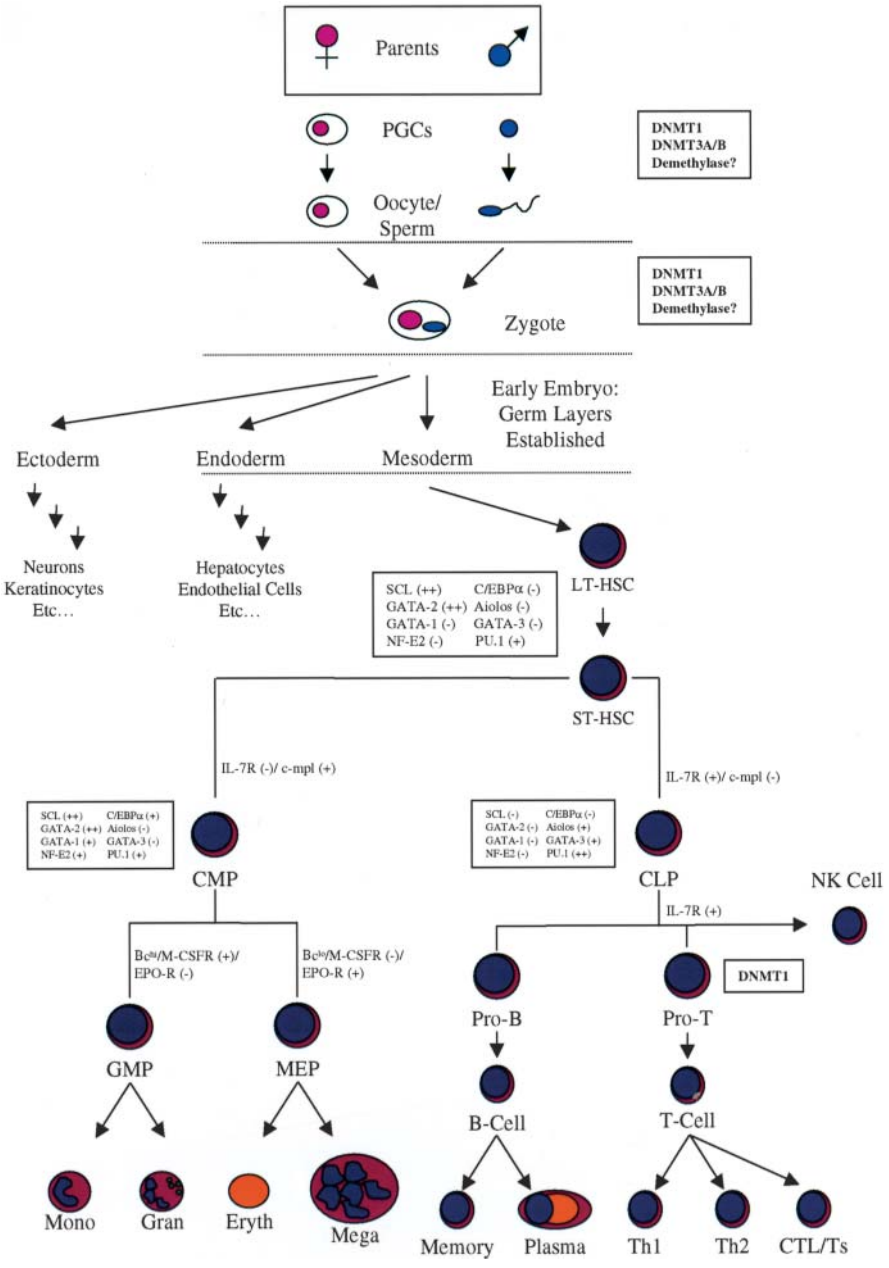

Fig. 1. **The germ layer model of developmental organization.** Transcription factors implicated in hematopoietic lineage differentiation are listed in boxes alongside the cells they regulate. DNA methylating enzymes along with demethylating activity required for specific developmental stages are depicted in bold-face type. Adult-type hematopoiesis has origins in the mesodermal germ layer of embryos. One study has shown the requirement for DNMT1 activity in thymocyte development to T-cell effectors (176). PGCs, primordial germ cells; LT-HSC, long-term hematopoietic stem cell; ST-HSC, short-term-HSC; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte/monocyte progenitor; MEP, megakaryocyte/erythrocyte progenitor.

through self-renewal coupled to the generation of differentiated progeny (2, 3). Specific transcription factors necessary to drive progeny through hematopoietic differentiation into a spectrum of terminally differentiated blood-borne effector cell-types have been identified (reviewed in 4–11). By contrast, the regulatory machinery and decision processes that control self-renewal vs. daughter cell production remain obscure.

According to the germ layer model, an HSC in the hierarchy of hematopoietic development would be positioned at a branch bifurcation with its potential restricted to generating common lymphoid precursors (CLPs) or common myeloid precursors (CMPs). In turn, each of these progenitors would then generate appropriate functional cell types through committed intermediates (*Fig. 1*).

Over the past two decades, however, results from *in vitro* studies have challenged the notion of a strictly hierarchical branching model of hematopoiesis. Numerous investigations have shown that both nontransformed and malignant hematopoietic effectors can switch cell-types within the hematopoietic lineage (recently reviewed in 12). Although every type of switch combination has not been demonstrated, many distinct lymphoid-to-myeloid and myeloid-to-erythroid switches were shown by forced transcription factor expression, cytokine or drug treatments and broad changes in environmental conditions (8, 13–32). These observations are at odds with a unidirectional branching model of hematopoiesis, but they could simply represent *in vitro* adaptations to experimental treatments without physiological relevance.

Recent *in vivo* demonstrations of interconversions between HSCs and nonhematopoietic effector cell-types have further challenged the concept of cell autonomous hematopoietic lineage commitment and differentiation. It now seems that HSCs are able to respond to tissue damage or transplantation by generating progeny that differentiate into myocytes, neurons, hepatocytes, myocardial and endothelial cell-types (33–47) (*Table 2*). Similarly, committed neuronal stem cells (NSCs) also appear to cross lineage barriers and differentiate into hematopoietic effectors in response to specific changes in local conditions (48). Tempering these striking findings, cautious reanalysis of the data has indicated that contaminating nonhematopoietic or non-neuronal precursor cell types might be the ultimate cell sources for the lineage switched phenotypes in most studies (11, 12, 33, 49–56).

It has been known for some time that the ability of committed cell types to switch lineages is tissue-type and organism specific. In amphibians, differentiated pigmented epithelial cells of the eye readily convert into retina or lens following damage (reviewed in 57). Also, rodent pancreatic duct epithelium yields functional islet cells in response to injury (reviewed in 58). These examples of cell-type switching occur within and not between germ layers and are similar to *in vitro* lineage switches seen in the hematopoietic system as alluded to earlier (12). They are impressive examples, as end-stage effector cells are reprogrammed to yield cells with a new function. More spectacular, however, are switches between germ layers, even from committed blood precursors to liver or brain precursors to blood, in which the genomic configuration and transcription factor machinery between cell-types is separated by more divergent developmental histories. Common to both intra- and intergerm layer transitions in organisms of all types is that they occur in response to changes in local microenvironments, tissue damage or regeneration. This suggests that the familiar stability associated with committed hematopoietic precursors and effectors is evident only when cells are examined in their natural surroundings under usual conditions. In these situations, active undefined mechanisms might provide phenotypic permanence and suppress hematopoietic lineage transitions. Prior experiments, mainly in muscle cell fusion with heterokaryon formation causing changes in cell phenotypes and gene expression patterns without DNA replication, support the notion that active control mechanisms are present inside cells to maintain committed and/or differentiated states (59–63). Combined with the emerging *in vivo* data on cell-type switching, such internal control mechanisms would almost certainly depend on transduced signals from the local microenvironment. Supporting this idea are results from Hakelien et al. that show that 293T fibroblasts are induced to express T-cell lineage-specific antigens and to signal through newly expressed interleukin-2 (IL-2) receptor pathway molecules upon incubation with stimulated Jurkat T-cell extracts (64).

These remarkable findings raise important questions about the model and the process of lineage dedication and irreversible progression of differentiation in hematopoiesis. For instance, do cell-type conversions in unnatural surroundings or during tissue repair represent violations of the germ layer theory of development? Or does the germ layer hypothesis depend upon undefined extrinsic environmental signals as well as cell-intrinsic mechanisms for fate restriction? In other words, are HSCs less committed to hematopoiesis than previously realized and only become dedicated with the influence of the local microenvironment? Do certain adult-type stem cells (ASCs), such as those in brain and muscle, arise from direct seeding of circulating HSCs (34, 65, 66)? Or, are these results the unfortunate product of contamination

by unsuspected nonhematopoietic pluripotent precursors and possibly cell fusion in highly enriched HSC populations (11, 54–56, 67, 68)? Do these observations of cross-lineage capability indicate a need to re-evaluate the basic concept of a lineage-committed and tissue-specific progenitor cell (69)? For example, can HSCs arise from advanced cell types within and across lineages by activation of a specific developmental program?

Commitment, differentiation and transdifferentiation

Commitment refers to the rigid dedication of a cell to a particular lineage (*Table 1*). An example would be an HSC, which until recently has been considered to be a committed self-renewing precursor that forms cells of the hematopoietic lineage to the exclusion of all other lineages. Differentiation is defined as the sequential execution of a developmental program of a cell committed to a particular lineage. An example of hematopoietic differentiation is the maturation of a normoblast to an erythrocyte. The term 'transdifferentiation' was coined in the mid-1970s to define a switch in differentiation during normal insect development (70). Transdifferentiation is distinct from lineage infidelity in which distinct mechanisms might also create unique cell phenotypes, such as simultaneous partial expression of two differentiation programs in biphenotypic hematologic malignancies (discussed later). It has been suggested that two main criteria must be satisfied to determine whether a potential lineage switch is genuine (71). First, the differentiated state before and after conversion must be distinct and clearly defined. Second, a direct relationship between cells in these two states must be established. A 'gold standard' for hematopoietic transdifferentiation consisting of three components has recently been proposed: (i) purified bone marrow cells must reconstitute irradiated mice and also form nonhematopoietic tissue components; (ii) single cells or clonal reconstitutions of both hematopoietic and nonhematopoietic tissues must be demonstrated; and (iii) bone marrow-derived nonhematopoietic cells must be differentiated and functional (12).

In recent years transdifferentiation has been used to describe irreversible switches of one functional cell type to another in multicellular organisms of all types (71–75). It is a term that has also been applied to tissue-specific stem cells yielding cross-lineage, differentiated progeny (*Fig. 2*). These processes are likely to occur by similar mechanisms and differ in the extent of nuclear reprogramming needed to affect a lineage switch. Large differences between two cell types resulting from transdifferentiation, such as transitions across germ layers, probably require more complicated and lengthier reprogramming events than smaller changes within germ layers.

Here, transdifferentiation is defined as reprogramming of the nucleus with gene extinction in committed precursor or functional effector cells coupled to the activation of new genes resulting in a lineage switch. This reprogramming process is probably cell-type dependent with unique time courses and degrees of overlap between gene extinction and distinct expression components. Transdifferentiation is not equated with dedifferentiation coupled to divergent redifferentiation. If they were equivalent, intermediate stages of de- or redifferentiated phenotypes between the original and final switched cell type should be observed. Supporting the idea that these

***Table 2.* Hematopoietic lineage switching in vivo**

Organism	Origin	Manipulation	New cell type	Reference
Mouse	Whole BM	Induce muscle regeneration, engraft into scid	Muscle (meso)	(33)
Mouse	Cultured NSC	Irradiate and engraft	Blood cells (meso)	(48)
Rat	Whole BM	Injure liver and engraft	Hepatocyte (endo)	(42)
Mouse	SP BM-HSC	Engraft SP cells into irradiated *mdx* mice	Muscle (meso)	(34)
Mouse	*lin*(–)BM-HSC	Irradiate and engraft	Hepatocyte (endo)	(40)
Human	Whole BM	Transplant for disease; evaluate archive tissue	Hepatocyte (endo)	(39)
Mouse	Whole BM	Irradiate and engraft	Neuron (ecto)	(37)
Mouse	Whole BM	I.P. injection into *PU1*$^{-/-}$ mice	Neuron (ecto)	(36)
Mouse	*lin*(–)BM-HSC	Coronary ligation and heart injection	Myocardium (meso)/ endothelium (endo)	(45)
Mouse	*lin*(–)BM-HSC	Irradiate, single-cell transfer and serial engraft	Multiepithelium (ecto)	(44)
Mouse	SP BM-HSC	Irradiate, engraft SP cells, ligate coronary	Myocardium (meso)/ endothelium (endo)	(65)
Mouse	Mobilized BM	Coronary ligation and cytokine infusion	Myocardium (meso)/ endothelium (endo)	(46)

Listed are reports of switching into or out of the hematopoietic lineage and the germ layer type of the switched cells. The species and cell(s) of origin for the donor are listed, along with the manipulations of environment performed on the recipient and the resultant cell type detected. It should be noted that sources of HSC for transfer vary greatly in purity from unfractionated bone marrow to highly enriched precursors from state-of-the-art separation procedures. *In vitro* and *in vivo* studies have shown that bone marrow contains non-HSC as well as HSC precursors (33, 50–52). The purity of cells yielding a lineage switch is not established in each example with the possible exception of the limit dilution analysis performed by Krause and colleagues in which the ultimate cell-of-origin still remains uncertain (11, 12, 54–56). SP = side populations; BM = bone marrow.

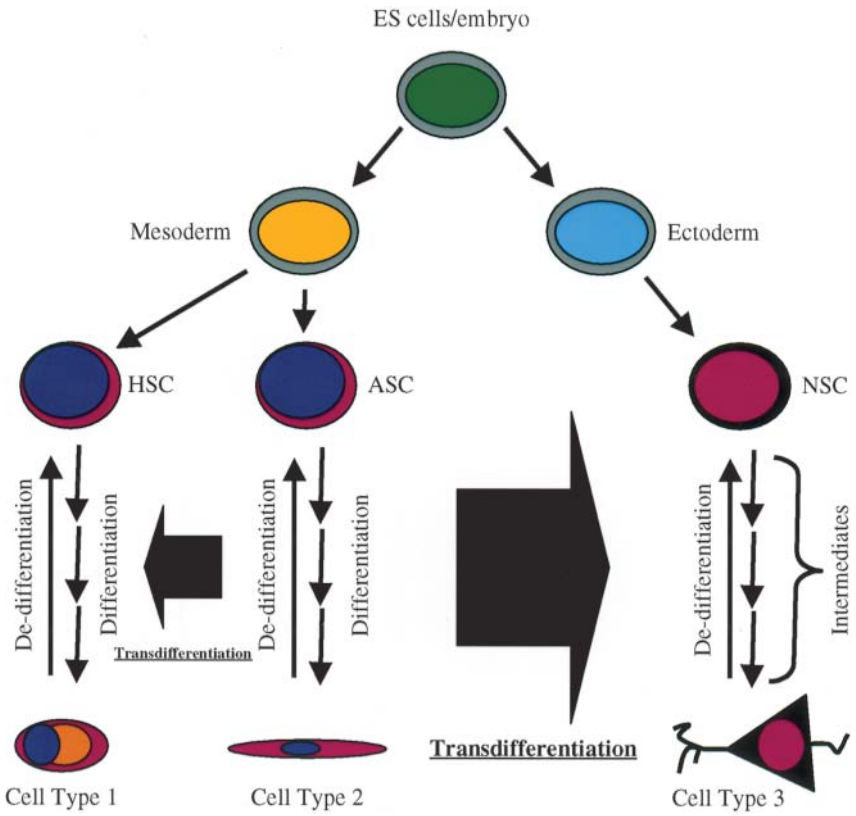

***Fig. 2.* Transdifferentiation.** Detection of cell-type switching requires that the differentiated state before and after cell-type conversion be distinguished and that a direct ancestor–descendent relationship between cells before and after the switch be confirmed (71). Most recent examples have described HSC conversions to nonhematopoietic effector cells. Because of the continuum in cell differentiation, there is an almost endless number of possibilities for cell-type conversions between original and converted lineage cells. However, unknown restrictions could prevent this from happening to an appreciable extent *in vivo*. Mechanistically, the nuclear program of lineage 2 in the example is extinguished and the program for lineage 1 or 3 is expressed with an unknown degree of temporal overlap between these processes. The extent of reprogramming needed to affect a lineage 2–3 switch is greater than that needed for a lineage 2–1 switch in which the developmental histories of the two lineages are more closely related. Transdifferentiation probably does not require dedifferentiation and distinct redifferentiation as precursor cell stages or differentiated intermediates have not been identified when sought (11, 12, 18, 25). A terminally differentiated effector cell type might also directly convert to a distinct effector cell type without detectable precursor stages, as best exemplified for amphibian transdifferentiation. Biphenotypic malignancies are distinct from transdifferentiation processes because they stably coexpress two differentiation programs, probably resulting from transformation of a pluripotent reserve or biphenotypic precursor cell type.

processes are distinct, GATA-1-induced expression of eosinophil markers in myeloid cells is not preceded by upregulation of granulocyte/monocyte progenitor (GMP)-specific markers (25, 26). Also, colony-stimulating factor-1 (CSF-1) induction of macrophages from Pax-5$^{-/-}$ pre-B-cells is not preceded by a macrophage progenitor stage (18). Furthermore, conversion of HSCs to nonhematopoietic effector cells does not result in a series of prefunctional intermediate cell-types, although a formal search for such intermediates was not undertaken (33–47).

Germ layer theory violations?

Transitions of lineage-committed cells in various stages of differentiation from one seemingly inevitable fate to another across germ layers appears to be a direct violation of the germ layer model of development. However, there are caveats. A major consideration is that apparent cross-lineage changes could represent contamination of highly enriched HSCs with additional types of ASCs (reviewed in 11, 54–56). In addition to HSCs, bone marrow contains mesenchymal stem cells and probably ASCs committed to additional germ layers (51, 53, 55). Methods used to isolate HSCs, such as cell-sorting based on the presence or absence of surface markers (Sca-1/CD34, c-Kit, Thy-1, CD38, HLA-DR and CD45) and efflux of Hoeschst 33342 or rhodamine 123 tracer dyes, might not yield pure HSCs (34, 41, 43, 65). In addition, highly purified HSCs are heterogeneous for the few cell-surface markers they express and unsuspected HSC precursors with multilineage potential could be present within this population (56, 76). It is also unlikely that the cellular markers typically used to determine lineage and state of differentiation are invariably

associated with a particular cellular phenotype. Cell markers have narrow to broad patterns of tissue and cell-type expression and can be anomalously expressed in neoplastic conditions. This implies that partial activation of multiple, distinct gene expression programs is possible, such as can be seen in biphenotypic hematologic tumors. If any of these scenarios have physiologic relevance, then the germ layer mechanism would remain intact and limiting-dilution clonal isolation of definitive HSCs would be required to determine their developmental potential. Results from limiting-dilution assays themselves should be interpreted cautiously, because the conditions used might also select for permissive stages of differentiation or elicit nonphysiologic qualities that exaggerate the *in vivo* biological potential of the cells being examined.

Despite these caveats, 'committed HSCs' might yield one of many distinct nonhematopoietic lineages, indicating that histologically and functionally equivalent end-stage cells of a specific organ might not originate entirely from a specific germ layer. Supporting this occurrence, one group used highly enriched pluripotent HSCs to transplant mice that contained a fatal liver enzyme defect leading to tyrosinemia type I disease (41). Transplanted mice that received these highly enriched HSC formed both hematopoietic and regenerative nonhematopoietic cell types that corrected the metabolic defect in the liver (44). This study meets two of the three conditions of the recently proposed gold standard criteria for transdifferentiation of HSCs mentioned earlier, yet it lacks the crucial limiting-dilution analysis to rule out contaminating nonhematopoietic precursors in the transplanted mixture (12). If verified with limiting-dilution studies, this finding would be remarkable, as hepatocytes and supporting stromal cells of the liver have been traditionally assumed to arise strictly from endoderm. It therefore seems critical to firmly establish that liver stem cells in early fetal development are indeed of endodermal vs. mesodermal origin (11). In a separate study, a limiting-dilution repopulation assay was performed in which Y-chromosome-marked single HSCs were transplanted into lethally irradiated female mice. These HSCs reconstituted the bone marrow and made low-level contributions to a variety of epithelial tissues including liver, lung, gastrointestinal tract and skin (44). Again, this study meets only two of the three proposed criteria for HSC transdifferentiation because functional testing of the transdifferentiated cells was not performed. However, it is also perhaps the most convincing example of transdifferentiation to date, as single-cell reconstitutions yielded both hematopoietic and nonhematopoietic effector cell-types of incompletely determined functional competence.

Circulating HSCs infiltrate solid organs

A complementary hypothesis is that circulating HSCs might themselves be progenitors for certain tissue-specific ASCs by seeding from the blood, followed by a change in lineage commitment and redifferentiation in response to newly acquired local microenvironments (56, 66, 69). This idea also suggests that reports of muscle and brain progenitor conversions to hematopoietic lineage cells could represent contamination from mobile HSCs within these organs that have not yet been reprogrammed to ASCs. For instance, a recent study tested the ability of muscle-derived stem (satellite) cells (MSCs) to repopulate whole bone marrow (34, 65). The observed repopulation could have been due either to MSC transdifferentiation to hematopoietic lineage effectors, skeletal muscle containing two distinct stem cell populations, or to primitive precursors capable of generating both HSCs and MSCs. Fractionation of skeletal muscle precursors by CD45 and Sca-1 HSC marker expression yielded distinct populations that could repopulate either muscle or hematopoietic lineages but not both concurrently (66). Only the CD45/Sca-1 expressing precursors were able to generate functional marrow cells. Because CD45 expression has only been reported in hematopoietic lineage cells, the data suggest that muscle-specific HSCs exist and are probably derived from circulating HSCs that seeded the muscle.

Is an HSC or an ASC a developmental program rather than a distinct cell type?

Alternative interpretations in addition to unsuspected HSC population heterogeneity or transdifferentiation between committed progenitors and progeny might explain recent lineage switching results as well. It is possible that committed lineage precursors, such as tissue-specific ASCs and HSCs, retain pluripotency during normal development, regeneration or repair and yield differentiated cells of multiple distinct lineages. In agreement with this notion is the idea that an ASC or an HSC represents a developmental program, rather than a committed cell-type, that can be activated in a variety of cells at certain stages of differentiation under specific conditions (69). In this model, tissue-specific ASCs represent cells in which this developmental program has been activated, even from advanced functional cell types through dedifferentiation. Direct testing will be required to establish or refute this intriguing theory.

Biphenotypic vs. transdifferentiated hematologic malignancies

All of these reports of changes in cell fate are observed in

systems designed to elicit this potential and do not determine its biologic relevance. In fact, transdifferentiation might never be observed in normal hematopoietic development, indicating difficulties in detection and/or the existence of passive or active mechanisms to suppress this potential. Are there any naturally occurring examples of hematopoietic transdifferentiation? It has been known for some time that infrequent hematologic malignancies express surface proteins derived from both myeloid and lymphoid lineages, creating so-called biphenotypic malignancies (77–81). Biphenotypic cancers probably contain transcription factors and a permissive chromatin conformation from parts of two distinct developmental programs. However, these tumors probably result not from transdifferentiation but from multilineage differentiation or lineage infidelity. An argument can be made that one cell phenotype is being acquired while another is being lost and these tumors are merely caught during this transition. However, cells from most biphenotypic tumors stably express markers from both lineages over extended periods of time. This occurrence suggests that nuclear programming or reprogramming might have been interrupted by cellular transformation at a crucial juncture. In addition, this type of lineage infidelity occurs within and not between germ layers, which might represent relatively small program modifications in contrast to marked programmatic alterations required from transitions across germ layers.

Biphenotypic hematologic tumors from biphenotypic or multipotential precursors?

Recent studies have shown that a subpopulation of normal, nonmalignant $CD5^+$ B-cells express markers of myeloid lineage cells (22, 82–84). Also, committed nontransformed CLP and pro-T1 stage thymocytes both maintain a latent granulocyte/macrophage (GM) differentiation program that can be initiated by stimulation of exogenously expressed IL-2 or GM-CSF receptors (20, 21). Furthermore, embryoid body cells arising from embryonic stem (ES) cell differentiation *in vitro* contain both embryonic erythroid and definitive adult-type lymphoid–myeloid differentiation potentials (84). These observations support the notion that biphenotypic tumors might result from transformation of biphenotypic or multipotential normal precursor cells, and that they might not even represent an example of lineage infidelity.

Lack of hematologic/solid tissue biphenotypic tumors

Overexpression of the v-raf oncogene in B-leukemia cells already overexpressing c-myc causes a lineage switch to malignant mature or immature macrophages (14). Unlike biphenotypic tumors, the B-lymphocyte program is lost while the macrophage program is gained, as evidenced by the extinction of B-cell markers and functions and the acquisition of macrophage markers and functions. This nuclear reprogramming occurs in the context of a malignant phenotype, suggesting that distinct subprograms can be manipulated without having to reprogram the entire nucleus.

Whether combinations of other oncogenes, tumor suppressors or transcription factors could yield hematologic malignancies from nonhematologic cell types or vice versa is unknown. Interestingly, intergerm layer biphenotypic malignancies involving hematopoietic lineage cells, such as monocyte/muscle cancers, have not been reported, whereas numerous examples of biphenotypic soft tissue and bone tumors containing both mesenchymal (mesoderm-derived) and epithelial (ectoderm-derived) malignant cell-types have been reported. A notable exception is the co-occurrence of hematologic and germ cell tumors, such as malignant mastocytosis combined with ovarian germ cell neoplasms (reviewed in 85). However, germ cell neoplasms often contain pluripotent elements, such as those present in teratomas, suggesting that a permissive hematologic component could arise with minimal nuclear reprogramming.

Epigenetic regulation of nuclear programs

Early theories on somatic cell differentiation suggested irreversible alterations, such as gains and losses of the genetic material, to explain the generation of distinct cell types present in multicellular animals (86). Subsequent cell fusion studies demonstrated that some inactive genes in one differentiated cell type might become activated by hybridization with a second distinct cell type (59–63, 87). The transfer of somatic cell nuclei into mammalian oocytes further showed that differentiated nuclei might be reprogrammed to generate entire organisms (88–96). Today we know that each of the roughly 200 distinct somatic cell types forming a human contain roughly the same set of gene sequences. Small exceptions include changes from viral infection and the loss of small intervening DNA regions in lymphocytes undergoing gene segment rearrangements to generate antigen receptors. This genomic stability indicates that cell diversity does not arise by irreversible gains, losses or reshuffling of the genetic material. Nevertheless, the genome of each cell is in a continual state of flux. In dividing cells, DNA must be reconfigured for replication, packaged for distribution to daughter cells and reorganized to facilitate gene expression or silencing during development and in response to transduced environmental sig-

nals. Furthermore, the genetic material must also be manipulated in such a way as to maintain the integrity of evolving differentiation programs throughout all phases of the cell cycle. For example, in lymphocyte division, some silenced genes remain silent through relocation to pericentromeric heterochromatin, where an unknown type of molecular memory prior to mitosis is established (97, 98).

In order to affect these and other dramatic changes during the cell cycle, DNA physically interacts with nongenetic protein components of the nucleus to create chromatin. It is the geometric configuration of chromatin within nuclei that permits or restricts access of transcription factors to regions within the genome and to particular genes within those regions. Although access is important, additional factors are required to regulate the expression of genes within accessible clusters, as evidenced by differential gene expression within the IL-4/IL-5/IL-13 T-helper 2 (Th2) cytokine gene cluster (reviewed in 99). Modifications of either DNA or protein components within chromatin that result in heritable changes in gene expression are termed epigenetic alterations. Enormous combinations of epigenetic instructions are responsible for the unique gene expression profiles of the 40 000–100 000 genes present in every human cell. These instructions result in multiple distinct nuclear programs that direct the development of independent cell lineages. Loosely defined, nuclear programs consist of specific temporal, spatial and geometric chromatin configurations of the genome (*Table 1*). These programs are re-established following cell division to maintain active or repressed patterns of gene transcription.

Epigenetic modifications of histones

Hematopoietic commitment and differentiation depend upon chromatin organization. Studies of intranuclear gene localization (100–103), chromatin compaction (reviewed in 99, 104), nucleosome positioning (105, 106) and chromatin modifier concentrations (reviewed in 107–111) are beyond the scope of discussion for this review. However, epigenetic modifications that are generated in response to changing microenvironments regulate these features of chromatin structure and are critical for transdifferentiation. To date, these modifications have been shown to target multiple chromatin-associated proteins and DNA. Protein modifications of specific amino acid residues within exposed histone tail motifs have been particularly well studied during the past 6 years. These residues are modified by enzymes that regulate DNA compaction and positioning of nucleosomes along the DNA (reviewed in 99). Histone tail modifications include phosphorylation, acetylation, methylation, ubiquitination and adenosine diphosphate (ADP) ribosylation (reviewed in 112, 113). Multiple types of modification occur simultaneously or sequentially on specific histone tails, creating an intricate 'histone code' with combinations of alterations resulting in synergistic or antagonistic effects on chromatin configuration and gene expression levels (reviewed in 114, 115). The first enzyme shown to modify the histone tail was a *Tetrahymena* ortholog of the yeast transcriptional coactivator protein Gcn5, a protein with histone acetyl transferase (HAT) activity (116). Enzymes with histone deacetylase (HDAC), methyltransferase and phosphorylation activity have subsequently been identified and associated with specific chromatin remodeling complexes (117–120). Despite an expanding knowledge of modification types and their effects on chromatin structure, little is known about the signals that control histone modifying enzyme production, stability, function and targeting. In fact, even less is known about control mechanisms in specific lineage rather than general developmental contexts, such as in mammalian hematopoiesis. Some of the best examples of epigenetic modification have in fact been described for DNA methylation and demethylation in germ cell and early embryonic development and are discussed here briefly.

DNA methylation in early development: parallels in hematopoiesis?

DNA methylation patterns form a major type of epigenetic regulation that creates distinct nuclear programs for lineage specification. In mammals, DNA methylation patterns are initially erased, and then parent-specific imprinting of about 50 genes is re-established in primordial germ cells (PGCs) by targeted methylation (reviewed in 121). DNA methylation provides an instructional mark of incompletely known significance that is usually associated with gene silencing and results in monoallelic expression at imprinted loci (122, 123). The enzymes responsible for this methylation, DNA methyltransferases DNMT3A/3B and DNMT1, transfer a methyl group from *S*-adenosylmethionine to the carbon-5 position of the pyrimidine ring of cytosines mainly in cytosine–phosphate–guanosine (CpG) dinucleotides (reviewed in 124, 125). In the period following establishment of imprints, methylation of the genome continues, and germ cells develop to mature sperm and oocytes.

Egg fertilization results in a diploid single-celled zygote containing gamete-imprinted genes, methylation of nonimprinted DNA regions and the full potential to create an entire organism. To reach this potential, a mitotically active zygote

is cleaved into smaller cells (blastomeres), resulting in a mass of about a dozen totipotent cells, the morula. These totipotent blastomeres subsequently undergo a series of decisions driven by developmental programs and biochemical processes responding to environmental signals that restrict their fates. Successful reproduction requires that a distinct nuclear program be established in selected blastomeres to facilitate histogenesis of trophectoderm and creation of the early placenta. The manufacture of trophectoderm from the morula signals the phenotypic loss of blastomere totipotency, the establishment of embryonic and extraembryonic tissue types and the initiation of cellular commitment and differentiation before implantation (reviewed in 126–128). In the embryo proper, loss of blastomere totipotency occurs with gastrulation and formation of the blastocyst with subsequent development from epiblast cells of the inner cell mass (129, 130). Specific epiblast cells rely on bone morphogenic protein (BMP)-4 and BMP8b signals transmitted from the trophectoderm for their differentiation into PGCs and repetition of the sexual cycle in mammals (131, 132). As such, these transduced signals are among the earliest described environmental effectors regulating cell-fate determination in mammalian development and serve as an example of signal transduction mechanisms that might be replicated during hematopoietic lineage commitment and differentiation. Consistent with this idea, distinct extracellular signals cause nucleosome remodeling on the IL-12 p40 and interferon-β (IFN-β) promoters and reprogramming of 293T fibroblasts into cells that express functional T-cell-specific effector proteins (99, 103, 133).

Global DNA demethylation, notably excluding the parent-specific imprinted genes, occurs a second time at the one-celled zygote stage and is complete by blastocyst formation (134, 135). This stage is followed by resumption of global, *de novo* DNA methylation. Cytosines within CpG-rich regions of the genome, in so-called CpG islands, escape methylation unless they reside on an inactivated X chromosome (136, 137). Promoters and exons of 50–60% of all human and mouse genes contain CpG islands, including almost all constitutively expressed housekeeping genes (138, 139). The absence of methylation within CpG islands permits continued gene expression. Clearly, dynamic changes in programming of the nucleus are occurring in early development to allow for subsequent lineage specification, as exemplified by rapid and specific changes in DNA methylation. These dynamic changes in DNA methylation during embryogenesis could mimic or exaggerate the extent of changes taking place in DNA methylation during hematopoiesis.

In general, silenced genes are methylated predominantly at CpG dinucleotides and are associated with stable and heritable changes in local chromatin structure (140–142). DNA methylation also helps to inactivate and silence exogenous viral and parasitic genomes and repetitive DNA elements in hematopoietic cells (122, 123, 143). Transcription repression by methylated CpG might occur directly, through the inhibition of essential transcription factor binding, or indirectly, through the recruitment of methyl-CpG-binding proteins that subsequently recruit HDAC-containing corepressor complexes (144–154). These HDAC corepressor complexes deacetylate histones H3 and H4 and promote repressive or so-called closed nucleosome structures. HAT-containing complexes are targeted to undermethylated genomic regions to effect acetylation of histones H3 and H4 and result in local chromatin structures with open nucleosomal arrangements that facilitate gene transcription (140, 141).

CpG methylation, histone modifications and chromatin structures are radically different between expressed vs. silenced genes. In contrast to the well-studied features of chromatin structure at these two extremes, the dynamic changes that occur in either activating or silencing genes are not so well resolved (155). The progression of changes in CpG methylation, histone deacetylation and nucleosome structure has been analyzed in the silencing of transfected chromosomally integrated reporter genes and exogenous retroviral genomes in infected cells (156–158). Gene silencing in both systems showed progressive increases in CpG methylation and histone deacetylation and decreases in chromatin accessibility over time. Remarkably, in both systems, gene silencing occurred early and preceded the appearance of appreciable CpG methylation (156, 158). Gene silencing at early stages could be reversed by addition of HDAC inhibitors, implying that histone deacetylation precedes extensive CpG methylation (158). However, when CpG methylation had reached dense methylation patterns at later times in silencing, 5-azacytidine pretreatment to inhibit DNA methyltransferase enzyme activity and decrease DNA methylation was required for HDAC inhibitors to reverse gene silencing (156, 158). These results suggest that increased levels of methyl-CpG fix and stabilize the state of silenced genes. Hence, one function of dense CpG methylation might be to lock genes in a silenced chromatin state, such as heterochromatin.

Most of the CpG sites that become methylated in early embryos are stably maintained in this state, except for CpG islands and other linked regions of genes that become actively expressed during subsequent extrauterine development (121, 159–162). Hematopoietic cells in children and adults might

respond to internal developmental and environmental cues through specific rather than global changes in DNA methylation, including specific demethylation, leading to cell type-specific methylation patterns that potentially regulate effector cell-specific gene expression (121, 160–162). Supporting this concept, specific regions of the IL-4 gene locus undergo either DNA methylation or demethylation preceding IL-4 promoter activation with cytokine induction of Th2-type cells (163–165).

Connecting histone and DNA epigenetic modifications

Within the past year, links between gene silencing, DNA methylation and histone modifications have emerged in genetic studies of the filamentous fungus *Neurospora crassa* and the plant *Arabidopsis thaliana*. These results should be interpreted cautiously, however, because epigenetic mechanisms discovered in nonmammals might or might not be applicable to mammals. For example, in contrast to mammals, yeasts have no demonstrable DNA methylation yet apparently contain a histone modification code (166, 167). Similarly, plants differ from mammals in that they have abundant non-CpG DNA methylation (168, 169). Given this caveat, studies in *Neurospora* demonstrate that the product of the *dim-5* gene is essential for DNA methylation, gene silencing and normal growth (170, reviewed in 171). The *Neurospora dim-5* gene encodes a SET-domain containing histone methyltransferase that transfers a methyl group to lysine 9 on the exposed tail of histone H3. Also, Jackson et al. showed that loss of function mutations of the SET-domain containing *KRYPTONITE* gene in *Arabidopsis* caused loss of methylation at CpNpG trinucleotide sites (172). It was then shown that *KRYPTONITE* also encodes a histone methyltransferase that transfers a methyl group to lysine 9 of histone H3. These authors further showed that methylated lysine 9 of H3 binds the *Arabidopsis* homolog of heterochromatin protein 1 (HP1), which in turn recruits the gene product of the *CHROMOMETHYLASE3* gene leading to DNA methylation of cytosines within CpNpG motifs (*Fig.* 3). Combined, these data suggest that methylation on lysine 9 of histone H3 directs sites of CpG and non-CpG DNA methylation in *Neurospora* and *Arabidopsis*. This immediately begs the question of whether this sequence of events beginning with histone H3 methylation is also responsible for DNA methylation, gene silencing and heterochromatinization in mammalian development and hematopoiesis. Interestingly, SET-domain containing Su(var)3–9 proteins in mammals are homologs of *dim-5* and *KRYPTONITE* gene products and are themselves lysine 9 histone H3 methyltransferases (118, 173). These results also raise the important question of how selective targeting to specific genes or regions of the genome is achieved during hematopoiesis or transdifferentiation.

Targeting DNA methylation by an aberrant transcription factor

One hint about a potential targeting mechanism for DNA methylation has been provided by studies of the PML-RAR fusion protein in patient samples of acute promyelocytic leukemia. Di Croce and colleagues found that PML-RAR fusion proteins could directly bind and recruit DNMT1 and DNMT3A DNA methyltransferases to the RARβ2 promoter, causing dense CpG methylation and gene silencing (174). Although this fusion protein acts as an aberrant transcription factor that targets the PML portion of the molecule to retinoic acid response element (RARE) binding motifs, it suggests the intriguing possibility that transcription factors themselves normally recruit the DNA methylation machinery to specific genes and regions of the genome during development. In cancer, aberrant targeting due to chromosomal translocations with resultant transcription factor fusion proteins could establish nonphysiologic patterns of DNA methylation and abnormal gene silencing (reviewed in 175). This mechanism also reveals a potential distinction between mammals and plants or fungi for the recruitment of the DNA methyltransferases that is independent of preceding histone methylation (*Fig.* 3). Careful examination of the timing and status of histone methylation in genes silenced by a PML-RAR fusion protein or additional transcription factors is required to exclude the possibility that histone methyltransferases have an initial role in this recruitment.

Do latent differentiation programs mediate hematopoietic transdifferentiation?

Hematopoietic cells undergoing transdifferentiation require signaling pathways and possibly surface receptors that are not normally expressed, functional or activated. Recently, CLPs were shown to maintain a latent granulocyte/macrophage differentiation program (21). Exogenous expression and stimulation of IL-2 or GM-CSF receptors activated this latent program and caused a switch from committed pro-T cell to myeloid lineage cells containing rearranged T-cell receptor-β (TCR-β)-locus genes (20). It will be highly instructive to determine what changes in chromatin structure induce this lineage switch and if histone modifications, DNA methylation/demethylation, ATP-dependent chromatin remodeling complex activation or additional epigenetic mechanisms are responsible for the required nuclear reprogramming.

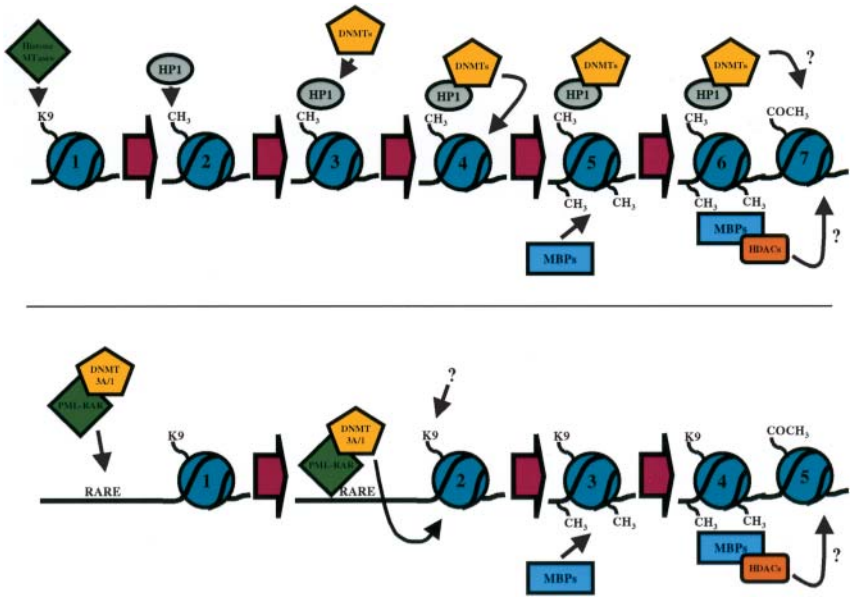

***Fig. 3.* Epigenetic regulation of chromatin structure.** *Top panel:* Methylation on lysine 9 of histone H3 (by KRYPTONITE – green diamond) recruits DNA methyltransferases (DNMTs – yellow pentagon) in *Arabidopsis* (172). Heterochromatin protein 1 (HP1 – gray oval) might directly facilitate binding of DNMTs and subsequent DNA methylation. In turn, methyl-binding proteins (MBPs – blue rectangle) are recruited to the methylated DNA, which recruits HDAC-containing corepressor complexes (orange rectangle) to further deacetylate histones H3 and H4 (144–154). It is not known whether this process of DNMT recruitment based on histone methyltransferase activity also occurs in mammalian development and hematopoiesis. Furthermore, the signals that target the histone methyltransferases to a specific genetic locus and whether or how the process spreads to flanking nucleosomes (i.e. nucleosome 7 in the figure) are unknown. *Bottom panel:* An aberrant transcription factor caused by the generation of a PML-RAR fusion protein (green diamond) recruits DNMT3A and DNMT1 (yellow pentagon) to the RARβ2 gene retinoic acid receptor element (RARE) (174). Targeted recruitment leads to DNA methylation and subsequent recruitment of MBPs (blue rectangle) and HDAC-containing corepressor complexes (orange rectangle) that foster chromatin condensation and gene silencing. It is unknown whether this targeted recruitment mechanism also functions for additional transcription factors and whether there is a dependence on histone modifications (i.e. methylation) during this process.

Nuclear programming in hematopoiesis and reprogramming in transdifferentiation

With the notable exception of studies on single genes during induced differentiation or gene recombination, little is known about the epigenetic factors responsible for global nuclear programming and changes in chromatin configuration that drive hematopoiesis. The hematopoietic nuclear program contains features that overlap with programs regulating the development of other lineages, such as instructions for mitosis, as well as features unique to hematopoietic cells. Two recent areas of investigation suggest that, similar to early embryonic development, changes in DNA methylation patterns and chromatin structure in hematopoiesis and stem cell differentiation are required (121, 162). In one set of studies progressive lysozyme gene demethylation at a CpG dinucleotide within the 5′ flanking region of the gene was correlated with its upregulated expression in differentiating myeloid precursor cells (161). Also, expression in macrophages was associated with complete demethylation at two additional, intragenic CpG sites. A similar correlation between CpG demethylation and myeloperoxidase or *c-fms* gene expression was also documented during macrophage maturation. In separate studies of T-cell differentiation, DNMT1 expression appeared essential for normal development, fate determination and effector function as shown by lineage-specific Cre/lox deletion of DNMT1 (176). Deletion of DNMT1 in double-negative thymocytes led to impaired survival of TCR-αβ-positive T cells and the generation of atypical CD8-positive TCR-γδ-positive T cells. Also, deletion of DNMT1 in CD4/CD8 double-positive thymocytes impaired activation-induced proliferation and caused enhanced cytokine mRNA expression by naive peripheral T cells.

Nuclear transfer, reprogramming and monoclonal mice

Recent whole nucleus transfer studies have demonstrated the ability of differentiated lymphocyte genomes to be forcibly reprogrammed to totipotency. Somatic cell nuclear transfer was developed in amphibians over 40 years ago. The basic technique involves replacing the nucleus of an egg with that from a specialized cell, either by cell fusion or direct nuclear microinjection, to determine whether the transferred nucleus can direct the development of an entire new organism. Unknown factors from the egg cytoplasm and surrounding environment act to reprogram the genome, facilitating the generation of a new individual that is a clone of the organism from which the nucleus was derived.

Advances in nuclear transfer and cell fusion techniques have paralleled an increased interest in cloning animals for a variety of purposes and in cloning human organs for replacement of diseased or worn-out body parts (177, 178). Despite improvements, these processes still remain technically challenging, inefficient and morally complicated. However, even low-efficiency nuclear transfer studies have shown that differentiated mammalian genomes can be reprogrammed to totipotency under specific circumstances. In several highly publicized examples, cloned sheep and mice were created from transfer of embryonic and adult nuclei. Initially, success was achieved with nuclei from a cultured cell line derived from early sheep embryos (88, 89). The degree of genome reprogramming needed to create a whole animal from these already totipotent nuclei could be modest compared with that required from a differentiated nucleus of a specialized somatic cell. The generation of 'Dolly' by electrofusion of adult sheep mammary-derived cells with enucleated sheep oocytes partially addressed this issue (90). Cloned mice were also produced by injection of terminally differentiated oocyte cumulus cell nuclei into enucleated eggs (91). However, in these and other examples the actual state of differentiation of the nucleus used in the transfer experiments could not be determined with certainty. Isolation procedures and/or cell culture and selection conditions could have yielded unsuspected tissue reserve or tissue-specific stem cells for transfer. Because these donor nuclei were not distinctly marked, their developmental histories could not be established.

An advantage of using lymphocytes to determine genome-reprogramming potential in nuclear transfer experiments is that their genomes are uniquely marked. Cells committed to the B-lineage rearrange immunoglobulin (Ig) heavy and light chain genes in the bone marrow to produce functional antigen receptors. Similarly, immature thymocytes rearrange TCR α,β,γ, and/or δ chain genes as they differentiate into functional T cells for export to the peripheral circulation and seeding of secondary lymphoid organs. Therefore, nuclei derived from peripheral lymphocytes are irreversibly marked by B- or T-cell antigen receptor gene rearrangements. Utilizing these markings, Hochedlinger and Jaenisch performed nuclear transfer experiments from mature B and T cells (179). Prior attempts to derive mice from lymphocytes by direct nuclear transfer into enucleated eggs, followed by *in vitro* blastocyst formation and implantation into uteri of recipient female mice, had failed (180). Therefore, a modified 2-step cloning procedure with a tetraploid complementation step was used (181, 182) (*Fig.* 4). In this procedure lymphocyte nuclei were transferred into enucleated mouse eggs that were cultured to the blastocyst stage. Unfortunately, lymphocyte nuclei reprogram at very low frequencies with only 4% of nucleus-transferred oocytes (41 out of 1000 attempts) surviving to the early blastocyst stage of development. Only two ES cell lines were generated from these cloned blastocysts. ES cells from these lines were then aggregated with tetraploid embryos generated from electrofusion of 2-cell embryos. This procedure produced chimeric mice in which the embryonic tissues were all derived from the isolated ES cells while the extraembryonic tissues were derived exclusively from the tetraploid cells. This approach was successful possibly because it avoided problems related to reprogramming of genomic imprints for monoallelic expression and development of placental tissues that require passage through parental germ cells (reviewed in 183). Two lines of mice were generated, one containing a nucleus transferred from a T cell and the other from a B cell. Only one macrosomic stillborn mouse was derived from the line generated with a T-cell nucleus. By contrast, 19 live-born fertile animals were derived from mice generated with the donor B-cell nucleus. This donor B cell originated from at least an immature transitional stage of development based upon Ig heavy and light chain gene rearrangements. Analysis of somatic hypermutation in the rearranged Ig genes from nonimmune cells of these mice would further refine the maturation stage of the transferred B-cell nucleus.

In addition to providing nonmanipulated placental tissues through tetraploid complementation, this strategy might have been successful for several other reasons. There was increased time for nuclear reprogramming to occur compared to straight transfer of nuclei into enucleated eggs followed by blastocyst implantation. There was also exposure to potential reprogramming factors in the ES cell culture and tetraploid complementation environments. The difficulty of reprogram-

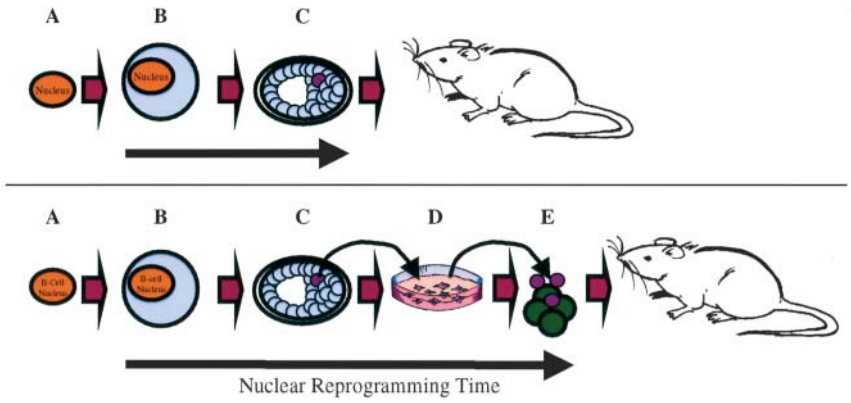

***Fig. 4.* Nuclear reprogramming and monoclonal mice.** *Top panel:* Nuclear reprogramming is initiated by injection of a somatic cell nucleus **(A)** into an enucleated ES cell **(B)** followed by injection of the hybrid ES cell into a blastocyst **(C)**. Implantation of the engineered blastocyst into carrier female uteri results in the generation of chimeric mice that have germ cell lineage reconstitution by the hybrid ES cell clone at a low frequency. *Bottom panel:* Transfer of a B-cell nucleus **(A)** into an enucleated egg **(B)** is followed by *in vitro* blastocyst formation **(C)** in which all contributing cells contain the injected B-cell nucleus. Culture of ES cells derived from such a cloned blastocyst **(D)** is followed by tetraploid complementation **(E)** in which modified ES cells (purple circles) establish all of the cells of resultant animals and the tetraploid embryos (green circles) contribute all of the extraembryonic placental tissues (182). The extra nuclear reprogramming time and multiple microenvironments to which the B-cell nucleus is exposed might hold the key to success, albeit at low frequency, of the tetraploid complementation technique vs. standard ES cell transfer methodologies (180).

ming mature B and T cells (only 2 ES lines were derived from 1000 nuclear injections) further suggests that transferred nuclei from previously cloned animals might have originated from stem cells present in the mature donor tissues rather than from terminally differentiated effector cell types. Most importantly for the current discussion, the derivation of any monoclonal mice clearly demonstrated the ability for nuclei from T and B cells to be reprogrammed for totipotency, leading to the development of a whole animal. This demonstration provides at least conceptual support for the ability of nuclear reprogramming to affect transdifferentiation of hematopoietic lineage cells given an appropriate set of reprogramming conditions.

Cell fusion vs. transdifferentiation

It has been largely assumed that nuclear reprogramming and transdifferentiation in response to environmental changes is the mechanism by which committed HSCs generate multiple effector cell types, such as myocytes, neurons and hepatocytes. However, a recent *in vitro* test of HSC transdifferentiation potential yielded a surprising result (67). Green fluorescent protein (GFP)-labeled, puromycin-resistant bone marrow HSCs from female mice were cocultured with totipotent male ES cells to determine the effect of secreted factors or cell contact from ES cells on HSCs. The resulting cells expressed GFP, were puromycin-resistant and acquired ES cell-like pluripotent properties. *In vitro* differentiation assays showed expression of Oct3/4 and UTF1, both markers of undifferentiated ES cells. Changing culture conditions, such as removing leukemia inhibitory factor, caused the cells to acquire mesodermal, endodermal and ectodermal differentiation features. Furthermore, upon transfer into NOD/SCID mice, conditioned HSCs formed teratomas, further indicating the acquisition of pluripotency. These transdifferentiated cells were then checked for DNA content by fluorescent-activated cell sorter (FACS) and showed polyploidy with a 4n or 6n chromosome composition. In addition, two examined cell lines contained Y chromosomes that must have originated from ES cells in the conditioning coculture. These surprising results were attributed to cell fusions between HSC and ES cells resulting in heterokaryons with ES cell-like totipotency, rather than transdifferentiation. In a separate study, similar fusion results using tagged cells explained the acquisition of ES cell-like totipotency by coculture of NSCs with ES cells rather than by transdifferentiation of NSCs (68). For years it has been known that cell fusion results in heterokaryons with features of one cell partner dominating the resultant hybridoma. Cell fusions are also

capable of yielding differentiated cell types representing any of the three germ layers and are routinely used in the production of monoclonal antibodies (59, 184, 185). In fusions between ES or embryonic germ cells and somatic cells a pluripotent phenotype typically dominates, as suggested for the fusion results between an HSC (or NSC) and ES cell (186, 187).

A major question is whether these *in vitro* fusion results negate transdifferentiation as a mechanism for *in vivo* HSC cell-type switching. So far, no observations of cell fusion between committed HSCs or ASCs and other cell types have been reported. If such fusions occur, they would presumably need to take place with a less differentiated precursor to HSCs or ASCs to increase potency for differentiation into a distinct lineage. Some HSC isolation protocols require manipulations that could expose highly enriched HSC to concentrated pluripotent precursors and potentially foster cell fusion. These very same cells are the potential contaminants that make clonality studies essential to determine whether hematopoietic cells transdifferentiate (11, 54–56). The common feature in both HSC–ES cell coculture and HSC isolation protocols is the exposure of HSC to pluripotent precursor types that might mediate cell fusion. It would therefore be prudent to check the DNA content even in clonal assays using HSC isolation protocols that test transdifferentiation potential. In addition, one transplantation study in mice showed a robust 30–50% efficiency of HSC reconstitution of hepatocytes, which is far greater than the frequency (~ 1 in 500 000) of HSC–ES cell fusion observed (41, 67). This discrepancy makes it unlikely that lineage switching is solely due to *in vivo* cell fusion rather than transdifferentiation, although drug selection and *in vitro* coculture conditions might have lowered the natural frequency of cell fusion events.

Concluding remarks

If a B cell and a T cell can be forcibly reprogrammed to generate an entire mouse, can a hematopoietic stem cell undergo transdifferentiation in physiological rather than experimental settings? Can a hematopoietic effector cell dedifferentiate and change fate and function in response to its environment? If so, what are the barriers and are they usually too high? Are unsuspected cell fusions a part of the transdifferentiation phenomenon being studied? Over the next few years, improved procedures and limiting-dilution studies will undoubtedly help to sort out the mechanisms of nuclear reprogramming that control the extent and situations in which transdifferentiation occurs within the hematopoietic system.

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